



Experiments with *Drosophila* for Biology Courses

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Editor-in-Chief

S. C. Lakhotia

Banaras Hindu University
Varanasi

Co-Editor

H. A. Ranganath

Centre for Human Genetics
Bangalore



Indian Academy of Sciences
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Experiments with *Drosophila* for Biology Courses

An e-resource book for laboratory experiments at under- and post-graduate levels and for research projects in Biology courses

Editor-in-Chief:

S. C. Lakhotia

Co-Editor:

H. A. Ranganath

Review Editors:

Pradeep K. Burma

Department of Genetics, University of Delhi, South Camps, New Delhi

Bimalendu B. Nath

Department of Zoology, S. P. Pune University, Pune

B. V. Shyamala

Department of Studies in Zoology, University of Mysore, Mysore.

Madhu G. Tapadia

Department of Zoology, Banaras Hindu University, Varanasi

Richa Arya

Department of Zoology, Banaras Hindu University, Varanasi

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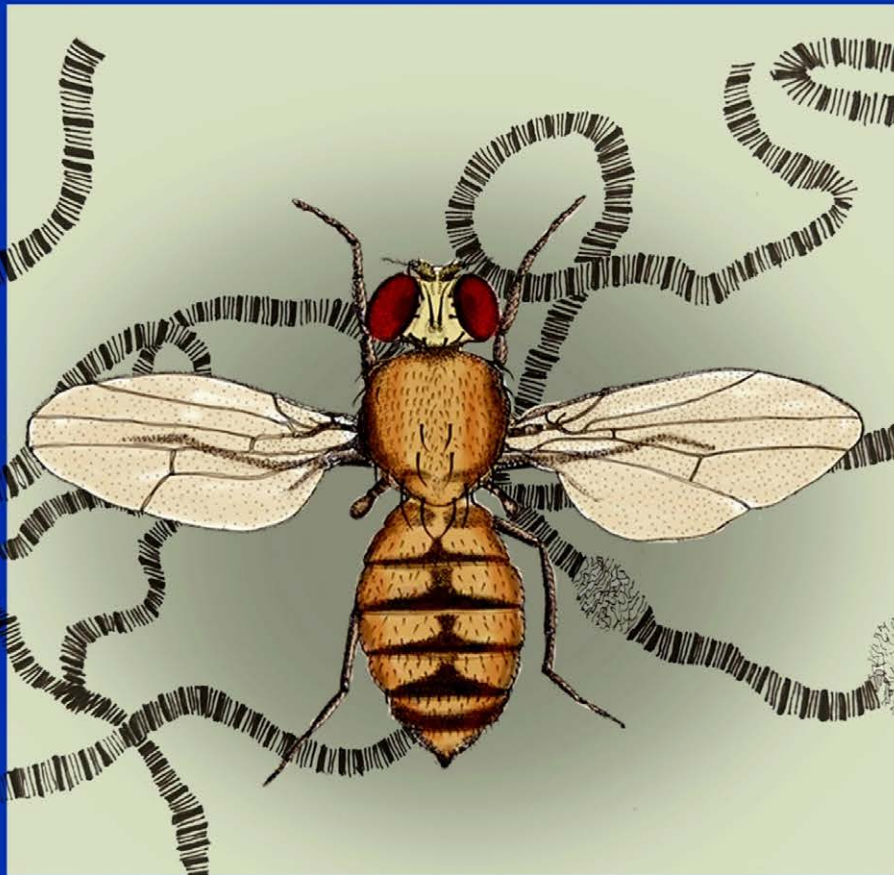
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EVER since the formulation of the chromosome theory of heredity, cytologists and geneticists alike have dreamed of the day when some one would find somewhere an organism in which the chromosomes were so large that it would be possible to see qualitative differences along their lengths corresponding to the different genes which we know must reside there.

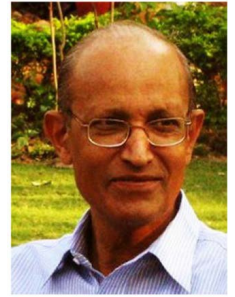
(T. S. Painter, 1933. Salivary chromosomes and the attack on the gene. *J. Heredity*)



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About Editors and Review Editors

Editor-in-Chief: Prof. S. C. Lakhotia (lakhotia@bhu.ac.in) is a Distinguished Professor and SERB Distinguished Fellow at the Banaras Hindu University. He has been a passionate teacher for >50 years and is engaged in research using the *Drosophila* model for >55 years with pioneering contributions in dosage compensation, polytene chromosome replication, non-coding RNA, stress response and Ayurvedic Biology. He is recipient of the S.S. Bhatnagar Prize, and is a Fellow of all the Science Academies in India



Co-Editor: Prof. H. A. Ranganath (haranganath@gmail.com) is a Distinguished Professor at the University of Mysore and Visiting Professor at Centre for Human Genetics, Bangalore. He has been working with *Drosophila* for over five decades with pioneering studies on experimental evolution in *Drosophila* species in India and established the *Drosophila* Stock Centre at University of Mysore in early 1990s. He is Founder Vice President of Indian Society of Evolutionary Biologists and is a Fellow of all the Science Academies in India

Review Editors

Prof. Bimalendu B. Nath (bbnath@gmail.com) is an Emeritus Professor at S. P. Pune University, Pune. He has been teaching genetics, evolution, and biodiversity for nearly 30 years and is actively involved in science popularization and pedagogy. His research involves integrative studies of stress-induced adaptive processes using *Chironomus* and *Drosophila* as model systems



Prof. Pradeep Kumar Burma (pburma@south.du.ac.in) is Professor at Department of Genetics, Delhi University (South Campus), New Delhi. With research experience of >30 years and initial training in *Drosophila* genetics, he switched to plant biology in mid 1990s to analyze promoters for crop improvement through transgenesis. He has deep interest in developing teaching approaches and remains enamored with the genetic tricks possible with *Drosophila*

Prof. B. V. Shyamala (shyamalabaragur@gmail.com) is a former Professor at Department of Studies in Zoology, University of Mysore, Mysore, with teaching experience of 25 years. She is engaged in research for >35 years, initially in evolutionary genetics and later in developmental genetics, neurodegeneration and drug discovery using the fly model



Prof. Madhu G. Tapadia (madhu@bhu.ac.in) is a Professor at Zoology Department, Banaras Hindu University, Varanasi, with 18 years of teaching experience and >25 years of research experience in developmental genetics, neurodegeneration, innate immune response, and Ayurvedic Biology using *Drosophila* as the model organism

Dr. Richa Arya (aryaricha@gmail.com) is an Assistant Professor and a former Ramalingaswami Fellow at Zoology Department, Banaras Hindu University, Varanasi, with >15 years of research experience using *Drosophila* as model for studies in developmental genetics and stem cell biology



Preface to First Edition

The fruit fly *Drosophila* is conventionally associated with Genetics because of the dominance of post-Mendelian Genetics, since 1910, by studies that used this ‘*Cinderella of Genetics*’. During the past 110 years, the subject of Genetics has evolved and has become central to all areas in Biology. It is significant that during this period, *Drosophila* has also become a favourite model for studies in almost all aspects of biology, including human diseases.

Experimental studies are essential learning tools for students of Biology. Besides providing better understanding and appreciation of what is learnt through lectures and reading, experimental studies provide hands-on experience and expertise, reinforce the learning, and arouse curiosity. With the astounding progress in biological sciences during the past several decades, the domain of experimental work has also substantially expanded. Unfortunately, the laboratory work, especially with live animals, is not getting the due share in most under- and post-graduate life sciences curricula.

Use of the fly model in research has expanded and contributed significantly to the progress in understanding of diverse life processes. Sometimes in May 2020, we felt that there is a need to popularize the use of *Drosophila* for experimental studies at under- and post-graduate laboratories beyond its limited use in conventional Genetics curricula. Since the underlying principles for many biological processes are now fairly well understood from phenotypic to molecular dimensions and since they share a basic unity among different living beings, the fruit fly *Drosophila* can be a powerful teaching tool to bring *LIFE* into LIFE SCIENCE courses.

The fly-community in India, which has vastly expanded during the past 2 decades, responded enthusiastically to this idea and, as a first step, contributed protocols for laboratory exercises that can be used at various levels in life-science related courses of study. The laboratory exercises included in this collection indeed reflect the diversity that the fly model can be used for. Each chapter has been prepared in a laboratory manual format providing a background for the objective of the given exercise, list of materials required, step-wise description of the experimental procedure, hints about what is to be observed. This is followed by a few leading questions to let students expand their understanding of the given topic. A small list of references to published papers and web pages is provided in each case. Appropriate cross-reference links to other relevant chapters in the book have also been provided.

Drosophila literature, much of which is available in public domain, provides excellent teaching material with historical anecdotes and experiments for concepts in Biology. The *Drosophila* lab exercises are simple, inexpensive and without any ethical issues. Use of the fly may not need any additional equipment and infrastructure, except for stereo-binocular microscopes and a BOD incubator (if the ambient temperature goes beyond 24-25°C). Therefore, we believe that this model can be used for classroom experiments even in institutions with limited resources.

The fly-based laboratory exercises included in this collection cover genetics, cytogenetics, developmental biology, cell and molecular biology, cancer biology, anatomy, physiology, immunology, neurobiology, behavioral biology, evolution, systematics, field studies, bioinformatics etc. The potential subject domains in which the experiment can be utilized are indicated in each chapter. Many of these laboratory exercises can be completed in one day but several need a longer time for completion, which would require adequate planning on part of teachers and students. Some of the protocols need a few months and thus are more appropriate as small research projects which the under- and post-graduate students can undertake as part of their curriculum. Most of the techniques would also be found useful by researchers.

The first chapter provides basics of the fly’s life cycle, its rearing and handling in lab. This chapter also includes information about the conventions, initially developed in Morgan’s “Fly Room”, and still followed by fly researchers, for naming genes and genotypes. Another chapter carries hand-drawings of fly embryos, larval and adult structures to encourage students, especially in this age of computer graphics and smart phones, to make use of their innate capacity and talent to draw what they see. A brief pictorial history of the fly research community in India, which started in a very modest way in the 1960s but which has now become a vibrant community, is presented in Chapter 88. A partial list of *Drosophila* researchers in the country is included in Chapter 89.

All the chapters have been written by researchers and/or faculty who are actually using the described methodology in their own laboratories. Authors have also agreed to provide the specific fly stocks that may be needed for conducting specific experiments. Teachers and students in different educational institutions can approach the fly researchers listed in Appendix 2. It is expected that the teachers and students in India, and elsewhere, would find this collection useful and would enthusiastically make use of the diverse laboratory exercises keeping in view the curricular relevance and feasibility.

We are thankful to the Indian Academy of Sciences, Bangalore, for readily agreeing to support this project and to host this e-book at its web page for full free access. We thank our Review Editors, Prof. Bimalendu B. Nath (Pune), Prof. Pradip Burma (New Delhi), Prof. B. V. Shyamala (Mysore), Prof. Madhu G. Tapadia (Varanasi) and Dr. Richa Arya (Varanasi) for reviewing different chapters and providing constructive comments which have helped make each chapter more comprehensive and user-friendly. We are grateful to all the authors who worked hard to prepare the contributions. We thank the entire fly community in India for their encouragement.

We thank the young research students, Mr. Govind R Chaudhary and Ms. Vaishali Yadav for the cover image of eclosing fly, Dr. Deepti Trivedi for the painting of fly and polytene chromosomes on the inner cover, and Dr. Smita Jain for the cross-stitch image of fly for the back cover. We acknowledge expert help of Ms. Deepti Banerjee in preparing the lay-out of different chapters.

We would be happy to get critical feed-back from users of this laboratory book about any errors/omissions that may exist, and suggestions for other lab protocols that can be used by students. We would like to keep this compilation as a dynamic online tool.

We hope that like the symbolic image of an eclosing fly on the cover, this collection would trigger a resurgence of laboratory exercises using *Drosophila* across a much wider spectrum of Biology curricula, beyond the classically inspired use of this fly in Genetics classes.

Preface to Second Edition

March 2024

The wide usage of the 1st edition of this e-book encouraged us to prepare the present expanded edition, which includes 33 additional laboratory exercises covering diverse areas in Biology. Small modifications and corrections in some of the earlier Chapters have also been made in the present version.

Chapters on pictorial history of fly genetics and current fly labs in India in the 1st edition have been removed from this edition. Information about these and other fly-related resources in India can be seen at: [Indian Drosophila Community Resources](#) web page.

We hope that the expanded collection would further enhance the utility of *Drosophila* for classroom and research experiments.

We thank all the authors for their enthusiastic contributions, and reviewers for their critical comments and helpful suggestions.

We acknowledge the continuing support of the Indian Academy of Sciences (Bengaluru) for this activity. We thank Mr. Varun D. Upadhyaya for preparing the lay-out of this edition.

Subhash C. Lakhota

Editor-in-Chief

Hassan A. Ranganath

Co-Editor

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Chapter 1

Rearing and handling of *Drosophila* – A primer for laboratory experiments

Govind, R. Chaudhary (govind.my.bhu@gmail.com), Akanksha Pandey (akankshapandey0002@gmail.com), Anjali Singh (anjalee01singh1993@gmail.com), Vaishali Yadav (yvaishali81@gmail.com), Vasudha Dwivedi (vasudha.dwivedi@gmail.com), Richa Arya* (aryaricha@bhu.ac.in) and S. C. Lakhotia* (lakhotia@bhu.ac.in)

* For correspondence

This chapter provides basic information about its life cycle, maintenance and handling in the laboratory, and the general conventions followed by fly geneticists while naming different classes of mutations, etc.

***Drosophila melanogaster*: from a little known Pomace fly to Cinderella of Genetics:**

Out of the ~1500 known *Drosophila* species, *D. melanogaster*, the Pomace fly or fruit fly has been the most extensively used and versatile experimental model for a little more than a century for studies in diverse areas in biomedical research. C. W. Woodworth first cultivated the fruit fly in 1900 at Harvard Zoological laboratory. Realizing the advantage of its short generation time, he suggested to one of his colleagues, William Castle, to use flies instead of mammals for his inbreeding studies. Castle developed the banana technique for culturing *Drosophila melanogaster* in the laboratory. His student Frank Lutz studied *Drosophila*'s biology and inheritance of wing variation at the Cold Spring Harbor (Davenport, 1941; Carlson, 2013). Thomas Hunt Morgan, an experimental embryologist by training, and interested at that time in the 'Mutation theory' of de Vries (Kohler, 1993), visited Lutz and got interested in this insect because of its short generation time and easy rearing in laboratory. Morgan took the red-eyed wild type stock from Lutz and started his research at Columbia University using this fly for studies on heredity. Morgan did not initially believe in the chromosomal basis of inheritance or even in evolution by Darwin's theory of natural selection of small variations. He and his student F. Payne exposed flies to diverse treatments like magnetic field, X-rays, UV light, centrifugation, heating, cooling, absence of light etc. to induce mutations. In about one year's time, the lab had obtained several flies with variations in phenotypes. However, they could not identify their distinct inheritance patterns at that time. Morgan's discovery of the white eyed fly in 1910, and its rapid identification as a sex-linked character followed by the subsequent mapping of mutant alleles of several other genes by his remarkable students (F. Payne, A. Sturtevant, C. B. Bridges, H. J. Muller, **Fig. 1**) convinced Morgan of validity of chromosomal basis of inheritance and natural selection. T. H. Morgan, his students and his wife L. V. Morgan, working with the little fruit fly provided direct experimental evidence for the chromosomal theory of inheritance by confirming that the genes are indeed located on chromosomes. Research in the 'Fly room' (a 16 x 23 feet size room) at Columbia University, where Morgan worked from 1910 to 1928, established the new post-Mendelian Genetics as an essential component of biological studies.



Fig. 1. The Fly Room. A. Thomas Hunt Morgan; B. Alfred Sturtevant; C. Herman Joseph Muller, and D. Calvin Blackman Bridges (Credit: Caltech Archives).

Many fundamental discoveries made in the ‘Fly room’ attracted large number of visitors, and very soon, *Drosophila melanogaster* became popular and famous for Genetic and other studies. Soon the little fruit fly was nicknamed as *Cinderella of Genetics*.

What makes *Drosophila melanogaster* an efficient and versatile model for laboratory studies?

Following are some of the features of biology of *Drosophila melanogaster* that contributed to its historical importance as the best model system for studies in Genetics:

1. It is cosmopolitan in distribution, and can be readily collected from nature for easy and less expensive rearing in laboratory in large numbers. Its small body size requires little space and facilitates handling.
2. It has a short generation time (<2 weeks) with large numbers of progeny (an important feature for studies on the inheritance of genes and phenotypes).
3. It has sharply defined body phenotypes which facilitate identification of numerous mutants (the number of systematically defined and listed mutants that are available now for *Drosophila melanogaster* is the largest among all model organisms, see www.flybase.org for listing of the mutants).
4. Has only four pairs of chromosomes in somatic cells.
5. Presence of ‘giant’ polytene chromosomes enables easy localization of genes on specific chromosome regions (physical maps) in parallel to linkage maps, and examination of gene activity.

6. It is neither a vector for any disease-causing pathogens nor is a pest.

As the fly genetics progressed in Morgan's lab and elsewhere, novel features of its biology were discovered and these added to the advantages offered by this model for research in Genetics and other biomedical domains. Some of these are:

1. **The practice of free sharing of newly discovered mutants** across the world was started by Morgan and is continuing to date. It is internationally agreed that *Drosophila melanogaster* can be sent across the world through postal services (in recent years different countries have introduced requirements of authorization by competent authorities for such shipments). The stock centers established in different countries have greatly facilitated the exchange of new genetic resources globally. No other model organism matches the genetic resources available for *Drosophila melanogaster*.

2. **Publication of *Drosophila* Information Service (DIS)** since 1934 as an informal medium greatly facilitated sharing of information about stocks, new mutants, new methods and research findings using the fly model (<https://www.ou.edu/journals/dis/>). DIS is like a "preprint journal", a practice that has become common in recent years in other disciplines. As noted in preface of volume 1 of DIS, "An appreciable share of credit for the fine accomplishments in *Drosophila* genetics is due to the broad mindedness of the original *Drosophila* workers who established the policy of a free exchange of material and information among all actively interested in *Drosophila* research. This policy has proved to be a great stimulus for the use of *Drosophila* material in genetic research and is directly responsible for many important contributions". DIS was the major source of information about new mutants and genetic stocks available in different labs across the world in the pre-internet era.

3. **Identification of imaginal discs in larvae** as undifferentiated precursors of adult cuticular structures led to their extensive use for studies on mechanisms of development, differentiation and malignancy (see [Chapters 18, 19, 80, 81](#)).

4. **Polytene chromosomes**, which permit direct visualization of gene activity (see [Chapter 31](#)), have been extensively used for studying chromosomal polymorphism and rearrangements at evolutionary scale, and for visualizing and understanding gene expression (Lakhotia, 1997) and regulatory systems. Some examples of the pioneering studies with these chromosomes include those on mechanism of steroid hormone action, heat shock response (see [Chapter 46](#)), dosage compensation, RNA pol II pausing.

5. **Discovery and characterization of the P-transposon** in the 1970s has been rapidly and ingeniously exploited as a versatile mutagen, a vehicle for germline transformation, conditional expression of the desired transgene and gene editing (see [Chapters 37, 38, 39, 40, 41, 42, 104](#)).

6. **Balanced lethal system and balancer chromosomes**, developed in the early stages of fly genetics greatly facilitated genetic studies ([Chapter 8, 10](#)).

7. **Unusual embryonic development** with syncytial blastoderm stage and the early delineation of pole cells ([Chapter 2, 32](#)), which are the exclusive precursors of germ cells in males and females: permitted a revolutionary but simple germline transformation strategy using the P-transposon.

8. **Identification of genes controlling early patterning of body axes** through mutational screening (see [Chapters 32, 33](#)), followed rapidly by their cloning, and discovery of their homologs in other eukaryotes. This revolutionized developmental biology (Chandrashekar and Lakhotia, 1991; Lakhotia 1994).

9. **Resources for *Drosophila*:** The following resources have significantly enhanced the advantages and utility of *Drosophila* as an experimental model in life sciences.

a. **Flybase:** The Flybase (<https://flybase.org>) is an online repository of genetic and molecular data for *Drosophila*, especially for *Drosophila melanogaster*. The data types include information about the gene name, synonyms, its linkage and cytological map positions, its mutant alleles and phenotypes, developmental expression patterns, nucleotide sequence (with genomic coordinates), known transcripts, polypeptide product/s (if a protein coding gene), its interactions with other gene/gene products, homologs/orthologs in other organisms, transgenes and other constructs available for the given gene, available mutant stocks, and references relating to the given gene. It is extensively hyperlinked to many other bioinformatics and other databases.

Historically, T. H. Morgan initiated publication of *Drosophila* Information Service (DIS) as an annual newsletter for informal communication between fly researchers across the globe. With increasingly improving computer infrastructure, beginning in 1980s, email exchanges of information supplemented DIS. Genetic and genomic data for *D. melanogaster* and a few related species became electronically available over the Internet. In 1992, the National Institute of Health (USA) funded the FlyBase project with the objective of designing, building and releasing a database of genetic and molecular information concerning *Drosophila melanogaster*. With continued expansion, the Flybase has become a major online source for comprehensive information about genes, genetics, genomics and many other features of the family Drosophilidae.

b. **Flybook: Genetics**, a journal published by the Genetics Society of America, has been periodically publishing historical perspectives about significant developments in fly genetics (and other disciplines). In 2015, the journal started publishing comprehensive review articles spanning the breadth of biology, genetics, genomics, and evolution of *Drosophila* as part of an encyclopedic 'Flybook' (see <https://www.genetics.org/content/flybook> for more details).

c. **Bloomington *Drosophila* Stock Centre, (BDSC, <https://bdsc.indiana.edu/>):** Free sharing of flies and their mutants has been a common practice since the inception of *Drosophila* genetics in the 'Fly Room'. As the users and the repertoire of mutant stocks increased, need for stock centre, which could serve as a repository for diverse stocks and provide them to desiring users, was felt. Calvin Bridges and Alfred Sturtevant established a *Drosophila* Stock Center at California Institute of Technology (CalTech) at Pasadena (USA). This collection was moved to the Indiana University, Bloomington (USA) in 1986, which now has become the major source for *Drosophila* stocks (see <https://bdsc.indiana.edu/about/history.html> for more details). Besides the BDSC, several other stock centres also exist in different countries.

Contributions of this little star in biological sciences are not limited to Genetics. This model has also been extensively used in disciplines like evolutionary biology, developmental biology, cell biology, neurobiology, behavioural biology, immunology, etc. Many investigators across the globe (with nearly 150 in India) are using *D. melanogaster* as a robust model for research in diverse areas in the bio-medical domain. The diversity and significance of research with this model is well illustrated by the six Nobel prizes for fundamental discoveries using the fly model (**Table 1**).

Table 1. Nobel prizes for research using *Drosophila* as the model organism

Awardees	Year	Major discovery	Reference
T. H Morgan	1933	Role of chromosomes in heredity	https://www.nobelprize.org/uploads/2018/06/morgan-lecture.pdf
H. J. Muller	1946	Production of mutations by X-ray irradiation	https://www.nobelprize.org/prizes/medicine/1946/muller/lecture/
E. B. Lewis, C. N Volhard, E. F. Wieschaus	1995	Genetic control of early embryonic development	https://www.nobelprize.org/prizes/medicine/1995/summary/
R. Axel, L. Buck	2004	Odorant receptors and organization of the olfactory system	https://www.nobelprize.org/prizes/medicine/2004/summary/
J. A. Hoffmann, B. Beutler, R. Steiman	2011	J. A. Hoffmann & B. Beutler: for activation of innate immunity. R. Steiman- for dendritic cells and their role in adaptive immunity.	https://www.nobelprize.org/prizes/medicine/2011/summary/
J.C. Hall, M. Rosbash, M. W. Young	2017	Molecular mechanisms of Circadian rhythm	https://www.nobelprize.org/prizes/medicine/2017/summary/

Short life cycle with well-defined developmental stages

Drosophila being a holometabolous dipteran insect has distinct stages of development viz., egg, larva, pupa, adult or imago (see **Fig. 2** and **Chapter 2**). Duration of these stages varies with the temperature (see **Chapter 49**). At 25°C, adult emerges in about 10 days, but at 20° C about 15 days are required.

Egg of *Drosophila melanogaster* is white in colour and is about 0.5 mm in length. The outer chorion membrane carries hexagonal marks (see **Chapter 2**) of the underlying follicle cells that secreted the chorion layer but died during oogenesis. A pair of egg filaments or dorsal appendages extends from its anterior end (**Fig. 3A**). These keep the egg from sinking into soft substratum on which it is laid. Between the chorion layer and the egg proper is an impervious vitelline membrane. The various events during embryonic development of *Drosophila* and their gene regulatory processes are well understood (see **Chapters 32, 33 34**).

The tiny larva, which hatches from the egg undergoes two moults, so that the larval period consists of three motile stages or instars, the first, second and third instars. Larva sheds the old cuticle at each of these two moults. The different larval instars can be identified by their age, body size (see **Fig. 3**), spiracles and mouth hooks (see **Chapter 14**). The moults and inter-moult larval growth are regulated, respectively, by ecdysone (see **Chapter 29**) and juvenile hormone. The 1st, 2nd and early 3rd instar larvae largely remain within the food, actively feed and increase their body mass (**Fig. 3B-D**). Late third instar larvae come out of food and crawl in search of a dry place for pupation, which is usually the wall of vial/bottle or the upper cover of Petri dish, where they are grown in laboratory. Larvae adhere at dry place using the glue protein secreted by their salivary glands (see **Chapter 7**) following which they undergo the last moult to begin the pupal stage. Pupa is formed within the last larval cuticle, which in the beginning is soft and white (white pupa, **Fig. 3E**). Gradually the cuticle hardens and becomes darker in color due to chitinization and sclerotization (brown pupa, **Fig. 3F**). During the next 4-5 days, the pupa undergoes complete metamorphosis within the pupal case. During the metamorphosis, most of the larval tissues undergo histolysis while the adult structures differentiate from the various imaginal discs (see **Chapters 2, 18, 19**) and other imaginal cells present in other tissues in the 3rd instar larval body.

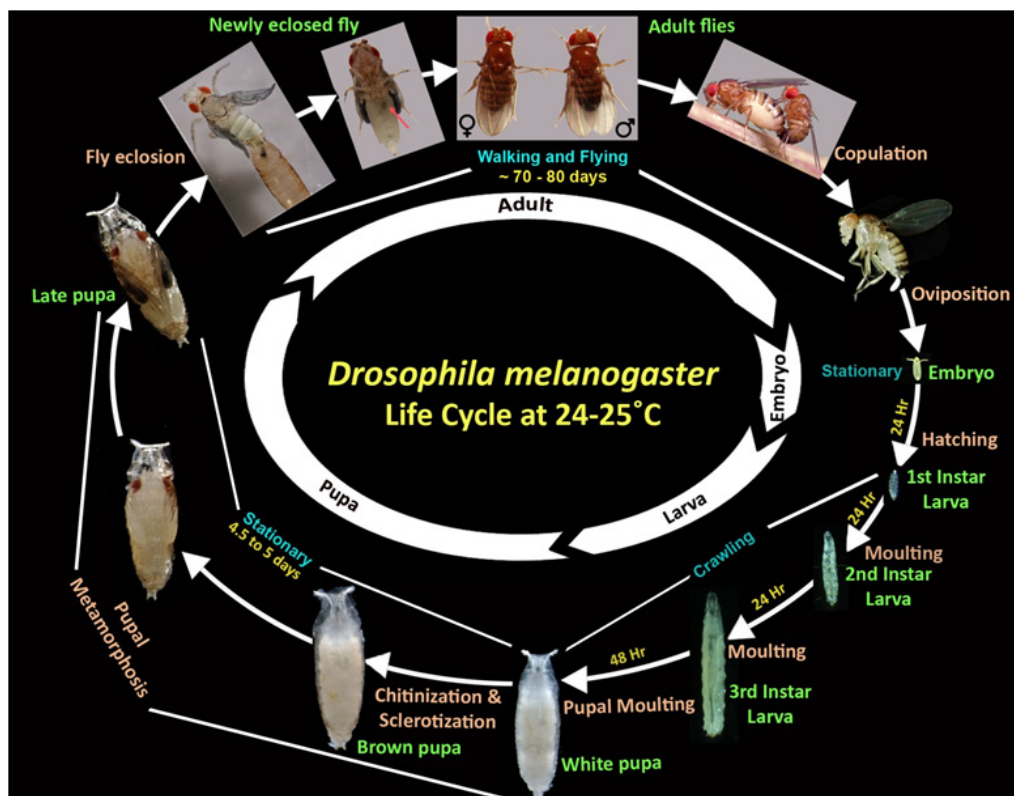


Fig. 2. Life cycle of *Drosophila melanogaster* showing major events, motility and average durations (at 24° – 25°C) of different stages. Red arrow points to the ‘meconium’ in newly emerged fly.

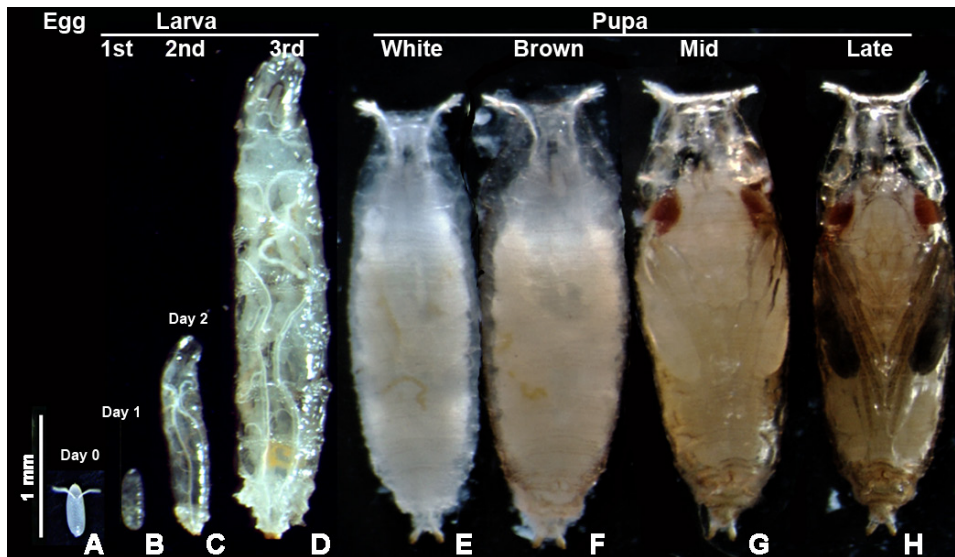


Fig. 3. Pre-adult stages of *D. melanogaster* and their relative sizes.

At the end of pupal metamorphosis, the imago ecloses by forcing its way through the anterior end of the pupal case (see **Fig. 2** and **cover page**). Freshly eclosed fly is elongated, lighter in colour, and with unexpanded wings. A small dark-greenish mark of ‘meconium’, representing the decaying larval gut, is visible on the anterior ventral left side through the young fly’s semi-translucent abdominal wall (red arrow in the panel for the newly emerged fly in **Fig.2**). These characteristic features of freshly emerged flies are used to isolate virgin females, when needed for setting up a desired genetic cross. Within the next 2-3 hr, the wings expand; body darkens and attains the typical adult body shape while the meconium disappears. Female flies become ready to mate in 4-6 hr after eclosion.

Identification of sex

Drosophila is sexually dimorphic. Male and female *D. melanogaster* flies (**Fig. 4**) can be distinguished by the following features:

1. Males are smaller than females.
2. Tip of the abdomen is elongated in female and somewhat rounded in male. As the female ages, its abdomen becomes distended with maturing eggs.
3. The abdomen of female has eight segments, with seven being clearly visible. In male, the last three abdominal segments are fused, so that only five segments are clearly visible.
4. In *D. melanogaster*, and several other *Drosophila* species, the abdominal tip in males is considerably darker than in females of the species (see **Chapters 43, 45**). The newly emerged flies, however, do not show such distinct difference in the abdominal pigmentation.
5. The male and female genitalia, visible on the ventral face of abdominal tip, are distinctly different, with the male genital plate appearing rougher and darker (see **Chapter 45**).
6. The males possess a sex comb (**Fig. 4 E'**) as a row of about ten stout, black bristles on the first tarsal segment of first pair of legs. The patterns and presence of sex-comb in males varies in different *Drosophila* species (see **Chapter 44**).

Males and females can also be distinguished during the larval stage by examining their gonads through the semi-transparent larval cuticle. The testes in male larvae and early pupae are larger than the ovaries in females (see **Chapter 28**). In the late pupal stage, male pupae can be identified by the sex-comb present on the 1st tarsal segment of prothoracic leg and visible through the pupal case (**Fig. 5**).

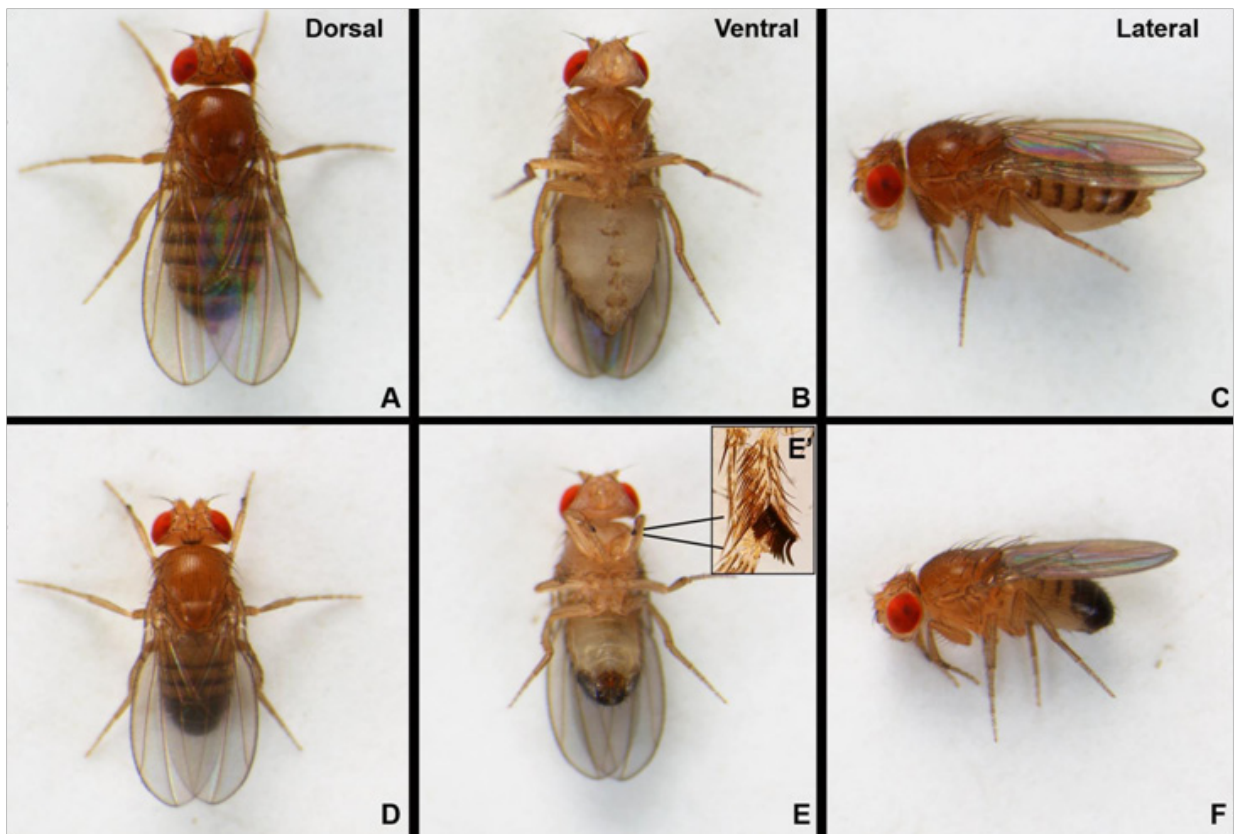


Fig. 4. Wild type *D. melanogaster* female (A-C) and male (D-F) flies as seen from dorsal, ventral and lateral views. The inset in E (E') shows a magnified view of sex-comb on the 1st tarsal segment of prothoracic pair of legs.

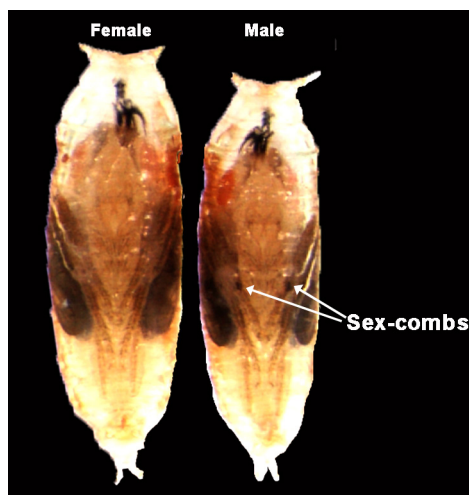


Fig. 5. Sex-combs on prothoracic legs as seen through the pupal case on ventral side of late stage male pupa.

Collection of virgins:

Many experiments require crosses between males and females of specifically desired genotypes (see [Chapter 5](#)). As is common in many insects, *Drosophila* female also stores sperms transferred during the mating in its spermatheca (see [Chapter 2](#)) and uses them lifelong. Therefore, it becomes imperative to select unmated virgin females to set crosses between flies of the desired genotypes. This can be achieved by any of the following methods:

1. **Selecting virgin adult females:** Female flies mate only 4-6 hr after eclosion (at 24°-25°C). Therefore, segregation of males and females in separate vials/bottles within a window of two hr after emergence ensures that the females remain virgins.
2. **Segregating male and female pupa:** Mature pupae are taken on a glass slide in a drop of water and their ventral side is viewed under a stereo-binocular microscope. Their legs can be seen through semi-transparent pupal case. Those pupae that do not have the sex comb on their first pair of legs (**Fig. 5**) are selected as females and transferred to a separate food vial. The flies emerging from these pupae remain virgin till they are allowed to mate.

Rearing flies in laboratory

Ideally, flies are reared and handled in rooms that maintain temperature between 20° to 25°C. Fly can also be reared in a B. O. D. incubator with temperature maintained in this range. Higher or lower temperature has adverse effects on fly viability and fecundity. Continued exposure to 30°C or above may cause sterility or death, whereas, temperatures lower than 18°C delay the life cycle and may impair viability.

Appropriate food medium in suitable containers is required for maintaining *Drosophila* in laboratory. Some of the recipes for the fly food used in different labs are provided in Appendix I of this Chapter. Glass ‘milk bottles’ (250 mL or 500 mL capacity) and glass vials (2.5 cm diameter and ~ 7.5 cm long) have been conventionally used for culturing. In recent decades, reusable and autoclavable semi-transparent plastic bottles and vials have become more popular (**Fig. 6**). They are plugged with non-absorbent cotton (reusable a few times after sterilization) or disposable synthetic foam plugs.



Fig. 6. Food bottles, vials and Petri dishes for culturing *Drosophila* in laboratory. A. Fresh food and B. with growing cultures.

Maintenance of fly cultures in laboratory requires care and vigilance to ensure that flies are healthy and of appropriate genotype in a given culture. Overcrowding is a common concern since flies keep laying eggs and expand their numbers within the limited space and resources available in the bottle/vial. Furthermore, the build-up of dead flies adversely affects the fitness of flies. Decaying food and fly debris attract infecting agents like bacteria, fungi, and mites (see below). Therefore, the flies must be transferred regularly to fresh bottles/vials with food to keep them healthy. As mentioned above, younger larvae

eat actively and mostly remain inside the food until they prepare for pupation (**Fig. 6**). However, overcrowding forces even the first and second instar larvae to crawl out of the food. Starting a new culture with about 10-20 flies in vials and <50 flies in bottles avoids overcrowding and maintains a healthy culture. As a good practice, flies reared at 24°/25°C must be transferred to fresh food within 25-30 days if cultured in bottles and 15-20 days if cultured in vials. It is also recommended to maintain all fly cultures in duplicate to provide a backup. A proper record of fly stock transfers should be maintained. *It is a good practice to carefully examine the flies before starting a new culture so that unwanted flies do not get mixed and thus 'contaminate' the culture and seriously jeopardize experiments.*

Care should be taken to not let flies escape from the culture vials and bottles during the maintenance process. These flies may contaminate other growing cultures in the laboratory or fresh food vials/bottles. To get rid of such unwanted flies in the lab, Fly traps should be set up in the lab area. A simple Fly trap can be prepared with a fresh food bottle with a funnel placed at its open end. A dilute solution of activated yeast with a few drops of propionic acid as a thin fluid layer on the food surface attracts the unwanted escapee flies to enter the trap through the funnel. Replace the fly traps weekly, if not earlier.

Major infections in *Drosophila* cultures:

Drosophila is relatively free of pests. However, in the absence of good care and practices, molds, bacteria, and mites become a serious problem in lab cultures. Some of the most common infections and the practical measures for avoiding and eradicating such infections are discussed in the section below.

A. Fungi

Filamentous fungi absorb nutrients from the food through their mycelia. The fungal spores spread via air and grow readily under humid conditions. The enhanced moisture content in the culture vial/bottle because of larval feeding provides good conditions for fungal growth.

Generally, the fly food recipes (see Appendix I) include an antifungal agent like Nipagin (Methyl 4-hydroxybenzoate, also known as Methylparaben). However, this cannot prevent all types of fungal growth. Common fungi like *Penicillium* and *Aspergillus sp.* can be eradicated by daily transfer of adult flies to a new vial for 7-10 days. If the cultures continue to have fungal growth, the daily transfer process should be extended for a few more days. Adding a small amount of baker's yeast (*Saccharomyces cerevisiae*) to the culture bottles and vials also reduces fungal growth (Lawrence and Goldstein, 1994).

B. Bacteria

Warm and moist places help in rapid growth of various bacteria. Mucus-producing bacteria (like *Acinebacter/Acinetobacter*) are a common problem in growing *Drosophila* cultures. These bacteria deposit a thick layer of mucus that coats the surface of food. Although not toxic to the flies, the mucus layer is detrimental since the larvae and adults get trapped and die (Ashburner and Roote, 2007). The bacterial growth can be inhibited by treating the stocks with antibiotics for one generation (Matthews, 1994) as follows:

- i. Add 100 µL of Penicillin (10,000 µg/mL) and Streptomycin (10,000 µg/mL) solution on food surface in a fresh vial and allow it to get absorbed.
- ii. Add a small amount of yeast and transfer flies to the treated medium.
- iii. Discard the parents before the progeny emerge and subculture progeny on untreated medium.

If the problem is recurrent, the source of contamination should be investigated. Prolonged use of antibiotics is not recommended as it may lead to resistance. Adding

dextrose in place of sucrose in the fly food medium may also help in preventing bacterial growth (Ashburner and Roote, 2007).

C. Mites

Mites are a major concern in any fly lab (Fig. 7). The ecto-parasitic mites can be easily spotted in older cultures as they wander around empty pupal cases. They lay a pearly strand of 10-20 eggs around pupae. Mites pose a serious threat to the growing cultures. Besides eating dead flies, they also attack live embryos and pupae, resulting in destruction of the fly stock. Since the adult mites are smaller than *Drosophila* eggs, it is challenging to detect them when they are fewer in number. They multiply rapidly and soon outnumber the growing flies in a culture. The mite infection spreads rapidly from one culture to another through dirty brushes, etherizer, or fly pads. The litter accumulated in trays and incubators also provides a good source of food for mites and thus facilitates their spread from one culture to the other.



Fig. 7. Mites infesting *Drosophila* cultures. A. Mites crawling on food tray; B. Magnified image of a mite; C-D. Mites on larva (C) pupa (D) and adult (E).

Maintaining a hygienic lab environment is essential to ensure mite-free conditions. Clean the fly working areas and utilities with 70% ethanol before and after use.

To rescue a mite-infested poorly performing stock, examine the flies and remove any adhering mites or their eggs using a soft brush and transfer the cleaned flies to a fresh mite-free food vial for further culturing. Monitor the culture regularly. Alternatively, collect pupae from a mite-infested culture, physically clean with a wet brush followed by a rinse in 70% ethanol before transferring to a mite-free fresh food vial. All the discarded culture bottles and vials should be frozen overnight at -20°C or immediately autoclaved.

To keep the fly facility mite free, all the flies that arrive from other labs should go through a quarantine process before they enter the fly room. A typical quarantine process for new stocks is as below:

- i. Designate a small quarantine area in the lab, a little away from the main working fly lab.
- ii. Immediately inspect the newly arrived culture/s for mites under binocular microscope. Examine surface of the culture and walls of the bottle/vial, and around pupae near the cotton plugs and dead flies. If mites are spotted, take out some larvae and pupae, clean them with water and 70% ethanol and transfer them to fresh vials.
- iii. Wait for the adult flies to eclose, examine them to be mite-free and then transfer to a new culture vial. Repeat the transfer process for five consecutive days. Flies have a self-cleaning habit, making it drop all the mite eggs, which stick to its body. Therefore, multiple transfers help build a mite free culture.

iv. Vials from the above first to fourth transfers should be discarded. Keep the fifth transfer and let the progeny grow for 10-15 days in a clean area. The original culture bottle/vial should also be separately retained for about 20 days as a backup and should also be monitored regularly.

v. Inspect the culture in the 5th vial after 15 days. It should be mite-free and ready to be moved to the fly room. Repeat the process if the cultures still have mites.

Tools for Handling of flie

A stereo-binocular microscope is typically needed to examine the flies. To examine flies under the binocular microscope (Fig. 8), they are taken out of the bottle/vial and put to sleep (see Appendix II for various methods used to immobilize flies during examination) so that they do not move or fly away while being examined. The common tools needed for handling flies in laboratories are shown in Fig. 9.



Fig. 8. Flies on the observation plate of a stereo-binocular microscope.



Fig. 9. Typical tools used for handling flies in the lab.

Naming Genes and Mutations:

Mutant phenotypes are essential for any conventional genetic study. The mutant alleles need to be distinctively named for convenient communication between researchers and others. Gregor, J. Mendel used single letter symbols to designate specific phenotypes of his different pure-breeding Pea strains. William Bateson followed the same principle and introduced single letter symbols like *T* for tall and *t* for short height pea lines in his 1909 book “Mendel’s Principles of Heredity’. With the start of fly work in Morgan’s Fly lab and with continuing discovery of new mutations, the system of single letter symbol for a phenotype soon became grossly limiting. Therefore, two and three letter symbols began to be used. With time, a combination of letters and digits to symbolize a gene also has become common. Morgan’s fly lab followed some conventions for naming new mutant alleles. These ensured uniformity in naming the new mutants. These have largely remained in practice till date.

The ‘wild type’ is ascribed to the most common phenotype seen in natural populations. Various natural fly populations have been collected from different geographical areas by researchers at different times and maintained in the laboratory as distinct ‘wild type’ populations since the original time of collection. These have generally been named according to the place from where they were initially collected (e.g., Oregon R, Canton-S, Samarkand etc.). It is believed that these stocks carry the most common alleles of different genes as present in the given natural population at the time of collection. Neither are they completely homozygous for all the ‘wild type’ alleles nor all the flies in a collection are identical in their genotype to each other. In other words, unless special effort has been made to generate and maintain selected isogenised lines, most of the ‘wild type’ stocks maintained in a laboratory are polymorphic for one or the other gene (e.g., see **Chapters 66, 113, 114**).

Genetic studies rely on variations/mutations present either in nature or induced in the laboratory. Identification of a gene responsible for a given mutant phenotype has been the traditional approach, often termed as ‘Forward Genetics’. On the contrary, ‘Reverse Genetics, which became possible with the advent of genetic engineering that permitted manipulation of genes as desired, starts with direct identification of a gene at the level of DNA or its product (RNA and/or protein) followed by subsequent characterization of its function and/or phenotype. Either of these approaches requires assignment of a unique identity to the gene. The basics of conventions used in naming a fly gene and its alleles are described below.

A. Genes named according to the associated mutant phenotype:

In these cases, name of a gene is based on the first identified mutant phenotype without necessarily knowing the location or other characteristics of the concerned gene. Historically, most mutant alleles identified in Morgan’s lab and later mapped on linkage maps were based on altered visible (morphological) phenotypes. They were named according to the discoverer’s perception of the mutant phenotype’s similarity with some known objects. For example the famous *white eye* mutation (see **Chapters 3, 5, 6**) was so named because eye of the fly was white and the mutant allele was symbolized as *w*. Bar eye (the adult fly’s eye shape is like a bar instead of the usual oval) mutation was symbolized as *B*. Bristles in a mutant fly looked like superficially burnt hair (singed) and, therefore, this mutation was named singed and symbolized as *sn*. If the first identified mutant phenotype is inherited as a dominant trait, the gene symbol starts with a capital letter (e.g., *B* for Bar eye, *Cy* for Curly wings) but if the first identified mutant allele behaves like a recessive allele, the symbol starts with a small letter (e.g., *w*, *y*, *cn* etc). In all cases, the wild type

allele is indicated by the gene name followed by ‘+’ as superscript (e.g., B^+ , w^+ , y^+ , sn^+ etc.).

Note: The b symbol used for the recessive black body mutant phenotype is example of a rare exception in fly genetics to the above rule of using caps and small letters for dominant and recessive mutant alleles (b is not an allele of the B or *Bar eye* gene).

B. Genes named according to their effect on other mutant phenotype/s:

Mutant alleles of several genes were identified in genetic screens to modify (suppress or enhance) the phenotype caused by a mutation in some other gene. Based on the effect of the modifier on the expression of candidate gene or mutant phenotype, the modifier gene name is prefixed with *enhancer* (en) or *suppressor* (su) followed by target mutant allele’s name whose phenotype is modified. For example, *suppressor of forked* ($su(f)$) name refers to a mutant allele of a gene that acts as a *suppressor* (prefix) of the *forked* (candidate gene) mutant allele so that in the presence of $su(f)$ allele, the forked bristle phenotype caused by the *forked* (f) mutant allele is suppressed. The prefix en is used when the phenotype of target mutant allele is enhanced.

C. Genes named according to the product:

When the encoded product of a gene is known before a mutation has been identified, the gene is named according to the product name. For example, the symbol *Sod* indicates that the gene encodes Superoxide dismutase enzyme. Such gene names do not reflect a mutant phenotype. In some cases, the gene symbol includes the known location of the gene on polytene chromosome map (see **Chapter 31**). For example, the name α -*tubulin*(3)67C (symbolized as α *Tub67C*) indicates that the gene encodes α -*Tubulin* and is located on chromosome 3 in the 67C region of polytene chromosome maps.

D. Genes named on bioinformatic analyses:

Bioinformatic analysis of genome sequences permits prediction of a functional gene and its possible homolog/ortholog in other organisms. In such cases, the gene may be named after its homolog’s name in other organisms or may be assigned a **Gene ID** (see later).

E. General guidelines for naming and symbolizing genes:

- a. **Uniqueness:** Names and symbols for new genes identified in *Drosophila* must differ from all the existing FlyBase-approved names and symbols.
- b. **Short symbols:** Allele symbols should be short and generally no more than three characters long.
- c. **Relevance:** Gene’s name should indicate the gene function, the mutant phenotype or any other relevant information. The symbols are generally abbreviations of English words but names in other languages are also given (e.g., some gene names like *Brahma*, *Rudhira*, *Radha*, *Maheshwara* are Sanskrit/Hindi words).
- d. **Restricted characters:** Characters like {, }, [,], ;, \, /, *, <, > are not allowed as part of the gene symbol.
- e. **Gene symbol should not contain spaces:** If a separator is needed within the gene symbol, a hyphen “-” is used rather than a blank space.
- f. **Use of Capital letter:** If the phenotype of the mutant allele for which gene is named is dominant, the first letter of the symbol will be in upper case (capital) while the subsequent letter/s will be in lower case.
- g. **Superscripts and subscripts:** Subscripts are generally not included in gene symbols. Superscripts commonly indicate a specific allele when more than one allele is identified. A “+” superscript after the gene name or symbol indicates the wild type allele. For designating mutant alleles other letters and/or digits are used as superscript. For example, $white^+(w^+)$ represents wild type allele of the *white* eye gene, the superscript in symbols w^a (*white-apricot*) or w^e (*white-eosin*) refer to specific alleles of the $white^+(w^+)$ gene.

h. **Italicization:** Gene symbol/name are printed in italic letters or these should be underlined if hand-written. However, while indicating phenotype or the protein product, full gene name/symbol is written in non-italic. For example, white eye (non-italicized) indicates phenotype but *white* or *w* (italicized) represents the gene.

i. **Inoffensive** Gene name/symbol should not be objectionable.

j. **Ambiguous allele:** A mutant allele whose specific identity is unknown, is usually designated by an asterisk. For example, *w** indicates an allele of *white* but which of its many alleles is not defined.

k. **Some common prefixes used in gene names:** Besides, the *su* or *en* prefixes noted above, a few other prefixes are used to indicate some common properties shared by different genes (see Table 2).

Table 2. Common prefixed used in gene symbols.

Mutant class	Common prefix in gene symbo
Recessive Lethal	<i>l(n)m</i>
Minute	<i>M(n)m</i>
Mutagen sensitive	<i>mus</i>
Ecdysone-inducible gene	<i>Eig</i>

Where *n* stands for chromosome (1, 2, 3 or 4 on which the gene is located) and *m* stands for the specific gene's symbol.

Naming of *Drosophila* chromosomes:

Drosophila melanogaster has four pairs of chromosomes, which include a pair of sex chromosomes (a pair of acrocentric X chromosomes in females and the X and sub-metacentric Y pair in males) and three pairs of autosomes (chromosomes 2, 3 and 4). Chromosome pairs 2 and 3 are large metacentric, while the 4th chromosome is very small and often named as 'dot' chromosome (see Chapter 30). The two arms of the long X, 2nd and 3rd, chromosomes are designated as left (XL, 2L, 3L) and right arms (XR, 2R, 3R) while those of the Y chromosome are designated as long (YL) and short (YS) arms (Fig. 10).

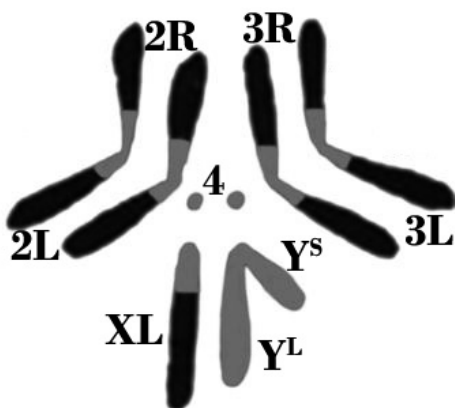


Fig. 10. Schematic of mitotic chromosomes of *Drosophila melanogaster*. L and R following the chromosome name (X, 2 and 3) indicate left and right arms, respectively; the very short right arm of the acrocentric X is not distinguishable. Y^S and Y^L represent the short and long arms of Y-chromosome, respectively. The 'dot' chromosome 4 does not show left or right arms. Light and dark shaded regions represent heterochromatic and euchromatic regions, respectively.

Reserved characters in nomenclature of genes and genotypes:

Certain characters are used to reflect specific features as noted in **Table 3**.

Table 3. Reserved symbolic characters used in naming genes and genotypes.

Symbolic character	Purpose
/	Used as a separator between homologous chromosomes: e. g., <i>y w/y f</i> indicates that one of the homolog carries <i>y</i> (<i>yellow body</i>) and <i>w</i> (<i>white eye</i>) mutant alleles while its partner (homolog) carries <i>y</i> and <i>f</i> (<i>forked bristles</i>) mutant alleles. A space is used as a separator between different mutant alleles present on the same chromosome, without any other punctuation mark or character in between.
;	Used as separator for non-homologous chromosomes: e.g. the <i>y w; Sp/Cy</i> genotype indicates that both (in females) or the single (in males) X-chromosome/s carry <i>y</i> and <i>w</i> mutant alleles while the chromosome 2 carries dominant <i>Sp</i> (<i>Sternoplural bristles</i>) and <i>Cy</i> (<i>Curly wings</i>) mutant alleles on the two homologs, respectively.
\	It is used as a separator after the gene symbol to indicate species identification. For example, <i>Rab11\Dmel</i> indicates <i>Rab11</i> gene of <i>Dmel</i> , representing <i>Drosophila melanogaster</i> .
	These parentheses marks enclose a character which is to be read as superscript: <i>w[e]</i> is equivalent to <i>w^e</i> .
{ }	These parentheses marks enclose details of transposon and transgene constructs in a transgenic line.
()	These parentheses marks enclose the chromosome number and is generally used in conjunction with a prefix symbol: e.g., <i>l(2)gl</i> indicates a recessive lethal mutant allele on chromosome 2 and causing giant larva phenotype.
:	This mark is used as part of the symbol for non-protein-coding genes, mitochondrial genome and in the symbol for fusion genes. <ol style="list-style-type: none"> i. Non-protein-coding genes like rRNA, tRNA, snRNA, snoRNA: for example rRNA gene is represented as 'nSrRNA:X', where <i>n</i> indicates the specific rRNA's sedimentation rate and X represents annotation ID. e.g., <i>18SrRNA:CR41548</i> (http://flybase.org/reports/FBgn0085802.html). ii. Gene encoded by mitochondrial genome are represented by symbol '<i>mt.</i>' e.g., <i>mt:ND4</i>, which stands for the gene encoding Mitochondrial NADH-ubiquinone oxidoreductase chain 4 (http://flybase.org/reports/FBgn0262952.html). iii. While naming a fusion gene, names of the component gene symbols are separated by two colons, e.g. <i>.Antp::Scr</i> (http://flybase.org/reports/FBgn0012038.html)

Symbols used for Gene Annotations:

i. **Gene annotation IDs:** During the annotation of genomic sequences, each known or bioinformatically predicted gene is assigned a unique identifier, the **Gene ID**. A Gene ID consists of a two-letter species-specific prefix followed by a four- or five-digit integer number. For Gene ID of *Drosophila melanogaster*, two types of prefixes, CG and CR are used. CG is used for protein-coding while CR is used for non-protein-coding genes. For other *Drosophila* species, a 2-letter species-specific prefix, common for protein-coding and non-coding genes, is used. For example, GG is used for *Drosophila erecta*, GJ for *Drosophila virilis* and GE for *Drosophila yakuba* Gene IDs.

ii. **Transposons and Transgene construct symbols:** Specific symbols have been assigned to indicate the transposons and transposon-based transgenes in flies. **Transposons** are symbolized as *ends{}*, where *ends* indicates the transposon's symbolic name: e.g., *P* indicates a *P-element*. The **Transgene** constructs are symbolized as *ends{construct-symbol}*: e.g., *P{Tub-Rab11.Venus}*, where *P{}* represents P-element and *Tub-Rab11.Venus* indicates that tubulin (*Tub*) promoter drives *Rab11* expression, whose N-terminal is tagged with Venus (yellow fluorescent protein) (<https://flybase.org/reports/FBtp0021368>).

iii. **Telomere symbols:** Telomeres of different chromosomes are indicated by the symbol *nAt*, where *n* represents chromosome number, *A* denotes chromosome arm and *t* is for telomere. Thus *1Lt* is telomere of left arm of X-chromosome, *2Rt*, is telomere of right arm of chromosome 2. If the origin of telomere is not known, *?t* symbol is used to signify undefined telomere.

iv. **Naming Chromosomal aberrations:** A large number of numerical and structural aberrations are known in *Drosophila melanogaster*. To provide an unique identity for each class, the symbol to define a chromosomal aberration (see **Table 4**) includes information about: i) type of rearrangement (deficiency, duplication, inversion, translocation, etc.), ii) chromosome/s involved (written within parenthesis), and iii) unique identifier. As for the mutant gene symbols, symbols for chromosomal rearrangements are also italicized. Spaces or subscripts are not used.

Table 4. Symbols for different types of chromosome aberrations

Chromosomal aberration type	Abbreviation
Deficiency	Df
Duplication	Dp
Inversion	In
Ring Chromosome	R
Translocation	T
Transposition	Tp

v. **Translocation (T):** Involves an interchange of chromosomal segments between two non-homologous chromosomes. The symbol used to define a translocation is *T(n1;n2)m*, where *n1*, and *n2* are participating chromosomes involved in translocation and *m* is a unique identifier.

Rules for naming a translocation: The involved chromosomes named within the parenthesis marks are listed in the following order 1, Y, 2, 3, 4 (example: *1;2*, *1;2;3*, *1;2;4*,

1;3,1;Y, 1;Y;3) and the involved chromosomes are separated by a semicolon, without any space in between. Example: $T(1;4)B^S$ represents translocation between chromosomes 1 and 4, and B^S represents the *Bar of Stone* allele that produces Bar eye phenotype.

vi. **Inversion (In):** Occurs when a broken chromosome segment reinserts between the same broken ends but after 180° turn resulting in reversal of the gene order in the affected region. Inversions may be **paracentric** (occurring within one chromosomal arm without involving the centromere) or **pericentric** (the inversion spans the centromere and thus, affects both arms of the chromosome).

Rules for naming an inversion: The symbol *In* is followed by parenthesis marks enclosing name of the chromosome arm or arms and then a unique designation. Example: $In(2L)Cy$ represents inversion in the left arm of chromosome 2, and *Cy* represents its name since this inversion is associated with Curly wings phenotype.

vii. **Deficiency (Df):** The term deficiency applies for a deletion visible at chromosome level as well as for those too small to be identifiable under the light microscope.

Rules for naming a deficiency Deficiency is symbolized as $Df(nA)m$, where *n* indicates the chromosome affected, *A* stands for chromosome arm, and *m* stands for unique designator. For example, $Df(3R)93D$ represents a deletion in the right arm of chromosome 3, involving the *93D* region of polytene chromosomes.

viii. **Duplication (Dp):** Presence of an additional chromosomal segment derived from a sister chromatid or homologous chromosome or non-homologous chromosome is known as duplication.

Rules for naming a duplication: A duplication is symbolized as $Dp(n1;n2)m$, where *n1* indicates donor chromosome, *n2* indicates the recipient chromosome and *m* specifies the duplication. For example, $Dp(3;1)O5$ represents duplication where chromosome 3 is donor and chromosome 1 is recipient; *O5* is this duplication's specific name, referring to its discovery by Oliver. A higher copy number of the duplicated region is also symbolized as *Dp*, but with the number of repeats indicated in parentheses. For example, $Dp(1:1)$ represent duplication while $Dp(1:1:1)$ represents triplication with donor and recipient chromosomes being Chromosome 1.

ix. **Transposition (Tp):** This type of chromosomal aberration is different from translocation, since in such cases, a segment is removed from one chromosome and attached to or inserted in another chromosome without a reciprocal exchange.

Rules for naming a transposition: The symbol used for transposition is $Tp(n1;n2)m$, where *n1* represents donor chromosome, *n2* represents recipient chromosome, and *m* stands for its specific name. In intra-chromosomal transpositions, the chromosome segment moves from one place to another in the same chromosome so that $n1 = n2$. For example: $Tp(3;1)ry35$ represents transposition of a piece of chromosome 3 into the X chromosome, which produces a mutant phenotype in the eye-color gene *rosy*.

Balancer Chromosomes:

Recombination between homologous chromosomes is critical in generating diversity for natural selection to act upon. In experimental genetic studies, recombination mapping has been extensively used in classical genetics for generating linkage maps. However, under certain experimental conditions, recombination between homologs is not desired. For example, when two mutant alleles have been brought together on the same chromosome

through homologous recombination, keeping them together on the same homolog becomes a challenge when the two linked mutant alleles are in a heterozygous state. Interestingly, the homologous recombination during meiosis is nearly completely absent in *Drosophila melanogaster* males. However, passage of the same chromosome with two or more mutant alleles in *cis*- condition may cause them to separate by recombination between non-sister chromatids of the paired homologs during meiosis in females, which makes it difficult to maintain the mutant alleles together in heterozygous combination (see **Chapter 11**). A solution to this was found in **balancer chromosomes**.

The balancer chromosomes, first described almost 100 years ago, remain a fundamental tool for *Drosophila* geneticists. In 1918 H. J. Muller found that a stock with a recessive lethal mutation could be maintained conveniently when a recessive lethal mutation in another gene is present on the homolog. Muller called them ‘Balanced lethals’ (see **Chapter 8**) and suggested that the homologous recombination between the two recessive lethal mutations generates homozygosity for one or the other mutant allele leading to death of such progeny. As a result, only heterozygous individuals for both the recessive alleles survive in the stock. Later, in 1921 Sturtevant, Muller’s colleague in Morgan’s lab, noted that inversion reduces recombination frequency within the inverted segment of a chromosome. These two observations led to the principle underlying the use of balancer chromosomes. Following the ‘balanced lethals’, a chromosome that prevented birth of progeny with recombined alleles on its homolog was designated as ‘**balancer chromosome**.’ A similar strategy is now being used in other models like *Caenorhabditis elegans*.

A balancer chromosome carries multiple inversions which prevent birth of progeny which is recombinant for alleles between the inversion carrying chromosome and its normal homolog. The balancer chromosomes also carry **markers** in form of dominant and recessive mutant alleles. Inversion does not generally inhibit crossing over between the inverted and non-inverted homologs in heterozygotes, but prevents recovery of progeny that carry chromatids derived from homologous recombination between the inverted chromosome and its non-inverted homolog. Such recombined chromatids carry duplications and/or deletions (Kaufman, 2017). In addition, recessive lethal and/or female sterile mutation is/are also generally present on the balancer chromosomes so that progeny homozygous for the balancer chromosome either would not survive or the homozygous females remain sterile. Therefore, the balancer chromosomes permit easy maintenance of chromosomes that carry multiple visible, lethal and/or sterility causing mutations without any need for selection of parents for each generation. Further, since they maintain the linear integrity of the homolog, they are also used while screening for new mutations (Kaufman, 2017). Most of the balancer chromosomes that are commonly used by fly researchers today were constructed by E. B. Lewis and his colleagues (Duncan and Celniker, 2004).

The three main features of **balancer chromosomes** are:

1. Primarily used to obtain only the progeny in which the balancer chromosome and its homolog retain their linear integrity without any homologous recombination. Heterozygosity for chromosomal inversion prevents recovery of recombinant progeny firstly because crossovers in the vicinity of inversion breakpoints are greatly reduced and secondly because the chromatids with single crossovers within the inverted regions carry duplications/deficiencies. The resulting zygotes receiving such chromatids become aneuploid and fail to develop normally.
2. A balancer chromosome can be identified in heterozygous conditions due to presence of one or more dominant mutant allele/s on it. In addition, they also carry one or more

recessive mutations for different morphological features of the fly.

3. Balancer chromosomes also carry recessive lethal or sterility inducing mutations. Consequently, progeny homozygous for the balancer chromosome do not survive as adults or remain sterile and thus, do not contribute to the next generation.

A general convention for naming the balancer chromosomes is '*nMx, a*' where *n* indicates the chromosome and can be F (for first), S (for second) or T (for third chromosome), *M* stands for multiply-marked chromosome, *x* is a digit only or a digit and an alphabet indicating the specific name of the chromosome while '*a*' after the comma separator stands for the list of dominant and recessive markers present on the given balancer chromosome.

Some common balancers:

Several different balancer chromosomes are widely available. The user should select the most appropriate for a given set of experiments. Information about a few common balancer chromosomes is given in the **Table 5** and **Fig. 11**. More details about various balancer chromosomes used in *Drosophila melanogaster* can be seen at Flybase and the Red book (Lindsley and Zimm, 1992).

Table 5: Some common balancers and associated markers

Chromosome	Balancer Name	Genotype	Dominant marker/s	Recessive marker/s
Chromosome 1	<i>ClB</i> : Homozygous or hemizygous lethal.	$B^1 In(1)C^1 sc^1 sl^1 t^2 v^1$	<i>B</i> (<i>Bar eye</i>)	<i>sc</i> (<i>scute</i>) <i>sl</i> (<i>small wing</i>) <i>t</i> (<i>tan</i>) <i>v</i> (<i>vermillion</i>)
Chromosome 1	<i>FM3</i> (<i>First Multiple 3</i>).	$In(1)FM3(=In(1)sc^8 + dl-49), B^1 Myc^{dm-1} sc^8 y^{31d}$	<i>B</i> (<i>Bar eye</i>)	<i>y</i> <i>sc</i> <i>dm</i> (<i>diminutive</i> , <i>dMyc</i>)
Chromosome 1	<i>FM7c</i> (<i>First Multiple 7c</i>): Homozygous females sterile.	$In(1)FM7, y^{31d} sc w^a sn^{X2} vOf g^4 B$	<i>B</i> (<i>Bar eye</i>)	<i>y</i> <i>sc</i> <i>w^a</i> <i>sn</i> <i>v</i> <i>g</i> (<i>garnet eye</i>)
Chromosome 2	<i>CyO</i> (<i>Curly of Oster</i>): Homozygous lethal.	$In(2LR)O, Duox^{Cy} dp^{IV1} pr^1 cn^2$	<i>Cy</i> (<i>Curly wings</i>)	dp^{IV1} (<i>dpy</i> , <i>dumpy wings</i>) recessive lethal allele <i>pr</i> (<i>purple eye</i>) <i>cn</i> (<i>cinnabar eye</i>)
Chromosome 2	<i>SM5</i> (<i>Second Multiple 5</i>): Homozygous lethal.	$In(2LR)SM5, al^2 ds^{55} Duox^{Cy} It^v cn^2 sp^2$	<i>Cy</i> (<i>Curly wings</i>)	<i>al</i> (<i>aristaless</i>) <i>ds</i> (<i>dachsous</i>) <i>cn</i> (<i>cinnabar</i>) <i>sp</i> (<i>speck</i>)

Chromosome 2	<i>SM6 (Second Multiple 6):</i> Homozygous lethal.	<i>In(2LR)SM6, al²Duox^{Cy}</i> <i>dp^{IV1} cn²p sp²</i>	<i>Cy (Curly wings)</i>	<i>al (aristaless)</i> <i>dy (dpy, dumpy wings)</i> <i>cn (cinnabar)</i> <i>sp (speck)</i>
Chromosome 3	<i>TM3, Ser (Third multiple 3):</i> Homozygous lethal	<i>In(3LR)</i> <i>TM3, Ser¹Ubx^{bx-34e}</i> <i>e¹kni^{ri-1} AdamTS-</i> <i>A^{89Aa-1} p^pvvl^{sep}</i>	<i>Ser (Serrate wings)</i>	<i>Ubx^{bx-34e}</i> <i>e (ebony)</i> <i>kni (knirps),</i> <i>recessive lethal</i> <i>AdamTS- A</i> <i>(l(3)89Aa)</i> <i>p (pink eye)</i> <i>vvl (ventral veins lacking)</i> <i>As in TM3 above</i>
Chromosome 3	<i>TM3, Sb</i>	<i>In(3LR)TM3, Sb¹</i> <i>Ubx^{bx-34e}e¹ kni^{ri-1}</i> <i>AdamTS-A^{89Aa-1}p^P</i> <i>vvl^{sep}</i>	<i>Sb (Stubble bristles)</i>	
Chromosome 3	<i>TM6 (Third Multiple 6):</i> Homozygous lethal	<i>In(3LR)TM6, Hn^p ss^{p88}</i> <i>Ubx^{p15} e¹</i>	<i>Hn (Henna)</i> (dark dull brown eye color)	<i>ss (spineless)</i> <i>Ubx^{p15}</i> <i>e (ebony)</i>
Chromosome 3	<i>TM6B (Third Multiple 6B):</i> Homozygous lethal	<i>In(3LR)TM6B, Antp^{Hu}</i> <i>e¹ Tb¹</i>	<i>Antp^{Hu}</i> (Humeral) <i>Tb (Tubby)</i>	<i>e (ebony)</i>

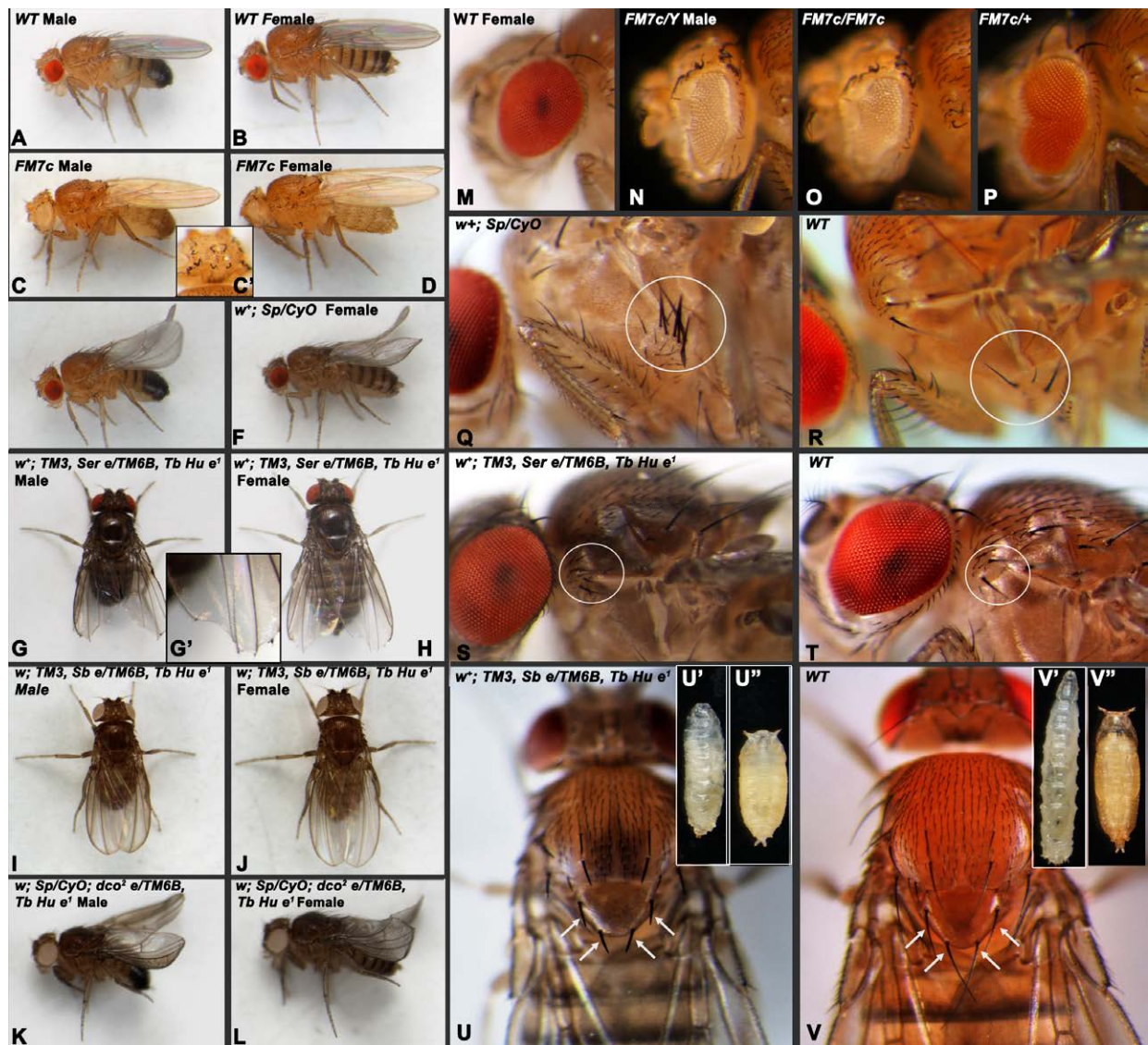


Fig. 11. Phenotypes associated with some balancer chromosome carrying stocks. A-B. Wild type (*WT*) male and female flies. C-L. Male and female flies of different genotypes (noted on top in each panel); C' shows the singed bristles at a higher magnification in thorax of the fly in C; G' shows the serrate wing phenotype. M-P. Head regions of *WT* (M) and *FM7c* males and females showing white apricot eye color, singed bristles and the Bar eye phenotypes. Q-R. Thorax of *Sp/CyO* (Q) and *WT* (R) to show the Sternopleural bristles (white circle). S-T. Thorax of *TM3, Ser e/TM6B, Tb Hu e¹* (S) and *WT* (T) flies to show Humeral bristles (white circle) and ebony body colour in S. U-V. Dorsal views of parts of *TM3, Sb e/TM6B, Tb Hu e¹* (U) and *WT* (V) flies to show Stubble bristles and ebony body colour (U). U' and V' are *TM3, Ser e/TM6B, Tb Hu e¹* (U') and *WT* (V') larvae showing tubby phenotype due to the *Tb* dominant allele (U'). U'' and V'' are *TM3, Ser e/TM6B, Tb Hu e¹* (U'') and *WT* (V'') pupae showing tubby body and lighter pupal case due to homozygosity of *ebony* allele (U'').

Points to remember while selecting and using balancers:

It is critical to understand the properties, advantages and limitations of balancer chromosomes to ensure their optimal use (Kaufman, 2017; Miller *et al.*, 2016). A few major points are noted below.

- i. A balancer chromosome may not completely suppress recombination across the entire

length of the homologs. Location of the gene, which is to be balanced, relative to the inversion breakpoints in the balancer should be considered (Kaufman, 2017). A breakpoint in the balancer should be close to the mutation. A mutation present in the centre of a large inverted segment may recombine because of double crossovers within the segment.

ii. Familiarity with the dominant marker/s present on a given balancer chromosome is essential to identify the progeny carrying that balancer chromosome. It is important to note that each balancer chromosome carries, besides the multiple inversions and the dominant marker mutation/s, several known recessive mutations (see examples in **Table 5**) and other as yet unknown gene disruptions (Miller *et al.*, 2016). Therefore, familiarization with the other mutations present on the given balancer chromosome is necessary, especially when genetic interactions between different genes are being examined. The many genetic lesions in the balancer chromosome may introduce unknown modifying effects.

iii. Stocks carrying multiple balancers have reduced fecundity due to early death of embryos that carry any of the balancers in homozygous condition. Stocks that carry balancers for more than one chromosome are useful in many genetic studies where mutants on different chromosomes need to be brought together. However, such doubly or triply balanced stocks need great care because of their significantly reduced fecundity and viability.

iv. Presence of multiple balancers creates additional difficulties in maintaining stocks. When meiotic recombination is inhibited in one region, the frequency of recombination in other regions increases due to “inter-chromosomal effect”. Thus, the stock may breakdown, especially when recombination is increased near the distal breakpoints of the balancer and within the inverted segments.

Concluding Remarks: The increasingly rich repertoire of classical mutants, chromosome rearrangements and the continuously expanding diversity of transgenes have helped *Drosophila melanogaster* to remain a prime model organism for a large diversity of studies, not only in Genetics but also in other domains of biological and bio-medical science. This is reflected in the various laboratory experiments described in the following chapters of this book. The present chapter about the basics of fly’s biology, and the processes and practices followed for this model is to prepare a potential user to take full of advantages of the innumerable features and privileges of this model. Further details of these can be seen in the resources and references listed below.

References and Further Reading:

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Miller, D. E., Cook, K. R., Yeganeh Kazemi, N., *et al.* (2016) Rare recombination events generate sequence diversity among balancer chromosomes in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA. 113: E1352–E1361. <https://doi.org/10.1073/pnas.1601232113>.

Web resources:

Bloomington *Drosophila* Stock Centre (BDSC): <https://bdsc.indiana.edu/>

Flybase: <https://flybase.org>

Flybook: <https://www.genetics.org/content/flybook>

The Interactive fly: <https://www.sdbonline.org/sites/fly/aimain/1aahome.htm>

Appendix I.

Food for flies

Historically, *Drosophila* cultures in lab have been maintained on bananas. However, since this kind of food becomes very soft and difficult to handle, agar-based culture media with maize powder or wheat cream etc, have become common. Some of the recipes used for preparation of fly food in different laboratories in India are given below.

A. Culture media for maintenance of flies in laboratory

i. Agar-Maize powder-sugar food (Cytogenetics Lab, BHU)

Ingredients:

Agar powder (bacteriological grade)	3 gm
Maize powder	17 gm
Sugar	15 gm
Yeast (dried)	6 gm
Nipagin (Methyl 4-hydroxybenzoate)	1 gm (dissolve in 1-2 mL of 90% ethanol)
Propionic acid	1 mL
Distilled water	360 mL

Mix the agar powder in 100 mL of warm water and then let it boil on heater till agar is completely dissolved. Separately, prepare a paste of maize powder and sugar in 150 mL water and add to the boiling agar solution with continuous stirring for thorough mixing. While it is boiling, prepare a paste of yeast powder in the remaining 110 mL water and add to the food being cooked. Bring it to boil again with regular stirring. Turned off the heat when adequately cooked, add Nipagin, dissolved in ethanol, and propionic acid and mixed well. Pour the cooked food in vials (~10 mL each) or bottles (~ 30-40 mL each) following which cover them with clean fine muslin cloth to prevent entry of unwanted flies. Allow the food to solidify as it cools. After 4-5 hr, wipe dry any moisture deposited on side walls of the bottles with cotton and plug with non-absorbent cotton.

ii. Corn meal-Agar medium (Dr. Javed Ahsan, Central Univ South Bihar)

Ingredients:

Maize flour	90.0 gm
Dextrose	50.0 gm
Sucrose	30.0 gm
Agar	10.0 gm
Yeast tablet powder	10.0 gm
Propionic acid	5 mL
Ortho phosphoric acid	0.6 mL
Benzoic acid (10 %)	0.7 mL
Distilled water	1 L.

Cook as in (i) above.

iii. Wheat Cream-Jaggery Medium (Dr. N.B. Ramachandra, Mysore University)

Ingredients:

Wheat cream	100 gm
White jaggery	100 gm
Agar-agar	10 gm
Propionic acid	7.5 mL
Dist. Water	1 L

Mix the ingredients and boil in 1 L water till a creamy consistency is reached. Pour into

sterilized bottle/vials and plug when cooled and solidified. Sprinkle a few granules of Baker's yeast over the medium before plugging with sterile cotton.

iv. Corn flour-Jaggery Medium (Dr. N.B. Ramachandra, Mysore University)

Ingredients:

Corn flour	80 gm
Jaggery	70 gm
Agar	9 gm
Yeast powder	15 gm
Propionic acid	4.4 mL
Methyl parahydroxybenzoate	1.25 gm

Weigh corn flour, jaggery, agar and yeast powder separately and keep them aside. Take the required quantity of water in a suitable container. Warm the water to ~ 35°C and add corn flour. Mix thoroughly to remove any clumps. Add other ingredients (Jaggery, agar and yeast powder) and mix again. Close the container lid and keep on high heat for 5 min. Once cooked, turn the heat off and wait to cool to 50-55°C (may be checked with a thermometer). Add Methyl parahydroxy benzoate and propionic acid. Mix thoroughly and pour into vials/bottles. When fully cooled and solidified, plug with sterile cotton or other plug.

v. Banana- Barley-jaggery medium (Dr. Sheeba Vasu, JNCASR, Bangalore):

Ingredients for about 100 food vials:

Banana	205 gm
Barley	25 gm
Ethanol (100%)	22 mL
Jaggery	35 gm
Agar (bacteriological grade)	12.4 gm
Yeast	36 gm
Sodium Benzoate	2.4 gm dissolved in 23 mL Ethanol
Water	1 L + 180 mL

Remove skins of bananas and mash the pulp in 500 mL of water in a mixie. Mix the barley and ethanol to the banana mash. Avoid clumps. In a separate cooking vessel, add jaggery to 500 mL of water and stir on low heat until completely dissolved. Slowly add Agar powder to the jaggery solution with stirring to avoid clumping. Set vessel on high heat and wait for the Jaggery-Agar mix to boil. While it is boiling, slowly add yeast to the banana-barley pulp in a mixer ensuring that no clumps are formed. Since addition of yeast may cause release of some CO₂, whisk or grind in the mixer again to release the trapped gas. If the jaggery-agar mix is boiling for too long while yeast is being dissolved, reduce the heat. Add the banana-barley-yeast-ethanol mix, when well ground up into a slurry to the boiling jaggery-agar syrup with stirring. Add 180 mL of water (to compensate for the loss that occurs during boiling). Let the combined media be on high heat for 5-7 min. with infrequent stirring, till it starts to boil again. Remove from flame and let it cool to below 65°C. Add Benzoic Acid mix which serves as an anti-fungal agent. Stir well and pour into glass vials (~10 mL each) quickly before the food solidifies. Take care to pour directly into the vial and not on the sides. The media should be brown-greyish in colour, though colour varies based on the kind of jaggery/agar used.

Vials with solidified food may be plugged for immediate use or may be stored in double-layered plastic bags for storage and later retrieval. Care must be taken to ensure that no stray flies are trapped in the bags as females will contaminate fresh vials by laying eggs in them.

Note: Seasonal variations in the ambient relative humidity and temperature, may need some adjustment in the amount of water used during cooking so that the final food medium is neither too hard/dry nor too soft. Longer storage may also lead to food becoming drier. If the food in vials/bottle becomes too dry, judicious addition of a small amount of distilled water or dilute yeast solution in water can make them usable. Sprinkling of a few dry yeast granules or application of a dab of thick yeast paste in centre of the food surface in prepared bottles/vials prior to use helps in better growth of the culture.

B. Media for egg collection:

Several experiments require collection of relatively large number of eggs in a limited time. Typically, eggs are collected in Petri dishes, because of the ease with which they can be harvested from the food surface (**Fig. 6**). The food medium containing dishes are conjoined with an egg collection chamber containing the desired flies (see **Chapters 32, 34, 70**). Eggs can be collected on Petri dishes containing any of the regular food medium described above. More commonly, however, simplified food media are prepared for this purpose, some of which are given below.

i. Agar-Sugar medium (Uendra Nongthomba, IISc, Bangalore):

Ingredients for ~10 numbers of 100 mm diameter Petri dishes:

Agar powder (bacteriological grade)	15 gm
Sugar	5 gm
Propionic acid	1 mL
Dist. Water	360 mL

Add the Agar powder and sugar to 360 mL water in a suitable vessel (e. g., a 500 mL Erlenmeyer flask. Cover the mouth of flask with aluminium foil (if using heater to boil) or plastic wrap (if using a microwave oven) and start heating with periodic vigorous swirling to mix the solid components. Continue boiling to make sure the agar dissolves completely. Switch off heating and let the flask cool to about 50 - 60°C. Add Propionic acid at this point and mix well without generating bubbles. Pour about 30 mL of the liquid medium in clean 100 mm (diameter) plastic Petri dishes and cover with its cap. Leave the plates to dry and cool. They may be used immediately or may be stored in a refrigerator for later use.

ii. Agar-Sucrose medium (Anuradha Ratnaparakhi, Agharkar Research Inst., Pune)
Prepared as above but with 0.8% Agar and 1% Sucrose.

iii. Semolina-Jaggery medium (Dr. Girish Chandran, SBRR Mahajana First Grade College, Mysuru)

Boil 10 gm jaggery in 80 mL dist. water in a 250 mL glass beaker. Add 10 mg Semolina and 1 gm Agar powder. Stir well and boil for ~10 min. Reduce the heat and add 0.75 mL Propionic acid. Stir well and add 20 mL distilled water followed by heating till it starts boiling. Put off the heat and immediately pour required amount of the medium in Petri dishes/vials as needed and immediately cover/plug.

Note: Addition of a small amount of charcoal powder to the egg collection medium during cooking makes the medium dark coloured which facilitates visibility of the white eggs. Keeping flies on any of these simplified egg collection media for a long time (> 6-8 hr.) should be avoided since this may subject flies to a starvation stress due to absence of the required nutrients in the medium. Eventhough 2-4 hr. starvation of otherwise well-fed flies can stimulate oviposition.

Appendix II.

Putting flies to sleep (immobilization or anaesthetization):

Typically the flies are put to sleep by briefly exposing them to anaesthetic di-ethyl ether (etherization) or through continued exposure to CO₂ or cold. Details of the processes are described below:

A. Immobilization of flies using anaesthetic ether

i. In order to examine and count flies, they are immobilized with a light dose of ether in an etherizer (**Fig. 9**). The etherizer is prepared from a small wide mouth transparent or semi-transparent glass or plastic bottle or tube with a small funnel placed over its wide mouth. A thin strip of cotton is securely wrapped around the funnel tube with thread or rubber bung. The cotton strip is wetted with a few drops of anaesthetic ether using a dispensing bottle and the funnel is quickly replaced over the mouth of the tube/bottle. The bottle or vial from which the flies are to be taken and anaesthetized, is tapped a few time on a sponge pad so that the flies get collected near the food surface. The plug is quickly removed and the bottle/vial immediately inverted over the funnel of the etherizer. The etherizer with the inverted bottle/vial in place are together tapped a few time on the sponge pad to force the flies fall through the funnel tube into the etherizer chamber. When done, the bottle/vial is quickly returned to normal upright position and re-plugged.

ii. In a few seconds, the flies stop movements in the etherizer (**Fig. II.1A**). At this time, the funnel is removed and using a fine soft dry brush the immobilized/anaesthetized flies are transferred on the observation plate placed under the stereo-binocular microscope.

iii. If the flies begin to revive before observation is complete, they can be re-etherized. The re-etherizer consists of a small transparent Petri-dish cap with a strip of blotting paper/cotton fastened to the inside with a tape (**Fig. 9**). A few drops of ether are placed on the paper/cotton and the re-etherizer inverted over the flies on the plate so that ether vapours would re-etherize and immobilize the flies on the observation plate. Examine the flies through the transparent Petri dish base and remove the re-etherizer, as soon as flies become immobile, If required during a prolonger examination of flies, this process may be repeated a few times.

iv. Excessively long exposure to ether would kill the flies which can be identified by their wings being extended at right angles to the body and folded legs (**Fig. II.1B**)

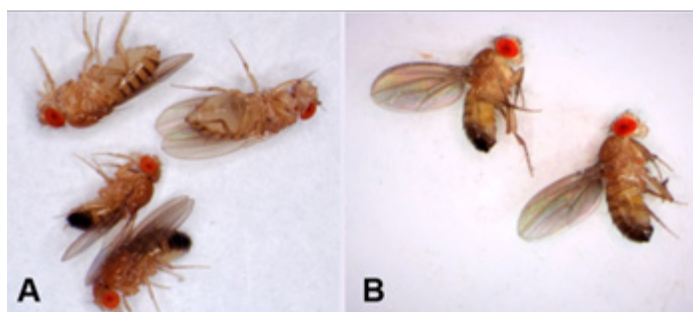


Fig. II. 1. A. Optimally etherized flies, and **B.** Over-etherized flies with stretched wings and folded legs.

v. After observation, the immobile are transferred to a fresh food vial or bottle and the same is kept horizontal till the flies ‘wake’ and begin to move. This ensures that the immobile flies (especially their delicate wings) do not get stuck on soft food. If the examined flies are no longer needed, they are discarded into a ‘morgue’ (a beaker or

flask containing non-evaporating oil or soap water; **Fig. 9**). The morgue needs periodic cleaning.

Note: Exposure of lab personnel to excessive ether vapours must be avoided.

B. Use of CO₂ for anesthesia (contributed by Sheeba Vasu, JNCASR, Bangalore):

Carbon-dioxide is also used to put flies to sleep. In this case, the flies remain paralyzed as long as they are exposed to controlled levels of CO₂, with only a minor risk of dying. The system primarily requires a source of CO₂ (typically from a gas cylinder) which is either introduced into the culture bottle/vial through a flexible tubing carrying a broad gauge hypodermic needle or to a flat porous pad on which the flies are placed for instant but temporary paralysis.

i. *Commercial CO₂ pad:* The commercially available anaesthetizing CO₂ pad has a surface made of porous polyethylene and a flow maze tubing underneath it for optimal and consistent diffusion of CO₂. The CO₂ reaches the fly pad via an input flexible tubing (**Fig. II.2**) connected to a CO₂ source (e.g., a cylinder or a CO₂ supply line) and its flow can be controlled via a foot-switch. For anaesthetizing flies, the CO₂ flow is turned on and the flies transferred from the bottle/vial directly onto the CO₂ pad, where they get instantly paralyzed and remain so till the carbon-dioxide supply remains on.

In addition to the CO₂ pad, availability of a CO₂ blowgun is useful for pre-anaesthetizing flies while they are still in the bottle or vial (**Fig. II.2**). The steel needle tip can be inserted into the vial or bottle by pushing the needle between the cotton or sponge plug and sides of the vial/bottle, while flow of CO₂ from the source is controlled by pressing the trigger of the **CO₂ blowgun**. This helps in controlling dosage and conserving CO₂ in comparison with a continuous flow system.

ii. *Do-it yourself fabricated CO₂ pad:* A working CO₂ pad can also be fabricated with easily available components (**Fig. II.3**). The essential parts of this are i) a CO₂ cylinder with a regulator, ii) base of a 95-100 mm Petri dish with a hole bored in its side wall and filled with layers of perfectly fitting Whatman filter papers (circular), iii) flexible tubing, iv) a T-junction, v) a regulator or tap to control the direction of flow of the gas, vi) a hypodermic needle (see **Fig. II.3**). The attachment of a needle with the help of T-junction can be used to direct flow of CO₂ into vials or bottles before transferring them on the CO₂ pad. The gas is supplied to the CO₂ pad via the tubing connected to the T-junction with the rate of flow of CO₂ maintained at the desired level via the regulator close to the Petri dish. The Petri dish is layered with 3-4 disks of Whatman filter paper (the pore size of the filter paper is not critical, but they must easily allow gas to diffuse through); the filter paper pad must be securely taped to the mouth of the dish using strong tape. Optionally, one can place a humidifier, consisting of a flat-bottomed flask with a 2-bore plug fitted with 2 tubes, in-line with the gas cylinder. The humidifier is useful in arid environments for reducing static charges on the fly pad. The CO₂ is bubbled through the tap water so that humidified gas passes out through the other tube to the plate or the needle. **Note:** Tubing, valves, T-junction, tap etc. can be purchased from aquaria or pet shops. CO₂ gas can be purchased from vendors who supply compressed gas cylinders to chemistry labs or hospitals.

Caution: Using CO₂ anaesthesia for studies involving behaviour analyses needs caution since exposure of flies to CO₂ can affect behaviour. Therefore, one must wait for about a day between the CO₂ exposure and collecting behavioural data. Prolonged exposure to CO₂ can also cause sterility in male flies. A limited exposure to CO₂ is not

known to be harmful to humans, but prolonged exposure may induce drowsiness in some people. Therefore, sufficient ventilation in laboratory is essential.



Fig. II. 2. Commercial CO₂ Fly pad placed at the base of a stereobinocular microscope enables viewing the immobilized flies.

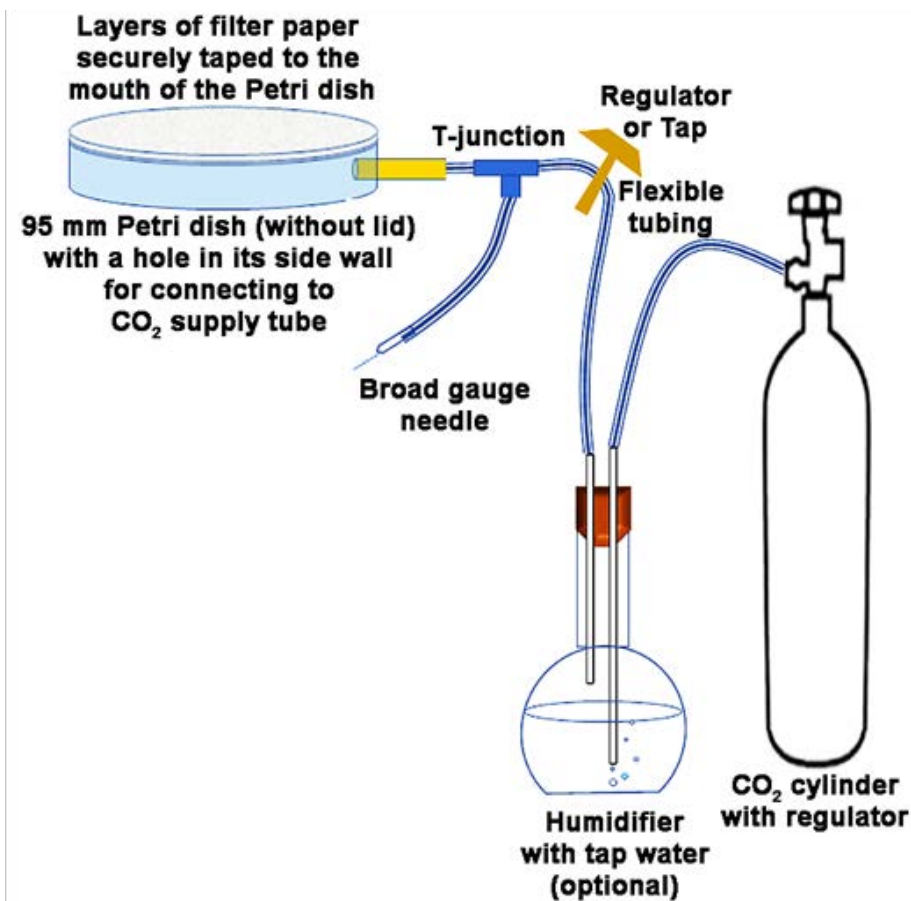


Fig. II. 3. Design of a do-it-yourself CO₂ pad.

C. Immobilizing flies and larvae by cold (contributed by Priya Dutta and Krishanu Ray, TIFR, Mumbai):

A brief exposure to cold temperature is used to reversibly immobilize flies and larvae (see [Chapter 69](#)). Briefly, it involves the following steps ([Fig. II.4](#)):

- i. Take a bottle ([Fig. II i](#)) and a collector tube ([Fig. II. B](#)), which consists of a 15 mL transparent plastic tube with attached funnel/adaptor ([Fig. II ii](#)).

- ii. Tap flies from the bottle/vial into the collector (Fig. II iii). Once the flies are transferred to the collector tube, remove the bottle/vial and replace its plug (Fig. II iv).
- iii. Stick the collector tube with flies into ice flakes kept in an ice bucket and keep tapping the tube periodically to dislodge climbing flies to the bottom (Fig. II vi) until all flies are collected at the bottom of the plastic tube.
- iv. As soon as the flies are immobilized, transfer them on an aluminum plate kept on ice (Fig. II C) by tapping the tube (Fig. II vii, viii), and sort them under a stereo binocular microscope using a fine hair brush (Fig. II ix).

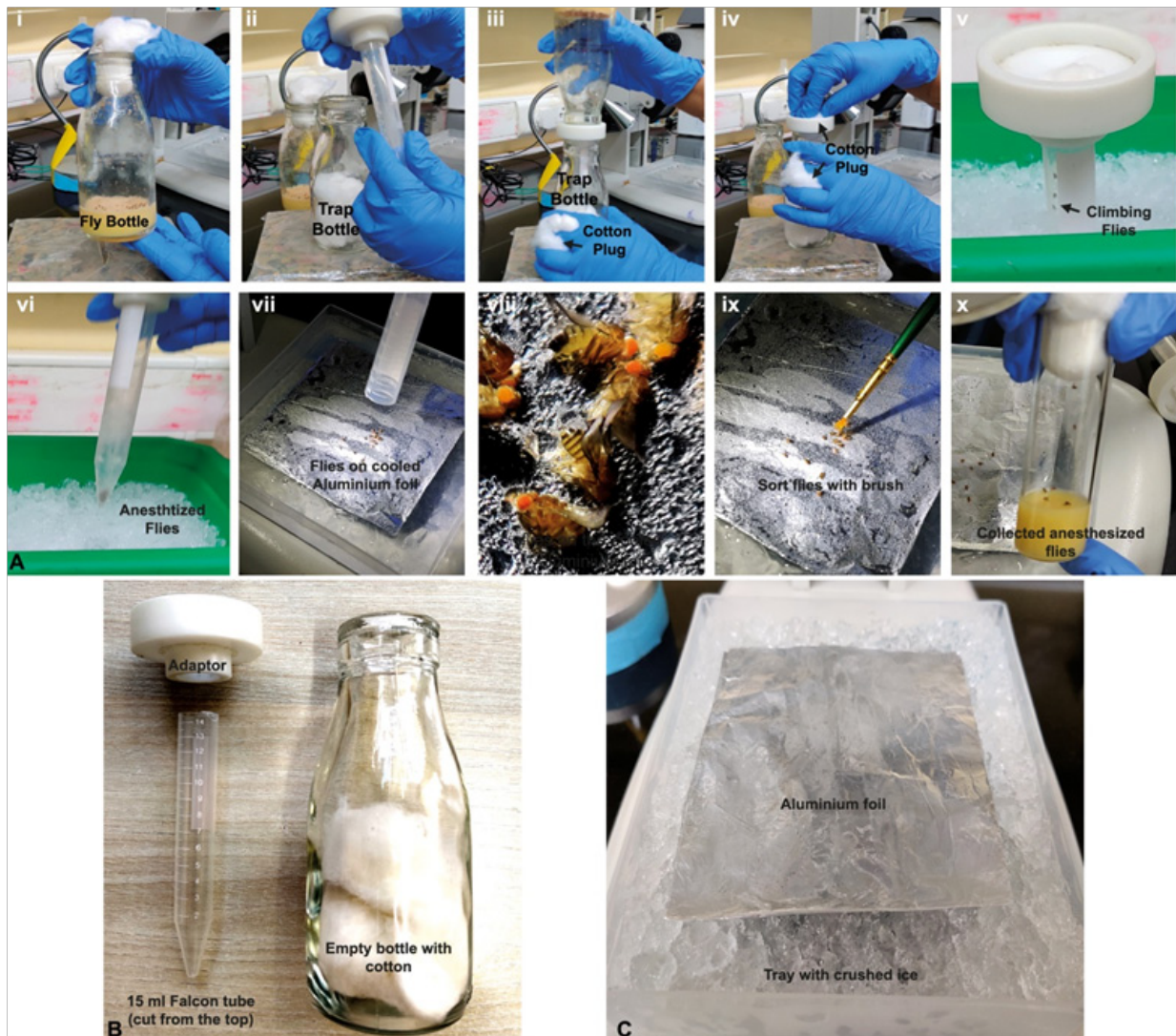


Fig. II. 4. Immobilizing flies on ice. A. A pictorial summary of the procedure (steps i – ix described in text B. Fly collector setup. C. The cooling setup to maintain cooled aluminum sheet for sorting the flies.

Error or Misconceptions in Science

One of the most important, but generally neglected, aspects of teaching about the process of science is the role of error, or misconceptions in science—that is, the portrayal of the ways scientists, especially well-known scientists, were often wrong even by the standards of their day, but for very good reasons. Too frequently science is presented as a steady march toward truth, with the figures we honor being the ones who “got it right”, while those who opposed them are dismissed as is guided or biased, and thus marginalized. Even those scientists who got it right (eventually) along the way sometimes held “wrong” ideas that are conveniently ignored or when discussed, present embarrassments. In textbooks it is seldom noted that Isaac Newton wrote more on alchemy than on dynamics, that the sixteenth-century astronomer Johannes Kepler was motivated as much by a belief in Pythagorean numerology as by empirical data on the positions of Mars, or that Darwin believed in forms of the inheritance of acquired characters. Yet, in teaching science as a process there is much to be said for examining how and why particular scientists came to hold what later came to be considered wrong ideas. Quite often these ideas engendered controversies that if presented to students can be useful in clarifying what are the truly critical issues involved with a particular concept or the different takes on the proper methodology involved in a particular research program.

(In: Garland, E. A. 2013. How Many Times Can You Be Wrong and Still Be Right? T. H. Morgan, Evolution, Chromosomes and the Origins of Modern Genetics. Science & Education.
https://nosyevolucion.files.wordpress.com/2015/09/allen_morgan-and-hos.pdf)

Chapter 2

Illustrated anatomy of *Drosophila melanogaster*

Homica Arya and Deepti Trivedi* (dtrivedi@ncbs.res.in)
National Centre for Biological Sciences, Bangalore, India.

* For correspondence

Drosophila melanogaster is extensively used for a very wide variety of studies in Biology. A good understanding of development and anatomy of this model is necessary to make the best of this versatile experimental model. This chapter presents some aspects of anatomy of *Drosophila melanogaster* through **hand-drawings** to help students visualize how *Drosophila* develops and how different organs and organ systems are organized within the fly's body. For more about development and anatomy of *Drosophila*, refer to the Atlas of *Drosophila* Development (<https://www.sdbonline.org/sites/fly/atlas/00atlas.htm>).

A. Life cycle and developmental stages of *Drosophila*

Drosophila melanogaster exists in four distinct forms during its lifetime, viz. embryo, larva, pupa and the adult fly (also see **Chapter 1**). A healthy mated female *Drosophila* stores sperms. The peak oviposition period of healthy females is between days 4-7 after emergence. However, the actual numbers of eggs laid per day varies over a wide range, being dependent upon diverse environmental factors. An embryo goes through different developmental stages (as described below) and takes ~10 days at 25°C to develop into an adult fly (**Fig. 1**).

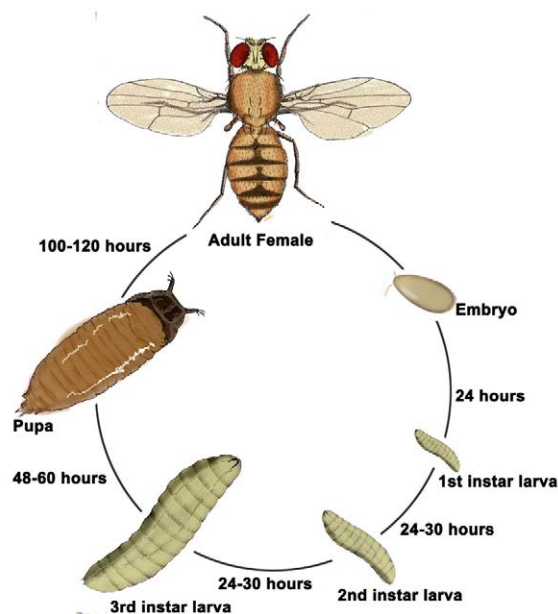


Fig. 1. Life cycle of *Drosophila melanogaster*.

i. Embryo

A fertilized egg (zygote) is oviposited by a female fly after internal fertilization. The zygote is white, ovoid, about 0.5 mm in length, and encased by a thin inner vitelline membrane and a tough outer chorion (**Fig. 2**). A pair of respiratory filaments (Dorsal appendages) extends from the chorion at the anterior end. These help in gas exchange and also prevent drowning of the embryo in the soft substratum where it is laid. After seventeen distinct embryonic developmental stages (**Fig. 3**) over the course of about 21-24 hr (25°C), the first instar larva hatches out of the egg case. The images show embryos as seen after the external chorion layer is removed (see **Chapters 32, 33**).

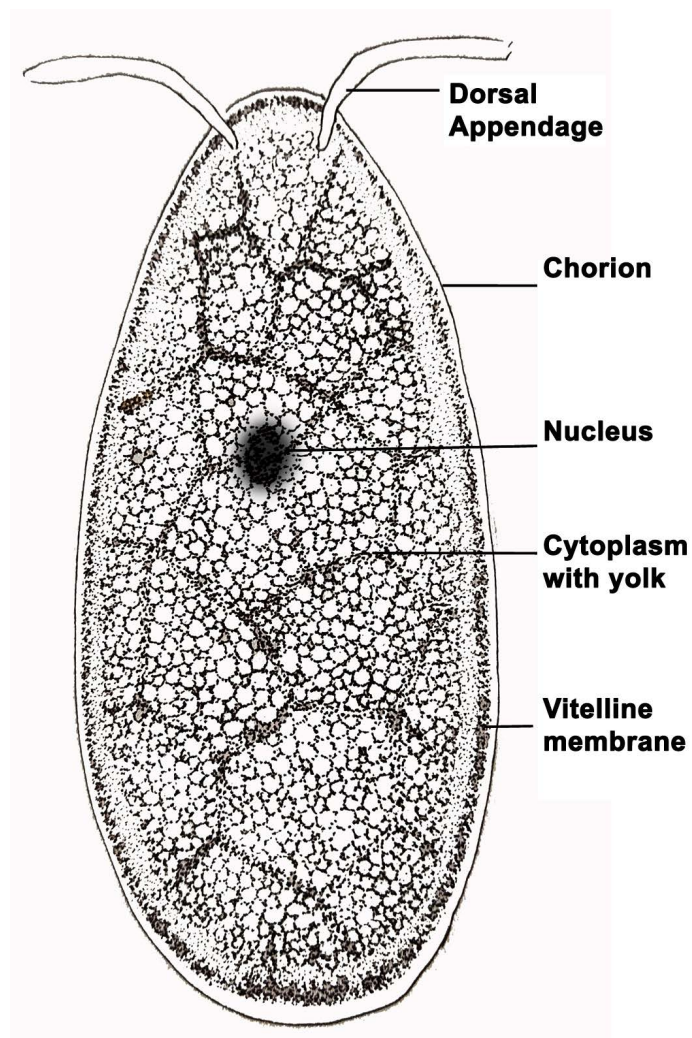


Fig. 2. Freshly laid *Drosophila* egg.

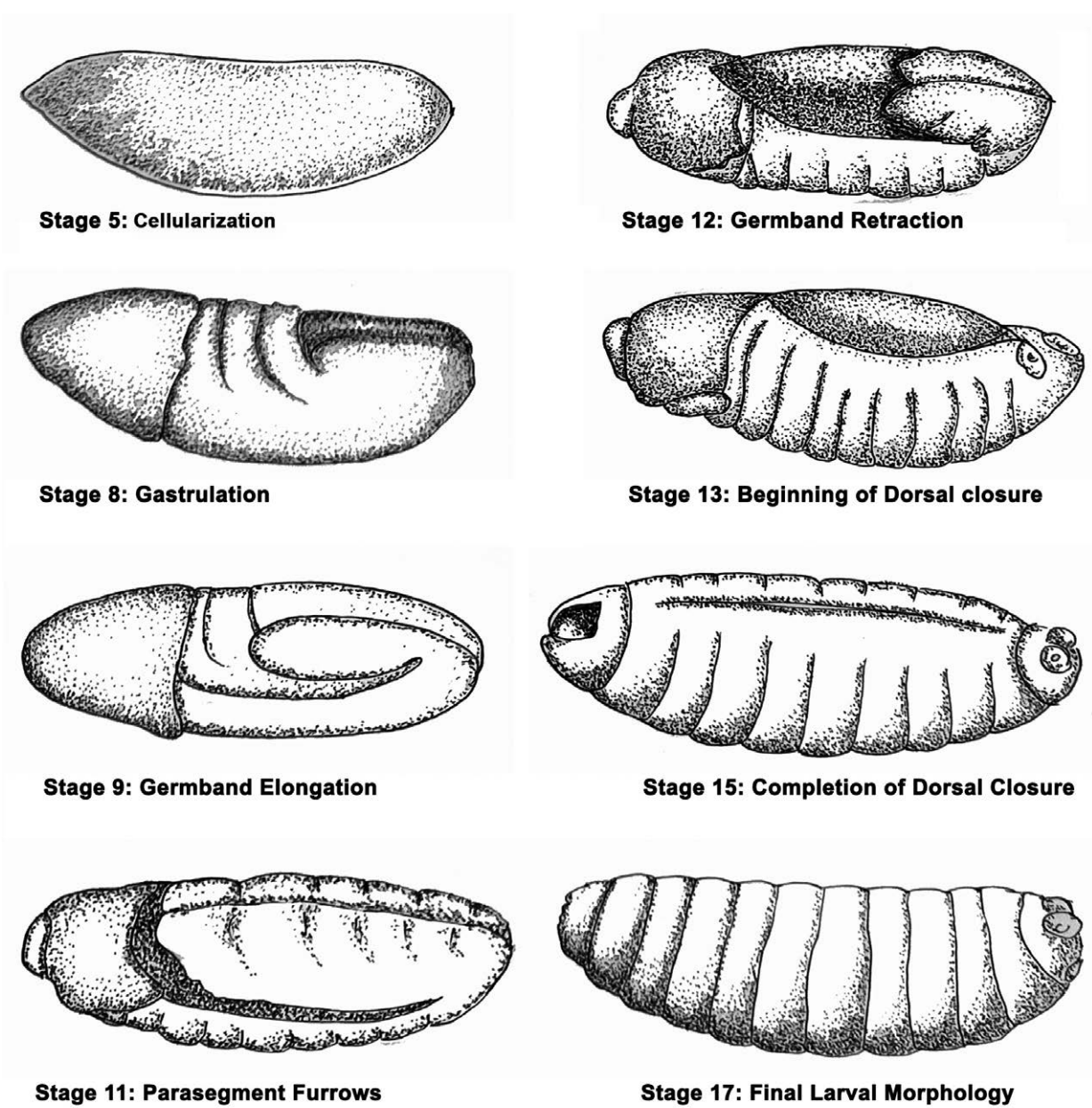


Fig. 3. Embryogenesis showing some developmental land-mark stages.

ii. Larva

Drosophila larva is a white-colored worm like burrower with a segmented body and black mouthparts or jaw hooks in the head region (**Fig. 4A**). A pair of respiratory spiracles at both ends enables tracheal breathing. The hatched larva is referred to as the first instar larva (**Fig. 1**). This moults twice by shedding the cuticle and mouthparts to produce the third instar larva (see **Chapters 1, 14**). The first and the second instar larvae exist for about 24 hr each (at 25°C) during which they feed constantly. The third instar larva, lasting around 48 hr, crawls out of the food and after active feeding and wandering for some time, moves to a relatively dry location for pupation. A remarkable increase in body mass of approximately 200-fold occurs during these juvenile larval stages (**Fig. 1**).

iii. Imaginal Discs

Epidermal organs of an adult fly develop from nineteen imaginal discs inclusive of a single genital disc that develops into all the genital structures except the gonads and nine pairs that develop into organs such as the eyes, legs and wings (**Fig. 4B**). The imaginal discs start out as epidermal thickenings in the embryo that later invaginate to become vesicles. In larvae, they dangle in the hemolymph from the tracheal tree and undergo growth but no differentiation. They appear as tiny transparent tear drop-shaped assemblies containing a tubular tissue that eventually develops into the adult structures. It is only during pupation that cellular elongation and differentiation takes place, finally resulting in organ formation. **Fig. 4C** depicts the eventual fate of each disc in the adult flies.

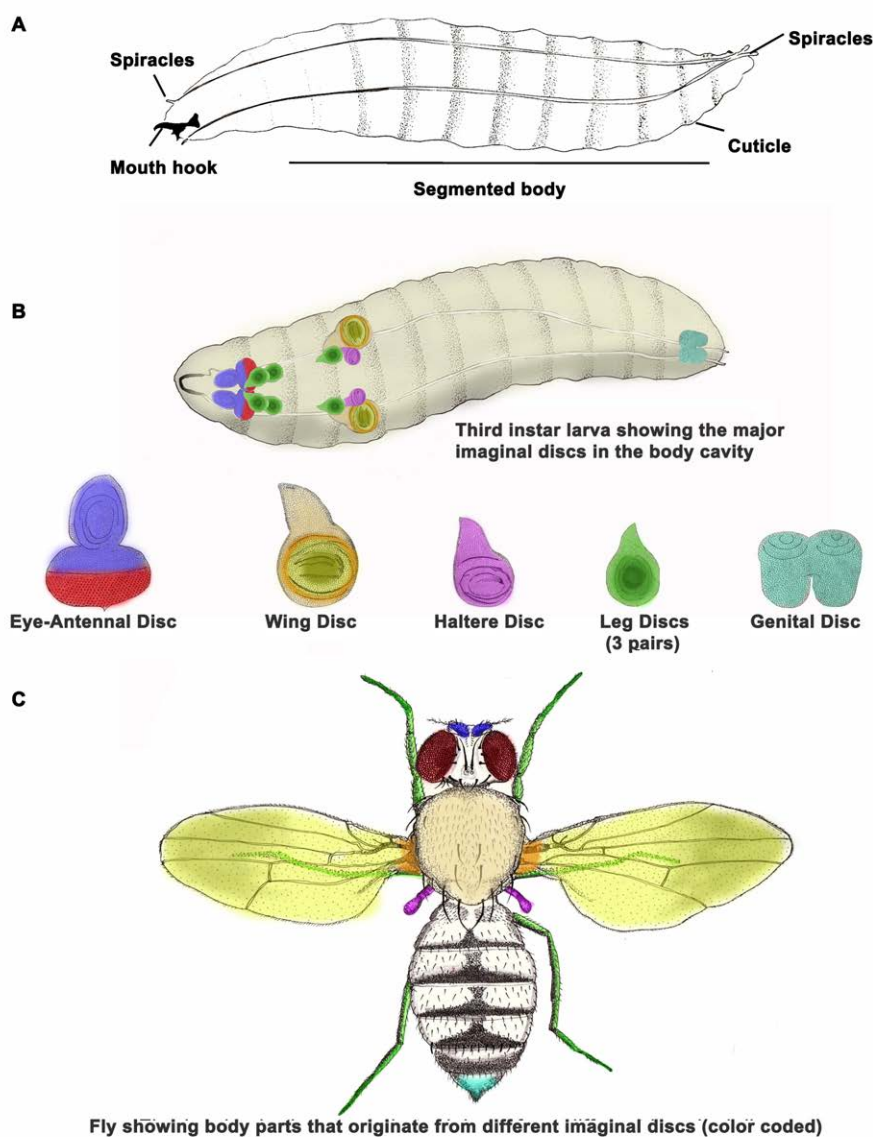


Fig. 4. Third instar larva and imaginal discs. **A.** Third instar *Drosophila* larva. **B.** Larval imaginal discs with their eventual fate in the adult fly shown in **C**. The smaller labial and clypeolabral pairs of discs that generate the adult mouth parts are not shown.

iv. Pupa

At the beginning of pupariation, the larval body shortens in length, the spiracles are everted, and hardening and sclerotization of the pupal cuticle begin. The metamorphosis of the larva into an adult occurs during this stage, with the breakdown of most larval tissues and formation of adult organs from the imaginal discs and other imaginal cells. The pupa gets progressively more pigmented on the outside. **Fig. 5A** depicts the external morphology of the pupa and **Fig. 5B** depicts various stages of pupal development. This stationary phase, lasting for about 4-5 days, eventually culminates in eclosion and emergence of the adult fly.

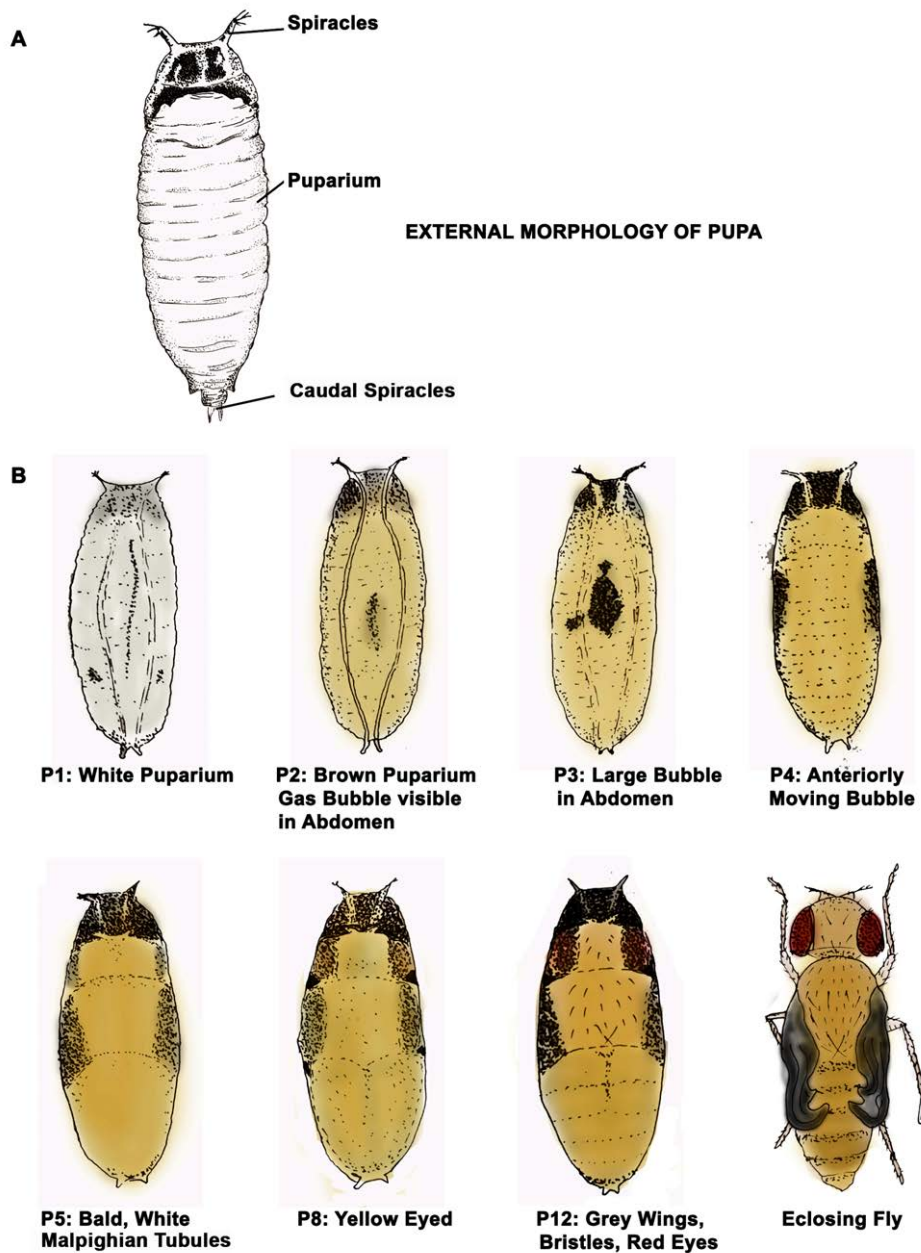


Fig. 5. Pupa and its development. A. External morphology of *Drosophila* pupa. **B.** Some stages of pupal development

v. Female and male adult flie

Drosophila displays sexual dimorphism, i.e., males and females appear distinct beyond the differences in their sex organs. Females (**Fig. 6A**) have a pointed abdomen whereas males (**Fig. 6B**) have a rounded abdomen. Males have greater and more uniform pigmentation of the abdominal tip. Females are typically larger than males, with a light coloured, pointed genitalia, which are more rounded in male (see **Chapter 45**). Males characteristically exhibit a small patch of dense bristles on the forelegs, called the sex comb, which aids in grasping the female during mating (see **Chapter 44**).

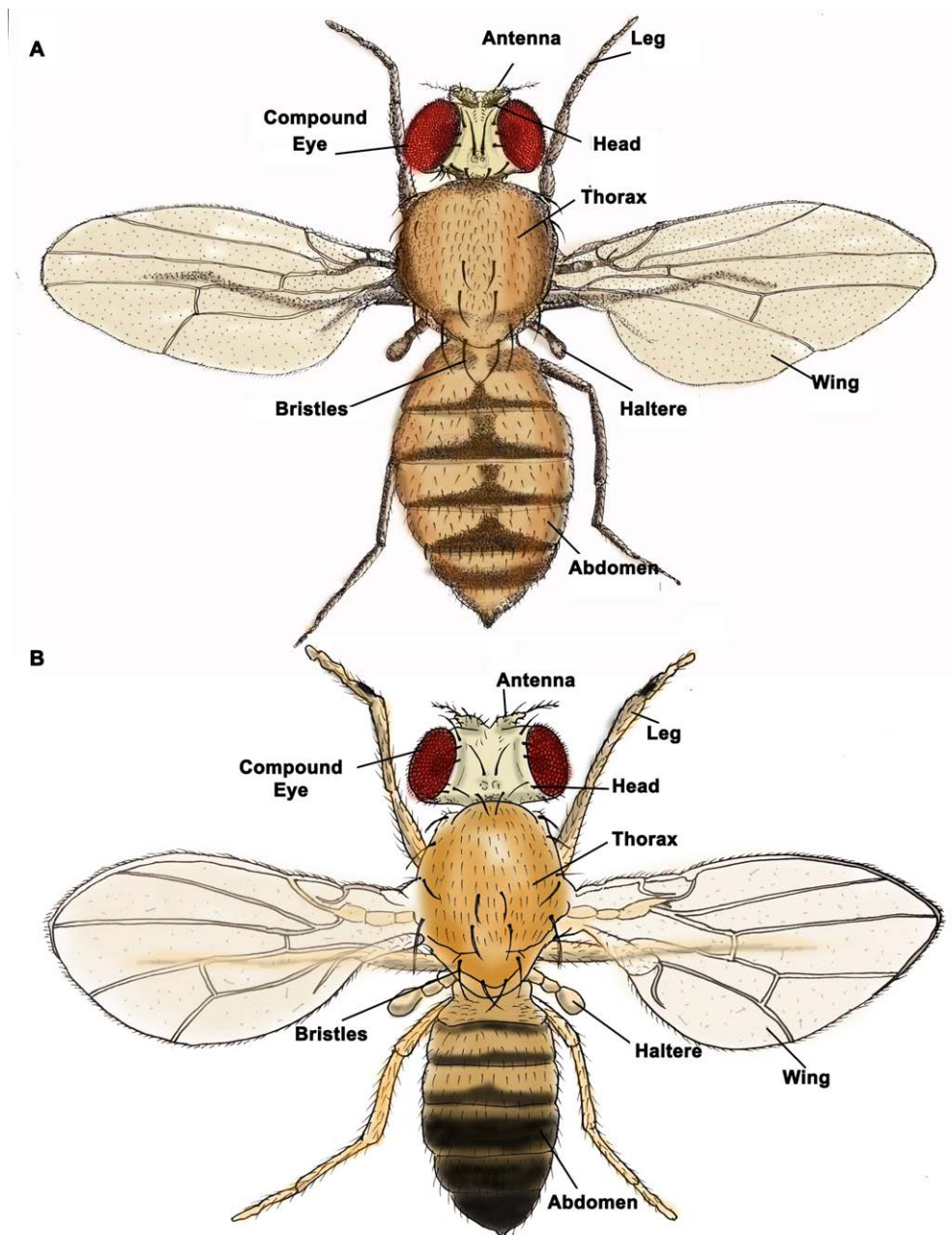


Fig. 6. Wild type adult female (A) and male (B) *Drosophila melanogaster*.

Further resources for life cycle, development and anatomy of *Drosophila*

***Drosophila* development and anatomy:** Atlas of *Drosophila* development (<https://www.sdbonline.org/sites/fly/atlas/00atlas.htm>).

BSCI 1511L Statistics Manual: Life Cycle and Anatomy of *Drosophila*; Vanderbilt University. <https://researchguides.library.vanderbilt.edu/c.php?g=156859&p=1161911>.

Chapter 8: Development of the Fruit Fly *Drosophila melanogaster*; University of Kentucky College of Arts & Sciences. <http://web.as.uky.edu/Biology/faculty/cooper/Population%20dynamics%20examples%20with%20fruit%20flies/08Drosophila.pdf>

Zar (2016) *Drosophila* embryo development; **YouTube:** <https://www.youtube.com/watch?v=B0ZlvCINozs>.

Yashaswi Sharma (2020) *Drosophila* Development Stages, Significance; Microbe Notes <https://microbenotes.com/Drosophila-development/>.

Stages of Development and Mitotic Domains; The Interactive Fly Society for Developmental Biology. <https://www.sdbonline.org/sites/fly/aimain/2stages.htm>.

Table of Pupal Stages; Dev Bio.net. <http://www.devbio.net/node/46>

Silvia Aldaz and Luis, M. Escudero (2010) Imaginal discs. *Current Biology*; 20(10): PR429431. [https://www.cell.com/current-biology/comments/S0960-9822\(10\)00291-5](https://www.cell.com/current-biology/comments/S0960-9822(10)00291-5).

The Fly Manual: A guide to working with *Drosophila*; Tauber Research Lab, University of Leicester. <https://www2.le.ac.uk/departments/genetics/research/staff-research-interests/tauber-research-lab/livegene/fly-manual>

B. Anatomy:

i. Nervous system

The central nervous system in a *Drosophila* embryo consists of neural stem cells, neurons and the glial cells (see **Chapter 34**), which together give rise to the two hemispheres and the sub-esophageal ganglion of the larval brain. Histologically, the central nervous system can be categorized into the neuronal cell cortex composed of the neuronal cell bodies and the neuropil, containing the axons and dendrites. The key structures of the *Drosophila* central nervous system in larva as well as adult (**Fig. 7**) are: (a) the mushroom body (see **Chapter 40**), associated with memory and learning, (b) the optical lobes, housing the neurons and neuropils concerned with vision, (c) the antennal lobes, consisting of neurons and neuropils of the olfactory chemosensory

pathway (see **Chapter 96**), and (d) the ventral nerve cord, which enables most motor behaviors of the fly.

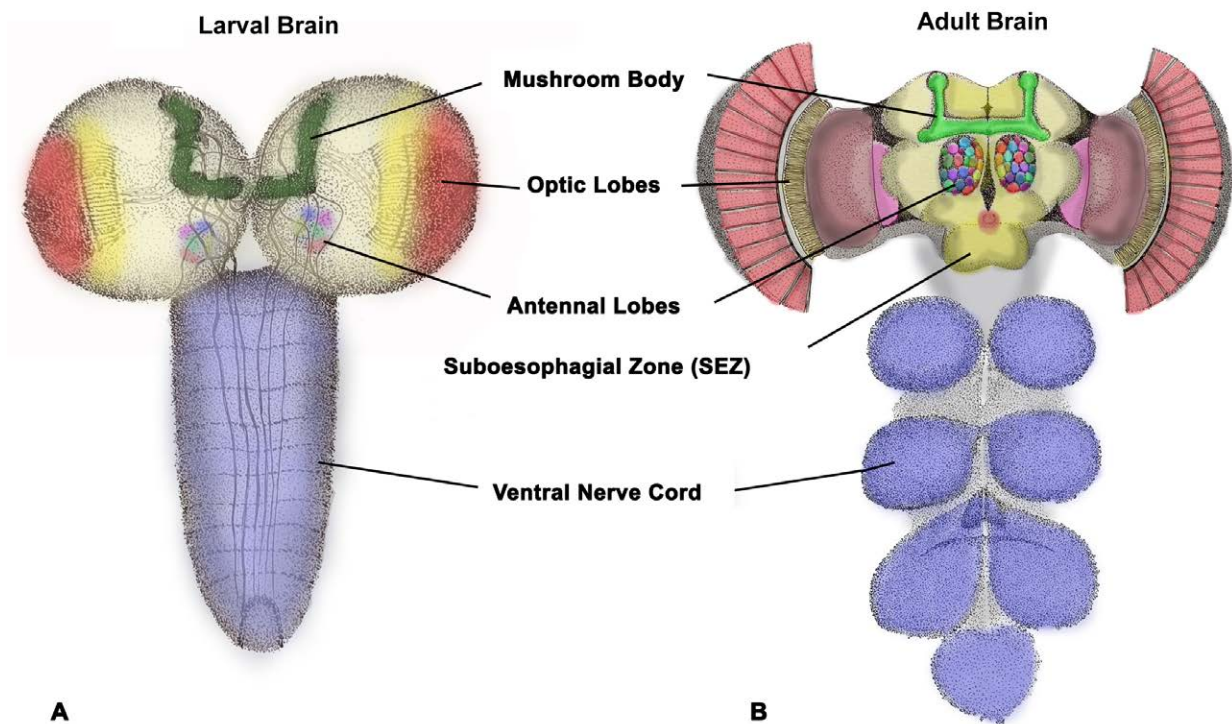


Fig. 7. Central nervous system of larva (A) and adult (B).

Further resources for nervous system:

Karlheinz Rein, Malte Zöckler, Michael, T., Mader, Cornelia Grübel and Martin Heisenberg (2002) The *Drosophila* standard brain. *Current Biology*; 12(3): 22731 <https://pubmed.ncbi.nlm.nih.gov/11839276/>.

Marc, R. Freeman (2015) *Drosophila* Central Nervous System Glia; Cold Spring Harbor Perspectives in Biology. <https://pubmed.ncbi.nlm.nih.gov/25722465/>.

Fly Brain: An online atlas and database of the *Drosophila* nervous system; University of Arizona. <http://flybrain.neurobio.arizona.edu/>.

Lalanti Venkata subramanian and Richard, S. Mann (2019) The Development and Assembly of the *Drosophila* Adult Ventral Cord. *Current Opinion in Neurobiology*; 56:135143. <https://www.sciencedirect.com/science/article/pii/S0959438819300170>.

S. J. View (2018) *Drosophila* Central Nervous System; YouTube: <https://www.youtube.com/watch?v=RmnZTOWlJT8>.

ii. Olfactory system

Drosophila olfactory receptors are present in the antennae and maxillary palp (**Fig. 8A**) located on the head. Each antenna comprises three large proximal segments and a thin arista (**Fig. 8B**). The third antennal segments and the maxillary palps are covered with sensory hairs or sensilla (**Fig. 8C**). These sensilla house the odorant receptor neurons (ORNs) that express one or more odorant receptors in a stereotyped pattern. The ORNs project to the olfactory lobes in the central brain (**Fig. 7**). In addition to an olfactory organ, the antenna also serves the purpose of mechanosensation and auditory reception. This involves arista and the third antennal segment (see **Chapter 96**).

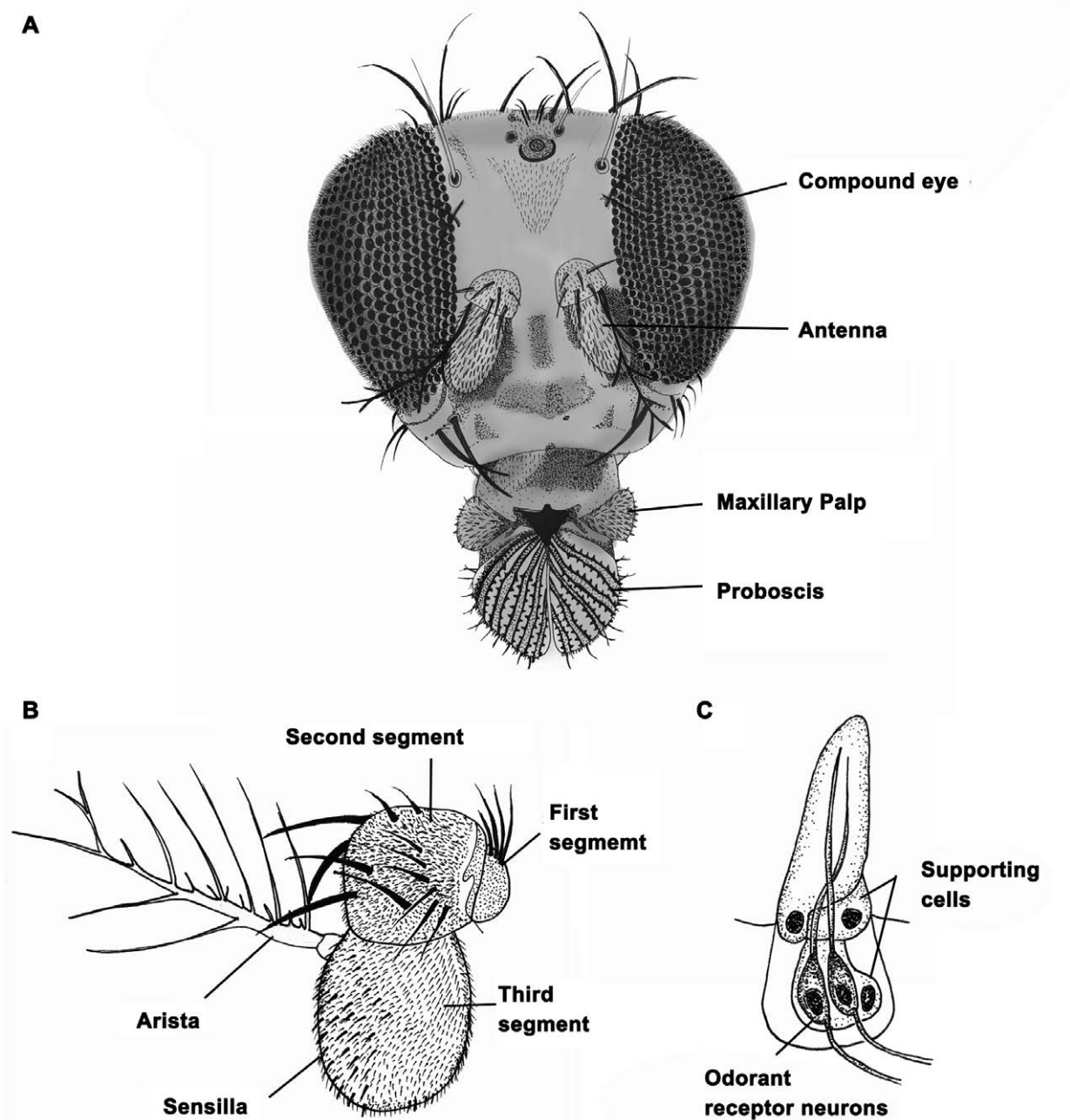


Fig. 8. Olfactory organs on fly head A. Head of adult fly. B. Antenna. C. Sensillum.

Further resources for olfactory and mechanosensory system:

Scott Barish, Pelin, C. Volkan (2015) Mechanisms of olfactory receptor neuron specification in *Drosophila*. Wiley Interdisciplinary Reviews Developmental Biology; 4(6):60921. <https://pubmed.ncbi.nlm.nih.gov/26088441/>.

Biao He and Paul, N. Adler (2001) Cellular mechanisms in the development of the *Drosophila* arista. Mechanisms of Development; 104(12):6978. <https://www.sciencedirect.com/science/article/pii/S0925477301003689>

Sokol, V. Todi, Yashoda Sharma and Daniel, F. Eberl (2004) Anatomical and Molecular Design of the *Drosophila* Antenna as a Flagellar Auditory Organ. Microscopy Research & Technique; 62(6):388399. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1805627/>.

iii. Visual system

The eye-antennal imaginal disc gives rise to the compound eye of *Drosophila* comprising 700-800 subunits called ommatidia (**Fig. 9A**). Each ommatidium consists of eight photoreceptor cells [six outer (R1-R6) and two inner (R7 and R8)] and additional support cells including cone cells and pigment cells. Each photoreceptor cell is highly specialized and contains a microvillar structure called rhabdomere (**Fig. 9B**) where the photoreception takes place and is converted to electrical signals. This information is then relayed to higher brain centers through lamina and optic lobes (**Fig. 9C**).

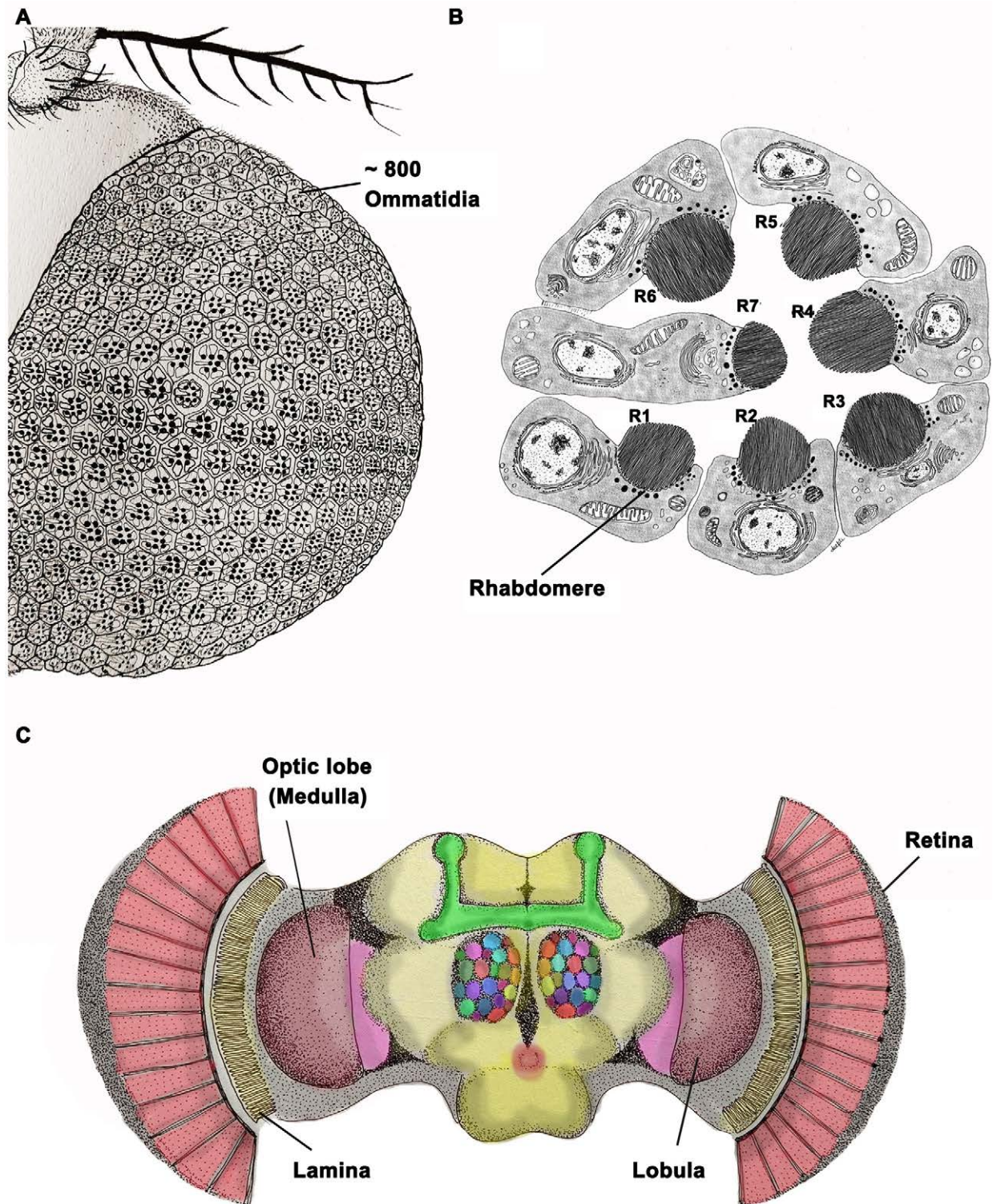


Fig. 9. Visual system in adult fl . A Ommatidia of the compound eye. **B.** Rhabdomeres in an ommatidium. **C.** Structures in brain associated with visual reception and signaling.

Further resources for visual system:

Nathalie Nériec and Claude Desplan (2016) From the Eye to the Brain: Development of the *Drosophila* Visual System. *Current Topics in Developmental Biology*; 116:247271. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5174189/>.

Ross Cagan (2009) Principles of *Drosophila* Eye Differentiation. *Current Topics in Developmental Biology*; 89:115135. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2890271/>.

Maria Tsachaki and Simon, G. Sprecher (2011) Genetic and developmental mechanisms underlying the formation of the *Drosophila* compound eye. *Developmental Dynamics*; 241(1):4056. <https://anatomypubs.onlinelibrary.wiley.com/doi/full/10.1002/dvdy.22738>

Martin Heisenberg (2003) Mushroom body memoir: from maps to models. *Nature Reviews Neuroscience*; 4:266-275. <https://www.nature.com/articles/nrn1074>

iv. Gustatory system

The fly gustatory system includes proboscis, upper margin of wings and the legs (**Fig. 10A and B**). The proboscis consists of three parts (**Fig. 10A**): the ventral lip-like labellum, a distal haustellum and a proximal rostrum, which interact during the process of feeding, helping in extension and retraction of the proboscis. Sensory hairs (Sensilla) on the labella, wings and legs have gustatory neurons important for taste (see **Chapter 84**), in addition to the support cells (**Fig. 10C**). The subesophageal zone (SEZ) (**Fig. 7**) directly controls the movements involved in feeding behavior.

Further resources for gustatory system:

Shivam Kaushik and Pinky Kain (2019) Understanding taste using *Drosophila melanogaster*. *Drosophila* Little, but Powerful Tool for Discovery. <https://www.intechopen.com/books/animal-models-in-medicine-and-biology/understanding-taste-using-em-Drosophila-melanogaster-em->.

Claire, E. Mckellar (2016) Motor control of fly feeding. *Journal of Neurogenetics*; 30(2):111. <https://www.tandfonline.com/doi/full/10.1080/01677063.2016.1177047>.

Olivia Schwarz, Ali, A. Bohra, Xinyu Liu, Heinrich Reichert, Krishnaswamy Vijay Raghavan and Jan Pielage (2017) Motor control of *Drosophila* feeding behavior. *eLife*; 6:e19892. <https://elifesciences.org/articles/19892>.

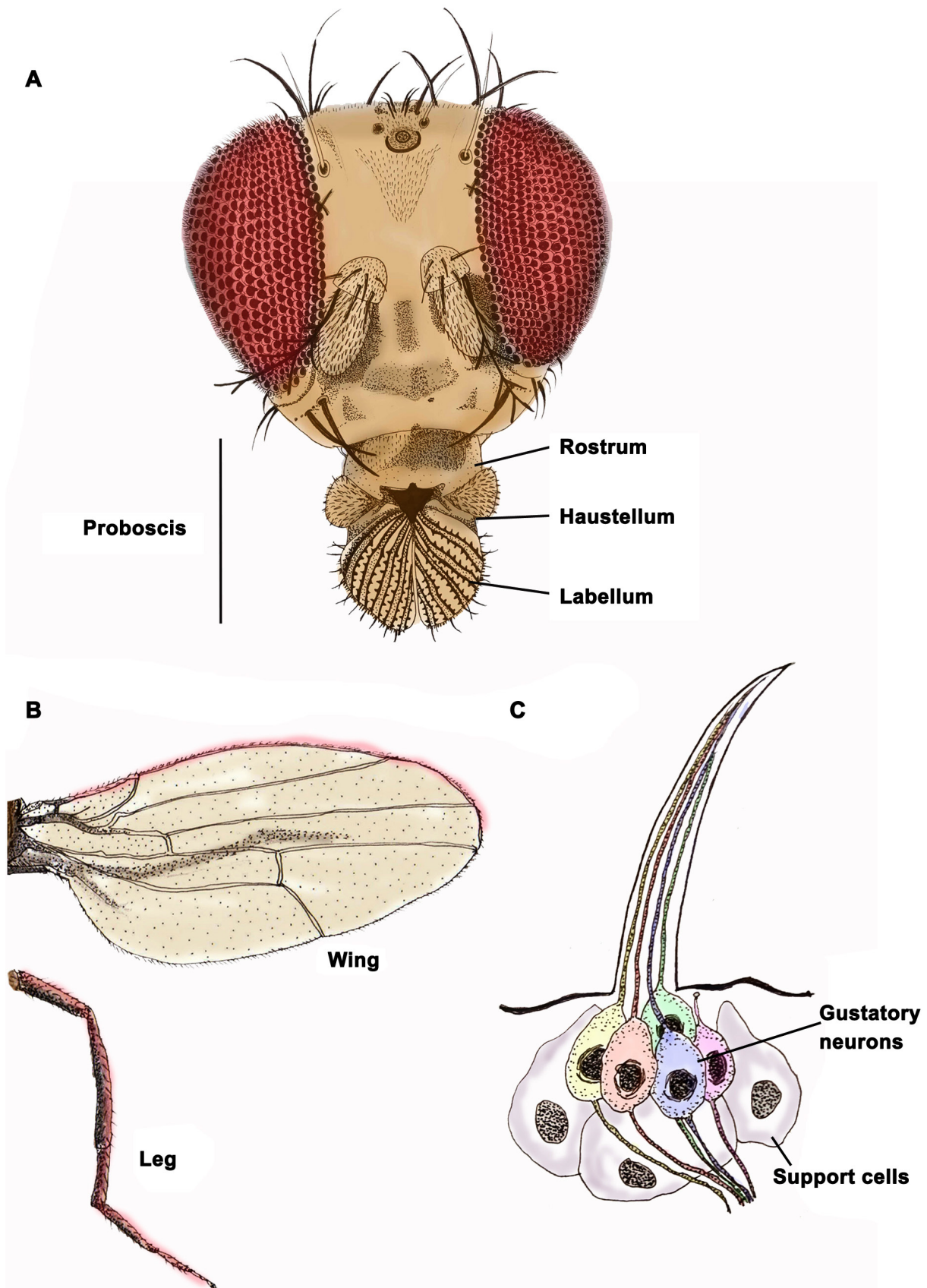


Fig. 10. Gustatory system. A. Gustatory structures on adult head. **B.** Gustatory sensillae on wings and legs. **C.** Structure of a chemo-sensory sensillum.

v. Lymphatic system and the heart

The larval lymph gland is divided into multiple lobes situated around the cardiac tube. The anterior most lobe is called the primary lobe. Posterior to the primary lobe are the secondary, tertiary and quaternary lobes (see [Chapter 22, 55](#)). The primary lobe is divided into zones that have functionally distinct cell populations (**Fig. 11A**). These zones include the Posterior Signaling Center (PSC), which acts as a niche to regulate progenitor maintenance; the Medullary Zone (MZ) consisting of medially located hemocyte progenitors; and the distal Cortical Zone (CZ), which is populated by maturing hemocytes.

Posterior to the larval lymph gland is the heart. *Drosophila* exhibits an open circulatory system composed of a simple tubelike heart (**Fig. 11B**) or dorsal vessel that pumps hemolymph, an interstitial fluid which bathes the internal organs. Located beneath the dorsal epidermis, the heart is formed during early embryogenesis by two opposing rows of contractile cardiomyocytes that pump the hemolymph towards the anterior portion of the body. Circulation of the hemolymph is also aided by wall muscles of the body cavity and additional pulsatile organs that aid in circulation of hemolymph in the appendages like the wings, legs and antenna.

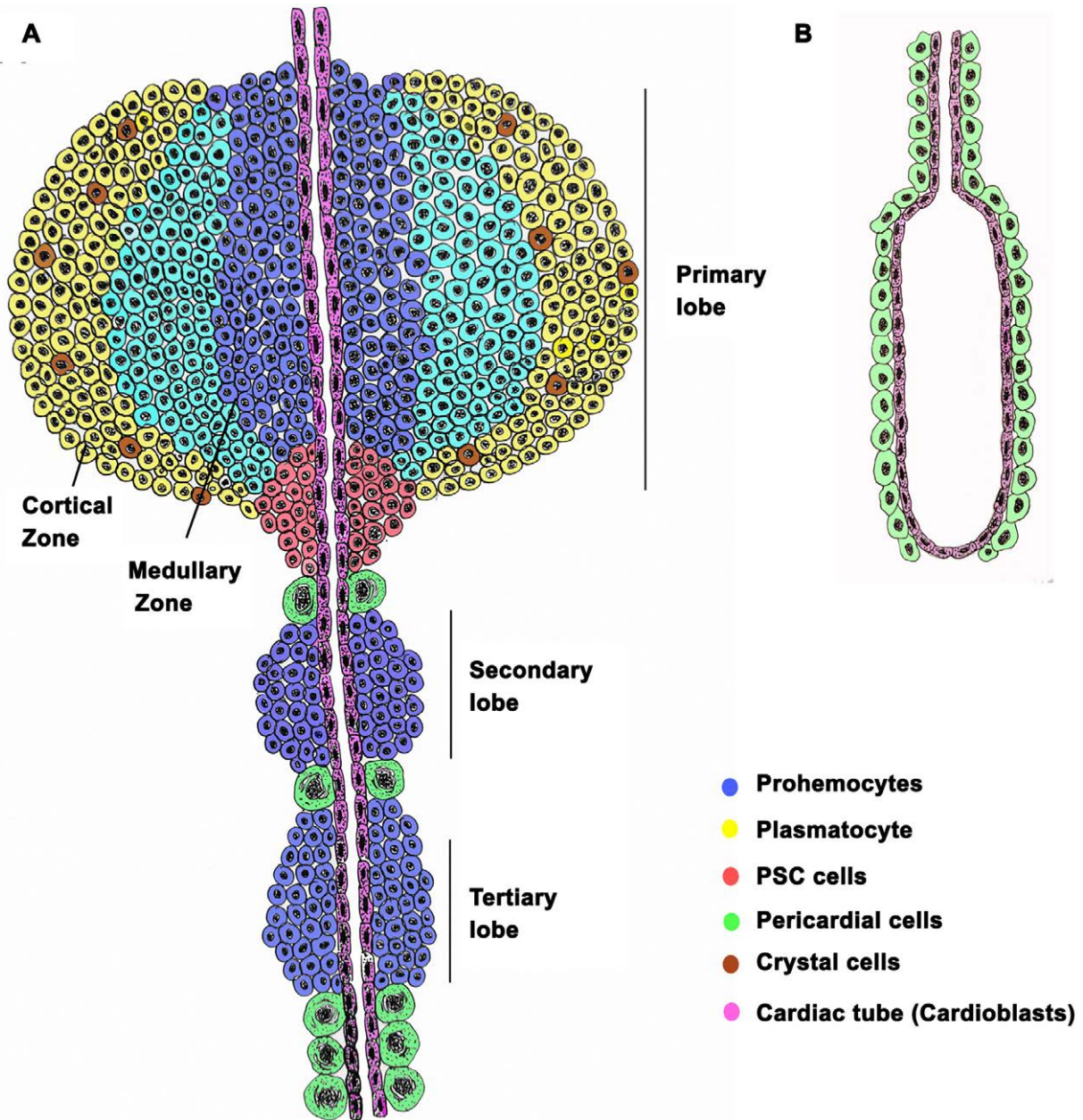


Fig. 11. Larval lymphatic and circulatory system. A. Lymph gland. B Heart.

Further resources for lymphatic system and the heart:

Utpal Banerjee, Juliet R. Girard, Lauren M. Goins and Carrie M. Spratford (2019) *Drosophila* as a Genetic Model for Hematopoiesis. *Genetics*; 211(2):367417. <https://www.genetics.org/content/211/2/367>

Barbara Rotstein and Achim Paululat (2016) On the Morphology of the *Drosophila* Heart. *Journal of Cardiovascular Development and Disease*; 3(2):15. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5715677/>

vi. Reproductive System

The first cells that form at the posterior end of an otherwise syncytial embryo are relatively large, RNA rich pole cells, situated between the vitelline membrane and blastoderm, which develop into the primordial germ cells and finally into the gametes. The gonads are already present as distinct transparent vesicular structures in the body cavity of the first instar larva, encased by structures known as fat bodies. Since the ovary is notably smaller than the testis and is more compactly surrounded by the fat bodies (see **Chapter 28**), the sex of the would be fly can be determined even at larval stage. Ahead of this, the primordial gonads increase rapidly in size and become more oblong ellipsoidal, retaining this shape through the larval and initial pupal stages.

The genital ducts (oviducts in females and vas deferens in males) develop from the genital imaginal disc which grows significantly after pupation. About 36-42 hr after pupation, the female gonads become attached to the oviducts. Differentiation of adult testis in male is more rapid.

a. Female Reproductive system:

The adult female reproductive system consists of a pair of ovaries, the oviduct, uterus, three sperm storing organs (a seminal receptacle and two spermathecae) and a pair of accessory glands (**Fig. 12**). A cluster of parallel ovarioles are present in each ovary, with each ovariole divided into a posterior vitellarium and an anterior sausage-shaped germarium. Each egg chamber comprises an oocyte and 15 nurse cells (both derived from the germ layer lineage) enveloped by a single cell thick follicle layer (somatic cell lineage). The first instar larva contains 8-12 oogonia. A remarkable fifty-fold growth occurs in the volume of ovaries during larval development with each larval ovary consisting of a dense cluster of mitotic cells at the apical and basal poles. The oogonia number increases to ~90-120 by end of the third instar stage. Differentiation into the ovarioles takes place during pupation.

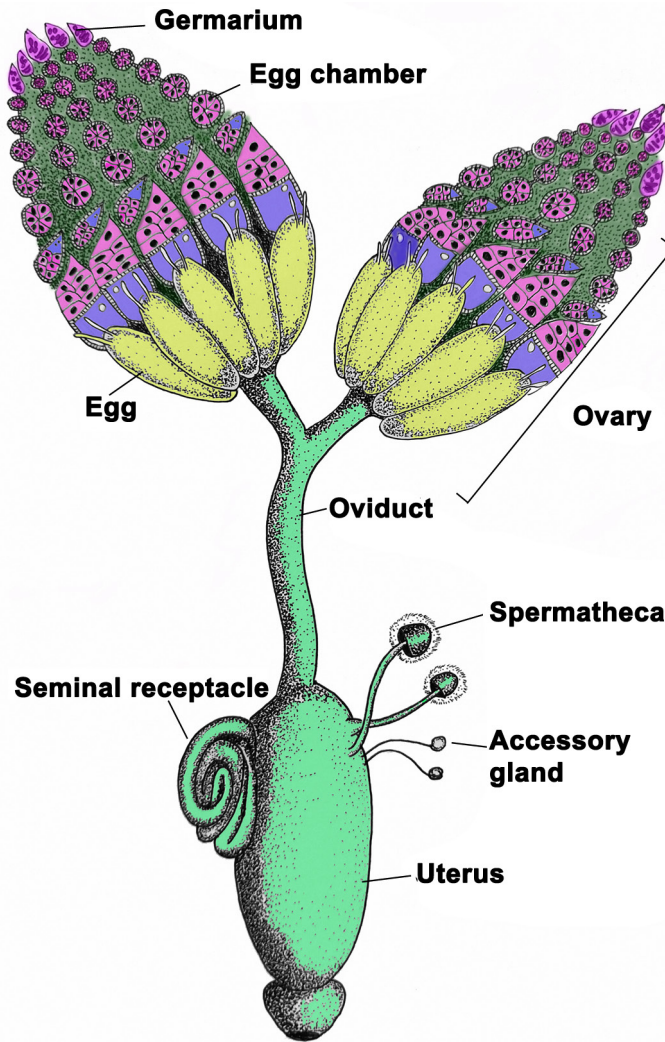


Fig. 12. Reproductive system of female fl

b. Male Reproductive system

The reproductive system in adult male *Drosophila* (see [Chapter 107](#)) includes of a pair of coiled testes, the vas deferens, a pair of large accessory glands, two ejaculatory ducts and an ejaculation bulb (**Fig. 13**). The accessory glands open in the anterior ejaculatory duct. The spermatozoa are released in large quantities from the testis into the vas deferens.

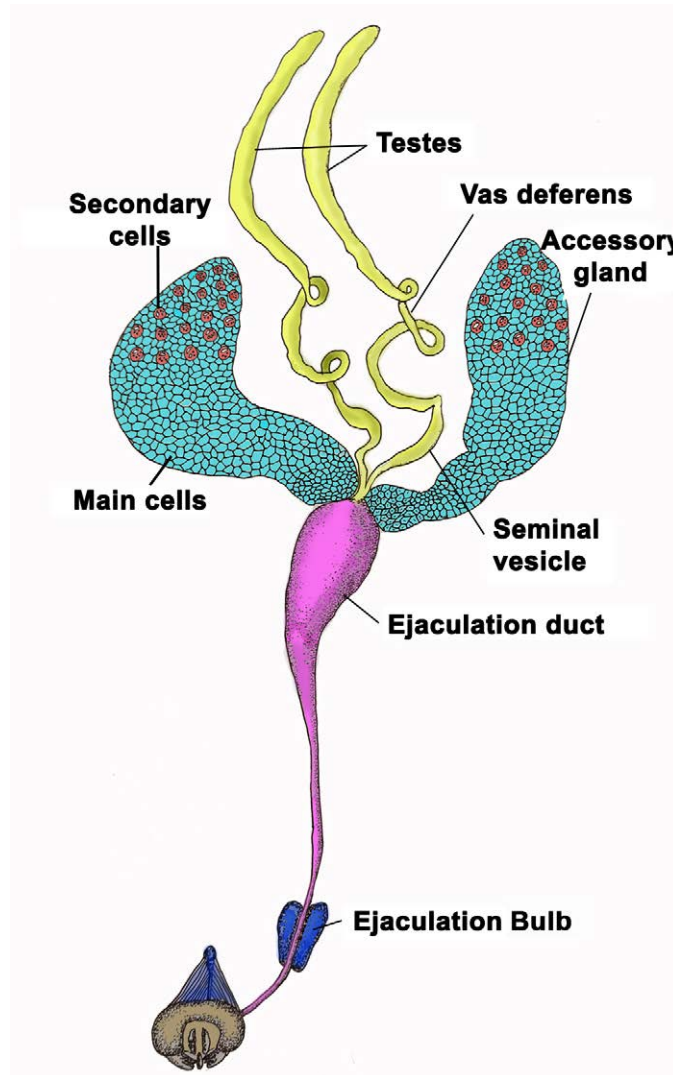


Fig. 13. Reproductive system of male fl

Further resources for *Drosophila* reproductive system:

Julius Kerkis (1931) The growth of the gonads in *Drosophila melanogaster*. *Genetics*; 16(3):212224. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1201094/pdf/212.pdf>.

Margaret C. Bloch Qazi, Yael Heifetz and Mariana F. Wolfner (2003) The developments between gametogenesis and fertilization: ovulation and female sperm storage in *Drosophila melanogaster*. *Developmental Biology*; 256(2):195211. <https://www.sciencedirect.com/science/article/pii/S0012160602001252>.

Aurelio Bairati (1968) Structure and ultrastructure of the male reproductive system in *Drosophila melanogaster*. *meig. Monitore Zoologico Italiano Italian Journal of Zoology*; 2(34):105182. <https://www.tandfonline.com/doi/abs/10.1080/00269786.1968.10736126>.

Chapter 3

Familiarization with a few mutant phenotypes of *Drosophila melanogaster*

H.A. Ranganath^{*1, 2} (haranganath@gmail.com) and V. Shakunthala² (drshakunthalav2@gmail.com)

1. Centre for Human Genetics, Bangalore;

2. *Drosophila* Stock Centre, University of Mysore, Mysore

* For correspondence

Recommended Level: UG and PG

Sub-discipline: Genetics/Cytogenetics

Background:

Systematic breeding experiments with pea plants, *Pisum sativum*, by Gregor Mendel through led to conceptualization of the fundamental rules of inheritance, namely, the law of dominance, law of segregation and law of independent assortment, which reflect behaviour of the ‘factors’ during their journey from one generation to the next. The availability of true breeding strains *Pisum sativum* for 7 pairs alternative characters was a great advantage. After the rediscovery of Mendelian principles, investigations on patterns of inheritance, mainly using *Drosophila melanogaster* revealed many more facets of inheritance than what Mendel visualized: these include incomplete dominance, codominance, interaction of genes, epistasis, hypostasis, polygenic-inheritance, sex-linkage, linkage, etc. The increasing availability of new mutant alleles in *Drosophila melanogaster* (see **Chapter 1**) has been the major factor leading to rapid transformation of the field of Genetics from merely examining patterns of inheritance of genes to understand how genes function.

Historically, the famous ‘Fly Room’ at Columbia University, USA had *Drosophila* doyens like T. H. Morgan, A.H. Sturtevant, C. B. Bridges and others who had the ‘sharpest eyes’ to recognise mutant phenotypes so that within a few years of *Drosophila* genetics, the ‘Fly Room’ had a large number of mutations isolated from various wild type populations. They evolved a system of naming and symbolising each mutation (see **Chapter 1**) and their discoveries laid the foundation for science of Genetics.

Characterization and analysis of mutations has been the guiding principle of genetic analysis of any biological process during 20th century and it is more so today. Information on the large number of mutations in *Drosophila* is available at “FlyBase” (www.flybase.org). Such information, together with the practice of sharing mutant stocks by the fly researchers across the globe through stock centres [e.g., the Bloomington *Drosophila*

Stock Center at Indiana Univ., USA (<https://bdsc.indiana.edu/>)] are research resources for *Drosophila* geneticists (see **Chapter 1**).

Mutations occur spontaneously at a low rate (spontaneous mutations) or may be induced by exposure to a mutagen (chemicals like EMS, EMMS, ENU etc. or ionizing radiations like X-rays and gamma rays, or UV-rays) or by insertion/mobilization of transposable elements (see **Chapter 37**). All of these methods induce random mutations. Recently developed methods for genome editing, for example by CRISPER/Cas technique (see **Chapter 38**), permit creation of very specific mutations in the desired gene or chromosome regions. With appropriate genetic crosses among different mutant strains thus generated, and by selecting the desired progeny flies with new combination of mutant markers, double, triple and multiple mutant stocks can be generated. Such stocks are extremely useful for experimental studies on gene expression/interactions etc, but also for conducting classroom experiments to understand basic patterns of inheritance like, independent assortment of genes, interaction of genes, linkage and crossing over etc. The present chapter would familiarize students with some common mutations that are used for a variety of classroom experiments to understand the basic principles of inheritance.

Objectives:

To familiarize with common visible mutations affecting different parts of *D. melanogaster* and with the genetic basis of these mutant phenotypes.

Materials Required:

A. Flies: Wild type *D. melanogaster*, and different mutant strains, which can be obtained free of cost from the *Drosophila* Stock Centre, Department of Zoology, University of Mysore, Manasagangotri, Mysore, 570006 (also from some other fly labs in the country). If flies cannot be maintained on long-term basis, these can be obtained every year from the stock centre with sufficient advance information.

B. Equipment and other supplies:

- i. Stereo binocular microscope.
- ii. Food bottles and vials (see **Chapter 1**).
- iii. Fine hair brushes, forceps, ethyl-ether, etherizer, re-etherizer (see **Chapter 1**) etc.

Experimental Details:

The wild type (**Fig. 1**) and different mutant flies (**Figs. 2-4**) provided are to be examined to identify the mutant character/s externally visible in each and to match them with the phenotypes illustrated in the examples given below. The description below provides the gene name, its symbol, linkage position and a brief description of the mutant phenotype.



Fig. 1. Wild type female (A) and male (B) flies as seen in lateral view.

A. White eye

Gene name: *white*; **symbol:** *w*; **Linkage position:** 1-1.5 (Fig. 2A)

Recessive. Eyes without any pigment and, therefore, the eyes and ocelli are white in colour. The larval and adult Malpighian tubules, and the testes sheath in adult are also colourless. The w^l was the first mutant characterized in *Drosophila melanogaster* (Morgan, 1910; Morgan and Bridges, 1916).

B. White-apricot eye

Gene name: *white apricot* allele of *white*; **symbol:** w^a ; **Linkage position:** 1-1.5 (Fig. 2B)

Recessive and an allele of w^l . Yellowish pink eye colour with colourless larval and adult Malpighian tubules and testes in adult.

C. Brown eye

Gene name: *brown*; **symbol:** *bw*; **Linkage position:** 3-52.4 (Fig. 2C)

Recessive. Eye colour light brownish wine on emergence, darkening to garnet. Adult testes and vasa colourless. Larval Malpighian tubules pale yellow. Produces white eyes in combination with *v*, *cn*, or *st*.

D. Rosy eye

Gene name: *rosy*; **symbol:** *ry*; **Linkage position:** 3-52.4 (Fig. 2D)

Recessive. Deep ruby eye colour.

E. Scarlet eye

Gene name: *scarlet*; **symbol:** *st*; **Linkage position:** 3-44.0 (Fig. 2E)

Recessive. Eyes bright vermilion, darkening with age. Ocelli colourless, even in old fly; Produces white eyes in combination with *bw*. Larval Malpighian tubules pale yellow.

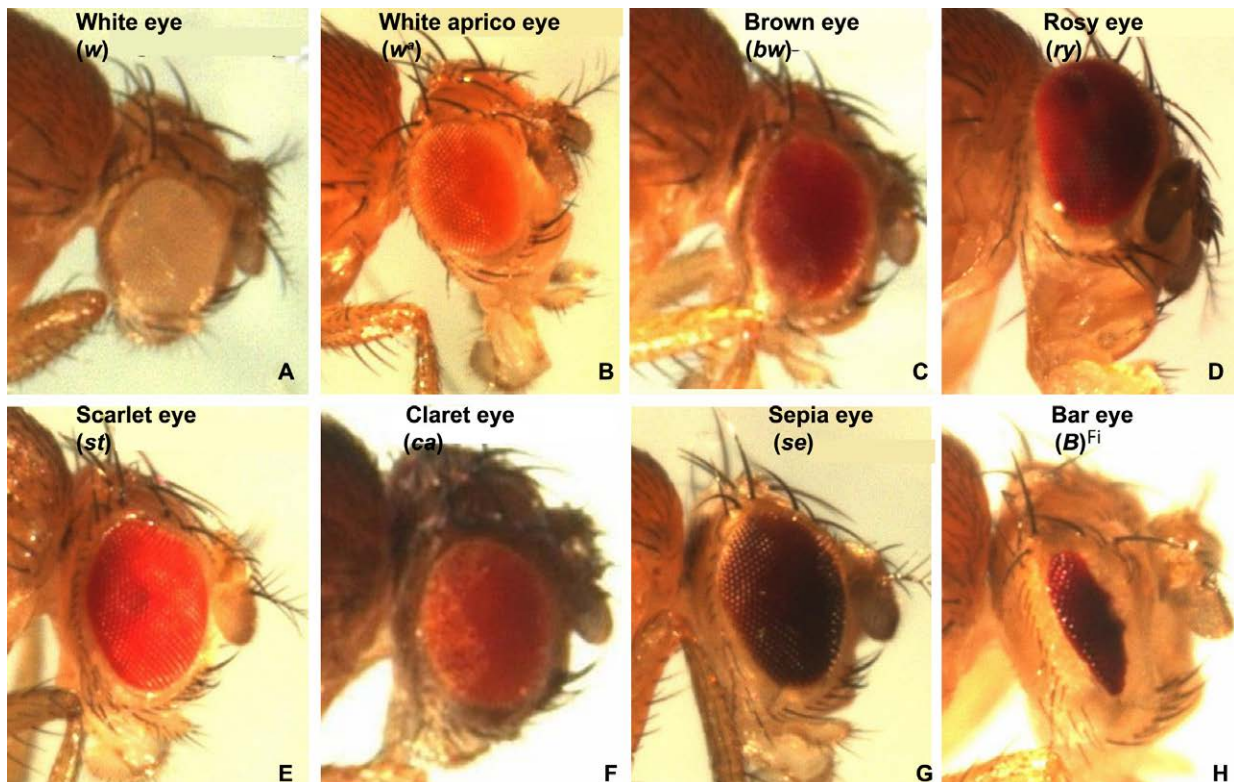


Fig. 2. Mutations affecting eye colour or shape in adult flies. Name of the mutation and symbol of the mutant gene (in parentheses) are noted at top in each panel.

F. Claret eye

Gene name: *claret*; **symbol:** *ca*; **Linkage position:** 3-100.7 (Fig. 2F)

Recessive. Eye colour ruby. In combination with *cinnabar* (*cn*), eye colour is deep reddish yellow while with *bw*, it is translucent brownish yellow. Larval Malpighian tubules colourless.

G. Sepia eye

Gene name: *sepia*; **symbol:** *se*; **Linkage position:** 3-26.0 (Fig. 2G)

Recessive. Eye color brown at eclosion, darkening to sepia, and becoming black with age.

H. Bar eye

Gene name: *Bar*; **symbol:** *B*; **Linkage position:** 1-57.0 (Fig. 2H)

Semi-Dominant. The ommatidial array in eyes is restricted to a narrow vertical bar like area with only about 90 facets in the male and 70 facets in homozygous female, in contrast with the normal numbers of about 740 facets in males and 780 in females. Heterozygous females (*B/B*⁺) show a 'kidney-shaped' eye with ~360 facets in each eye. This mutation is due to a tandem duplication of a small part (16A1-7 chromosome region) of the X-chromosome and is an example of stable position effect.

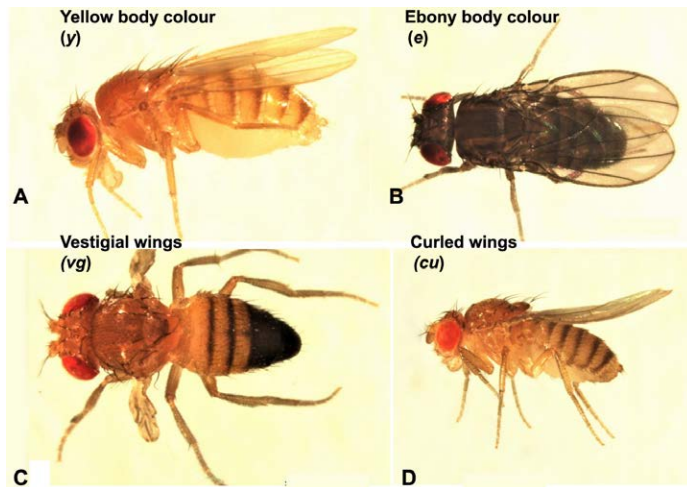
I. Yellow body:

Gene name: *yellow*; **symbol:** *y*; **Linkage position:** 1-0.0 (Fig. 3A)

Recessive. The body colour is yellow, with body hairs and bristles brown but with yellow tips. The wing hairs and veins are yellow.

J. Ebony body:**Gene name:** *ebony*; **symbol:** *e*; **Linkage position:** 3-70.7 (**Fig. 3B**)

Recessive. Body color varies from shining black to slightly darker than wild type.

**Fig. 3. Mutations affecting body colour or wing size/shape.** Name of the phenotype and symbol of the mutant gene (in parentheses) are noted at top in each panel.**K. Vestigial wings****Gene name:** *vestigial*; **symbol:** *vg*; **Linkage position** 2-67.0 (**Fig. 3C**)Recessive. In the classical *vg* mutants, the wings of homozygotes are reduced to vestiges and are usually held at right angles to the body; wing veins still visible. Halteres may be reduced or absent. Temperatures of 29°C or greater appreciably increase wing size.**L. Curled wings****Gene name:** *curled*; **symbol:** *cu*; **Linkage position** 3-50.0 (**Fig. 3D**)

Recessive. Wings curved upward throughout length and slightly divergent. Good nutrition of larvae enhances curled character as does high temperature in last day of pupal life.

M. Curly wings**Gene name:** *Curly*; **symbol:** *Cy*; (**Fig. 4C**)Dominant. Wings strongly curled upward and forward; rarely overlaps with wild type at 25°C, but frequently overlaps at 19°C. Curly phenotype decreased by larval crowding but increased by elevated temperature during pupal development. Homozygous lethal. Associated with the Curly inversion, *In(2L)Cy*, on left arm of chromosome 2. Often used as a balancer chromosome (see [Chapter 1](#)) because of its dominant visible phenotype but recessive lethality.**N. Bristle****Gene name:** *Bristle*; **Symbol:** *Bl*; **Linkage position:** 2-54.8 (**Fig. 4C**)Dominant. Bristles one-half to two-thirds of the normal length, blunt, thicker, and beaded in outline. Overlaps wild type when reared at 20°C. Homozygotes lethal. With a dominant visible but recessive lethal phenotype, it can be maintained only in heterozygous condition; often used as part of a balanced lethal stock (see [Chapter 1](#)).**O. Forked bristles****Gene name:** *forked*; **Symbol:** *f*; **Linkage position:** 1-56.7 (**Fig. 4C**)

Recessive. Macrochaetae, microchaetae, and trichomes affected to various degrees - gnarled, and bent with ends split or sharply bent.

P. Roughoid

Gene name: *roughoid*; **Symbol:** *ru*; **Linkage position:** 3-0.0 (Fig. 4E)

Recessive. Eyes small and rough, have irregular facets and hairs, and have black specks from erupted facets. Expression variable; sometimes overlaps wild type.

Q. Stripe

Gene name: *stripe*; **Symbol:** *sr*; **Linkage position:** 3-62.0 (Fig. 4E)

Recessive. Trident pattern on thorax replaced by broad light gray stripe at 25°C; stripe is dark at 18°C.

R. Dumpy wings

Gene name: *dumpy*; **Symbol:** *dp*; **Linkage position:** 3-62.0 (Fig. 4E)

Recessive. Wings reduced to two-thirds normal length giving a truncated cut-off appearance.

S. Cinnabar eye

Gene name: *cinnabar*; **Symbol:** *cn*; **Linkage position:** 2-57.5 (Fig. 4F)

Recessive. Eye colour bright red, Ocelli colourless. Eye colour darkens and becomes duller with age, but ocelli remain colourless. Larval Malpighian tubules pale yellow. In combination with *bw*, produces white eye (Fig. 4F).

T. Black body

Gene name: *black*; **Symbol:** *b*; **Linkage position:** 2-48.5 (Fig. 4F)

Black pigment on body, tarsi and along wing veins. Body colour darker at low temperature.

Note: A given fly may carry a single mutation, more than one mutations, which may be on same chromosome in cis- (Fig. 4B, D,E) or trans- configuration (e.g., Fig. 4C) or on different chromosomes (e.g., Fig. 4A, 4F).

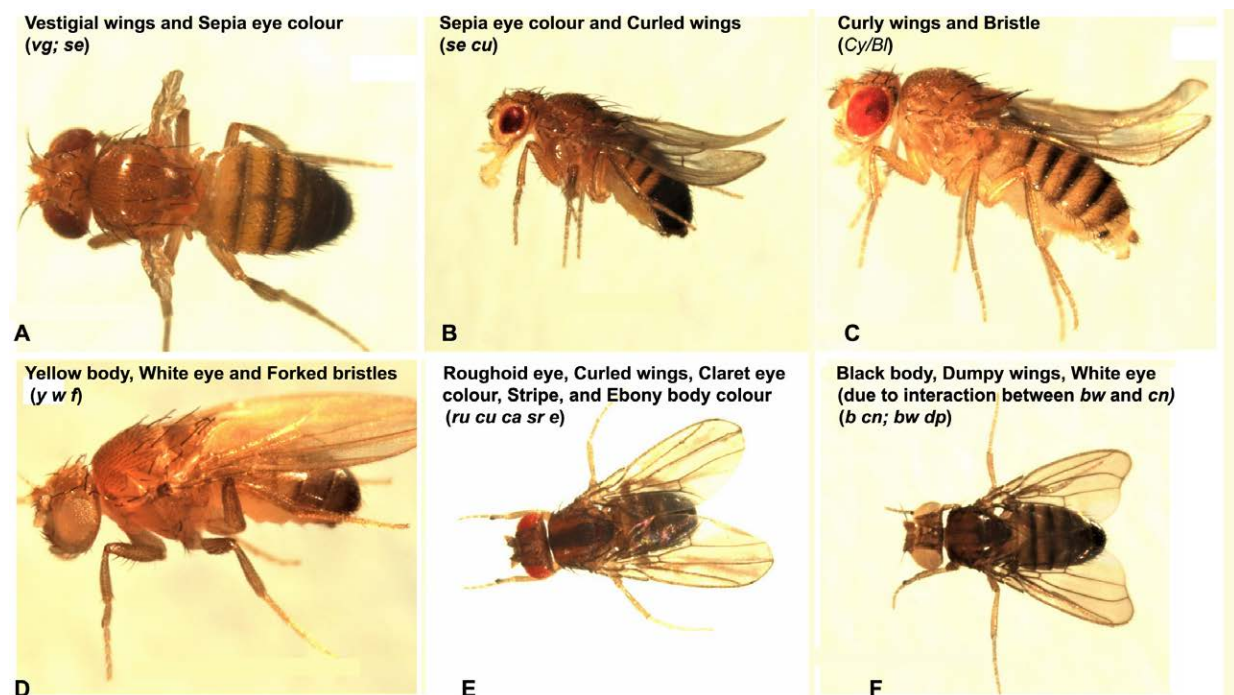


Fig. 4. Flies with multiple mutations. Names of the phenotypes and symbols of the mutant genes (in parentheses) are noted at top in each panel.

Observations:

Compare the morphology of normal flies with each of the provided mutant strains. Draw images of the flies and comment on the nature of mutations.

Note for instructors: Students are not to be informed of the nature of the mutant strains provided to them). Students are expected to recognise the specific mutant phenotypes and name of the mutated gene by referring to the above description and to the Flybase (www.Flybase.org).

Questions for self-study and interpretation:

1. Are such mutant flies available in natural populations?
2. Can mutant phenotypes be induced in laboratory?
3. Are mutant phenotypes restricted to morphology only?
4. Why do mutations in different genes result in different eye colours?
5. Why flies carrying cinnabar and brown eye mutations together show white eye colour (**Fig. 4 F**) when the wild type allele for red eye (w^+) is present on their X-chromosome? Find out the nature of gene interaction.

References:

Ranganath, H. A. (1999) Teaching and learning genetics with *Drosophila* as a model system, *Resonance*. 4: 48–52.

Ranganath, H. A. and Tanuja, M. T. (1999) Teaching and learning genetics with *Drosophila*: 2 Mutant phenotypes of *Drosophila melanogaster*. *Resonance*. 4: 95–104.

From Wild Type to Diverse Mutants

Within the last five or six years, however, from a common wild species of fly, the fruit fly, *Drosophila ampelophila*, which we have brought into the laboratory, have arisen over a hundred and twenty-five new types whose origin is completely known.

(In: Morgan, T. H. 1916. A Critique of the Theory of Evolution. Oxford University Press. <https://www.gutenberg.org/files/30701/30701-h/30701-h.htm>)

Chapter 4

Analysis of *Drosophila* eye pigments by paper and thin layer chromatography

Girish Chandran^{1*} (changirish86@gmail.com) and S. R. Ramesh² (rameshuom@gmail.com)

1. Department of Life Sciences, Pooja Bhagavat Memorial Mahajana Education Centre, Mysuru, 570016.

2. Department of Studies in Zoology, University of Mysore, Manasagangotri, Mysuru 570006; Current address: Chief Scientist, Department of Life Sciences, Pooja Bhagavat Memorial Mahajana Education Centre, K. R. S. Road, Mysuru 570016.

* For correspondence

Recommended Level: UG/ PG/ Research

Sub-discipline: Genetics/Biochemistry

Background:

Adult *Drosophila melanogaster* bears two red colored compound eyes. Eye color mutants hold a pivotal space in the history of *Drosophila* genetics. Discovery of the white-eye mutation by T. H. Morgan marked the advent of *Drosophila* as a genetic model organism. Since then many eye color mutants have been identified in *D. melanogaster* (see [Chapter 3](#)) and other species of *Drosophila*. Identification of pigments responsible for color of *Drosophila* eyes dates back to 1940s (Ephrussi and Herold, 1944; Wald and Allen, 1946). The ease in recognition of the eye color variants paved way for the development of biochemical genetics since variations in eye color indicate changes in activities of one or more enzymes involved in the pigment synthesis. A series of reactions, mediated by specific enzymes, lead to the formation of different pigments that together produce the wild type eye colour.

Eye pigments in *Drosophila* belong to two major classes, viz., ommochromes (brown) and pteridines or drosopterins (red). The ommochromes are synthesized from tryptophan; while drosopterins are synthesized from Guanosine 5'-triphosphate (GTP). The enzymes involved in the biosynthetic pathways of these pigments are different (Ryall and Howells, 1974; Kim *et al.*, 2013). Ommochromes, the brown pigments, are synthesized and stored in specialized organelles called Ommochromosomes, which are related to lysosomes and vertebrate melanosomes (Figon and Casas, 2018).

An adult eye of wild type *Drosophila melanogaster* has seven sub classes of pteridines viz., Drosopterin, Sepiapterin, Isolepiaterin, Biopterin, 2-amino-4-hydroxy Pteridine, Xanthopterin and Isoxanthopterin. There are many eye color mutants of *Drosophila melanogaster* viz., red, white, sepia, cherry, blood, brown, eosin, yellow, apricot, ivory and cream (see [Chapter 3](#)). A mutation in any of the genes involved in ommochrome synthesis results in limited or no ommochrome synthesis so that the eye color appears bright red. In contrast, a loss of function mutation in the enzymes of pteridine pathway results in a darker eye color. Analysis of eye pigments in different mutants thus, provides a simple demonstration of gene expression. The chromatographic techniques permit characterization of small molecules in biological samples.

All chromatographic systems comprise two phases:

- i) Stationary phase which can be a solid or immobilized solid/liquid mixture or gel, and
- ii) Mobile phase which can be gaseous or liquid that flows through or over the stationary phase.

Paper chromatography is a technique of separating organic molecules based on their solubility partition coefficient among two liquid phases namely, mobile and stationary phase. The water molecules trapped in the cellulose fibers of chromatographic paper act as stationary phase while the organic solvent mixture acts as mobile phase. The molecules get separated based on their difference in the partition coefficients. Two types of paper chromatography viz., radial (circular) and ascending paper chromatography can be carried out.

Thin layer chromatography (TLC) is also called ‘adsorption chromatography’ which separates molecules based on their distribution coefficient or partition coefficient. The test compounds compete between the liquid mobile phase and solid stationary phase. Differences in the adsorption efficiency among the molecules influences their separation as spots on the chromatogram.

The chromatographic separation of the components in a sample can be visualized using ultra violet illumination and iodination. Generally, the unknown compounds are identified using retention factors in comparison to known standards. However in the case of chromatography of *Drosophila* eye pigments; usually the various pigments are compared with those of wildtype samples owing to the fact that the pigments follow the same pattern in resolving on chromatograms. Here we describe paper and thin layer chromatography methods for analysis of *Drosophila* eye pigments.

Objectives:

To compare eye pigments in wild type *Drosophila melanogaster* and some eye color mutants.

Materials Required:

A. Flies: Adult males of wild type and some eye color mutants. In the example here, flies homozygous for sepia, scarlet, white and brown mutant alleles can be used. (The experiment can be repeated with female flies as well; however, flies of only one gender should be used for preparing the samples for a given run). Pure pigments can also be obtained and used as reference standard for the sake of comparison.

B. Equipment:

- i. Stereo-binocular microscope
- ii. Table top microcentrifuge
- iii. Ultra-violet illuminator
- iv. Chromatography chamber or a glass chamber to hold the chromatography assembly
- v. TLC chamber

C. Fly handling materials:

- i. Entomological needles and fine brush (size ‘0’ or ‘1’).

- ii. Counting board: 15 cm X 15 cm white vitrified tile or white plastic board (sturdy) or a ground glass slab.
- iii. 1.5 mL micro-centrifuge tubes
- iv. Micropipette (10 μ L, 100 μ L) and corresponding tips
- v. Petri plate (2 cm depth, 12 cm diameter)
- vi. Tissue paper
- vii. Micro-tissue homogenizer
- viii. Whatman No. 1 paper circular 12 cm diameter
- ix. Whatman No. 1 paper rectangular: 16 cm X 16 cm
- x. Silica coated plates 16 cm X 16 cm for TLC with 0.5 mm thick coating

D. Reagents

- i. Distilled water
- ii. Diethyl ether
- iii. N-Butanol
- iv. Glacial Acetic acid
- v. Solvent mixture: A mixture of Butanol: Acetic acid: Water is prepared in 4:1:5 ratio, by mixing 400 mL butanol, 100 mL acetic acid and 500 mL distilled water. Mix well in a separating funnel and set it aside for separation of the two phases. Collect the lower aqueous phase in Petri plates and keep in chromatography chamber. The aqueous phase saturates the chamber. The upper organic phase is collected in a different set of Petri plates for use as the mobile phase.

Experimental Details:

A. Preparation of samples:

- i. Etherize and collect at least 20 male flies of each genotype on the counting plate, sever their heads from the body using entomological needles.
- ii. Collect the heads in a microcentrifuge tube (1.5 mL) using fine brush.
- iii. Add 50 μ L of the solvent mixture.
- iv. Homogenize the heads using a microhomogenizer.
- v. Centrifuge at 500x g or 2000 rpm for 10 min.
- vi. Collect the supernatant in a fresh and labeled 1.5 mL tube

Samples can also be prepared by homogenizing heads in 100 μ l of 30% alcohol acidified with HCl (pH 2.0).

B. Paper chromatography:

- i. Circular paper chromatography:
 - a. Take a Whatman No. 1 circular paper disc (12 cm) and make a hole at the centre using a needle. Gently draw a small circle of 3 cm diameter around the hole with a pencil. Draw and divide the whole paper into 5 equal parts using a pencil and a ruler and mark each zone as D1, D2, D3, D4 and D5 at the outer edges and note

down in the log-book about labels and *Drosophila* strain (5 samples are taken in the present example (if different number of samples are taken, change the numbering accordingly).

b. Prepare a wick in the shape of a cone with flat base (3 cm height) using a strip (7.5 cm X 3.5 cm) of Whatman filter paper to hold the circular paper in place while the solvent runs through (**Fig. 1A**).

c. Load atleast 10 μL of the sample in each of the marked points on the circle drawn in centre of the circular filter paper (**Note:** 1 μL of the sample solution is to be loaded at a time and allowed to dry; reload 1 μL of the same sample at the same place; repeat these steps to load 10 μL of the sample).

d. Load all the samples following the steps above.

e. After completion of loading of all samples, securely position the Whatman paper disc on the wick and place the assembly in a Petri plate containing solvent mixture to atleast 1.5 cm height.

f. Place the entire set up in a chromatography chamber (**Fig. 1B**). Allow the solvent to run up and through the samples, until the solvent front is 1 cm away from the periphery of the filter disc.

g. Pick up the filter paper disc using forceps and mark the final solvent front using a pencil in all the zones and allow the paper to dry.

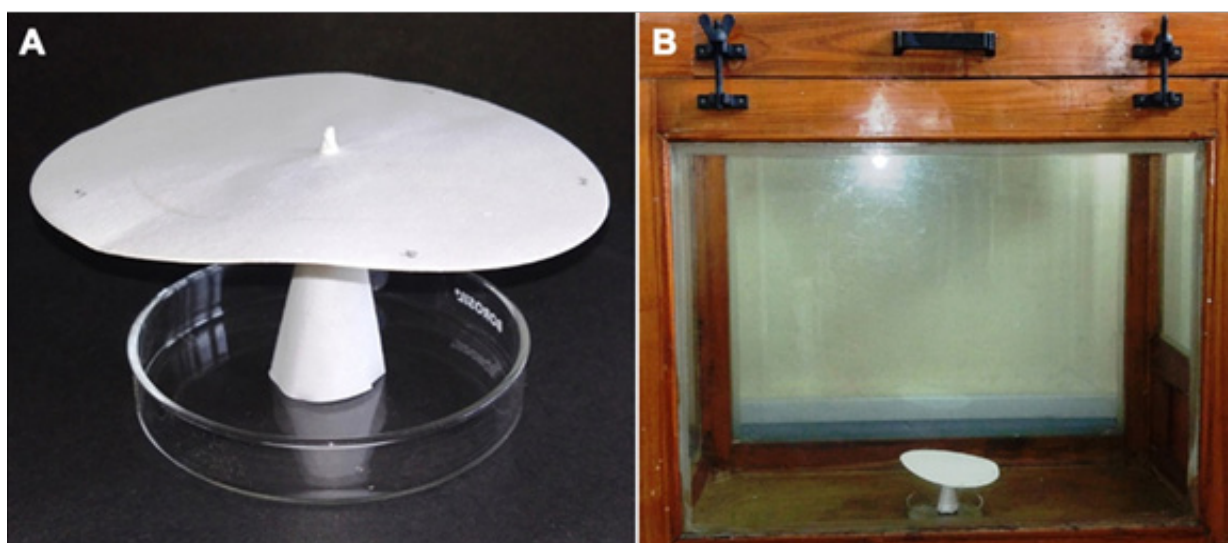


Fig. 1. Circular paper chromatography. A. Setting up of Whatman filter paper disc on a paper wick. B. Circular chromatography assembly in the chromatography chamber

ii. **Ascending paper chromatography:**

a. Take a 10 cm X 15 cm size Whatman chromatography paper. Mark the direction of solvent movement. Draw a horizontal line using a pencil 2.5 cm away from the edge which will be immersed in the solvent. Mark D1, D2, D3, D4 and D5 (corresponding to as many samples as are to be loaded) on the line which are at least 2 cm apart from each other.

b. Load at least 10 μL of the sample on each spot on the line in steps of 1 μL each as described earlier for circular paper chromatography.

c. After loading all the samples, place the paper in a rectangular trough having solvent mixture poured to a height of 1.5 to 2.0 cm (**Fig. 2**) and close the trough with a glass lid.

d. Apply petroleum jelly or grease on the edges of the trough where the lid is placed in order to make it air tight. The edge of the paper nearer to the loaded samples should be dipped in the solvent mixture, taking care that the loaded sample area remains above the solvent mixture.

e. Allow the solvent to run through the paper. When the solvent is about 2 cm away from the top edge, take out the chromatographic paper and allow it to dry in shade away from bright light.

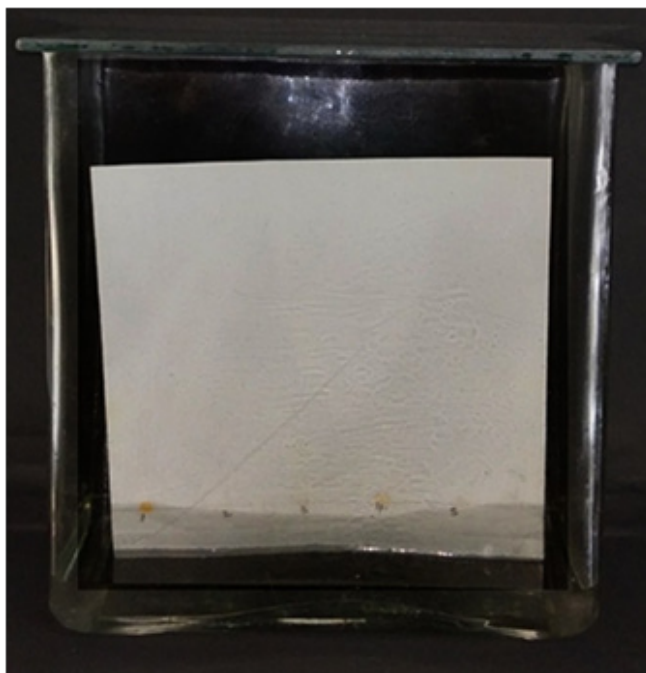


Fig. 2. Ascending paper chromatography setup.

iii. Thin layer chromatography:

a. Take a pre-coated silica plate and draw a line 2.5 cm away from the edge using pencil. Very gently mark D1, D2, D3, D4 and D5 on the line with a spacing of at least 2 cm between each.

b. Load at least 10 μL of the sample on each spot on the line in steps of 1 μL at a time as described earlier.

c. Place the TLC plate in a glass chamber (as described in ascending paper chromatography) containing the solvent mixture poured to a height of 1.5 cm. The edge nearer to the samples (bottom edge) should be dipped in the solvent mixture but the loaded samples must remain outside the solvent mixture.

d. Allow the solvent to run through the samples. When the solvent is nearly 2 cm away from the top edge, take out the TLC plate and allow it to dry overnight.

iv. Detection of pigments under UV illumination:

a. The dried chromatograms are placed in a UV illuminator to visualize and document the pigments (**Fig. 3**). A dark room can also be used for working with UV light.

b. Draw margin around each of the bright spots on the chromatograms and note the color of different spots as visible under the UV illumination.

c. Identify the central point of the marked area and measure the distance from the point where sample was loaded. Likewise, measure the solvent front from the point where samples were loaded. Using these measurements, calculate the **Retention factor (Rf)** values for each spot using the formula:

$$\text{Retention factor} = \frac{\text{Distance travelled by the spot (in cm)}}{\text{Total distance travelled by solvent (in cm)}}$$

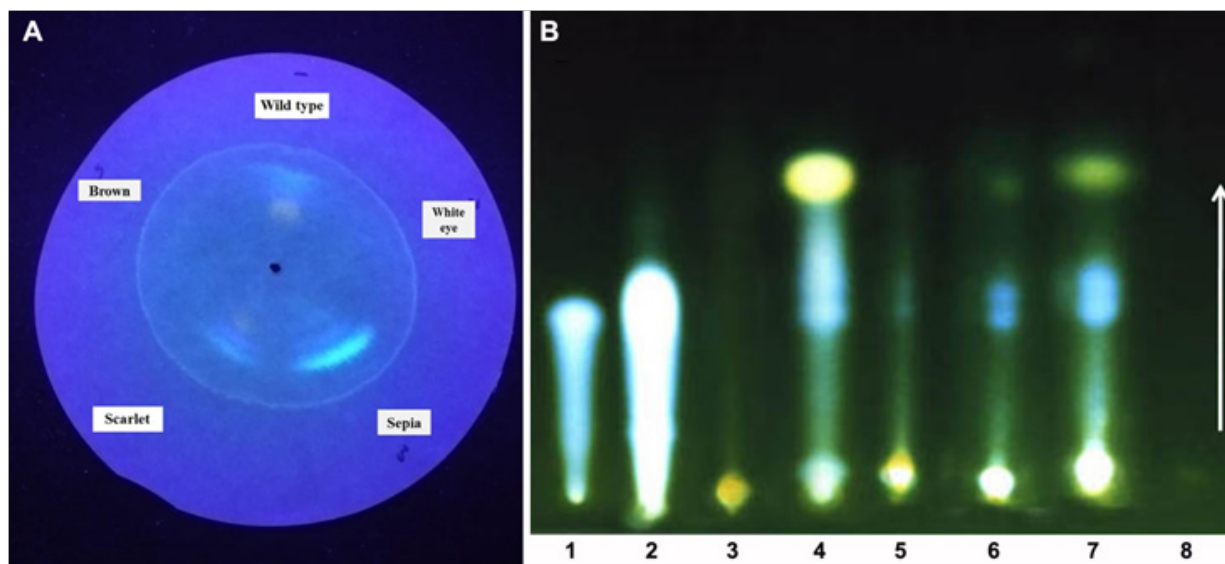


Fig. 3. A. Circular paper chromatogram of eye color mutants of *D. melanogaster* (Wild type, White, Sepia, Scarlet and Brown represent the eye color phenotypes). **B. Thin Layer Chromatogram of different samples:** 1 Pure pigment Pterin; 2 Pure pigment Xanthopterin; 3-4 Eyes from *D. melanogaster* Scarlet (3) and Sepia (4) mutants; 5-8 Eyes from *D. n. nasuta* Wild type (5), Sepia (6), Brown (7) and White (8) mutants. The arrow shows the direction of solvent movement (TLC image in B courtesy: Dr. J. S. Ashadevi, Yuvaraja's College, University of Mysore, Mysuru)

C. Precautions:

- i. Use of surgical gloves (latex) throughout the experiment especially while handling the TLC plates and Whatman papers is essential to avoid any finger prints on the chromatograms.
- ii. Gloves should also be used while handling organic solvents used in the protocol.
- iii. Solvent vapors should not be inhaled.
- iv. Location of the solvent front should be marked immediately after removing the chromatography paper/TLC plate from the solvent chambers before it dries out.
- v. Wear UV eye protection glasses/face shield while using the UV light for observing the chromatograms.
- vi. Never see the UV light directly with naked eyes or even with goggles on.

Results/Observations:

The resolution of eye pigments in paper chromatography would be less when compared with the resolution that can be obtained with TLC. The specific R_f values can be calculated when we use pure pigments as reference standards (**Fig. 3B**).

Mark the boundaries and note the color of individual spots of wild type sample to identify the pigments. In wild type sample, pigments are identified in order from origin to the solvent front: Drosopterin (Orange), Isoxanthopterin (Violet-Blue), Xanthopterin (Green-Blue), Sepiapterin (Yellow), 2-amino-4-hydroxyPteridine (Blue), Biopterin (Blue) and Isosepiaterin (Yellow). Note the absence of any kind of pigments in samples prepared from white eye mutant (**lane 8, Fig. 3B**). Compare the wild type pattern with those of the patterns in different mutants. Record your observations in a Table (a sample is provided in **Table 1**) and note which pigments are more abundant or less abundant in different eye mutants used for sample preparation.

Table 1. Presence of different eye pigments in flies of different genotypes

Pigment	Color of the spot	Wild type D1	Strain 2 D2	Strain 3 D3	Strain 4 D4	Strain 5 D5
Isosepiaterin	Yellow	+				
Biopterin	Blue	+				
2-amino-4-hydroxy Pteridine	Blue	+				
Sepiapterin	Yellow	+				
Xanthopterin	Green-Blue	+				
Isoxanthopterin	Violet-Blue	+				
Drosopterin	Orange	+				

Questions for self-study and interpretation:

1. How did the classical studies on eye pigments in *Drosophila* contribute to understanding of how gene mutations affect the phenotype?
2. Why no pigments are seen in eyes of the white eye mutant flies?
3. What is partition coefficient?
4. Which pigments are absent in eyes of sepia mutant flies?

References and further reading:

Ephrussi, B. and Herold, J. L. (1944) Studies of eye pigments of *Drosophila*. I. Methods of extraction and quantitative estimation of the pigment components. *Genetics*. 29: 148-175.

Figon, F. and Casas, J. (2018) Ommochromes in invertebrates: biochemistry and cell biology. *Biol. Rev.* 94: 156-183.

Kim, H., Kim, K. and Yim, J. (2013) Biosynthesis of Drospterins, the red eye pigments of *Drosophila melanogaster*. IUBMB Life.65: 334–340. <https://iubmb.onlinelibrary.wiley.com/doi/pdf/10.1002/iub.1145>.

Ryall, R. L. and Howells, A. J. (1974) Ommochrome biosynthetic pathway of *Drosophila melanogaster*: Variations in levels of enzyme activities and intermediates during adult development. Insect Biochem. 4: 47-61. [https://doi.org/10.1016/0020-1790\(74\)90041-9](https://doi.org/10.1016/0020-1790(74)90041-9).

Wald, G. and Allen, G. (1946) Fractionation of the eye pigments of *Drosophila melanogaster*. J. General Physiol. 30: 41–46.

Web links:

<https://biophysics.sbg.ac.at/protocol/drosophila.pdf>

Two Pigments Make Fly's Eyes Red

Microscopic observation, as well as solubility tests and studies of the effects of gene substitutions, have shown that the normal, wild type eye color of *Drosophila melanogaster* is due to the presence of two pigments, red and brown. These two pigments are the end products of two largely independent chains of reactions. Recent work on the genetic control of eye color in *Drosophila* was almost entirely confined to the reaction chain leading to the formation of brown pigment. The hormone-like diffusible substances derived from tryptophane and representing intermediate links of this chain were the most intensively studied phase of this process. The study of the reaction chain resulting in the formation of red pigment was on the contrary very much neglected and so was the study of the end products

(In: Ephrussi, B. & Herold, J. L. 1944. Studies of eye pigments of *Drosophila*. I. Methods of extraction and quantitative estimation of the pigment components. Genetics. 29: 148-175

<https://www.genetics.org/content/genetics/29/2/148.full.pdf>).

Chapter 5

Genetic crosses with *Drosophila melanogaster* to understand Mendelian inheritance

Bimalendu B. Nath^{1*} (bbnath@gmail.com), J. K. Roy² (jagatkroy@gmail.com), S. C. Lakhotia^{2*} (lakhotia@bhu.ac.in) and H. A. Ranganath^{3*} (haranganath@gmail.com)

1. Department of Zoology, S. P. Pune University, Pune-411007

2. Cytogenetics Laboratory, Department of Zoology, Banaras Hindu University, Varanasi 221005

3. Centre for Human Genetics, Bangalore 560100 & *Drosophila* Stock Centre, University of Mysore, Mysore 570006

* For correspondence

Recommended Level: UG/PG

Sub-discipline: Genetics

Background:

The conceptual and experimental framework of controlled mating of a model organism for genetic analysis began with Gregor Johann Mendel in 1865 but it was not fully appreciated until Thomas Hunt Morgan started his pioneering work with *Drosophila* in 1908. Morgan's introduction of *Drosophila* to Genetics has been a 'trend-setter' for other genetic model systems in the post-Morgan era. Although availability of complete sequence of the *Drosophila* genome has facilitated rapid characterization of genes, application of classical genetic methodologies, mating schemes and genetic screenings continue as essential tools in current times. The continuing popularity of *Drosophila* is primarily due to the availability of very rich resources (see [Chapter 1](#)) and its amenability to forward genetic screening and analysis (Ranganath 1999; Ray and Lakhotia 2014). This chapter introduces different types of genetic crosses and how to infer genotypes of parents on the basis of progeny phenotypes and inheritance patterns.

In a diploid organism, all chromosomes (except the sex-chromosomes in many cases) are paired, one being contributed by the father and its partner by the mother. The two members of a pair are called homologous chromosomes. An individual is either homozygous if the same allele is present at the same locus on each **homologous chromosome** or is designated as **heterozygous** if alleles at a particular locus on the homologs are different. To indicate the **genotype** (the genetic makeup) of an individual, the general conventions discussed in [Chapter 1](#) are followed. For example, a *Drosophila* mutant fly with 'vestigial' wings is symbolized as vg/vg when homozygous for the vg mutant allele. A fly with normal wings (generally referred to as wild type or WT) can be either **homozygous** (vg^+/vg^+) for the **dominant** vg^+ WT allele or vg^+/vg **heterozygous**. Since the vg^+ allele is dominant over the recessive vg allele, the vestigial wing phenotype is not visible, due to recessive nature of the mutant allele, in vg^+/vg heterozygous flies.

Across involving only one character/trait at a time is referred to as a **monohybrid cross** while in a **dihybrid cross**, inheritance of two different characters/ traits is examined. Mendel's postulated **laws of segregation** and **independent assortment** parallel the behavior of homologous and non-homologous pairs of chromosomes during meiosis, which provides

the chromosomal basis of inheritance. The Mendelian 9:3:1:1 ratio expected in dihybrid cross is not realized if the concerned genes exhibit phenomena like ‘linkage’ (<https://www.nature.com/scitable/definition/linkage-51/>) or ‘epistasis’ (<https://www.nature.com/scitable/definition/epistasis-222/>). Other types of genetic crosses like test and reciprocal crosses (see below) are carried out to understand the basis for such deviations from the expected Mendelian ratios (see **Chapters 6, 8**).

In a typical monohybrid cross (e.g., WT and vestigial winged flies), sorting of F_2 flies on the basis of their genotypes is not feasible since both the homozygous vg^+/vg^+ and heterozygous vg^+/vg flies display normal wing. A **test cross** is used in such cases to determine if the individual in question is heterozygous or homozygous for a particular dominant allele. In a test cross, an individual of unknown genotype is crossed with an individual homozygous for the recessive allele (for the character/trait under consideration). Thus a cross between normal winged F_2 flies in the above example of WT and vestigial winged monohybrid cross and a homozygous recessive vestigial winged (vg/vg) fly would be a test cross. On the other hand, if an individual from the progeny generation is crossed with any one of the two parental genotypes, it is called a *backcross*.

There are 8 chromosomes (4 pairs) in the somatic cells of *Drosophila melanogaster* (see **Chapters 1, 23**). Males and females differ in their **sex-chromosome** pair (XX and XY pairs in females and males, respectively) while they are similar for other 3 pairs, which are called autosomes. Genes located on X-chromosomes (**X-linked genes**) show different patterns of inheritance compared to genes on the **autosomes**, which becomes obvious when the phenotypic ratios seen in monohybrid cross involving an X-linked gene (e.g. *white eye* mutant) are compared in reciprocal crosses (see **Chapter 6**). For example, reciprocal crosses for a sex-linked ‘a’ allele (X^a) would involve examining progeny from X^a/Y male x X^{a+}/X^{a+} female and ii) X^{a+}/Y male x X^a/X^a female parents. A diploid male fly can never be heterozygous for an X-linked allele because they possess only a single X chromosome. Therefore, male flies are called **hemizygous** for X-linked genes.

It is a common experience that results (e.g., phenotypic ratios of progeny flies) obtained in a genetic cross may not match exactly with what is expected under a particular hypothesis. Mendel first introduced the concept of probability and how to ‘think mathematically’ in genetics or rather in biology per se. The legacy has continued and statistical analysis in genetics remains an essential component. For all the exercises described in this chapter, an appropriate statistical analysis (e.g. chi (χ^2)-squared test) is required to be carried out to compare the observed and expected results, and validate the hypothesis on which the expected values are derived (<https://www.nature.com/scitable/topicpage/genetics-and-statistical-analysis-34592/>).

Objective:

To set up and execute genetic crosses using mutant and WT *Drosophila melanogaster* flies to infer patterns of inheritance and to predict genotypes and phenotypes of parents and progeny.

Materials Required:

A. Flies: WT *Drosophila melanogaster* and mutant flies carrying mutant allele for a single trait or mutant alleles for two or more different traits (see **Chapter 3**).

Note for Instructor: genotypes of mutant stocks should not be disclosed to students at the beginning of the crosses; however, they should be informed about the mutant phenotype/s

(single or multiple) that they need to examine in the given set of crosses. Students should infer the parental genotypes and pattern of inheritance in each case by analysis of the progeny data.

B. Equipment and other fly handling items:

- i. Stereo-binocular microscope
- ii. Biological Oxygen Demand (B.O.D.) incubator or a room maintained at $23 \pm 1^\circ\text{C}$.
- iii. Etherizer, re-etherizer.
- iv. Fine hair brushes, forceps.
- v. White board for examining flies

Experimental Details:

A. Common steps for any genetic cross: For any type of genetic cross, the following basic steps are to be followed:

- i. Collect virgin female flies of the desired genotype following the methods described in **Chapter 1**. The mutant phenotypes are arbitrarily named here as **A** and **B** with respective gene symbols as a^+ (wild type or WT allele) or a (mutant allele), and b^+ (WT allele) or b (mutant allele), respectively (+ superscript indicating the dominant wild type allele). The mutant stock may carry mutant alleles for only one character **A** or **B** (single mutant) or may carry both a and b mutant alleles (double mutant). In each case, also set reciprocal cross by reversing sex of the WT and mutant (single or double mutant, as required) flies.
- ii. For setting up crosses take 5-10 pairs of male and female flies of the desired genotypes in food bottles, label and place them either in a B.O.D. incubator or in a room maintained at $23/24 \pm 1^\circ\text{C}$ temperature.
- iii. Remove the parent flies after 5-7 days when sufficient numbers of fertilized eggs are observed in the cross bottles. This is essential to avoid them from mating with the F_1 progeny flies.
- iv. Examine the F_1 flies after ~10-15 days and note down their phenotypes and numbers (record data for males and females separately) in the datasheet.
- v. Make a self-cross (brother-sister mating) by keeping 5-10 pairs of F_1 males and females in a fresh food bottle to obtain the F_2 generation.
- vi. After ~5-7 days, discard the parent (F_1) flies and wait for another 10-15 days to collect the F_2 progeny flies for examination of their phenotypes. The F_2 flies should be collected over a period of 7-8 days until all the F_2 progeny pupal cases are seen to be empty (this is necessary to avoid collection of any F_3 progeny flies).
- vii. Screen all the F_2 flies for their phenotypes and tabulate your data. Discard the F_2 flies after phenotypic examination.
- viii. Record the data and interpretations following the templates provided below.

B. Templates for observation tables:

Table 1: Template for all monohybrid crosses involving single gene difference (Phenotype A considered here).

P Generation cross Wild type (a^+/a^+) Female X Mutant (a/a) Male				
Date	Number of F₁ Progeny			
	WT Phenotype A		Mutant Phenotype A	
	Male	Female	Male	Female
F₁ cross (brother-sister mating) F₁ Male X F₁ Female				
Date	Number of F₂ Progeny			
	WT Phenotype A		Mutant Phenotype A	
	Male	Female	Male	Female
No. of flies				
Observed ratio				
Expected ratio				
Remarks after Statistical test				

- i. Prepare similar data sheet for reciprocal cross [Wild type (a^+/a^+) Male X Mutant (a/a) Female].
- ii. Based on the data and their statistical analyses, determine i) if the inheritance of mutant phenotype follows the law of segregation and ii) if the responsible gene is X-linked or not.

Table 2: Template for all dihybrid crosses involving two gene differences (Phenotypes A and B)

P Generation cross								
Wild type ($a^+/a^+ b^+/b^+$) Female X Double Mutant ($a/a b/b$) Male								
Expected gametes ↓ $a b$	a^+b^+							
	No. of Progeny and phenotype							
	WT A & WT B		WT A & Mutant B		Mutant A & WT B		Mutant A & B	
	Male	Female	Male	Female	Male	Female	Male	Female
F ₁ cross (brother-sister or self-cross)								
Wild type X Wild type								
Expected gametes ↓	a^+b^+		a^+b		$a b^+$		$a b$	
	No. of Progeny and phenotype							
	WT A & WT B		WT A & Mutant B		Mutant A & WT B		Mutant A & B	
	Male	Female	Male	Female	Male	Female	Male	Female
a^+b^+								
a^+b								
$a b^+$								
$a b$								
Total no. of flies								
Observed ratio								
Expected ratio								
Remarks after Statistical test								

i. Prepare similar data sheet for reciprocal cross [Wild type ($a^+/a^+ b^+/b^+$) Male X Double Mutant ($a/a b/b$) Female].

ii. Based on the data and their statistical analyses, determine a) if the inheritance of mutant phenotypes follows the law of independent assortment, and b) if anyone or both the responsible gene/s is/are X-linked.

iii. If the observed data do not agree with the law of independent assortment, is there evidence for a) linkage or b) epistatic or other interaction (see **Chapters 6, 8**)?

C. Test Cross for dihybrid crosses: If the results do not agree with Independent Assortment, carry out test cross between F₁ double heterozygous male/female ($a^+/a b^+/b$) with double recessive female/male ($a/a b/b$) and record the ratios of different phenotypic

classes for the characters **A** and **B** in the progeny (use template in **Table 3**). When two mutant alleles are considered, as in a test cross involving the dihybrid progeny, it is designated as 2-point test cross.

Table 3: Template for recording data for 2-point test cross using the F₁ progeny in a dihybrid cross

Test cross:	Test cross progeny		Conclusion/ Inference
	Expected genotypes*	Observed Phenotypes and their numbers	
F₁ double heterozygous progeny Female from cross in Table 2 $(a^+/a b^+/b)$ X Double recessive homozygous Male $(a/a b/b)$	1. $a^+/a b^+/b$:		
	2. $a/a b^+/b$:		
	3. $a^+/a b/b$:		
	4. $a/a b/b$:		

* If assorting independently, the four phenotypes [two parental (genotypes 1 and 4) and two recombinant (genotypes 2 and 3) types] in the above 2-point test cross are expected in 1:1:1:1 ratio.

i. If linkage is suspected on basis of observed results of the 2-point text-cross, calculate the map distances between genes *a* and *b* from frequencies of the recombinant classes ($a^+/a b/b$ and $a/a b^+/b$) in progeny of the 2-point test cross (cross between $a^+/a b^+/b$ and $a/a b/b$).

ii. Carry out a reciprocal test cross (mating between F₁ progeny male from cross in **Table 2** with the double recessive homozygous female). Compare results of the two test crosses.

Observations:

Enter the data in observation tables. Analyze and draw inferences about the patterns of inheritance involved in different crosses.

Additional Experiments:

An extension of the 2-point test cross, the 3-point test cross, first devised by A. H. Sturtevant in 1913, can be carried out between triple heterozygous (e.g. $a^+/a b^+/b c^+/c$) female and triple recessive ($a/a b/b c/c$) male individuals (see **Chapter 3** for triple mutants). In this case, 8 phenotypic classes are expected in the test cross progeny. If the three genes are assorting independently, all the 8 phenotypes in the test-cross progeny would be present with nearly equal frequency. However, if the three genes (*a*, *b* and *c*) are linked, there would be two parental types and six recombinant types (four single cross-over and two double cross-over) types in different frequencies. Use the observed frequencies of these different classes to determine the order of the three genes and the distances between them on the linkage map.

Questions for self-study and interpretation:

1. How does a test cross permit inference about genotype of the parents of the tested progeny?
2. Design appropriate genetic cross/es to determine if the Bar eye trait in *Drosophila melanogaster* is due to a dominant or incompletely dominant or recessive allele of an autosomal or sex-linked gene.
3. If a male fly with yellow body and curled wings (the yellow body and curled wing phenotypes are due to recessive mutations) is crossed with a grey-bodied and normal wing (phenotypically wild type) female, what would be the expected phenotypes of male and female F_1 progeny if the wild type female used in this cross was: a) homozygous for wild type alleles for both the traits; b) heterozygous for both mutant alleles, c) heterozygous for *yellow* but homozygous for wild type allele for *curled* wing gene, or d) homozygous for *yellow*⁺ but heterozygous for *curled* wing allele?
4. Under 'Additional Experiments' mentioned above, it is suggested to cross triple heterozygous (e.g. $a^+/a b^+/b c^+/c$) female and triple recessive ($a/a b/b c/c$) male individuals. Would you get same result if triple heterozygous (e.g. $a^+/a b^+/b c^+/c$) male and triple recessive ($a/a b/b c/c$) female individuals were crossed? Why or why not?

References:

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- Ranganath, H. A., Tanuja, M. T. (1999) Teaching and learning genetics with *Drosophila*. 3. Pattern of inheritance of autosome and sex chromosome linked genes/characters. Resonance 4: 78-87. <https://www.ias.ac.in/article/fulltext/reso/004/10/0078-0087>.
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Multitude of Effects of a Gene

Certain students of genetics inferred that the Mendelian units responsible for the selected character were genes producing only a single effect. This was careless logic. It took a good deal of hammering to get rid of this erroneous idea. As facts accumulated it became evident that each gene produces not a single effect, but in some cases a multitude of effects on the characters of the individual. It is true that in most genetic work only one of these character-effects is selected for study—the one that is most sharply defined and separable from its contrasted character—but in most cases minor differences also are recognizable that are just as much the product of the same gene as is the major effect.

(In: Morgan, T. H. 1935. The Relation of Genetics to Physiology and Medicine, *The Scientific Monthly*. 4: 5-18 https://www.jstor.org/stable/pdf/15918.pdf?casa_token=Ilgc-6rPx9kAAAAA:zkaNrdEzeZqyzw cxornb9Te2n3TqYPy_o8u8kEwOoNs4btYnbZLTsVkf4nKoTedthf_L5j8Ih79lxOEYpuwrXW6fTxoBFcwcLTSUpq6Ebb82fzOQdq8ZQg0).

Chapter 6

To study the pattern of inheritance of white eye phenotype in *Drosophila*

H.A. Ranganath (haranganath@gmail.com)

Centre for Human Genetics, Bangalore; *Drosophila* Stock Centre, University of Mysore, Mysore.

Recommended Level: UG/PG

Sub-discipline: Genetics/Cytogenetics

Background:

Mutations, the heritable changes, result in different phenotypes. The contrasting phenotypes of a given character, because of mutation, are a prerequisite to identify the gene/s and inheritance patterns. Over the years, geneticists have identified mutations for a very large number of characters in *D. melanogaster*. Consequently, *D. melanogaster* provides a very rich collection of mutant forms to study various patterns of inheritance. Pure breeding mutant lines are available for different mutant forms (see [Chapters 1](#) and [3](#)), which permits desired selective mating between flies with different contrasting characters and thus understand their inheritance pattern and how a given phenotype is generated. Genetically defined stocks are available at *Drosophila* Stock Centre, Department of Zoology, University of Mysore, Manasagangotri, Mysore 570006.

The present chapter takes the white eye phenotype in *Drosophila melanogaster* as a model to explore some basic aspects of inheritance, like segregation, independent assortment, sex-linkage and gene interaction (Ranganath, 1999; Ranganath and Tanuja, 1999 a, b). This supplements the lessons learnt from experiments about various crosses (see [Chapter 5](#)).

Objectives:

Uncovering genetic features of mutations and deriving genotypes of parents by following their inheritance patterns and generation of phenotypes, using the white eye phenotype.

Materials Required:

A. Flies:

- i. Wild type (WT) *D. melanogaster* flies (e.g., Oregon S or Oregon K or Oregon R or Canton S or any other with red eyes).

- ii. Two different stocks showing the white eye phenotype, coded here as **Stock I** and **Stock II**.

Note for instructor: Genotypes of the two stocks are not to be disclosed to the students before they interpret results of the crosses performed by them. The two white eye phenotype stocks can be obtained from the Stock Centre at University of Mysore and must be maintained separately, without any cross-contamination.

B. Equipment and other items:

- i. Stereo-binocular microscope.
- ii. Food bottles and other routine items for fly handling.

Experimental Details:

A. Experiment 1: Wild type (WT) flies (red eyes) X Flies with white eyes (Mutant Stock I)

- i. Set up reciprocal crosses between WT (red-eyed) males/females (virgin) with white eyed **Stock I** females/males.
- ii. Discard parental flies after a week so that they do not get mixed up with F₁ progeny.
- iii. Collect F₁ progeny. Count and record the numbers of flies with different eye colours in each sex as in the **Table. 1** below.

Table 1. Table for recording data on progeny in Experiment I.

Generation	Phenotypes		Phenotypes	
Parental cross	<i>Cross a.</i> WT ♀ X Stock I (White eye) ♂		<i>Cross b.</i> WT ♂ X Stock I (White eye) ♀	
F ₁ Progeny	Red eye♀: ? White eye♀: ?	Red eye♂: ? White eye♂: ?	Red eye♀: ? White eye♀: ?	Red eye♂: ? White eye♂: ?
F ₁ Cross	F ₁ Red eye ♀ X F ₁ Red eye ♂#		<i>Cross b.</i> F ₁ Red eye ♀ X F ₁ Red eye ♂ #	
F ₂ Progeny	Red eye♀: ? White eye♀: ? Other eye colour/ s♀: ?*	Red eye♂: ? White eye♂: ? Other eye colour/ s♂: ?*	Red eye♀: ? White eye♀: ? Other eye colour/ s♀: ?*	Red eye♂: ? White eye♂: ? ?Other eye colour/s♂: ?*
<p>? = Record the numbers of flies of the given phenotype in the F₁ and F₂ progenies. # = If in the F₁ progeny of a given cross, no red eyed males are available, use white eye F₁ males to cross with F₁ Red eye female and record the same. * = Record the number of F₂ progeny. If flies with eye colours other than red or white are present, record the numbers of each colour type in males and females.</p>				

B. Experiment 2: WT flies (Red eyes) X Flies with white eyes (Mutant Stock II

- i. Set up reciprocal crosses between wild type (WT, red-eyed) males/females (virgin) with white eyed **Stock II** females/males.
- ii. Discard parental flies after a week so that they do not get mixed up with F₁ progeny.
- iii. Collect F₁ progeny. Count and record the numbers of flies with different eye colours in each sex as in the **Table. 2** below.

Table 21. Table for recording data on progeny in Experiment 2.

Generation	Phenotypes		Phenotypes	
Parental cross	<i>Cross a.</i> WT ♀ X Stock II (White eye) ♂		<i>Cross b.</i> WT ♂ X Stock II (White eye) ♀	
F ₁ Progeny	Red eye♀: ? White eye♀: ?	Red eye♂: ? White eye♂: ?	Red eye♀: ? White eye♀: ?	Red eye♂: ? White eye♂: ?
F ₁ Cross	F ₁ Red eye ♀ X F ₁ Red eye ♂ #		F ₁ Red eye ♀ X F ₁ Red eye ♂ #	
F ₂ Progeny	Red eye♀: ? White eye♀: ? Other eye colour/s ♀: ?*	Red eye♂: ? White eye♂: ? Other eye colour/s ♂: ?*	Red eye♀: ? White eye♀: ? Other eye colour/s ♀: ?*	Red eye♂: ? White eye♂: ? Other eye colour/s ♂: ?*
? = Record the numbers of flies of the given phenotype in the F ₁ and F ₂ progenies. # = If in the F ₁ progeny of a given cross, no red eyed males are available, use white eye F ₁ males to cross with F ₁ red eye female and record the same. * = Record the number of F ₁ progeny with red and white eyes. If flies with eye colours other than red or white are present, record the numbers of each colour type in males and females.				

Observations:

1. Analyse the data for F₁ and F₂ progeny and interpret the observations with respect to the following:

- i. Is there evidence for dominance of any allele? If yes, which one and why?
- ii. Do the F₂ progeny data in any of the **Experiments 1 and 2** above provide evidence for **Segregation** or **Independent Assortment** of alleles/genes?
- iii. Is there evidence for sex-linked pattern of inheritance in any of the above crosses?
- iv. In one of the above two experiments, some of the F₂ progeny show eye colours different from red and white. How many types of eye colours are seen in this set of progeny? Using the description and images of mutant flies in **Chapters 1, 3**), identify the mutant genes that may be responsible for the different eye colours in these flies.

- v. What are the genotypes of the parental generations in each of the two sets of crosses?
- vi. Why are different eye colours (that were not present in Parental or F₁ flies) seen in some of the F₂ progeny in one the above crosses? Which principle/s of inheritance and gene expression can explain these results?

Questions for self-study and interpretation:

1. How is the inheritance of autosomal and sex linked genes different?
2. How many classes of pigments are present in wild type 'red' eyes?
3. Why should the *w* mutant allele (when homozygous in females and hemizygous in males) generate white eye colour?
4. Learn about the biochemical pathways that are responsible for the production of eye pigments in *Drosophila*.

References and Further Reading:

Ranganath, H. A. (1999) Teaching and learning genetics with *Drosophila* as a model system. Resonance. 4: 48–52.

Ranganath, H. A. and Tanuja, M. T. (1999a) Teaching and learning genetics with *Drosophila*: 2. Mutant phenotypes of *Drosophila melanogaster*. Resonance. 4: 95–104.

Ranganath, H. A. and Tanuja, M. T. (1999b) Teaching and learning genetics with *Drosophila*: 3. Pattern of inheritance of autosome and sex chromosome linked genes/characters. Resonance. 4: 78–87.

Chapter 7

Demonstration of X-linkage of tissue specific proteins

S. R. Ramesh¹* (rameshuom@gmail.com) and K. Ravi Ram² (raviram@iitr.res.in)

1. Department of Studies in Zoology, University of Mysore, Manasagangotri, Mysuru 570006;
Current address: Chief Scientist, Department of Life Sciences, Pooja Bhagavat Memorial Mahajana Education Centre,, K. R. S. Road, Mysuru 570016.

2. Embryotoxicology Laboratory, Environmental Toxicology Group, CSIR- Indian Institute of Toxicology Research, Vishvgyan Bhavan, 31 MG Marg, Lucknow 226001

* For correspondence

Recommended Level: PG/Research

Sub-discipline: Genetics/Molecular Biology

Background:

The chromosomal sex-determining mechanism in *Drosophila* is XX-XY type, with males being heterogametic and females homogametic (XX). Thus the progeny males inherit their X-linked genes exclusively from the female parent, while the females receive their X-linked genes from both the parents. Classical procedure to validate the inheritance of X-chromosomal genes include conducting reciprocal crosses to compare the phenotypes of heterozygous F₁ females and hemizygous F₁ males (see [Chapters 5, 6](#)). If the phenotypes observed in reciprocal crosses are different, it suggests that the trait may be sex-linked.

The experiment described here for demonstrating X-linkage of tissue-specific proteins has been designed by taking advantage of the following:

1. *D. nasuta nasuta* and *D. nasuta albomicans* sub-species can be crossed to produce F₁ hybrid progeny.
2. The glue proteins in *Drosophila* are expressed in a tissue and stage specific manner in the larval salivary gland cells, and are stored as granules in the cytoplasm. At the end of third instar larval stage, these granules are secreted into lumen of the gland. The secretions from the lumen are extruded to the exterior shortly before pupariation. These proteins are supposed to be involved in affixing the puparium to the substratum (Prithi *et al.*, 2016).
3. The major glue proteins in *D. n nasuta* and *D. n albomicans*, show differential mobility on SDS-PAGE; as a result of which the parental origin of the glue proteins can be easily identified in the F₁ hybrid progeny (Ramesh and Kalisch, 1989).
4. Since the major glue protein fractions are very prominent, one pair of larval or prepupal salivary glands is sufficient for preparing the sample and visualizing their electrophoretic patterns (Ramesh and Kalisch, 1987).

5. Accessory glands in adult male fly are part of the male reproductive system in *Drosophila* (see **Chapter 2**). The accessory glands synthesize and secrete a variety of proteins that constitute seminal fluids and along with sperm are transferred to the female during mating. The accessory gland protein fractions in *D. n. nasuta* and *D. n. albomicans* are categorized into three groups (Group I, II & III) based on their mobility in SDS-polyacrylamide gels (Ravi Ram and Ramesh, 2001).

6. The 25 kDa accessory gland secretory protein fraction (member of the group III) is seen in *D. n. albomicans* but not in *D. n. nasuta*. Likewise, the 26 kDa fraction is seen only in *D. n. nasuta* but not in *D. n. albomicans* (Ravi Ram and Ramesh, 2001).

7. Analysis of these differential protein fractions in the F₁ progeny can provide information about sex-chromosomal linkage of genes encoding these fractions.

The given protocol is based on the original methods described in Ramesh and Kalisch (1988) and Ravi Ram and Ramesh (2001).

Objective:

Identification of sex-linkage of genes that encode major glue proteins or accessory gland protein fractions in *D. nasuta nasuta* and *D. nasuta albomicans* by SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) technique.

Materials Required:

A. *Drosophila* Stocks:

i. *D. nasuta nasuta* (*Drosophila* Stock Centre, Mysore University) Catalogue No. 201.001 or 201.009).

ii. *D. nasuta albomicans* (*Drosophila* Stock Centre, Mysore University) Catalogue No. 202.001).

These stocks can be obtained from The Chairman, Department of Studies in Zoology, University of Mysore, Manasagangotri, MYSURU – 570 006, Karnataka.

B. Ringer's solution:

i. Sodium chloride 750 mg

ii. Potassium chloride 35 mg

iii. Calcium chloride 20 mg

iv. Sodium bicarbonate 20 mg

Dissolve in 100 mL distilled water

C. Sample buffer

Following sample buffers have been standardized for optimal resolution of the protein patterns from the two different tissues:

i. For larval salivary glands: 0.0625 M Tris-HCl (pH 6.8) with 1% SDS, 1% β-mercaptoethanol, 10% Glycerol, and 0.001% Bromophenol Blue dye.

ii. For male accessory glands: 0.0625 M Tris-HCl (pH 6.8) with 5% SDS, 5% β-Mercaptoethanol, 10% Glycerol and 0.001% Bromophenol Blue dye. The increased

quantities of SDS and Mercaptoethanol are basically to facilitate complete denaturation of higher protein levels in the accessory glands.

D. Solutions for preparation of gels:

- i. *Lower gel buffer* 18.15 gm of Tris (hydroxymethyl) aminomethane (Tris) dissolved in and made up to 100 mL with glass distilled water, pH adjusted to 8.8 with conc. HCl.
- ii. *Upper gel buffer* 6.0 gm of Tris dissolved in and made up to 100 mL with glass distilled water, pH adjusted to 6.8 with conc. HCl.
- iii. *Acrylamide solution*: 22 gm Acrylamide and 0.8 gm bis-acrylamide dissolved in and made up to 100 mL with glass distilled water.
- iv. *Ammonium persulfate*: 1% solution (to be prepared fresh).
- v. *Sodium dodecyl sulfate*: 20% solution in glass distilled water.

E. Tray or Running (0.05 M Tris, 0.384 M Glycine) buffer 6.057 gm of Tris, 28.8 gm of Lysine and 1 gm of SDS dissolved in 500 mL glass distilled water and the volume is made up to 1 Lt. using glass distilled water. pH adjusted to 8.3.

F. Pre-staining solution: 50% TCA and isopropanol (1:1) to enhance resolution of the low molecular weight protein fragments.

F. Stain: 0.175% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid.

G. Destaining solution: Equal volumes of 25% methanol and 7.5% acetic acid

H. Equipment:

- i. Electrophoresis unit (including gel casting unit, notched and rectangular glass plates, combs, spacers, etc.).
- ii. Electrophoresis power supply along with electrode cables.
- iii. Orbital/Dancing shaker.
- iv. Stereo binocular microscope.
- v. Microcentrifuge.

I. Plasticware/Glassware:

- i. Petri plates or plastic tray.
- ii. Micropipettes (20 and 100 μ L and corresponding disposable tips).
- iii. Conical flasks.
- iv. Dilution flasks.
- v. Measuring cylinders.

Experimental Details:

A. Preparation of protein samples from larval salivary glands:

- i. Collect 5 days old unmated males and virgin females from the *D. nasuta nasuta* and *D. nasuta albomicans* parental stocks to set reciprocal inter-specific crosses (*D. n. nasuta* males X *D. n. albomicans* females and *D. n. nasuta* females X *D. n. albomicans* males). Grow the two parental species and collect progeny in parallel.
- ii. Grow the progeny larvae (two parental species and two inter-specific hybrids) on standard food at 22/24±1°C.
- iii. Prepare protein samples for SDS-PAGE from third-instar larval salivary glands of parental and F₁ hybrid larvae as follows:
 - a. Wash well-grown third instar larvae with distilled water to remove adhering food particles.
 - b. Segregate male and female larvae based on the size of gonads (Males have large gonads; while females have smaller gonads (see [Chapter 28](#)).
 - c. Dissect larvae to isolate salivary glands in the Ringer solution.
 - d. Transfer a pair of salivary glands into a 1.5 mL microcentrifuge tube in ~0.5 mL cold 10% Trichloroacetic acid (TCA) and keep on ice for 20 min.
 - e. Replace TCA with 95% ethanol and shake/vortex the microcentrifuge gently for 15 sec; leave it for 20 min.
 - f. Replace 95% ethanol with 0.5 mL of a mixture of methanol and chloroform (1:1), shake/vortex for 15 sec and leave it for 20 min.
 - g. Discard methanol: chloroform mixture and dry the tissue at 37°C (15 min).
 - h. Add 30 µL of sample buffer to the dried tissue and leave it for 2-3 hr.
 - i. Heat the sample in boiling water bath for 10 min and cool to room temperature.
 - j. Centrifuge for 3 min at 1,000 rpm.

Note: The above prepared sample can be stored overnight at room temperature. Next day it is heated in boiling water bath for 10 min, cooled to room temperature and then centrifuged for 3 min at 1,000 rpm. If required, the samples can be stored at -20°C for 8-10 days before use as above.

B. Preparation of protein samples from prepupal salivary glands:

- i. Set up different crosses as above for larval salivary glands.
- ii. Grow the progeny larvae (two parental species and inter-specific hybrid) on standard food at 22/24±1°C and collect freshly formed prepupae.
- iii. Dissect a pre-pupa to take out salivary glands, which are filled with secretions (**Fig. 1A**) in the Ringer's solution and treat the glands with 95% ethanol to precipitate the secretions as a plug within the salivary gland (**Fig. 1B**).
- iv. Using fine needles, carefully take out the plug and discard the salivary gland debris (**Fig. 1C**).

Note: By using the plugs, the salivary gland cellular proteins are eliminated from the sample.

- v. Proceed further following the steps iii. f-j above for larval salivary gland sample preparation.

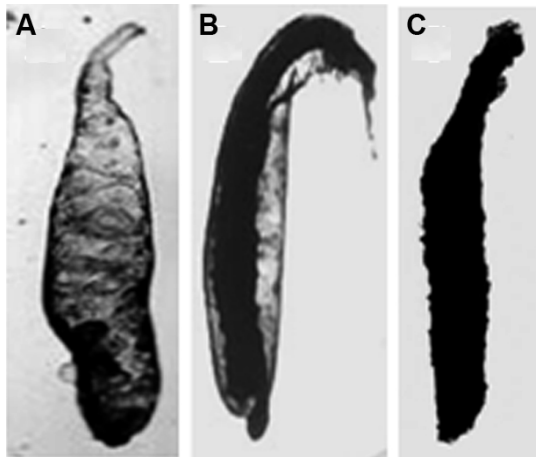


Fig. 1. Preparation of prepupal salivary gland secretion plug. A. Pre-pupal salivary gland of *D. n. nasuta* filled with secretion. B. Pre-pupal salivary gland treated with 95% ethanol to precipitate in situ the secretion which is seen as a plug. C. Secretion removed from the salivary glands as a plug.

C. Preparation of protein sample from adult male accessory glands:

- i. Set up reciprocal crosses as above. After mating for a day, remove the males and allow the female to lay eggs to obtain the F₁ progeny.
- ii. Isolate unmated F₁ male flies from the cross vials within 2-3 hr of their eclosion from the puparium. Transfer these F₁ males to culture vials containing fresh food and age them for 7 days.
- iii. Transfer the 7 days old F₁ progeny males to an empty glass vial and plug with cotton. Place the vial in -20°C freezer or stick the vial in ice bucket (ensuring that cotton plug does not sink into the ice) for 20 min to immobilize them.
- iv. Prepare the protein samples from accessory glands of these males using the following steps:
 - a. Place a male on a slide with the ventral surface facing up in a drop of chilled Ringer's solution. Using a stereo-binocular microscope, hold the first abdominal segment of the fly gently with a needle/forceps, and cut open and pull the last abdominal segment/genital plate of the male with another needle/forceps so that the male reproductive system comprising of the coiled testes, accessory gland, ejaculatory duct, etc (see [Chapter 2, 107](#)) becomes visible. With the help of needle/forceps, dissect out the accessory glands (transparent S shaped structures as in [Fig. 13](#) in [Chapter 2](#)) by cutting at the ejaculatory duct and clearing the testes. Care should be taken not to puncture the accessory glands as that would lead to leakage of proteins from the gland and loss of the sample.
 - b. With the help of needle/forceps, carefully transfer the glands from the slide to the microfuge tube containing 95% ethanol. Upto five pairs of accessory glands can be transferred into 50 µL of ethanol. Allow the glands to remain in 95% ethanol for 20 min.
 - c. Replace 95% ethanol with 100 µL of a mixture of methanol and chloroform (1:1) 3 times at an interval of 10 min each.
 - d. Discard the methanol: chloroform mixture and dry the tissue by keeping microcentrifuge tubes, with lids open, at 37°C for 30 min.
 - e. Add sample buffer to the dried tissue (20 µL per pair of accessory glands).
 - f. Leave overnight at room temperature.
 - g. Before loading samples into the gel slots, heat the sample tubes in a boiling water bath for 10 min, cool at room temperature, centrifuge for 10 min at 1000 rpm

D. Electrophoresis:

i. Prepare the stacking gel or upper gel or large pore gel, and separating gel or lower gel or small pore gel solutions by mixing different components as in **Table 1**.

Table 1. Composition of upper and lower gel solutions

	Lower gel	Upper gel
Lower gel buffer	7.5 mL	---
Upper gel buffer	---	4.0 mL
Acrylamide solution	18.0 mL	4.0 mL
Distilled water	4.5 mL	7.76 mL
Ammonium persulfate	200 μ L	160 μ L
SDS	225 μ L	240 μ L
TEMED	20 μ L	16 μ L

Note: The amounts of gel solution mixtures shown in **Table 1** are enough for casting a 15 (height) X 10 (width) X 1 (thickness) cm gel (for preparation and casting of gel, see **Chapter 66**, and Ostermann, 1984). Since this mixture produces a high percentage gel, it is preferable to cast the gels at an ambient temperature below 25°C. The gel thus prepared may be used immediately or may be stored at 8-10°C for 24-48 hr.

ii. Assemble the vertical gel apparatus (see **Chapter 66**) and load 20 μ L of the above prepared samples into different slots of the gel using a micropipette (use fresh tips for loading each sample). Carefully add the tray buffer (running buffer) in the lower and upper chambers.

iii. Perform electrophoresis at 100 volts (35 mA maximum) until the Bromophenol Blue dye front reaches near the end of gel.

iv. Stop the power supply, remove the gel assembly from the tank. Remove the gel by separating the glass plates and carefully place the slippery gel in a suitable tray for fixing and staining.

v. Treat the gel overnight with pre-staining solution to fix and better detection of low molecular weight protein fractions.

vi. Next day, remove the pre-staining solution and add 0.175% Coomassie Brilliant Blue R-250 solution for 2 hr.

vii. Destain the gel in destaining solution until the protein bands become distinct.

viii. Record the protein patterns using a Camera.

Note: Carrying out the prestaining, staining and destaining steps on an orbital/dancing shaker provides uniformity in staining and destaining and thus provides a better resolution of protein patterns.

Results/Observations:

A. Inheritance patterns of larval salivary gland Glue proteins:

- i. Examine the staining patterns of the gel (for comparison, the SDS-PAGE patterns of protein fractions from the salivary glands is shown in Fig. 2). The prominent bands (43, 35, 28 and 23 kDa) are the glue protein bands.
- ii. Compare the patterns of these bands in F_1 male and female hybrid progeny with those in the parental species.
- iii. Interpret the mode of inheritance of the 43 kDa and 28 kDa glue proteins in *D. n. nasuta* and 35 kDa and 23 kDa glue proteins in *D. n. albomicans* and their dominance relationship.

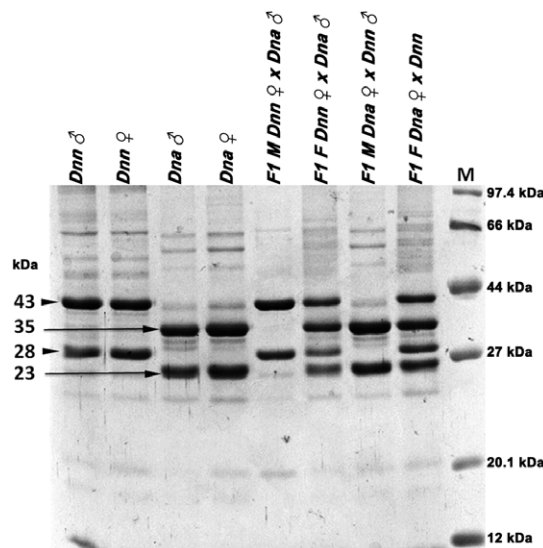


Fig. 2. Determining linkage of major glue protein fractions in *D. n. nasuta* (*Dnn*) and *D. n. albomicans* (*Dna*). Patterns of Coomassie Brilliant Blue stained proteins from third instar larval salivary glands of male and female larvae of the two parental species (lanes 1-4) and their F_1 hybrids (lanes 5-8) as indicated above each lane. $F_1 M$ and $F_1 F$ (above lanes 5-8) represent F_1 hybrid male and female progeny, respectively, of the crosses. Lane **M** shows the Marker proteins (sizes in kilodaltons, kDa). Sizes (in kDa) of the four major glue proteins are indicated on left with arrow heads pointing to *D. nasuta nasuta* (*Dnn*) and long arrows to the *D. nasuta albomicans* (*Dna*) specific glue protein bands. Image modified from Prithi and Ramesh (2016).

B. Inheritance patterns of male accessory gland protein:

- i. Examine staining patterns of the gel (for comparison, the SDS-PAGE patterns of protein fractions from the male accessory glands are shown in Fig. 3).
- ii. Compare the patterns of these bands in F_1 male and female hybrid progeny with those in the parental species.
- iii. Interpret the mode of inheritance of the 26 kDa and 25 kDa accessory gland proteins in *D. n. nasuta* and *D. n. albomicans* and their dominance relationship.

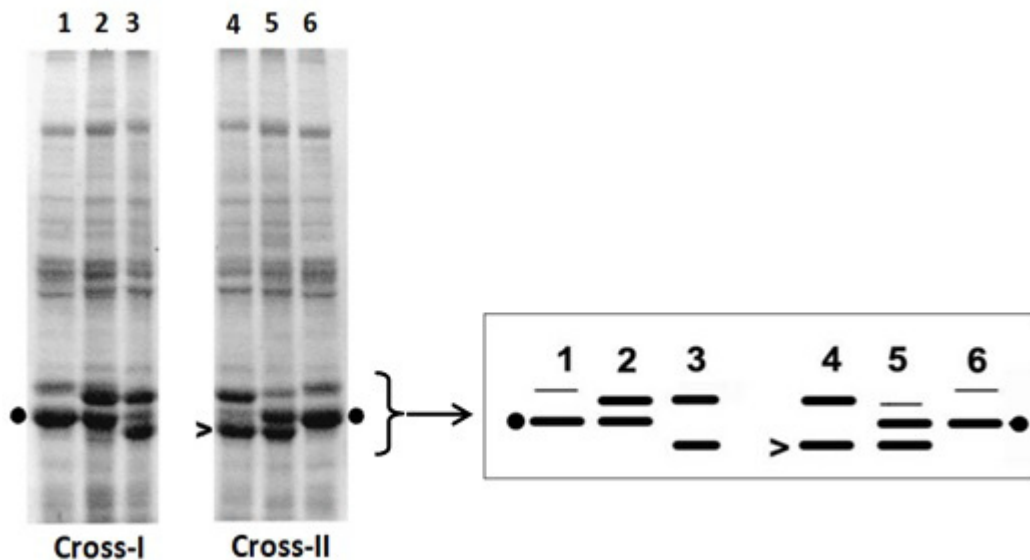


Fig. 3. SDS-PAGE protein patterns showing X-linked or autosomal protein fractions from the male accessory glands of *D. n. nasuta*, *D. n. albomicans* and their hybrids. **Lanes 1 and 6:** *D. n. nasuta* male; **lane 2:** F₁ male from the *D. n. nasuta* ♀♀ X *D. n. albomicans* ♂♂ cross [Cross-I]; **lanes 3 and 4:** *D. n. albomicans* male; **lane 5:** F₁ male from the *D. n. nasuta* ♂♂ X *D. n. albomicans* ♀♀ cross [Cross-II]. A schematic of the protein bands of interest is shown on the right. ● indicates the 26 kDa and > indicates the 25 kDa protein bands. Figure partially modified and reproduced with permission from Ram and Ramesh, 2001).

Questions for self-study and interpretation:

1. How does the inheritance pattern of sex-linked genes differ from that of an autosomal gene?
2. What factors influence mobility of proteins on a SDS-polyacrylamide gel?
3. Why is SDS used in these gels?
4. What are functions of glue proteins?
5. What are functions of male accessory gland proteins?

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Morgan on Chromosomes and Heredity in 1910

WE have come to look upon the problem of heredity as identical with the problem of development. The word heredity stands for those properties of the germ-cells that find their expression in the developing and developed organism. When we speak of the transmission of characters from parent to offspring, we are speaking metaphorically; for we now realize that it is not characters that are transmitted to the child from the body of the parent, but that the parent carries over the material common to both parent and offspring. This point of view is so generally accepted to-day that I hesitate to restate it. It will serve at least to show that in what I am about to say regarding heredity and the germ-cells. I shall ignore entirely the possibility that characters first acquired by the body are transmitted to the germ.

.....

The case of color blindness in man appears to follow the same rule, for here also females may transmit the character without developing it, while males, if they have it at all, develop color blindness. One dose of color blindness in males makes the male color blind; one dose in females is insufficient. The rarity of color-blind females is explicable on this view.

.....

Science advances by carefully weighing all of the evidence at her command. When a decision is not warranted by the facts, experience teaches that it is wise to suspend judgment, until the evidence can be put to further test. This is the position we are in to-day concerning the interpretation of the mechanism that we have found by means of which sex is determined

(In: Morgan, T. H. 1910. Chromosomes and Heredity. *The American Naturalist*. 44: 449-496

<https://www.jstor.org/stable/pdf/2455783.pdf>)

Chapter 8

Studying the pattern of inheritance of linked genes and preparing linkage map

H. A. Ranganath (haranganath@gmail.com)

Centre for Human Genetics, Bengaluru; *Drosophila* Stock Centre, University of Mysore.

Recommended Level: UG/PG

Sub-discipline: Genetics/Cytogenetics

Background:

After the rediscovery of Mendelian principles, Cytologists and Geneticists began to see parallels between the behaviour of chromosomes during cell division and the expected behaviour of Mendel's Factors. Around 1902, Walter S. Sutton, Theodor Boveri and others independently noted these parallels which led to the *Chromosomal Theory of Inheritance*, which implied that Mendelian genes are located on chromosomes and the laws of Segregation and Independent Assortment depended upon the behavior of chromosomes during meiosis. The important evidence for establishing the correspondence between the pattern of inheritance of a phenotype with the transmission of a particular chromosome through meiosis was provided by studies following T.H. Morgan's isolation of a white eye mutant in *D. melanogaster*, and subsequent uncovering of the pattern of this mutant's inheritance. He suggested that the inheritance of the white and red eye colour phenotype is linked to X-chromosome, with white and red phenotypes being due to different alleles namely 'w' and 'w⁺', respectively. C.B. Bridges of Morgan's team, through his elegant experiments involving non-disjunction of chromosomes during meiosis, provided the definitive proof for the association of X-chromosome and the eye color gene. Thus, the abstract genetic factors postulated by Mendel were finally localised on the visible structures (chromosomes) within cells. With this, the principles of segregation and independent assortment could be explained in terms of behaviour of chromosomes during meiosis, as also previously suggested by Sutton and Boveri.

T.H. Morgan's team isolated and studied pattern of inheritance of many mutations for different phenotypes of the fly. These studies laid a strong foundation for the fundamentals of genetics, including the concepts of linkage, crossing over and genetic maps. Earlier Bateson and Punnett had used the terms 'coupling and repulsion' to indicate linkage and crossing over.

D. melanogaster has 4 pairs of chromosomes ((X/Y, 2, 3 and 4; 2n=8). Each chromosome, represents one linkage group so that this species has 4 linkage groups. Since the number of genes is much higher than the linkage groups, each chromosome or linkage group of the fly must carry many genes. Therefore, it was necessary to learn about inheritance and arrangement of different genes on chromosomes. A.H. Sturtevant, a graduate student

of T. H. Morgan's team, designed experimental strategy to uncover the sequence and approximate distance between genes located on a given chromosome. He devised the 3-point test cross and constructed the first genetic map of a chromosome in 1913.

This chapter describes setting up a 3-point test cross to determine the order of three autosomal genes on a linkage map and knowing the map distances between them. For mapping of X-linked genes, see [Chapter 9](#).

Objective: To understand the pattern of inheritance of linked genes and to construct linkage maps.

Materials Required:

A. Flies:

- i. Wild type (WT) *D. melanogaster* flies (e.g., Oregon S or Oregon K or Oregon R or Canton S).
- ii. Triple mutant strains of *D. melanogaster* carrying mutant alleles for three different genes (see [Chapter 3](#)). In the present example, the chromosome 3 (autosome) linked mutants, namely curled wings (*cu*), stripes (*sr*) on the thorax, and ebony body colour (*e*) are used. The genotype of this stock is *cu sr e*.

B. Equipment and other items:

Stereo-binocular microscope and other items required for fly handling.

Experimental Details:

The basic steps for performing genetic crosses are as given in [Chapter 5](#).

The example taken here is for determining linkage between three autosomal genes (for a summary of the workflow see Tables 1 and 2).

- i. Set up reciprocal crosses: **Cross A-** WT males (normal wing, no stripes, gray body; genotype $cu^+ sr^+ e^+ / cu^+ sr^+ e^+$) X triple mutant virgin females (curled wing, stripes, ebony body; genotype $cu sr e / cu sr e$), and **Cross B-** WT virgin females (normal wing, no stripes, gray body; genotype $cu^+ sr^+ e^+ / cu^+ sr^+ e^+$) X triple mutant males (curled wing, stripes, ebony body; genotype $cu sr e / cu sr e$)
- ii. Discard parental flies after a week so that they do not get mixed up with the F₁ progeny.
- iii. Collect F₁ progeny. Count and record the numbers of flies with different phenotypes in each sex as in Tables 1 and 2.
- iv. Set up Test crosses (for details, see [Chapter 5](#)) as in the Tables 1 and 2.
- v. Discard F₁ parental flies after a week so that they do not get mixed up with the test cross progeny.
- vi. Collect the test cross progeny.

Observations

- Count and record the numbers of flies with different phenotypes in each sex for Crosses A and B as in the Tables 1 and 2, respectively.
- Analyse the progeny of the 4 test crosses, 2 from each of the parental reciprocal crosses.
- Compare the types of progenies of the Crosses A and B.
- Statistically compare the observed and expected (on independent assortment of the three mutant phenotypes) ratios (see Chapter 5).
- Are some combinations of phenotypes statistically more frequent than others?
- Are the types of progenies from the two test crosses for each cross (A and B) similar?
- Among the test cross progeny flies, which category of flies with new combinations of phenotypes are more frequent, less frequent, and least frequent.
- Based on the above, identify the single cross-over and double cross-over classes.
- Using the above data, determine the sequence in which these three genes are placed on the chromosome and the map distances between them.

Table 1 : Workflow for Parental Cross A

Parental Cross					
WT males: (normal wing, no stripes, gray body: $cu^+ sr^+ e^+ / cu^+ sr^+ e^+$)					
X					
Triple mutant virgin females: (curled wing, stripes, ebony body: $cu sr e / cu sr e$)					
F ₁ Progeny: ($cu^+ sr^+ e^+ / cu sr e$)					
Phenotypes	Numbers of progeny				
	Males		Females		
Wild type	?		?		
Mutant	?		?		
Test Cross					
F ₁ Male X Mutant parental type virgin female ($cu sr e / cu sr e$)		F ₁ Virgin female X Mutant parental type male ($cu sr e / cu sr e$)			
Numbers of test cross progeny of different phenotypes					
Males	Females	Progeny Phenotypes		Males	Females
		Normal wing, no stripes, gray body			
		Curled wing, stripes, ebony body			
		Curled wing, no stripes, gray body			
		Normal wing, stripes, ebony body			
		Curled wing, stripes, gray body			
		Normal wing, no stripes, ebony body			
		Normal wing, stripes, gray body			
		Curled wing, no stripes, ebony body			
TOTAL					

TABLE 2: Workflow fo Parental Cross B

Parental Cross					
WT virgin females: (normal wing, no stripes, gray body: $cu^+ sr^+ e^+ / cu^+ sr^+ e^+$)					
X					
Triple mutant males: (curled wing, stripes, ebony body: $cu sr e / cu sr e$)					
F ₁ Progeny: ($cu^+ sr^+ e^+ / cu sr e$)					
Phenotypes	Numbers of progeny				
	Males		Females		
Wild type	?		?		
Mutant	?		?		
Test Cross					
F ₁ Male X Mutant parental type virgin female ($cu sr e / cu sr e$)		F ₁ Virgin female X Mutant parental type male ($cu sr e / cu sr e$)			
Numbers of test cross progeny of different phenotypes					
Males	Females	Progeny Phenotypes		Males	Females
		Normal wing, no stripes, gray body			
		Curled wing, stripes, ebony body			
		Curled wing, no stripes, gray body			
		Normal wing, stripes, ebony body			
		Curled wing, stripes, gray body			
		Normal wing, no stripes, ebony body			
		Normal wing, stripes, gray body			
		Curled wing, no stripes, ebony body			
		TOTAL			

Questions for discussion and interpretations

1. Is there a difference in the types of progenies of the reciprocal test crosses? Why or why not?
2. Which combination of phenotypes are statistically least frequent among the test cross progenies, and why?
3. Is the linkage map and the map distances determined from your results in agreement with the standard genetic maps of *Drosophila melanogaster* (see Chapter 3).
4. How are the results from the 3-point testcross obtained in this exercise different from the results of exercise in Chapter 9.
5. What are the cytogenetic mechanisms responsible for the formation of new combinations of phenotypes (different from the parental types) among test cross progeny?
6. Would the results of similar crosses using the sex-linked genes ($y w f$) follow the same pattern? Why or why not?

7. What is 'co-efficient of co-incidence'? Calculate the same for your data.
8. What is learnt from the progenies of reciprocal crosses made in the above workflow?
9. How would you determine if the three different mutant phenotypes are controlled by one gene or different genes?
10. If the three genes were assorting independently, how many phenotypes of progeny you would expect in the test crosses and what would be the relative frequency of each phenotype?

References and Further Reading:

Ranganath, H. A. (1999) Teaching and learning genetics with *Drosophila*. 1. *Drosophila* as a model system. Resonance 4: 48-52. <https://www.ias.ac.in/article/fulltext/reso/004/02/0048-0052>.

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Strickberger, M. W. (1962) Experiments in genetics with *Drosophila*, John Wiley and Sons Inc., New York, London, Sydney.

Exception Establishes Rule

When Mendel observed an “independent assortment of traits” (Mendel’s second law), he was fortunate to have chosen traits that were not localized close to one another on the same chromosome. Subsequent studies revealed that many genes were indeed linked, ie., that traits did not assort or segregate independently, but that traits encoded by these linked genes were inherited together. Studying *Drosophila* genetics, T. H. Morgan showed that the degree of linkage increased with physical proximity of the genes and that the 4 genetic linkage groups actually corresponded to the presence of 4 chromosomes in *Drosophila*.

(In: Pulst, S. M. (1999). Genetic Linkage Analysis. JAMA Neurology. 56: 667– 672., DOI: 10.1001/archneur.56.6.667. <https://pubmed.ncbi.nlm.nih.gov/10369304/>).

Chapter 9

Calculating genetic distances by analyzing recombination frequencies from a trihybrid cross

Pradeep Kumar Burma (pburma@south.du.ac.in)

Department of Genetics, University of Delhi South Campus, Benito Juarez Road, New Delhi 110021

Recommended level: PG

Sub Discipline: Genetics

Background:

The ‘*three-point test cross*’ is a basic approach developed by Alfred H. Sturtevant in 1913 (Sturtevant, 1913) to prepare linkage maps (see Chapters 5, 8). This laid the foundation for mapping relative locations of genes on chromosomes not only in *Drosophila* but for all organisms.

An important component of a 3-point test cross is a strain that carries mutant alleles for three different genes. This has to be generated by recombining the different mutant alleles (see Chapter 11) before the genetic mapping can be carried out. Generating a triple mutant is a bottleneck in several organisms, which limits application of this strategy. In such cases genetic maps are created from F_2 and other types of mapping populations. In recent times genetic maps are also developed using molecular/DNA markers that could be dominant or codominant. A 3-point test cross, however, remains a good starting point to understand the basic nuances of genetic mapping:

- i. A test cross (see Chapter 1) allows analysis of genotypes of the gametes formed from a heterozygous parent, and thus permits a direct estimation of the percentage of parental-type (non-recombinant) and recombinant gametes.
- ii. An important feature of genetic maps, as proposed by Sturtevant, is its additive nature, i.e. if three genes were linked in the order $A...B...C$ and the distance between $A - B$ is ‘x’ and $B - C$ is ‘y’, then the distance between $A - C$ would be ‘x + y’. Three-point test cross also helps one to understand how double (even number) cross overs can lead to underestimation of genetic distances.
- iii. The phenomenon of ‘interference’, which arises because of a crossover inhibiting another nearby crossover event (McPeck and Speed, 1995) can also be assayed in this analysis.
- iv. Haldane (1919) suggested the use of term ‘Morgan’ to indicate the distance between linked genes: “It is suggested that the unit of distance in a chromosome as defined

above be termed as 'morgan', on the analogy of ohm, volt, etc. Morgan's unit of distance is therefore a centimorgan" (Haldane, 1919). The term centimorgan (cM) is as a unit of distance represented by 1% recombination between two linked genes

- v. The underestimation of distances between a pair of genes due to even crossovers can be corrected using mapping functions like that proposed by J. B. S. Haldane or that by D. D. Kosambi (Vinod, 2011)

This chapter considers genetic mapping of the X-chromosome linked (sex-linked) genes, which involves reciprocal parental crosses (I and II) between wild type/triple recessive mutant male and triple recessive mutant/wild type female flies. The F_1 progeny are sib-mated and the F_2 progeny analyzed to calculate genetic distances. In this experiment, the phenotypes have been chosen in a manner such that the example in cross I is a test cross, although F_1 progeny are being sib-mated. The cross II in the example allows analysis from progenies that are not resulting from a test cross. A previous chapter ([Chapter 8](#)) describes preparation of genetic maps using three-point test cross for autosome-linked mutants

Objective: To understand the concept of genetic maps through a trihybrid cross.

Materials Required

A. Flies

- i. *Drosophila melanogaster* wild type strain (e.g. Canton-S, Oregon-R)
- ii. A triple mutant strain (*y cv f*) which shows Yellow body (*y*), Crossveinless (*cv*), and Forked (*f*) bristles phenotypes ([see Chapters 1, 3](#)).

B. Equipment and other materials

Stereo-binocular microscope and other routine fly-handling materials for setting up and maintenance of crosses and their examination.

Experimental Details

A. Week 1 (Setting up the parental cross)

- i. Observe the wild type and the triple mutant flies (*y cv f*) under the stereo-binocular microscope. Draw the phenotypes observed. Use colour pencils if needed.
- ii. Set up reciprocal crosses in food vials or bottles between the wild and mutant (*y cv f*) flies. Use 5 virgin females and 3 males for each cross.
- iii. Mark the cross between the triple mutant females and wild type males as **Cross I** and the reciprocal cross (triple mutant males and wild type females) as **Cross II**. Record their phenotypes in **Table 1**.

B. Week 2

- i. After a week, discard the parental flies from vials/bottles of each set so that they do not get mixed up with the F_1 progeny.

- ii. Observe the vials/bottles under stereo-binocular microscope for presence of eggs and larvae. Record your observation.

C. Week 3 (analysis of the F₁ progeny and setting up the sib-mating crosses)

- i. Collect F₁ progeny. Count and record the numbers of flies with different phenotypes in each sex in **Table 1**.
- ii. Compare the F₁ progeny from the reciprocal crosses and record your inference in **Table 1**.
- iii. Record the **expected numbers** of males and females for each cross in **Table 1**.
- iv. Carry out Chi-squared test to check if the observed numbers are significantly different from the expected numbers. Note the Chi-square values, degrees of freedom and the associated p-value in **Table 1**.
- v. Record your inference based on the above in **Table 1**.
- vi. Separately sib-mate the F₁ progeny obtained from Crosses I and II. Take 5 females and 3 males for each cross.

D. Week 4

- i. After a week, discard F₁ flies so that they do not get mixed up with the F₂ progeny.
- ii. Keeping in mind that these crosses involve three different mutant phenotypes, write the different phenotypic combinations expected in the F₂ progeny in **Table 2** and **3** for Crosses I and II, respectively

E. Week 5 (analysis of the F₂ progeny)

- i. Observe the F₂ progeny for each of the two crosses, recording the number of flies observed for each phenotype. Record the results of cross I in **Table 2** and that of cross II in **Table 3**.
- ii. The tasks iii-vi below are for results from **cross I**. Based on information in Table 1, identify the parental (non-recombinant) phenotypic combinations and note the number of progenies with the parental genotypes in **Table 4** for **cross I**.
- iii. Identify phenotypes which have resulted due to recombination between i) *yellow* and *crossveinless*, ii) *crossveinless* and *forked*, and iii) between *yellow* and *forked*. Record the numbers of flies for each phenotype and their genotype in **Table 4**.
- iv. For each of the above recombinant classes (at S. No. **E.iv**), calculate the percentage of recombinants and record in **Table 4**.
- v. Based on observation in the step **E.iv**, determine the map distances between each pair of the characters in **Table 4**.
- vi. Based on the above determined map distances (recombination frequencies), infer the order of the genes responsible for the three mutant phenotypes. Mark the distances between each of the genes (**Table 4**).

- vii. Carry out the following tasks to complete the analysis.
- Is the distance between the outer-most genes equal to the additive distance between the first and the middle gene and that of the middle and third genes?
 - If not, identify the reason and calculate the corrected map distances.
 - The established locations (<https://www.flybase.org>) of the three genes on the genetic map are: $y - 0.0$; $cv - 13.7$; $f - 56.7$. Using this information check if your observed genetic distances match that from the published genetic map. If not, identify possible reasons for the difference.
 - Some of the expected phenotypes recorded in Table 2 arise due to recombination. For each of the phenotypes, identify those resulting from (i) no crossover events, (ii) single cross over (SCO) between y and cv , (iii) SCO between cv and f , and (iv) double crossover (DCO) between y and f . Record the recombination frequencies for each case. Also record the expected genotypes for each class.
 - Based on the observed frequencies of the two SCO classes, calculate the expected frequency of the DCO classes. Does the observed frequency of DCO match the expected DCO frequency? If not, calculate the Co-efficient of coincidence (COC) and Interference (I), using the formulae: $COC = \text{observed frequency of DCO} / \text{expected frequency of DCO}$, and $I = 1 - COC$.
 - Which among the Crosses I and II is a test cross?
 - Calculate the genetic distances from cross I based on cumulative observation of the whole class. Comment on any differences in results from individual versus cumulative observations.
- viii. Calculate the genetic distances between the three mutants on the basis of results (Table 3) obtained from cross II. Prepare a table similar to Table 4 and record your analysis.
- ix. Carry out the following tasks to complete the analysis.
- Is there any difference between the genetic distances obtained from cross I and II? If yes, what could be possible reasons?
 - Schematically represent the crosses carried out and the progeny obtained, mentioning their phenotype and genotypes.

Observations:

- Draw figures of wild type and various combinations of the mutant phenotypes.
- Record the data in following tables.

Table 1: Observations of parental crosses and F₁ progeny (Step No. in this and subsequent tables refers to the task in Experimental Details)

	Parental Cross I			Parental Cross II			
	Female		Male	Female		Male	
Phenotype (Step No. A.iii)							
F₁ progeny: observed phenotypes and genotypes (Step No. C.i)							
Cross I				Cross II			
Females		Males		Females		Males	
Phenotype	No.	Phenotype	No.	Phenotype	No.	Phenotype	No.
Genotype:		Genotype:		Genotype:		Genotype:	
Inference from comparison of the results of the reciprocal cross (Step No. C. ii):							
Chi- squared test (Steps. No. C.iii-v)							
	Cross I			Cross II			
Observed no.	Female =		Male =	Female =		Male =	
Expected no.	Female =		Male =	Female =		Male =	
Chi-square value							
Degree of freedom							
p- value (refer to the Chi-Square P-value Table)							
Inference							

Table 2: Observation of F₂ progeny from Cross I

Expected phenotypes (Step No. D.ii)	No. of progeny females for each phenotype (Step No. E.i)	No. of progeny males for each phenotype (Step No E.i)	Total No. of observed progeny flies fo each phenotype
Total No. of Progeny			

Table 3: Observation of F₂ progeny from **cross II**

Expected phenotypes (Step No D.ii)	No. of progeny females for each phenotype (Step No. E.i)	No. of progeny males for each phenotype (Step No. E.i)	Total No. of observed progeny flies for each phenotype
Total No. of Progeny			

Table 4: Calculating genetic distances based on results from **cross I**

	Parental (non-recombinant)	Recombinant between <i>y</i> and <i>cv</i>	Recombinant between <i>cv</i> and <i>f</i>	Recombinant between <i>y</i> and <i>f</i>
No. of progeny (Step Nos. E. ii and E. iii)				
% Recombinants (Step E.iv)				
Genetic distance (Step E.v)				
Order of genes (Step E.vi)				

Questions for self-study:

1. Collection of virgin females for the parental crosses is crucial for the success of the experiment. How can it be determined that the collected females are virgin?
2. What would be the major changes in experimental design of the current protocol, if a map were being developed for autosomal genes? (**Note:** There is no crossing over in males of *D. melanogaster*).
3. The fly strain used in this experiment has three mutant genes. The strain would have been developed by making appropriate crosses. Outline a strategy of developing this strain from stocks, each of which carries only one of the three mutations.
4. How many linkage groups are there in *D. melanogaster*?

5. It is said that the maximum percentage of recombinants that can be obtained from a test cross is fifty. However, the map lengths for any of the linkage groups in *D. melanogaster* are more than 50cM. How are these map lengths derived?
6. Some of the alleles of *crossveinless* (*cv*) arise due to P-element induced deletion. Assume that a 500bp deletion in the coding region of the wild type (*cv*⁺) leads to the Crossveinless phenotype. Design a protocol that can differentiate a mutant from a wild type allele at the level of the DNA.
7. Using the above DNA marker (Q. No. 6), how can one study the inheritance of the gene in the crosses carried out in the experiment. Is the designed DNA marker, dominant or co-dominant?
8. *Sepia* (*se*) and *curled* (*cu*) are mutant alleles of two genes affecting eye color and wing shape respectively. *se* and *cu* are located on chromosome 3 at map positions 26 and 50, respectively. These mutations give rise to recessive phenotypes. A cross is made between wild type and *sepia*, *curled* mutant flies. The F₁ progeny is sib-mated. What percentage of the progeny is expected to show (i) both Sepia and Curled phenotypes and (ii) only Sepia phenotype?

References and further readings:

Griffiths, A. J. F, Doebley J., Peichel, C., Wassarman, D. A. (2020) Introduction to Genetic Analysis 12th Edition, Macmillan International, New York, USA pg. 125 (*All editions of this book have a section titled 'Accounting for unseen multiple crossover' which deals with mapping function*).

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An Undergraduate's Insight Established the Foundations of Classical Genetics

In 1909 Castle published diagrams to show the interrelations of genes affecting the color of rabbits. It seems possible now that these diagrams were intended to represent developmental interactions, but they were taken (at Columbia) as an attempt to show the spatial relations in the nucleus. In the latter part of 1911, in conversation with Morgan about this attempt— which we agreed had nothing in its favor— I suddenly realized that the variations in strength of linkage, already attributed by Morgan to differences in the spatial separation of the genes, offered the possibility of determining sequences in the linear dimension of a chromosome. I went home and spent most of the night (to the neglect of my undergraduate homework) in producing the first chromosome map, which included the sex-linked genes *y*, *w*, *v*, *m*, and *r*, in the order and approximately the relative spacing that they still appear on the standard maps (Sturtevant, 1913).

(In: A. H. Sturtevant: A History of Genetics (California Institute of Technology), Chapter 7). <http://www.esp.org/books/sturt/history>).

Chapter 10

To study the balanced lethal system in *Drosophila melanogaster*

H. A. Ranganath (haranganath@gmail.com)

Centre for Human Genetics, Bangalore; *Drosophila* Stock Centre, University of Mysore.

Recommended Level: UG/PG

Sub-discipline: Genetics/Cytogenetics

Background:

Mutations are the raw materials for studying patterns of inheritance and other genetic studies. Many of the mutant strains used in the experiments to learn Mendelian principles such as law of segregation, independent assortment, linkage, interaction of genes, sex linkage etc. are homozygous for the concerned mutant alleles. They are pure/true breeding stocks. On the other hand, some mutations cannot be maintained in homozygous condition because they have recessive lethal effect on the flies so that they do not survive as homozygotes. Such mutations are called ‘recessive lethal’ mutations, and have to be maintained in heterozygous condition. Consequently, such stocks generate both normal as well as mutant progeny in each generation. Therefore, establishing a pure/true breeding strain for a recessive lethal allele is challenging.

The tools and techniques of the *Drosophila* genetics have evolved dramatically over the last century. One such tool that has stood the test of time is the ‘balancer chromosome’ (Miller *et.al.*, 2019). Muller (1917, 1918) pioneered balancer chromosomes (see [Chapter 1](#)). One of the homologous chromosomes carries an inversion while the other has the standard sequence. In this heterozygous state, if a meiotic recombination occurs between the two, the recombinant chromatids carry duplications and deletions so that the zygotes produced by these gametes are not viable but the gametes carrying chromatids which did not participate in the homologous recombination produce viable zygotes. The net effect is retention of the linear integrity of the parental combinations of alleles of different genes in this part of the chromosome. A chromosome with inversion is thus, designated as a ‘balancer chromosome’. A recessive lethal or sterility causing mutation in one gene can be maintained in a population if it is balanced with its homolog that also carries a recessive lethal or sterile mutation in another gene. This arrangement of mutations is called a “balanced system”. However, the meiotic recombination between these two mutations needs to be avoided since otherwise, a recombinant chromosome without any of these mutant alleles will be generated and the stock will be unstable. The Balancer chromosomes serve this purpose (see [Chapter 1](#)). Many mutations are known which have a dominant visible phenotype but are recessive for their lethality or sterility effect. Mutant alleles of this class affect the ratios of different

phenotypic/genotypic classes expected in the progeny on typical Mendelian inheritance but are important components of a balanced lethal system (see **Chapter 1**) and thus of great use in diverse genetic studies.

Objective:

To learn about balanced lethal system for maintenance of a recessive lethal mutation as a pure breeding strain.

Materials required:

A. Flies:

- i. Any wild type *D. melanogaster* stock maintained in laboratory.
- ii. Mutant stocks of *D. melanogaster* with “Curly Wings and Bristle” phenotype or strain with other mutant phenotype combination, e.g., “Lyra -Dichaete”.

Note for instructor: Genotypes of the stocks are not to be disclosed to the students before they interpret results of the crosses performed by them. These stocks can be obtained from the *Drosophila* Stock Centre at University of Mysore.

A. Equipment and other items:

- i. Stereo-binocular microscope.
- ii. Food bottles and other routine items for fly handling.

Experimental Details:

1. Start with a true breeding strain of *D. melanogaster* showing Curly wings and short thick bristle phenotypes and a wild type strain with normal wings and bristles.
2. Set up reciprocal crosses between wild type flies (with normal wings and bristles) and flies with Curly wings and Bristle phenotypes and obtain F₁ progeny.
3. Proceed further as instructed in the **Box 1** below:

Box 1. Analysis of the F₁ progeny and further crosses

Parents : Wild type <i>D. melanogaster</i> and Curly Bristle mutant (Genotypes of these are to be deduced from analysis of the results obtained)	
Reciprocal Parental crosses	
Curly wings and mutant bristle virgin Females X Wild type males	Curly wings and mutant bristle males X Wild type virgin females
F₁ Progeny	
Possibility 1 (all progeny showing wild type phenotypes)	
Males and females with wild type wings and wild type bristles only.	Males and females with wild type wings and wild type bristles only.
Possibility 2 (all progeny showing mutant phenotypes)	
Males and females with Curly wings and mutant bristles.	Males and females with Curly wings and mutant bristles.

Possibility 3 (progeny with various phenotypes)	
a) Males and females with Curly wings & wild type bristles.	a) Males and females with Curly wings & wild type bristles.
b) Males and females with wild type wings and mutant bristles.	b) Males and females with wild type wings and mutant bristles.
c) Males and females with wild type phenotypes for both the characters.	c) Males and females with wild type phenotypes for both the characters.
d) Males and females with mutant phenotypes for both the characters.	d) Males and females with mutant phenotypes for both the characters.

4. Depending on which one of the above three possibilities is **REALISED**, set up the $F_1 \times F_1$ crosses to obtain F_2 progeny. Depending upon the above, this cross/es can be one or more of the following type/s:

A. **Possibility 1:** $F_1 \times F_1$, (both parents with wild type phenotypes).

B. **Possibility 2:** $F_1 \times F_1$, (both parents with double mutant phenotypes).

C. **Possibility 3:** Due to presence of multiple possible phenotypes in F_1 progeny in this case, set up one or more of the following possible F_1 crosses:

i. Curly wings, wild type bristles X Curly wings, wild type bristles;

ii. Wild type wings, mutant bristles X Wild type wings, mutant Bristles;

iii. Curly wings, wild type Bristles X Wild type wings, mutant Bristles.

Observations/Results:

1. Record the wing and bristle phenotypes of F_1 and F_2 progeny males and females.

2. Interpret the inheritance patterns of the Curly wings and short thick bristle phenotypes with regard to the following:

i. Is any of the two phenotypes showing autosomal or sex linked inheritance?

ii. Are the two mutant phenotypes due to mutation(s) in a single/same gene or due to mutations in two different genes?

iii. What is the nature of the mutant allele/s with respect to being dominant or recessive?

iv. Based on above, predict genotype of the parents and F_1 progeny.

v. If two different genes are involved, are they linked or assorting independently?

vi. If linked, are the two mutant alleles in cis- or trans-configuration in mutant stock?

Questions for self-study and interpretation:

1. Did the F_2 progeny show all the four types of phenotypes that may be expected on Independent Assortment or on linkage and crossing over? If not, what could be possible reasons?

2. What are the genetic mechanisms underlying the true breeding nature of Curly Bristle parent?

3. Why is the balanced lethal system important in genetic studies?

4. Can you give an example of a major study with far-reaching implications that depended upon the balanced lethal system?

5. Since recombination between homologous chromosomes is avoided within the stretch of Balancer, is there a scope for accumulation of mutations in this region?

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Chapter 11

Generation and establishment of genetic recombinant stocks in *Drosophila melanogaster*

Vanshika Kaushik (vanshikakaushik0@gmail.com) and S. C. Lakhotia* (lakhotia@bhu.ac.in)
Cytogenetics Laboratory, Department of Zoology, Banaras Hindu University, Varanasi
221005

* For correspondence

Recommended level: PG/Research

Sub-Discipline: Genetics

Background

A geneticist's primary approach to understand a gene's function is to examine the phenotypic change/s associated with its mutant allele. The next step in functional genetic analysis involves gene interaction studies that seek to find if mutant allele of one gene affects expression of mutant allele of another gene. Following the wider use of transgenesis (see Chapter 1), one or more transgene/s is/are required in many instances to be introduced in the genome of a fly carrying another transgene or conventional mutant allele of same or another gene. These studies require bringing the two mutant alleles and/or transgenes in one individual through genetic crosses. It is relatively easier to bring two mutant alleles/transgenes together if they are not syntenic, i.e., are located on non-homologous chromosomes; the process of bringing together two mutants/transgenes in same individual through hybridization is often termed as 'introgression'. Bringing the desired two mutants/transgenes that map on the same chromosome, i.e., are syntenic, together requires homologous recombination between them, which is a stochastic event during meiosis and whose probability of occurrence correlates with the physical distance between the concerned genes/transgenes on the chromosome (see Chapters 8, 9). It is to be noted that genes that are far away (>50cM linkage map distance) from each other on a chromosome show 'independent assortment'. Therefore, two syntenic genes may appear 'linked' or 'unlinked' in genetic crosses on the basis of map distances between them.

Two features of the toolkit available to fly geneticist facilitate generation of recombinants between genes located on homologous chromosomes. The first is a 'natural gift' that homologous recombination is absent in male *Drosophila melanogaster* (Morgan, 1914; Roberts, 2006) and the second toolkit created by fly geneticists is the battery of the available balancer chromosomes (see Chapter 1 and https://bdsc.indiana.edu/stocks/balancers/balancer_intro.html). Balancer chromosomes permit perpetuation of the newly

generated syntenic condition by preventing different mutant alleles/transgenes on the same chromosome (syntenic) from being separated by the meiotic recombination events.

The sets of genetic crosses required to recombine two mutant genes/transgenes that map to a given homolog described here is based on example of two recessive mutants (say *a* and *b* mutant alleles of genes *a*⁺ and *b*⁺, respectively), which are linked to chromosome 3. The first step in these series of crosses is to bring *a* and *b* in heterozygous condition (*a b*⁺/*a*⁺ *b*) in female progeny so that recombination between homologs can generate some *a b* and *a*⁺ *b*⁺ progeny, the frequency of which depends upon the map distance between the two genes. The next set of crosses require identification of the rare, recombined individual/s and establishing a stock in which the recombined genotype can persist either in homozygous condition or as heterozygote with a balancer chromosome. If none of the mutants has a recessive lethal phenotype, the recombined genotype can be maintained in homozygous condition; otherwise, the recombined chromosome is maintained against an appropriate balancer chromosome.

Objective:

To carry out genetic crosses between desired mutants on homologous chromosomes to generate and maintain recombinant progeny carrying the desired combination of mutant alleles/transgenes.

Materials required:

A. Flies:

- i. Fly stocks of desired mutant allele/transgenes that need to be recombined. In the example here, we name chromosome 3 linked mutant alleles that are to be recombined as *a* and *b* and their wild type alleles as *a*⁺ and *b*⁺, respectively. Accordingly, the two mutant stocks are designated as *a/a* and *b/b*.
- ii. A chromosome 3 balancer stock like *dco*² *e/TM6B* (see Chapter 1), in which *dco*² (*disc overgrown*) is a recessive lethal allele, while *e* (*ebony*) gives ebony or dark-bodied flies when homozygous; since the *TM6B* balancer chromosome, besides carrying the dominant *Tubby* mutation also carries *e* mutant allele, the *dco*² *e/TM6B* males and females show ebony and Tubby phenotype.

B. Equipment and other fly handling items

- i. Stereo-binocular microscope, food vials and bottles and other fly handling items.
- ii. B.O.D. incubator (or room) maintained at 24°C.

Experimental details:

A. Setting up the genetic crosses:

- i. Collect freshly eclosed virgin female flies from one of the fly stocks, *a* or *b* and males of the other mutant. Age the flies for 4-5 days.

- ii. For setting up the parental cross (Cross 1), place anesthetized 4–5 days old virgin female flies of one genotype (e.g., $a b^+$) and 2-3 males of the other genotype (e.g., $a^+ b$) in a food vial. Setup such crosses in 2-3 vials. Once the flies recover from anesthesia, keep the food vials in the B.O.D incubator or room maintained at 24°C.
- iii. Transfer the parental flies to a new food vial after 5-6 days so that a larger number of $a b^+/a^+ b$ (a/b) heterozygous F1 progeny can be obtained.
- iv. After 11-12 days, when the F1 progeny flies begin to eclose, select 15-20 $a b^+/a^+ b$ (a/b) heterozygous progeny virgin females. Age them for 4-5 days.
- v. Set multiple F1 (Cross 2) crosses in food vials with 4-5 days old a/b heterozygous progeny females (obtained in step 4 above) and $dco^2 e/TM6B$ males (4-5 virgin females and 2-3 males per food vial).

Notes:

- a. a/b heterozygous male progeny is not taken since homologous recombination does not occur in male *Drosophila melanogaster*.
- b. Multiple crosses are set so that a large number of male progeny carrying the *TM6B* balancer are obtained.
- vi. Select the Tubby male progeny (from the cross at step A.v above), which may be i) $a b^+/TM6B$ or ii) $a^+ b/TM6B$, iii) $a^+ b^+/TM6B$, or iv) $a b/TM6B$. It is the last category (iv) of males which is of interest as they have inherited a chromosome 3 homolog with a and b mutant alleles recombined.

Notes: In most cases, the recessive alleles do not show any phenotype when in heterozygous combination with the balancer chromosome (except when the balancer chromosome happens to carry recessive mutant allele/s for one or both the genes being recombined). Therefore, in most cases, the progeny types i, ii iii or iv above cannot be distinguished. Their genotypes are determined by the appearance of homozygotes in subsequent generations. To ensure that no further recombination occurs between the recombined mutants during subsequent meiotic division, only males are taken as parents, and each is crossed with a single female fly carrying the balancer chromosome through pair-mating.

- vii. Set up 50-100 pair mating (one male and one female in one vial) F2 crosses (Cross 3) using males obtained at the step A.vi above with $dco^2 e/TM6B$ virgin females.

Notes: Large number of pair-mating vials at step A.vii is required to ensure that at least some of the male parents would be carrying the desired recombined genotype. If the two mutants to be recombined are closely located on the genetic map, more than 100 crosses may be required to be set up.

- viii. Setup F3 (Cross 4) self-crosses (as many as the F2 pair-mating crosses set up in step A.vii,) using the male and female sibs collected from F2 cross vials.
- ix. Examine the Cross 4 (F3) progeny in each vial and select those vials in which the non-Tb flies are homozygous for both the mutants.
- x. Generate homozygous recombined stock by crossing the male and female (virgin) sibs from such vials

Notes:

- i. Prepare separate tables to predict the expected F3 progeny with different genotypes depending upon the $? ?/TM6B$ male being $a b^+/TM6B$ or $a^+ b/TM6B$ or $a^+ b^+/TM6B$ or $a b/TM6B$. The $dco^2 e$ homozygotes are larval lethal due to homozygosity of the dco^2 . The $TM6B$ homozygotes also die as embryo due to the recessive lethal mutation associated with the $TM6B$ balancer chromosome.
- ii. *Cross 4* (F3 progeny): set large number of self-crosses ($? ?/TM6B \times ? ?/TM6B$) using the F3 non-ebony Tubby progeny from one vial in each case. The number of F2 pair-mating crosses to be set up may be reduced or increased if the map-positions of the two mutant genes are known. Greater the distance in their map locations, greater is the expected recombination frequency and therefore, fewer crosses are also expected to generate the desired recombinants.
- iii. Setting up of large numbers of *Cross 4* (F3) will not be required if each of the two mutant alleles/transgenes has a dominant phenotype so that the *Cross 3* (F2) progeny carrying the desired recombined mutant alleles/transgenes can be distinguished by their phenotype. In such case, these parents can be selected from brother-sister mating and establishment of the desired stock.
- iv. Setting up of many sets of *Cross 4* (F3) will not be required if presence of each of the two mutant alleles/transgene can be identified through PCR amplicons. In this case, males to be used as parents for *Cross 3* (F2) can be DNA genotyped, using the non-lethal DNA extraction method from wings or 2nd pair of legs (see Chapter 63) for PCR analysis. Those males that show the presence of all the expected PCR amplicons are crossed with the balancer-carrying female to establish the desired recombined stock.
- v. If presence of each of the two mutants can be identified through PCR, but for some reason the non-lethal DNA extraction (see Chapter 63) is inconvenient, the males can be removed from each cross vial 2-3 days after setting up of the *Cross 3* (F2) pair-mating (after ascertaining that the females have oviposited fertile eggs), and single-fly DNA extraction followed by PCR (see Chapter 62) can be used to determine which of the F2 male carried the desired recombination. Accordingly, progeny of that specific vial/s only is examined and used to establish the recombined stock. This substantially reduces the number of crosses that need to be made and examined further.

Summary of scheme of crosses:

Cross 1 (P): $a b^+/a b^+ \text{♀} \times a^+ b/a^+ b \text{♂}$



F1 Progeny: $a b^+/a^+ b$



Cross 2 (using the F1 progeny): $a b^+/a^+ b \text{♀} \times dco^2 e/TM6B \text{♂}$ (group-mating)

Tabulate the F2 Progeny data as follows:

Gametes	$a b^+$	$a^+ b$	$a b$ (recombined)
$dco^2 e$			
TM6B			

(Fill in the genotypes in the table)

Cross 3 (using the F2 progeny): $? ?/TM6B \text{ ♂ } \times dco^2 e/TM6B \text{ ♀}$
(large number of pair mating crosses)



Select non-ebony and Tubby male and female (virgin) F3 progeny ($? ?/TM6B$)



Cross 4 (using the F3 progeny): $? ?/TM6B \times ? ?/TM6B$
(sib-mating, as many as Cross 3 pair-mating vials). Label each vial as M1...M_n.

Record the F4 progeny phenotype data as follows:

Serial number of F2 males used for pair-mating (M1- M _n)	F4 progeny phenotype			Remarks
	Phenotype	Presence of allele 'a'	Presence of allele 'b'	
M1				
M2				
M3				
-				
-				
M _n				



Cross 5 (using the F4 progeny): Select the F4 progeny males and virgin females which are homozygous for both the mutant alleles and establish the homozygous double mutant stock.

Precautions:

1. Each cross vial must be carefully labelled and recorded to avoid any confusion or cross-contamination.
2. If any one or both the mutant alleles reduce viability in homozygous condition, the recombined stock should be maintained with an appropriate balancer chromosome

Question for self-study and interpretations:

1. Why is heterozygous female fly used as one of parents in Cross 2 to obtain some progeny with recombined mutants in next generations?
2. Why are male flies selected from the Cross 2 progeny?

3. What is the need of introducing a balancer chromosome against the recombined homolog chromosome during Cross 3 above?
4. Design a scheme of crosses if the desired mutant alleles to be recombined are present on chromosome 1 (X-chromosome).
5. Design a scheme of crosses to bring in a chromosome 2-linked mutant allele *c* in the background of the above generated *a b/a b* recombined stock (*Hint*: Chromosome 2 and 3 double balancer stock will be required).
6. Design a scheme of crosses if the one of the desired mutant alleles to be recombined has a recessive lethal phenotype.

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Chapter 12

To study inheritance of quantitative traits: Numbers of sex comb teeth and sternopleural bristles in *Drosophila melanogaster*

H. A. Ranganath (haranganath@gmail.com)

Centre for Human Genetics, Bangalore, and *Drosophila* Stock Centre, University of Mysore,
Mysore

Recommended Level: UG/PG

Sub-discipline: Genetics/Cytogenetics

Background:

Mutants of *Drosophila* have helped us to understand the transmission of genes from one generation to the next. In such Mendelian studies, the phenotypes are discrete, like colour of the eyes or features of wings or body pigmentation. Studies on the inheritance of such traits present precise statistical ratios such as 3:1 or 9:3:3:1 or 1:1:1:1, or some variant of these ratios. However, many phenotypic traits do not have variants that fall into discrete qualitative categories, but are rather continuously distributed, so that the variants form a spectrum of phenotypic measurements. Thus, traits, like body size, wing-length, lifespan, fecundity, bristle number etc., are termed quantitative or metric traits because they need to be measured, not just scored in terms of ratios of qualitative phenotypes. Since many non-allelic genes contribute to such traits, this situation is also called 'polygenic inheritance'.

The study of polygenic inheritance and its consequences for evolution of such quantitative traits is the domain of quantitative genetics. In such cases, it is difficult to detect effects of the many genes that affect the phenotype, or even to identify the genes that are involved. The extent of manifestation and transmission of quantitative traits is extremely sensitive to environmental influences. One of the great achievements of quantitative genetics has been the development of methods to overcome the problem of not knowing which or how many genes affect any given quantitative phenotype. This is done through the concept of heritability, first developed by Francis Galton, and later given its present interpretation in genetic terms by Ronald A Fisher. Heritability is the proportion of total phenotypic variation in a population that is due to variation in breeding values for that trait among individuals. It reflects the aspect of the phenotype that is transmissible to offspring, without requiring knowledge of the specific genes involved in generating the phenotype. Heritability data also provide clues as to how much relative influence of intrinsic and/or extrinsic environmental factor/s operate/s for a polygenic trait. Thus, to predict evolutionary change in mean trait values under a given intensity of selection, a measure of heritability is all that is required from the point of view of genetics.

Just as in Mendelian genetics, in quantitative genetics also, information about the inheritance patterns of polygenic traits can be obtained from reciprocal crosses at the

populational level. By comparing the mean trait values in the two kinds of reciprocal hybrids and their parents, we can assess the degree to which the trait is under additive genetic control versus dominance, epistatic deviations i.e. non-additive interaction between alleles at different genes, and whether it is largely autosomal or X-linked, and whether there are maternal effects.

Objective:

To study inheritance of quantitative, continuously distributed phenotypes in *D. melanogaster*.

Materials Required:

A. **Flies:** Cultures of *Drosophila melanogaster* collected from nature (see [Chapter 108](#)).

B. **Equipment and fly handling items:**

- i. Stereo-binocular and bright-field microscope
- ii. microscope
- iii. Food bottles
- iv. Cotton
- v. Dissection needles and fine hair brushes
- vi. Glass plates
- vii. Anaesthetic ether and etherizer,
- viii. Mouth aspirator

Experimental Procedure:

The steps detailed below are for studying inheritance of the number of bristles/teeth in the sex comb, present on the first tarsal segment of the first pair of legs of male *Drosophila melanogaster* (see [Chapter 44](#)). A similar approach can be taken for several other traits such as bristle numbers on the sternopleural plate located on the lateral side of the thorax (see [Fig. 11Q, R](#) in [Chapter 1](#)) of both males and females.

A. **Mix *D. melanogaster* males and females collected from field and establish a stock.**

- i. Count the number of teeth in the sex comb of 25 males in this stock to know the spectrum of variability in the stock.
- ii. Establish the following two subcultures/lines from this stock:
 - a. Select 5 males with highest number of teeth and mate them with virgin females drawn from the stock. Label this as “**High line**”.
 - b. Select 5 males with lowest number of teeth and mate them with 5 virgin females drawn from the stock in a fresh food bottle. Label this as “**Low line**”.
- iii. If there has been good egg-laying and larvae are seen to be active in the original parental bottles, discard the parent flies after 5 days while retaining the progeny in the form of eggs, larvae and pupae. However, if the progeny growth is sub-optimal, subculture these two lines by transferring the parental flies to fresh food bottles after 5 days.

- iv. When progeny flies appear, sample 25 males from each line (**High** and **Low**), and record the number of teeth in sex combs of each of them.
 - v. Select 5 males with highest number of teeth among the progeny of **High line**, and mate them with 5 virgin females from the same culture.
 - vi. Similarly, select 5 males with lowest number of teeth among the progeny of **Low line**, and mate them with 5 virgin females from the same culture.
 - vii. Repeat the steps iii to vi above for at least for 5 subsequent generations. At the end of 5 or more generations, set up reciprocal crosses between males and virgin females of **High** and **Low**.
 - viii. Record the number of teeth in the sex combs of these two sets of hybrid progenies.
 - ix. Perform following statistical tests:
 - a. Calculate the ‘Arithmetic mean’, ‘Standard deviation’ and ‘Standard Error’ for each generation of **High** and **Low** lines.
 - b. Apply “*t*-test” to determine whether (a) the differences between the means at successive generations and (b) between **High** and **Low** lines are statistically significant.
 - c. Carry out a regression analysis on the line Means as obtained for successive generations.
 - d. Compare the mean values of each hybrid progenies with each of the parental lines.
- Note:** If the chosen quantitative trait is the number of bristles on sternopleural plate, both males and females have to be selected to establish **High** and **Low** lines and screened in each generation since these bristles are present in both the sexes.

Questions for self-study and interpretation:

1. What are the significance and relevance of Arithmetic Mean, Standard Deviation and Standard Error?
2. Based on the outcome of (a) regression analysis and (b) of pattern of inheritance of the phenotype in the reciprocal hybrids, discuss whether the trait is under additive genetic control or is due to dominance, and whether it is largely autosomal or X-linked, and whether there are non-genetic maternal effects.
3. Compare the findings of pattern of inheritance of teeth of the sex comb and bristles on the sternopleural plate.

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Geneticists' Discrete Genes and Biometricians' Continuous Variations

After the rediscovery of Mendel's work in 1900, bitter disputes erupted between the first geneticists and the biometricians who studied quantitative traits. How could the discrete genes of the geneticists explain the continuous variation observed by biometricians? And could natural selection shape variation in these genes? Eventually, the two camps came to understand that quantitative variation is due to multiple Mendelian genes of small effect, and selection on this variation is highly effective. Yet in 1931, very few attempts had been made to formally describe the genetics of evolving populations. By explicitly reconciling Mendel's and Darwin's theories, Sewall Wright and the other pioneers of population genetics laid an enduring mathematical foundation for understanding evolution

(In: Barton. N. H. 2016 Sewall Wright on Evolution in Mendelian Populations and the "Shifting Balance". *Genetics*. 202: 3–4. <https://doi.org/10.1534/genetics.115.184796>)

Chapter 13

Use of paper microscope (Foldscope) for classroom teaching of genetics

Kelevikho Neikha (peleikha@gmail.com), Nukshimenla Jamir (nukshimenlajamir@gmail.com), Abuno Thepa (abunzthep@gmail.com), Bendangtula Walling (atuwalling7@gmail.com), Abhik Das (dasabhik91@gmail.com) and Sarat Chandra Yeniseti* (yschandrays@rediffmail.com)
Drosophila Neurobiology Laboratory, Department of Zoology, Nagaland University (Central), Lumami, 798627, Nagaland, India.

*For correspondence

Recommended Level: School/UG/PG

Sub-discipline: Cytogenetics/Biodiversity

Background:

Invention of microscope changed the perspective of humans towards nature since it permitted looking at objects that could not be seen with human eyes because of their small sizes. It has also helped in identification of the causative micro-organisms responsible for various diseases. Most microscopes are rather expensive and, therefore, often beyond the reach of many students and institutions. An affordable and sturdy microscope was developed by Dr. Manu Prakash and his team at Stanford University (USA) in 2012 (Cybulski *et al.*, 2014), which can provide a magnification from 140X to 2000X with submicron resolution. This “Foldscope” microscope is made of paper and uses the principle of “Origami”. Although punched only on a flat sheet of paper, it is robust enough for field study and because of its low cost, it can reach in resource-limited conditions (Neikha *et al.*, 2020; Walling *et al.*, 2020).

Foldscope is a simple and inexpensive, yet powerful tool which can be used to observe and understand microscopic living and non-living forms and also can be helpful in classroom teaching and biomedical research. The assembly of the commercially available (including online) “Foldscope” is described here. Exposure of students to Foldscope in biology courses could channel their interest for further experimental activities.

Objectives:

To learn, to assemble the simple and inexpensive paper microscope (“Foldscope”) and use it for examining *Drosophila* flies and tissues.

Material Required:

- A. **Flies:** wild type and mutants of *Drosophila*.
- B. Foldscope (can also be purchased online).
- C. Mobile ‘smart’ phone with camera.

D. LED Magnifier.

E. Glass slides, cover-slips (22 mm²), Petri dish, fine hair brush etc. for handling flies.

F. Solutions:

i. Phosphate buffered saline (pH-7.4)

ii. Glycerol

iii. 0.7% NaCl

iv. 1N HCl

v. 2% Lacto-aceto orcein

vi. 45% acetic acid

vii. Distilled water

Experimental Details:

A. Assembling the Foldscope:

i. Following the instruction manual received with the Foldscope, get familiar with its different parts like lens stage, sample stage, panning guide, focus ramp, magnetic coupler, lens, ring sticker and ID sticker (**Fig. 1**).

ii. Insert the coupler into the lens stage on the front side by keeping the silver side UP, with alignment holes to the right and inserting it into the curved slots with “dog ears” by sliding to the right and pushing the corners into the slots.

iii. Flip the lens stage back side and place the coupler, silver side down, so that the alignment is on the left side. Insert the coupler into the curve slots by sliding to the left.

iv. Fold and lock the bottom flap by folding the dashed line and inserting the 3 tabs into the slots.

v. Now take the lens and a ring sticker. Attach the ring sticker on the back of the first magnetic coupler, clean the lens with a soft cotton swab very gently on both sides. Paste the lens on top of the ring sticker with its bump side facing down and gently press it around the lens. Avoid touching the lens.

vi. Take the focus ramp and flip it over the grey side. Follow the dotted lines, fold and unfold starting from the shorter side all the way to the top. After that, starting from the shorter end, fold the ramp following the crease created.

vii. Take the lens stage. Hold the ramp on the thicker end with tabs facing up and insert the thin side in the right slot, push down till it comes on the left slot. Once inserted, fold the tabs on each side of the focus ramp.

viii. Place the sample stage on a table, allowing the blue coloured side to face you. Fold the flaps using the dotted lines and insert it into the slots.

ix. Assemble panning guide to sample stage by first flipping both into the yellow side and weave the sample stage through the panning guide.

x. Connect the lens stage to the assembly by placing the lens stage on top of the assembly and snap all 8 panning guide flaps forward, locking in the lens stage beneath.

xi. Lastly paste your ID sticker on the labeled area.

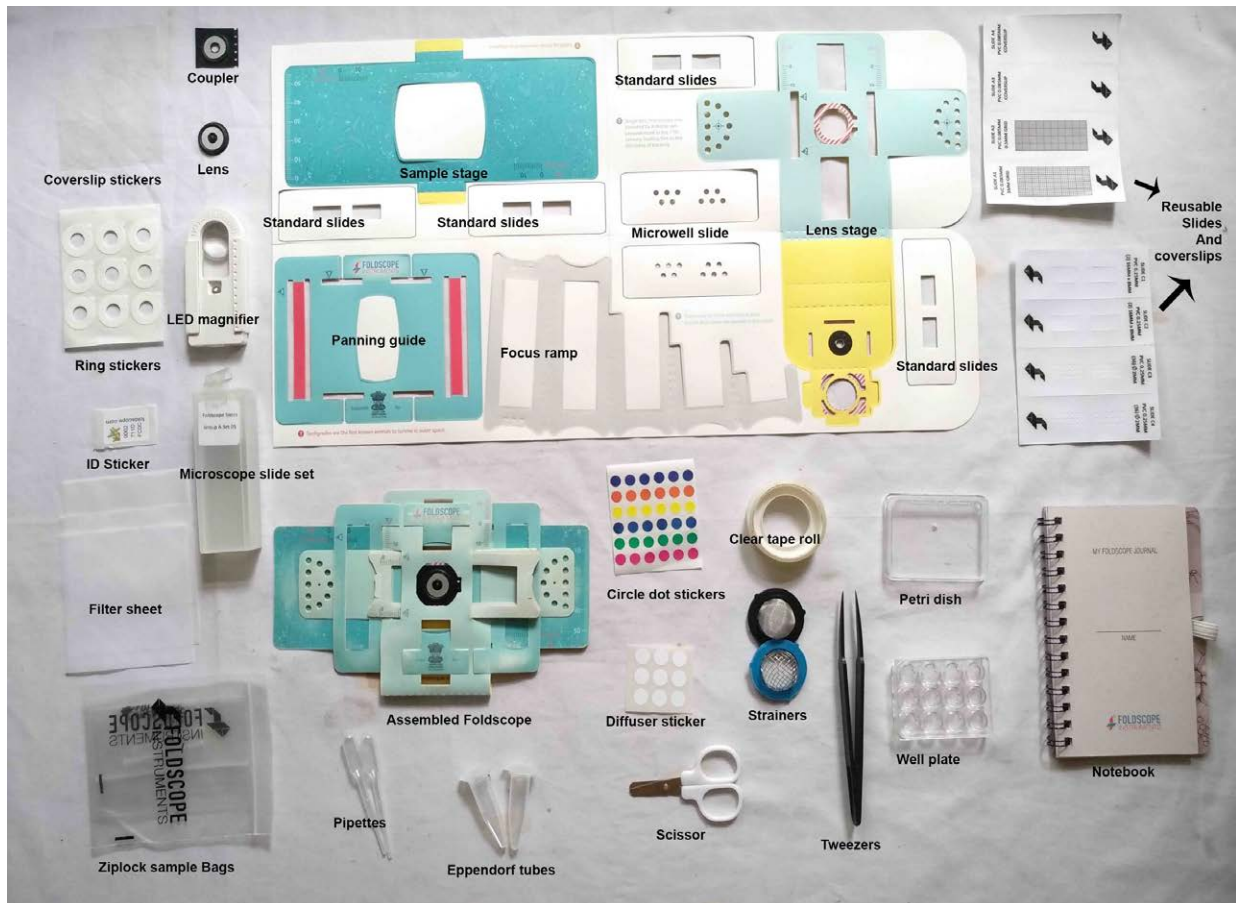


Fig 1. Parts of Foldscope and the assembled Foldscope.

B. Placing the sample under Foldscope and viewing it:

i. Insertion of slides:

- a. Flip the Foldscope to the back side and open the back tab.
- b. Take the sample slide and turn it over so that the coverslip faces down and comes close to the lens.
- c. Open the tab and insert the slide following the arrow mark in both the tabs and adjust the specimen by aligning with the lens.
- d. Close the tabs, flip it over to the front side and proceed further for observation.
- e. The LED magnifier, 3X/10X can be used as a light source by first sliding a coupler (silver side facing up) into the back, following the dotted alignment. Flip the magnifier and then mount to the yellow side of Foldscope.

ii. Panning and Focusing:

- a. Panning of the lens stage can be done by moving X-axis right and left and, Y-axis up and down.
- b. For focusing, the Z-axis of the focus ramp can be panned left and right.

iii. Direct view:

- a. If using daylight or artificial lighting source, hold the Foldscope by placing your thumbs on the half moons and point the microscope lens towards the light source.

b. Bring the lens close to the eye and observe the specimen.

iv. Phone view:

a. Place a double ring sticker over the center of the smart phone's camera and place the magnetic coupler on top of the sticker with the silver side facing down.

b. Take the Foldscope and mount the mobile phone on top of it to view and capture images and videos.

v. Projection:

a. Place a double ring sticker over the center of the phone's flashlight, remove the sticker cover and place the coupler on top of the sticker with the silver side facing up.

b. Mount the phone on the yellow side of the Foldscope.

c. Open the flashlight and project it on a flat surface in a dark room.

Note: Instead of mobile flash light, LED magnifiers can also be used as light source for projection by mounting the yellow side of the Foldscope.

C. Observing wild type and white eye mutant flies of *Drosophila melanogaster*:

Wild type and white eye mutant flies (or any other available eye colour mutant, (see **Chapter 3**) are etherized and taken on a clean slide. For examining eyes or any other feature of the head, separate the head from rest of the body with a sharp dissecting scalpel or needle and transfer it onto a cavity slide filled with glycerol. Cover with a coverslip and observe under the Foldscope (**Fig. 2A, B**).

D. Observing wild type and mutant wing:

For examining wings of wild type and mutant (e.g. vestigial wings, see **Chapter 3**) flies, etherize flies and keep on a clean slide in a drop of water. Gently cut the wings from the body using fine dissecting needles. Discard the body carcass and soak excess water with a filter/blotting paper. Add a small drop of glycerol on wings, cover with a coverslip and examine under the Foldscope (**Fig. 2C, D**).

E. Study of salivary glands and polytene chromosomes:

Prepare larval salivary glands and squashes of their polytene chromosomes as described in (**Chapter 31**). Examine the prepared slides under Foldscope (**Fig. 2E, F**).

Results/ Observations:

Observe the various preparations (see **Fig. 2**) and record your observations.

Limitations of Foldscope:

1. Because of its low depth of focus, it can sometimes be difficult to get as sharp images as with a conventional microscope.

2. When examining material related to infectious diseases, close contact of eyes of the observer with Foldscope has to be avoided. In such cases, smart mobile phones become essential for observation.

3. Although the Foldscope is not expensive, the smart mobile phone is expensive.

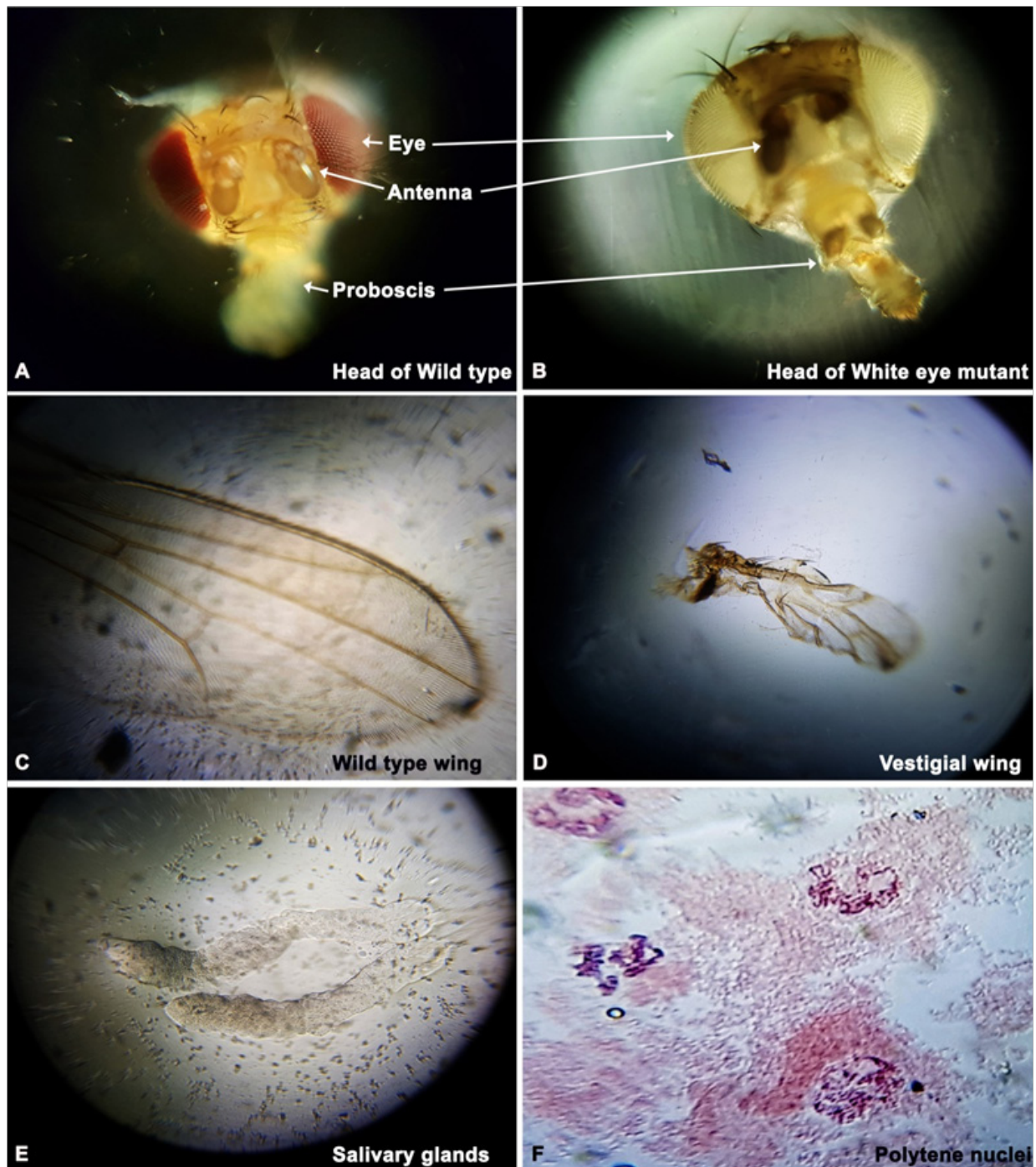


Fig. 2. Images of *Drosophila melanogaster* as seen through the Foldscope. Head of wild type (A) and white eye mutant fly (B).Wings of wild type (C) and vestigial mutant fly (D). Larval salivary glands (E). Polytene nuclei in squash preparation of larval salivary glands (F).

Questions for Self-Study and Interpretation:

1. How do you think the Foldscope can be a great tool for observing things in nature?
2. What is the difference between a traditional microscope and a Foldscope?
3. Why is the image quality of a Foldscope poorer than a typical compound microscope?

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Chapter 14

Identification of *Drosophila* larval stages

Kiran Nilangekar^{1,2} (knilangekar@aripune.org), Nidhi Murmu^{1,2} (nidhimurmu@aripune.org), Minal Ayachit^{1,2} (minalayachit@aripune.org), and Bhupendra Shrivage^{1,2*} (bvshrivage@aripune.org)

1. Developmental Biology Group, Agharkar Research Institute, G.G. Agarkar Road, Pune -411004

2. Savitribai Phule Pune University, Ganeshkhind, Pune -411004.

* For correspondence

Recommended Level: UG/PG

Sub-discipline: Developmental Biology/Anatomy

Background:

Drosophila melanogaster is a very popular and powerful model for Developmental Biology. *Drosophila* completes its life cycle in about ten days under standard laboratory conditions. Its life cycle is divided into egg, larva, pupa and adult stages. Being a holometabolous insect, it undergoes complete metamorphosis during the pupal period. The larval stage is broadly subdivided into three molting stages as first, second and third instar larva. Compared to the adult, larvae exhibit a simpler organization of the different functional systems (Bate and Arias, 1993).

Drosophila larvae have been widely used to study diverse aspects in biology ranging from physiology, developmental biology, gene expression, neurobiology, toxicology etc. Many of these studies require knowledge of the specific developmental stage of larvae that are used as experimental system. Experimental conditions like temperature, food, drugs, mutations and other genetic alterations can change the development and phenotype of larva (Alpatov, 1929; Ashburner, 1989; James and Partridge, 1995; Roberts, 1998). This chapter describes morphological characteristics associated with normal development of larvae (under standard laboratory conditions) which help in identifying the specific stage of its development.

Objectives:

To obtain larvae of a certain stage based on developmental time and morphological characteristics.

Materials required:

A. Equipment and other supplies for fly handling

- i. Stereo - binocular microscope.

- ii. Microscope slides and cavity slides
- iii. Blunt pairs of forceps and fine hair paint brushes
- iv. Micropipettes (1000 μ L, 100 μ L) and corresponding micropipette tips
- v. Petri dishes
- vi. 1.5 mL microfuge tubes
- vii. A 50 - 100 mL glass container which can be sealed with a lid; 50 mL beaker and Petri dish or 100 mL screw cap glass bottle
- viii. Absorbent cotton
- ix. Anesthetizing setup for larvae (**Fig. 1 A**): 100 mL wide-mouthed and screw-capped transparent plastic or glass bottle and caps of 1.5 mL microfuge tubes (prepared by cutting off the joint of the cap and tube of a 1.5 mL microcentrifuge tube using scissors (**Fig. 1 B**)).

B. Reagents

- i. 1X Phosphate Buffer Saline (PBS pH 7.4)
 - a. NaCl 8 gm
 - b. KCl 200 mg
 - c. Na₂HPO₄ 144 gm
 - d. KH₂PO₄ 272 mgSerially dissolve the above in 800 mL distilled water; when fully dissolved, adjust to pH 7.4 and make the volume to 1000 mL with more distilled water. Autoclave the solution and store at room temperature or 4^oC.
- ii. 20% Sucrose solution in distilled water
- iii. Diethyl ether
- iv. 80% Glycerol (mix 8 mL Glycerol and 2 mL dist. water)
- v. Bromophenol Blue dye

Experimental Details:

A. Collecting and staging larva according to developmental time.

(Ashburner, 1989; James & Partridge, 1995; Roberts, 1998)

- i. Anesthetize healthy 4-6 day old wild type flies and transfer ~ 10-15 pairs of males and females to a fresh food vial, supplemented with dry yeast pellets (covering not more than one third of the food surface or a pea sized drop of freshly prepared yeast paste). Keep the vial in horizontal position till the flies wake up to avoid the unconscious flies from sticking to the food.
- ii. After 24 hr, transfer the flies to a fresh vial containing food with yeast. Perform this transfer preferably in the morning. Note the exact time of the transfer and let the flies lay eggs in the vial for 3 - 4 hr.

- iii. At the end of 3 - 4 hr, remove flies from the vial. Incubate this vial containing eggs at standard conditions for different durations to obtain larvae of the desired developmental stage as indicated in **Table 1**.
- iv. Take out larvae of desired age from the food vial by adding 1-2 mL of 20% sterile sucrose solution and incubating for at least 3 min.
- v. Gently swirl the vial so that any larvae still stuck in the food will detach and float in the sucrose solution. Decant the sucrose solution onto a Petri dish gently so as to avoid the detached food from falling along with solution and the floating larvae.
- vi. Wash the larvae by transferring them to a dish containing 1X PBS using a fine brush or forceps.

Table 1: Developmental times for different larval stages at 24-25°C

Duration of larval stages (in hr after egg laying (approximate))			
First instar	Second instar	Third instar	
24 - 48	48 - 72	Early third instar (Active feeding stage): 72 - 108	Late third instar (wandering stage): 108 - 116

B. Immobilizing larvae:

All the larval stages are highly kinetic. Their microscopic examination requires temporary immobilization, which can be achieved by exposure to cold or to Diethyl ether (Ashburner, 1989; Roberts, 1998).

- i. Immobilization by exposure to cold: Pre-chill 1X PBS in ice and dispense the ice cold PBS in a Petri dish kept on ice. Place the larvae into the ice-cold PBS in a Petri dish using forceps or brush. In about 5 min, the larvae become immobile. (**Note:** Volume of PBS in the Petri dish should be minimal so that the larvae do not get drowned.)
- ii. Immobilization by exposure to anesthetic ether: Cover the bottom 1 cm of the 100 mL glass wide-mouthed and screw-capped transparent plastic bottle with cotton (**Fig. 1 E**). Add 5-6 mL of diethyl ether onto the cotton. Keep the chamber closed to avoid losing the diethyl ether vapors.
 - a. Add a drop of PBS in the cut lid of the microfuge tube and transfer 3-5 larvae in it using forceps or fine brush (**Fig. 1 C**).
 - b. Aspirate the PBS with a micropipette with attached tip (**Fig. 1 D**), taking care that the larvae do not get hurt during aspiration.
 - c. Open the lid of the bottle containing ether-soaked cotton and with the help of forceps, place the microcentrifuge tube cap with larvae on surface of cotton in the chamber. Close the lid of the anesthetizing chamber (**Fig. 1 E**).

d. Leave for two minutes.

e. Remove lid of the chamber and retrieve the microfuge tube caps using forceps, and using forceps or fine brush transfer the larvae onto a cavity slide containing 1X PBS.

Note: 1X PBS should be at 25°C so that larvae can recover rapidly from anesthesia.



Fig. 1. Setup for immobilizing larvae by ether anesthesia. **A.** Components of the setup for larval anesthesia. **B.** Cutting off the cap of 1.5 mL microfuge tube with scissors. **C.** Larvae in PBS in the space in microfuge tube cap. **D.** Larvae in the cap after aspirating out of the PBS. **E.** Larvae in the cap (arrow) being exposed to ether in the closed bottle with ether-soaked cotton.

iii. Examining larvae for developmental staging:

a. Transfer larvae onto a slide with 1X PBS using a brush as gently as possible. A blunt forceps may also be used.

b. Observe larvae under stereo-binocular microscope (preferably equipped with both transmitted and incident light systems).

c. Transmitted light can be used to differentiate translucent and opaque structures within the larval body (i.e, fat body, trachea, anterior spiracles etc).

d. Observe the dorsal, ventral and lateral sides of the larvae by rotating them on the slide.

e. Stage them using the criteria listed in **Table 2** and **Figs. 2** and **3**. **Note:** After the examination and when the larvae have resumed movement, transfer them to a vial containing food so that they can resume development.

iv. Preparing mouth hooks for microscopic examination: A more precise staging can be attained based on microscopic examination of the anterior end of a larva under bright-field microscope and counting the number of teeth present on the larval hooks (**Fig. 2 D-F**) (Alpatov, 1929; Ashburner, 1989; Roberts, 1998). For more clarity, prepare the larval mouth hooks as follows:

a. Tear off the larvae using fine forceps along the body length (see **Chapter 21**) taking care not to damage the pair of chitinous, dark and hard mouth hooks in the mouth region (**Fig. 2 D-F**).

- b. Remove as much tissue (cuticle, muscles, trachea etc.) as possible to expose the mouth hooks.
- c. Using a pair of fine forceps, transfer the hooks onto another slide in 40-50 μ L of 80% glycerol.
- d. Add a coverslip, taking care to avoid trapping air bubbles.
- e. Remove excess glycerol using tissue paper.
- f. Examine and draw mouth hooks using 10X/40X objective on a bright-field microscope (Fig. 2 G-I).

Table 2: Distinguishing morphological characters of the three larval stages of wild type *D. melanogaster* (Fig. 2)

Character	First instar	Second instar	Third instar
Size (Fig. 2 A-C)	1 - 1.5 mm	2.1 - 2.6 mm	3.0 - 3.8 mm
Mouth hooks (Fig. 2 D-I)	Mouth hooks with only one tooth	Mouth hooks with 3-4 teeth	Mouth hooks with 9-10 teeth
Paired Anterior spiracles (Fig. 2 J-L)	Absent	Appear rounded or club shaped. They do not extrude out of the body of the larva.	Appear branched and are periodically extruded out and retracted back in the larval body.
Paired Posterior spiracles (Fig. 2 M-O)	White in color	Light orange in color	Dark orange in color

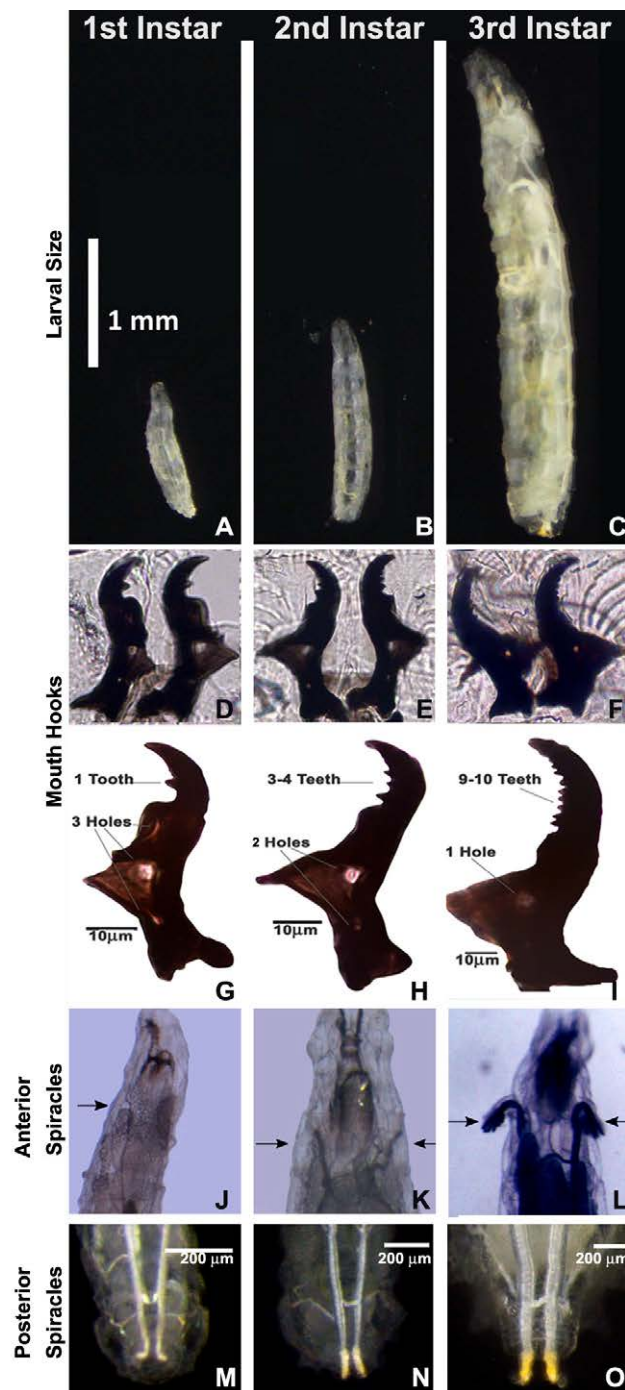


Fig. 2. Distinguishing features of 1st, 2nd and 3rd instar larvae of *Drosophila melanogaster*. **A-C.** Relative larval sizes of different instars (scale represents 1 mm). **D-F.** Mouth hooks in situ in different larval instars; **G-I.** Mouth hooks in different larval instars after their cleaning and mounting (scale bars represent 10 μm in **G-I**); **J-L** anterior (arrows) and **M-O.** posterior pairs of spiracles of the three larval instars (scale bars in **M-O.** represent 200 μm).

- v. Differentiating between feeding (foraging) and wandering 3rd instar larvae:
 - a. To a vial containing mixed stages of feeding larvae, add 200-300 μL of 0.5 % (w/v in water) bromophenol blue (BPB) dye such that the solution covers entire surface of the food. The blue color should stain about 5 mm of the top layer of the food by diffusion in about half an hour (Roberts, 1998).

- b. Continue incubating the vial at standard conditions.
- c. After 24 hr, observe the larvae which are crawling on the walls of the vial. Pick them with a moistened fine hair brush and place them into a Petri dish containing 1X PBS to wash off sticking food and dye.
- d. Examine the larvae under stereo-binocular microscope for presence of blue colour in their gut (**Fig. 3**).

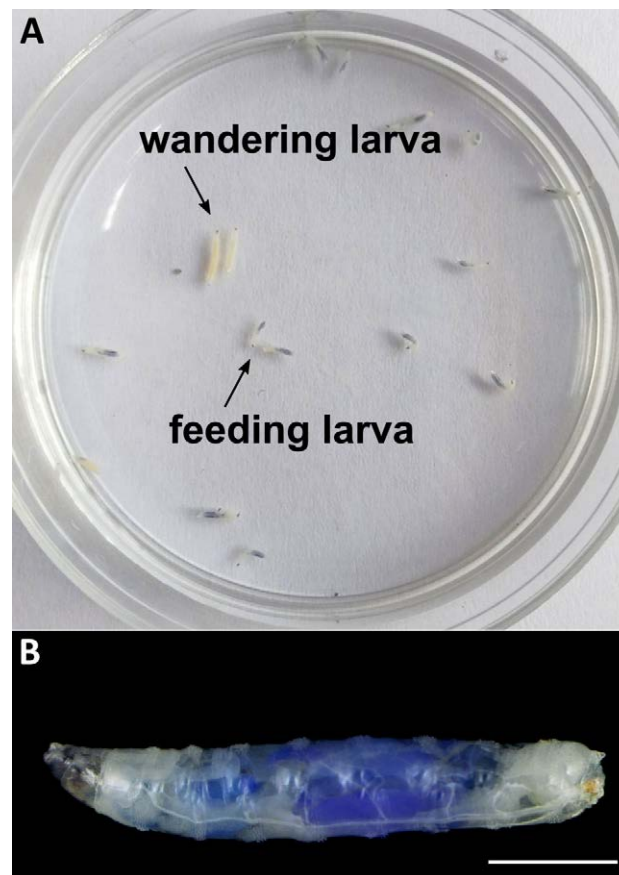


Fig. 3. Feeding and wandering larval stages of *Drosophila*. **A.** Feeding and wandering stages of 3rd instar larvae on food Petri dish. **B.** Feeding larvae show blue colored gut visible through the translucent larval cuticle. Scale bar in **B** = 1 mm.

Observations:

Examine larvae of different ages. Identify and draw their different features (as listed in **Tables 1** and **2**).

Notes:

1. Durations of different life cycle stages are subject to many factors like temperature, humidity, nature of food, genotype, etc.
2. In order to obtain larvae of a specific age, larval development can be substantially synchronized by collecting 1st instar larvae that hatch within an interval of 1 hr.

3. After exposure to diethyl ether, the larvae are kept in PBS during recovery to prevent them from drying. The amount of PBS should be such that the larvae do not drown.
4. In order to observe movement of the anterior spiracles, larvae should not be immobilized but gently held in the field of view with a pair of blunt forceps.
5. Prolonged exposure to diethyl ether can cause dizziness to the experimenter. Anesthetizing should be carried out in lab space with good ventilation.
6. Detailed drawings of larvae and their anatomical structures can be found out at <https://www.flybase.org/imagebrowse/>.

Questions:

1. Design an experiment to find out if developmental timings are affected by nutrition and temperature at which *Drosophila* is grown.
2. What should be properties of a dye that can be used for differentiating feeding and wandering (non-feeding) 3rd instar stages?
3. What is the function of spiracles? Design an experiment to demonstrate the function of spiracles.
4. The larvae go through an exponential growth in volume. Design an experiment to measure the volume of larvae at different stages.
5. Does the pattern of motility of larvae vary at different stages?
6. How would you examine if *Drosophila* larvae show a chemosensory behavior?

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<http://flybase.org/imagebrowse/>

<http://flybase.org/>

Chapter 15

Analysis of minimum threshold/critical size of *Drosophila melanogaster* larvae for pupation in response to ecological conditions

Khushboo Sharma (khushboo.zoology@bhu.ac.in)*^{1,2} and Mallikarjun N. Shakarad (beelab.ms@gmail.com)¹.

ORCID id- 0000-0003-2278-3635 (KS) and 0000-0003-1134-3001(MS)

1- Evolutionary Biology Laboratory, Lab no. 205, Department of Zoology, University of Delhi Delhi 110007.

2- Present address: Cytogenetics Laboratory, Department of Zoology, Institute of Science, Banaras Hindu University, Varanasi- 221005.

* For correspondence

Recommendation level- PG/Research

Sub-discipline- Ecology and Evolution

Background:

The adult size in insects with complete metamorphosis is a vital fitness trait that impacts the fecundity and the lifespan (Collins *et al.*, 2023). Growth conditions during the larval stage often influence the adult size. Temperature during growth is an important environmental condition that affects adult body size (Ghosh *et al.*, 2013). Temperature and body size are inversely related since higher temperature is associated with short developmental duration that in turn results in smaller adult body size. The minimal size at which the larvae irreversibly commit to the process of metamorphosis, is called the critical size since once this is attained, even starvation does not affect the time course to undergo metamorphosis (De Moed *et al.*, 1999; Mirth *et al.*, 2005). Attainment of critical size during larval growth initiates the stop-growth signal and commits the larva to the process of metamorphosis (Sharma *et al.*, 2020; Sharma and Shakarad, 2021).

Drosophila larvae are semi-aquatic as they grow in over-ripe fruits that rapidly progress towards a rotting stage. Therefore, it is important for the larvae to metamorphose quickly. The total larval growth duration can be split into pre-critical and post-critical periods. Once the larva attains the critical size, hormonal cascade is initiated to increase the levels of circulating moulting hormone, which inhibits growth and triggers metamorphosis (Mirth and Riddiford, 2007). *Drosophila* has species-specific critical sizes, with higher growth temperature leading to smaller critical size, that in turn results in reduced adult size.

In the present example, different growth temperatures (24°C and 29°C) are employed to affect the larval size. The following protocol discusses a method to examine the significance of critical size and its relationship with adult body size in *Drosophila*.

Objective:

To ascertain effect of rearing temperature on the minimum threshold or critical size in *Drosophila melanogaster* larvae.

Material required:

A. Flies: Wild type *Drosophila melanogaster* (e.g., Oregon R+ strain).

B. Equipment and other supplies

- i. BOD Incubators maintained at 24 °C and at 29 °C.
- ii. Stereo-binocular microscope and other fly-handling materials like fly-culture bottles with regular food prepared with agar, cornmeal, sucrose yeast, fine hairbrush, tissue paper (see Chapter 1).
- iii. Transparent plastic Petri-plates (60 X 15 mm).
- iv. Stopwatch.
- v. Microbalance with sensitivity up to 0.1 or 1 mg.
- vi. **Non-nutritive Agar Base food:** Add 2.5g agar powder, 7.5g Sucrose in 180 mL water and boil to dissolve. Let it cool, add 0.5 mL propionic acid, and mix well. Dispense while still fluid into bottles or vials of size 9.5cm x 2.3 cm.
- vii. **Liquid food:** Prepare regular food (with maize powder, sucrose, yeast paste as in Chapter 1) without adding agar powder so that the food remains fluid even when cooled to room temperature. Dispense the liquid food in 9 cm and 5.5 cm diameter petri plates. Keep them covered till use.
Note: Collection of larvae from liquid food is more convenient than from solid fly food, especially when large numbers are required.
- viii. Distilled/RO water.

Experimental details

A. Synchronization of larval development

1. To get synchronously developing larvae, take 100 mated 4-6 days old female flies in a milk bottle with fly media supplemented with a teaspoon of yeast paste.
Note: Yeast supplement enhances oviposition in *Drosophila melanogaster*.
2. Keep the flies on yeast supplemented food for three days.
3. On the 3rd day, transfer the flies to fresh food bottles at 1 hour interval over a period of 3 hours.

Note: *Drosophila* females tend to hold fertilised and developing eggs in the oviduct if a suitable substrate is unavailable. The hourly transfer to fresh food facilitates the flushing of eggs stored in the female genital tract, which would have developed to varying stages. This ensures the subsequent batches of eggs laid are freshly fertilized, and therefore, they develop synchronously.

4. At the end of the 3rd hour, transfer the flies to a food bottle containing non-nutritive agar base food for 1 hour for collecting ‘synchronized’ eggs. Discard the adult flies and incubate the bottles at 24°C for 24 h. **Note:** The non-nutritive agar media provide a transparent background, which facilitates collection of eggs on its surface.
5. After 24 hours, transfer the hatched larvae to 5 cm petri-plates with 5 mL liquid food media (food prepared without agar powder) at a density of 30 larvae per plate and incubate at 24°C for 48 hours. Prepare at least 20 such plates from the collected synchronized eggs.
6. Incubate another set of 20 plates, prepared as in step 5 above, at 29°C to examine the effect of growth temperature on larval critical size.

Notes:

- i. A greater number of larvae per petri-plate leads to crowding which adversely affects the larval physiology and development.
 - ii. The critical size time point for larvae reared at 24°±1°C is known to occur during the early 3rd instar stage, i.e., after 48 hours growth as larvae for *Drosophila melanogaster* (De Moed *et al.*, 1999). Since the larvae reared at 29°C grow faster, they are expected to reach the critical size at an earlier time point. Therefore, the time points for sampling of larvae during the next steps of the experiment will be different for larvae reared at 24°±1°C or at 29°±1°C.
7. **Estimation of larval weight at different time points of growth in relation to the rearing temperature:**
 1. For larvae reared at the control temperature (24°±1°C), start sampling at 44 hr after transfer of newly hatched eggs to liquid media plates (68 hr after egg collection i.e., 24h+44h). Beginning at 44 hr after larval growth at 24°±1°C, collect batches of 60 larvae (4 plates with 30 larvae each) at 2 hr intervals, i.e., at 44, 46, 48 and 50 hr after larval growth at 24°C.
 2. For the higher temperature at 29°±1°C, start collecting larvae (4 plates with 30 larvae each) at 2 hr intervals beginning at 34 hr after larval growth in liquid food, i.e., collect larvae at 34, 36, 38 and 40 hr after larval growth at 29°C. **Note:** Larvae are collected from the liquid food by filtering the food through a mesh.
 3. Wash each batch of the collected larvae with distilled water using a fine paint brush to remove the adhering food.
 4. Roll the washed larvae on a paper towel to remove excess water.
 5. Weigh five replicates of 20 larvae, each using a fine balance, and record the weights of larvae of different ages reared at two different temperature conditions (24°C and 29°C).

C. Assessment of critical size time point for larvae reared at different temperatures:

1. Following weighing of each set of larvae, transfer all the 20 weighed larvae from each batch (N = 100 larvae for each time point sample) into non-nutrient agar vials and incubate them at respective temperatures, i.e., at 24°C or at 29°C, for further development under starvation condition.
2. Count the number of larvae that pupated and later eclosed as adults in each of the above vials.
3. Collect and sort the emerging male and female flies, anesthetize and weigh them (in groups of 5). Record data in Table 2, similar to the Table 1 for larval weights.

Observation/Calculations:

1. Record the weights for each batch of larval samples as in Sample Table 1.
2. For determining the Larval Critical Weight (average weight of larva that permits successful pupation and adult eclosion of 50% individuals despite being in non-nutrient agar food is defined as critical weight):

Calculate

- i) Mean weight (\pm S. D.) of larvae at a given time point, and
- ii) % Adult eclosion in each sample.
3. Find the Mean Larval Weight at which 50% of the starved larvae emerge as adults. This larval weight is the Larval Critical Weight for the given larval growth temperature.
4. Compare the Larval Critical Weight when larvae are reared at 24°C or 29°C.

Table 1 : Sample table for recording larval weights at different time points.

Rearing temperature	Time point (hours after transfer of freshly hatched larvae to liquid food)	Mean weights (in μg) of single larva in different replicates					Grand Mean \pm S.D. of 5 replicates
		R1	R2	R3	R4	R5	
24°C	44h						
	46h						
	48h						
	50h						
	52h						
29°C	34h						
	36h						
	38h						
	40h						
	42h						

Table 2 : Sample table for recording adult weight corresponding to different larval samples.

Rearing temperature	Time point (hours after transfer of freshly hatched to liquid food)	Mean weight (in µg) of single fly in different replicates					Grand Mean ± S.D. of 5 replicates
		R1	R2	R3	R4	R5	
24°C	44h						
	46h						
	48h						
	50h						
	52h						
29°C	34h						
	36h						
	38h						
	40h						
	42h						

Use appropriate statistical test to determine if the Larval Critical Weight is different at the two growth temperatures and if this affects the adult body weight.

Questions for self-study and Interpretations:

1. Why should smaller Larval Critical Weight affect adult body size? Does this also affect life span of adult flies?
2. Does the duration of pupal stage differ between samples?
3. Why is the difference in Larval Critical Weight not compensated during pupal period so that the emerging flies are comparable in size irrespective of the larval weight?
4. Do *Drosophila* species inhabiting different ecological niche with varying altitudes show difference in their Larval Critical Weight?
5. Will other ecological parameters like crowding, nutritional status, etc. also affect Larval Critical Weight?

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Chapter 16

pH profile of the gastrointestinal tract in *Drosophila melanogaster* larvae

Shamprasad Varija Raghu (shamprasadvarijaraghu@gmail.com), Sanmitha S Jujare and Rajashekhar K Patil* (patilsirmu@gmail.com)

Neurogenetics Lab, Dept of Applied Zoology, Mangalore University, Karnataka 574199

* For correspondence

Recommended Level: UG/PG/Research

Sub-discipline: Anatomy/Physiology

Background:

The gastrointestinal (GI) tract is one of the largest organ systems in humans and other animals. It plays a significant role in the digestion and absorption of nutrients, movement of the bolus through the GI tract via peristalsis. In addition, the inner lining of the digestive tract serves as the first line of defense against a wide range of pathogens (Lemaitre and Miguel-Aliaga, 2013). To guard against microbial infections, the stomach is acidic in nature. This is a common feature among animals. The enzymes involved in digestion in different parts of the GI tract also have their pH optima in accordance with the pattern of pH in the respective part of gut.

In *Drosophila melanogaster*, the GI tract is segmented into an anterior neutral region, acid-secreting mid zone and a base secreting posterior region, similar to humans. The *Drosophila* GI tract is, therefore, also used for modeling different human gastrointestinal disorders (Pandey and Nichols, 2011).

In the current experiment, pH indicators are used to physiologically differentiate acidic and alkaline regions in the GI tract of the *Drosophila* larvae (Shanbhag and Tripathi, 2009). The indicators are non-toxic and show different colors at different pH ranges. The Bromophenol Blue dye gives light yellow color to acidic and blue to alkaline region (**Fig. 1A**). Phenol Red shows yellow color for pH around 6.4 and red in the alkaline region (**Fig. 1B**).

Objectives:

To study variations in the pH across the gastrointestinal tract in *Drosophila* larva.

Materials Required:

1. Healthy wild type *Drosophila* larvae (late 3rd instar stage)
2. pH indicator dyes: Bromophenol Blue and Phenol Red
3. Yeast
4. 1 X Phosphate Buffered Saline (1X PBS): To prepare 1000 mL of 1× PBS solution, dissolve 8 gm NaCl, 0.2 g KCl, 1.42 g Na₂HPO₄ and 0.272 g KH₂PO₄ in 800 mL of H₂O. Adjust the pH to 7.4 with HCl and then add H₂O to make the volume to 1000 mL. Autoclave and store at RT or 4°C.
5. Dissecting needles
6. Microscope slides and 22 mm² coverslips.
7. Stereo-binocular and light microscopes.

Experimental Details:

1. 25 mg of yeast granules are dissolved in 500 µL of distilled water together with 2 µL of 2% solution of one of the pH indicator dyes (Bromophenol Blue or Phenol Red, as desired) to make a thick paste. The yeast paste along with pH indicator is added to fresh food vial and 10-15 third instar larvae are introduced into the tube. The larvae are allowed to feed on the thick yeast paste, containing the desired pH indicators, for one to two hours.
2. After the feeding, place a larva on a slide in a drop of PBS and observe under a stereo-binocular microscope.
3. Take two dissecting needles and gently place each at two ends of the larva.
4. Slowly pull apart the needles to let the internal organs come out of the cuticle. Using the same dissecting needles, carefully straighten out the digestive tract (see **Fig. 1**).
5. Observe the dissected larva under the microscope.

Results/Observations:

The GI tract is divided into different anatomical compartments with different functional properties. These compartments can be identified based on their pH range using different pH indicators that can physiologically differentiate regions with acidic and alkaline pH. In the case of bromophenol blue fed larval gut, the acidic regions appear light yellow while the alkaline regions show blue color (**Fig. 1**). Phenol red fed larval gut shows yellow color in regions with pH around 6.4 (arrow in **Fig. 2**) and red in regions with pH around 8 (**Fig. 2**).

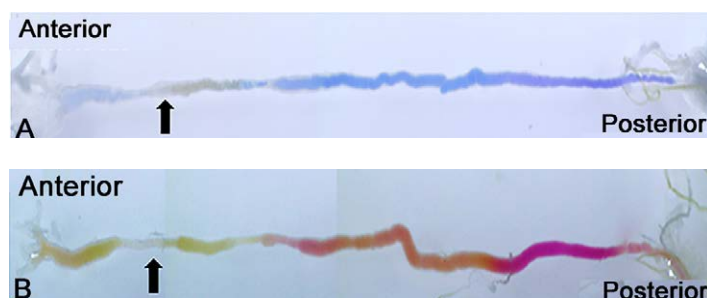


Fig. 1. GI tract of larvae fed with Bromophenol Blue (A) or Phenol Red (B) dye containing food. The arrow in each case indicates the regions of acidic pH while the blue (A) or red (B) regions of gut have alkaline pH (about 8). Anterior and Posterior refer to anterior and posterior ends, respectively, of the gut.

Questions for self-study and interpretation:

1. How is the digestive system organized in insects/*Drosophila*?
2. Is insect GI tract pH profile comparable to human GI tract?.
3. If yes, what is the physiological significance and the mechanism of acid secretion/generation?
4. What are the different digestive disorders in human? How can *Drosophila* be used as a animal model to understand etiology of human digestive disorders?

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Shanbhag, S. and Tripathi, S. (2009) Epithelial ultrastructure and cellular mechanisms of acid and base transport in the *Drosophila* midgut. *J. Exp. Biol*. 212: 1731-1744.

Skeptical Approach to Hypothesis

The investigator must . . . cultivate a skeptical state of mind toward all hypothesis – especially his own- and be ready to abandon them the moment the evidence points the other way.

(In: Morgan, T. H. 1927. *Experimental Embryology*. New York, NY: Columbia University Press)

Chapter 17

Study of *in situ* fluorescence “on/off” switch mechanisms using novel fluorescent probes inside the gut tissues of *Drosophila*

Brijesh Singh Chauhan and S. Srikrishna* (skrishna@bhu.ac.in)

Department of Biochemistry, Institute of Science, Banaras Hindu University, Varanasi-221005

*For correspondence

Recommended Level: PG/Research

Sub-discipline: Cell Biology

Background:

Fluorescent probes are small fluorophores which are excited with specific wavelength of light and emit light of different wavelength. They have multiple applications in biological research ranging from cell imaging, live tracking of intracellular dynamics, drug discovery, analysis of environmental contagions disease diagnosis etc. (Fu and Finney, 2018). Fluorescent probes are also employed to detect target proteins in tissues by crosslinking with antibodies, biotin or avidin to monitor biological processes specific to the given cellular pathway (Jain and Cheng, 2017). Structure of the probe can be modified to create a range of excitation and emission spectra, target-binding affinity, chemical reactivity, and subcellular localization (Valeur and Berberan-Santos, 2011). Some important fluorophores used in biological science as fluorescent probes include coumarins, naphthalimides, fluorescein and its derivative fluorescein isothiocyanate (FITC), rhodamine and its derivative tetra methyl rhodamine isothiocyanate (TRITC) and cyanines (Fu and Finney, 2018). Fluorescent probes are also used to detect specific metal ions whose presence can turn on or turn off the fluorescence. Earlier, a novel probe Rhodamine B salicylaldehyde hydrazone bearing allyl group was synthesized to exhibit “turn on” fluorescence in presence of Al^{3+} ion, which opens the spirolactum ring to cause color change and enhanced fluorescence. Several other Rhodamine based probes have also been synthesized for detection of biologically important metal ions like Hg^{2+} , Cu^{2+} , Fe^{3+} and Al^{3+} (Rai *et al.*, 2018). Recently a novel boronic acid derivative fluorescent probe 4-(3-(4-(4,5-diphenyl-1H-imidazole-2-yl)phenyl)-1,2,4-oxadiazol-5-yl)phenylboronic acid (IOPBA) has been explored to examine ‘on-off-on’ fluorescence activity in HeLa cells in presence of Fe^{3+}/F^{-} ions. Fluorescence of the probe IOPBA was found to be quenched in presence of Fe^{3+} and recovered in the presence of F^{-} (Fluoride) ions (Selvaraj *et al.*, 2019).

Here, we describe an experimental procedure based on a novel fluorescent probe 3, 3'-(pyridine-2,6-diylbis(azanediyl))bis(methylene)dinaphthalen-2-ol (RAPHN), which can be used to specifically detect Fe^{3+} ions of ferric chloride and fluoride (F^-) ions of sodium fluoride in the gut of *Drosophila*. The RAPHN and its Fe^{3+} complex show selectivity in the detection of iron and fluoride ions in *Drosophila*. The activity of fluorescent probe depends upon the presence or absence of $\text{Fe}^{3+}/\text{F}^-$ because of $-\text{CH}=\text{N}$ isomerization and probe to metal charge transfer (PMCT). The strong fluorescence of RAPHN is seen when $\text{CH}=\text{N}$ isomerization is inhibited.

Fluorescence of the RAPHN probe is ‘turned off’ in presence of Fe^{3+} while it is ‘turned on’ in presence of F^- (Fig.1) (Dwivedi *et al.*, 2019). In RAPHN- Fe^{3+} complex, the quenching property of fluorescent probe RAPHN is achieved due to partially filled d shell of Fe^{3+} which induces to unlock a non-radiative deactivation channel of fluorescent probe and assists the PMCT route (Warrier and Kharkar, 2018). The addition of F^- in RAPHN- Fe^{3+} complex causes demetallation of Fe^{3+} from the probe by the formation of $[\text{FeF}_6]^{3-}$ complex and thereby disrupting the probe-metal interaction which ultimately releases the free fluorescent probe (Dwivedi *et al.*, 2018). Here, the fluorescent probe to metal ratio of 1:2 is used for tracing the metal ions in the gut tissues. The present exercise demonstrates in situ on/off switch behavior of RAPHN probe in presence of specific metal ions, which can be effectively used in understanding metal dys-homeostasis conditions associated with various human neurodegenerative diseases such as Alzheimer’s and Huntington’s disease (Qian and Xu, 2015). Thus, different fluorescent probes could be designed to identify specific intra-cellular metal dys-homeostasis in a wide array of biological systems.

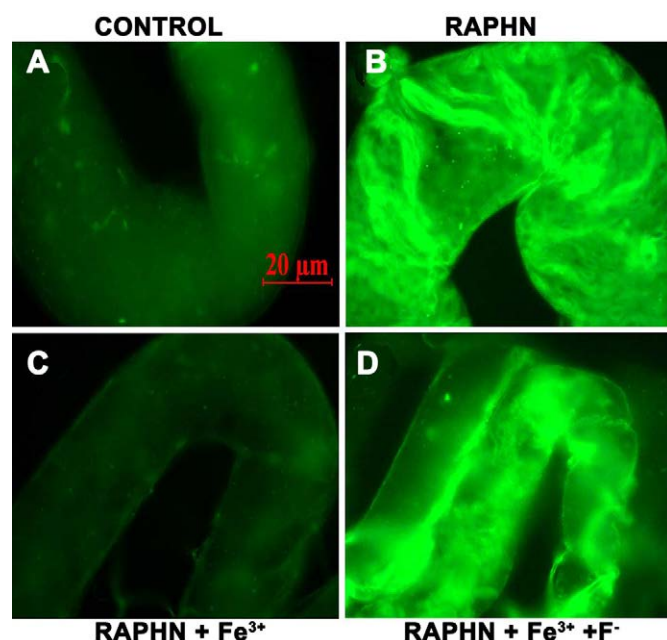


Fig. 1. Fluorescence intensity of the RAPHN probe in third instar larval gut tissues of *Drosophila* is modulated by Fe^{3+} and F^- ions. A. Control gut cells treated with 1X PBS show no fluorescence, B. RAPHN containing gut tissue shows ‘turned on’ fluorescence. C. RAPHN fluorescence is ‘turned off’ in presence of $200\ \mu\text{M}\ \text{Fe}^{3+}$. D. Addition of $200\ \mu\text{M}\ \text{F}^-$ ‘turns on’ the RAPHN fluorescence is again.

Objective:

To examine the fluorescence switch “on/off” mechanisms of a probe in gut tissues of *Drosophila*.

Materials Required:

A. Fruit flies: Wild type *Drosophila* third instar larvae (or flies if desired).

B. Equipments and other accessories:

- i. Stereo binocular microscope
- ii. Fluorescence microscope
- iii. Fine hair brush
- iv. Fine dissection needles, forceps, cavity or Maximov slides
- v. Cover slips (22 mm²) and fresh glass-slides
- vi. Micro-Pipettes

C. Reagents:

- i. Fluorescent probe (RAPHN): To prepare a 10 mM stock solution, dissolve 0.842 gm RAPHN in 5 mL ethanol through slow heating on hot plate for 60 sec and store at room temperature (RT, 20 to 25°C).
- ii. Ferric chloride (FeCl₃): To prepare 5 mM stock solution, dissolve 0.0814 gm FeCl₃ in 5 mL ethanol and store at RT.
- iii. Sodium fluoride (NaF) solution: To prepare 5 mM stock solution dissolve 0.00105 gm NaF in 5 mL ethanol with slow heating on hot plate for 60 sec and store at RT.
- iv. 1 X Phosphate Buffered Saline (1X PBS): Dissolve 8 g NaCl, 0.2 g KCl, 1.42 g Na₂HPO₄ and 0.272 g KH₂PO₄ in 800 mL of distilled H₂O. Adjust the pH to 7.4 with HCl and then add distilled H₂O to make the volume to 1000 mL. Autoclave and store at RT or at 4°C.
- v. Fixative: 4% paraformaldehyde in 1X PBS.
- vi. Founting medium: 1, 4-Diazabicyclo-2, 2, 2-octane (DABCO).
- vii. Transparent nail polish.

Experimental Details:

The experimental samples are of three types, viz., **a.** Gut tissues from RAPHN fed *Drosophila* larvae; **b.** Gut tissues from RAPHN-fed *Drosophila* larvae treated with Fe³⁺, and **c.** Gut tissues from RAPHN fed *Drosophila* larvae treated with Fe³⁺ followed by F⁻ treatment.

1. Prepare RAPHN fluorescent probe supplemented food by thoroughly mixing the RAPHN probe (at 100 μM final concentration) in standard fly food after its cooking and when it has cooled to ~45-55°C.

2. Transfer synchronized first instar larvae to this food and grow them at 24±1°C till third instar stage. Grow parallel control larvae on standard food. **Note:** Although RAPHN is stable under visible light, it is advisable to maintain low light conditions to reduce photo-bleaching of the probe.

3. Dissect gut from control and experimental third instar larvae and/or adult flies in PBS under stereo binocular microscope with low light illumination.
4. Transfer the gut tissues (n=10) in Maximov or cavity slide containing PBS at room temperature (RT). Wash the tissues 2 x 5 min each with PBS.
5. After washing, process some gut tissues (as in the later step 11 onwards) to examine the 'turned on' RAPHN signal under fluorescence microscope.
6. Treat the remaining (after step 4) gut tissues from the RAPHN probe fed larvae with 200 μM FeCl_3 solution (donor for Fe^{3+} quencher ions) for 15 min.
7. After incubation with quencher, briefly wash the gut tissues twice with PBS.
8. After step 7, process some of the tissues as in step 11 onwards for examining the 'turned off' RAPHN signal by fluorescence microscopy.
9. Incubate the remaining gut tissue (after step 7) in 200 μM NaF solution (donor for F^-) for 15 min for complete recovery of the fluorescence.
10. Wash the tissues twice with PBS for 5 min.
11. Fix all the tissues (from steps 5, 7 and 10) with 4% paraformaldehyde at RT for 15 min.
12. Wash the tissues twice in PBS for 5 min each.
13. Mount tissues from control and RAPHN probe fed larvae/fly on slides in 50 μL DABCO under coverslip and seal the edges with nail polish.
14. Examine under a fluorescence microscope using blue excitation and green emission filter to record the *in situ* fluorescence On/Off signal.

Observations:

1. Gut from control larvae show only a background fluorescence (**Fig. 1A**), while gut cells from RAPHN-fed larvae/flies show intense green fluorescence ('turn on' behavior) (**Fig. 1B**).
2. RAPHN-fed gut samples treated with the FeCl_3 solution (Fe^{3+} quencher) show little fluorescence because the RAPHN probe is 'turned off' by the quencher.
3. In the gut tissue from RAPHN-fed larvae treated with FeCl_3 solution and then with NaF (F^- ion donor), the initially quenched fluorescence is 'turned on' again (**Fig. 1D**).

Questions for self-study and interpretation:

1. What is the chemical basis of fluorescence 'turn on' and 'turn off' phenomenon in presence of different metal ions?
2. How can we determine the behaviour of fluorescent probes inside the cells or tissues?
3. Can metal sensing fluorescence probes be used in diagnosing disease condition?
4. Can we design tissue compatible novel fluorescent probes of choice to understand transient dynamic molecular mechanisms through bio-imaging?

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Warrier, S. and Kharkar, P. S. (2018) Highly selective on-off fluorescence recognition of Fe^{3+} based on a coumarin derivative and its application in live-cell imaging. *Spectrochimica Acta Part A: Molecular Biomolecular Spectroscopy*. 188: 659-665.

Cell Genetics & Cell Mechanics

Cell genetics led us to investigate cell mechanics. Cell mechanics now compels us to infer the structures underlying it. In seeking the mechanism of heredity and variation we are thus discovering the molecular basis of growth and reproduction. The theory of the cell revealed the unity of living processes; the study of the cell is beginning to reveal their physical foundations.

(In: Darlington, C. D. 1937. Recent Advances in Cytology, CAB Direct)

Chapter 18

Dissection and mounting of imaginal discs of *Drosophila*

B. V. Shyamala (shyamalabv@yahoo.com)

Developmental Genetics Lab, *Drosophila* Stock Centre, Department of Studies in Zoology, University of Mysore, Manasagangotri, Mysore-570006.

Recommended Level: PG/Research

Sub-discipline: Developmental Biology/ Genetics/ Cell Biology/Anatomy

Background:

Drosophila, is a holometabolous insect. It passes through distinct larval, pupal and adult stages during its life cycle. There is a dramatic change in the form and function, between larva and the adult, which is brought about within the pupal case during metamorphosis. A special class of cells in the larva are organized into distinct bags of cells called 'imaginal discs'. These cells do not have any specific function in the larva, but are precursors of adult integumental structures, set aside and awaiting a signal to differentiate into definite structures of "Imago" or the adult.

Imaginal disc cell clusters, specified during embryonic development, appear as local thickenings in the just hatched larvae, and transform later during larval development into bilayered epithelial sac like structures connected by a stalk to the larval epidermis. One of the layers undergoes extensive proliferation generating a large number of columnar cells. The extended layer folds on itself into a characteristic pattern of whorls, in the compact and flattened disc. Preceding the pupal metamorphosis, ecdysone hormone triggers each disc to start differentiating, which involves opening of the folds, changing into a preliminary form representing the corresponding adult structure, following which dramatic cellular differentiation begins. This event is referred to as "Disc Eversion" (Gilbert and Barresi, 2016).

Imaginal discs offer very special systems for developmental biologists to probe into the genetic and molecular aspects of cellular processes during development. The uniqueness lies in the fact that these discs are physically isolated from each other and differ from the remainder of the larval tissues with respect to their developmental and functional status (Bryant, 1974). Thus each imaginal disc serves as a distinct unit of developmental field presenting independent and unique pattern of gene expression, pattern formation and differentiation programs. The precursor cells in a given disc undergo a wide variety of specializations in a complex predetermined pattern into different elements. For example,

In: Experiments with *Drosophila* for Biology Courses. 2024. (eds: S.C. Lakhotia & H. A. Ranganath). Second Edition, Indian Academy of Sciences, Bengaluru, India

the unilayered cells of the leg disc differentiate into coxa, femur, tibia and tarsal segments of the leg consisting of cuticle forming cells, sensory neurons, supporting bristles, sex-comb teeth, claws at the distal tip etc., organized in a definite pattern. Similarly, the monolayer cells of the eye disc (see Chapters 2, 19) form lens forming cells, pigment cells, photoreceptor neurons, inter-ommatidial bristles etc. in a stereotypic pattern. Another useful feature of the imaginal discs is that they can be induced to undergo differentiation in exogenous conditions, e.g, by transplanting them into a host larva which is ready to undergo pupation (Murphy, 1967) or in an *in vitro* culture with the addition of ecdysone (Guillermet and Mandaron, 1980; Shyamala and Bhat, 2002). Interestingly they retain their predetermined / restricted developmental capabilities in these conditions as well.

Extensive gene expression studies carried out using imaginal discs, specially the wing and the eye discs, have taught us immensely about the molecular cross talks and signaling pathways which regulate compartmentalization, cell fate specification and differentiation processes within a defined developmental unit. With the fast evolving genetic tools in recent years, and the ability for genetic manipulations of specific cell clusters within a normal cellular context of the disc, they have also proved to be good models for better understanding of cellular interactions in growth regulation, tissue regeneration, cancer and other diseases (Bedra and Paro, 2016). This chapter provides protocol for dissection, preparation and identification of imaginal discs from third instar larvae.

Objectives:

To dissect and mount imaginal discs from *Drosophila* larvae.

Materials Required:

1. Well fed third instar (or any other stage as per requirement) larvae of *D.melanogaster* or any other *Drosophila* species of interest.
2. 1X Phosphate Buffered Saline (PBS), pH 7.4: Dissolve 8 g NaCl, 0.2 g KCl, 1.42 g Na₂HPO₄ and 0.272 g KH₂PO₄ in 800 mL of distilled H₂O. Adjust the pH to 7.4 with HCl and then add distilled H₂O to make the volume to 1000 mL. Autoclave and store at RT or at 4°C.
3. 0.3% PBTx : Measure 300 µL of TritonX-100, dispense it into 99.7 mL of 1X PBS. Stir well on a magnetic stirrer for 1-2 hr to obtain a uniform solution.
4. 4% Paraformaldehyde solution in PBS (To be prepared fresh)
5. 50% Glycerol
6. Fine needles, brush, microscope slides (cavity and plane) and coverslips.
7. Stereo-binocular microscope and compound microscopes with control for illumination and sub-stage iris diaphragm.

Experimental Details:

1. Select well fed active third instar larvae from the *Drosophila* culture with the help of a fine brush and wash in PBS to remove any adhering food particles.
2. Place a larva in a drop of PBS in a clean cavity slide. Carry out the dissections under stereo-binocular microscope, keeping black plate as background.
3. Hold the larva with one needle at about 1/3rd the length from its anterior end, place another needle firmly on the black mouth hook and pull the two needles in opposite directions. Mouth hook along with the brain, salivary glands and cluster of the head, pro and mesothoracic imaginal discs will get separated from rest of the larva (Fig. 1A), while the paired metathoracic leg, haltere and wing discs will usually be found attached as two clusters to the inner side of the body wall (Fig. 1B) at the cut end of the larva (also see Chapter 2).

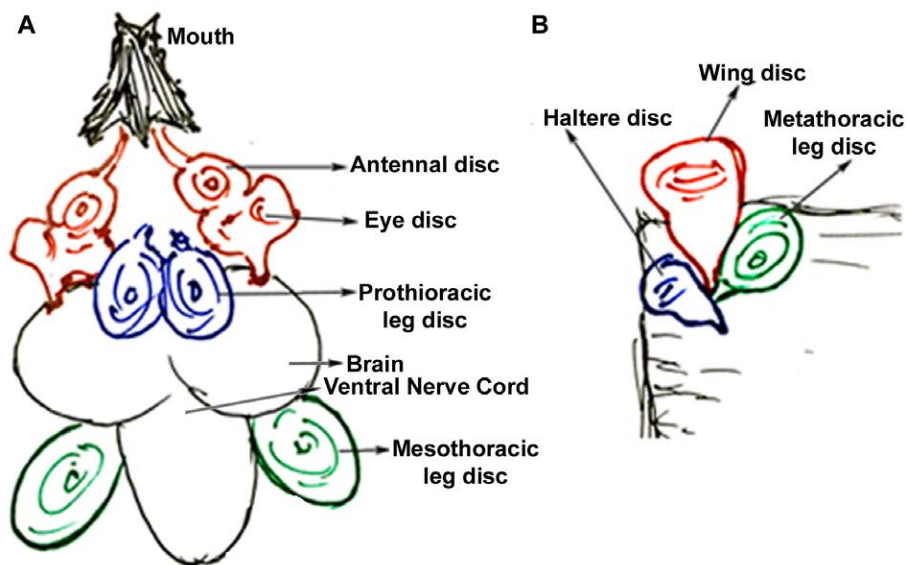


Fig. 1. Schematic showing the positions of different imaginal discs. A. cluster of discs found associated with the larval brain. **B.** Discs found attached to the body wall at the cut end.

4. Fixation: Remove the salivary glands along with their attached fat body, and transfer the cluster of anterior discs along with the mouth hook and brain into freshly prepared 4% paraformaldehyde fixative in a cavity slide. Transfer the wing, metathoracic leg and haltere discs together with a small piece of attached body wall also into the fixative. Keep the sample covered with a petriplate to minimize evaporation. Fix the discs for 10-15 min at room temperature. The discs now become relatively more rigid and easy to handle.
5. Following the fixation, separate individual discs from the other attached tissues (mouth hook, brain lobes, body wall etc.) using fine dissecting needles.
6. Drain out /blot the fixative from the slide carefully without losing the discs. Wash the discs with 0.3% PBTx once (10 min).
7. Transfer individual discs carefully into a drop of 50% glycerol taken on a plane slide.

Allow the discs to settle flat on the slide and slowly place a coverslip, avoiding trapping of air bubble.

8. Examine the unstained discs under a phase contrast microscope. In the absence of phase-contrast optics, observe the discs under bright-field microscope by reducing the aperture size of the sub-stage iris diaphragm and light intensity.

Observations:

A total of 10 pairs of imaginal discs are present. Among these, the ones giving rise to adult mouth parts (Labial disc, Clypeo-labrum disc) and the genitalia (genital discs) are relatively smaller in size. The following larger discs can be more easily prepared and identified (**Fig. 2**):

IMAGINAL DISC

- Eye-Antennal
- Prothoracic Leg disc
- Mesothoracic leg disc
- Wing disc
- Metathoracic leg disc
- Haltere disc

ADULT STRUCTURES FORMED

- Eye, Antenna and Head case
- First pair of legs
- Second pair of legs
- Wing blade, flight muscles
- Third pair of legs
- Haltere and its supporting muscles

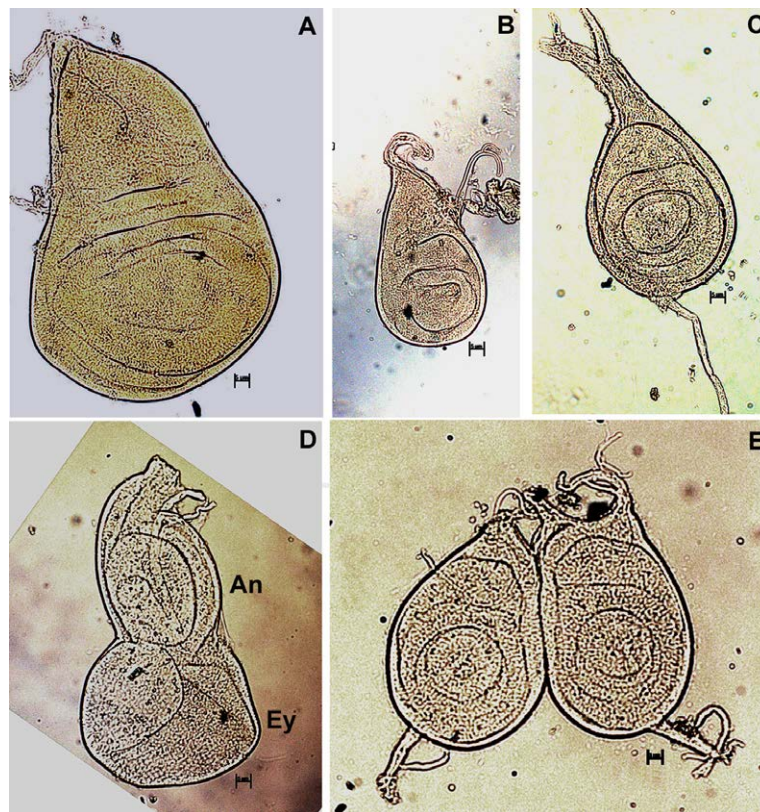


Fig. 2. Unstained imaginal discs observed under bright-field microscope with regulated iris-diaphragm and illumination. A. Wing disc B. Haltere disc C. Mesothoracic leg disc D. Eye (Ey) – Antennal (An) discs and E. Prothoracic leg discs. Scale bars in each image represents 5 μ m.

Questions for self-study and interpretation:

1. **Fig. 3** below shows leg and wing imaginal discs from **A.** *en-lacZ/CyO*, and **B.** *vgM-lacZ* enhancer trap strains, respectively, which have been stained histochemically for β -galactosidase enzyme activity, which is used as a reporter (see **Chapter 39**). What is the principle and importance of this technique in studying gene expression and pattern formation? Why are the staining patterns in the two images in **Fig. 3.** **A** and **B** different and what do such differences indicate?

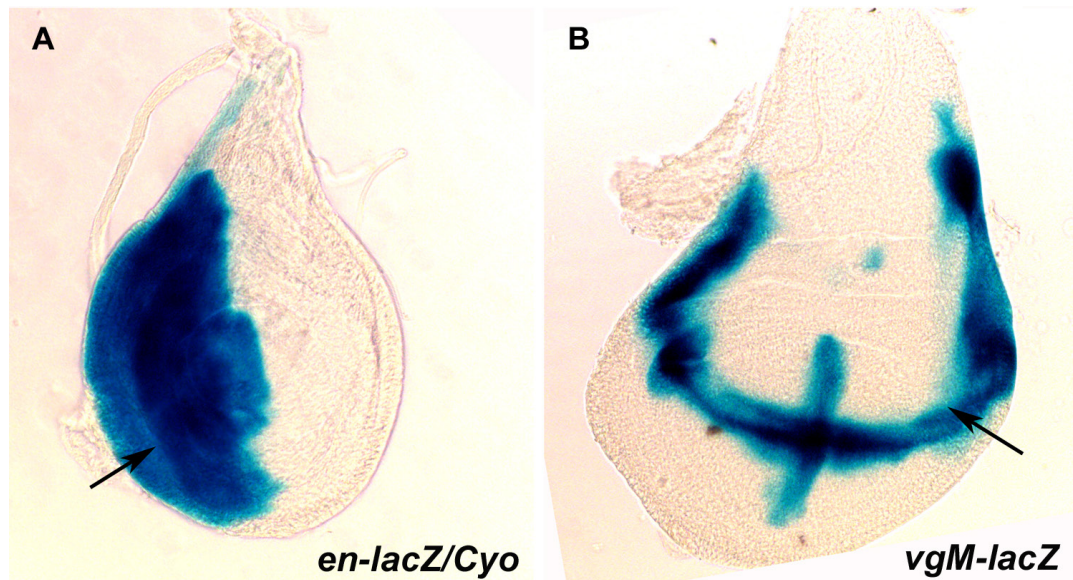


Fig. 3. Imaginal discs from 3rd instar larvae stained for β -galactosidase enzyme activity. **A.** Leg disc from *engrailed-lacZ*, and **B.** Wing disc from *vestigial-lacZ* transgenic late third instar larvae. Blue stained regions (arrows) show the β -galactosidase reporter activity.

2. Pupal prothoracic leg discs immuno stained with antibodies against Bric-a-brac and Sex comb reduced gene products are shown in **Fig. 4.** **A** and **B**, respectively. How is this technique different from that of the method employed in **Fig. 3** with reference to the information it provides?

Why are these prothoracic leg discs (**Fig. 4**) different in their shape and structure compared to the ones in **Fig. 2E**? Read more about the developmental event involved.

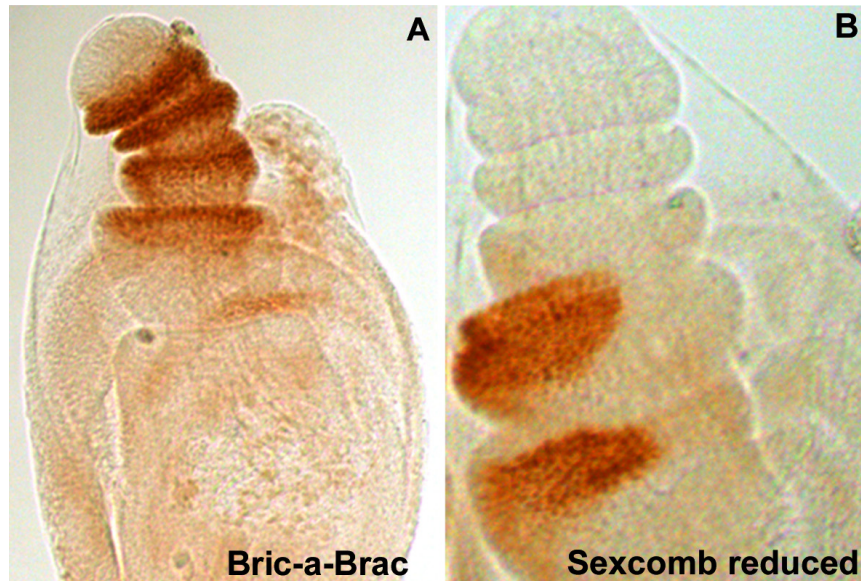


Fig. 4. Pupal leg discs immunostained for A. Bric-a-brac, and B. Sex comb reduced proteins (images reproduced from Devi *et al.*, 2013).

3. How are imaginal discs transplanted and how did such experiments lead to the discovery of the phenomenon of ‘trans-determination’?

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Chapter 19

Development of eye imaginal disc during larval and pupal stages of *Drosophila melanogaster*

Vanshika Kaushik (vanshikakaushik0@gmail.com) and S C Lakhota* (lakhotia@bhu.ac.in)

Cytogenetics Laboratory, Department of Zoology, Banaras Hindu University, Varanasi
221005

*For correspondence

Recommended level: UG/ PG/ Research

Sub-discipline: Anatomy/Developmental Biology

Background:

Drosophila melanogaster or fruit fly is a holometabolous insect in which the larvae undergo complete metamorphosis during the pupal stage to produce the imago or adult stage (see Chapter 1). The primordia of different adult appendages are represented in larvae as imaginal discs within the larval body cavity (see Chapter 18). The larval imaginal discs are sac like structures of epithelial cells. All imaginal discs, except the genital discs, originate during embryonic development by invagination of ectoderm. The genital discs are mesodermal in origin. *Drosophila* has a total of 19 imaginal discs that give rise to specific adult appendages (see Chapter 18). Each imaginal disc has an outer peripodial layer and the inner disc proper cells (Haynie and Bryant, 1986; Beira and Paro, 2016). During later larval period, the imaginal disc cells undergo extensive cell proliferation, accompanied by cell fate determination. Their differentiation into specific adult external appendages occurs during the pupal metamorphosis. However, in the case of eye discs, the nearly 800 ommatidial units that make up the adult compound eyes are progressively established during the third instar larval stage.

The visual system, head epidermis, and olfactory organs of the adult fly develop from a pair of composite eye-antennal imaginal discs (Gehring, 1966; Baker, 1978; Haynie and Bryant, 1986). The eye-antennal disc remains very small till the first instar stage with only about 20-70 cells (Madhavan and Schneiderman, 1977; Kaliss, 1939). Active cell division begins during the second instar stage so that the compound disc develops an oval shape for the eye region and rounded appearance for the antennal region (Held, 2002). Amidst continuing cell division, differentiation of ommatidial units occurs in an orderly manner in the eye disc during the third instar stage (Ready et al 1976). The eye part of each third instar larval eye-antennal disc is associated with the optic lobe of the cerebral hemisphere through the optic nerve on each side (Fig. 1). The anterior antennal part of the compound eye-antennal disc is near the mouth-hooks. A morphogenetic furrow (MF) expressing the Dpp signaling molecule forms at the posterior end of eye disc soon after the beginning of the third instar stage (Chanut and Heberlein, 1997). The MF appears as an indentation in the epithelium which moves forward (anteriorly) as a wave spanning

the width of the disc (Fig. 1). During the third instar stage the anteriorly advancing MF recruits undifferentiated cells anterior to it and initiates signaling cascade that leads to their progressive differentiation and organization into the patterned array of nearly 700 unit eyes or ommatidia posterior to it (Fig. 1, Ready et al., 1976; Chanut and Heberlein, 1997). Due to the progressive differentiation, the ommatidial units in posterior rows that are further away from the advancing MF are larger and more developed than those closer to posterior boundary of the MF (see the patterned green fluorescence of differentiating ommatidial array in each eye disc in Fig. 1). The ommatidia patterned during the larval stage mature into the adult compound eye during the pupal period. Each ommatidial unit includes cone cells, pigment cells, 8 photoreceptor neurons, and ommatidial bristles. During the pupal stages, extra cells within each ommatidium undergo cell death through apoptosis while the eye pigments are deposited after the mid-pupal stage (Cagan and Ready, 1989).

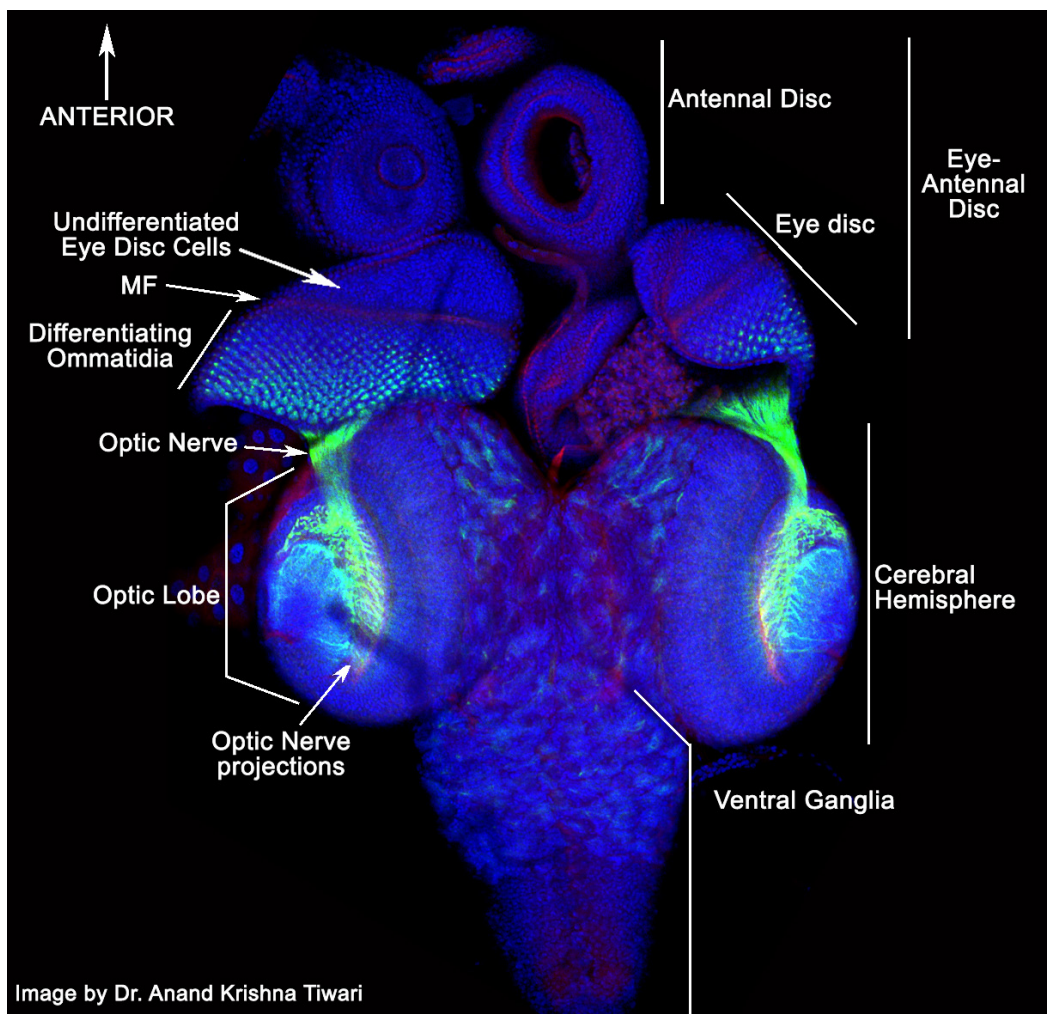


Fig. 1 : Eye discs and central nervous system of late third instar larva of *Drosophila melanogaster*. Confocal image of eye-antennal discs, cerebral hemispheres, and ventral ganglia. Nuclei are in blue (DAPI), neuronal photoreceptor cells in differentiating ommatidia, the optic nerve and optic neurons in the optic lobe of cerebral hemispheres are in green (GFP) and F-actin in red (Phalloidin-Rhodamine). MF = Morphogenetic Furrow (red fluorescence due to high F-Actin). We thank Dr. Anand Krishna Tiwari (Institute of Advanced Research, Gandhinagar, India) for providing this confocal image collected by him in 2009 while working in our Laboratory at BHU.

Development and differentiation of ommatidial lattice in the large larval eye discs have been widely studied and documented. Most of the signaling pathways that determine the eye development in flies are well understood and are conserved in mammals (Mishra *et al.*, 2021; Catalani *et al.*, 2022). Thus, *Drosophila* eye disc and adult eye have become very good models for examining the basics of development and differentiation. Since the eye includes neuronal photoreceptor cells, the fly eye model is also widely used for understanding many human neurodegenerative disorders, especially because the GAL4-UAS system (see Chapters 39, 40) permits targeted expression of neurodegeneration-inducing transgenes in the neuronal photoreceptor cells of eye discs (Mallik and Lakhota, 2010; Nitta and Sugi, 2022).

The present chapter describes methods to dissect eye-antennal imaginal disc from second and third instar larvae and the developing eye form early, mid, and late pupal stages. A simple method for aceto-orcein staining of eye-antennal imaginal discs for easy visualization of the ordered pattern of developing ommatidial units during late larval and pupal stages is also described.

Objective:

To observe development of eye-antennal imaginal discs from second instar larvae to late pupal stages and to examine formation of ommatidial units.

Materials required:

- A. **Flies:** Healthy wild type (or any desired genotype) larvae and pupae
- B. **Equipment and other supplies:**
 - i. Stereo zoom-binocular microscope.
 - ii. Needles for dissection: pair of 6 mm/31-gauge insulin syringes (fine needle) and a pair of tuberculin syringes with 26-gauge needle (coarse needle, Fig. 2).
 - iii. Fine hairbrush.
 - iv. Cavity block/ cavity slides/ Maximov slides (Fig. 2).
 - v. Slides and coverslips.
- C. **Reagents:**
 - i. 1x Phosphate Buffer Saline (PBS pH 7.4):

NaCl	8 gm
KCl	0.2 gm
Na ₂ HPO ₄	1.44 gm
KH ₂ PO ₄	0.24 gm

Serially dissolve the above in 800 mL distilled water; when fully dissolved, adjust pH to 7.4 with 1N HCl as required. Finally make the volume to 1000 ml with distilled water, autoclave the solution and store at room temperature.

- ii. 4% w/v Paraformaldehyde: Dissolve 400 mg Paraformaldehyde powder in 10 mL 1X PBS at 37°C for 60 min with periodic shaking. Cool and store at 4°C, for a few days, if required.
- iii. Aceto-Orcein stain: Dissolve 2 gm Orcein powder in 100 mL of 50% Acetic acid by boiling for 2 hr under a reflux condenser. Filter when cool. The prepared stain can be used for several months but requires periodic filtration before use to avoid accumulation of stain crystals (2% Aceto-Carmine stain, which can also be used, is prepared in a similar manner; commercially available stains can also be used).

Note: Aceto-Orcein/Aceto-Carmine stains are generally used to stain nuclei/chromosomes.

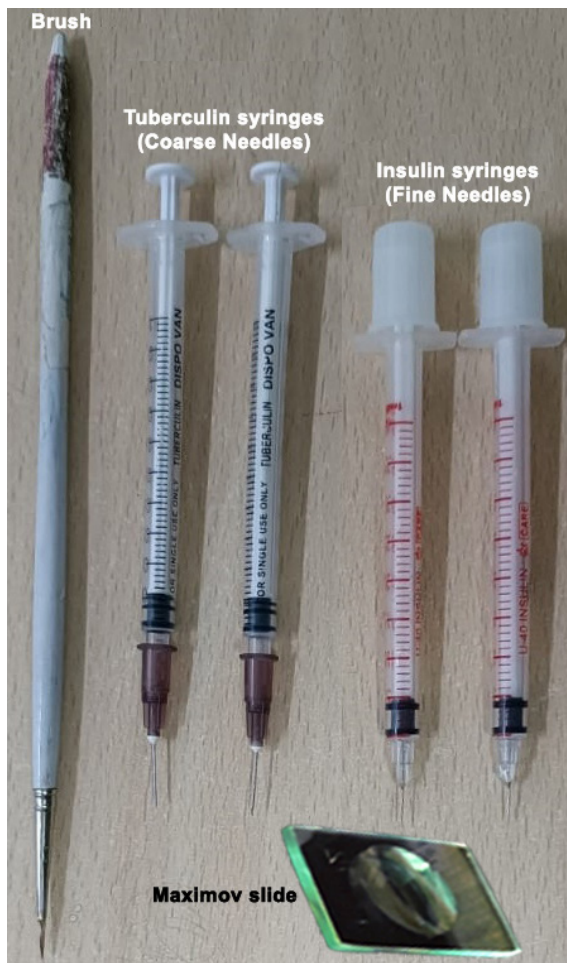


Fig. 2. Fine hairbrush, needles and Maximov slide required for dissection of eye discs

Experimental details:

1. Dissection of eye-antennal imaginal discs/developing eyes from larvae and pupal stages

- A. Late second and early third instar larva (46-48 hr and 48-50 hour after egg-laying, Fig. 3)
 - i. Wash healthy 4-5 late second/early third instar larvae with distilled water to remove adhering food particles
 - ii. Place a larva on a slide in a small drop of PBS.

- iii. Observing through the stereo-binocular microscope and using 5X or higher magnification objective, hold the posterior end of the larva with tip of one fine needle and the anterior end near mouth hook with the other fine needle (Fig. 3A).
Note: Initial practice with dissection of the larger mid- or late-third instar larvae helps in dissecting out the tiny eye-antennal discs from small late 2nd/early 3rd instar larvae.
- iv. Gently pull the needles in opposite directions to liberate inner tissues (Fig. 3B).
Note: The antennal part of the very small eye-antennal discs remains associated with the esophagus at anterior end of larva close to the mouth hook while the posterior eye region of the disc is associated with the optic lobe of brain/central nervous system (CNS) through the optic nerve on each side.
- v. Carefully pull out the spherical pair of cerebral ganglia (CNS) so that the eye-antennal discs become visible (Fig. 3C).
- vi. Using the fine pair of needles, pull the eye disc away from the optic lobe so that the optic nerve is severed, and the disc becomes free (Fig. 3C).
- vii. To separate the eye-antennal disc from the mouth region, hold the hard cuticular mouth hook tip with one fine needle and using the other fine needle, delicately sever the anterior stalk of the eye antennal disc to release it from the oesophagus and the mouth hook (Fig. 3D).

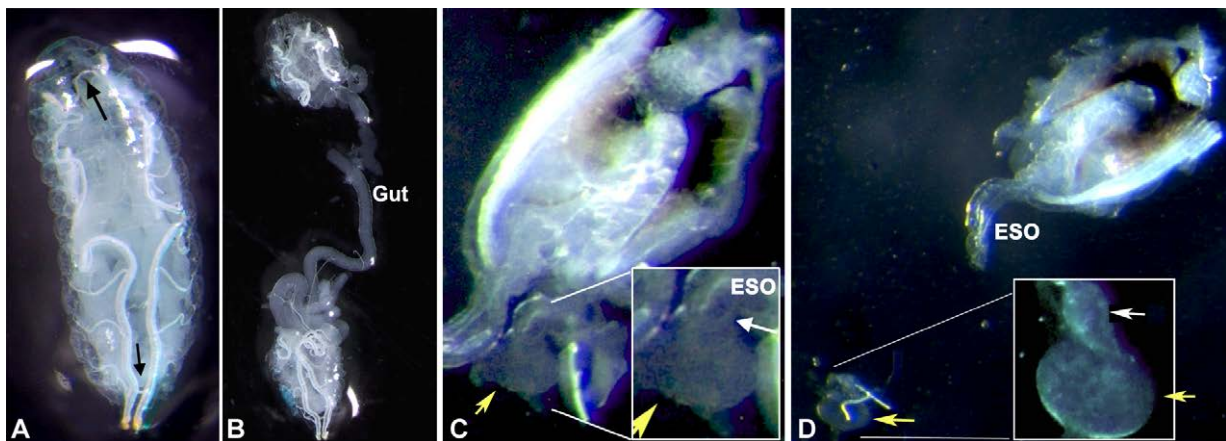


Fig. 3. Steps in dissection of eye discs from late second/early third instar larvae. **A.** Intact larva; black arrows indicate the directions of pulling the larval cuticle with fine needles. **B.** Liberated viscera after a cut at the anterior one third of body and pulling out of the anterior and posterior ends of larva in opposite directions. **C.** Anterior end of the dissected larva after the eye disc part (yellow arrow) has been freed from the optic ganglia while the antennal part (white arrow) is still attached with the esophagus (ESO): the inset in **C** shows the eye-antennal disc at a higher magnification. **D.** The eye-antennal disc (lower left corner) after removal of its antennal region's attachment (white arrow) with the esophagus (ESO); the inset in **D** shows the eye-antennal disc at a higher magnification. Yellow arrows in **C** and **D** indicate the eye part while the white arrows indicate the antennal part.

- viii. Carefully remove any adhering tracheal tubes with fine needles from the separated eye-antennal disc.
 - ix. Examine the disc under microscope without or after fixation and aceto-carmin staining (see later)
- B. Mid/Late third instar larva (96-120-hr after egg laying, Fig. 4)
- i. Wash 4-5 third instar larvae as in step A.1.
 - ii. Place one larva in a drop of PBS on a slide and using two needles, hold the larva in the middle with one needle and at the mouth hook with another needle. Pull the two needles in opposite directions. The CNS and anterior part of the gut are pulled out anteriorly (Fig. 4B).

Notes:

- a. In some cases, while pulling out the mouth hooks at step B.2, most of the viscera and fat bodies flow out anteriorly. In such cases, use fine needles to gently spread the internal larval tissues on the slide and look for the brain complex and mouth hooks before proceeding to step 3.
 - b. The eye-antennal discs may remain associated with the mouth region or may be attached with the optic lobes of the CNS (Fig. 4B).
- iii. If the disc is associated with the mouth region, use fine needles to separate the disc from mouth region as in step A.7 and sever the optic nerve to free the eye-antennal disc (Fig. 4B-D).
 - iv. If the disc is associated with the optic lobes, use fine needles to hold the optic lobe with one needle, and carefully remove the larval CNS from the eye region of the disc without poking it with the needle. (Fig. 4C, E, F).
 - v. Mount the separated disc (Fig. 4G) in PBS and examine under microscope to identify the advancing morphogenetic furrow or fix and stain as described later.

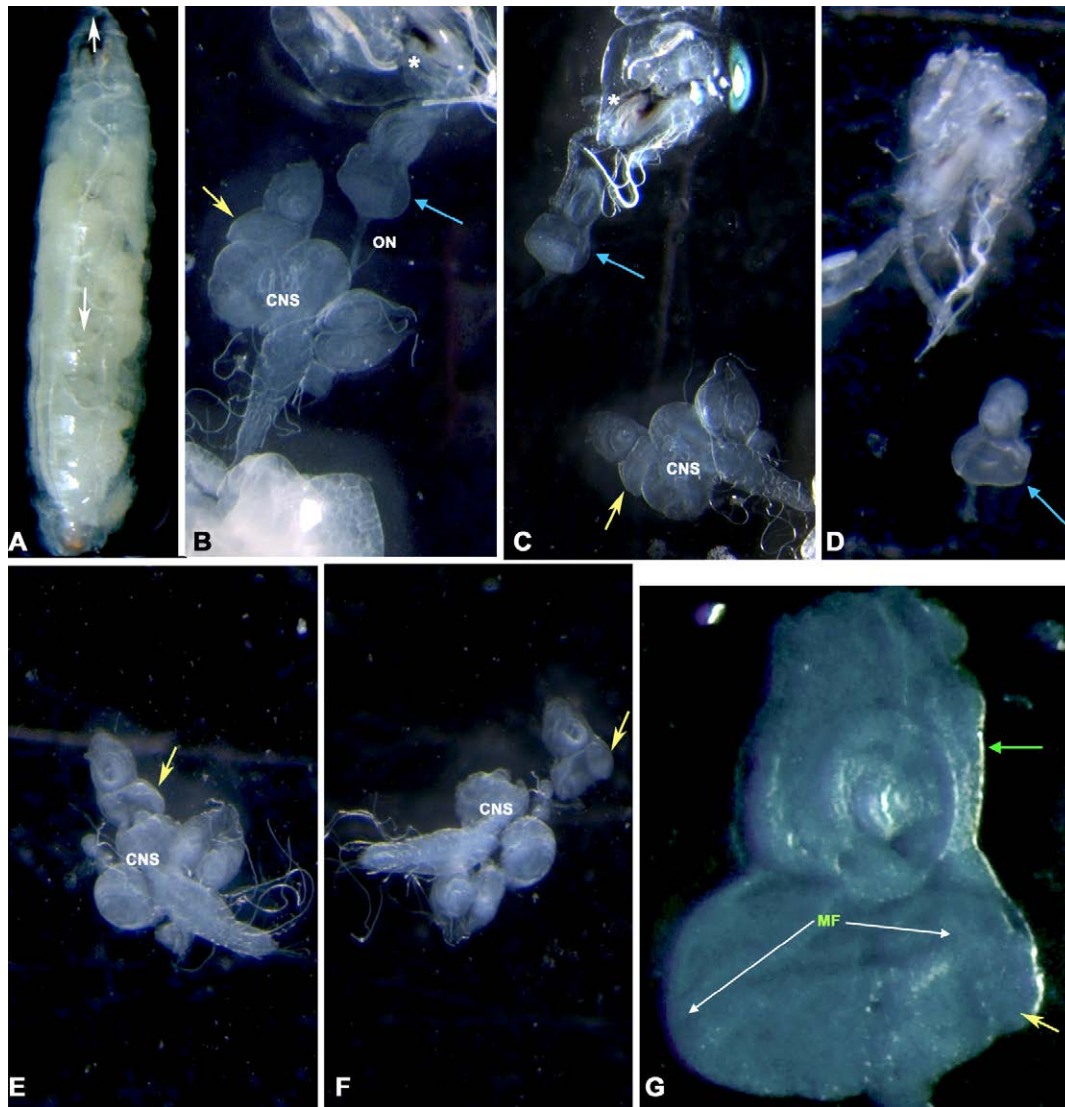


Fig. 4. Steps in dissection of eye discs from mid/late third instar larvae. **A.** Intact larva: white arrows indicate the directions of pulling of the larval cuticle with needles. **B.** Released tissues at the anterior end of larva viscera with one eye-antennal disc (yellow arrow) attached to the optic lobe of the brain (CNS) and another (blue arrow) near the mouth region with its optic nerve (ON) connected to the optic lobe of brain stretched out. **C. D.** Separation of the eye-antennal disc (blue arrow) near the mouth hook after the optic nerve and connection with the mouth hook are severed. **D. E.** Separation of the eye-antennal disc (yellow arrow) attached to the optic lobe of CNS. **G.** Magnified view of the cleaned eye-antennal disc; green and yellow arrows indicate the antennal and eye parts, respectively; white arrows indicate the dark-appearing morphogenetic furrow (MF) ahead of the differentiating ommatidial units in posterior part of the eye disc. Images not to scale.

C. Early Pupa (Fig. 5)

- i. Collect a few early pupae (8-10 hr after the formation of white pupa), wash them in 1x PBS and place one pupa on a slide in a drop of PBS.
- ii. While viewing under the stereo-binocular microscope, hold the posterior end of the pupa with one fine needle and make a lateral cut (Fig. 5B) with another fine needle on the cuticle just behind the head region visible through the pupal case.

- iii. Carefully open the cuticle, without damaging the soft pupal tissues, from the point of cut to remove the pupal case from the head region (head capsule, Fig. 5C), following which remove the posterior part of the pupal case to expose the soft pupal body from the chitinous case (Fig. 5D).
- iv. With a fine needle, cut off the pupal head region from the posterior part of body and discard the latter (Fig. 5D).
- v. Place one needle in the center of the head region and apply slight pressure with side of the fine needle so that the pair of translucent oval eyes ooze out and become visible on posterior side of the head region (Fig.4D-F). Cut the eyes from sides using another needle (Fig. 5G-H).
- vi. Remove excess fat bodies from sides and other structures and use the dissected eye (Fig. 5I) for further staining/mounting.

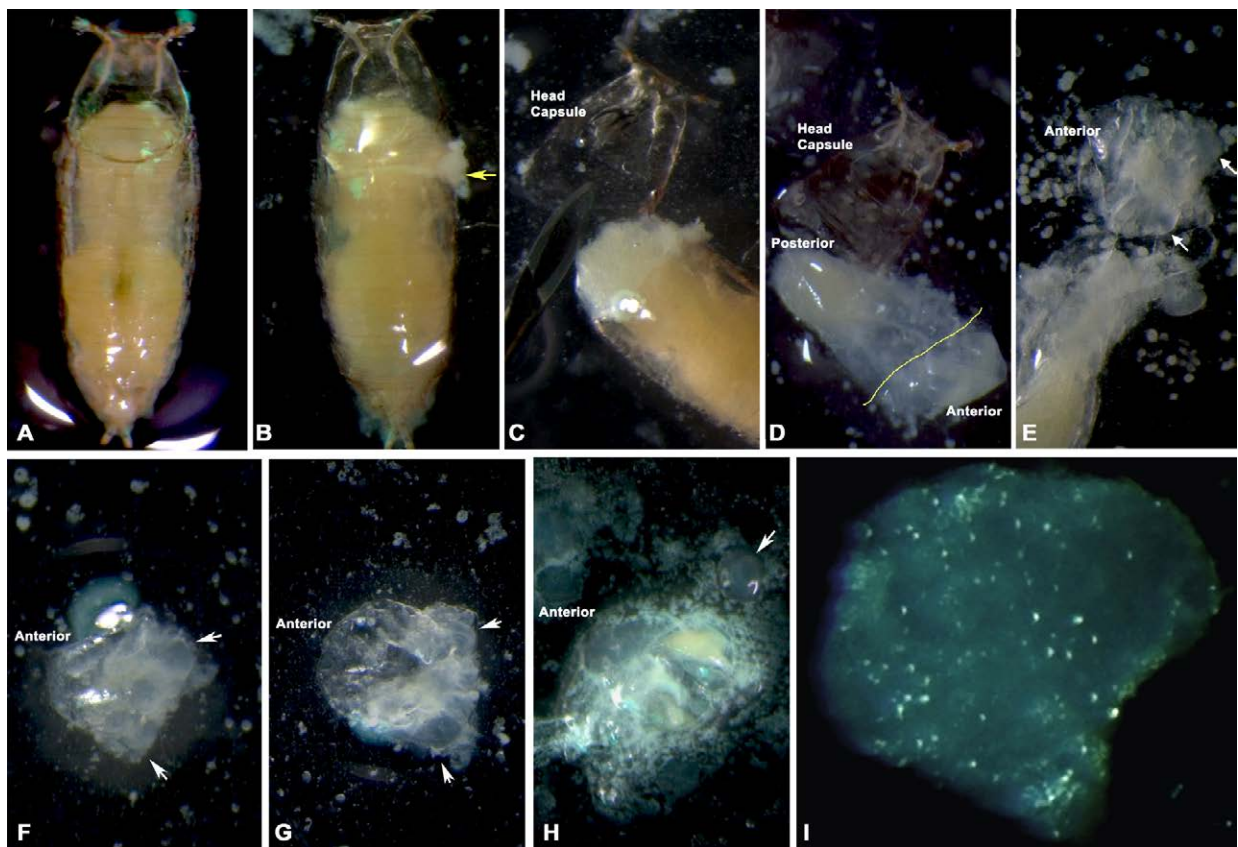


Fig. 5. Steps in dissection of developing eyes from early pupa. A. Intact early brown pupa. B. Sharp cut on lateral side at the anterior one third of pupal cuticle (yellow arrow) with internal tissues oozing out. C. Anterior cuticle covering the head (Head Capsule) separated away from rest of the pupa. D-E. Cuticle covering the posterior pupa removed; yellow line indicates the plane for incision by fine needle to separate head from the posterior region of pupal body to expose the pigment-less oval eyes (white arrows). F-H. Steps in clearing the oval translucent pair of eyes from attached brain and other tissues. I. Magnified view of one eye which still does not show any external indication of the ommatidial organization. Images not to scale.

D. Mid Pupa (Fig. 6)

- i. Take a washed mid stage pupa (45-50 hr after white pupa formation) in a drop of PBS on a slide under a stereo-binocular microscope.

- ii. Hold the posterior end of the pupa with a fine needle, and using another fine needle, make a lateral cut on cuticle just behind head region of the pupa (Fig. 5B) and carefully open the cuticle from the cut point with the same fine needle. Remove the head cuticle away from internal tissue (Fig. 6C). **Note:** Pigmented eyes are clearly visible by mid-pupal stage through the chitinous pupal case.
- iii. Carefully remove the thin delicate transparent membrane covering the pupal tissues with needles.
- iv. Using the fine needles, make a central antero-posterior incision in the head to separate the two lateral halves (Fig. 6G-H). **Note:** Avoid excess pressure from needles at this stage as it may damage the soft eyes.

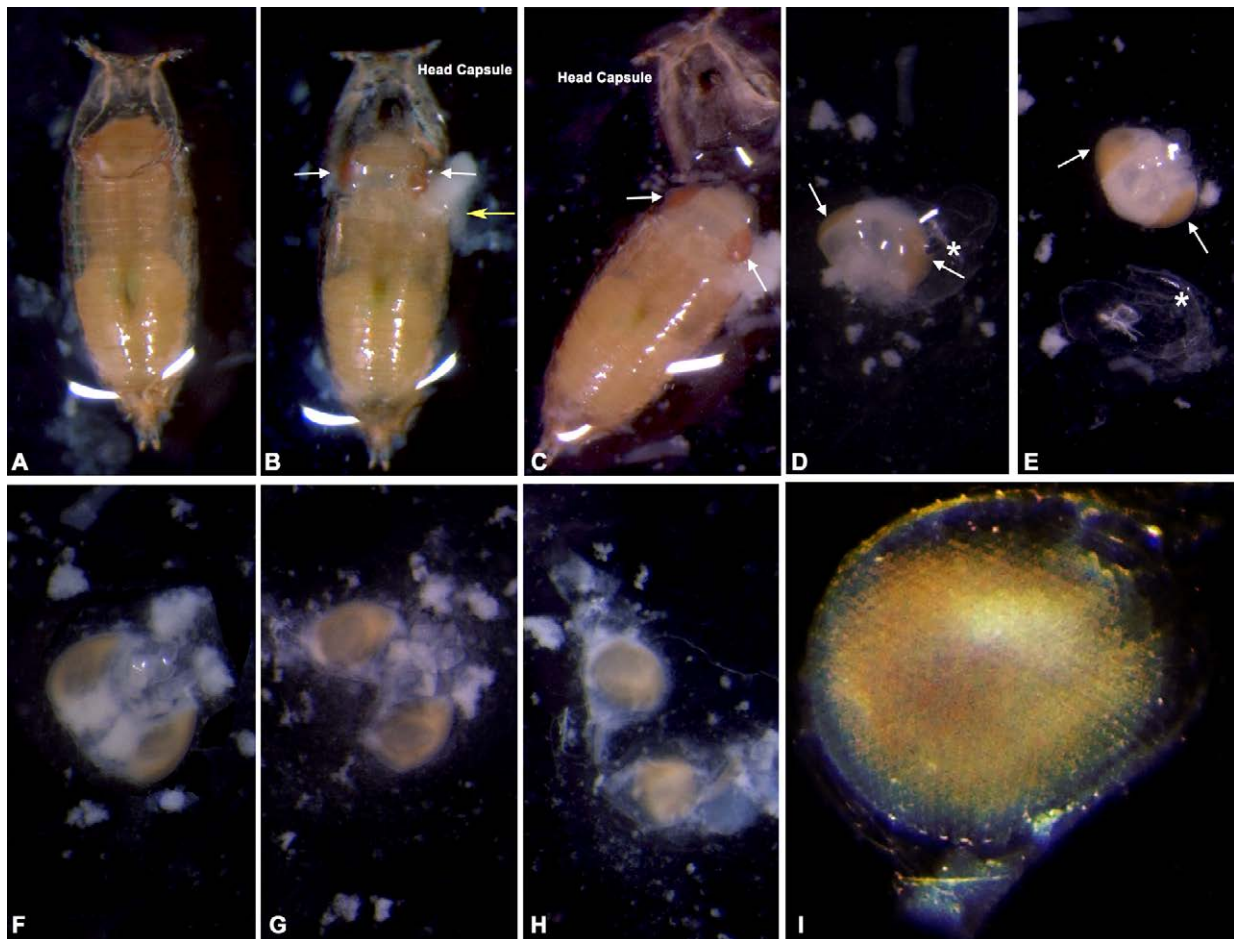


Fig. 6. Steps in dissection of developing eyes from mid pupa. A. Intact pupa. B. Sharp cut (yellow arrow) on lateral side at the anterior one third of pupal cuticle (just behind the Head Capsule region) with internal tissues oozing out. C. Anterior cuticle (Head Capsule) covering the head separated away from rest of the pupa. D. Cuticle covering the posterior pupa removed and the head region separated. E. Removal of thin transparent membrane covering the pupal tissues. F-H. Steps in clearing the oval translucent pair of eyes from attached brain and other tissues. I. Magnified view of the mid pupal eye which begins to show an indication of the ommatidial organization. Images not to scale.

- v. Carefully clean the fat bodies and other tissues surrounding the eyes.
 - vi. Process eyes for further direct examination under microscope or after fixation and staining.
- E. Late Pupa (Fig. 7)
- i. Take one washed late-stage pupa in a drop of PBS on a slide under stereo-binocular microscope.
 - ii. Using one coarse needle, hold the posterior end of the pupa, and make a lateral cut on cuticle just behind head region of the pupa with another coarse needle. Open the cuticle from the point of cut and remove the head cuticle as in Step **D.2** above (Fig. 7B, C).
 - iii. Using coarse needles, cut off the pupal head where it joins the thorax (Fig. 7D).
 - iv. Place two coarse needles on the region between the two eyes and pull in opposite directions to separate two eyes from each other (Fig. 7E, F).
 - v. Clean the excess tissue around each eye with fine needles.
 - vi. Process the eyes for further direct examination under microscope or after fixation and staining.
- F. **Aceto-Orcein staining of eye-antennal imaginal discs of early, mid and late third instar larvae, and eyes of early, mid and late pupae.**
- i. Carefully pick the dissected eye disc/eye on tip of a fine needle and transfer to a drop of PBS on a cavity/Maximov slide.
 - ii. Remove the PBS and add a small drop of paraformaldehyde fixative.
 - iii. After 10-15 min (for larval disc and early pupal eyes) or 20-30 minutes (for mid-late pupal eyes), remove PFA and wash the tissue with PBS 3 times (15 minutes each).
 - iv. Remove PBS and add 2 drops of Aceto- Orcein stain (Aceto-Carmine stain can also be used), cover the slide/Maximov, and leave for 5-10 min.
 - v. Remove the stain and wash the discs/eyes with 45% acetic acid 2-3 times, 10 seconds each, to remove excess stain.
 - vi. Transfer the tissues to a small drop of 45% acetic acid on a clean slide and carefully place coverslip without applying any pressure. Seal the slide using a transparent nail enamel (to prevent the tissue from drying).
 - vii. Observe the tissues under a bright field microscope, using 10X/20X and 40X objectives. Record your observations (Fig. 8).



Fig. 7. Steps in dissection of developing eyes from late pupa. **A.** Intact pupa with fully formed red pigmented eyes (yellow arrows) and other external structures of the adult body visible through the pupal case. **B.** Sharp cut with coarse needles on the pupal case just behind the visible eyes (yellow arrows). **C.** Anterior cuticle covering the head (Head Capsule) separated away from rest of the pupal body. **D.** Head region severed from the thorax with coarse needles. **E-F.** Steps in clearing the oval pair of pigmented eyes from attached brain and other mouth pieces. **G.** Magnified view of one eye with typical ommatidial organization. Images not to scale

General precautions:

1. Do not let the tissue dry during dissection.
2. Wash larvae or pupae before dissection so that the adhering food particles do not interfere.
3. Take only a small amount (50 -100 μ L) of 1X PBS for dissection to avoid floating of the small tissues during dissection steps.
4. Avoid puncturing larvae/pupae to avoid spilling the fat bodies on slide.

Observations:

1. Note the changes in size and shape of eye-discs during larval growth and the advancing morphogenetic furrow (Fig. 4G, 8B) during the third instar stage.
2. Observe the appearance of pigment, ommatidial organization and bristles at corners of the ommatidia as the pupal differentiation progresses with age.
3. Compare the appearance of unstained and Aceto-Orcein (or Aceto-Carmine) stained late third instar larval/pupal eye discs.
4. In the stained discs, record the appearance of ommatidial lattice, behind the morphogenetic furrow, as the larval age increases. Ommatidial lattice becomes more distinct with pupal development.

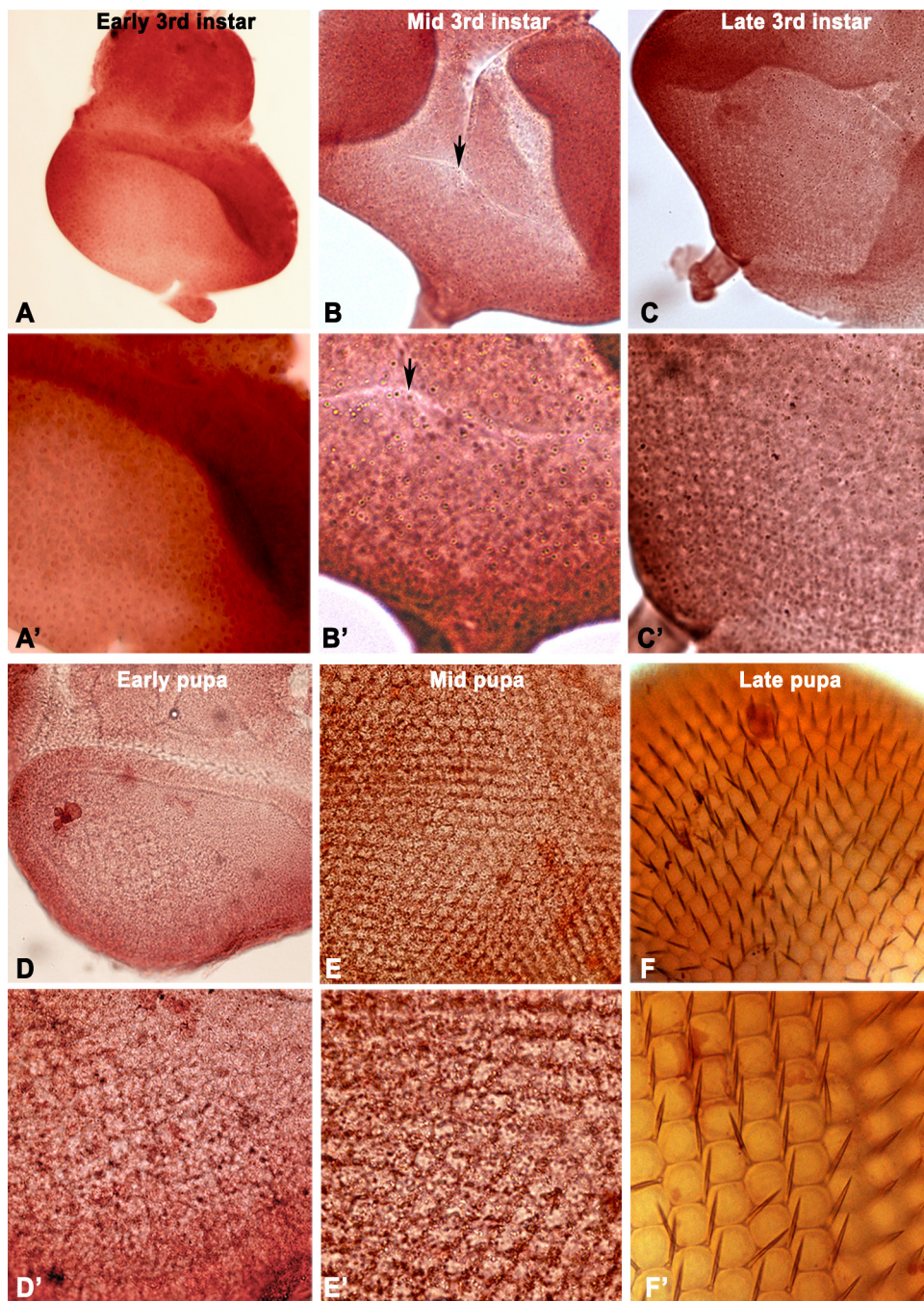


Fig. 8 : Aceto-orcein stained larval eye discs/developing eyes. A-C. Eye discs from early (A), mid (B) and late third instar larvae (C); images in A'-C' are magnified views of parts of posterior regions of discs in A-C, respectively; arrows in B and B' indicate the morphogenetic furrow. **D-F.** Developing eyes from early (D), mid (E) and late (F) pupal stages; images in D'-F' are magnified views of parts of posterior regions of eyes in D-F, respectively. Images not to scale.

Questions for self-study and interpretation:

1. What are the different cell types in the differentiating ommatidial units?
2. What is the significance of morphogenetic furrow? Why are ommatidial units formed only posterior to it.
3. Which signaling drives the wave of differentiation in developing eye disc and how?
4. What are the different types of photoreceptors in an ommatidia? Do their times of differentiation vary?
5. How and which pigments accumulate in the developing eyes during mid-late pupal stage?

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Chapter 20

Malpighian tubules at different life stages of *Drosophila melanogaster*

Saurabh Chand Sagar (schand027@bhu.ac.in), Neha Tiwari (tiwarin495@gmail.com) and Madhu G. Tapadia* (madhu@bhu.ac.in)

Cytogenetics Laboratory, Department of Zoology, Institute of Science, Banaras Hindu University, Varanasi, 221005.

* Corresponding author

Recommendation level: Undergraduate/Postgraduate/Research

Sub-discipline: Anatomy/Developmental biology/Cell biology

Background:

Malpighian tubules (MTs) in *Drosophila* are involved in osmoregulation, immune response and excretion in larval as well as adult flies (see Chapters 23, 25, 26, 27). MTs in *Drosophila* were discovered first by the Italian scientist Marcello Malpighi in the 17th century and have, therefore, been named after him as Malpighian tubules (Dow and Davies, 2001). During metamorphosis almost every larval organ, except MTs, undergoes histolysis and the adult organs differentiate anew from the different sets of imaginal cells present in larvae. The larval MTs, however, persist through the morphogenesis process and continue with some changes as the adult MTs (Shukla and Tapadia, 2011). Being involved in osmoregulation and excretion (see Chapters 25, 26 and 27), the MTs are functionally analogous to the human kidneys (Dow and Davies, 2001). In addition, they also have a role in the innate immune response (see Chapter 23). Development of the MTs starts at the embryonic stage 10 at the anterior part of the ectodermal proctodeum or hindgut. During embryonic stage 11, buds start protruding from the hindgut and by embryonic stage 13, cell proliferation is nearly completed to form the somewhat cylindrical MT (Denholm *et al.*, 2013). During stages 14-15, cellular rearrangement occurs so that by stage 16, the MTs are extended to their final length and positioned inside the developing embryo. Normally, no major changes in the cellular architecture of MTs occur after this stage.

Drosophila has two pairs of the MTs, i.e., an anterior and a posterior pair, each branching out from a short ureter on either side from the junction of the midgut and hindgut (Fig. 1). The anterior tubules are slightly longer in length and extend anteriorly in hemolymph around the midgut whereas posterior tubules are extended posteriorly around the hindgut. Both the pairs of tubules are divided into three main regions: i) the distal initial segment which appears white due to uric acid deposition, ii) transitional segment and iii) the proximal main segment. The transitional segment, joining the initial and main segments, is comparatively short and thin (Dubey *et al.*, 2002). These subdivisions are not as prominent in the posterior tubules as in the anterior pair. The main segments of the anterior pairs of tubules join the mid- and hind-gut junction on one side through a common ureter; likewise, the main segments of posterior pairs of tubules join the gut through the common ureter on the other side (Fig. 1). Both the pairs of tubules are majorly comprised of two main cell types i.e., the large columnar ectoderm-derived and endo-replicated

epithelial Principal cells (PCs) and the intercalated smaller Stellate cells (SCs) of mesodermal origin. The large PC nuclei contain poorly developed polytene chromosomes as they undergo only a few cycles of endoreplication. The PCs maintain ion balance while the SCs are responsible for fluid secretion and water reabsorption (Sözen *et. al.*, 1997).

The present chapter provides a simple method to visualize anatomy and cellular organization of MTs during different developmental stages. A knowledge of the anatomy of this tissue is useful for diverse developmental and functional studies related to transportation, excretion, osmoregulatory and immune-responsive activities in the body of *Drosophila melanogaster* at different developmental stages (see Chapters 23, 25, 26 and 27).

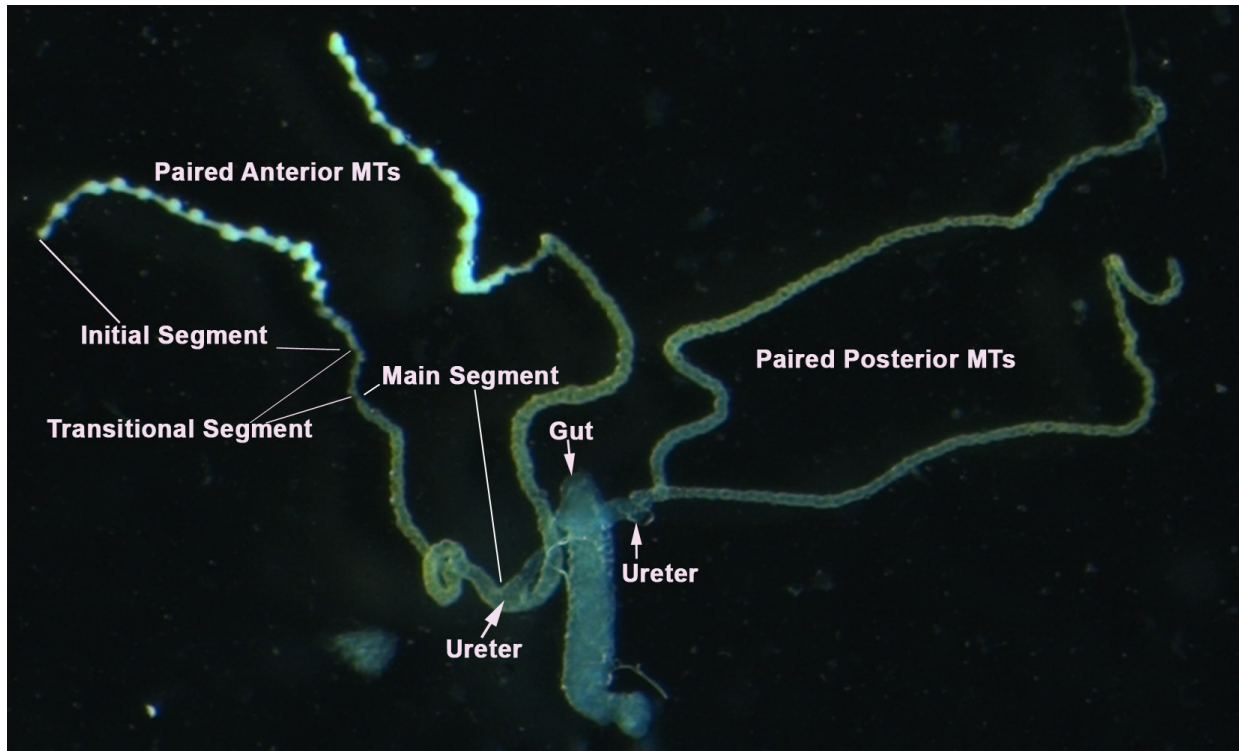


Fig. 1. Unstained anterior and posterior pairs of Malpighian tubules of late third instar larvae of *Drosophila melanogaster* connected with the gut through short ureters at the junction of mid- and hindgut.

Objective:

To follow development of MTs during larval, pupal and adult stages of *Drosophila melanogaster*.

Material Required:

A. Flies

Wild type healthy larvae, pupae and adults of wild type (or any other mutant of interest) *Drosophila melanogaster*.

B. Reagents:

- i. 1X Phosphate Buffered Saline (PBS), pH 7.4:

Sodium Chloride (1.37 M)	8.0 g
Potassium Chloride (27 mM)	0.2 g
Disodium hydrogen phosphate (100 mM)	1.42 g
Potassium dihydrogen phosphate (20 mM)	0.272 g

Dissolve the salts in 800 ml distilled water and adjust pH to 7.4 by adding 1N-HCl, as required. Finally add distilled water to make the volume to 1000 mL. Autoclave and store at 4°C.
- ii. 4% w/v Paraformaldehyde: Dissolve 4g Paraformaldehyde powder in 100 ml 1X PBS at 37°C for 60 min with periodic shaking. Cool and store at 4°C.
- iii. 0.5% v/v PBST: Add 500 µL Triton-X in 100 ml 1X PBS.
- iv. Aceto-Orcein stain: Dissolve 2 gm Orcein powder in 100 mL of 50% Acetic acid by boiling for 2 hr under a reflux condenser. Filter when cool. The prepared stain can be used for several months but requires periodic filtration before use to avoid accumulation of stain crystals (2% aceto-carmin stain, which can also be used, is prepared in a similar manner; commercially available stains can be used).
- v. 50% v/v Acetic acid

C. Equipment and other supplies:

- i. Stereo-binocular microscope, and other fly handling materials
- ii. Bright-field microscope
- iii. Fine needles and forceps
- iv. Wash bottle
- v. Slides and coverslips
- vi. Micropipettes (100/200 µL) and corresponding microtips
- vii. Petri dish

Experimental details:

A. Dissection of MT from larvae (Fig. 2):

1. Take a clean slide and put a small volume of chilled 1X PBS (pH 7.4) solution onto the slide with the help of a micropipette or dropper.
Note: Chilled PBS helps minimize larval movements.
2. Select well fed larvae of the desired developmental stage with the help of a fine brush and wash them in 1X PBS to remove attached food particles and debris.
3. Place a larva in the small drop of 1X PBS on slide to carry out the dissection under stereo-binocular microscope.
4. Using two fine needles, one placed at the posterior end and another at its anterior region (behind the black mouth hooks), pull them in opposite directions (Fig. 1A). Internal organs, including the gut along with MTs, will squeeze out of the larval cuticle.
5. Carefully separate MTs from the gut with the help of fine needles and remove any attached fat bodies (Fig. 1B, C).

6. Mount the tissue in PBS for examining under microscope, or fix and stain the MTs (see sub-section **D** below) before microscopic examination

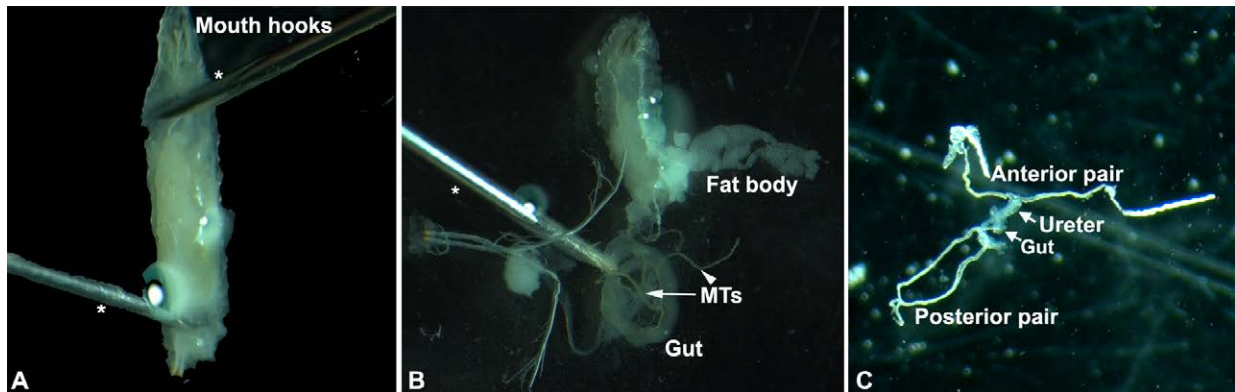


Fig 2. Steps in dissection of a larva to separate Malpighian tubules. **A.** Positions of needles/forceps (marked with *) on anterior and posterior regions of larva for pulling them in opposite directions. **B.** The squeezed-out gut and MTs. **C.** MTs connected through ureters with the gut after removal of other tissues.

B. Dissection of MT from pupae (Fig. 3):

1. Take a pupa of the desired stage on a clean slide in a drop of 1 x PBS.
2. While looking through the stereo-binocular microscope, place the tip of one fine needle at the anterior end to hold the pupa; use another fine needle to carefully cut the pupal case a little forward to the posterior end (Fig. 2A) to let the viscera squeeze out of the pupal case (Fig. 2B).
3. Using fine needles, carefully separate the gut and the attached MTs from other tissues.
4. Separate the MTs from the gut and remove the adhering fat bodies.
5. Mount the tissue in PBS for examination under microscope or fix and stain (see sub-section **D** below) before microscopic examination.

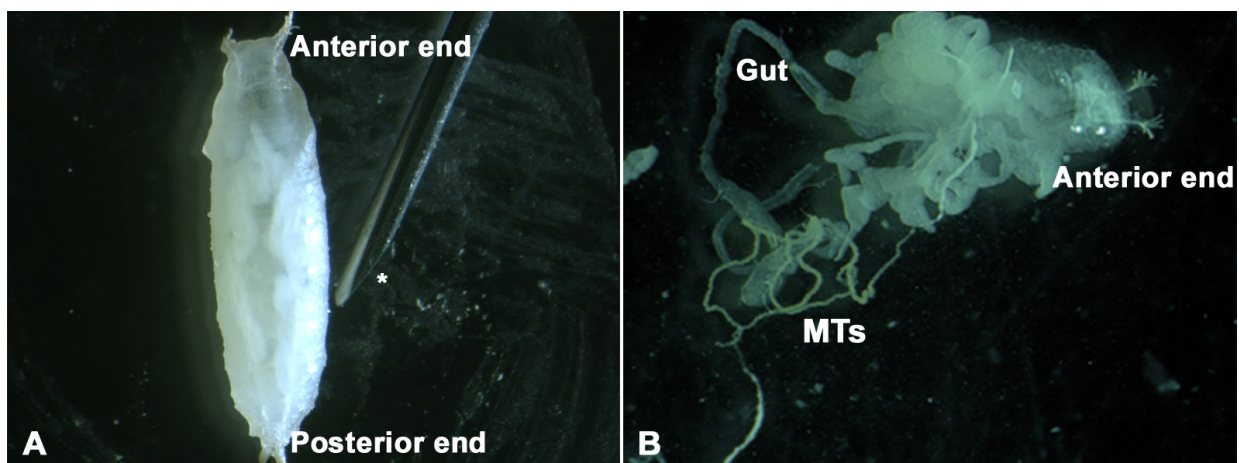


Fig. 3 : Steps in dissection of pupa to separate the MTs. **A.** Position of needle (indicated by a * mark) near the posterior one third position for cutting the pupal case. **B.** Squeezed out gut, MTs and the other internal tissues.

C. Dissection of MT from adult (Fig. 4):

1. Anesthetize the desired fly and keep it in a drop of 1 x PBS on a slide (Fig. 4A)
2. While looking through the stereo-binocular microscope and using fine needles, remove wings and legs of the fly (Fig. 4B)
3. Make a cut with needle tip on the ventral side of the abdomen of the fly so that the viscera squeeze out from the cut abdomen (Fig. 4C, D).
4. Separate the gut and the attached MT from the viscera. (Fig. 4E)
5. Carefully separate MTs from the gut and remove any adhering fat body tissue.
6. Mount the tissue in PBS for examination under microscope or fix and stain (see sub-section **D** below) before microscopic examination.

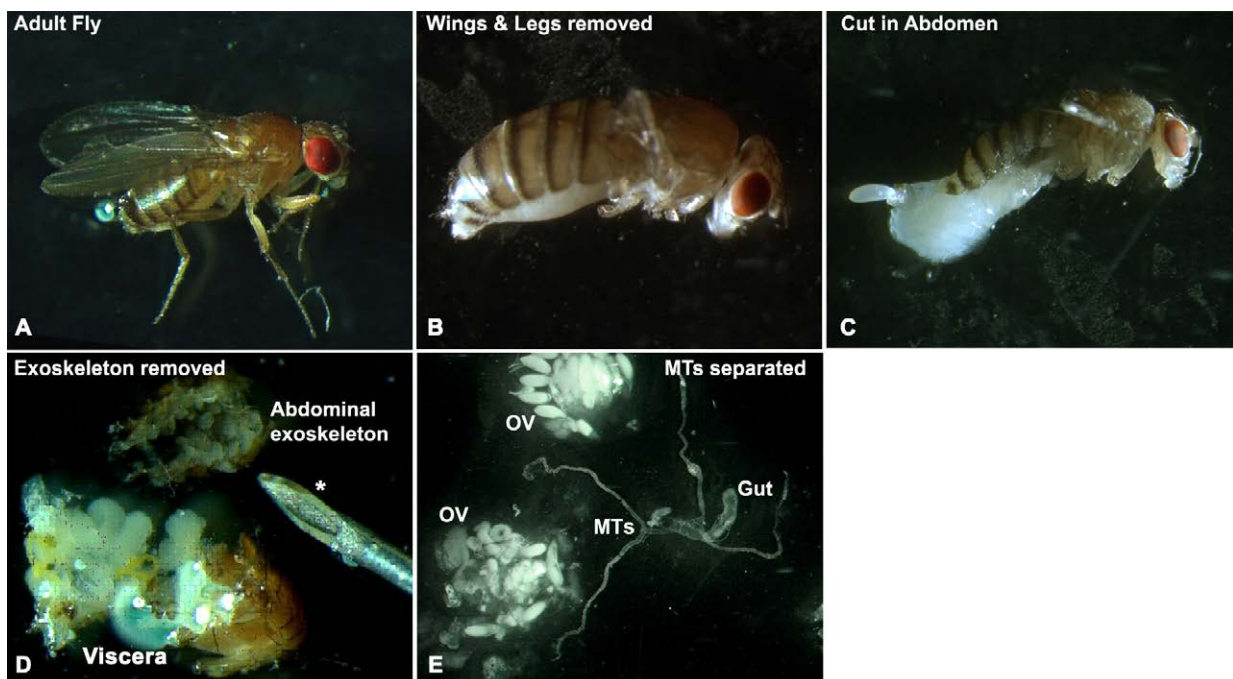


Fig 4. Steps in dissection of MTs from adult fl . **A.** Healthy adult on a slide. **B.** Fly after removal of legs and wings, **C.** Squeezed out viscera following a cut on abdominal cuticle with a fine needle. **D.** Abdominal cuticle and viscera separated with a needle tip (* mark). **E.** Cleaned MTs attached to a part of the midgut and hindgut junction; the pair of ovaries (**OV**) can be seen on the left side.

D. Staining of MT:

1. Following the dissection of MT from the desired developmental stage, fix the dissected tissue in 4% paraformaldehyde for 20-30 min.
2. After fixation, wash the tissue 3X with 0.5% PBS for 10 min each.
3. Remove the PBST and add a drop of 2% aceto-orcein (or 2% aceto-carmin) on the tissue and leave for 5-10 min.
4. Wash stained tissue with 50% acetic acid 3X for ~1 min each.

5. Mount the tissue in 50% acetic acid and observe under microscope using 10/20X or 40X objective, as desired.

Observations:

1. Morphology of MTs at various developmental stages (Fig. 5):

Examine and record changes in size and morphology of MTs during different larval instars, early (white), mid (yellowish with visible red eyes), and late pupae/pharate (dark brown pupal case with distinguishable organs within) and adult stages. Note the more prominent white and swollen initial segments in mid pupal MTs (fig 5E, F) due to high uric acid deposition. In the late pupae/pharate stage (Fig 5F), the previously accumulated uric acid disappears and the MTs become more coiled as they rearrange around other internal organs. The adult MTs (Fig 5G) do not show any major changes, although they become more coiled and a little shortened than in the late larval and early/mid pupal stages.

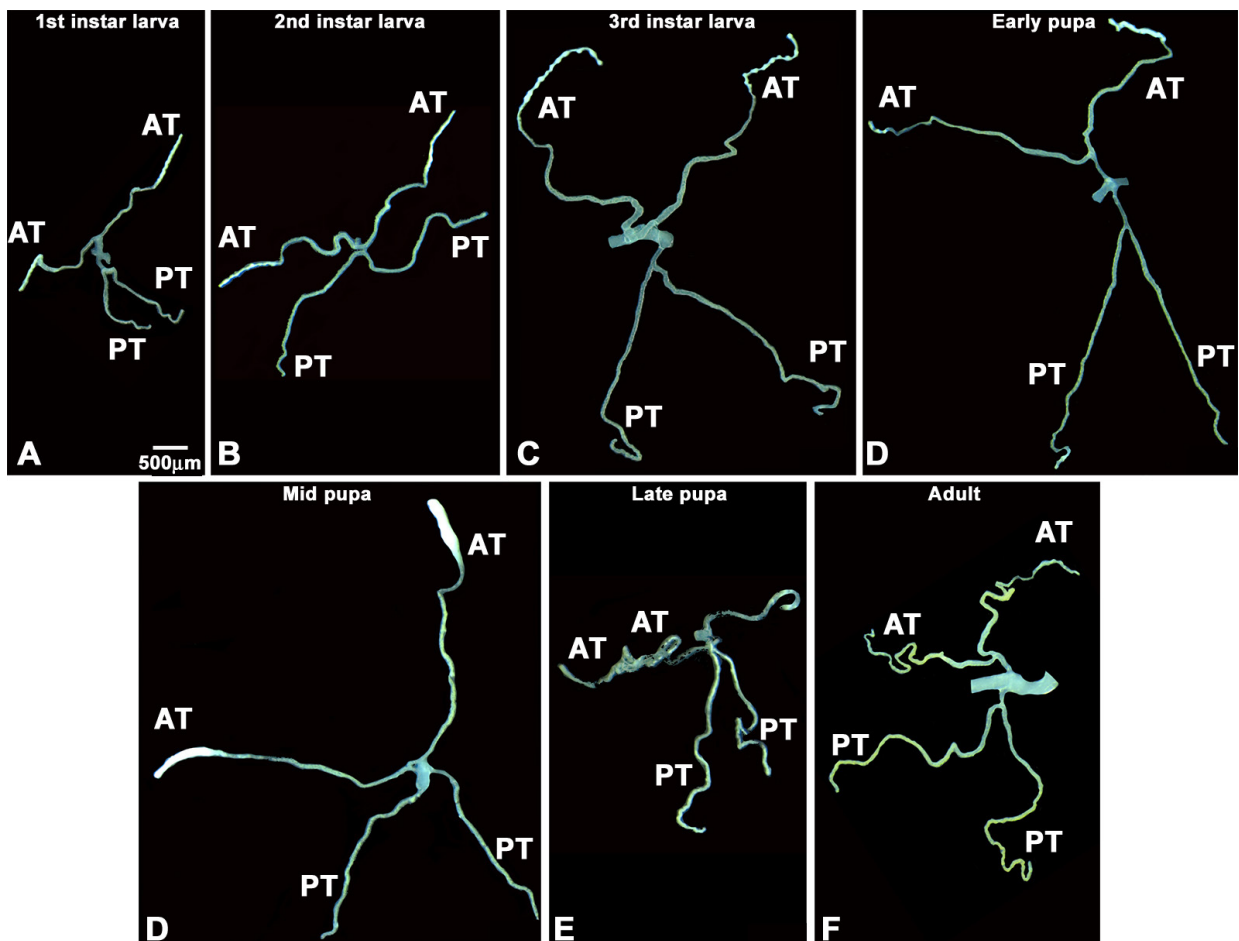


Fig. 5: Malpighian tubules (MTs) from different developmental stages (indicated on top in each panel). AT = anterior and PT = posterior tubules. Scale bar (500μm) in A applies to all panels.

2. Cell types in the MTs

Examine the stained MTs from different stages (Fig. 6) and identify the large principal cells with large endo-replicated nuclei and the smaller stellate cells, which have smaller and less stained nuclei. The stellate cells, intercalated between the large principal cells, are more common in the initial segment. In larval and pupal MTs, the

stellate cells, with less stained cytoplasm, are generally rectangular to cuboidal in appearance (Fig. 6A-D), while a few are star-shaped. In the adult MTs, most stellate cells are star-shaped (Fig. 6E, F).

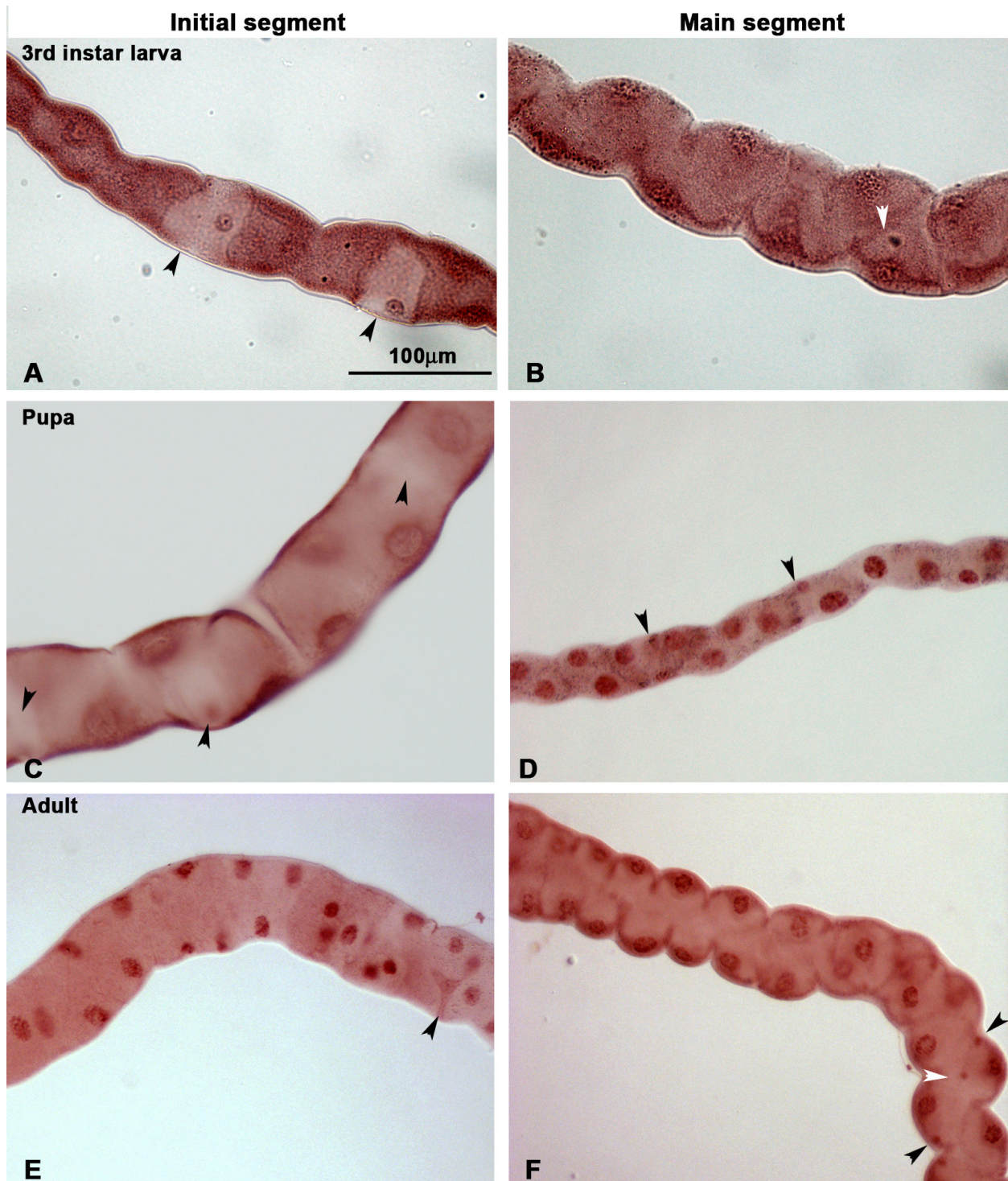


Fig. 6. Aceto-Orcein stained MTs showing large Principal and smaller Stellate cells (indicated by black/white arrowheads) in Initial (A, C and E) and Main (B, D and F) segments of MTs from different developmental stages (noted on top left of each row). Scale bar (100µm) in A applies to all panels.

Questions for self-study and interpretation:

1. What are the main anatomical differences between MTs of different developmental stages?
2. Why do mid-pupal stage MTs show very high uric acid deposition in initial segments of the anterior pair?
3. Most larval tissues undergo apoptosis during metamorphosis. How do MTs avoid cell death during metamorphosis? What could be the possible reason for absence of cell death in MTs during pupal metamorphosis?

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Chapter 21

Anatomy of fat body of wild type *Drosophila melanogaster* third instar larvae

Indira Paddibhatla¹ (paddibhatlaindira@gmail.com)

Department of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad

1. Current Address: Cardiology Department, School of Medicine, Johns Hopkins University, Baltimore, MD, USA

Recommended Level: UG/PG/Research

Sub-discipline: Anatomy/Physiology

Background:

The fat body in *Drosophila*, as in other insects, is derived from the mesoderm and is functionally comparable to human liver (Yongmei, 2015). Fat body in the larval stages of *Drosophila* is a monolayer of endoreplicated (~256C DNA content) large cells that extends continuously throughout the body. It is covered with an outer layer of extracellular matrix separating it from the other larval parts including hemolymph. Fat body, besides being main fat storage organ performing metabolic functions in *Drosophila* larva, is also an immune tissue, participating in immune responses triggered against bacterial, parasitoid wasp and viral infections (Kim-Jo *et al.*, 2019). Fat body tissue cells also perform nutritional signaling (Musselman and Kühnlein, 2018). The larval fat body stores fats, proteins and carbohydrates that are utilized during pupariation period for the development of adult body parts (Mattila and Hietakangas, 2017). Apart from triglycerides and diacylglycerol, trehalose and glycogen are also present as per the energy demands of the fat body cells (Arrese and Soulages, 2010). Various hemolymph proteins are found in fat body cells such as juvenile hormone binding proteins, lipid binding proteins, esterases, peptidases etc that contribute to the innate immune responses (Flatt *et al.*, 2008). Understanding the anatomy of fat body (see Fig. 1) is therefore, useful for diverse studies using the *Drosophila* model.

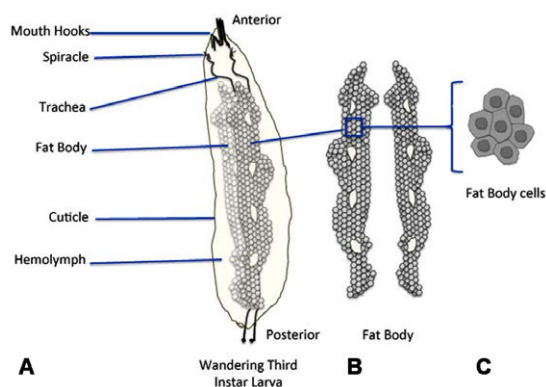


Fig. 1. Schematic of the fat body tissue of third instar *Drosophila* larva. A. Distribution of the fat body tissue in larval body cavity; **B.** Schematic of the dissected out fat body. **C.** Schematic of hexagonal and large fat body cells.

Objectives:

To understand anatomy of the fat body in third instar *Drosophila* larva.

Materials Required:

A. Wild type flies

B. Equipment and other supplies:

- i. Stereo-binocular microscope with incident light illumination or table lamp
- ii. Incubator,
- iii. Fly food vials, Yeast and other fly handling items
- iv. Spatula
- v. Maximov or Cavity slide
- vi. Cleaned microscope slides and coverslips (22 mm²)
- vii. Pasteur Pipettes
- viii. Fine forceps and fine dissecting needles
- ix. Wash Bottle
- x. Tissue paper

C. Reagents:

- i. Phosphate-Buffered Saline (1X PBS): Dissolve 8 g NaCl, 0.2 g KCl, 1.42 g Na₂HPO₄ and 0.272 g KH₂PO₄ in 800 mL of distilled H₂O. Adjust the pH to 7.4 with HCl and then add distilled H₂O to make the volume to 1000 mL. Autoclave and store at RT or at 4⁰C.
- ii. Ethanol

Experimental Details:

1. Place 50-60 two to four days old adult flies (wild type or mutant, as desired) in food vials for regular maintenance. Collect eggs in fresh food vial for 6 hr (to ensure that the off springs are similarly aged) at 24⁰C by transferring flies from the vial into fresh vial after six hr.
2. Collect late third instar larvae (day 5-6) from the vial and wash them in a Petri dish sequentially with 1X PBS, dist. water, ethyl alcohol, dist. water and 1X PBS to clean the traces of attached food.
3. Transfer a larva on a ethanol cleaned slide in a drop of 1X PBS for dissection of fat body under a stereo-binocular microscope using incident light illumination.
4. Using forceps, position the larva such that the posterior of the larva is towards you.
5. Hold the left side of the anterior cuticle present near the mouth hooks using forceps in left hand, tear the cuticle on right side below the mouth hooks and pull the tear till the posterior end using fine needles or forceps in right hand. The internal organs float out in the PBS.

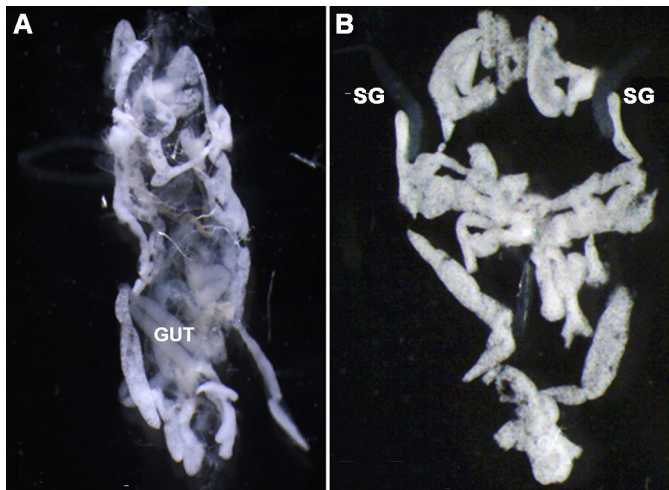


Fig. 2: Dissected fat body from wandering third instar larva. A. Fat body (whitish) and other internal organs of larva immediately after dissection. **B.** Fat body tissue (whitish) cleaned of other organs except the nearly transparent salivary glands (SG) which are still attached with fat body

6. Through the cuticle tear, slowly and gently remove the gut to one side on the slide. Pay attention to the proventriculus and be gentle while removing this region.
7. Slowly and gently remove imaginal discs, brain, ring gland, and lymph gland associated with the dorsal vessel located near the mouth hooks in anterior region of the larva where the cuticle was torn. Ensure that the fat body tissue is not damaged (**Fig. 2A**).
8. Since the nearly transparent salivary glands are attached with whitish fat body tissue, do not remove them at this stage (**Fig. 2B**).
9. Remove the cuticle gently from either sides and the other separated out parts so that the fat body tissue from anterior to posterior region only is left in the PBS drop. Salivary glands can also be carefully removed at this stage with fine needles.
10. The dissected fat body may be mounted in PBS under a coverslip or may be processed further for other uses, as required.

Results/Observations:

1. Fat body is seen as a continuous tissue extending from anterior to posterior end of the wandering third instar larva. Fat body cells are attached to each other in single layer but when the wandering third instar larva is about to pupariate, these cells fall apart and get rearranged in the pupa to give rise to adult fat body cells. Therefore, the fat body tissue from very late third instar larvae tends to fall apart easily during dissection.
2. We can observe the fat globules falling into the 1X PBS if the fat body cells are damaged while dissecting.

Questions for self-study and interpretation:

1. What type of fat is found in the fat body cells of wandering third instar *Drosophila* larva?
2. How do we know that fat body is involved in the immune response of *Drosophila* larvae?
3. How do cells in the monolayered fat body tissue of larvae remain adhering to each other?

Link for the video for the methodology: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3466936/> (Small *et al.*, 2012)

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Chapter 22

Understanding anatomy of wild type *Drosophila melanogaster* third instar larval blood cells (hemolymph and lymph gland)

Indira Paddibhatla1 (paddibhatlaindira@gmail.com)

Department of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad

1. Current Address: Cardiology Department, School of Medicine, Johns Hopkins, University, Baltimore, MD, USA

Recommended Level: PG/Research

Sub-discipline: Developmental Biology/ Physiology/ Behavioural Biology/ Ecology/ Toxicology

Background:

Hemocytes/blood cells are found in two regions in *Drosophila* larva, namely in the circulating hemolymph and in the hematopoietic organ, the lymph gland (Banerjee *et al.*, 2019; Jung, 2005; Yu *et al.*, 2018). In these hematopoietic compartments, we can observe all the three mature blood cell types, i.e. plasmatocytes, crystal cells and the lamellocytes. Blood cells are immune defensive (see [Chapter 55](#)) and are crucial during wound healing, phagocytosis and encapsulation of the parasitoid wasp egg found in the circulating hemolymph (Paddibhatla *et al.*, 2010). Lymph gland is the hematopoietic organ found in the larval stages of the *Drosophila* larva (Jung, 2005; Small *et al.*, 2012). It harbors, besides the progenitor cells, mature hemocytes in the larval stages. The lymph gland is composed of anterior lobes, posterior lobes and the pericardial cells that are found on either sides of the dorsal vessel. Each anterior lobe can be further divided into cortical zone and medullary zone. There is a posterior signaling center (PSC) on the posterior end of the anterior lobes towards the dorsal vessel. Posterior signaling center serves as a hematopoietic niche in *Drosophila* larva. Medullary zone is the inner region of the anterior lobe where the progenitor blood cells are found while the matured cells and their progenitors are present in the cortical zone (Krzemień *et al.*, 2007; Minakhina and Steward, 2010).

Objectives:

To understand the anatomy of the hematopoietic tissues, the circulating hemolymph and the lymph gland in third instar *Drosophila* larva.

Materials Required:

A. Wild type flies

- B. Fly food vials
- C. Yeast
- D. Spatula
- E. Cavity slide or other dissecting dish
- F. Cleaned microscope slides and coverslips (22 mm²)
- G. Pasteur Pipettes
- H. Fine forceps and dissecting fine needles
- I. Wash Bottle
- J. Tissue paper
- K. Anesthetizing ether or CO₂ tank with anesthetizing set up
- L. Stereo-binocular microscope with incident light illumination
- M. Incubator
- N. Phosphate-Buffered Saline (1X PBS)

Component	Amount	Concentration
NaCl	8 gm	0.137 M
KCl	200 mg	0.0027 M
Na ₂ HPO ₄	1.44 gm	0.01 M
KH ₂ PO ₄	245 mg	0.0018 M

Take 800 mL of distilled H₂O in a suitable container and serially add the above components. Mix well using a magnetic stirrer and adjust to pH to 7.4. Finally, bring the total volume to 1 L using distilled H₂O.

O. Fixative: Freshly prepared 4% paraformaldehyde (PFA) in 1X PBS (4% formaldehyde in 1X PBS can also be used). Weigh 400 mg of paraformaldehyde powder and add to 2 mL of 1X PBS. Stir using magnetic stirrer in a beaker at 60°C till paraformaldehyde is completely mixed. Slowly add 8 mL of the 1X PBS to make 10 mL volume.

Note: PFA is toxic. It causes irritation to skin, nose and throat. Handle carefully. Do not breath the fumes from PFA.

P. Ethanol

Experimental Details:

1. Making blood smears:

- i. Place 50-60 two to four days old adult flies (wild type or mutant, as desired) in food vials for regular maintenance. Collect eggs in fresh food vial for 6 hr (to ensure that the off springs are similarly aged) at 24°C by transferring flies from the vial into fresh vial after six hr.

ii. Collect late third instar larvae (day 5-6) from the vial and wash them in a Petri dish sequentially with 1X PBS, dist. water, ethyl alcohol, dist. water and 1X PBS to clean the traces of attached food.

iii. Position one washed larva in 200 μ L of 1X PBS on a microscope slide (cleaned in ethanol) on the stage of stereo-binocular microscope using incident illumination.

iv. Ensure that the source of light is not directly falling on the larva (Tilt away the source of light focusing on the sample). This will make the larva appear somewhat transparent and, therefore, easy to visualize the internal organs including the fat body.

v. Using forceps, position the larva so that its posterior end is towards you with ventral side upwards.

vi. Using forceps hold the animal at either side of the posterior end. Using another forceps make a gentle tear in the cuticle.

vii. Hemolymph oozes out of the larva at the tear site onto the slide. *Drosophila* larva has an open circulatory system so that the hematopoietic precursors, progenitors and the matured blood cells are freely circulating in the hemolymph. Gently tap the dissected larva on the region where it was dissected to ensure the entire hemolymph is falling on the slide. Gently remove the larva and let the hemolymph on the slide air dry

Note: Brief air drying helps in adherence of blood cells to the slide.

viii. Fix the blood cells by adding 100 μ L of 4% PFA (in 1X PBS) on the dried hemolymph. Remove the 4% PFA after 5-8 min and gently wash the slide with 1X PBS by adding a few drops of 1X PBS on the sample area on the slide and leaving it undisturbed for 5 min, following which the PBS is removed by slanting the slide to let the PBS drain down. Repeat this washing step three times. Air dry the slide after washing is complete. Further processing of the hemocytes fixed on the slide can be as required e.g., immunostaining of blood cells or staining with fluorescent dyes like phalloidin/DAPI etc. to visualize the blood cells.

Note: The blood cells can also be directly examined in fresh unfixed hemolymph under phase-contrast or DIC optics.

2. Lymph gland dissection:

i. Follow steps i-iv as above.

ii. Using both the forceps, gently hold the cuticle on the right anterior side of the larva and make a gentle tear.

iii. Repeat the same step on left anterior side of the larva.

iv. Hold the larva using the left side forceps and gently peel off the cuticle by pulling it downwards to the posterior side. Internal parts of the larva like the brain, fat body, imaginal discs, salivary glands, proventriculus and rest of the gut will become visible

v. The anterior lobes of the lymph gland are present (as they appear on either side of the dorsal vessel) below the proventriculus, and therefore, gently move the proventriculus away and then gently remove the entire gut with fine dissecting needles.

vi. Gently remove the brain and the associated imaginal discs.

vii. Slowly and carefully remove the fat body so that the entire lymph gland is visible. Be extra-gentle to ensure that the anterior lobes do not detach from the ring gland present at anterior side along the same dorsal vessel.

viii. Remove the cuticle from the left side as well, along with the fat body tissue if anything is still remaining. Now the entire lymph gland is visible on the microscope slide (**Fig. 1**).

ix. Let the LG air-dry for a few minutes (it adheres to the slide as it air dries).

x. Fix the LG using 4% PFA following the same steps as mentioned in the section 1 hr above. Remove the 4% PFA and wash with 1X PBS three times for five min at each wash step. Further processing of the fixed lymph gland on slide can be as required for experiment of interest, e.g., immunostaining of the proteins or staining with fluorescent dyes like phalloidin/DAPI etc. to visualize the tissue.

Results/Observations:

1. Normal wild type blood cells (**Fig.1B**) in circulating hemolymph appear circular and approximately 8 μM in diameter. Upon infection (microbial or parasitoid), the differentiated lamellocytes, which are larger than other blood cells, also become common (**Fig. 1C**, white arrow).

2. The intact lymph gland shows a pair of anterior lobes, two to three pairs of posterior lobes and pericardial cells running on either side of the dorsal vessel (**Fig. 1D-E**). As observed in the **Fig. 1D** and **1E**, the anterior lobes are bigger than the posterior lobes. If the lymph gland is detached from the cuticle on the posterior side, it will not appear straight but will curl-up.

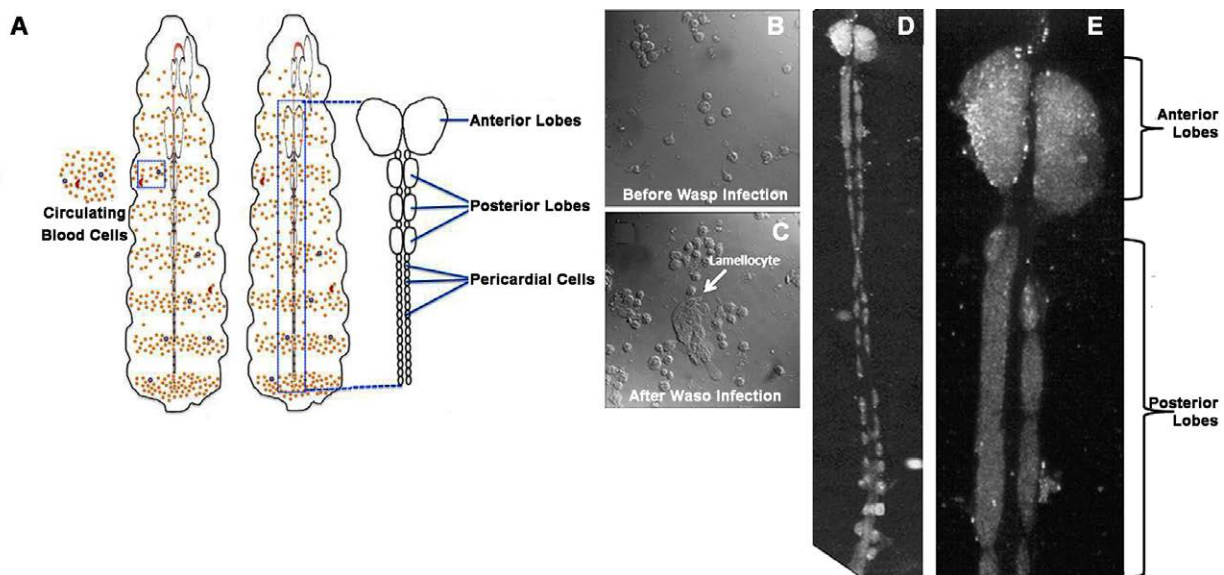


Fig. 1. **A.** Schematic of hematopoietic tissues (circulating blood cells and the lymph gland) in third instar larva. **B.** *Drosophila* blood cells from the circulating hemolymph of wild type larva. **C.** Blood cells from an infected larva showing higher number and presence of large lamellocytes (white arrow). **D-E.** Lymph gland dissected from third instar larva (**E** shows the anterior region and a pair of posterior lobes at higher magnification).

Questions for self-study and interpretation:

1. Why are the anterior lobes bigger than the posterior lobes?
2. What possible types of blood cells would be residing in the posterior lobes of the LG?
3. Why is the number of blood cells, especially the lamelocytes, higher in hemolymph of an infected larva?

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Link for the video about the methodology: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3466936/> (Small *et al.*, 2012).

Taxonomy, Anatomy, Phylogeny and Evolution will Reassert

As long as museums and universities send out expeditions to bring to light new forms of living and extinct animals and new data illustrating the interrelations of organisms and their environments, as long as anatomists desire a broad comparative basis for human anatomy, as long as even a few students feel a strong curiosity to learn about the course of evolution and the relationships of animals, the old problems of taxonomy, phylogeny and evolution will gradually reassert themselves even in competition with brilliant and highly fruitful laboratory studies in cytology, genetics and physiological chemistry.

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Chapter 23

Malpighian tubules of *Drosophila melanogaster* as a model to study systemic infection by detecting antimicrobial peptide expression

Puja Verma (pujaaiu@gmail.com) and Madhu G Tapadia* (madhu@bhu.ac.in)

Cytogenetics Laboratory, Department of Zoology, Banaras Hindu University, Varanasi

*For correspondence

Recommended Level: Postgraduate/ Research

Sub-discipline: Developmental Biology/ Immunology/Parasitology

Background:

Immune response against microbial infection is a prerequisite for survival of host organisms. Metazoans do this specifically by activating the innate immune response, which is evolutionarily conserved first line of defence. The jawed vertebrates are additionally endowed with an adaptive immune response, which retains the memory of earlier infection. Innate immune system is activated in response to infection by microbes (viruses, bacteria, fungi) and other parasites (see [Chapter 24, 55](#)), and has a humoral as well as cellular components. The humoral response includes expression of Antimicrobial peptides (AMPs, see [Chapter 23](#)), while the cellular response is carried out by specialized cells (see [Chapter 55](#)). AMPs are effective against bacteria (Gram-negative, Gram-positive), fungi (yeasts and filamentous), parasites and in some cases, the enveloped viruses. In insects, fat bodies (see [Chapter 21](#)) are the primary organs that produce AMPs, which are divided into seven major groups, namely Dipterin, Cecropin, Drosocin, Defensin, Drosomycin and Metchnikowin (ImLer and Bulet, 2005, Lemaitre *et al.*, 2007). Apart from the fat bodies, other tissues like barrier epithelia including the epidermis, digestive tract, trachea, reproductive tissues and Malpighian tubules also produces AMPs locally in response to infections (Tzou *et al.*, 2000). Malpighian tubules (MT), besides their primary function of excretion and osmoregulation (see [Chapter 20](#)), are also autonomous immune organs, which sense and respond to pathogenic challenges, independent of the fat bodies (McGettigan *et al.*, 2005). The AMPs are released in the hemolymph where they interact with the pathogens.

MT of *Drosophila* can be used as a model to study the early innate immune response of the host (Verma and Tapadia, 2012). Several transgenic strains are available, wherein a given AMP is tagged with reporter genes like *LacZ* or *GFP* which makes it very convenient to assess the pathogenic challenges through assay for the reporter gene expression (see [Chapters 39, 40](#)). Alternatively RT-PCR (see [Chapter 65](#)), using AMP specific primers, can be employed for detection of levels of the given AMP gene transcripts.

Lipopolysaccharides (LPS) are cell wall components of Gram-negative bacteria and are strongly immunogenic. Their commercial availability makes it very convenient to examine the immune response of *Drosophila* following feeding larvae/flies on food containing LPS.

Objective:

To evaluate AMP expression in Malpighian tubules of *Drosophila* as a readout of pathogenicity of infection using transgenic flies carrying: *cecropinA-LacZ* reporter gene.

Materials Required:

1. **Fly Stocks:** *w**; *P{y83pw103}/TM3, Ser*: A P element insertion of a transgene carrying *cecropinA* tagged with *LacZ* gene sequence encoding the bacterial β -galactosidase. The transgene is inserted on chromosome 3, which is balanced with the *TM3, Ser* balancer chromosome (Verma and Tapadia, 2012). This stock, referred to here as *CecropinA-LacZ*, can be requested from the corresponding author. Several other transgenic reporter stocks of AMPs are available from the Bloomington *Drosophila* Stock Centre.

2. Equipment:

- i. Stereo-binocular microscope and other routine fly handling items.
- ii. Bright-field microscope.
- iii. 37°C incubator.

3. Reagents and other items:

- i. Phosphate buffered saline (1X PBS, pH 7.4): Dissolve 8 g NaCl, 0.2 g KCl, 1.42 g Na₂HPO₄ and 0.272 g KH₂PO₄ in 800 mL of distilled H₂O. Adjust the pH to 7.4 with HCl and then add distilled H₂O to make the volume to 1000 mL. Autoclave and store at RT or at 4°C.
- ii. PBST (0.1% Triton X-100 in 1X PBS).
- iii. Schneider's medium for *Drosophila* cells (available commercially).
- iv. LPS: Prepare a stock solution (2 mg LPS dissolved in 1 mL of 1x PBS or Schneider's medium and store at -20°C. Use this for preparing the working solution (0.02 μ g/mL) by dilution with Schneider's medium.
- v. Reagents for β -galactosidase activity staining (for details see [Chapter 39](#)):
 - 4% Paraformaldehyde in 1X PBS.
 - Prestaining buffer: 0.1 M phosphate buffer (pH 7.3), 114.5 mL 0.1 M Na₂HP_O₄ (6.9 gm in 500 mL H₂O), 385 mL 0.1 M NaH₂PO₄ (14.2 gm in 500 mL H₂O), 0.4 mL 1 M MgCl₂ in 500 mL total volume.
 - X-gal staining solution: Add 0.106 gm K₃[Fe(CN)₆], 0.082 gm K₄[Fe(CN)₆], 2 mL of 25 mg/mL X-gal stock in 0.1 M phosphate buffer (pH7.5) to 48 mL prestaining buffer.
- vi. 8 0% glycerol.
- vi. Other items: Maximov or Cavity slides, microscope slides and 22 mm² cover slips.

Experimental Details:

β -Galactosidase staining assay for *Cecropin* expression:

i. Collect healthy transgenic *CecropinA-LacZ* third instar larvae (prepupae, pupae or adults can also be taken if desired).

ii. Place larvae in a small drop of 1X PBS on slide. Working with stereo-binocular microscope, place one needle at the posterior end of third instar larva and another at its mouth region, and pull apart the two ends. The MT (and other organs in the body cavity) come out. The long slender MT are attached at the junction between midgut and hindgut.

Note: For dissecting MT from adults, decapitate them and using fine needles, pull apart the anterior and posterior ends as for larvae to expose the slender MT attached at the junction of mid- and hind-gut. For pupae, place one needle on anterior side of pupa and cut the pupal case at posterior end very carefully with another needle.

i. Squeeze the viscera inside out from pupal case and separate MT from the fat body.

ii. Collect MT from 4-5 larvae in a Maximov or cavity slide in 100 μ L Schneider's medium.

iii. For immune challenge, add LPS in Schneider's medium (0.02 μ g/mL) and incubate MT (experimental) for 3 hr at 24-25°C. Keep another set of MT in Schneider's medium without LPS, as parallel control, for comparison.

iv. Remove media from control and experimental samples after 3 hr and wash the MT with 1X PBS twice for 5-10 min each, following which add 4% PFA to fix the tissue at room temperature for 20 min.

v. Wash the tissue with pre-staining buffer three times for 15 min each.

vi. Remove the pre-staining buffer and add 50 μ L of the X-Gal staining solution (this volume may vary in relation to the number of MT being stained). Ensure that the MT are fully covered by the solution. Keep the covered Maximov/cavity slides in a moist chamber at 37°C till the magenta/deep blue colour appears (usually 5-6 hr).

vii. Wash the tissue in PBS. Mount in 80% glycerol on a clean slide, cover with a coverslip and seal all sides with transparent nail polish.

viii. Examine the MT from control and experimental sets on a bright-field microscope.

Observations:

Compare the X-gal staining in control and LPS-immune-challenged MT. The blue colour in X-Gal stained tissues from the *CecropinA-LacZ* reporter strain provides a measure of the β -galactosidase activity as a readout for the *CecropinA* gene activity (**Fig. 1**). Light blue stain of β -Galactosidase in control MT shows basal level of *Cecropin* expression at all developmental stages. Intense blue stain after immune challenge indicates enhanced expression of *CecropinA-LacZ* transgene in comparison to control at all stages.

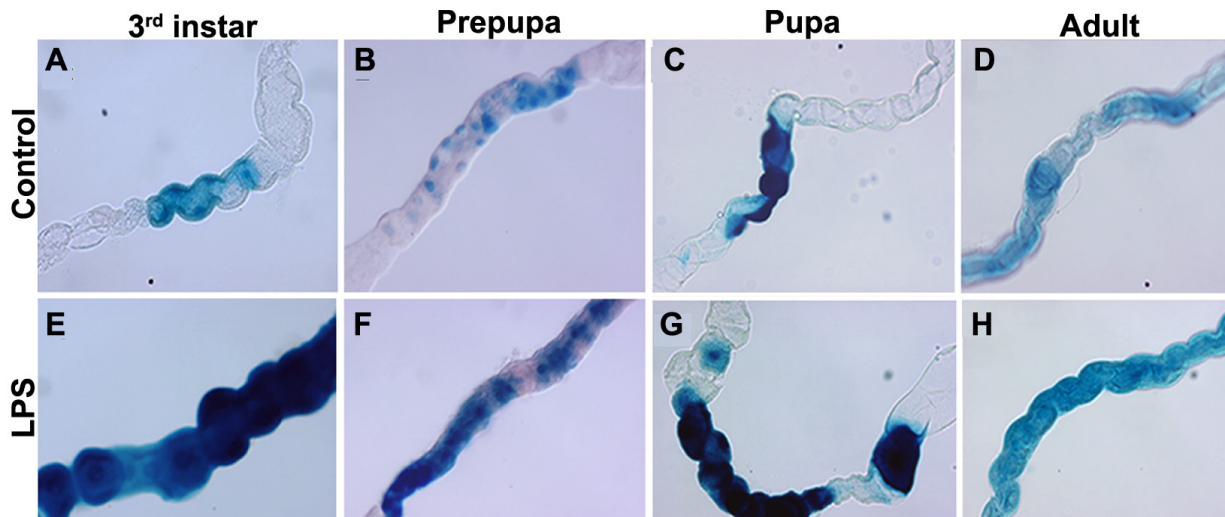


Fig. 1. X-Gal staining for β -galactosidase activity reflecting expression of the *CecropinA-LacZ* transgene in MT (small segments only shown) at different developmental stages (third instar larvae, Prepupa, Pupa and Adult) under control condition (A-D) and after LPS immune challenge (E-H).

Questions for self-study and interpretation:

1. Is immune response the primary function of Malpighian tubules?
2. Is there a cross-talk between innate and adaptive immune responses?
3. What could be the immune challenge to Malpighian tubules under normal development for them to produce AMPs?

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Chapter 24

Demonstration of arms race between *Drosophila* and its parasitoid wasp and the haplo-diploid mode of sex determination in wasps

Indira Paddibhatla (paddibhatla@gmail.com; ichilukuri@gmail.com)

Department of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad

Recommended Level: PG/Research

Sub-discipline: Parasitology

Background:

Leptopilina bouvardi-17 (*Lb-17*) is a parasitoid wasp belonging to the family Figitidae. *Leptopilina heterotoma* (*L. h.*), *Leptopilina victoria* (*L. v.*) and *Leptopilina clavipes* (*L. c.*) also belong to same genus and family (Dubuffet, *et al.*, 2007). All these *Leptopilina* species are widely used to study host-parasitoid interaction with *Drosophila* as the host (Dubuffet *et al.*, 2009). Sex-determination in *Leptopilina* follows the haplo-diploidy mode, also referred to as Arrhenotoky. In this system, unfertilized eggs develop into males while the fertilized eggs develop into females. All members of the insect order Hymenoptera that includes bees, ants and wasps, follow haplo-diploidy mode of sex determination (Heimpel and de Boer, 2008). Based on the range of hosts of the *Leptopilina* they are classified as specialists and generalists (Hedlund *et al.*, 1996). While both infect *Drosophila* larvae, *Lb-17* is a specialist wasp found in tropical and sub-tropical regions where its main host is *Drosophila melanogaster*, although it can also infect *Drosophila simulans* with less success (Schlenke *et al.*, 2007; Debuffet *et al.*, 2009).

Host-parasitoid interaction can be used in the classroom to study the arms race between the two where the immune response of host is triggered against wasp infection. Thus infestation with parasitoid wasp, *Lb-17* also triggers an immune response in *Drosophila melanogaster* larvae (Debuffet *et al.*, 2007; Small *et al.*, 2012). Depending upon when the immune response is triggered, outcome of the infection can be predicted. Infection of early larval stages by the parasite leads to suppression of the host's development while infection of the later larval stages (third instar) leads to suppression of the parasitoid egg through encapsulation response; the wasp egg is approximately 100 μm in size which is encapsulated by specialized cells, the lamellocytes, of the host and later melanized by crystal cells resulting in the blackening of the wasp egg inside the host body (Small *et al.*, 2012). Therefore, the host-parasitoid interaction between *Drosophila* and *Leptopilina* can be used experimentally to understand and display the arms race between the two. It is an easy model to understand

immune responses triggered by the parasite in the host. This system can also be used to show haplo-diploidy mode of sex determination.

Objectives:

1. To determine outcome of the arms race between the host and parasite upon infection at different larval stages of the host.
2. To examine the haplo-diploidy mode of sex determination in *Lb-17* wasps

Materials Required:

A. Flies and wasp

- i. Wild type *Drosophila melanogaster*
- ii. Wasp – *Leptopilina boulardi-17* (these can be obtained from Dr. Indira Paddibhatla)

B. Equipment and other supplies:

- i. Stereo binocular microscope with incident illumination or table lamp
- ii. B.O.D. Incubator set at 24°C
- iii. Fly food bottles/vials with standard food and other fly handling materials
- iv. Yeast
- v. Honey
- vi. Plastic vials and cotton plugs/foam stoppers for vials
- vii. Spatula
- viii. Maximov or cavity slides
- ix. Microscope slides and coverslips (22 mm²)
- x. Pasteur Pipettes
- xi. Fine Tweezers/forceps and dissecting needles
- xii. Wash Bottle
- xiii. Tissue paper and paper Towel

C. Reagents

- i. Phosphate-Buffered Salt Solution (1X PBS):

Component	Amount
NaCl	8 g
KCl	200 mg
Na ₂ HPO ₄	142 mg
KH ₂ PO ₄	272 mg

Take 800 mL of distilled H₂O in a suitable container and serially add the above components. Mix well using a magnetic stirrer and adjust pH to 7.4. Finally, bring the total volume to 1 L using distilled H₂O. Autoclave and store at RT or at 4°C.

- ii. Ethanol
- iii. Formaldehyde (37% w/w)

Experimental Details:

A. Objective 1: Outcome of the arms race between the *Drosophila melanogaster* host and parasite upon infection

i. Infestation of the host in early second instar larval stage:

1. STEP 1: Place 50-60, 2-4 day old flies in food vials for egg laying for 24 hr at 24°C. Remove the adult flies and transfer them into fresh food vials.
2. STEP 2: 48 hr after removal of the parental flies (the fly larvae are at second instar stage at this time), add 2-3 day old 6 female and 6 male wasps into the food vial in which the fly larvae are growing. Do not use the normal cotton to plug the vials but use a cotton plug with a drop of 2:1 diluted honey (diluted with distilled water) placed on the inner side of the cotton plug. Wasps feed on the honey drop. Parallel vials with *Drosophila* larvae but without any wasp are maintained as control. **Note:** The wasps may be anaesthetized, like the flies, using CO₂ or ether. Place the anaesthetized wasps into the vial with second instar *Drosophila* larvae and leave the food vial in a sleeping position to avoid the wasps getting stuck in the food at the bottom. The tube is turned upright when the wasps recover from anesthesia and start moving.
3. STEP 3: Once the wasps begin to move freely in the vials, shift them to the 24°C incubator. Let the wasps remain in the vials till eclosion of progeny *Drosophila* flies. **Note:** Depending on the wasp species and temperature, wasp eclosion happens in 25-30 days after laying eggs in the host larvae. The uninfected *Drosophila* larvae will develop and eclose ~10 days after the egg laying. Next to emerge would be male wasps (with longer antennae) followed by the female wasps.
4. STEP 4: Count and remove the emerging *Drosophila* adult flies from the wasp-infected (in which wasps are still developing) and control vials. Compare the number of host flies that emerge in wasp-infected vials and uninfected control vials. Later, when the wasps begin to eclose, count the numbers of male and female wasps in the infected vials.

Note: The *Drosophila* flies that eclose are referred to as escapees. These emerge because either some larvae were never infected with wasp egg or the infected larvae mounted a stronger immune response and **encapsulated the wasp egg** and successfully developed into the adults. Such infected escapee adults carry the encapsulated wasp egg in their abdomen, which can be seen under a stereo-binocular microscope.

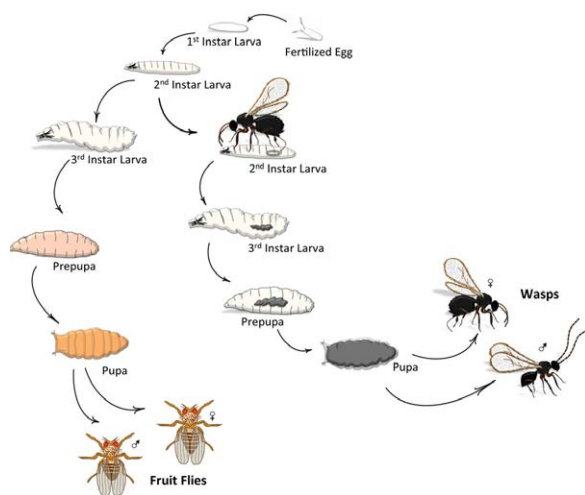


Fig. 1. Life cycles of *Drosophila* (left) and *Leptopilina bouvardi-17* wasp (right).

ii. Infestation of the host in third instar larval stage:

1. STEP 1: Place 50-60, 2-4 day old flies in food vials for egg laying for 6 hr at 24°C. Remove the adult flies after egg collection by transferring them into fresh food vials.
2. STEP 2: 96 hr after removal of the parental flies (the fly larvae are at third instar stage at this time), add 2-3 day old 6 female and 6 male wasps into the food vial in which the third instar fly larvae are growing. Do not use the normal cotton to plug the vials but use a cotton plug with a drop of 2:1 diluted honey (diluted with distilled water) placed on the inner side of the cotton plug (wasps feed on honey). **Note:** The wasps may be anaesthetized, like the flies, using CO₂ or ether. Place the anaesthetized wasps into the vial with third instar *Drosophila* larvae and leave the food vial in a sleeping position to avoid the wasps getting stuck in the food at the bottom. The tube is turned upright when the wasps recover from anesthesia and start moving.
3. STEP 3: Once the wasps are seen to be moving freely in the vials, shift them into the 24°C incubator. Remove the wasps from the vials after 12 hr of infection.
4. STEP 4: Examine the infected host larvae, before they pupate, for presence of black bodies visible through their cuticle. The third instar larval host's immune response encapsulates the wasp egg resulting in the formation of the black bodies under the cuticle. Despite the immune response, some of the infected third instar larvae die while others survive to adult stage.
5. STEP 5: Count and remove the emerging *Drosophila* adult flies from the wasp-infected (in which wasps are still developing) and control vials.
6. STEP 6: Later, when the wasps begin to eclose, count the numbers of male and female wasps that emerge in the infected vials.
7. Tabulate all the data appropriately and compare them for statistically significant differences between the different sets and parameters.

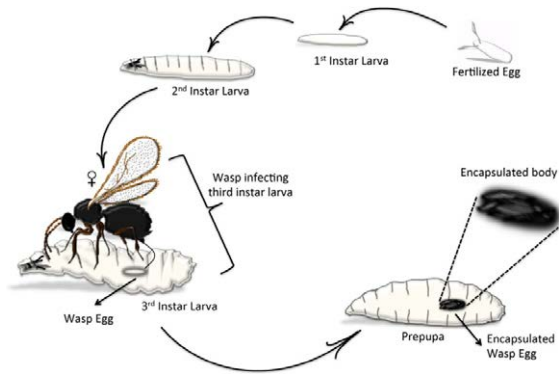


Fig. 2: Immune response of third instar *Drosophila* larva to *Leptopilina bouvardi-17* wasp infection.

B. Objective 2: To examine the haplo-diploidy type of sex determination in wasps by infestation of *Drosophila* using virgin *Lb-17* wasps.

1. STEP 1:

- a. Collect freshly eclosed female wasps which are virgin.
Note: Since we cannot easily determine if the female wasp is a virgin, it is helpful to place each wasp pupa in a separate vial and collect females as they eclose from the pupa and keep them separate from males. This eliminates the possibility of females mating with male wasps.
- b. In another set, collect the emerging female wasps and keep them with male wasps for a day to let them mate.

2. STEP 2: Set up two separate infections (follow the steps as described above in **(Objective 1)**). In the first case, the virgin female wasps are allowed to infect second instar *Drosophila* larvae and in the second, the mated females are used to infect second instar *Drosophila* larvae. Ensure that the numbers of host larvae (preferably ~100 larvae) are similar in both the cases.

3. STEP: Collect the eclosed flies and wasps and record the numbers of flies and male and female wasps that eclosed in each condition.

Results:

A. Objective 1:

1. Record i) the numbers of male and female flies and of male and female wasps that eclose in each of the above conditions, and ii) the number of flies that show black encapsulated wasp egg through their cuticle.

Note: Depending upon the immune response of the host *Drosophila* larvae, the relative numbers of flies and wasps that emerge in each condition is expected to be different. When wasps infect the younger first or second instar larvae, the wasps (parasite) survive and more wasp adults emerge but when the third instar larvae are infected, the host larvae show encapsulation response. Thus in the host-parasite arms race, the wasp wins when the host's immune response is weaker at the early larval stages. On the other hand,

because of the stronger immune response of older host larvae, the parasitoid wasp eggs are successfully encapsulated so that the egg dies without further development.

B. Objective 2:

1. Document the number of flies and male/female wasps that eclose in each condition. A greater number of male wasps eclose upon infestation with virgin female wasps compared to the infestations with mated female wasps.

Note: Virgin females lay unfertilized eggs, all of which develop into male progeny while the mated females lay fertilized as well as some unfertilized eggs which emerge as female and male progeny, respectively (**Fig. 3**).

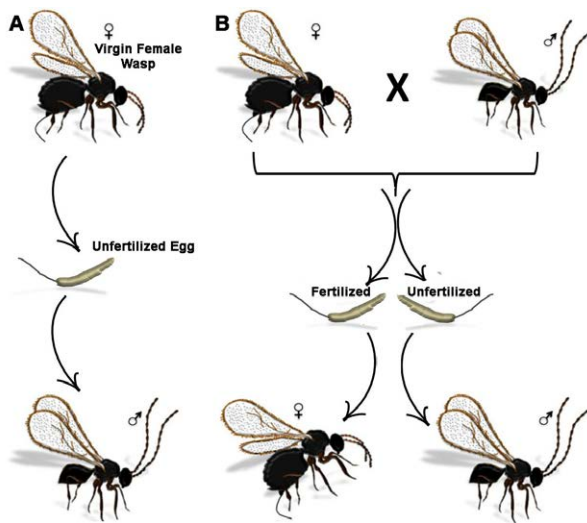


Fig. 3: Schematic of haplo-diploidy type of sex determination in *Leptopilina boulardi-17*. **A.** Virgin females lay unfertilized eggs, all of which develop into male progeny. **B.** Mated females lay unfertilized as well as fertilized eggs which emerge as male and female progeny, respectively.

Questions for self-study and interpretation:

1. How can the male and female wasps be differentiated?
2. Would the males and females of this wasp species have equal amount of DNA in their body cells?
3. How do wasps recognize their hosts? Why are parasitoid wasps categorized as generalists and specialists?
4. Why does the response of host larvae differ when infected in early second instar versus when infected in third instar stages of development?
5. Why do the unfertilized wasp eggs develop into male wasps only?

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Link for the video about the methodology: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3466936/>

Costs and Benefits of Investment in Resistance to Parasitoids

The extensive additive genetic variation in resistance to parasitoids found in *D. melanogaster* is most likely to be maintained by a combination of fluctuating selection pressures, both temporal and spatial, and costs of resistance. The latter are central to most arguments about evolutionary interactions between hosts and parasites, or predators and prey, and insect parasitoids have proved a valuable model system for studying these issues.

There are two main issues in assessing the costs and benefits of investing in resistance. The first concerns what happens after a parasitoid attack: are there costs to the successful encapsulation of the parasitoid (or, equivalently, what are the benefits of resistance)? The second concerns the costs of maintaining the resistance machinery, costs that are borne irrespective of whether the host is attacked or not. By analogy, the two types of costs can be thought of as the penalties of fighting a war (actual defence) and of maintaining a standing army (resistance ability).

(In: Fellowes, M. and Godfray, H. 2000. The evolutionary ecology of resistance to parasitoids by *Drosophila*. *Heredity*. 84: 1–8. <https://doi.org/10.1046/j.1365-2540.2000.00685.x>)

Chapter 25

Demonstration of efflux activity of Malpighian tubules of *Drosophila* larvae

Sanjay Saini¹ (sanju21brd@gmail.com), Madhu G. Tapadia^{2*} (madhu@bhu.ac.in), and Naveen Kumar Gautam^{3*} (nkgautam@sgpgi.ac.in; gautam.naveen6@gmail.com)

1. Embryotoxicology Laboratory, Environmental Toxicology Group, CSIR-Indian Institute of Toxicology Research (CSIR-IITR), Vishvighyan Bhavan, 31 Mahatma Gandhi Marg, Lucknow- 226001

2. Cytogenetics Laboratory, Department of Zoology, Banaras Hindu University, Varanasi.

3. Department of Urology and Renal Transplantation, Sanjay Gandhi Post Graduate Institute of Medical Sciences (SGPGIMS), Raebareli Road, Lucknow 226014.

* For correspondence

Recommended Level: Postgraduate /Research

Sub-discipline: Toxicology/Physiology

Background:

Kidneys play critical roles in excreting waste and harmful chemicals, as well as maintaining the ionic balance. The functional unit of the kidney is nephron, and the precise functioning of multiple transporters determines a nephron's optimal activity. Exposure to xenobiotic (heavy metals, pesticides and drugs) greatly impacts their function. A suitable animal model helps in identifying potential xenobiotics that can alter functioning of nephrons. Malpighian tubules (MT) of *Drosophila melanogaster* (see Chapter 20) perform excretory and osmo regulatory functions analogous to vertebrate renal tubules and are enriched in similar set of transporters as vertebrate nephrons (Gautam *et al.*, 2017). Thus, fluid secretion by MT can be used as a simple assay to judge its proper functioning.

Rhodamine 123 is a red fluorescent dye, which easily enters MT cells by passive and active processes. Healthy MT normally effluxes out this dye within 15 min so that the dye does not accumulate in the lumen. Thus, a disruption in MT functioning can be assayed by examining accumulation of this dye in MT lumen (Yadav *et al.*, 2016). This chapter describes Rhodamine 123 accumulation in MT as an assay to examine effects

of Cadmium chloride (CdCl_2), a known nephrotoxicant compound, on efflux activity of MT. This system can be used to assess effects of different heavy metals or pollutants or mutations that affect MT function.

Objective:

To study the effect of the Cadmium chloride (CdCl_2) exposure on efflux activity of MT using Rhodamine 123 fluorescent dye.

Materials Required:

A. **Flies:** Healthy wild type *Drosophila melanogaster* reared at $24 \pm 1^\circ\text{C}$.

B. Equipment and other supplies:

- i. Stereo-Binocular microscope.
- ii. Dissecting needles. fine paint brush, forceps and other items needed for fly handling.
- iii. Glass slides and cover slips (22 mm²).
- iv. Fluorescence or confocal microscope.

C. Chemicals and reagents:

- i. Cadmium chloride (CdCl₂).
- ii. Rhodamine 123 (stock solution: 1 mg/mL in absolute ethanol; dilute the stock 1:1000 with 1X PBS to obtain 1 µg/mL Rhodamine 123 working solution).
- iii. 1X Phosphate Buffered Saline (1X PBS): Dissolve 8 g NaCl, 0.2 g KCl, 1.42 g Na₂HPO₄ and 0.272 g KH₂PO₄ in 800 mL of distilled H₂O. Adjust pH to 7.4 with HCl and then add distilled H₂O to make the volume to 1000 mL. Autoclave and store at RT or at 4°C.
- iv. Vectashield antifade mounting medium.

Experimental details:

1. **CdCl₂ containing (experimental) food:** Add CdCl₂ salt to freshly prepared normal fly food when it is still warm and fluid to a concentration of 50 µM (this is 1/10th of the reported Lc₅₀ of CdCl₂ for *Drosophila*, Saini *et al.*, 2020) and mix thoroughly by brief vortexing. Use normal food as control. Prepare food vials with control and the CdCl₂ mixed experimental food at the same time.
2. Collect eggs from healthy wild type flies and transfer freshly hatched 15-20 first instar larvae to control and CdCl₂ (50 µM) supplemented experimental food vials. Prepare 3 replicates of control and experimental vials each.
3. Let the larvae grow up to late third instar stage (approximately 96 hr of exposure to CdCl₂).
4. Dissect out the MT from the CdCl₂ exposed and control larvae in 1X PBS and transfer to cavity/Maximov slides, containing 200 µL Rhodamine 123 (1 µg/mL).
5. incubate in dark for 15 min at 37°C, followed by immediate chilling on ice.
6. After chilling, wash the tissues with PBS three times for 5 min each at 37°C to allow the dye to efflux out.
7. Transfer the control and experimental (CdCl₂ treated) MT on slides in Vectashield mounting medium, cover with coverslip and seal with transparent nail polish.
8. Examine the preparations under fluorescence or confocal microscope using excitation/emission maxima at 546/568 nm, respectively.

Observation/Result:

Compare the Rhodamine123 fluorescence in MT from control and experimental larvae (Fig. 1).

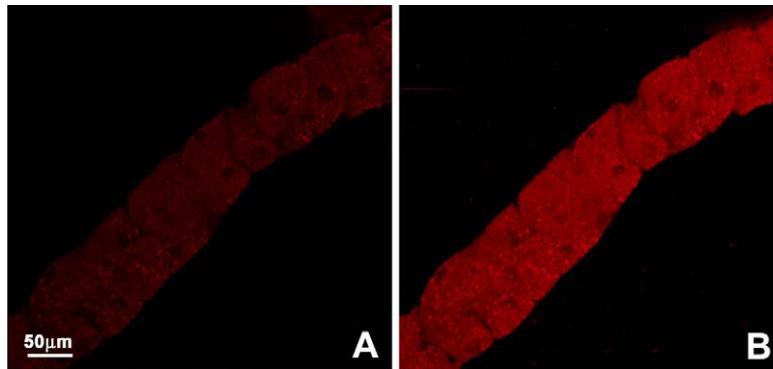


Fig. 1. Cadmium chloride affects efflux activity of Malpighian tubules in *Drosophila*. Rhodamine 123 fluorescence patterns of MT from control (A) and CdCl₂ fed experimental (B) larvae. The brighter fluorescence signal in the treated MT indicates impaired efflux of Rhodamine 123 dye.

Questions for self-study and interpretation:

1. What is function of the efflux activity in kidney or Malpighian tubules and what happens if this is inhibited?
2. Activity of which transporters can be assessed by this assay?

References and further reading:

Gautam, N. K., Verma, P. and Tapadia, M. G. (2017). *Drosophila* Malpighian tubule: A model for understanding kidney development, function and disease. In: *Kidney Development and Disease*, R. K. Miller (ed.). Springer Nature. Springer International Publishing AG, Switzerland. DOI 10.1007/978-3-319-51436-9.

Saini, S., Rani, L., Shukla, *et al.* (2020). Development of a *Drosophila melanogaster* based model for the assessment of cadmium and mercury mediated renal tubular toxicity. *Ecotoxicology Environmental Safety*. 201:110811. DOI: 10.1016/j.ecoenv.2020.110811.

Yadav, S. and Tapadia, M .G. (2016) Expression of polyQ aggregates in Malpighian tubules leads to degeneration in *Drosophila melanogaster*. *Dev. Biol.* 409: 166-180. DOI: 10.1016/j.ydbio.2015.10.028.

Why Study Malpighian Tubules in *Drosophila*?

Why then, when they have been studied so intensively, is it necessary to extend studies to another species like *Drosophila rnelanogaster*, and one with no obvious biomedical or agricultural significance? The reasons are threefold: we know more about the genetics of *Drosophila* than another other insect; we can manipulate *Drosophila* genetically better than any insect; and *Drosophila* tubules are similar enough to those of other insects to allow general inferences to be drawn.

(In: Dow, J.A.T. and Davies, S. A. 2001. The *Drosophila melanogaster* Malpighian Tubule. *Advances In Insect Physiology*. 28: 1-83)

Chapter 26

Assessing functionality of *Drosophila* nephrocytes using silver nitrate

Lavi Rani² (laviverma2100@gmail.com), Sanjay Saini² (sanju21brd@gmail.com), Neha Shukla¹ (nehashukla400@gmail.com), Madhu G. Tapadia³ (madhu@bhu.ac.in) and Naveen Kumar Gautam^{1*} (nkgautam@sppgi.ac.in; gautam.naveen6@gmail.com)

1. Department of Urology and Renal Transplantation, Sanjay Gandhi Post Graduate Institute of Medical Sciences (SGPGIMS), Raebareli Road, Lucknow 226014

2. Embryotoxicology Laboratory, Environmental Toxicology Group, CSIR-Indian Institute of Toxicology Research (CSIR-IITR), VishvgyanBhavan, 31 Mahatma Gandhi Marg, Lucknow 226001

3. Cytogenetics Laboratory, Department of Zoology, Banaras Hindu University, Varanasi 221005

* For correspondence

Recommended Level: PG/Research

Sub-discipline: Toxicology/Physiology

Background:

A nephron is the basic structural and functional unit of the mammalian kidney that comprises of a glomerulus where filtration of blood takes place, and a renal tubule, where the filtrate is modified and urine is excreted. It has been shown (Weavers *et al.*, 2009) that the nephrocytes in *Drosophila* have anatomical, molecular, and functional similarities with the mammalian podocyte, a layer of cells in the glomerulus of the mammalian nephron that forms the glomerular filtration barrier (GFB). The GFB is made up of three layers, viz., endothelium, glomerular basement membrane (GBM), and the podocytes. The podocytes form a tight layer around the top of the GBM with their interdigitating foot processes that are joined by a ~30 nm slit diaphragm. The slit diaphragm forms a charge and size selective barrier that allows movement of water and solute molecules, but does not allow the large size molecules such as blood protein albumin (Weavers *et al.*, 2009).

Drosophila nephrocytes are spherical cells that remove harmful or toxic substances from the hemolymph through filtration and endocytosis. *Drosophila* nephrocytes are present in two clusters, namely the large garland cell nephrocytes (located near proventriculus region of the foregut) and the pericardial nephrocytes (located near the heart tube). The plasma membrane in nephrocytes forms many infoldings leading to the formation of ~30 nm wide lacunae, which are flanked by the foot processes. Therefore, nephrocytes form a nephrocyte diaphragm that provides a size and charge selective filter to take up molecules from hemolymph (Weaver *et al.*, 2009). The waste or toxic materials taken from hemolymph by the nephrocytes are stored intracellularly in these cells through endocytosis. Their high endocytic activity is analogous to “excretory” activity. Failure or reduced uptake of waste or toxic materials from hemolymph by the nephrocytes suggests deformities in the nephrocyte filtration barrier. Deformities in the nephrocyte filtration barrier can be demonstrated by feeding unwanted molecules like silver nitrate (AgNO₃) to larvae and examining their uptake in

the garland cells. If the nephrocytes have optimally functional filtration barrier, they display normal uptake of the AgNO_3 (Fig. 1). However, if the nephrocyte filtration barrier is affected because of some pathological conditions or mutations, AgNO_3 uptake by the nephrocytes from the haemolymph is reduced (Weavers *et al.*, 2009).

Objective:

To assess nephrocyte activity using AgNO_3 up take assay.

Materials required:

A. Healthy first instar larvae of wild type *Drosophila* reared on standard *Drosophila* food medium at $23 \pm 1^\circ\text{C}$.

B. Equipment:

i. Stereo-binocular and bright-field (preferably with phase-contrast optics) microscope.

C. Other supplies:

- i. Fly-handling items and food vials.
- ii. Micro-pipettes and corresponding tips.
- iii. Gloves.
- iv. Dissecting needles.
- v. Glass slides and coverslips.
- vi. Tissue Paper.

D. Chemicals and reagents:

- i. Silver nitrate (AgNO_3 : 0.003% solution in distilled water).
- ii. Live *Saccharomyces cerevisiae* (Baker's Yeast).
- iii. Schneider's medium (Insect medium).
- iv. 1X Phosphate buffered saline (PBS pH 7.4): .Dissolve 8 g NaCl, 0.2 g KCl, 1.42 g Na_2HPO_4 and 0.272 g KH_2PO_4 in 800 mL of distilled H_2O . Adjust the pH to 7.4 with HCl. Finally add distilled water to make the final volume 1000 mL. Autoclave and store at RT or 4°C .

Experimental details:

1. Prepare the agar-sucrose food vials: heat 5 gm agar, 15 gm Sulphur-free sugar, 1 g Nipagin and 1 mL propionic acid in 360 mL water in a conical flask until agar and sugar dissolve completely; pour 5 mL in each vial and leave them to cool and solidify before plugging.
2. Collect first instar larvae growing on standard food and wash them with distilled water to remove remnants of food particles.
3. Transfer 15 larvae on agar-sucrose vials supplemented with a dab of yeast paste containing AgNO_3 (experimental).

4. Feed the first instar larvae till the late third instar wandering stage.
5. Collect third instar larvae and wash them with distilled water to remove food remnants.
6. Dissect the proventriculus region of gut (see **Fig. 1** in **Chapter 30**), with which the nephrocytes remain attached, in 1X PBS.
7. Transfer the dissected proventriculus region of gut on clean slide in a drop of Schneider's medium (for short-term observations, PBS can also be used in place of the Schneider's medium) and mount with coverslip.

Observations:

Observe the AgNO₃ present in nephrocytes, attached with proventriculus (**Fig. 1**) under a microscope using a 40X objective (phase-contrast optics is useful in examining unstained cells).

For comparison, examine levels of AgNO₃ in nephrocytes from larvae fed with any toxicant added to the agarose-sugar food supplemented with yeast-AgNO₃ solution.

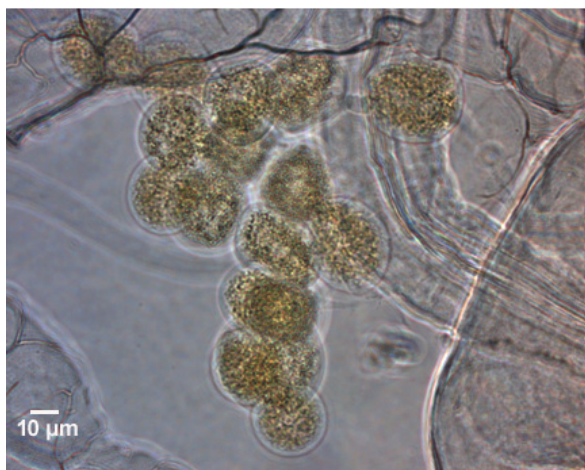


Fig. 1. Presence of AgNO₃ (brown inclusions) in larval nephrocytes attached to proventriculus (phase-contrast image).

Questions for self-study and interpretation:

1. *Drosophila* nephrocytes are functionally similar to human podocytes. Is it possible to use *Drosophila* nephrocytes to screen the environmental chemicals that can affect the function of human podocytes?
2. What would be the impact of disrupted nephrocyte diaphragm on the survival of *Drosophila*?

References:

- Denhom, B. and Skaer, H. (2009). Bringing together components of the fly renal system. *Current Opinion Genetics Development*. 19: 526-532.
- Weavers, H., Prieto-Sanchez, S., Grawe, F., Garcia-Lopez, A. *et al.* (2009). The insect nephrocyte is a podocyte-like cell with a filtration slit diaphragm. *Nature*. 457: 322-326.

Marcello Malpighi – A Pioneer Microscopic Anatomist

It is reasonable to start with Marcello Malpighi (1628-1649), professor of medicine and personal physician to Pope Innocent XII, and a pioneer of microscopic anatomy. Accordingly, a number of anatomical structures still bear his name: Malpighian corpuscles in the circulatory and lymphatic systems, the Malpighian layer of the epidermis, and the Malpighian tube in insects. Since then, Malpighian tubules have been documented by morphologists, but it was in the twentieth century that modern physiology was brought to bear.

(In: Dow, J.A.T. and Davies, S. A. 2001. The *Drosophila melanogaster* Malpighian Tubule. *Advances In Insect Physiology*. 28: 1-83)

Chapter 27

Fluid secretion assay to assess the rate of secretion by Malpighian tubules in *Drosophila*

Priya Singh (priya.singh10@bhu.ac.in) and Madhu G Tapadia* (madhu@bhu.ac.in)

Cytogenetics Lab, Department of Zoology, Banaras Hindu University, Varanasi-221005, India

* For correspondence

Recommended Level: PG/Research

Sub-discipline: Cell Biology/Developmental Biology/Physiology

Background:

Malpighian tubules are the excretory organs of insects that function like the kidneys in vertebrates. They are involved in the osmoregulation, excretion, ion balance and water balance. The relatively simple structure and genetic tractability of *Drosophila* Malpighian tubules (see Chapters 20, 25, 26) make them an excellent model system to study various aspects of renal physiology.

The Fluid secretion assay described in this chapter allows to understand the role of specific genes, signalling pathways, environmental factors or drugs that may affect the secretion rate. Several drugs that affect the secretion rate of mammalian kidney also have similar action on *Drosophila* tubules as well (Dow and Maddrell *et al.*, 1998). The knowledge of factors that affect the rate of secretion can be extrapolated to gain insight into renal physiology and related human disorders. This method can be used not only to determine the rate of secretion but also the composition of the secreted fluid (Dow and Maddrell *et al.*, 1998; Schellinger and Rodan, 2015).

Objectives: To determine the rate of fluid secretion by Malpighian tubules of *Drosophila*.

Materials Required:

- A. **Flies:** Late third instar larvae of Wild type or other desired mutant *Drosophila melanogaster* (for comparative study, use age matched larvae).
- B. **Equipment and other items:**
 - i. Stereo-binocular microscopes and other fly handling materials.
 - ii. Surgical scalpel.
 - iii. Petri dish (60 mm X 15 mm).
 - iv. Fine stainless all-pins (paper pins).

- v. Glass beaker (100 ml).
- vi. Micro-Pipettes (10 μ l) and corresponding tips.

Reagents:

- i. Insect saline (Poels' salt solution (PSS), pH 7.0)

NaCl	86 mg
KCl	313 mg
CaCl ₂ .2H ₂ O	116 mg
NaH ₂ PO ₄ .2H ₂ O	88 mg
KHCO ₃	18 mg
MgSO ₄ .7H ₂ O	513 mg
Distilled H ₂ O	100 mL

Mix the above salts in distilled H₂O, adjust pH to 7.0 and filter.

- ii. Silicon elastomer reagents.
- iii. Mineral oil (laboratory purpose).

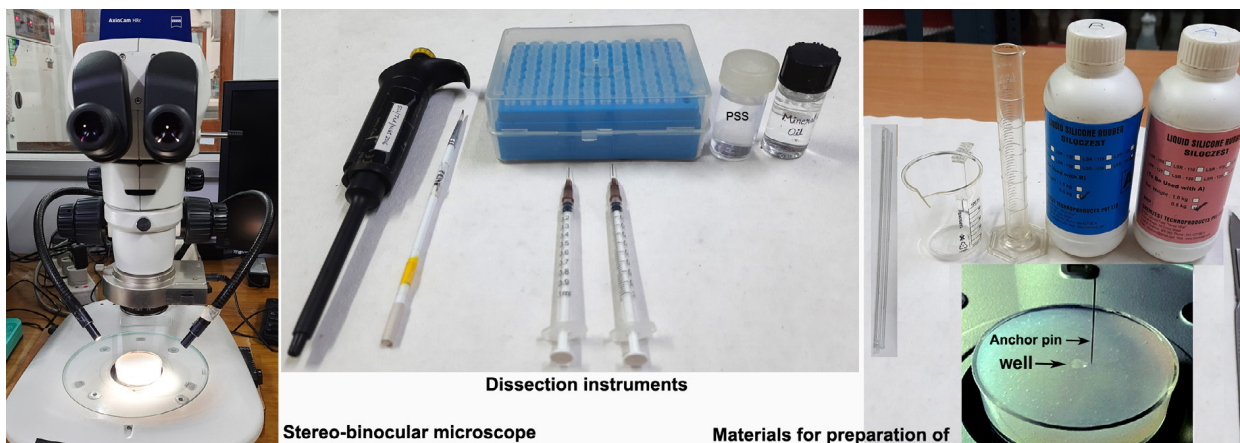


Fig. 1. Materials required for the Fluid secretion assay.

Experimental Details:

A. Preparation of silicon elastomer base for collection of secretion

Silicon elastomer is used as the provide a hydrophobic surface so that the secreted fluid does not disperse after its secretion from ureter. The small pore size of silicon elastomer base permits the secreted fluid to be retained on the surface without percolating down.

1. Pour equal amounts (25 mL of each solution for 5 dishes) of silicon elastomer base and silicon elastomer-cure in a glass beaker (or as directed by the manufacturer). Mix the two liquids manually using a glass rod, without creating any air bubbles.
2. Pour the mixture in a clean glass petri dish (60 X 10 mm) to provide ~6-7 mm depth of elastomer base in a 60 mm x 15 mm petri dish.
3. Cover and leave the dish on a bench at room temperature to cure (harden) the elastomer base for ~20-24 hr.

4. After the silicon elastomer has hardened, make wells of 3-4mm diameter and 3-4mm depth on the elastomer surface with a sharp scalpel. To make a well, insert the tip of scalpel in the elastomer to a depth of about 3-4 mm and using the opposite hand smoothly rotate the dish to 360° so that the wall of the well is as smooth as possible. **Note:** 3-4 wells can be prepared in a single petri dish. The wells may be re-used after thoroughly washing them with distilled water (until they tend to get dried). The prepared dishes can be stored in sealed polybags at 4°C.
5. Insert a fine paper pin 1mm away from the well while viewing the well under a stereo-binocular microscope.
6. Working under the stereo-binocular microscope, fill the well with 10-15 µL PSS or insect saline. Avoid over filling the well as it will result in the Malpighian tubules floating away from the well during the experiment.
7. Carefully layer 100-150 µl mineral oil on the top of the saline-filled well to prevent evaporation of saline.

B. Preparation of Malpighian tubules for secretion assay:

1. Dissect the tubules in PSS/saline and detach a pair of anterior tubules, including the ureter. The hindgut and midgut should be carefully removed completely. Retain only the ureter and the anterior pair of tubules (Fig.2).

Note: The anterior pair of tubules is distinct because of its whitish initial segment, which makes it easier to dissect the intact tubules. However, the posterior pair of tubules can be used as well.

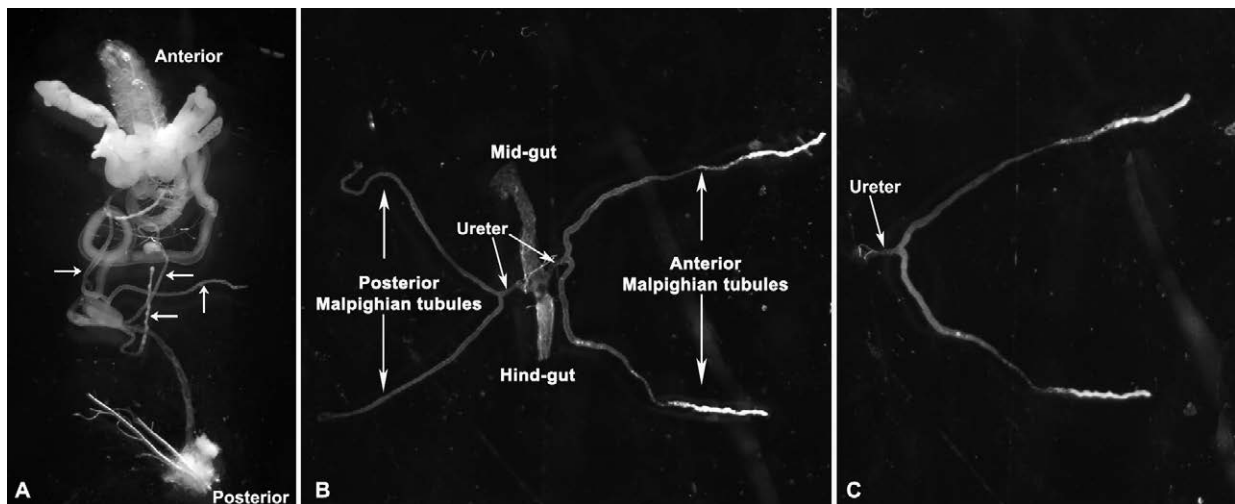


Fig. 2. **A.** Dissected larva with internal organs pulled out (arrows indicate the paired Malpighian tubules). **B.** Anterior and posterior pairs of Malpighian tubules attached to gut via ureters. **C.** Separated pair of anterior Malpighian tubules attached to the ureter.

2. Using a fine dissection needle, pick up the separated anterior tubule pair and transfer into the assay-well filled with PSS, prepared as in section A.6-7 above.
3. Immediately after placing the tubules, pick the free end of one of the tubules, pull it out of the well so that the cut end of the ureter is halfway between the anchoring pin and the well.

4. Now carefully wrap initial segment of the pulled-out tubule around the anchoring pin, avoiding any damage to the tubule, as illustrated in schematic in Fig. 3). Note the time immediately.

Note: In this arrangement, one tubule remains in the PSS-filled well while the other tubule acts as ‘anchor tubule’ (Fig. 3). Fluid secreted by the Malpighian tubule dipped in the PSS-filled well comes out of the cut end of the ureter on the dry surface of the silicon elastomer and accumulates as a drop on surface of the silicon elastomer.

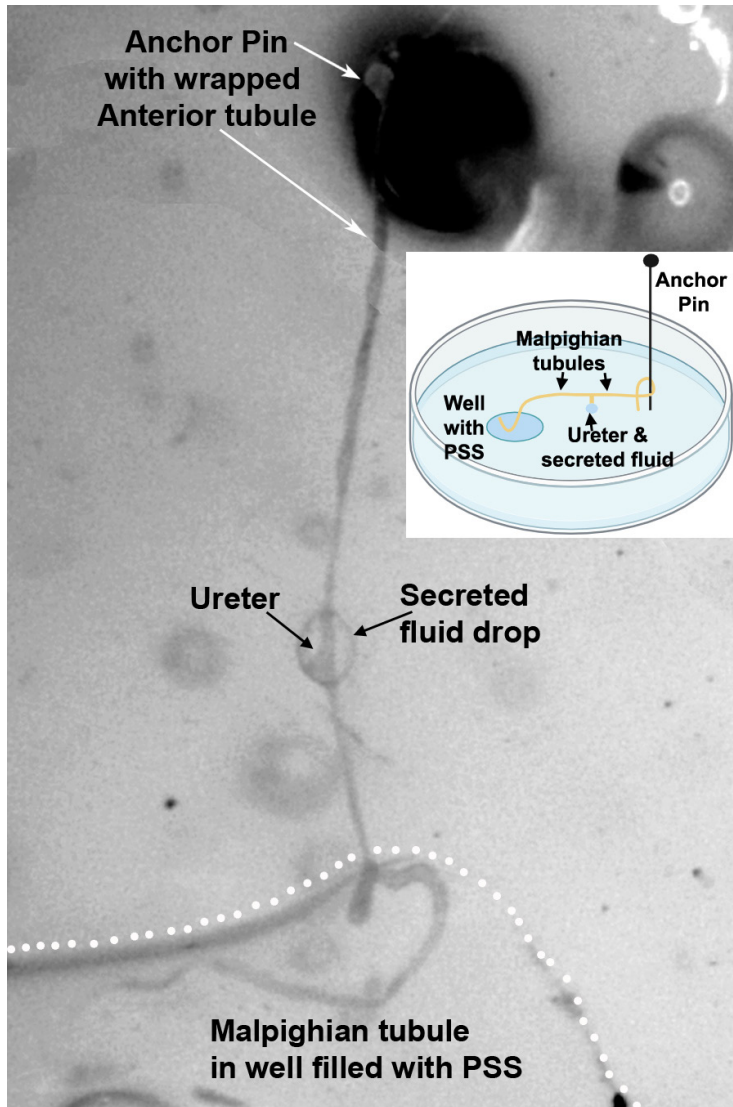


Fig. 3 : Setup for the fluid secretion assay with one of the anterior tubules (lower side) dipped in PSS filled well in the silicon elastomer base while the other anterior tubule acts as an anchor as it is wrapped around the anchor pin (a schematic in the inset). White dotted line indicates part of boundary of the well, filled with PSS. The fluid secreted by the Malpighian tubules collects at the ureter opening (arrows).

6. Take images of the secreted drop through the stereo-binocular microscope at intervals of 15 min over a period of 60-90 min to measure the rate of secretion.

Observations:

Measure the diameter of the secreted fluid drop with help of an ocular micrometer placed in the eye piece of the stereo-binocular microscope or use an image analysis software for calculating size of the secreted fluid drop. Determine if the size of secreted fluid drop changes during the period of observation.

Questions for self-study and interpretation:

1. Why is mineral oil layered on PSS/saline in the well?
2. What physiological conditions disturb the rate of secretion of Malpighian tubules in *Drosophila*?
3. Which human disorders disturb the fluid secretion and reabsorption in kidney?
4. What factors that affect the rate of fluid secretion in kidney?

References and further reading:

Dow, J. A., Maddrell, S. H., Görtz, A., Skaer, N. J., Brogan, S., and Kaiser, K. (1994) The Malpighian tubules of *Drosophila melanogaster*: A novel phenotype for studies of fluid secretion and its control. *Journal Experimental Biology*, 197: 421-428. <https://doi.org/10.1242/jeb.197.1.421>.

Schellinger, J. N., and Rodan, A. R. (2015) Use of the Ramsay assay to measure fluid secretion and ion flux rates in the *Drosophila melanogaster* Malpighian tubule. *Journal Visualized Experiments*. 105: e53144. <https://doi.org/10.3791/53144>.

Malpighian Tubules: Multi-Tasking Organ

The Malpighian Tubules (MTs) are the main excretory organs in most insects. They play a key role in the production of primary urine and osmoregulation, selectively reabsorbing water, ions, and solutes. Besides these functions conserved in most insects, MTs can serve some specialized tasks at different stages of some species' development. The specialized functions include the synthesis of mucopolysaccharides and proteins for the building of foam nests, mucofibrils for the construction of dwelling tubes, adhesive secretions to help the locomotion, and brochosomes for protection as well as the usage of inorganic salts to harden the puparia, eggs chorion, and pupal cells' closing lids. MTs are also the organs responsible for the astonishing bioluminescence of some Diptera glowworms and can go through some drastic histological changes to produce a silk-like fiber utilized to spin cocoons. The specialized functions are associated with modifications of cells within the entire tubules, in specific segments, or, more rarely, modified secretory cells scattered along the MTs. In this review, we attempted to summarize the observations and experiments made over more than a century concerning the non-excretive functions of insects' MTs, underlying the need for new investigations supported by the current, advanced technologies available to validate outdated theories and clarify some dubious aspects.

(Farina P, Bedini S, Conti B. (2022). Multiple functions of Malpighian tubules in insects: A review. *Insects*. 13: 1001. <https://doi.org/10.3390/insects13111001>).

Chapter 28

Dissection of larval gonads of *Drosophila*

Nidhi Murmu^{1,2} (nidhimurmu@aripune.org), Kiran Nilangekar^{1,2} (knilangekar@aripune.org), Minal Ayachit^{1,2} (minalayachit@aripune.org) and Bhupendra V. Shrivage^{1,2*} (bvshrivage@aripune.org)

1. Developmental Biology Group, Agharkar Research Institute, Pune -411004

2. Savitribai Phule Pune University, Ganeshkhind, Pune -411004.

* For correspondence

Recommended Level: PG/Research

Sub-discipline: Biology/Anatomy

Background:

Drosophila melanogaster is a widely used model system in biology. The insect exhibits high fecundity and a short life cycle spanning over four stages: embryo, larvae, pupae and imago (adult fly). The larval stage is subdivided into first, second and third instar larvae. The fruit fly completes its life cycle in about 10 days at 25°C (Silva, *et al.*, 2016) under laboratory conditions.

Sexual dimorphism is seen as morphological and behavioral differences between different sexes of the same species. It arises due to genetically and temporally coordinated tissue specific molecular cues during the course of development. The difference in the development and morphology of female and male reproductive organs is a prime example of sexual dimorphism.

The male and female gonads can be observed in the early to late third instars larvae of *Drosophila* as a highly transparent cluster of cells embedded within the fat body which appears to be translucent (Maimon and Gilboa *et al.*, 2011). The size of gonads in male is five times larger than that of female gonads during this stage (Greenspan, 2004). A simple protocol for visualization of the differences in gonads of male and female larvae and their separation from other organs is described here which can be useful for developmental and gene expression studies involving advanced assays like DNA, RNA, protein extraction, immuno-staining etc. Identification of gonads can also be used to separate male and female larvae that may be required for some other studies.

Objectives:

Examination of gonads of male and female larvae of *Drosophila melanogaster* and distinguishing male and female larvae.

Materials required:

1. Equipment and other supplies:

- i. Stereo-binocular microscope.
- ii. Bright-field microscope (optional: phase-contrast or differential interference contrast (DIC) optics).
- iii. Microscope slides (preferable: Cavity slides for dissection of larvae) and 22 x 22 mm coverslips.
- iv. Blunt and fine-tipped pairs of forceps and fine dissection needles/pins.
- v. Micropipettes (1000 μ L, 100 μ L) and corresponding micropipette tips.
- vi. Petri dishes, glass slides.
- vii. 1.5 mL microfuge tubes.
- viii. Transparent nail polish (**Fig. 1**).

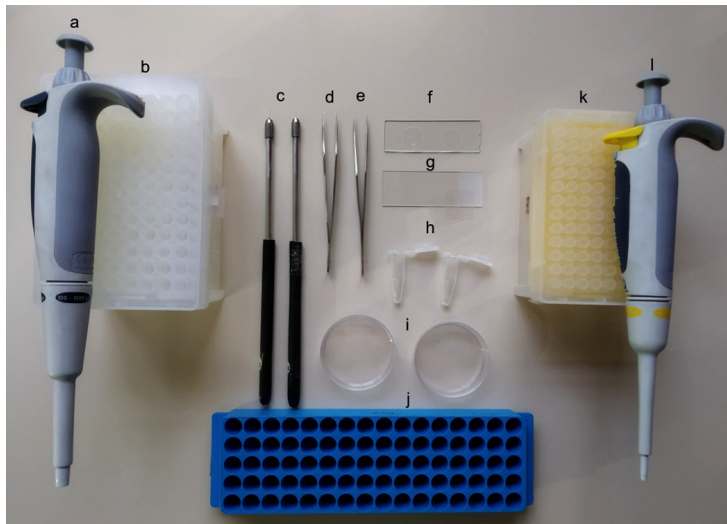


Fig. 1. Dissection instruments and plastic wares required: (a) 1000 μ L micropipette, (b) 1000 μ L micropipette tips, (c) Pair of insect dissecting pins, (d) Pair of blunt forceps, (e) Pair of sharp forceps, (f) Cavity slide, (g) plain glass slide and coverslips, (h) 1.5 mL microfuge tubes, (i) 60 mm Petri dish, (j) Microfuge tube stand, (k) 100 μ L micropipette tips, (l) 100 μ L micropipette.

2. Solutions:

- i. 1X Phosphate Buffer Saline (PBS pH 7.4)
 - a. NaCl 8 gm
 - b. KCl 200 mg
 - c. Na_2HPO_4 1.42 gm
 - d. KH_2PO_4 272 mg

Serially dissolve the above in 800 mL Distilled water; when fully dissolved, adjust to pH 7.4 and make the volume to 1000 mL with more distilled water. Autoclave the solution and store at room temperature

- ii. 20% Sucrose solution, in distilled water ; autoclave the solution and store at room temperature

iii. 4% Paraformaldehyde (PFA) pH 7.4 (to be prepared fresh)

- a. PFA 4 gm
- b. 1X PBS pH 7.4 80 mL

Heat to 60°C for 30 min, mix intermittently. Allow to cool and adjust pH to 7.0 with NaOH. Finally make up the volume to 100 mL with 1X PBS pH 7.4.

iv. 80% glycerol (mounting medium) 8 mL of 100% glycerol mixed with 2 mL of sterile 1X PBS

v. 0.1% PBTx solution (500 μ L of 10% Triton X-100 in 49.5 mL of sterile 1X PBS)

Experimental details:

1. Collecting *Drosophila* larvae from culture vials/bottles: Set up a cross between newly eclosed *Drosophila* males and at least one day old virgin females of the desired genotype (10 males + 15 females) in a vial. Let the females lay eggs for at least 24 hr. Remove the adults and transfer them to a fresh vial. Incubate the vials with eggs for 60-70 hr at 25°C. For isolating a large number of mid third instar larvae, which actively feed inside the food, add sterile 20% sucrose solution to the culture bottle/vial such that it forms a 0.5 - 1 cm layer on top of the food (**Fig. 2**). As the density of larvae is lower than that of the 20% sucrose solution, they will float on top of the sucrose solution, which makes it easier to retrieve them (**Fig. 2B, C**).

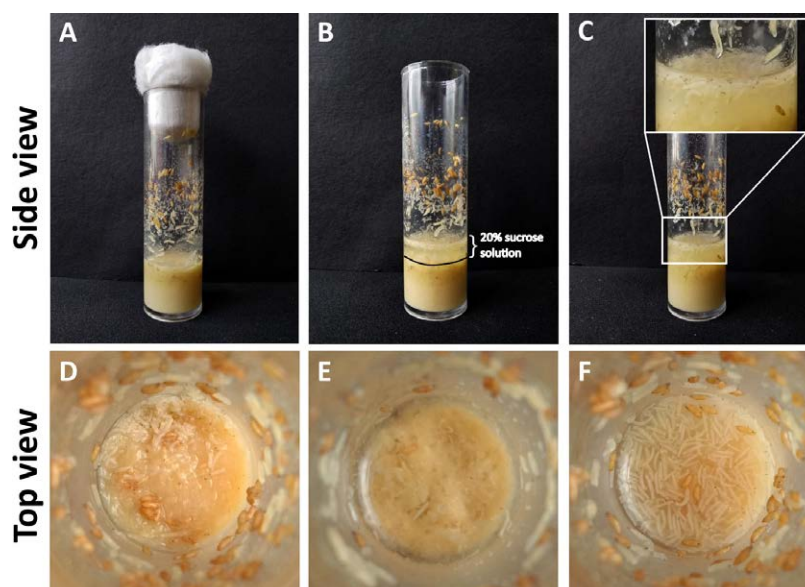


Fig. 2. **A.** *Drosophila* culture vial (5-6 days after egg collection) with larval stages and pupae. **B.** Culture vial immediately after addition of 20% sucrose solution on the food surface, and **C.** after 3 min of addition of sucrose solution. Inset in **C** shows a higher magnification of the larvae floating in the sucrose solution. Side view of vials is shown in **A-C**, while **D-F** show top view of **A-C**, respectively.

2. Gently swirl the vial so that the feeding larvae loosen and detach from the food (**Fig. 2C, F**).

Note: Avoid vigorous shaking/tapping as this will lead to some mixing of churned food into the sucrose solution.

3. After the larvae start floating in the sucrose, gently pour the sucrose solution in a clean Petri plate ensuring that the food is not getting dislodged (**Fig. 3A**).

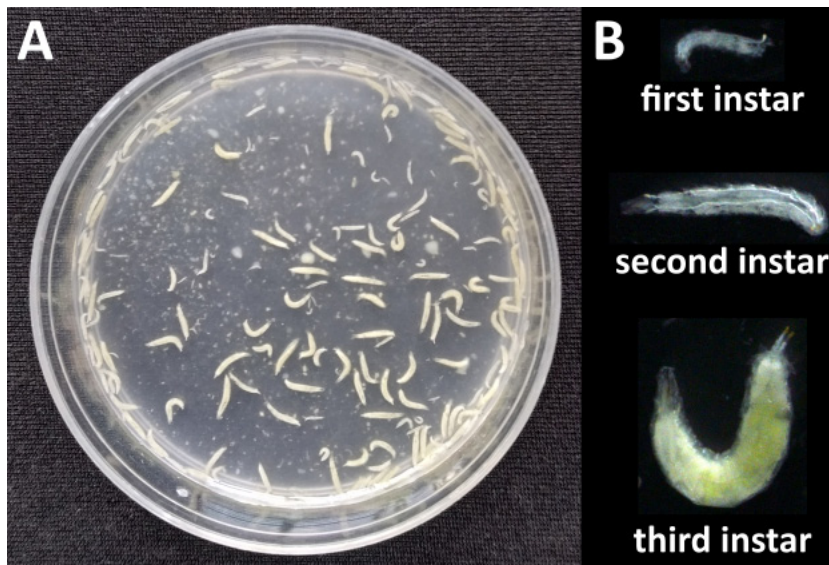


Fig. 3. A. The floating larvae transferred to a Petri plate. B. Different stages of *Drosophila* larvae.

Alternatively, when fewer larvae are needed, mid third instar larvae can be collected one by one with a soft brush from the food surface where they feed and crawl actively; the late third instar larvae stop feeding and wander on the dry surface of the Petri dish or the vial/bottle and thus can be directly collected with soft brush. For identifying larval stages of *Drosophila melanogaster*, see **Chapters 1, 14**. With the help of blunt forceps or soft brush, transfer third-instar larvae into a clean Petri dish containing a few drops of 1X PBS solution. Care should be taken while picking the larvae to prevent rupturing of the larval cuticle and damage to the internal organs. Additionally, it should be ensured that the posterior spiracles of the larvae (**Fig. 3**) are exposed to air for them to respire.

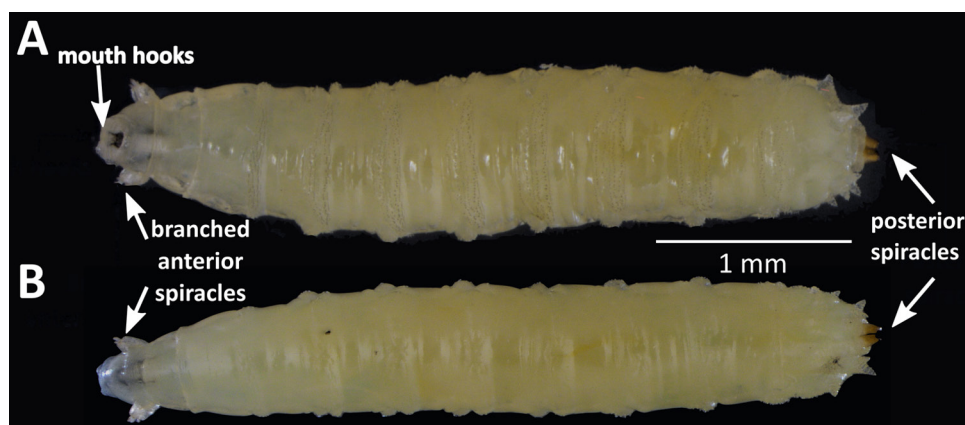


Fig. 4. A. Ventral and B. Dorsal views showing the distinguishing morphological characteristics of a third instar larva.

2. Differentiating between male and female larvae and larval gonads: Identification of the sex of the larva is relatively simple and requires a stereo-binocular microscope (for best viewing) preferably with a facility of transmitted light (illumination from top

with a table lamp can also serve the purpose). The larvae are distinguished into either sex depending on the size of the gonad which are visible through the cuticle in the posterior third region (fifth abdominal segment) of the larva as small spherical and more transparent structures. The female gonads are five times smaller in size and hence a little more difficult to visualize as compared to the male gonads (**Fig. 5**)

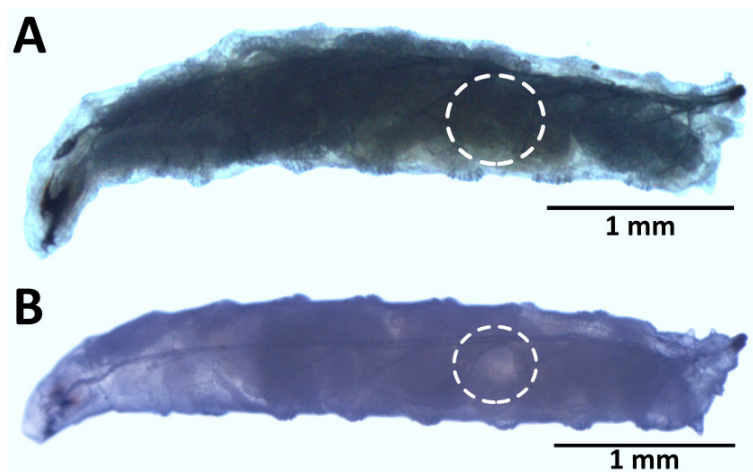


Fig. 5. **A.** Female and **B.** Male third instar larvae of *Drosophila melanogaster*. The gonads are located in the white encircled area with testes readily visible as a larger transparent spherical object through the cuticle (**B**).

3. Dissection of larvae: Place a clean cavity slide (or normal slide) and add 200 μ L of 1X PBS pH 7.4 solution onto the cavity with the help of a micropipette.

Hold the larva at the anterior end (where the mouth hooks lie) using forceps, hold the transparent cuticle by the sharp forceps near the grip of the first forceps (**Fig. 6A**) and tear it along the length of the larva (**Fig. 6B**). The internal organs will be pushed out of the larval cuticle (**Fig. 6C**). The gonads are embedded in the fat bodies which are long transparent sheets of single layer of large hexagonal cells and which run along the length of the larval body (**Fig. 7**).

Transfer the larval carcasses into a 1.5 mL microfuge tube containing 500 μ L 4% PFA. Allow the tissues to be fixed overnight at 4°C in a standing tube or for 2 hr at 25°C on a shaker (5 to 10 rpm).

Note: For studies requiring isolation of DNA, RNA and protein, the larval gonads should be dissected with minimal carry over of the fat tissue and transferred to the lysis buffer, rather than the above fixative, for further processing)

Following the fixation, discard the PFA solution from the tube and wash the tissue using 500 μ L of 1X 0.1% PBTx solution for 15 min on a shaker at 5-10 rpm. Repeat this step three times. Since the larval fat body tissue is very delicate and thus subject to easy damage, a gentle handling throughout the procedure is essential. Carefully aspirate the solutions from the tube without damaging the tissue. If required, the fixed gonads can be stored in 1X PBS at this stage at 4°C until the next step.

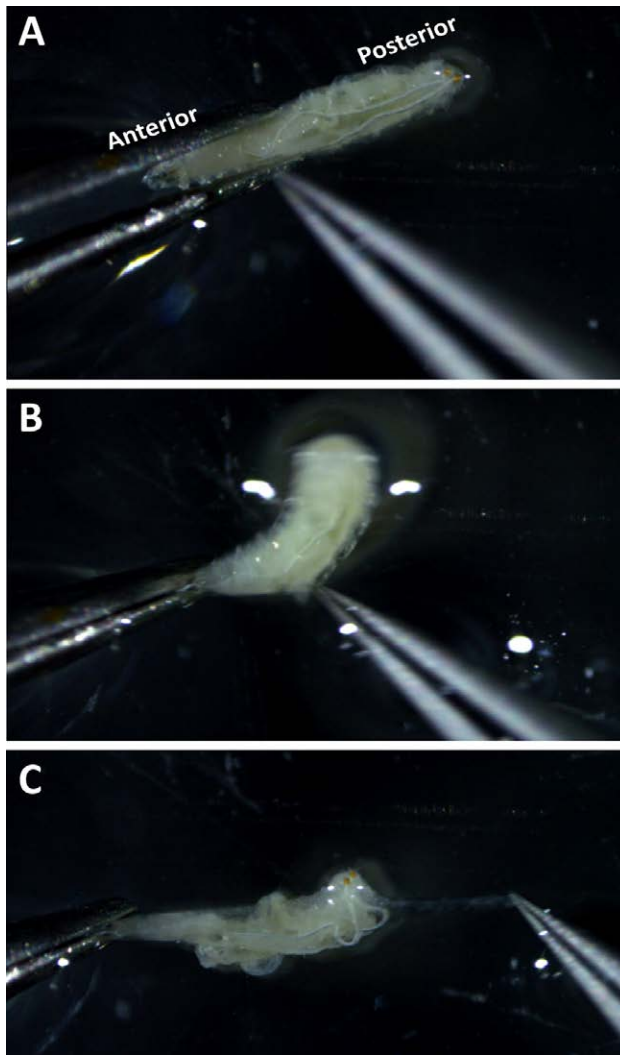


Fig. 6. Steps in the dissection of third instar larva. (A-C) Sequential tearing of larval cuticle. C shows a torn larva after removal of thin strip of larval cuticle.

1. Mounting of gonads: Transfer a few fixed larval carcasses (**Fig. 7**) in the PBS on a slide (cavity slide preferred). Separate the fat bodies from the other tissues using forceps or insect dissecting pins. Gonads are a pair of transparent structure embedded in the two lobes (left and right) of the translucent fat body tissue (**Fig. 7, 8**).

Place about 20-25 μL of 80% glycerol solution at the center of a clean glass slide and gently transfer the cleaned gonads with minimal amount of the surrounding fat body tissue with help of sharp forceps.

Space out and align the tissue fragments after all of the gonads have been transferred to the 80% glycerol drop on the slide.

Hold a 22 x 22 mm coverslip at 45° angle with one hand at the side of glycerol drop on the slide. Gradually lower it on the glycerol drop ensuring that no air bubbles are trapped. Wipe away the excess mounting medium with filter/tissue paper. Care should be taken to prevent the tissue from being pushed out of the coverslip area.

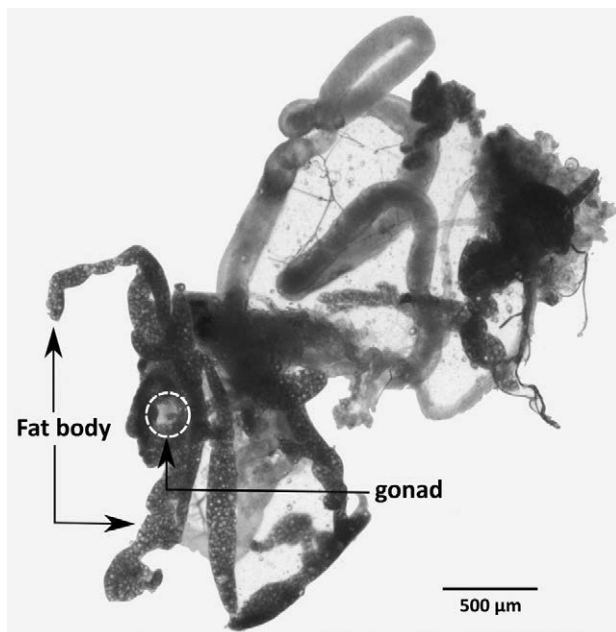


Fig. 7. Internal organs (unstained), including fat bodies, gut etc., of a dissected third instar larva. The fat bodies contain large fat droplets (optically clear circular structures within the fatbody). The white encircled area indicates the gonad embedded in fat body.

Seal all sides of the coverslip with transparent nail polish and allow it to dry. Examine the preparations under microscope (microscope with phase contrast or differential interference contrast optics is better; however, gonads can also be seen under normal bright-field optics with less intense illumination and substantially closed sub-stage iris diaphragm).

Observations:

The gonads can be identified as globular structures comprised of smaller cells relative to bigger and translucent cells of the fat body. Testis is much larger with more cells than ovary (**Fig. 8 E and F**).

With experience, it is possible to identify the gonads in live larvae and thus distinguish male and female larvae even without dissection.

Questions for self-study and interpretation:

1. Are there any other distinguishing characteristics of sexual dimorphism in *Drosophila* larvae?
2. What could be the reason for the difference between the size of male and female larval gonads?
3. Which kind of studies may need prior sexing of *Drosophila* larvae?
4. Why study development of gonads in *Drosophila*?
5. The male and female reproductive systems include structures other than the internal gonads. Are these structures formed by cells within the larval gonads?

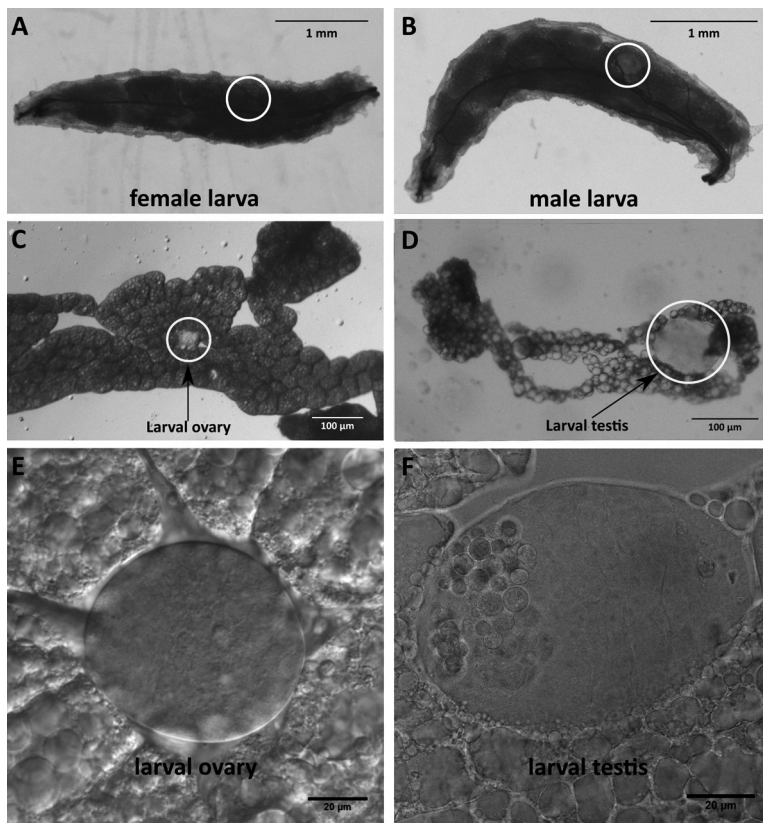


Fig. 8. Female (A) and Male (B) larvae with their gonads marked by the yellow circle. Larval gonads embedded in the fat body as seen during dissection (C and D). Differential interference contrast (DIC) microscope image of larval ovary (E) and bright field microscope image of larval testis (F).

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Chapter 29

Demonstration of the hormonal control of metamorphosis using *Drosophila* as a model

Leena Thorat*¹ (leenathorat@gmail.com) and Bimalendu B. Nath* (bbnath@gmail.com)

Department of Zoology, S.P. Pune University, Pune-411007, India

1. Current address: Department of Biology, York University, Toronto, ON M3J 1P3, Canada

* For correspondence

Recommended Level: PG/Research

Sub-discipline: Developmental Biology/ Physiology

Background:

More than a hundred years ago, the Polish biologist, Stefan Kopec discovered that hormones released from the anterior region (head) of insect larvae bring about changes in body form and structure, from immature to adult stage. He conducted a series of ligation experiments in gypsy moth larvae and concluded that pupation was induced by a 'circulating substance' that originated from the anterior portion and termed it the 'head factor'. Later, studies by Wigglesworth, Kuhn and Fukuda provided further insights on the role of insect hormones in metamorphosis (Karlson, 1996). Today we know that hormones produced and secreted by the brain and endocrine glands in the anterior part of the body play a major role in development, growth and metamorphosis of insects. These include the prothoracicotrophic hormone (PTTH), ecdysone and juvenile hormone. Insect growth and development is characterized by molting or ecdysis, a periodic process which involves shedding of the old exoskeleton and the secretion of a new exoskeleton (see [Chapter 1](#)). The hormone ecdysone is the major insect molting hormone, while the timing of ecdysis is influenced by other hormones and environmental cues. This protocol uses *Drosophila* larvae as a model to demonstrate the role of the hormonal components contributed by the anterior region in larval to pupal transformation.

Objective:

To study the hormonal control of metamorphosis in *Drosophila* based on the differences in developmental patterns in non-ligated versus ligated larvae.

Materials Required:

1. Healthy *Drosophila* larvae (~72 hr or 3-day old (late-second to early-third instar) larvae of a wild type strain reared at $23 \pm 1^\circ\text{C}$).
2. Tissue paper
3. Tap water
4. Two Petri dishes (100 mm diameter), half-filled with fresh *Drosophila* medium
5. Blunt stainless steel forceps
6. Fine paint brush (Camlin India, series 66, size 2)
7. Fine silk thread
8. Dissecting microscope (preferably Stereo-zoom binocular microscope)
9. Parafilm strips or any transparent covers such as plastic wrap/cling wrap to cover the Petri dish
10. Dissection needle or a similar tool to make holes into the parafilm or plastic film covering the Petri dish
11. Biological Oxygen Demand (B.O.D.) incubator or a room maintained at $23 \pm 1^\circ\text{C}$

Experimental Details:

1. Collect early-third instar larvae from the Petri dish (see [Chapter 14](#)). Using a fine brush and water, clean off the food particles attached on body surface. Blot the larvae on a tissue paper.
2. Pick one larva at a time for ligation. Make sure the other larvae do not dry out on the tissue paper. For this, keep the paper moist with tap water.
3. Gently handle the larva with a fine brush or blunt forceps and place it on one Petri dish containing the medium. Take a piece of a silk thread (approximately 15 cm long) and prepare a loop. Using blunt forceps insert the larva in the loop with the posterior end facing towards the investigator (**Fig. 1a**). This step can be carried out under a dissection microscope. Keeping the Petri dish containing larvae on ice for ~5-7 min reduces larval movements and helps in performing the ligation step without harming the larvae.
4. Once the larva is positioned as desired, pull apart both the ends of the thread in order to wrap the thread firmly but gently around the anterior part of the larva, just behind the head region (**Fig. 1b**). Make sure not to pull away forcefully, else it will lead to choking/death of the larva.

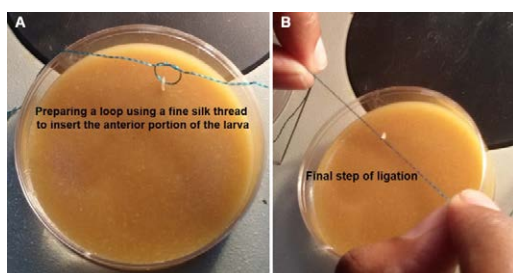


Fig. 1. Steps involved in ligation of *Drosophila* larvae. A. Preparing a loop and positioning the larva (described in step #3 above), **B.** Pulling apart both the ends of the thread and wrapping the thread around the larva (described in step #4 above). Images not to scale.

5. Cut off the extra portion of the thread and release the larva on a fresh Petri dish with food. Ligate a total of ten larvae and label the Petri dish as 'experimental'.
6. On the other food dish, release ten non-ligated larvae and label the dish as 'control'.
7. Observe the ligated larvae, through the transparent cover, for the next 30 min. The larvae should be able to move around. In case the movement ceases, it is likely that the larvae have suffered damage or are dead during the ligation procedure. Discard such larvae.
8. Keep the dishes inside a B.O.D. incubator or in a temperature controlled room. Record the time and date.
9. Over the next few days, monitor the dishes every 24 hr and note down the changes in control and experimental larvae.

Observations:

1) Control (non-ligated larvae): On post-ligation day 3 (± 0.5 d), the non-ligated larvae should appear as fully developed pupae on surface of the medium. 4-5 days after pupation, adults will be ready to, or would have already emerged from the pupal cases.

2) Experimental (ligated larvae): On post-ligation day 3 (± 0.5 d), the ligated larvae should still be seen in the larval stage (**Fig. 2**). These larvae are expected to be less active and may become motion-less, indicating that the larvae have died without undergoing pupation. In any case, no pupae and adults can be found even if a few larvae survive beyond 5-6 days post-ligation since they die without entering the pupal stage.



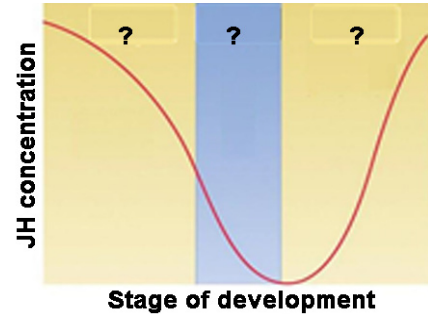
Fig. 2. A ligated larva which failed to pupate.

3) Interpretation: Due to ligation, the ecdysone hormone, secreted from the anterior region could not circulate through the body to reach all the target tissues and organs to initiate the developmental events that lead to larval to pupal transformation.

Questions for self-study and interpretation:

1. Which organs in *Drosophila* are responsible for the synthesis and secretion of PTTH, ecdysone and juvenile hormone? What are the roles of these hormones in molting and metamorphosis?

2. The adjacent figure shows levels of the juvenile hormone (JH concentration) during the various stages of development in an insect. Replace question marks in the figure with the appropriate developmental stage (larva/pupa/adult) corresponding with the rise and fall of the JH levels?



3. What results would one expect if ligation was performed using very late third instar larvae?

4. What results would you expect if the ligation was performed around the middle region of larval body?

Additional information:

Drosophila melanogaster undergoes two larval molts (see [Chapter 1](#)) which are characterized by an increase in ecdysone levels: (i) first molt indicates the end of the first instar and the beginning of the second instar stage, and (ii) second molt occurs at the transition from the second to the third instar. Since the second molt occurs at 72 hr of larval development, it is important to select 72 hr old larvae (after egg collection) to ensure that the larvae have entered the third instar stage. Normally, they would begin the pupariation molt (formation of the puparium/ pupal case) about 48 hr after (see [Chapter 14](#)). This molt is also triggered by a rise in ecdysone levels. Thus, age of the experimental larvae is crucial for success of this experiment which aims to track the third instar-pupal development in the context of the role of ecdysone released from the anterior part of the larval body.

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Chapter 30

Study of mitotic chromosomes of *Drosophila melanogaster*

J. K. Roy* (jagatkroy@gmail.com) and S. C. Lakhota (lakhota@bhu.ac.in)

Cytogenetics Laboratory, Department of Zoology, Banaras Hindu University, Varanasi 221005, India

* For correspondence

Recommended Level: UG /PG/Research

Sub-discipline: Cytogenetics/Genetics/Cell Biology

Background:

Walther Flemming, a German anatomist, described the cell division process for the first time in 1882 and named it “mitosis” since he found paired threads to be characteristic feature of dividing cell nucleus and the Greek word for paired threads is ‘*mitos*’. All multicellular organisms grow in size primarily by increase in cell numbers by the mitotic division process. Following the establishment of chromosomal basis of Mendelian inheritance, the discipline of Cytogenetics came into being. Studies on mitotic chromosomes have played pivotal roles in understanding evolution (changes in karyotypes through diverse chromosome rearrangements and modifications in heterochromatin regions in related species), role of chromosome rearrangements in diverse human diseases (including cancer), cell cycle regulation, organization of chromatin and regulation of its functions, including epigenetic changes (Swedlow and Hirano, 2003; Yanagida, 2014).

One of the advantages offered by *Drosophila melanogaster* as a model organism for genetic studies is its low diploid chromosome number (2N), which is 8, with a pair of sex-chromosomes (XX in female and XY in male) and 3 pairs of autosomes, comprising 2 pairs of large metacentric chromosomes and a pair of very small ‘dot’ chromosomes. Although *Drosophila* is better known for its polytene chromosomes (see [Chapter 31](#)), the mitotic chromosomes have also been widely used for diverse studies, including evolution of karyotype and heterochromatin in different species (Pimpinelli *et al.*, 1976) and effects of DNA-binding drugs on chromosome structure (Lakhota and Roy, 1983).

A feature associated with the holometabolous mode of development in *Drosophila* is that cells in the larvae are of two kinds, the larval and the imaginal cells. Most larval cells undergo varying degrees of endoreplication cycles while the imaginal cells, that give rise to adult structures during the pupal metamorphosis, remain undifferentiated and divide mitotically during most of the larval life. Actively dividing cells in the 3rd

instar larvae are found in the imaginal discs (see **Chapter 18**) and brain ganglia. The brain ganglia contain actively dividing neuroblast and neuron mother cells and thus provide a good material for studying mitotic chromosomes. Chromosomes in mitotically dividing somatic cells of *Drosophila* and many other dipteran insects display somatic pairing of homologs. Therefore, in metaphase chromosome spreads, the homologous chromosomes are nearly always seen adjacent to each other.

Conventionally, the mitotic chromosome spreads from *Drosophila* tissues were obtained by the squash method. Adoption of the air-dry method of chromosome preparation, commonly practiced for mammalian tissues, has provided a simple and convenient procedure to examine well-spread metaphase chromosomes from fly tissues.

Objective: To prepare metaphase chromosome spreads from brain ganglia of third instar larva

Materials Required:

A. Healthy late third instar *Drosophila* larvae reared at 24°C without crowding on food with additional yeast-supplement.

B. Equipment and Other Supplies

i. Stereo-binocular and Bright-field microscope (**optional:** phase-contrast optics)

ii. Clean slides and coverslips (22 mm²)

Note: It is essential that slides are totally free of any dust-particles, fibers and greasy material. A simple way to achieve this is to store fresh (or detergent-cleaned, if desired) slides in 90% ethanol in suitable containers and wipe them dry, immediately before use, with a clean soft cloth and stored in a covered dust-free box till used.

iii. Fine-tipped forceps, fine dissecting needles and soft brush for handling larvae.

iv. Glass droppers or other small volume liquid dispensing system.

v. Heating plate set at 60°C

C. Solutions

i. Poels' Salt Solution (pH 6.8):

a. NaCl	86 mg
b. KCl	313 mg
c. CaCl ₂ .2H ₂ O	116 mg
d. NaH ₂ PO ₄ .2H ₂ O	88 mg
e. KHCO ₃	18 mg
f. MgSO ₄ .7H ₂ O	513 mg
g. Dist. H ₂ O	100 mL

Adjust pH to 6.8 with 1M NaOH and filter.

ii. Hypotonic solution: 0.67% Tri-sodium citrate

iii. Colchicine stock solution: (1 $\mu\text{g}/\text{mL}$ in distilled water; can be stored in refrigerator for long time)

iv. Aceto-Methanol 1:3 (freshly prepared)

v. 60% Glacial acetic acid (freshly prepared)

vi. Giemsa stain:

Stock solution

Giemsa Powder 380 mg

Methanol 25 mL

Glycerol 25 mL

Leave overnight at 37°C. Filter the stain and store.

vii. Giemsa water

0.2 M Na_2HPO_4 80 mL

Distilled water 800 mL

Adjust pH to 6.8 with 0.1 M Citric acid (roughly 24 mL) and then make up the volume to 1 L

Note: Tap water, with pH 7 can also be used in place of Giemsa water. However, distilled water, which generally has more acidic pH is not recommended for making working solution of Giemsa or for washing the slides after staining.

Working Giemsa stain (to be prepared fresh just before use)

Stock solution 2.5 mL

Methanol 1.5 mL

Giemsa/Tap water 50.0 mL

Note: Commercially available Giemsa stain solution may also be used.

Experimental Details:

1. Collect late third instar larvae (about 5 day old if grown at 24°C) from a healthy culture (late third instar larvae crawl out of the food medium and move actively on surface of food or food-free surface of the Petri dish/vial/bottle), wash with water to remove adhering food particles and transfer to a clean slide in a drop of Poels' salt solution (alternatively, 1X PBS or simple Ringer's saline [0.67% NaCl] can also be used).

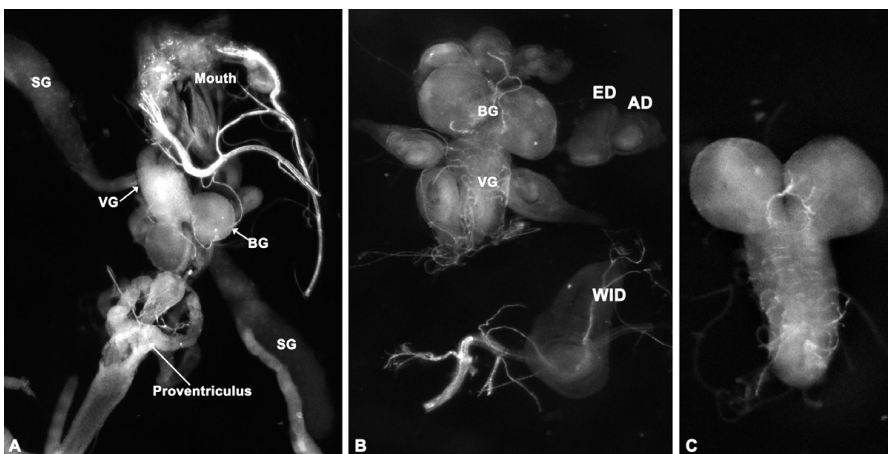


Fig. 1. **A.** Internal organs pulled out of larval cuticle. **B.** Brain and imaginal discs etc. after removal of the other larval organs and cuticle. **C.** Brain and ventral ganglia after cleaning of other tissues. **AD** = antennal disc; **BG** = cerebral ganglia; **ED** = eye disc; **SG** = salivary glands; **VG** = ventral ganglia; **WID** = wing disc; Mouth and Proventriculus are also marked. Images not to same scale.

2. Keep one pair of fine forceps and/or dissecting needle at the middle of the larval body and the other at the mouth region. Pull them in opposite directions so that all internal organs are forced out of the larval cuticle (**Fig. 1A**). Brain ganglia are seen as a whitish translucent bilobed structure located near the salivary glands (**Fig. 1A**). Using fine dissecting needles, remove the other adhering tissues (**Fig. 1B**) as much as possible without damaging the brain ganglia (**Fig. 1C**).
3. Using tips of the dissecting needles, transfer several (~20) brain ganglia to 1 mL of Poels' salt solution (or 1X PBS) in a cavity block, add 1 μ L Colchicine stock solution (to obtain final conc of 1 μ g/mL); cover the cavity block and incubate at room temperature (~24°C) for 1 hr.
4. Remove Poels' solution, add 1 mL of hypotonic solution in the same cavity block, cover with the lid and leave for 30 min at room temperature (24°C; if the room temperature is higher, the incubation time in hypotonic medium would need to be reduced accordingly).
5. Remove hypotonic solution and add a few drops of fixative holding the cavity block in a slanting position such that fixative washes out any trace of remaining hypotonic solution. Now immediately add 1 mL of fixative, cover with the lid and fix the tissue for 30 min at room temperature.
6. Pre-warm the clean slides on a hot plate at 60°C, transfer one brain ganglia complex in a small drop of 60% acetic acid on the warm slide, leave for 1 min for the cells to get loosened. Using fine dissecting needles or forceps, tease/ the tissue for about 1 min on slide in the acetic acid drop so that the loosened cells get dissociated from each other. **Note:** The upper surface of the slide may be marked with a diamond marker so that after the preparation is completed, the upper and lower faces of the slide can be easily identified. This would help in preventing accidental rubbing off of cells while cleaning the slide at any stage during its processing for staining, mounting etc. Marking with a marker pen may not stay during staining and washing steps.
7. Add the aceto-methanol fixative drop-by drop with the help of a Pasteur pipette/microtip on all sides of the acetic acid drop in such a way that the suspended cells in acetic acid drop come in contact with the fixative and get stuck to the slide. Let the slide air dry. The dissociated cells will form a ring, which can be easily seen even with naked eye.
8. Stain the slide with Giemsa stain for 5-10 min. Wash the slides well in Giemsa water or tap water to completely remove the excess stain and then leave them vertically to air dry. A blue/magenta coloured ring of cells on the slide can be seen with naked eye.
9. Check the staining under a microscope. When completely dried, mount a 18 or 22 mm² cover glass with a small drop of DPX. These permanent slides can be stored for years.

Observations:

1. Scan the slide under low power objective of microscope to locate the ring of stained cells. Identify interphase nuclei and metaphase plates under high power objective.
2. Examine and draw the metaphase chromosome spreads using a 40X dry or higher

power oil immersion objective to identify each chromosome (Fig. 2). The chromosomes 2 and 3 are differentiated on the basis of their relative sizes, with the chromosome 3 being a little larger than chromosome 2.

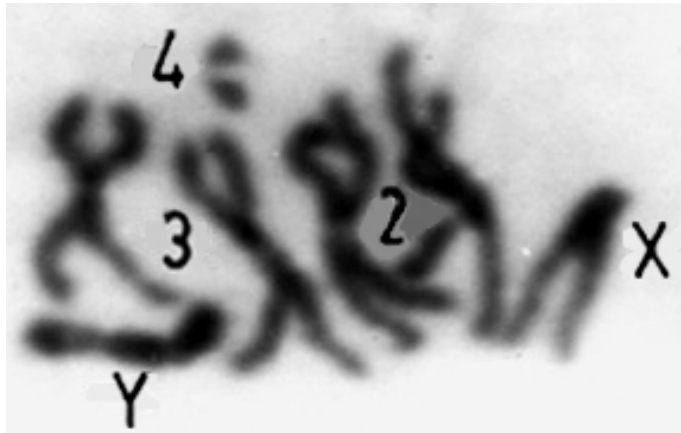


Fig. 2. A metaphase plate from brain ganglia of late third instar male larva of *D. melanogaster* stained with Giemsa. Note the nearly acrocentric (with very short right arm) X-chromosome, large sub-metacentric Y-chromosome, and three pairs of autosomes (two pairs of long metacentric chromosomes 2 and 3 and a pair of dot like chromosome 4)

Questions for self-study and interpretation:

1. Compare the locations of the two chromosomes of homologous pairs in mitotic metaphase plates from somatic cells of mammals (or plant) and *Drosophila*. Is there any strikingly different feature, which is seen only in metaphase plates of *Drosophila*?
2. Why do mitotic chromosomes differ in size from polytene chromosomes in the same individual?
3. Would the metaphase chromosome spreads from tumours in *Drosophila* mutants appear similar to those from wild type?
4. How can heterochromatic regions be identified in mitotic chromosomes?
5. Would different species of *Drosophila* display the same karyotype?

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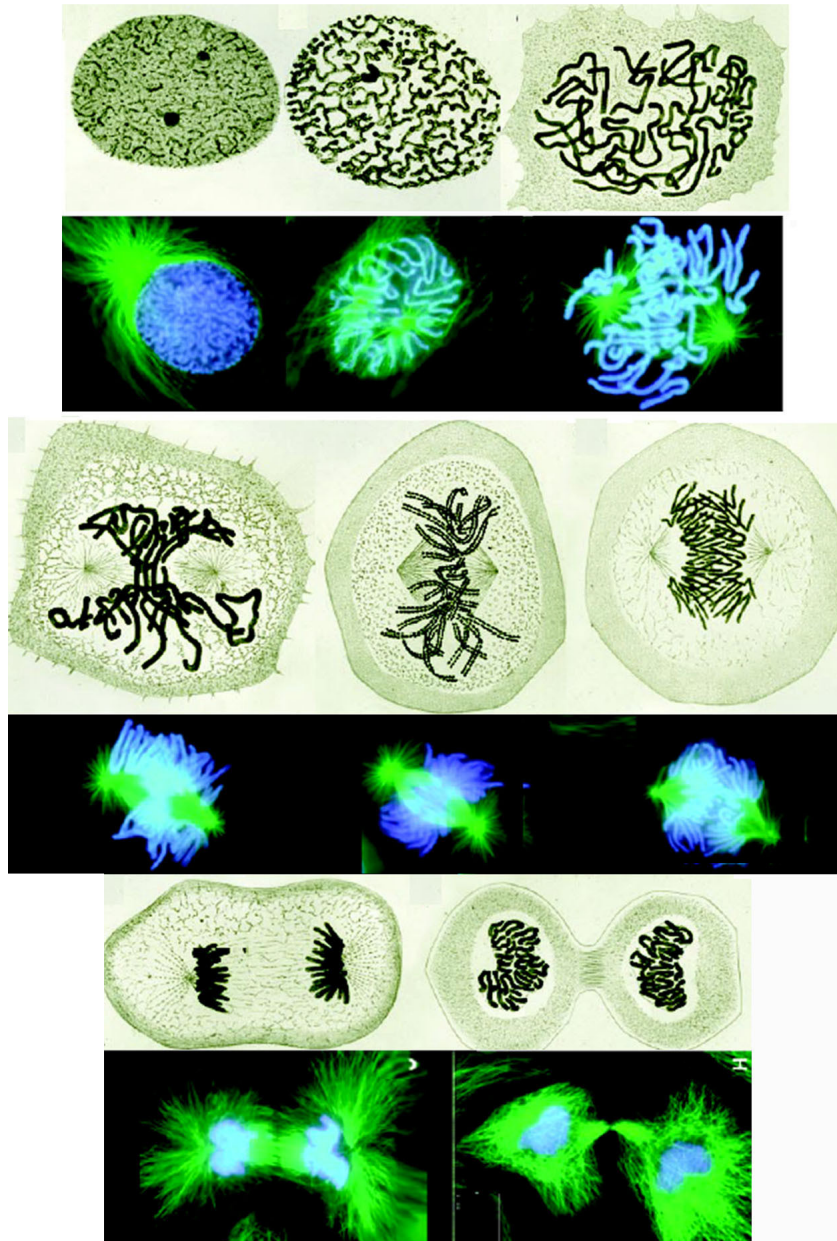
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Power of Observation & Beauty of Hand-Drawings

Mitosis - Then (W. Fleming 1882) & Now



(Flemming's hand-drawings of mitosis in newt cells in 1882 and contemporary confocal images; adapted from: Rieder, C. L. and Khodjakov, A. 2003 Mitosis through the microscope: advances in seeing inside live dividing cells. *Science*. 300: 91-96
<https://science.sciencemag.org/content/300/5616/91>)

Chapter 31

Study of polytene chromosomes of *Drosophila melanogaster*

J. K. Roy (jagatkroy@gmail.com) and S. C. Lakhotia* (lakhotia@bhu.ac.in) Cytogenetics Laboratory, Department of Zoology, Banaras Hindu University, Varanasi 221005, India

* For correspondence

Recommended Level: UG/PG/Research

Sub-discipline: Genetics/Cell Biology/Developmental Biology/Molecular Biology

Background:

Polytene chromosomes have been widely used in a variety of genetic, cyto-genetic and molecular studies on gene expression (Zhimulev *et al.*, 2009). These chromosomes remain in permanent interphase but due to repeated endoreduplication cycles and tight lateral association of all the daughter chromatids, each chromosome becomes thicker and distinctly visible as a cable-like structure with alternating dark and light regions, the bands and interbands, respectively. Polytene chromosomes are most commonly found in dipteran insects like *Chironomus*, *Rhynchosciara*, mosquitoes, *Drosophila*, etc. In addition, they are also seen in certain other insects, macronucleus of some ciliates and in certain plant tissues.

Salivary glands of late third instar *Drosophila* larvae provide excellent polytene chromosome spreads due to a high level of polyteny achieved by many cells in this tissue. These were first recognized as ‘giant’ polytene chromosomes by T. S. Painter in 1934 (see **Fig. 1**).

With increasing larval age, the level of polyteny of chromosomes in salivary gland cells increases. Each salivary gland has about 120 cells. Of these, the more posterior cells provide better chromosome spreads because by the late third instar stage they have undergone 8 or 9 rounds of endoreplication while the anterior cells endoreplicate fewer times. All the resulting chromatids (2^8 or 2^9 chromatid fibrils) maintain their lateral association in such a way that their differentially coiled regions remain in tight register along the full length of each chromosome: this results in the characteristic banding pattern of dark band regions (with more densely packed chromatin) alternating with lighter interbands (less packed chromatin). The most significant feature of polytene chromosomes is that these chromosomes permit visualization (Lakhotia, 1997) of transcriptionally active genes under light microscope in the form of “puffs” (see **Chapter 46**). The chromatin fibrils in a puff open up and the newly synthesized RNA accumulates at active gene sites resulting in the enlarged

diameter and lighter staining of puff regions compared to the transcriptionally inactive regions (bands). The pericentromeric heterochromatic regions of different chromosomes remain in close association with each other and together they form the chromocentre. The euchromatic arms of different chromosomes appear to radiate from this common chromocentre (in some dipterans, like *Chironomus*, chromocentre formation does not occur in polytene cells). A major part of the DNA in chromocentre region does not participate in endoreplication, i.e., it remains under-replicated.

Mitotically dividing cells of *Drosophila melanogaster* females show a pair of acrocentric X-chromosome, two pairs of metacentric chromosomes (chromosomes 2 and 3) and a pair of very small dot-like 4th chromosomes. Cells from males show one acrocentric X and a large sub-metacentric heterochromatic Y-chromosome besides the three pairs of autosomes. The Y-chromosome, like the centromeric heterochromatic regions of other chromosomes, remains under-replicated and buried within the chromocentre mass.

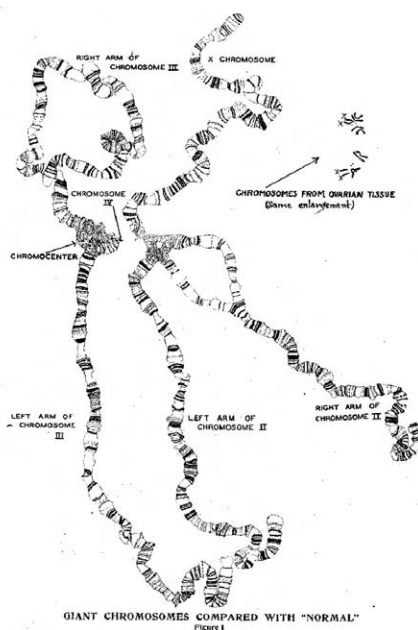


Fig. 1. T. S. Painter's camera-lucida (https://en.wikipedia.org/wiki/Camera_lucida) drawing (1934) of polytene chromosomes of *Drosophila melanogaster* in the classical paper entitled "Salivary gland chromosomes and the attack on the gene" (Journal of Heredity. 25: 465-476). For comparison of sizes of the 'giant' and "normal" mitotic chromosomes, drawings of metaphase chromosomes from male and female *Drosophila melanogaster* are also shown in the upper right corner. A region at the chromocentre-end of the right arm of polytene chromosome 2 shows asynapsis of the homologous arms (the figure is reproduced with permission of the Oxford University Press)

As in other somatic cells of *Drosophila* and many other diptera, the homologous chromosomes in polytene cells also remain tightly synapsed. Sometimes, however, the homologs can appear asynapsed (**Fig. 1**) for variable lengths either because of differences between the homologs due to small or large chromosome rearrangements or other unknown reasons. As a result of the normally extensive synapsis of homologous chromosomes, a wild type polytene nucleus of *D melanogaster* typically shows a common chromocentre (formed by centromeric and pericentromeric heterochromatic regions of all chromosomes) from which 5 long and a very short euchromatic banded chromosome arms radiate out. The 5 long arms represent the acrocentric X-chromosome, left and right arms of metacentric chromosomes 2 and 3, respectively, while the very short arm is formed by the very small 4th chromosome (also called 'dot' chromosome due to its appearance in mitotic nuclei, see **Chapter 30**). Each chromosome arm has a characteristic banding pattern due to which each region of every chromosome can be very easily distinguished and identified. Every band has been given a specific number identity: for *D melanogaster*, the polytene chromosome maps prepared by C. B. Bridges and P. N. Bridges in 1930s and 1940s are followed (**Fig. 2**) to identify each of the approximately 5000 bands seen in a salivary gland

polytene nucleus (<https://flybase.org/maps/chromosomes/maps>). Constancy of the banding pattern and the availability of diverse chromosome rearrangements facilitated correlation between genetic and cytological maps since the early 1930s. Thus studies on polytene chromosomes provided the first available ‘physical’ maps of genes on chromosomes. More recently, the polytene chromosome bands of *Drosophila melanogaster* have been mapped with reference to the genomic sequence coordinates (**Fig. 2**).

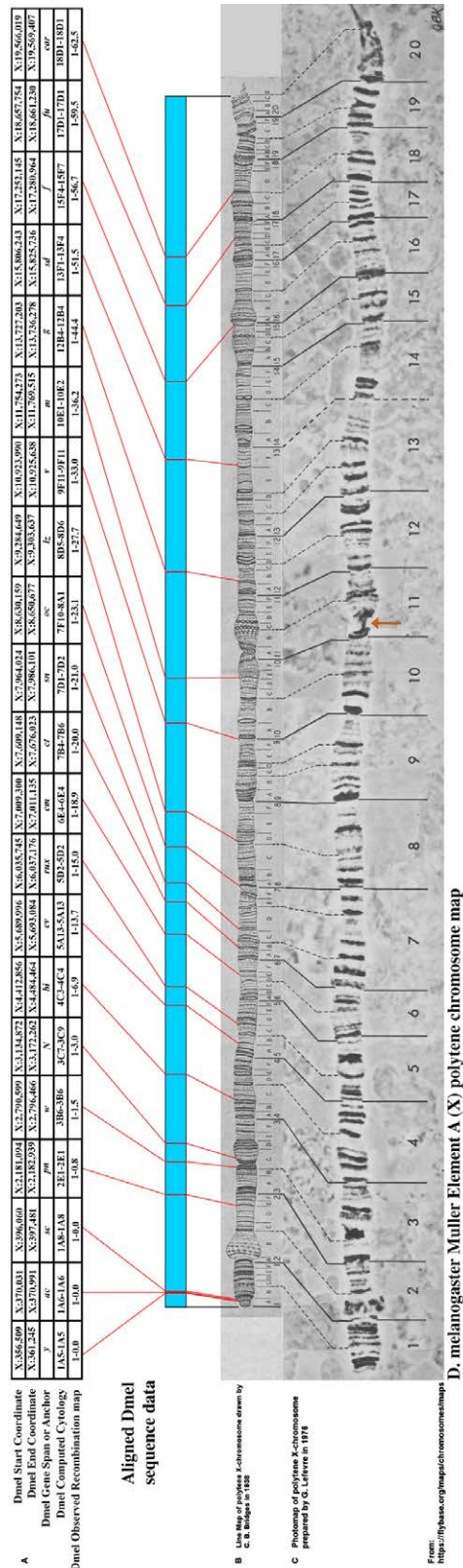


Fig. 2. Correlation of cytological maps of polytene chromosomes of *Drosophila melanogaster* prepared by C. B. Bridges in 1930s (B) with genetic and genomic sequence maps (A) of the X-chromosome or Element A, shown here as an example. Bridges’ hand-drawings used camera-lucida to draw morphology of every band and inter-band regions that could be seen in a given chromosome region in multiple preparations. Detailed drawings of small regions of each chromosome arm were assembled into composite chromosome maps (see panel B). In Bridges’ scheme of cytological maps, each of the long chromosome arms was divided into 20 divisions (1-20 on X-chromosome, 21-40 on left arm of chromosome 2 (2 L), 41-60 on right arm of chromosome 2 (2 R), 51-80 on left arm of chromosome 3 (3 L), 81-100 on right arm of chromosome 3 (3 R) and 2 divisions (101-102) on the tiny chromosome 4; each division was further sub-divided into 6 sections, A-F and each visible band was serially numbered as 1, 2, 3 and so on. Accordingly, each visible band has a definite cytological coordinate. For example, the white locus is mapped to band 3C6, meaning the white gene is physically located at the visible band number 6 in section C of division 3 of X-chromosome. With the availability of extensive genomic sequence data, the genomic base sequence coordinates for each band and the interband have also been now defined (A). Later in 1970s, G. Lefevre (1976) ‘stitched’ micro-photographic images of small regions of each well-spread and flattened chromosome to provide composite photo-maps of all the polytene chromosome arms of *D. melanogaster* (C). The thin red connecting lines between A-C panels indicate the location of a given gene at the given band seen in polytene chromosomes. A red arrow in C indicates the position of a characteristic constriction in X-chromosome (image from <https://flybase.org/maps/chromosomes/maps>).

It is interesting to note that assembly of chromosome maps using transmission electron microscopic images of squashed salivary gland polytene chromosomes by V. Sorsa and M. Sorsa's group in 1960s did not reveal even a single new band that was not already drawn by C. B. Bridges and P. N. Bridges prepared in late 1930s and early 1940s using the then available bright-field light microscopes. Cytological maps of polytene chromosomes of several other species of *Drosophila* and other Diptera are now available, but none of them match the detail and accuracy seen in the drawings of Bridges and Bridges. The digital availability of these standard maps (<https://flybase.org/maps/chromosomes/maps>) makes it greatly convenient for any researcher interested in studies on gene organization and expression to use the remarkably detailed chromosome maps.

As the salivary glands are histolysed after pupation, polytene chromosome spreads can be obtained from salivary glands of larvae only. Salivary glands of adult flies do not contain polytene cells. For cytological studies, temporary preparations of polytene chromosomes are made by the classical squashing technique.

Objective:

To prepare polytene chromosome spreads from salivary glands of late third instar larvae of *Drosophila melanogaster* by squashing method and study their features.

Materials Required:

A. Larvae: Healthy late third instar wild type *Drosophila melanogaster* larvae grown without crowding on food with additional yeast-supplement at 24°C.

B. Equipment and Other Supplies

- i. Stereo-binocular and Bright-field microscopes (optional: phase-contrast optics)
- ii. Slides and coverslips (22 mm²): To obtain good squash preparations, it is essential that the slides and coverslips are totally free of any dust-particles, fibers and greasy material.
- iii. Fine-tipped forceps, fine dissection needles and soft brush for handling larvae
- iv. Filter/blotting paper
- v. Plastic/glass droppers or Micropipette and tips

C. Solutions

- i. Poels' Salt Solution (pH 6.8)
 - a. NaCl 86 mg
 - b. KCl 313 mg
 - c. CaCl₂.2H₂O 116 mg
 - d. NaH₂PO₄.2H₂O 88 mg
 - e. KHCO₃ 18 mg
 - f. MgSO₄.7H₂O 513 mg
 - g. Dist. H₂O 100 mLAdjust pH to 6.8 with 1 M NaOH and filter.

- ii. Aceto-Methanol 1:3 (freshly prepared)
- iii. 50% Glacial acetic acid
- iv. Aceto-Orcein (2%) stain: Dissolve 2 gm Orcein in 100 mL of 50% Acetic acid by boiling for 30 min under a reflux condenser. Filter when cool. The prepared stain can be used for many months but it should be periodically filtered before use to avoid stain particles.
- v. Aceto-Carmine (2%) stain: Dissolve 2 gm Carmine powder in 100 mL of 50% Acetic acid by boiling for 2 hr under a reflux condenser. Filter when cool. The prepared stain can be used for many months but it should be periodically filtered before use to avoid stain particles.
- vi. Lacto-Aceto-Orcein (2%) (optional): Dissolve 2 gm Orcein powder in a solution containing 51 mL glacial acetic acid, 34 mL distilled water and 15 mL of 85% lactic acid by boiling for 2 hr on a very low flame in a flask fitted with a reflux condenser. Filter when cool. The prepared stain can be used for many months but it should be periodically filtered before use to avoid stain particles. **Note:** Though these stains are also commercially available, those properly made in the laboratory seem to work better.
- vii. Transparent nail polish

D. Cleaning of slides and coverslips

To obtain good squash preparations, it is essential that the slides and coverslips are totally free of any dust-particles, fibers and greasy material. A simple way to achieve this is to store the fresh (or soap-cleaned, if desired) slides and coverslips in 90% ethanol in suitable containers and wipe them dry, immediately before use, with a clean soft silken cloth. These are stored in a covered dust-free box till used.

Experimental Details:

1. Take late third instar larvae (about 5 day old if grown at 24°C) from a healthy culture (late third instar larvae crawl out of the food medium and move actively on surface of food or food-free surface of the Petri dish/ vial/ bottle), wash them free of adhering food particles etc with water and transfer to a clean slide in a drop of Poels' salt solution (1X PBS or simple Ringer's saline can also be used if the glands are fixed soon after their dissection).
2. Keep one pair of fine forceps and/or dissecting needle at the middle of the larval body and the other at the mouth region. Pull them in opposite directions so that all internal organs are forced out of the larval cuticle (**Fig. 3A**). Salivary glands are seen as a pair of whitish translucent elongated structures connected at their anterior ends with a common salivary duct. Remove fat bodies adhering to glands very carefully, (without damaging the glands) (**Fig. 3B** and **C**). Persistence of some small regions of fat bodies with salivary glands, however, would not seriously affect the quality of the final chromosome preparation.
3. Using tips of the dissecting needles, transfer the cleaned salivary glands (typically one pair) to a drop of Poels' salt solution on a clean slide. Drain out the salt solution by

keeping the slide in a slanting position without letting the glands dry. Continuing to keep the slide in slanting position, add drops (drop-by-drop) of freshly prepared fixative over a period of 45-60 sec. Wipe out excess fixative with a piece of filter/blotting paper.

4. Immediately, thereafter, keep the slide flat on the table and, before the remaining small volume of fixative dries, add two small drops of Aceto-Orcein and one drop of Aceto-Carmine stain and cover the slide with a watch glass to avoid drying of the stain solution. Let the glands be stained for about 5 min (only Aceto-Orcein stain can also be used).

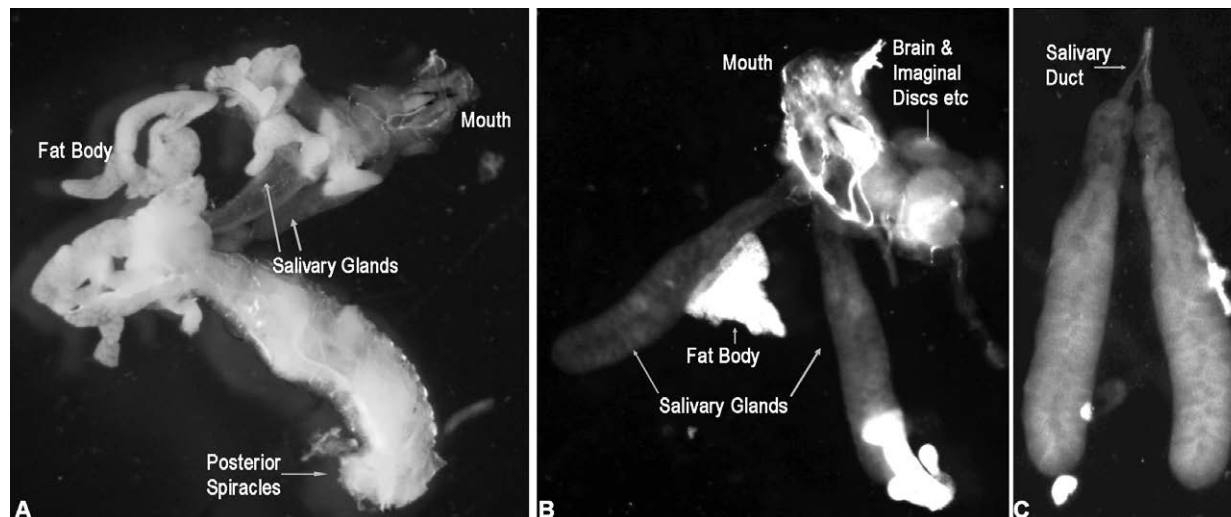


Fig. 3. Dissection of *Drosophila* larva to separate salivary glands. A. Internal organs pulled out of larval cuticle. B. Mouth parts, salivary glands, brain and imaginal discs etc. after removal of the other larval organs and cuticle. C. Pair of salivary glands with their common duct after cleaning of other organs and associated fat body.

5. Drain out the stain and add a few drops of 50% acetic acid to remove excess stain. Remove the coloured acetic acid solution by slanting the slide so that the acetic acid drains down in a waste-collection dish. Finally place a drop of fresh 50% acetic acid on the glands, which should appear brick-red in colour, and cover them with a clean 22 mm² coverslip.

6. For squashing, keep the slide with its coverslip between folds of a clean filter/blotting paper and lightly tap the paper immediately above the coverslip area either with the rubber-end of a pencil or with the blunt end of the needle-holder or with the needle tip (tapping breaks the cell and nuclear membranes and releases chromosomes free in cytoplasm; but too strong a tapping would break chromosomes in pieces), while holding the coverslip in position with thumb and a finger of one hand placed over the filter paper such that they press on two diagonal corners of the coverslip. After the ‘tapping’ continue to hold the coverslip in position over the slide and apply firm pressure with thumb of the other hand on the coverslip. This act of squashing spreads the polytene chromosome arms of a nucleus and makes them flat in one plane. The “optimum” thumb-pressure is learnt with experience.

7. After squashing, seal the coverslip with nail polish and observe under a microscope. **Note:** These preparations will be usable for a few days only; temporary preparations will last longer if after the 50% acetic acid washing step, a drop of Lacto-Aceto-Orcein is placed over the stained glands and then squashed as above).

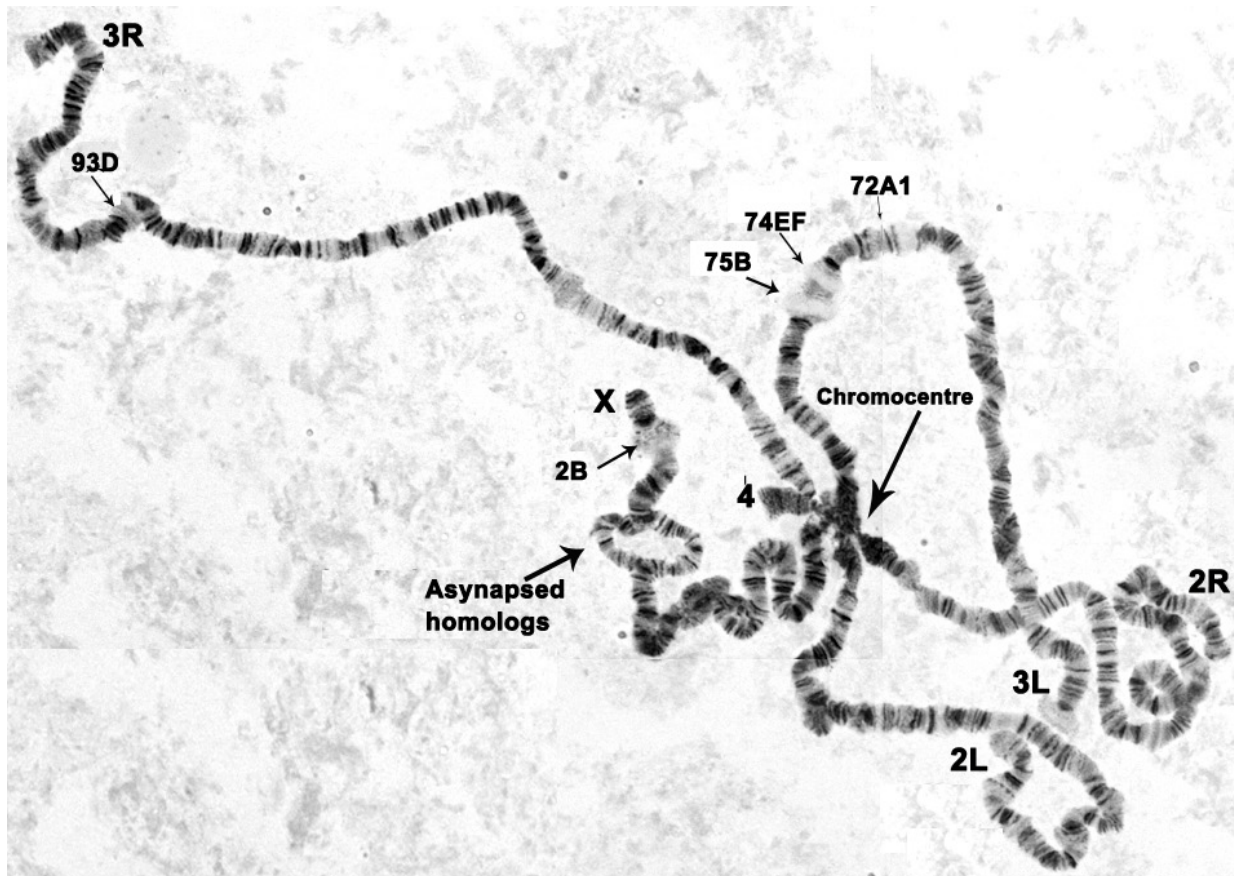


Fig. 4. A polytene nucleus from Aceto-Orcein stained squash preparation of salivary glands of late third instar female larva of *Drosophila melanogaster*. Note the five long chromosome arms (X, 2L, 2R, 3L, 3R) and the short chromosome 4 connected to a common chromocentre (large arrow). Small arrows indicate some of the developmental puffs identified by their locations on Bridges' map; a region showing asynapsis of the two X-homologs is also indicated. The chromosome arms are identified on basis of the standard maps available for these chromosomes (image provided by Dr. Rohit Kumar).

Observations:

1. Scan the slide under low power objective and count the number of polytene nuclei visible on the slide. How many of the nuclei are well spread like that represented in **Fig. 4**.
2. Examine the well spread polytene nuclei under 20 X or 40 X objective to count the number of long chromosome arms in each. Identify the chromocentre. Make a free-hand drawing of a well-spread nucleus and, using the landmarks in **Fig. 1, 2** and **4** as guide and the chromosome maps available at <https://flybase.org/maps/chromosomes/maps>, label each chromosome arm. Note bands of different shapes/sizes and staining intensity and some constrictions along the length of a chromosome arm.
3. Identify regions that are puffed.

4. Does one chromosome arm appear to be stained lighter than others in all polytene nuclei spreads from some glands? If yes, identify this chromosome. Could this differential staining be due to asynapsis of homologous chromosomes or related to sex of the larva from which the given salivary glands were taken out?

Troubleshooting:

Getting well-spread polytene chromosome squash preparation is an art and requires practice and care. Following are some of the commonly encountered problems.

1. Any damage to salivary glands by the dissecting needles during dissections must be avoided.
2. Excessive tapping may lead to breaking of chromosomes into small pieces and likewise, excessive pressure during squashing may cause over-stretching of some chromosome regions. In a well-spread preparation, all chromosome regions are equally flattened and thus remain in focus at one plane.
3. Any lateral movement of the coverslip relative to the slide during squashing results in chromosomes being “rolled” and fragmented: such chromosomes appear as small “rolls” of homogeneously stained material.
4. Imbalanced salt concentrations in the Ringers’ or the saline solution in which the glands are dissected or incubated may cause poor morphology of the chromosomes (the bands do not appear “crisp”) or most of the polytene nuclei remain rounded up with intact nuclear envelope.
5. Total duration of fixation of glands should not exceed 1 min since longer fixation makes chromosomes hard and difficult to spread.
6. Salivary glands from larvae that have not reached the late third instar stage or that are weak due to over-crowding or growth at higher temperature, do not provide well spread, thick and distinctly banded chromosomes because of their reduced levels of polyteny.
7. Any trace of grease on slides or coverslips hampers good spreading of chromosomes. The slides and coverslips must be kept in 95% alcohol for some time and should be wiped dry just before use with fresh clean and soft cloth - examination of the slide and coverslips under reflected light (from a lamp) will reveal if traces of oiliness or fibers etc. are present on their surface.
8. Any pieces of tracheae or fibers (e.g., from filter-paper) or stain particles or any cuticular structures (e.g., mouth parts) of larvae left on the slide will not permit good squashing. All these must be carefully removed before applying the coverslip.

Questions for self-study and interpretation:

1. How do polytene chromosomes differ from mitotic chromosomes?
2. Is there any relation between genes and the bands and interbands seen in polytene chromosomes?
3. How are polytene chromosomes useful in gene mapping and understanding the mechanism of gene activity?

4. *Chironomus* larvae show well developed polytene chromosomes in salivary glands as well as Malpighian tubules. Would you expect same set of puffs in polytene chromosomes from these two organs of a given *Chironomus* larva? Why or why not?

Further reading:

Lakhotia, S. C. (1997) Visualizing a concept - Methods of looking at active genes. *Resonance*. 2: 41-49. <https://link.springer.com/content/pdf/10.1007/BF02835000.pdf>.

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Painter, T. S. (1934) Salivary gland chromosomes and the attack on the gene. *J. Heredity*. 25: 465-476, <https://doi.org/10.1093/oxfordjournals.jhered.a103862>.

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Polytene Chromosomes & Highway to the Lair of the Gene

With these four discoveries before us (i. e., constant and distinctive patterns, somatic synapsis, the behavior of active and inactive regions and the separation of the arms of the large autosomes) it was clear that we had within our grasp the material of which everyone had been dreaming. We found ourselves out of the woods and upon a plainly marked highway with by-paths stretching in every direction. It was clear that the highway led to the lair of the gene.

(In: Painter, T. S. 1934. Salivary gland chromosomes and the attack on the gene. *J. Heredity*. 25: 465-476)

Chapter 32

Live observation of embryonic development of *Drosophila*

R. Ramya (ramya.r.mysore@gmail.com) and B. V. Shyamala* (shyamalabv@yahoo.com)

Institute of Excellence, Developmental Genetics lab, *Drosophila* Stock Centre, Department of Studies in Zoology, University of Mysore, Manasagangothri, Mysore, 570006

*For correspondence

Recommended Level: Post graduate/ Research

Sub-discipline: Developmental Biology

Background:

Drosophila embryos have been extensively used in recent decades as research material of choice to understand many basic aspects of developmental biology and their genetic regulation. Early embryologists used frog, sea urchin, newt or avian embryos as favourite experimental systems because of their large size, transparent vitelline membrane and accessibility for experimental manipulations. Though these studies were instrumental in the formulation of important fundamental concepts of development like fate maps, embryonic induction etc. understanding the molecular basis of these processes seemed infinitely complex. On the other hand, despite the rich genetic knowledge that was being generated, the eggs of *Drosophila* were thought to be obscure and difficult research objects, because of their small size, opaque and fragile nature, and with an unusual mode of development (Keller, 1996). *Drosophila* egg has two protective layers, the outer chorion and an inner vitelline membrane surrounding the “egg membrane”. Since the white chorion is opaque, the internal changes which occur during embryonic development are not visible from outside. The famous “Heidelberg Screening” for developmental mutations affecting embryonic development in *Drosophila*, carried out by Christine Nusslein-Volhard and Eric Wieschaus in 1980, kindled great interest in *Drosophila* embryogenesis. This screen led to isolation of nearly 20,000 mutants affecting about 5,000 genes with nearly 120 of them involved in early embryonic developmental events. Elucidation of genetic regulation of embryonic axis specification, segmentation etc. laid the foundation to our understanding of genetic and molecular regulations of developmental program. This study revolutionized the field of Developmental Biology and transformed *Drosophila* embryos into “Bridging objects” for integrating Genetics and Developmental Biology (Keller, 1996). Further innovations of more tools for genetic manipulations and molecular analysis (see [Chapters 33, 39](#)) have made *Drosophila* embryos favoured experimental systems, and the studies have paved way to understanding developmental regulators in other organisms including humans.

In the early studies special oils like Paraffin oil, Voltalef etc. were used to make the chorion transparent to facilitate examination of live embryos directly under a microscope without further processing (Wieshaus and Nusslein- Volhard, 1986). A simple method is presented here for collection, preparation and observation of live embryos of *Drosophila*. The method involves use of Halocarbon oil 700 which is a Polychlorotrifluoroethylene (PCTFE), chemically the same as Voltalef oil used by Wieschaus and Nusslein-Volhard (1986). Halocarbon oil is chemically inert, non-inflammable and has low viscosity. Its refractive index is similar to Glycerol and it has good optical properties. Most important property for the live embryo study is its ability to penetrate through the chorion and make it transparent. At the same time, being permeable to oxygen, it facilitates oxygen supply to the developing embryo. Halocarbon oil also prevents the embryo from desiccation during observation. Live embryos covered with Halocarbon oil and maintained under standard *Drosophila* culture conditions complete their development and hatch out as normal larvae. Some of the experimental conditions which require examination of live embryos of *Drosophila* are:

1. Study of morphogenetic changes in live embryos in real time under different genetic and/or environmental conditions.
2. Since timed collections can also have some variations in their developmental progress, morphological identification of eggs of the exact desired developmental stage is very useful for studies on gene expression or other experimental manipulations.
3. Selection of embryos of appropriate stage for microinjection of DNA to generate transgenic strains of *Drosophila*.
4. For live imaging of embryonic development with specific fluorescence tagged cellular markers.

Objective:

To collect, prepare and examine developing live embryos of *Drosophila*.

Materials required:

A. Fly: *Drosophila melanogaster* wild type /mutant strains or other species of interest.

B. Chemicals and other supplies:

- i. 2% Sucrose agar medium.
- ii. 50% v/v bleach (commercial Sodium hypochlorite).
- iii. Halocarbon oil-700 (Sigma Aldrich or other suppliers).
- iv. Baker's Yeast.
- v. Small sized transparent plastic bottle/ any other appropriate plastic container for use as egg collection chamber.
- vi. Petri plates of the same diameter as that of the mouth of the egg collection chamber.
- vii. Embryo strainer (fabricated as described below).

- viii. Fine hair brush.
- ix. Insulation/ Transparent sticky tape.
- x. Stereo-binocular and compound microscopes.

Experimental details:

A. Preparation of 2% Sucrose Agar plates:

- i. Add 2 gm of Sucrose and 2 gm of Agar (bacteriological grade) to 100 mL of boiling water. Stir well until the agar melts into solution.
- ii. Remove the beaker from heater and let it cool to $\sim 55^{\circ}\text{C}$. The molten medium is poured into Petri dishes and allowed to cool and solidify. The prepared agar plates can be stored in a plastic bag at 4°C for a few days.

B. Preparation of Yeast paste: Add water to a small amount of dry Baker's yeast to bring it to a consistency of thick paste and allow it to ferment for a few hours in a closed container.

C. Flies for embryo collection: Keep 4-6 days old flies in a fresh food bottle with additional dry yeast added for a day prior to egg collection. **Note:** A major fraction of the flies being transferred for embryo collection should be females. Feeding on yeast-supplemented food enhances egg-laying by females.

D. Setting up the embryo collection cups (Fig. 1): Modified from Delcour (1969).

- i. Take a transparent plastic container whose mouth matches the size of the sucrose agar plates. Using a heated needle pierce a number of small holes (should not allow the flies to move out) on the wall of the container, to help air flow in and out of the container.
- ii. Bring the 2% Sucrose Agar plates to room temperature (if stored in refrigerator) and place a small dab of yeast paste in the centre of the plate (**Fig. 1A**).
- iii. Transfer the well-fed flies from culture bottle into the plastic container without anaesthetising them by inverting the food bottle on a funnel placed on the mouth of the container.
- iv. Tap the container so that flies fall down to the bottom, and cover the mouth of the container with sucrose agar plate, keeping the agar side facing inward. **Note:** Sizes of the plate and the mouth of the plastic container should perfectly match with each other so that no gap is left when the two are conjoined.
- v. Seal the plate and the rim of the collection chamber with a sticky tape (**Fig.1B**).
- vi. Keep the set up with agar plate down position, preferably in a dark place.
- vii. Allow the flies to lay eggs on the plate for required number of hours at desired temperature depending on the experimental specifications. **Note:** In order to get embryos of different stages of development in a single collection, egg-collection may be continued for 18-20 hr.

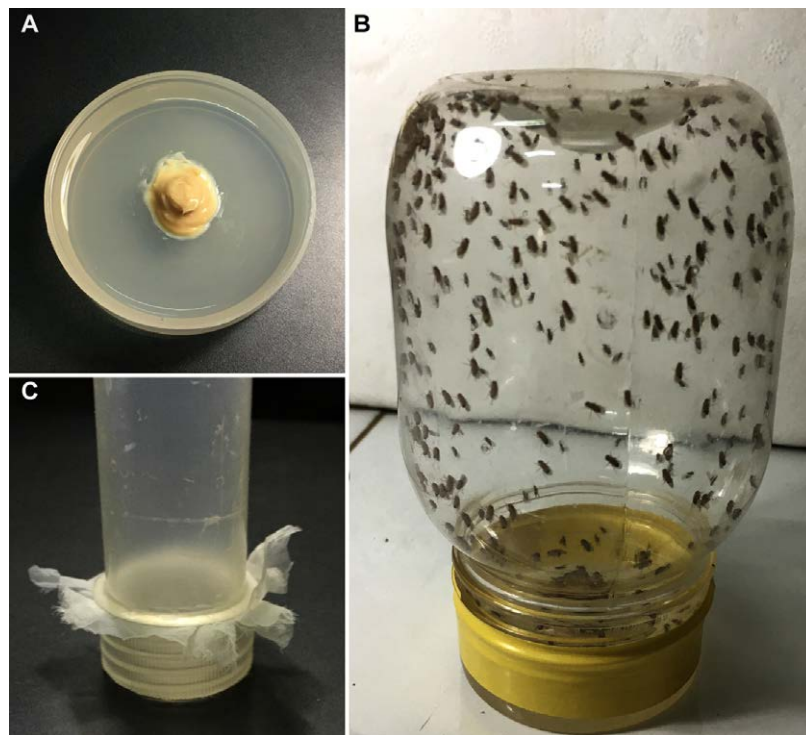


Fig. 1. Set up for egg collection and harvesting. A. Sucrose Agar plate with yeast paste. B. Collection chamber with flies, set on the sucrose agar plate. C. Embryo strainer (fabricated in lab).

E. Harvesting Embryos:

i. Preparation of Embryo strainer: Take a transparent plastic tube of 2.5 cm diameter and 6 cm long, which has a screw cap. Cut the base of the tube with a hot knife. Cut open the closed side of the lid to make it into a screwable ring. Place a clean ~6 cm² piece of a fine mesh cloth (with pore size smaller than fly eggs) on the open cap end of the tube and securely tighten the cut cap so that the mesh is snugly fixed between the tube and its cap (**Fig. 1C**).

ii. After the egg collection period is over, invert the embryo collection set up to bring the agar plate to the top. Tap the bottle to bring the flies to the bottom of the bottle. Quickly remove the plate with laid eggs. Immediately place a fresh plate and reseal the mouth of the bottle for collecting another batch of eggs. **Note:** if more eggs are not needed, the flies can be transferred to fresh food bottle.

iii. Add a small amount of tap water (should not overflow) into the embryo collection plate and gently release the embryos into water with the help of a fine hair brush.

iv. Transfer the water along with the embryos into the embryo strainer (**Fig. 1C**). Hold the strainer under running water from a squeeze bottle to wash off the yeast paste and agar particles.

Note: It is important to wash off the yeast completely; otherwise it will form a coat on the embryos and prevent the entry of Halocarbon oil.

E. Dechoriation: Treating the embryos with 50% v/v liquid bleach in water removes the chorion from the embryo leaving the embryos covered only with their vitelline membrane.

This step is optional for examination of live embryos. However, this is an essential step for experiments requiring penetration of the antibodies/ probes into the embryos.

- i. Place the strainer with washed embryos in a 50% solution of Sodium Hypochlorite for 2-3 min. (depending on the strength of the bleach), while monitoring under a stereobinocular microscope. Disappearance of the anterior egg filaments (arrow heads in **Fig. 2B**) marks a complete removal of the chorion layer (**Fig. 2. C-D**). It is important to make sure that embryos are not over treated with bleach.
- ii. Remove the egg-basket from the bleach solution and wash the embryos in the strainer thoroughly with running water to remove the residual bleach completely. Blot the excess water by placing the basket on a clean filter paper.

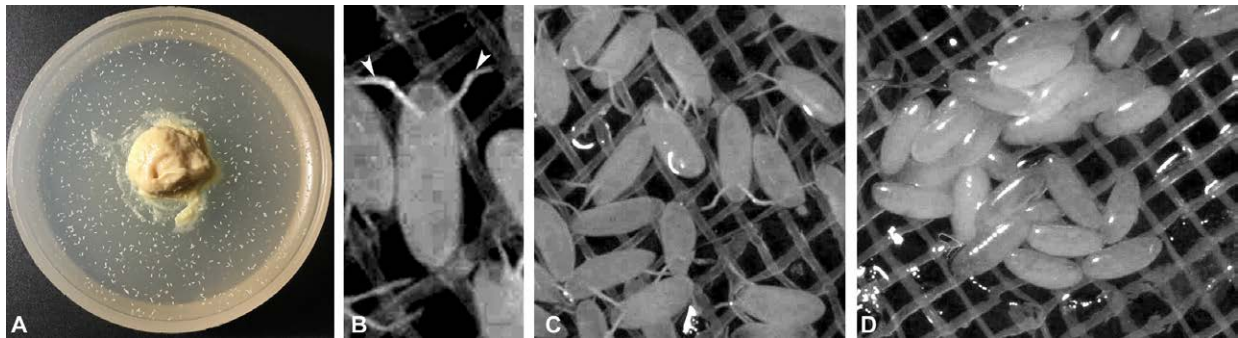


Fig. 2. Dechorionation of embryos. A. Sucrose agar plate with embryos, B. Single embryo showing chorion with egg filaments (arrow heads), C. Unbleached embryos in the strainer. D. Embryos after dechorionization.

G. Live observation:

- i. Using a fine brush, transfer a few embryos either after step E or F, onto a clean slide and add a drop of Halocarbon oil to cover the embryos. Break the clump of embryos gently with a fine needle to separate individual embryos in a single file. Allow the Halocarbon oil to penetrate through the embryos for about 2-5 min at 24°C.
- ii. Observe the embryos under microscope using phase contrast/DIC optics or under bright-field optics with partially closed sub-stage iris diaphragm to enhance contrast (**Fig. 3**).
- iii. Use reference images from Fullilove *et.al.*, (1978) and Hartenstein (1993) for identifying the stages of development (also see **Chapter 2**).

Observations:

Using the images in **Fig. 3** and the above references, identify and study embryos which have developed to different stages showing the following developmental events:

- i. Syncytial Blastoderm (**Fig. 3A**).
- ii. Cellular Blastoderm (**Fig. 3B**).
- iii. Ventral furrow formation.

- iv. Cephalic furrow formation (**Fig. 3C**)
- v. Germ band extension (**Fig. 3D**)
- vi. Germ band retraction
- vii. Intestine lobes formation
- viii. Trachea formation

Document the observable changes in the structure of the embryo at every stage.

Read about the developmental process involved in each of the stages, and note the time line.

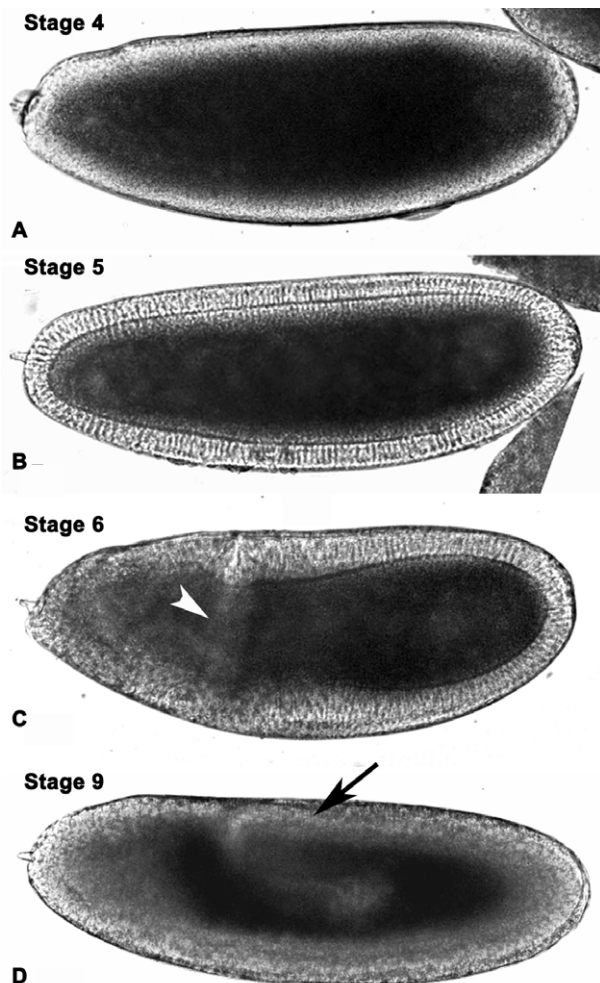


Fig. 3. Images of live early embryos (Stages numbers after Hartenstein, 1993). A. Syncytial blastoderm (Stage 4). B. Cellular Blastoderm (Stage 5). C. Cephalic furrow (arrow head) formation (Stage 6). D. Germ band extension (black arrow, Stage 9).

Questions for self-study and interpretation:

1. Given the time and staging of developmental events, which stage embryo would you select for processing if you have to study gene functions regulating i) gastrulation, ii) nerve chord development, iii) segmentation?
2. How are these embryos handled further for micro injections? Which stage of the embryos and what orientation is used for micro-injection of DNA /RNA? Why?
3. Think of an experimental system which can permit you to follow the very rapid early 'cleavage' nuclear divisions and their movements in live embryos leading to syncytial blastoderm and then cellular blastoderm formation.

References and further reading:

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From a Single Celled Egg to a Complex Living Being of Great Beauty

The three of us have worked on the development of the small and totally harmless fruit fly, *Drosophila*. This animal has been extremely cooperative in our hands - and has revealed to us some of its innermost secrets and tricks for developing from a single celled egg to a complex living being of great beauty and harmony. ... None of us expected that our work would be so successful or that our findings would ever have relevance to medicine.

(In: Nüsslein-Volhard, C. 1995. Banquet speech <https://www.nobelprize.org/prizes/medicine/1995/nusslein-volhard/speech/>)

Chapter 33

Study of genetic basis of segmentation in *Drosophila melanogaster* using embryonic cuticle patterns

Prachi Yadav¹ (pallu.prachi@gmail.com), S. C. Lakhotia^{2*} (lakhotia@bhu.ac.in) and Jagat K. Roy^{2*} (jagatkroy@gmail.com)

1. Division of Genetics, Indian Agricultural Research Institute, New Delhi 110 012, India

2. Cytogenetics Laboratory, Department of Zoology, Banaras Hindu University, Varanasi 221005, India

* For correspondence

Recommended Level: PG/Research

Sub-discipline: Genetics/Cell Biology/Developmental Biology/Molecular Biology

Background:

Drosophila, like other higher organisms, has a metamERICALLY segmented body. The adult fly's body is clearly distinguishable into the head, thorax and abdomen, although such demarcation is not easily identifiable in the larval stages (**Fig.1**). The adult head, although not visibly segmented, comprises of the clypeo-labrum, maxillary and labial palps, derived, respectively, from the anterior-most three larval segments. The adult thorax, which is also not visibly segmented, comprises three segments (pro-, meso- and meta-thorax) as indicated by the three pairs of legs on its ventral side and the paired wings on dorsal side of mesothorax and paired halteres on the metathorax. The abdomen of adult flies retains the larval metameric segmentation and shows eight segments as in larvae (**Fig. 1**).

An important question in embryology/developmental biology concerns the mechanism underlying the complex processes that govern the orderly segregation and spatial organization of the large number of cells that are produced when a zygote begins to develop. Studies on embryogenesis and its genetic regulation in *Drosophila* have led to the identification of the common sets of genes that govern the initial steps during embryonic development in all higher organisms.

The early cleavage stage in *Drosophila* (and other Dipteran insects) egg follows a different pattern than in most other animal groups. The single celled *Drosophila* zygote undergoes rapid incomplete 'cleavage' divisions resulting in a syncytial embryo with ~5000 nuclei distributed through the embryonic volume in common cytoplasm. Cellularization occurs after migration of nuclei to the periphery of the syncytium, generating a syncytial blastoderm, followed by cellular blastoderm. Gastrulation establishes the general body plan with three primary germ layers, the ectoderm, mesoderm, and endoderm. These events involve ordered changes in cell shapes, acquisition of cell polarity, migration etc. to set in motion the morphogenesis (Payre, 2004).

The embryonic development of *Drosophila melanogaster* has been divided into 17 stages on the basis of specific visible events (see <https://www.sdbonline.org/sites/fly/aimain/2stages.htm>). The blastoderm signifies stage 5, whereas morphogenetic movements of gastrulation mark the stage 6. The germ band elongation occurs at stage 7 and the morphological segmentation along the antero-posterior axis of embryo begins at stage 10 with delineation of parasegments. The germ band retraction occurs at stage 12 (see Chapters 2, 32). The externally visible segments in *Drosophila* embryos are repeating units, called para-segments (PS). The embryonic cuticle is deposited by the epidermal epithelium during stage 16. Each PS has 12 rows of epidermal cells of which the 6 anterior-most rows secrete smooth cuticle, while the posterior six rows secrete cuticle with hard protrusions, called denticles (Fig. 1A). The shape and size of each denticle is unique (Fig. 1A), which provides positional information of the underlying epidermal cells that secrete it. Thus the pattern of denticles in late embryos provides an easily scorable marker of final patterning decision made by different cells.

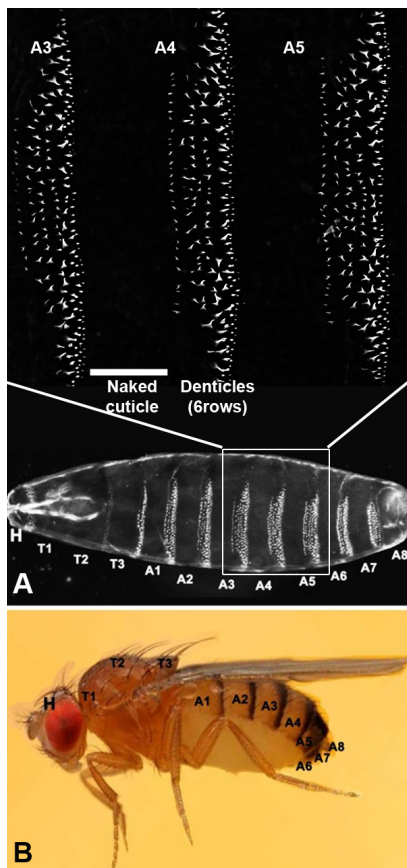


Fig. 1. Metameric units of segmentation in embryo/first instar larva (A) and (B) adult *Drosophila melanogaster* fly (B). Images in A show cuticular preparation of a fully developed embryo, as seen under dark-field microscope. The boxed area in the lower part of A is magnified in the top panel to show the six rows of highly refractile denticles in the posterior part of each para-segment and a region of naked cuticle in the anterior part. H = head region, T1-T3 = pro- meso- and meta-thoracic segments; A1-A8 = abdominal segments 1-8 (Images not to scale).

A remarkable advancement in the identification of genes and their mode of actions in early embryonic morphogenesis in higher organisms occurred when in 1980, Nusslein-Volhard and her colleagues identified, through simple mutagenesis experiments, the genes which when mutated affected the early segmentation of *Drosophila* embryo. A comparative study of cuticle in late embryos/early larvae in wild type and different mutants provided a simple read out of the effects of mutations in these embryonic patterning genes. This Nobel-prize winning study led to identification of the sets of genes which act in a hierarchical manner to produce the normal segmentation pattern (Chandrashekar and Lakhotia, 1991; Lakhotia, 1995). Mutations in the developmentally expressed genes which play key

roles in embryonic epithelial morphogenesis, give rise to an array of epithelial defects and these defects may be detected by the observation of the secreted cuticle which reflects the developmental decisions of the underlying epidermis. The method for obtaining cuticle preparations from late embryo/first instar larva of *Drosophila melanogaster* is described here. In addition, effects of mis-expression of a key patterning gene during the embryonic development on the denticle pattern are also illustrated.

Objective:

To prepare and examine cuticle from wild type and mutant late embryo/first instar larvae to study the segmental identity on the basis of denticular banding pattern.

Materials Required:

A. *D. melanogaster* Flies: Wild type and stocks carrying mutant alleles of some patterning genes. In the example presented here $w^*;P\{w^{+mC}=Act5C.Gal4\}17F01/TM6B$, $w^*;P\{w^{+mC}=UAS.DWnt4.G\}13\}17F01/TM6B$, Tb^1 and $UAS-DWnt4RNAi- y^1 v^1$; $P\{y[+t7.7] v[+t1.8]=TRiP.JF03378\}attP2$ stocks are used (see [Chapters 39, 40](#)).

B. Equipment and other supplies:

- i. Stereo-binocular and bright-field microscopes
- ii. Microcentrifuge
- iii. Embryo Wash Chamber: a nylon cloth/mesh is tied at one end of a both ends open 1" x 3" plastic/glass tube with a rubber band or with screw cap of the tube with the cap's top part cut away (see [Chapters 32, 34](#)).
- iv. Petri dishes (50 mm) with agar medium for egg collection
 - a. Mix 3.5 gm Agar and 1.5 gm sucrose in 100 mL water
 - b. Add a pinch of charcoal powder (to provide dark background against which the white eggs can be easily seen)
 - c. Bring this solution to boil and keep cooking till agar is completely dissolved
 - d. Allow the solution to cool to approximately to 60°C add 0.1 gm Methyl parabenzoate (Nepagin) and 200 µL Propionic acid
Pour the medium on petri-plate (50 mm plate) and allow to cool and solidify.
- v. Slides and coverslips (22 mm²)
- vi. Setup for collection of eggs in a Petri dish (see [Chapters 33, 34, 70](#)).
- vii. Yeast paste: Dissolve a small amount of commercial Baker's yeast in distilled water to make a thick paste and spot it at a few places on solidified agar medium or spread it on the plate in a thin layer.
- viii. Nail polish
- ix. Fine hair brush, fine needles and forceps
- x. 1.5 mL microfuge tubes

C. Solutions and Reagents:

- i. 5% Sodium hypochloride or commercial bleach solution
- ii. Devitellization solution: Methanol:n-Heptane (1:1)

iii. Glycerol-acetic acid (1:4) fixative

iv. Hoyer's mountant

- a. Add 30 gm of Gum Arabic to 50 mL water in a glass beaker let it dissolve overnight on a magnetic stirrer at 60°C in a chemical fume hood.
- b. Add 200 gm of chloral hydrate in small batches to above and mix.
- c. After the chloral hydrate has completely dissolved, add 20 gm of glycerol.
- d. Centrifuge for 30 min at 10000 rpm and then filter the supernatant through glass wool.
- e. Store the Hoyer's medium at room temperature. It can be diluted with little water during use, if necessary.

Experimental Details:

Collect eggs over a period of 2 hr using ~5 days old wild type flies or of the desired genotypes. Let the eggs develop for ~22 hr before the dechorionization step.

A. Dechorionization of Embryos: Any one of the following methods can be used.

i. Method I- A strip of cello tape is fixed on a glass slide with the sticky surface facing up (the two ends of the upwardly facing strip are fixed to slide by two small strips of cello tape; alternatively use a double sided adhesive tape). Clean eggs are transferred to this strip with a brush or forceps (caution: the eggs must not rupture) and are rolled on the sticky surface with a needle or tip of forceps. The chorion sticks and eggs with their shiny vitelline membrane are picked up for further work.

ii. Method II- In this method, which is more convenient when a larger number of eggs are needed, the chorion layer is removed by treatment with bleaching powder or Na-hypochlorite solution as follows.

a. Transfer eggs from the egg-laying chamber with a brush in the washing chamber. The chamber, with embryos supported on the nylon mesh, is repeatedly dipped in distilled water in a beaker to remove adhering food-particles.

b. Dechorionate embryos by dipping the wash chamber in the bleaching solution, kept in a suitable dish, for 2-3 min: monitor embryos under a stereo-binocular microscope for the gradual disappearance of egg filaments on their anterior end. Dechorionation is complete when the egg filaments completely disappear.

c. Wash embryos in the wash chamber by passing through several changes of distilled water to remove traces of bleaching solution.

B. Devitellization of Embryos: Transfer the dechorionated embryos to the devitellizing solution (n-heptane:methanol mixture) at room temperature with vigorous shaking for 20-30 sec. Collect the devitellized embryos which sink to the bottom and discard the embryos which are floating in the methanol phase.

C. Fixation of Embryos or Larvae: Fix the dechorionated and devitellinized embryos or freshly emerged first instar larvae (larvae do not require the above pre-treatments) in freshly prepared Glycerol-acetic acid (1:4) fixative at 45°C for at least 1 hr (overnight fixation at 45°C gives better results).

D. Mounting of Embryos/Larvae:

i. Put a drop of Hoyer's mountant mixed with lactic acid and water (2:1:1) on a clean slide and transfer the fixed embryos or larvae to this drop using a brush.

Note: If the embryos were not devitellinized by n-heptane/methanol earlier, they may now be popped out of their vitelline membranes. For this purpose, gently cover the embryos in Hoyer's medium with a coverglass and leave at room temperature (~25°C) for 1 hr. The mounting medium should not completely dry or harden. At this stage, working under stereobinocular microscope, pop the embryos out of the vitelline membrane by applying a little pressure on the coverglass).

ii. Transfer the mounted preparations to incubator at 40°C-50°C for overnight or longer (2-3 days) for them to become transparent enough for microscopy. The preparations may be examined immediately after mounting, but the quality will be inferior to those that have been optimally cleared overnight or longer.

iii. Using the right amount of Hoyer's mountant helps flatten the preparations as the solution is pulled out over the entire surface under coverglass. Addition of small weight over the coverglasses (2-5 gm) after partial clearing at 50°C also helps in flat cuticular preparations.

E. **Examining the preparations:** Examine the cleared preparations under low/high power dry objectives using dark-field illumination (**Fig. 1A**). The denticles and other high refractive index cuticular structures appear bright against a dark background. **Note:** The preparations may also be examined under bright-field optics. However, the details of cuticular patterns will not be as distinct as when using dark-field illumination (compare **Figs. 1A** and **Fig. 2**).

Observations:

1. Wild type embryos have eight abdominal segments with six of denticular bands alternating with naked cuticle (**Figs. 1A** and **2A**).
2. Embryos in which *Dwnt4* is knocked down through RNAi using the ubiquitously expressing *Actin5C-Gal-4* driver (*Actin5C-Gal-4>UAS-DWnt4-RNAi*), only 4 or 5 denticular bands are seen in each row (**Fig. 2B**).
3. Overexpression of *Dwnt4* in *Actin5C-Gal-4>UAS-DWnt4* embryos causes near complete loss of naked cuticle resulting in a 'lawn' of denticles (**Fig. 2C**).

Questions for self-study and interpretation:

1. How do defects in segmentation, revealed by an altered pattern of denticles in late embryos due to mutation in a given gene, help in understanding of the role of that gene in the establishment of body segmentation?
2. Why should knockdown or over-expression of the *Wnt4* affect the denticle development?

3. How have the initial studies by Nusslein-Vollhard and colleagues in the 1980s on genes that regulate the antero-posterior segmentation in *Drosophila*, contributed to understanding of genetic regulation of development in higher organisms, including humans?

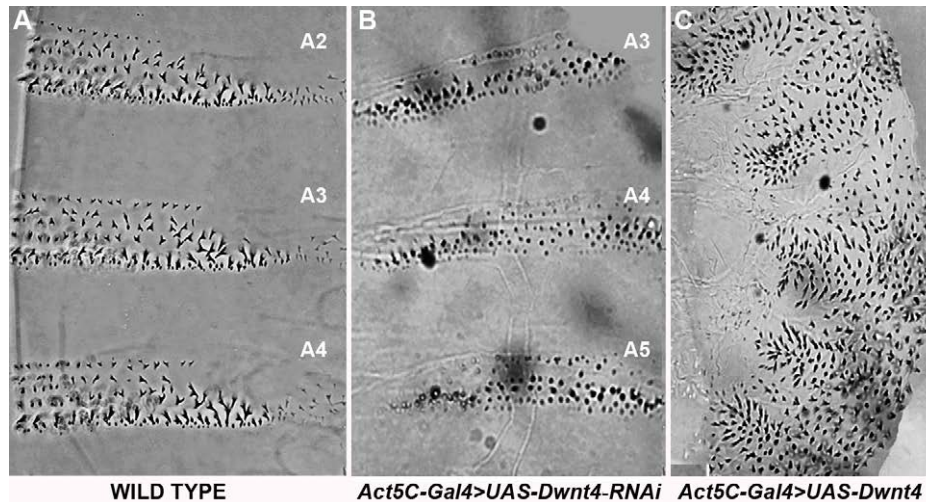


Fig. 2. Bright-field microscope images of denticular patterns in ventral region of abdominal segments in wild type (A), *Actin5C-Gal-4>UAS-DWnt4-RNAi* (B) and *Actin-5C-Gal-4>UAS-DWnt4* late embryos (C). Each segment in wild type embryo shows six denticle rows alternating with naked cuticle while knockdown of *DWnt4* leads to fewer denticle rows with expansion of naked cuticle (B). On the other hand, over-expression of *DWnt4* (C) results in a ‘lawn’ of denticles, with little naked cuticle because the segment boundaries are not established.

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Chapter 34

Introduction to the embryonic nervous system in *Drosophila*

Anuradha Ratnaparkhi (anu.aripune@gmail.com)

Developmental Biology Group, MACS-Agharkar Research Institute, Pune 411004

Recommended Level: PG/Research

Sub-discipline: Cell Biology/ Developmental Biology/ Neurobiology

Background:

The two major cell types in the nervous system are neurons and glia. Neurons form circuits that enable the organism to receive and respond appropriately to stimuli. During development, specific guidance cues present in the cellular environment help axons navigate and reach their target cells and establish synaptic connections. Studies in *Drosophila* have contributed significantly to our understanding of this process.

The embryonic neuromuscular system in *Drosophila* serves as an excellent model system to understand how motor neurons reach and innervate their target muscles. Each hemi-segment in the embryo is made up of 30 muscles which receive innervation in an invariant manner indicating that the process is genetically controlled (Keshishian and Chiba, 1993; Bate, 1993).

While neurons 'transmit' signals, glial cells play important roles during development and function of the nervous system. For example, glia present at the midline, are known to secrete attractive and repulsive cues that help axons navigate across the midline or stay away from it. Glia ensheath axons, neuronal cell bodies and the neuropil to protect and create distinct functional compartments in the central nervous system (CNS). *Drosophila* has many different types of glia which bear functional similarity to vertebrate glia. These can be identified based on their position and morphology. In the embryonic CNS, the longitudinal glia (LG) are present dorsally and ensheath axons of the CNS. A subset of these give rise to astrocytes in the larval stages. The other glial types include the ventrally placed cell body glia (CBG), and the sub-perineurial glia (SPG) which are large flat cells that ensheath the entire CNS towards the end of embryogenesis. SPG are polyploid and characterized by the presence of a large nucleus. In the larval and adult CNS, perineurial glia (PNG) are seen outside the sub-perineurial sheath (Hartenstein, 2011; Omoto *et. al.*, 2016).

In part one of this exercise, the goal is to introduce (a) the stereotyped organization of

the somatic musculature in *Drosophila* embryos, (b) the different motor nerves and their projection patterns and (c) the pattern of innervation during embryonic development. In part two, the organization of glia in the embryonic CNS will be studied using the nuclear marker Repo and different *GAL4* lines (see Chapters 39, 40) to understand correlation between morphology and function. The Fig. 1 shows images of an embryo stained with anti-FasciclinII that labels axons tracts in the CNS and motor axons in the periphery (Fig. 1A), and an embryo stained with anti-Repo, which labels all glial nuclei other than the midline glia (Fig. 1B).

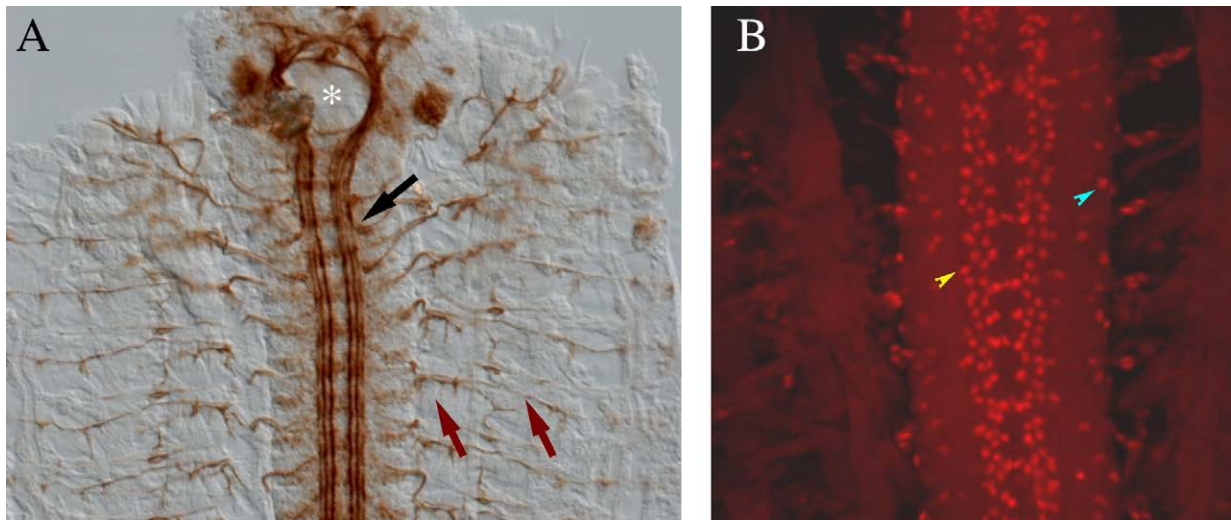


Fig. 1. Embryonic CNS. **A.** Embryo fillet stained with anti-Fasciclin II (mAb1D4) to show axons in the nerve ring (asterisk) and tracts (black arrow) in the brain and ventral nerve cord respectively; red arrows point to motor axons that exit the CNS and innervate the muscles. **B.** Embryonic CNS stained with anti-Repo showing organization of glia. The longitudinal glia (yellow arrowhead) sit atop the neuropil; blue arrowhead points to subperineurial glia.

Objectives:

1. To observe the stereotyped pattern of embryonic body wall muscles and their innervation at different embryonic stages.
2. Identification of the stereotyped organization of glia in the embryonic CNS and their different morphologies.

Materials Required:

A. Flystocks

- i. For Objective 1:
 - Canton-S or Oregon-R or *w¹¹¹⁸*
- ii. For Objective 2:
 - Canton-S or Oregon-R or *w¹¹¹⁸*
 - *repo-GAL4/TM3* (BDSC #7415; all glia except midline glia)
 - *MZ97-GAL4/Cyo* (BDSC# 9488; LG, Exit Glia and SPG)

- *UAS-mCD8GFP* or *UAS-mCD4tdGFP*
- *htl-GAL4/Cyo* (Hidalgo *et al.*, 2001; LG, exit glia, cell body glia;)
- *moody-GAL4/Cyo* or *moody-GAL4/TM3* (Baintonet *et al.*, 2005), or *nrxIV-GFP(SPG)*

B. Materials and reagents for embryo collection and fixation

i. Sucrose agar bottles

Cut the regular bottles used for culturing flies into half. Pour melted sucrose agar (2.5 gm agar and 3 gm sucrose dissolved by heating in 100 mL water) into the bottom half and allow it to set. Join the top and bottom halves of the bottle together using insulation tape.

ii. Fresh yeast paste

iii. Soft brush

iv. Egg basket

v. 50% bleach (without detergent)

vi. 20% para-formaldehyde made in 1X PBS or 37% formaldehyde solution

vii. 1M PIPES solution (Sigma # 3768, pH 7.0)

viii. 10 mM MgSO₄ solution

ix. 10 mM EGTA solution

x. n-heptane

xi. Methanol

xii. Screw capped glass scintillation vials or any other wide mouthed glass vials with cap

xiii. PEM buffer (PIPES, EGTA, MgSO₄; pH 7.0)

0.1 M PIPES (pH 7.0) 2 mM EGTA

1 mM MgSO₄

xiv. Fixative (4% Fixative in PEM Buffer)

0.1 M PIPES (pH 7.0)

2 mM EGTA

1 mM MgSO₄

4% Para-formaldehyde/ formaldehyde

C. Solutions for Immuno histochemistry:

i. 10X PBS

ii. 1X PBS+1% Tween-20 (PT)

iii. 1X PBS+ 0.1% BSA+0.1% Triton-X-100 (PBTX)

iv. Primary Antibodies

a. Part I

- Mouse anti-Fasciclin II/anti-FasII (labels motor neurons).

- Rabbit anti-HRP (labels neuronal membranes).

b. Part II

- Anti-Repo

- Anti-GFP

v. Secondary Antibodies

- Anti-mouse::HRP

- Anti-rabbit::HRP

vi. Solution for color reaction: 3, 3' Di-Amino-Benzidine (DAB)

Dissolve 0.3 mg/mL DAB + 0.05% Tween-20 in 1X PBS. Aliquots of the solution can be made and stored at -70°C.

Experimental Details:

1. Setting up embryo collection: Time Required: 3 days

- Day 1. Transfer young, healthy flies to the sucrose agar bottle (at least 25-40 flies) containing a small amount of yeast paste placed on the agar. Keep overnight for acclimatization.
- Day 2. Transfer the flies to a fresh yeasted sucrose agar bottle. Keep overnight at 25°C for egg laying.
- Day 3. Collect embryos for fixation. For an overnight collection, harvest the eggs 15-18 hr after transfer.

2. Embryo Fixation: Time Required: 30 min

- Dislodge embryos from the sucrose agar using water (from a squirt bottle) and a soft brush and transfer into a home-made egg basket (**Fig. 2**).

Note: To make an egg basket, take any old 50 mL/15 mL falcon tube (Tarsons or equivalent). Using a hot knife cut the tube into two at a distance of about 2 inches from the cap. Discard the bottom half. Cut out the central portion of the cap to create a hole. Place a small square piece of fine nylon mesh (bigger than the cap) on the inner side of the cap and tighten the cap onto the cut tube. The nylon mesh acts as a sieve for collecting embryos and can be removed, washed and reused several times.

- Wash the embryos in the basket thoroughly under running tap water or by swirling it several times in a beaker containing water. Next, dip the basket into a mild bleach solution (50% mild commercial bleach (without detergent) or 5% Sodium hypochlorite solution) for 3-5 min for dechoriation. Dechoriation is complete when the egg filaments are no longer visible and the embryos appear translucent.

- Dip the egg basket with embryos in water several times to remove all traces of bleach. Using a soft brush, transfer the dechoriated embryos into a transparent glass screw-capped vial containing 2 mL of fixative and 8 mL of n-heptane taking care to avoid pieces of agar and other debris from getting transferred into the fixative.

- Gently shake the vial on a rotating platform shaker for 20 min. At this stage, the embryos will be seen at the interface of the fixative and n-heptane layers.

- After 20 min, remove the lower phase (fixative) using a glass pasture pipette. Add 10 mL of methanol and shake the vial vigorously. Devitellinized embryos will sink to the bottom of the vial.

- Remove the heptane layer and transfer the embryos to a 0.5 mL/1.5 mL microfuge

tube.

g. Rinse the embryos twice with small volumes of methanol and store in methanol at -20°C . At this stage, the embryos may be stored for several weeks before use.

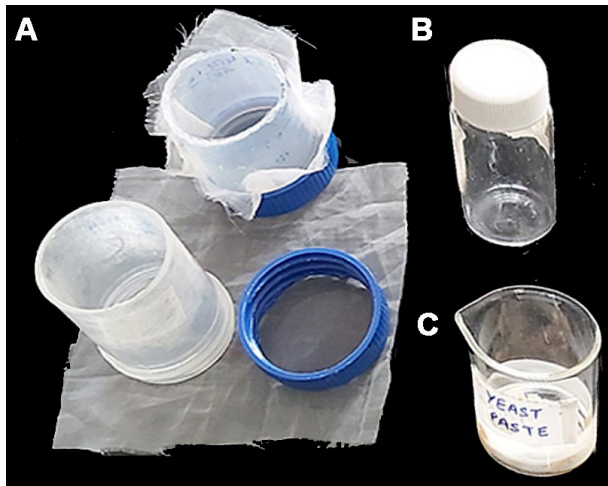


Fig. 2. The egg basket. **A.** Components and assembly of egg basket. **B.** Scintillation vial for fixing embryos. **C.** Fresh yeast paste to promote egg-laying.

3. Immunostaining

- a. Take approximately 20 μL volume of fixed embryos in a 0.5 mL microfuge tube (this will reduce the volume of diluted primary antibody required for staining).
 - b. Remove the methanol used for storage and rinse the embryos 3 x 5 min with PT.
 - c. Replace PT with PBTX and allow the blocking to proceed for 40-45 min by placing the microfuge tube in a rotatory shaker.
 - d. Take fresh PBTX (200 μL) in a separate microfuge tube to make a dilution of the primary antibody as per the manufacturer's instructions. (Anti-Fasciclin II).
 - e. Remove the PBTX after the blocking step and add the antibody solution. Allow the antibody reaction to proceed overnight at 4°C . (This step can be carried out for 4-5 hr at 24°C).
 - f. Remove the antibody solution and wash the embryos 3x 5 min with PBTX and 3 x 15 min with PBT. The used diluted primary antibody solution can be stored at 4°C for reuse.
 - g. Block the embryos with PBTX for 30 min.
 - h. Remove PBTX and add diluted secondary antibody (dilution in PBTX as recommended by manufacturer) to the embryos. Incubate on a rotatory shaker at room temperature for 2-3 hr. (The incubation can be carried out overnight at 4°C).
 - i. Wash 4 x 15 min with PBTX.
 - j. For development of color, incubate the embryos with DAB solution. Just before use, add H_2O_2 to a final concentration of 0.03%. Monitor the reaction under a stereo microscope.
 - k. When the colour has developed adequately, stop the reaction by removing the DAB solution and rinsing the embryos 3 X 5 min with PT.
- Note:** i. To prevent exposure to light, If fluorescent secondary antibodies are used instead of the HRP tagged one, the samples can be viewed directly without DAB staining.
- ii. Used and unused DAB solution needs to be inactivated in bleach before discard.

Used pipette tips can be dropped into a beaker containing 50% commercial bleach to inactivate any residual DAB present in the tips.

4. Mounting

- a. Rinse the embryos for 1 min with 1X PBS. (Do not store embryos in PT or PBTX)
- b. Remove PBS and add 70% glycerol (v/v in 1X PBS). Keep for 1 hr at room temperature (**Note:** Incubation time in glycerol can be adjusted to 15-20 min by examining tissue morphology).
- c. Place a few embryos in glycerol on a clean slide. Arrange the embryos to lie on their lateral side to view the dorsal innervation. For visualizing more ventral innervation, place embryos ventral side up while mounting. **Note:** A 70% glycerol solution can be used to clear and mount the colour immunostained embryos. If fluorescent secondary antibodies are used, add 5% (w/v) n-propyl gallate to glycerol.
- d. Place a coverslip (18 mm² or 22 mm²) gently on top of the embryos. Press lightly to flatten the tissue.
- e. If more glycerol needs to be added, a small drop can be placed at the edge of the coverslip. Excess glycerol should be removed by gently dabbing the edges with tissue paper.
- f. Seal the coverslip using colorless nail polish. Begin by placing a little nail polish on the 4 vertices of the coverslip before sealing the edges.
- g. Once dry, the slide is ready to be viewed under a bright-field microscope. If the secondary antibody is tagged with a fluorochrome, a fluorescence or confocal microscope is required for viewing the preparations.

Results/Observations:

1. Stereotyped pattern of embryonic body wall muscles and their innervation at different embryonic stages (Fig. 3 and 4):

- a. Note the stage at which motor neurons exit the CNS.
- b. Examine the projection of the ISN and SN axons and their branching at different stages of embryonic development.
- c. Note the pattern of innervation on muscles 6 and 7, muscle 12 and muscle 4 in late stage 16 embryos

2. Stereotyped organization and types of glia in the embryonic CNS (Fig. 4 and 5):

- a. Identify LG in the embryonic CNS. Note and draw the organization.
- b. Identify lateral SPG on the basis of nuclear size.
- c. Count the average number of Prospero positive LG glia per segment of the CNS. **Note:** A description of different embryonic stages can be found at <http://flybase.org>

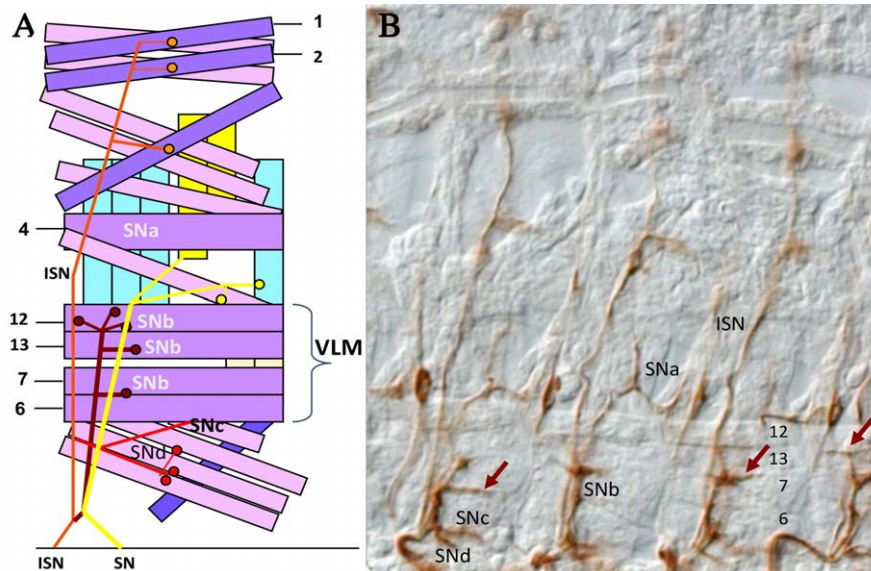


Fig. 3. **A.** Schematic of the bodywall muscles and the innervation pattern: the intersegmental nerve (ISN, orange) extends dorsally to innervate muscles 1 and 2; segmental nerve a (SNa, yellow) shows a characteristic bifurcation at a point dorsal to muscle 12 to innervate muscles 8 and 24; and 24; SNb (maroon) innervates ventro-lateral muscles 6, 7, 13, and 12. SNc and SNd (red) innervate along the cleft between muscle 15 and muscle 16. **B.** Innervation pattern of motor axons in an embryo detected using anti-FasII antibody (*mAb1D4*).

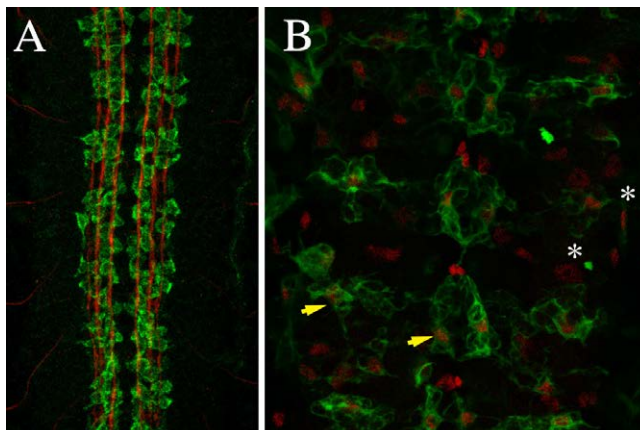


Fig. 4. Images of an embryonic CNS showing different types of glia. **A.** embryonic CNS stained with anti-FasII (red, axons) and anti-Nazgul (green, longitudinal glia). **B.** *htrl-GAL4 > UAS-CD4-tdGFP* embryo stained with anti-Repo (red) and anti-GFP (green). Arrows indicate the cell body glia while asterisk marks the SPG.

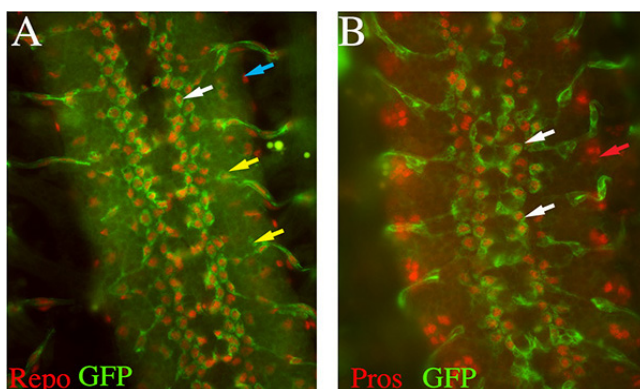


Fig. 5. Expression pattern of *MZ97-GAL4*, visualized through *UAS-mCD8-GFP* (green) expression. **A.** Embryo immunostained with anti-Repo (red) and anti-GFP (green). Embryo immunostained with anti-Prospero (red) and anti-GFP (green). LG and nerve root glia are indicated by white and yellow arrows, respectively. Blue arrow (**A**) indicates SPG; Red arrow (**B**) indicates Prospero positive neurons.

Questions for self-study and interpretation.

1. Is the timing of motor axon extension into the periphery an intrinsic property of the neuron?
2. How variable is the branching and innervation pattern of the motor neurons?
3. What do you think might influence the process of branching?
4. How variable is the distance between the two rows of LG present on either side of the midline?
5. Correlate morphology and function of different glia.
6. What might be the mechanisms involved in regulating this organization?
7. What approaches may be taken to identify genes that determine the regulated branching of different motor axons?

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<http://www.its.caltech.edu/~zinnlab/motoraxons/primer%20contents.html>

(An excellent resource for motor axon development with details on embryo dissections).

Chapter 35

Visualization of *Drosophila* larval body wall muscles and their innervations

Pooja Rai (pooja.rai@umassmed.edu)^{1,2}, Rima Saha (rimasaha6@gmail.com) and S. C. Lakhotia^{1*} (lakhotia@bhu.ac.in)

1. Cytogenetics Laboratory, Department of Zoology, Banaras Hindu University, Varanasi-221005, India

2. Current address: Department of Molecular, Cell and Cancer Biology, University of Massachusetts Chan Medical School, Massachusetts-01655, USA

* For correspondence

Recommended Level: UG/PG/Research

Sub-discipline: Anatomy/Physiology

Background:

Insects have three types of muscles, namely external body wall muscles (multi layered somatic musculature), visceral muscles associated with internal organs like digestive tract and gonads, and specialized myo-epithelial tubes forming the vascular system. The *D. melanogaster* wild type third instar larva is ~5 mm in length and highly mobile. Its body is metamERICALLY segmented (see **Chapters 1, 2**), which besides the head region, includes three thoracic and eight abdominal segments (A1-A8). The *Drosophila* larval body wall muscles provide a relatively simple system to study the somatic muscles, nervous system and their interface, the neuromuscular junctions. Different segments have a highly organized pattern of muscle fibers, with distinct variations in the thoracic and some abdominal segments. Each of the A1 to A7 hemi-segment shows a repeating pattern for position and orientation of about 30 muscles (Schulman *et al.*, 2015; Kohsaka *et al.*, 2017). Maintenance of muscle integrity is essential during their growth, as muscle functionality is required for larval mobility and later for pupation.

The CNS consists of two brain hemispheres or the brain lobes and the ventral nerve cord (VNC) comprising the thoracic and abdominal ganglia (Hartenstein *et al.*, 2008). The VNC corresponds to the vertebrate spinal cord. Motor neurons in the VNC send long axons to target muscles where they end at the neuromuscular junctions (NMJ). The neuromuscular junction is a synaptic connection between the terminal end of a motor nerve and the muscle (Menon *et al.*, 2013). The motor neurons in each hemi-segment of larva exit the CNS as two main fascicles namely, inter-segmental nerve (ISN, the anterior fascicle) innervating the dorsal targets and the segmental nerve (SN, posterior fascicle) innervating lateral and ventro-lateral targets (Bastiani *et al.*, 1984).

We describe here a simple procedure, a simplified version of the protocol described by Ramachandran and Budnik (2010), to visualize the segmental body wall muscles and

their innervations in the third instar larvae. This method would be useful in understanding effects of gene mutations or other conditions like the fly models for human disorders that affect skeletal muscle development, their innervations (see [Chapter 34](#)), and formation and activity of neuro-muscular junctions.

Objective:

Examination of the organization of body wall muscles and their innervations in third instar larvae of *Drosophila*.

Materials Required:

A. **Flies:** Wild type or any mutant, as desired

B. Equipment and ly handling materials:

- i. Stereo binocular and Bright-field microscopes
- ii. Food bottles for fly culture and Petri dishes for larval growth
- iii. Larval dissection tray: Melt paraffin wax by heating in a small beaker and pour into a clean small Petri dish (5 cm diameter) to ~ 3/4th of its height. Let the paraffin wax solidify as it cools. This is used as the larval dissection tray.
- iv. Insect pins
- v. Pair of fine dissecting needles or insulin syringe needles, fine pair of forceps, fine hair brush for handling and dissecting larvae
- vi. Iridectomy scissors (optional)
- vii. Clean slides and cover slips (22 mm²)

C. Reagents and solutions:

- i. Phosphate buffered saline (1X PBS): Dissolve 8 g NaCl, 0.2 g KCl, 1.42 g Na₂HPO₄ and 0.272 g KH₂PO₄ in 800 mL of distilled water. Adjust the pH to 7.4 with HCl and then add distilled water to make the volume to 1000 mL. Autoclave and store at RT or at 4°C.
- ii. Bouin's fixative: Add 0.75 mL of saturated picric acid solution in distilled water, 0.25 mL formalin (37% Formaldehyde), 0.05 mL glacial acetic acid and make the volume to 10 mL with 1X PBS.

Alternatively, dissolve 2 mg of Picric acid in 5 mL of 1X PBS (Picric Acid crystals are explosive in nature) and add 20 mg of para-formaldehyde powder in the Picric acid solution and dissolve by heating at 60°C in a fume hood; a few drops of 1N NaOH help in solubilization. Finally, add 1X PBS to make total volume to 10 mL. Mix well and store at room temperature.

Note: 4% Formaldehyde can also be used as fixative in place of Bouin's fixative.

- iii. Aceto-carmine stain in 50% acetic acid (see [Chapter 31](#)).

Experimental Details:

1. Thoroughly wash healthy third instar larvae of the desired genotype with 1X PBS to remove adhering food particles etc.
2. Place one larva in the Paraffin wax plate in a drop of 1X PBS.
3. Pin down the larva on the paraffin wax with insect pins inserted at the dorsal anterior and posterior ends (**Fig. 1A**).
4. While looking through the dissecting microscope, use fine sharp-tipped dissection or insulin needle to carefully make an incision (**Fig. 1B**) in the dorsal body wall of the pinned larva along its entire length (Iridectomy scissors can be more convenient for making this incision).
5. After making the incision, gently flush out the internal viscera with 1 mL 1X PBS using micropipette or a Pasteur pipette.
6. To make a stretched fillet, hold each corner of the dorsal body wall with fine forceps and gently stretch it sideways and fix them using fine pins. Stretch each corner evenly to ensure that all muscles are properly stretched (**Fig. 1D**).
7. Using 1X PBS and forceps/needles, remove the remaining internal tissues including gut, salivary glands, fat body, imaginal discs, Malpighian tubules etc., but carefully leave the nervous system and body wall muscles as they are.
8. Flatten the body wall muscles further by adjusting positions of the pins with the help of forceps so that the fillet assumes a near rectangular shape (**Fig. 1D**).
9. Remove the 1X PBS and debris from the wax plate and add a few drops of Bouin's fixative to completely submerge the larval fillet. Leave for 15-20 min at room temperature.
10. Examine the preparation under stereo-binocular microscope using higher magnification objective (5-8X) to determine the quality of preparation (**Fig. 1E**).
11. Wash the fillet in the wax plate with multiple rinses of PBS to remove the fixative.
12. Carefully remove the fixing pins from the dissection dish and, using a pair of fine forceps, gently transfer the larval fillet to a clean slide in a drop of 1X PBS.
13. Drain out or soak the PBS with filter paper and immediately place a few drops of Aceto-carmin stain on the fillet. Cover with a watch glass and leave it for 5-10 min for staining at room temperature.
14. Wash out excess stain with a few drops of 50% acetic acid.

15. Drain out excess of acetic acid, while holding the fillet in place with a needle. Carefully blot out 50% acetic acid and immediately place a drop of 50% glycerol on the stained fillet and cover with a coverslip, without trapping air bubbles.

16. Examine the preparation under a stereo-binocular microscope using 5x or higher objective magnification.

Note: In case the stereo-binocular is not equipped with a base light for observation with transmitted light, the preparation can be examined under a bright field microscope using 4/5x or 10x objective.

Bouin's fixative fixed preparation, without staining, can also be mounted in PBS and examined under a bright-field microscope. For a better contrasted image of the larval muscles and nerves in the Bouin's fluid fixed but unstained fillet, adjust the brightness of light and the iris diaphragm in the sub-stage condenser of the microscope. Observation of unstained preparation under DIC optics provides a better image of the nerves that exit the central nervous system to innervate the different muscles.

Results:

1. Observe and identify the segmentally repeated stereotyped patterns of various muscles in the thoracic and abdominal segments (**Fig. 1E**).
2. Identify the brain hemispheres and ventral nerve cord and the nerves that emerge from them to innervate the muscles and body segments (**Fig. 1F**).

Questions:

1. Why do the muscles become distinctly visible after fixation?
2. How do you think the differently oriented muscles help the larva move in a meandering path?
3. Are the larval body wall muscles similar to those seen in adult body?
4. Compare the body muscle patterns and their innervation in *Drosophila* embryo (see **Fig. 3** in **Chapter 34**). Are they similar or different?
5. How do motor neurons innervate the muscles and transmit the signal?

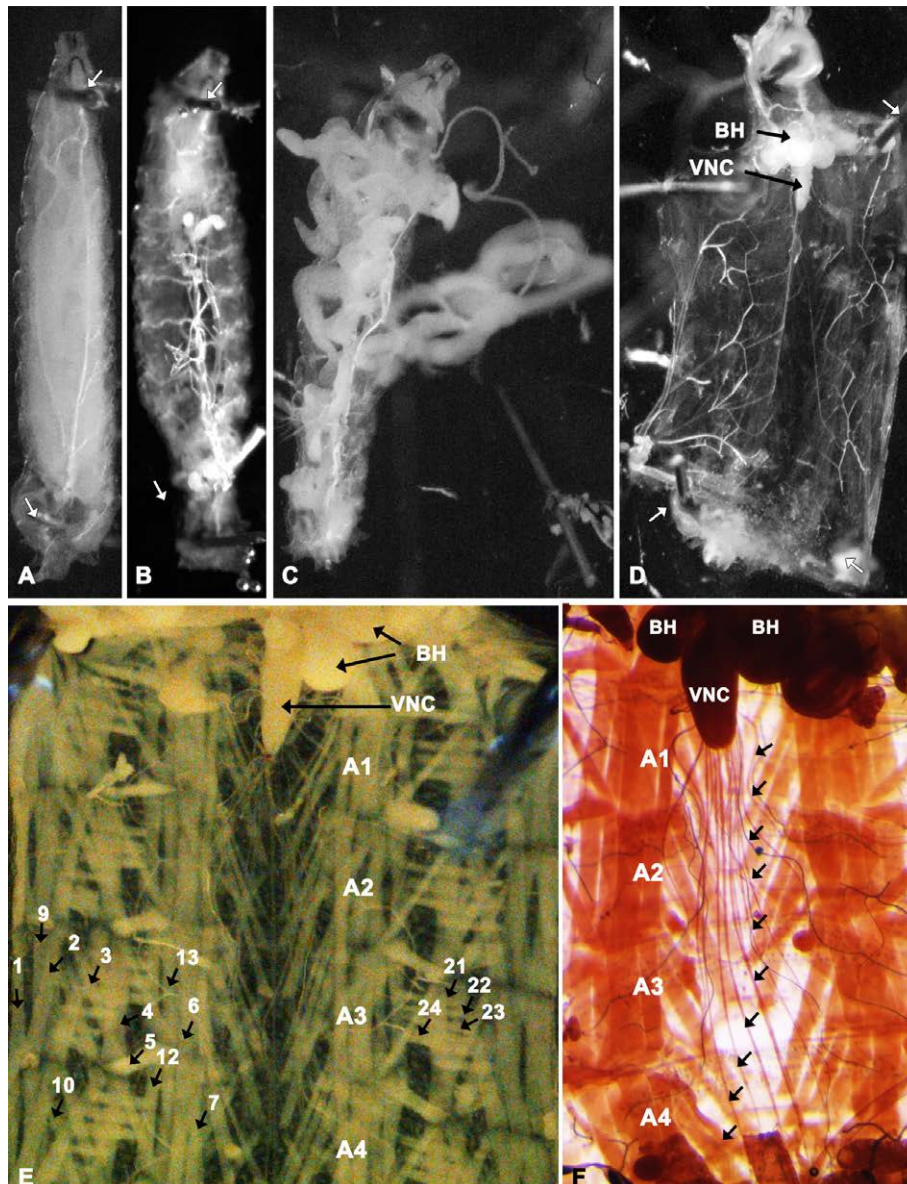


Fig. 1. Body wall muscles and their innervation in third instar *Drosophila* larval fillet. **A.** Larva pinned (white arrows) on dissecting tray (Petri dish [10 cm dia] with paraffin wax); **B.** Dorsal cuticle slit from anterior to posterior end; **C.** Internal organs coming out of the dorsally opened cuticle; **D.** Fillet with dorso-lateral cuticle flaps stretched out laterally and pinned (white arrows) after internal viscera is removed; **E.** Body wall muscles seen after fixation of the larval fillet as in **D** with Bouin's fixative; **F.** Aceto-carmine stained larval fillet with distinctly visible CNS and segmental nerves (black arrows) emerging from ventral nerve cord. **A1-A7** = abdominal segments 1 to 7; **BH** = brain hemispheres; **VNC** = ventral nerve cord. Numbers in **E** indicate the various muscles identified after Hooper (1986) and Schulman *et al.*, (2015): **1, 2, 3** = Dorsal Acute 1, 2 and 3; **4**= Lateral Longitudinal 1; **5** = Lateral Oblique 1; **6** = Ventral Longitudinal 3; **7** = Ventral Longitudinal 4; **9** and **10** = Dorsal Oblique 1 and 2; **12** and **13** = Ventral Longitudinal 1 and 2; **21, 22** and **23** = Lateral Transverse 1, 2 and 3. Images not to scale.

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Chapter 36

Visualization of thoracic indirect flight muscles of wild type *Drosophila melanogaster*

Pooja Rai (pooja.raai@umassmed.edu)^{1,2}*, S. C. Lakhotia (lakhotia@bhu.ac.in)¹*

1. Cytogenetics Laboratory, Department of Zoology, Banaras Hindu University, Varanasi-221005, India

2. Current address: Department of Molecular, Cell and Cancer Biology, University of Massachusetts Chan Medical School, Massachusetts-01655, USA

* For correspondence

Recommended level: PG/ Research

Sub-discipline: Anatomy/Developmental Biology/Physiology

Background:

Two sets of flight muscles in the thorax, the indirect and direct flight muscles, control wing movements during flight in *Drosophila*. The indirect flight muscles (IFMs) power the wing stroke while direct flight muscles (DFMs) control the angle of the wings. The IFMs constitute more than 70% of flight muscle mass and include two groups of large muscles: six Dorsal Longitudinal Muscles (DLM) extending along the anterior to the posterior axis of thorax, and three Dorsal Ventral Muscles (DVM) that extend along dorso-ventral axis (reviewed in Jawkar and Nongthomba., 2020; Swank 2012). The IFMs are not directly attached to wings, but to the cuticle whose deformation helps control wing beats.

In *Drosophila*, the larval muscles formed during embryogenesis are largely destroyed during pupal metamorphosis. The adult muscles develop mostly de novo during the late larval and pupal stages although some of the larval muscles act as scaffolds for adult muscle development. The DLMs develop through fusion of myoblasts to a set of persistent larval muscles in the thoracic segments while DVMs are generated de novo during the pupal stages (Fernandes et al, 1991). The IFMs provide a good model system for studying muscle development and regeneration, and for understanding muscle associated human diseases (Gunage et al., 2017; Jawkar and Nongthomba, 2020).

In this chapter a simple method for visualizing the IFMs in adult flies is described. A more detailed and refined protocol is described by Swank (2012).

Materials required:

- A. **Flies strain:** Wild type or any mutant, as desired
- B. Equipment and other items:
 - i. Stereo-binocular microscope and other fly handling materials

- ii. Fine needles (e.g., insulin needles), fine forceps, insect pins

Note: Fine Iridectomy scissors and forceps, if available, provide greater convenience in the dissection of fly thorax

- iii. Microscope slides, 22 mm² coverslips and Nail-polish

C. Reagents and solutions:

- i. 1X Phosphate buffered saline (1X PBS, pH 7.4):

NaCl	8 gm
KCl	0.2 gm
Na ₂ HPO ₄	1.44 gm
KH ₂ PO ₄	0.24 gm

Serially dissolve the above in 800 mL distilled water; when fully dissolved, adjust pH to 7.4 with 1N HCl or 0.4 N NaOH, as required. Finally make the volume to 1000 mL with distilled water, autoclave the solution and store at room temperature.

- ii. Bouin's fixative: Add 0.75 mL of saturated picric acid solution in distilled water, 0.25 mL formalin (37% Formaldehyde), 0.05 mL glacial acetic acid and make the volume to 10 mL with 1X PBS.

OR

Dissolve 2 mg of Picric acid in 5 mL of 1X PBS and add 20 mg of para-formaldehyde powder in the Picric acid solution and dissolve by heating at 60°C in a fume hood; a few drops of 1N NaOH help in solubilization. Finally, add 1X PBS to make the total volume to 10 mL. Mix well and store at room temperature.

Note: Picric Acid crystals in the storage bottle must always be under distilled water since dry picric acid crystals can explode by friction (associated with opening of the bottle cap) or shock (see <https://oag.ca.gov/sites/all/files/agweb/pdfs/cci/safety/picric.pdf>).

Experimental details:

1. Take 2-3 days old healthy female flies of desired genotype.

Note: Female flies are more convenient to dissect because of their larger body size; rearing larvae and flies at 18-20°C also increases the body size.

2. Anesthetize flies using ether or cold (place the vial with desired flies in refrigerator for 10 to 20 min)
3. Place the fly in a drop of PBS on a slide with its dorsal side up.
4. Viewing the fly under a stereo-binocular microscope, use fine needles to cut off the abdomen and head from the thorax and discard the head and abdomen regions. Keep the thorax only on the slide with its dorsal side up (Fig. 1A, B).

5. Insert the fine tip of a needle in the hole at the anterior end, where thorax joined the head and move the needle posteriorly to split the dorsal thoracic cuticle along the midline to open thorax (Fig. 1C).
6. Make a final incision through the ventral side to separate the left and right pairs of legs. This may separate the two thoracic hemi-sections (Fig. 1D).
7. Carefully remove the PBS and add ~200 μ L of Bouin's fixative on the thoracic hemi-sections to fix them and improve their visibility due to yellow color of the fixative.
8. Working with fine needle, locate and detach the array of dorsal longitudinal muscles (DLMs) in the dorsal part of each hemi-section. Make an incision underneath the DLM array to release a patch of cuticle with attached DLM fibers (Fig. 1E and F).
9. Wash out the extra fixative in 1X PBS for 15 minutes, 3 times.
10. Mount the tissue in 1X PBS on slide, gently cover with a coverslip and seal the edges with nail polish. Do not press the coverslip.

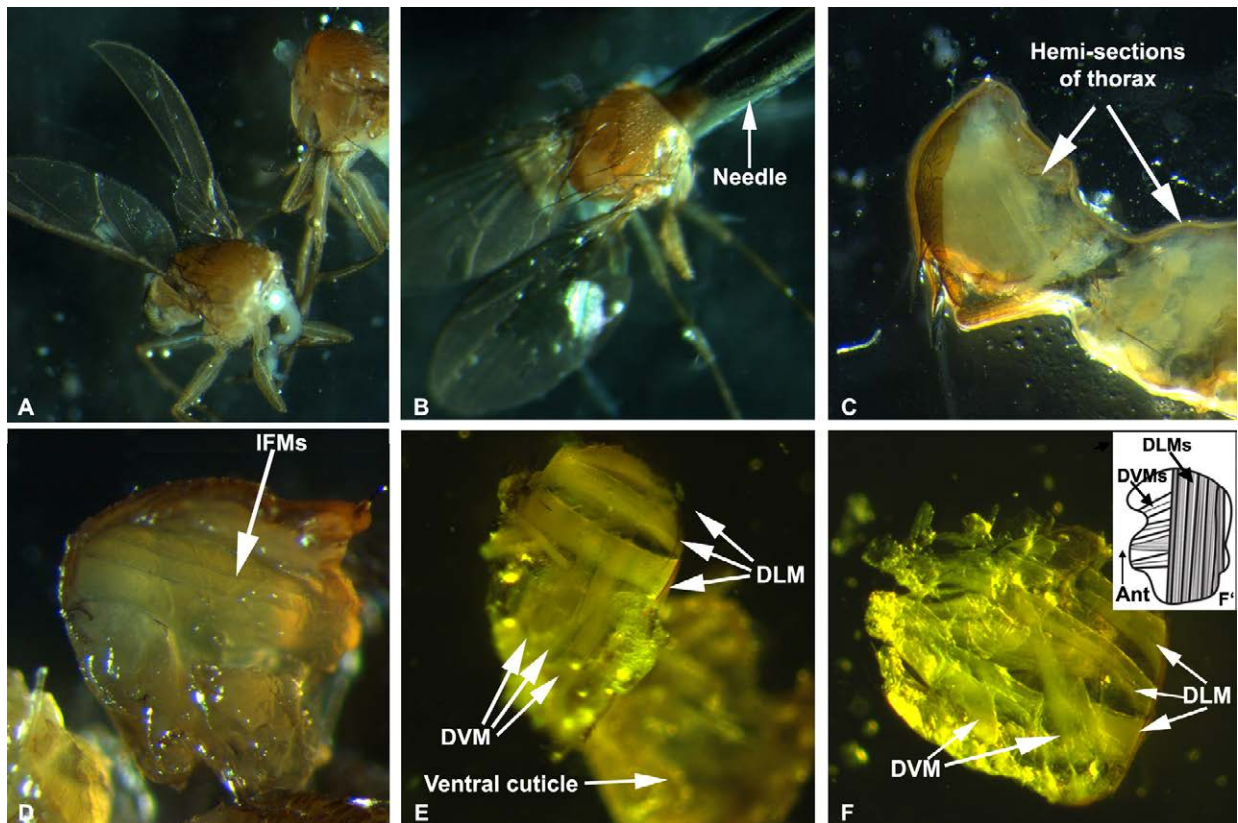


Figure 1: Steps in dissection and visualization of the thoracic Indirect Flight Muscles (IFMs) in adult fly . A. Head and abdomen removed using a fine needle, **B.** A needle inserted on anterior side of the thorax to make a vertical cut on dorsal side; **C.** Bisected thorax with two antero-posterior hemi-section; **D.** Addition of Bouin's fixative makes the **IFMs** more distinct; **E.** Dorsal-Ventral Muscles (**DVM**) underneath Dorsal Longitudinal Muscles (**DLM**); part of the **ventral cuticle** is seen on right; **F.** The **DLM** and **DVM** at higher magnification of a hemi-section; the inset in **F'** shows a schematic of **DLMs** and **DVMs**; Anterior side of thorax (**Ant** in **F'**) is towards upper-left in **E-F** and upwards in **F'** (**Ant**).

Observations:

Examine and draw the DLMs and DVMs under a stereo-binocular microscope using 5X or higher objective magnification.

Questions for self-study and interpretation:

1. How do the indirect flight muscles power the wing stroke?
2. Does poor development of wings, as in *vestigial* mutant flies, affect the development of IFMs?
3. Are the indirect flight muscles ‘voluntary’ or ‘involuntary’?
4. Why do some muscles become weak in certain neurodegeneration disorders/diseases?

References and further reading:

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Chapter 37

P-element mutagenesis and characterization of P-element insertional mutations

J. K. Roy* (jagatkroy@gmail.com) and S. C. Lakhotia (lakhotia@bhu.ac.in)

Cytogenetics Laboratory, Department of Zoology, Banaras Hindu University, Varanasi 221005, India

* For correspondence

Recommended Level: PG/Research

Sub-discipline: Genetics/Developmental Biology/Molecular Biology

Background:

P-element is a transposable DNA sequence in the genome of *Drosophila melanogaster*, which under certain conditions may be dislodged from its existing site and get inserted elsewhere in the genome. If it inserts in the regulatory or transcribed region of a gene, that gene may undergo insertional mutation with alterations in its expression, which may result in a mutant phenotype. Several other transposable elements (e.g., Copia, Hobo, Gypsy, etc.) are also present in the genome of *Drosophila melanogaster* (McCullers and Steiniger, 2017), which too can cause mutation in a gene due to their insertion or excision. In fact, many of the spontaneous mutant alleles known in *Drosophila melanogaster* are because of transposon induced mutation in the original wild type allele.

Exploitation of the P-element has revolutionized genetic studies. This transposon is 2907 bp long and is characterized by the presence of 31 bp long inverted terminal repeats and a gene coding for the transposase. The transposase enzyme, when active, causes excision of the P-element from one site and re-insertion at other site/s by acting on the terminal inverted repeat sequences. The site of re-insertion does not seem to require any specific target sequence. Since the P-element transposition requires only the transposase activity and the specific terminal repeat sequences, *Drosophila* geneticists devised elegant methods for controlled mobilization and/or insertion of a P-element sequence in *Drosophila melanogaster* genome. The basic principle for the use of P-element in genetic engineering is that any DNA sequence that has the P-element repeats on its two ends can be mobilized by the P-transposase, if available in the cell. The technique of P-element mediated germ line transformation of *Drosophila melanogaster*, developed in early 1980s by Spradling and Rubin, needs two classes of defective P-elements - one in which the internal transposase-coding sequence has been replaced by other DNA sequences desired to be introduced in *Drosophila* genome, and the other in which the terminal repeats are altered or deleted but the transposase coding sequences are intact (also nick-named as

‘wings-clipped’ P-element since this transposon cannot move because of the absence of the terminal repeats). The first category of P-element has the required terminal sequences to permit its excision and/or insertion by the transposase provided by the ‘wings-clipped’ P-element. In recent years, a large variety of P-element vectors have become available to suit specific requirements for germline transformation (see Ashburner, 1989).

Very efficient and versatile mutagenesis methods that use the above two type of defective P-elements have also been developed and employed with great success. The P-insertional mutagenesis does not present any environmental issues that accompany chemical or radiation mutagenesis. The general strategy of this mutagenesis is as follows. Two different stocks of *Drosophila melanogaster* carrying defective P-elements are used. One of them, called the “mutator” stock, carries a defective P-element which has the correct terminal repeat sequences but whose transposase-coding sequence has been replaced by the other desired marker and reporter gene sequences. The second stock, called “jump-starter” stock, carries a ‘wings-clipped’ P-element which cannot ‘jump’ but produces transposase. The “mutator” P-element by itself remains immobile due to lack of transposase. However, if the “mutator” and “jump-starter” elements are brought together in same genome through genetic crossing, the “mutator” element is mobilized by the transposase produced by the “jump-starter” in the same cell) and the mobilized “mutator” element may re-insert at any other site (on the same or different chromosomes) in the genome causing in its wake a mutation if the site of insertion happens in a functional (transcribed and/or regulatory) region. The presence of appropriate “markers” in the “mutator” element allows easy identification of the mutant individual and isolation of the mutant allele for its further characterization. Other versions of these mutator elements carry the essential parts of a bacterial cloning vector like the multiple cloning site, *ori* sequence and an antibiotic resistance marker. This allows direct cloning of the gene, or a part of it, after restriction digestion of the genomic DNA of the insertion mutant fly and transforming competent *E.coli*. Only the genomic DNA flanking the P-element insertion can be transformed in *E. coli* for growth as a plasmid (Bier *et al.*, 1981).

Another application of the insertion mutagenesis is to identify enhancers/promoters affecting specific structural gene and to study *in situ* expression of that gene (Enhancer trap mutagenesis). A typical enhancer detector P-transposon (e.g., the (*P-Lac*) w^+ developed by Bier *et al.*, 1989), which also permits plasmid rescue of flanking sequences, is shown in Fig. 1.

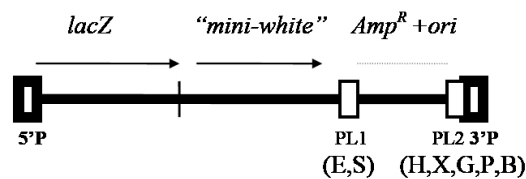


Fig. 1. Enhancer detector transposon in *Drosophila*: The 5'P and 3'P, the terminal repeats of the P-element enclose i) the *LacZ* gene of *E.coli* joined with the P-element's weak promoter, ii) the *mini-white* marker gene encoding a product for the normal red eye colour of *Drosophila*, iii) the *Amp^R* gene for beta-lactamase to confer ampicillin resistance, and iv) a bacterial origin of replication (*ori*) to allow replication and selection in *E.coli*; The PL1 (*EcoRI* and *SalI*) and PL2 (*HindIII*, *XhoI*, *BglII*, *PstI* and *BamHI*) are two multiple enzyme sites for cloning of the flanking genomic DNA by “plasmid rescue”.

The “reporter” gene (e.g., *LacZ* gene of *E.coli* coding for β -galactosidase enzyme) in such specially designed ‘enhancer-detector’ mutator P-element is close to the weak *P*-promoter at

the 5' terminal repeat of the P-element. Therefore, it has a high probability of being activated by a nearby genomic enhancer when this element gets inserted in the fly genome so that the *LacZ* gene would express wherever the original gene was expected to be active under the control of that enhancer/promoter. Expression of this reporter gene can be examined *in situ* by a simple cytochemical staining for β -galactosidase enzyme activity (see [Chapter 39](#)). Using this approach, a very large number of P-insert lines have been generated and maintained by individual investigators or *Drosophila* Stock Centers and many genes involved in different cellular activities or in pattern formation have been identified. An additional advantage offered by these lines, in the pre-genomic sequencing time, was the feasibility of cloning the flanking sequences of the mutated gene using the “plasmid-rescue” technique. The *GAL4-UAS* binary conditional expression system (see [Chapters 39, 40](#)) is a modification of the enhancer-trap P-transposon where the *LacZ* reporter is replaced by the yeast *GAL4* coding region.

Recent developments in genome editing through CASPER/R-CAS system (see [Chapter 38](#)) permit specifically desired mutations in the gene of interest. However, a large scale mutational screening still remains a method of choice when screening for modifiers of a given phenotype. P-element mediated mutational screening remains a powerful approach. The P-mutagenesis scheme is also useful in cases where the existing P-element based transgene may need to be mobilized to a new location in the fly genome. A basic scheme for generating insertional gene mutations using P-element is described here.

Objectives:

To induce random P-element mutagenesis and to chromosomally map the induced mutation.

Materials Required:

A. Fly stocks:

- i. *P-lac w⁺* or (also termed *P(w⁺)* or “Mutator” stock), developed by Bier *et al.*, (1989) in which the *P-Lac w⁺* transposon (**Fig. 1**) is inserted on a *y* and *w* (yellow body and white eye) mutant allele carrying X-chromosome so that these flies show yellow body and orangish-red eyes (due to the *mini-white* on the P-transposon). Two different mutator stocks, namely, *JSK1* and *JSK2* (JSK = ‘jump-starter kit’) are available, both showing yellow body and orangish eyes (darker in *JSK1* than in *JSK2*). The two stocks differ in the site of insertion of the *P-lac w⁺* on X-chromosome.
- ii. The “**Jump-Starter**” stock (also called *JSK17*) carries, on chromosome 3, the *delta 2-3 ($\Delta 2-3$)* P-element which has defective terminal repeats (‘wings-clipped’) and which also lacks intron 3 from the transposase gene. As a result, this P-element itself cannot jump but makes active transposase. The $\Delta 2-3$ carrying chromosome 3 is also marked with a dominant marker, *Stubble (Sb)* which causes body bristles to be short and thick; the other homologue of chromosome 3 is the *TM6B* balancer chromosome (see [Chapter 1](#)) marked with dominant *Tubby (Tb)* mutant allele. Thus the *JSK17* flies show white eyes, stubble bristles and tubby (short and fat) body.
- iii. The *JSK3* (*y w; TM3/TM6B*) stock carries chromosome 3 balancers (*TM3* and *TM6B*). They show white eye, Tubby body, Stubble bristles and dark body color (each of the balancer chromosomes carry the recessive *ebony (e)* mutant allele which lead to the dark body colour) (see [Chapter 1](#)).

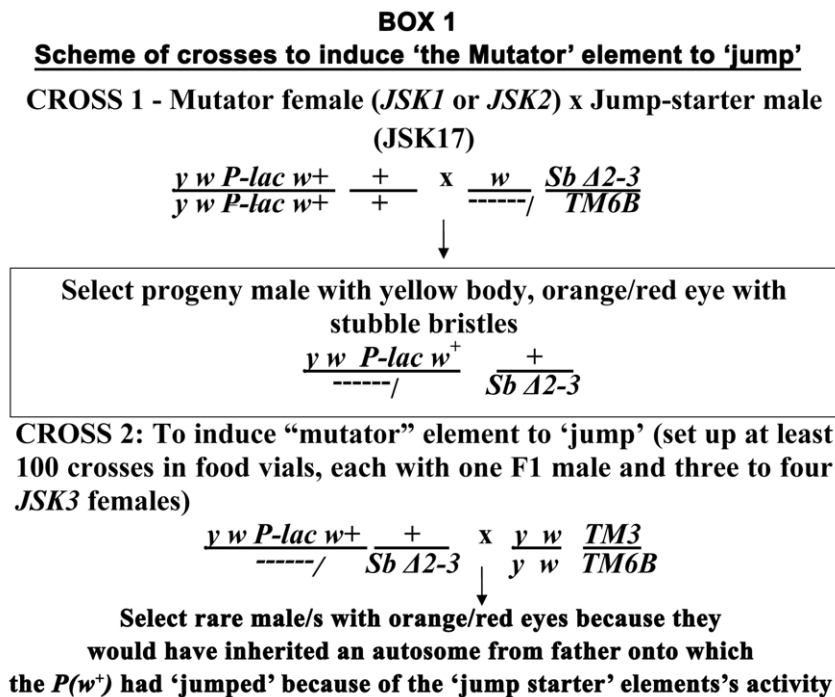
iv. *JSK4* (*y w*; *CyO/Sco*) stock carries chromosome 2 balancer *CyO*, with its homolog marked with *Sco*, which is a dominant second chromosome marker causing loss of scutellar bristles. They show yellow body, white eye, Curly wings and absence of scutellar bristles (see Chapter 1).

B. Equipment and other items

- i. Stereo-binocular microscope
- ii. *Drosophila* food bottles, vials and other items for fly handling.

Experimental Details:

1. Induction of P-insertional mutations: Set up the series of crosses shown in **Box 1**, firstly, to bring the “mutator” transposon (on X-chromosome) and the $\Delta 2-3$ *Jump-starter* transposon together in the F1 progeny (Cross 1), and secondly, to identify the rare male progeny which inherited an autosome from the male F1 parent with the “mutator” element inserted after ‘jumping’ out from the X-chromosome (Cross 2).



Note: The rare red eye males in the progeny of Cross 2 above may carry the *TM3* or *TM6B* or $\Delta 2-3$ chromosome as one of the chromosome 3 homologs. Since, both the *TM3* and the $\Delta 2-3$ chromosomes carry Stubble marker, an easy identification of presence of the $\Delta 2-3$ homolog in the rare male progeny may not be feasible. Therefore, to avoid any further destabilization of the *P-Lac w⁺* “mutator” element, it is better to select those rare red/orange eyed F2 males that carry the *TM6B* chromosome (i.e., who are “tubby”). However, if the available rare P males have stubble bristles (i.e. they carry either the *TM3* or the $\Delta 2-3$ chromosome), they may also be used for further crosses, but the *Sb*-marked chromosome will have to be eliminated during further crosses.

2. Ascertaining linkage of the jumped “mutator” P-transposon with Chromosome 3: Select one rare F2 male progeny from each of the Cross 2 vials above and cross each male with a group of three-four *JSK3* (*y w*; *TM3/TM6B*) virgin females in separate vials

(CROSS 3). Number each of these vials using the identification number given to each of the rare F2 male progeny. Follow the scheme of crosses as given in **Box 2**:

BOX 2
Scheme of crosses to determine linkage of the new P-insertion allele

A. Rare red/orange male with Tubby phenotype (carrying *TM6B* chromosome)

$$\begin{array}{ccc} \frac{y\ w\ +\ P-lac\ w^+(?)}{----/\ TM6B} \text{ Male} & \times & \frac{y\ w\ \underline{TM3}}{y\ w\ \underline{TM6B}} \text{ Female} \\ & \downarrow & \end{array}$$

PHENOTYPE OF PROGENY	RESULT	<i>P-lac w⁺</i> INSERTION
I. Are all <i>e Sb Tb</i> flies (<i>TM3/TM6B</i>) with white eyes (absence of <i>P-lac w⁺</i>)?	YES	On Chromosome 3
	NO	Not on Chromosome 3
II. Are all non-<i>ebony</i> and <i>Sb</i> (<i>TM3/+</i>) or <i>Tb</i> (<i>TM6B/+</i>) flies with red/orange eyes ?	YES	On Chromosome 3
	NO	Not on Chromosome 3

B. Rare red/orange male with Stubble bristles (carrying $\Delta 2-3$ or *TM3* chromosome)

$$\frac{y\ w\ +\ P-lac\ w^+(?)}{--/\ TM3} \times \frac{y\ w\ \underline{TM3}}{y\ w\ \underline{TM6B}}$$

OR

$$\frac{y\ w\ +\ P-lac\ w^+(?)}{--/\ \Delta 2-3} \times \frac{y\ w\ \underline{TM3}}{y\ w\ \underline{TM6B}}$$

PHENOTYPE OF PROGENY	RESULT	<i>P-lac w⁺</i> INSERTION
I. Are all non-<i>ebony</i>, non-<i>stubble</i> and <i>Tb</i> (<i>TM6B/+</i>) flies with red/orange eyes ?	YES	On Chromosome 3
	NO	Not on Chromosome 3
II. Are all non-<i>ebony</i>, <i>stubble</i> and non-<i>Tb</i> (<i>TM3/+</i>) flies with red/orange eyes ?	YES	On Chromosome 3
	NO	Not on Chromosome 3

Select the lines which on above criteria suggest the insertion to be on chromosome 3 and maintain them balanced with *TM6B* balancer.

Lines that are not on chromosome 3 by the above criteria, may have the *P-lac w⁺* transposon inserted on chromosome 2. This can be ascertained further by crossing them with the *JSK4* stock and examining segregation pattern in the progeny

Keep the new insertion lines on chromosome 2 and 3 balanced with the *CyO* or *TM6B* balancer chromosome, respectively.

Observations:

1. Record how many new ‘jump’ lines with the *P-Lac w⁺* transposon inserted on chromosome 2 and 3 were obtained.

Further studies:

1. Expression of the gene where the transposon is inserted can be monitored by the histo-chemical staining for β -galactosidase with X-Gal (see **Chapter 39**).

2. *In situ* hybridization of a labelled *white* gene probe to DNA in polytene chromosome preparations (see **Chapter 56**) can provide information about chromosomal location of the transposon insertion.

3. The conventional recombination mapping can be used to map the genetic region where the transposon is inserted.

4. The genomic sequences flanking the P-element can be identified by the ‘plasmid rescue’ cloning and sequencing of the cloned genomic DNA or by inverse PCR.

Questions:

1. Why are female progeny from *JSK1* (or *JSK2*) and *JSK17* cross (CROSS 1 above) not taken for identifying progeny flies in which the “mutator” transposon may have jumped?

2. Why does the appearance of rare orange/red eyed male in the F1 progeny indicate that the *P-Lac w⁺* transposon has jumped out of the X-chromosome?

3. Why do we take only one male and 2-3 females in a vial for crossing the F1 progeny with *JSK3* or *JSK4* female flies?

4. How can we identify the specific gene or chromosome region where the *P-Lac w⁺* transposon is inserted after the ‘jump’?

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Chapter 38

Gene knockouts in *Drosophila* using CRISPR-Cas9 based genome editing

Sushmitha Hegde¹ (sushmitha.hegde@students.iiserpune.ac.in), Prajna Nayak¹ (prajna.nayak@students.iiserpune.ac.in), Deepti Trivedi^{2*} (dtrivedi@ncbs.res.in), Girish Ratnaparkhi^{1*} (girish@iiserpune.ac.in)

1. Indian Institute of Science Education & Research, Pune

2. National Centre for Biological Sciences, Bengaluru

* For correspondence

Recommended Level: PG/Research

Sub-discipline: Genetics /Molecular Biology

Background:

Drosophila is extensively used for understanding the genetic basis of cellular and organismal function. To enable genetic analyses, fly researchers have relied on random mutants generated by either X-rays or chemicals or by the insertion of transposons (St Johnston, 2002, also see [Chapter 37](#)). Although these methods have been powerful in identifying novel functions of genes, they are labor-intensive and time-consuming, especially when a mutant/knockout allele for a specific gene is required. In recent years, several sequence directed endonucleases that induce double-stranded DNA breaks in a sequence-specific manner have been used to generate targeted mutations in the organism. The target-specific methods employing Zinc-finger nucleases (ZFNs) and Transcription activator-like effector nucleases (TALENs) require extensive designing and cloning, making them less lucrative as compared to the use of the more recent Clustered Regularly Interspaced Palindromic Repeats (CRISPR)/Cas9 method (Gupta *et al.*, 2019).

The CRISPR based genome engineering system is adapted from components of a naturally occurring mechanism of adaptive immunity in bacteria. It relies on Watson-Crick base pairing between the target genomic DNA and a target-specific 18-20 nucleotides (nt) region present in a single guide-RNA (sgRNA) for homing of the Cas9 endonuclease that generates a double-stranded break (DSB) at the target locus (Doudna and Charpentier, 2014; Gratz *et al.*, 2013). Since its first usage in 2013, this system has been optimized for genomic manipulations in a large number of organisms, including *Drosophila*.

Briefly, in this two-component system, a Single-Guide RNA (sgRNA) recognizes the target DNA and guides the Cas9 protein to induce a double-strand break (DSB). The sgRNA can be engineered to contain target-specific 20 nt at its 5' end and a constant double-stranded 3' structure that allows recruitment of the Cas9 protein. The only requirement for the DSB induced by Cas9 seems to be the presence of a

Protospacer Adjacent motif (PAM) at the target locus, in the form of an NGG trinucleotide sequence at the 3' end of the 20 nt sequence. The role of CRISPR-Cas9 ends once the DSB is generated. The DSB triggers two possible cellular DNA repair pathways. The error-prone Non-Homologous End Joining (NHEJ) repair pathway, which joins the cut ends together, sometimes leads to small insertions and/or deletions (indels), thereby disrupting gene function. On the other hand, the Homology Directed Repair (HDR) pathway is precise, and an exogenous donor repair template flanked by homology arms can be incorporated at the desired region (Bier *et al.*, 2018). The 2020 Nobel Prize in Chemistry (<https://www.nobelprize.org/prizes/chemistry/2020/summary/>) was awarded to Drs. Emmanuelle Charpentier and Jennifer Doudna for the discovery of the CRISPR/Cas9 gene-editing tool.

Here, we describe the design and methodology of creating loss-of-function (lof) alleles in *Drosophila melanogaster* using CRISPR-Cas9 via the NHEJ repair pathway (Fig. 1).

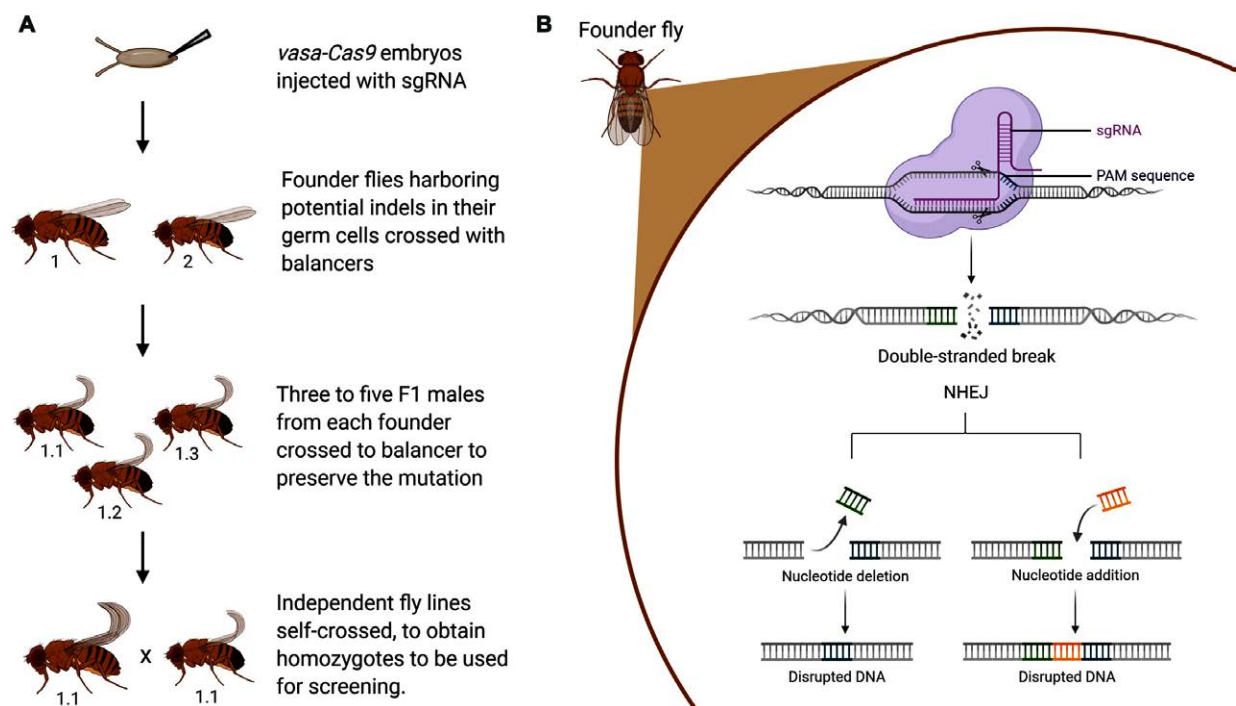


Fig. 1. Generating loss-of-function alleles using CRISPR-Cas9. **A.** Scheme of crosses, following micro-injection of the sgRNA, to obtain *lof* allele: the sgRNA, designed to target the desired gene's disruption, is injected in 2 hr old embryos in which the Cas9 is expressed in germ cells. **B.** Schematic of NHEJ dependent disruption of the target gene: the sgRNA, DNA and Cas9 form a complex, leading to a double-stranded break in the target DNA, which activates the DNA repair machinery, specifically the NHEJ pathway, leading to the incorporation of insertions/deletions, which disrupt the gene (graphics generated using BioRender, <https://biorender.com/>).

Objectives:

Learning principles of designing, generating and maintaining CRISPR edited allele of a desired target gene in *Drosophila melanogaster*.

Materials Required:

1. Flies: Suitable fly lines, expressing Cas9 protein in germ cells. These flies can be procured from the Bloomington *Drosophila* Stock Center (BDSC), and include *nos-Cas9*, *vasa-Cas9* and *Actin5C-Cas9*. See https://bdsc.indiana.edu/stocks/genome_editing/crispr_cas9.html, for detailed genotypes.

2. Equipment and other materials:

- i. Stereo-binocular microscope and other fly handling materials
- ii. Micro-injection setup for fly embryos
- iii. Thermocycler
- iv. Agarose gel running apparatus

3. Reagents:

- i. Sequence-specific primers for cloning gRNA
- ii. pBFv-U6.2 plasmid (<https://www.addgene.org/138400/>)
- iii. Standard PCR reagents (Taq polymerase, dNTPs, etc)
- iv. Competent cells
- v. LB Agar and Broth
- vi. Plasmid mini- and midi-prep kits

Experimental Details:

A. Designing a suitable gRNA: The following criteria are to be considered while designing the gRNA: (i) choice of target genomic locus based on specific requirement (ii) length of gRNA target, (iii) expected efficiency of the gRNA, and (iv) possibility of off-target effects.

Depending upon specific requirements, the choice of target site can be very specific or relatively broad based. For example, a simple method for generating a *loss of function* (*lof*) allele is to disrupt the coding region of the target gene. To achieve this, in most cases a single gRNA sufficiently proximal to the translation start site can be used to introduce indels via NHEJ. Alternatively, two gRNAs can be used to delete a larger intervening region. However, when a specific nucleotide needs to be deleted or changed, the choice is greatly reduced.

Once the target site is chosen, PAM sites are recognized in the region. For the *S. pyogenes* Cas9 enzyme, the PAM sequence is 'NGG'. 18-20 nt towards the 5' region of the PAM site is the target region for Cas9. If the region of interest contains multiple target sites, BLAST these gRNA options against the reference genome for possible off-targets. Any genomic region that has an identical sequence in 12 nucleotides most proximal to PAM is a potential off target and hence must be avoided. A large number of empirical data has provided some understanding of what makes an efficient gRNA. Based on this data, several algorithms have been developed and are available to use online. One such tool is CRISPR optimal target finder (<https://flycrispr.org/target-finder/>) that can be used to identify prospective

gRNAs with minimal off-target effects. Tools for efficiency prediction can also be used (<https://www.flyrnai.org/evaluateCrispr/>).

Once an optimal 20 nt target sequence is chosen, it can be cloned into the sgRNA plasmid under the appropriate promoter. The gRNA sequence should begin with a G, for efficient transcription under the U6 promoter. Alternatively, a G can be added at the 5' end while designing primers during cloning. Note that although it is an absolute requirement for the target genomic sequence to be preceded by a PAM sequence, the gRNA itself does not contain the PAM sequence.

B. Cloning the gRNA sequence into a plasmid:

The 20-nt sequence targeting the site of modification is cloned into the pU6-2-BbsI-gRNA vector using the following protocol (adapted from <https://flycrispr.org/protocols/grna/>).

1. Design of PCR primers:

forward primer: 5' – G (19 nt) GTTTTAGAGCTAGAAATAGCAAG – 3'

reverse primer: 5' – GAAGTATTGAGGAAAACATACCTATATAAATG – 3'

- i) The 19 nt forward primer sequence is the target sequence at the genomic locus.
- ii) The reverse primer is common/standard.

2. Amplify the pU6-2-BbsI-gRNA plasmid:

1.0 µL pU6-BbsI-gRNA (1 pg–1 ng)

2.5 µL 10 µM Forward Primer

2.5 µL 10 µM Reverse Primer

10 µL 5X Q5 Reaction Buffer

1.0 µL 10 mM dNTPs

0.5 µL Q5 High-Fidelity DNA Polymerase

Nuclease-Free Water to 50 µL

Thermocycling Conditions: Initial melting at 98°C for 30 sec, 35 cycles: 98°C for 10 sec, 62°C for 30 sec, 72°C for 2 min and final extension at 72°C for 10 min.

3. Run the PCR product on an agarose gel and purify, using standard protocols.

4. Ligate the PCR product.

5. Assemble the following reaction:

50 ng of PCR Product from above

2 µL of 10X T4 DNA ligase buffer

1 µL T4 Polynucleotide Kinase

1 µL DpnI restriction enzyme

Nuclease-free water to 20 µL total

6. Incubate at 37°C for 30 min.

7. Add PEG4000 to 5% final w/v and 1 µL of T4 DNA ligase. Mix thoroughly.

8. Incubate for 10 min at room temperature and then place on ice.

9. Transform competent *E. coli* with 4 µL of the reaction, screen and select the desired transformed colonies.

10. Purify the plasmid to use for micro-injection..

Note: Alternatively, transgenic flies expressing sgRNAs are available publically for a subset of genes in the *Drosophila* genome as part of the TRiP-CRISPR Knockout (TRiP-KO) repository (<https://fgr.hms.harvard.edu/trip-knockout>). These fly lines can be used to set up crosses with Cas9 expressing flies to yield mutant lines, which can be identified phenotypically or via molecular screening. A detailed protocol can be found at <https://fgr.hms.harvard.edu/vivo-crispr>

C. Injection of sgRNA plasmid into *Drosophila* embryos:

Fly stocks harboring stable insertions of Cas9 from BDSC (e.g. #51323, *Vasa-Cas9*:#54590, *Act5C-Cas9*:#78781, *nos-cas9*). The sgRNA plasmid is injected into Cas9 embryos (<https://flycrispr.org/protocols/injection/>).

D. Generation of balanced mutant lines:

1. Cross flies emerging from injected embryos with appropriate balancer lines, depending on the chromosome location of the targeted gene.
2. Balance three to five resultant F1 males (putative mutant/balancer) with a balancer to preserve the mutation; Once balanced, these individual lines can be propagated via self-crossing, and used for molecular screening.

Results/Observations:

1. If the CRISPR editing has resulted in a recessive lethal mutation, mutant homozygotes will not survive. Such mutation should be kept balanced with an appropriate balancer chromosome.
2. Any other recessive phenotypic (including behavioral) changes can be assessed visually in homozygous flies or in mutant/deficiency genotype.
3. Use the primers flanking the expected site of modification for PCR amplification and Sanger sequencing to verify the presence of the desired indel (**Fig. 2**).

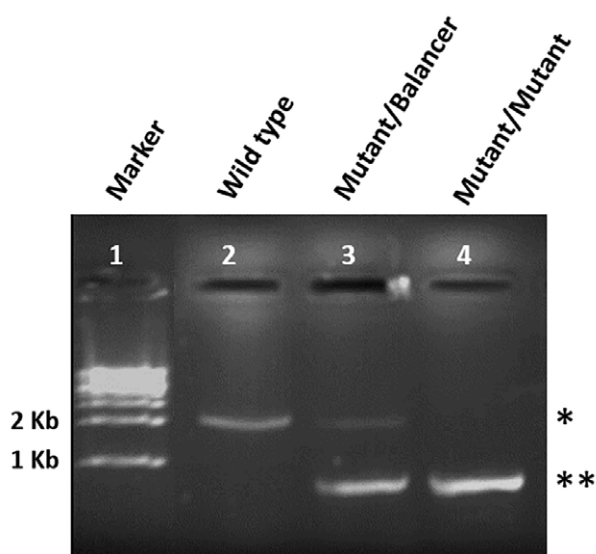


Fig. 2. PCR-based screening to detect a successful gene knockout after a CRISPR genome editing experiment. A deletion spanning ~1 kb in the gene CG3838 was generated using a dual gRNA strategy, creating a *lof* mutant allele. PCR Primers flanking the expected deletion were designed for detection of the *lof* allele in a population of flies such that the homozygous mutant fly (Lane 4) would generate an ~1 kb amplicon (**) while in the unmodified wild-type fly (Lane 2), a PCR product of 2 kb is generated (*), as expected for the intact gene. The mutant/Balancer heterozygous fly (Lane 3) yields both the PCR products (* and **). PCR products were run on a 1% agarose gel and stained with Ethidium Bromide for visualization.

Questions for self-study and interpretation:

1. Why is CRISPR/Cas9 better than other genome editing technologies?
2. Does CRISPR/Cas9 genome editing lead to off-target effects in the *Drosophila* genome?
3. Does the PAM motif, 'NGG' for Cas9 limit the choice of target sites? Can one engineer or discover Cas proteins that have other/diverse recognition motifs?
4. When would you use single versus dual gRNA for planning a genome editing experiment?

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Chapter 39

The *GAL4-UAS* system to monitor gene activity patterns and to induce ectopic expression in *Drosophila melanogaster*

Prachi Yadav¹ (pallu.prachi@gmail.com), Vanshika Kaushik² (vanshikakaushik@gmail.com), Neha Tiwari² (tiwarin495@gmail.com), S. C. Lakhotia^{2*} (lakhotia@bhu.ac.in) and Jagat K. Roy^{2*} (jagatkroy@gmail.com)

1. Division of Genetics, Indian Agricultural Research Institute, New Delhi, 110 012, India.

2. Cytogenetics Laboratory, Department of Zoology, Banaras Hindu University, Varanasi 221005, India.

*For correspondence

Recommended Level: UG/PG/Research

Sub-discipline: Genetics/Cell Biology/Developmental Biology/Molecular Biology

Background:

Visualizing expression of a gene in a tissue-specific manner is essential for understanding function/s of the gene. Development of the P-element based germline transformation and the enhancer-trap systems in *Drosophila* (see Chapter 37) have provided a versatile strategy to modulate expression of a gene as desired. The P-element system permits synthesis of chimeric genes comprising a well-defined promoter (with conditional or tissue-specific expressivity) and transcribed region of the desired gene of the same or different organism in a transgenic system. However, this strategy has certain limitations. The conditional promoter (like the heat shock promoter) may cause the given gene to express globally, rather than in a tissue-specific manner or the experimental expression of the reporter gene in a particular cell type may be grossly deleterious so that such lines cannot be established or maintained. Such situations can be avoided by using a conditional or tissue-specific promoter in a “binary” system where expression of gene of interest will take place in temporally and/or spatially regulated manner when the desired gene and its chimeric transcriptional regulator gene are brought together in the progeny by crossing two parental transgenic lines. The two transgenes are normally maintained in separate transgenic parental lines without any phenotypic consequence.

The *GAL4-UAS* system (Fig. 1) developed in *Drosophila melanogaster* (Brand and Perrimon, 1993) is a “Binary” system of gene expression where the desired gene can be selectively expressed in cell and developmental stage specific pattern. The *GAL4* is transcription factor identified in Baker’s yeast as a regulator of galactose induced genes. The *GAL4* operates through an Upstream Activating Sequence (*UASG* or *UAS*) present in promoters of the regulated target genes (Traven *et al.*, 2006). Generally, no endogenous gene in *Drosophila* is known to be activated by *GAL4* and the expression of

GAL4 protein has also not been found to have any effect in flies. However, the *GAL4* can activate transcription in fruit flies if a transgene carries the *UAS* sequence in its promoter region. Brand and Perrimon (1993) exploited this feature where a promoter (enhancer) of *Drosophila* regulates the expression of *GAL4* which in turn directs *GAL4* responsive expression of target gene carrying the *UAS* in the promoter region. Consequently, when a transgenic line carrying the *GAL4* gene downstream of a *Drosophila* gene's promoter or a promoter activated under certain conditions (conditional promoter like the heat shock inducible promoter) is crossed with another transgenic line carrying the desired gene (or a reporter gene like the coding sequences for the *E. coli lacZ* (producing the β -galactosidase protein) or the green fluorescent protein (*GFP*) downstream of the *UAS* promoter, the progeny shows abundant expression of the desired target gene (Fig. 1). Expression of the reporter gene can be easily monitored and, therefore, permits one to identify the spatial and temporal domains of expression of the promoter that drives the *GAL4* driver.

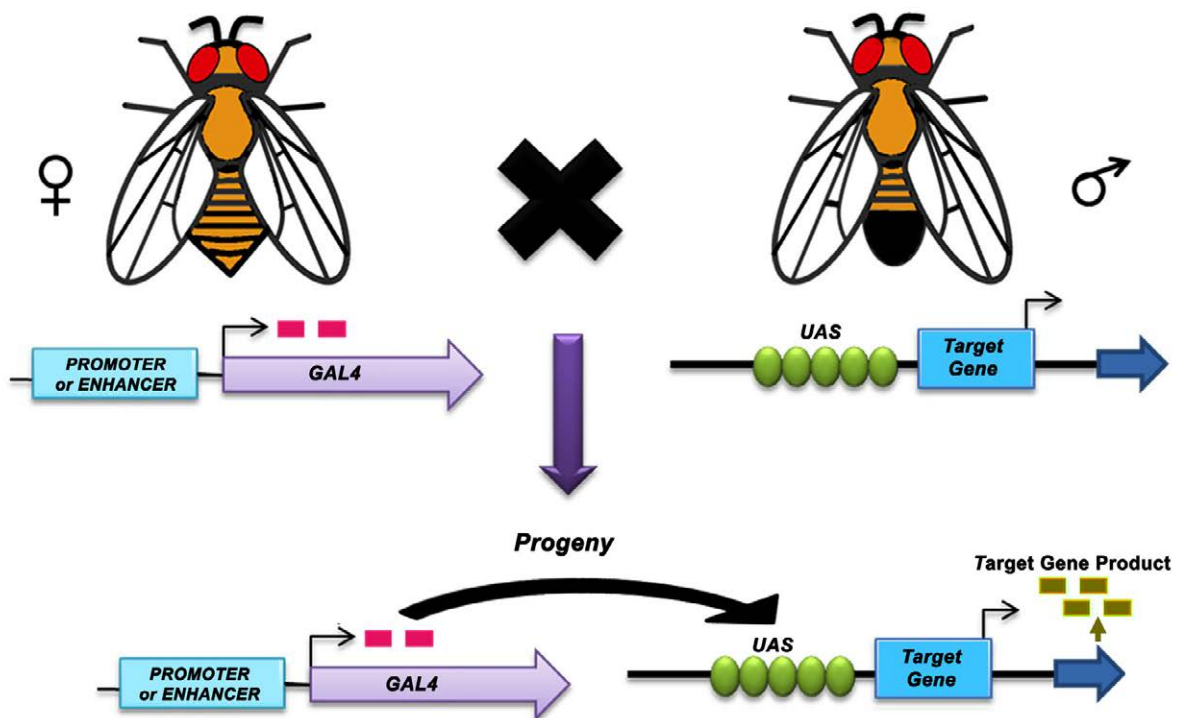


Fig. 1. The GAL4-UAS binary system. Progeny of the cross between the two transgenic parents brings the *GAL4* driver and the *UAS*-responder in same individual leading to the directed expression of the target as driven by the *GAL4* 'driver', which itself is expressed under control of the desired promoter.

The *GAL4-UAS* system has been further modified leading to development of more versatile "enhancer trap", system (Kaiser, 1993). Restricted expression of *GAL4* in certain cells of *Drosophila* can be achieved either by fusing the *GAL4* gene to a well characterized *Drosophila* promoter or by randomly integrating an enhancer-less *GAL4* gene in the fly genome. This is done through P-insertional mutagenesis scheme (see Chapter 37) using a *P-GAL4* transposon (Fig. 2), whose random integration in the fly genome brings the enhancer-less *GAL4* coding region under the control of a nearby genomic enhancer. Accordingly, the *GAL4* expression shows varying spatial and temporal patterns in different 'enhancer-trap' lines reflecting activity of the nearby enhancer. The

pattern of expression of the *GAL4* in the given ‘enhance trap’ line can be assayed by crossing the same to the *UAS*-reporter (e.g., *UAS-lacZ* or *UAS-GFP*) transgenic line and monitoring the reporter expression in the hybrid progeny. While monitoring of the *UAS-GFP* expression requires fluorescence (or confocal) microscopy (see Chapter 40), the *UAS-lacZ* expression can be easily monitored using the X-gal histochemical staining of tissues as described here. Cellular localization of the blue colored product provides information or ‘reports’ about the spatial and temporal expression of the specific promoter. This permits easy identification of cell types in which the *GAL4* is specifically expressed under the adjoining enhancer in the given ‘enhancer-trap’ line.

Once the ‘enhancer-trap’ line that expresses *GAL4* in the specific cells of one’s interest is identified, it is possible to express any desired gene in that tissue by making germ line transformants with a transposon vector carrying the desired target gene downstream of the *UAS* promoter. Appropriate P-vector (e.g., *pUAST*) carrying the *GAL4*-responsive *UAS* region upstream of the cloning sites is used for such transformation (Fig. 3). Using such strategies, a large number of *GAL4* driver lines have been generated and characterized in different laboratories around the world.

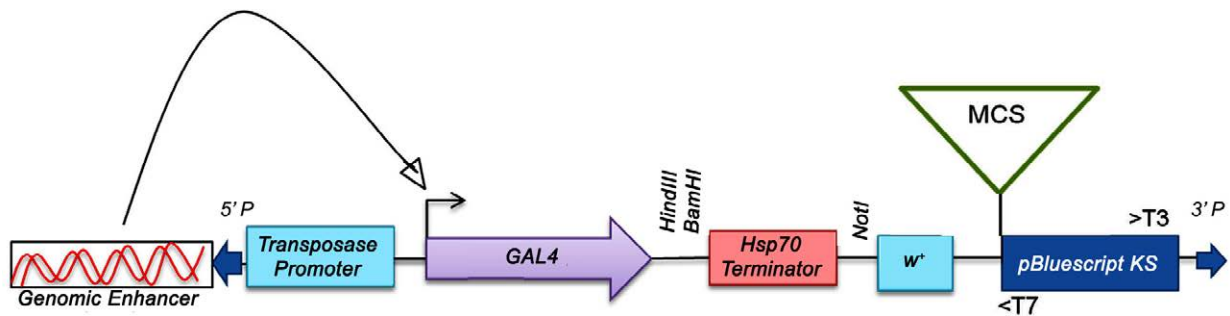


Fig. 2 : Schematic of the pGAWB vector commonly used as a p-GAL4 transposon for generating ‘enhancer-trap’ lines. A genomic enhancer, near the site of insertion of the transposon, regulates expression of the *GAL4* coding region via the basic P-transposase promoter. The w^+ is the ‘mini-white’ gene which generates red eyes (used as a selection marker) in the transgenic w -fly; the 5’P and 3’P represent the 5’ and 3’ ends, respectively, of the P-element; MCS, <T7 and >T3 indicate the multiple cloning site, T7 and T3 promoters, respectively, associated with the *pBluescript* vector backbone.

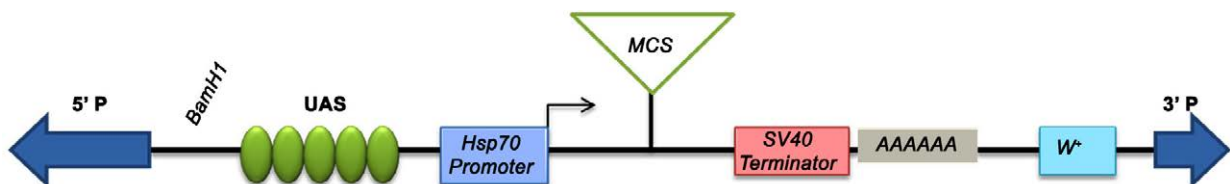


Fig. 3. Schematic of the *pUAST* vector commonly used for P element-based transformation to generate the *GAL4*-responder transgenic lines. This vector contains *UAS* sequence, *heat shock protein 70* (*Hsp70*) gene’s basal promoter (TATA box of *Hsp70* Promoter), the multiple cloning sites (MCS), SV40 small *t* intron polyadenylation signal (*SV40* Terminator and AAAAAA) and the ‘mini-white’ (w^+) gene; 5’ P and 3’ P represent the 5’ and 3’ ends, respectively, of the P-element.

This chapter describes two different approaches to examine expression domains of the *decapentaplegic* (*dpp*) gene which encodes the Dpp morphogen, a homolog of

mammalian TGF β , that regulates cell fate, patterning, and growth during *Drosophila* development (Padget et al 1987). One set of approaches (A and B) monitors the domain/s of expression of Dpp through *dpp-GAL4* driven reporter gene assay (e.g., the *LacZ* encoded β -galactosidase) using embryos or larval wing imaginal disc, In the other approach (C), developmental effects of *dpp-GAL4* driven ectopic expression of the *UAS-ey* transgene is examined. The *eyeless* (*ey*) gene product Eyeless is homolog of the mammalian Pax-6 transcription factor which plays a major role in development of the central nervous system and eye development (Tsonis and Fuentes, 2006). Development of ectopic eye/s in fly's other appendages following ectopic expression of the *ey* gene using the GAL4-UAS system (Halder *et al.*, 1995) provides an interesting model for developmental genetics studies.

Objectives:

- i. To identify cells that express *decapentaplegic* (*dpp*) gene in *Drosophila* embryos and in wing imaginal discs using *dpp-GAL4* driver and *UAS-lacZ* responder fly lines through X-Gal staining.
- ii. To induce development of ectopic eyes in *Drosophila melanogaster* using *dpp-GAL4* driver and *UAS-ey.H* responder transgene.

Materials Required:

A. Transgenic fl lines:

- i. *w¹¹¹⁸*; *P{w^{+mC}=UAS-lacZ.B}Bg4-1-2* (referred to as *UAS-lacZ*; transgene inserted on chromosome 2).
- ii. *w**; *dpp-Gal4/TM6B, Tb¹ e* (referred to as *dpp-GAL4*; transgene inserted on chromosome 3).
- iii. *y w**; *UAS-ey.H* (transgene inserted on chromosome 2).

B. Reagents for X-gal staining:

- i. Embryo wash buffer: 0.4% NaCl and 0.03% Triton X-100.
- ii. 5% Bleach solution (5% solution of bleaching powder in distilled water) for removal of the chorion layer of embryos.
- iii. Heptane (to remove the vitelline membrane from embryos to permeabilise them).
- iv. 1X Phosphate Buffered Saline (1X PBS):
 - a. Sodium Chloride (1.37 M) 8.0 g
 - b. Potassium Chloride (27 mM) 0.2 g
 - c. Disodium hydrogen phosphate (100 mM) 1.42 g
 - d. Potassium dihydrogen phosphate (20 mM) 0.272 g

Dissolve the salts in 800 mL distilled water, and adjust pH to 7.4 adding 1N-HCl, as required. Finally add distilled water to make the final volume 1000 mL. Autoclave and store at 4°C.

- v. 4% Paraformaldehyde: Dissolve 400 mg of paraformaldehyde in 10 mL 1X PBS by heating at 65°C with rapid stirring till completely dissolved. Store at 4°C (use within a week).
- vi. 1 M Na-Phosphate Buffer (pH 8.0)
 - a. 1 M Na₂HPO₄·2 H₂O (17.8 g in 100 mL H₂O) 3.2 mL
 - b. 1 M NaH₂PO₄·2H₂O (15.6 g in 100 mL H₂O) 6.8 mL
- vii. 1 M Na-Phosphate buffer (pH 8.0) with 0.2% Triton X-100.
- viii. 50 mM Na-Phosphate buffer (pH 8.0).
- ix. 1 mM Na-Phosphate buffer (pH 8.0).
- x. 80% Glycerol in PBS.
- xi. X-GAL (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining solution

Stock soln	Amount	Final Conc.
5% X-GAL (5 mg dissolved in 100 μL Dimethylformamide)	60 μL	0.3%
100 mM K ₃ [Fe(CN) ₆]	20 μL	2 mM
100 mM K ₄ [Fe(CN) ₆]	20 μL	2 mM
1 M Phosphate buffer (pH 8.0)	50 μL	50 mM
Dist. Water	850 μL	

- xii. Embryo Wash Chamber: a nylon cloth/mesh is tied at one end of a both-ends open 1" x 3" plastic/glass tube with a rubber band (see Chapters 32, 34).

Experimental Details:

A. X-gal staining of embryos:

1. Cross *w¹¹¹⁸*; *P{w^{+mC}=UAS-lacZ.B}Bg4-1-2* and *dpp-Gal4/TM6B, Tb¹ e* flies from the stocks and collect eggs for a period of 2-3 hr.
2. Transfer the eggs (12-16 hr after egg laying for this experiment) with a brush to the embryo wash chamber. The chamber with embryos supported on the nylon mesh is repeatedly dipped in distilled water in a beaker to remove any adhering food-particles (for details see Chapters 32, 33, 34).
3. Dechorionate embryos by dipping the wash chamber with embryos in a 5% bleaching solution kept in a suitable dish for 2-3 min. Monitor under a stereobinocular microscope to check when the egg filaments at the anterior end of embryos disappear, which indicates that the dechoriation is complete.
4. Wash embryos by passing the wash chamber with embryos supported on the nylon mesh through several changes of distilled water to remove traces of bleaching solution. Finally wash the embryos with the embryo wash buffer.

5. Transfer embryos with the help of a soft brush from the wash chamber to a 1.5 mL microfuge tube containing 500 μ L of heptane.
6. Immediately remove the heptane and add a 1:1 mixture of freshly prepared fixative (paraformaldehyde) and heptane. Shake the tube vigorously for ~5 sec and then let it stand for 10 min. Repeat vigorous shaking and standing two more times at 5 min intervals (total fixation time = 20 min). Adequately permeabilized embryos should form a single layer at the organic/aqueous interface.
7. Remove fixative and heptane layers as much as possible.
8. Add 200 μ L of 50 mM Na-Phosphate buffer with Triton X-100; briefly spin the tube and remove the liquid.
9. Add 50 μ L X-gal staining solution. Keep the tube in dark at 37°C until embryos show blue colour (a few hours to overnight).
10. After the staining, wash embryos with 50 mM Na-Phosphate buffer (pH 8.0).
11. Mount the embryos in 80% Glycerol in PBS: on a 'bridge' slide to prevent damage to embryos by pressure of coverslip. Place the embryos in the gap between the two supports on the slide and then place a 22 mm² coverslip on the two supporting coverslip pieces on the 'bridge' slide.

Note: A 'bridge' slide is prepared by fixing two small broken pieces of coverslip on a clean slide with DPX, leaving a gap of 1 cm between the two pieces. Let the DPX dry before using the 'bridge' slide for mounting embryos.

B. Procedure for X-gal staining of larval imaginal discs:

1. Collect eggs from healthy *w¹¹¹⁸; P{w^{+mC}=UAS-lacZ.B}Bg4-1-2; dpp-Gal4/TM6B* flies for a period of 2-3 hr and let the larvae grow to late third instar stage.
2. Select non-Tubby larvae (for Tubby phenotype [see Chapter 1](#)) since only these larvae would have both the *dpp-Gal4* (on chromosome 3) driver and the *UAS-lacZ* responder (on chromosome 2) transgenes.
3. Using fine needles, dissect out wing imaginal discs ([see Chapter 18](#)) from the non-Tubby late third instar larvae in 0.67% NaCl or in PBS and transfer the discs to a cavity block/Maximov slide/normal slide with fresh saline or PBS.
4. Remove saline and add 50-100 μ L of 4% paraformaldehyde; fix for 10 min.
5. Remove fixative and wash the discs 3x with 50 mM Na-Phosphate buffer.
6. Remove buffer and add 50 μ L X-gal staining solution to the cavity block/Maximov/normal slide (cover with a coverslip if the tissue is in cavity block/Maximov) and keep in a moist chamber in dark at 37°C for 20-30 min.
7. Wash the discs in 50 mM Na-Phosphate buffer and mount them on a clean glass slide using 80% glycerol. Examine under microscope.

C. Induction of ectopic eyes following *dpp-GAL4* driven expression of *UAS-ey.H*

1. Set a cross using 3-4 *dpp-GAL4* virgin females and 3-4 *UAS-ey.H* males in a standard food vial (a reverse cross can also be set). Keep the vials at 24°C.
2. Transfer the parental flies to a new food vial after 4-5 days to increase the progeny number and to avoid overcrowding in the vial.
3. Examine the progeny non-Tubby pupae.

Observations:

A. Pattern of *dpp-GAL4* driven expression of *UAS-lacZ* reporter gene in embryos:

Only some of the surviving progeny embryos from the $w^{1118}; P\{w^{+mC}=UAS-lacZ.B\}Bg4-1-2; dpp-Gal4/TM6B$ parents would show the X-Gal staining since embryos that inherit the *TM6B* balancer chromosome from parents would not carry the *dpp-Gal4* transgene carrying chromosome 3 and, therefore, would not express the *UAS-lacZ* transgene. Only the $w^{1118}; P\{w^{+mC}=UAS-lacZ.B\}Bg4-1-2/+; dpp-Gal4/+$ progeny embryos would show X-Gal staining.

Note: *TM6B* homozygous embryos die early.

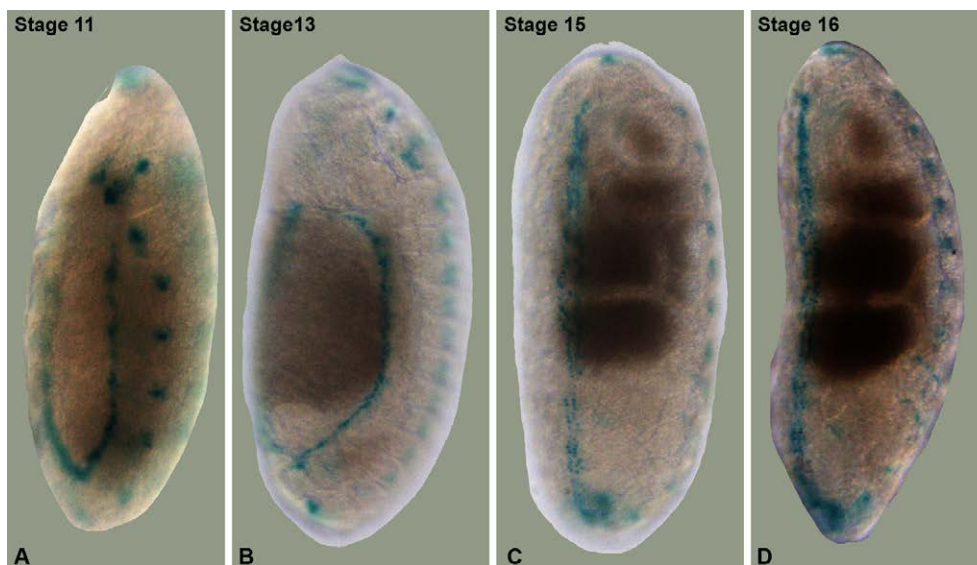


Fig. 4. The *dpp-GAL4* driven *UAS-lacZ* expression in $w^{1118}; P\{w^{+mC}=UAS-lacZ.B\}Bg4-1-2/+; dpp-Gal4/+$ embryos show stage-specific pattern. A, B, C and D represent embryonic development stages 11, 13, 15 and 16, respectively. The reporter blue color following X-Gal staining indicates activity of the *dpp* gene's promoter in defined domains at these stages of embryonic development.

The *dpp-GAL4* driven expression of *lacZ* gene (encoding β -galactosidase), placed downstream of the *UAS*-promoter, is seen along the dorsal most epithelial cells of the lateral epidermis at different stages of development like the germ band extended stage (Stage 11), dorsal closure stage (Stage 13), Stage 15 and Stage 16 (Fig. 4).

Note: A brief description of embryonic stages is given in [Chapters 2, 32](#).

B. Pattern of *dpp* expression in larval wing imaginal disc:

The late third instar larval wing discs show blue color along the cells of the anterior-posterior axis due to the *dpp-GAL4* driven expression of the *UAS-lacZ* reporter so that the β -galactosidase synthesized in these cells converts X-Gal into blue colored product (Fig. 5A). The image shown in Fig. 5B confirms that the X-Gal staining seen in Fig. 5A faithfully reproduces the normal developmental pattern of expression of the *dpp* gene since immunostaining (see Chapter 58) of *dpp-lacZ* wing discs, in which the *lacZ* reporter is expressed directly under the control of the *dpp* enhancer-promoter, shows the same pattern.

Rationale and chemistry underlying the X-GAL staining: Depending upon the cell types in which the *GAL4* driver is produced, the responder *UAS* causes transcription of the *lacZ* gene resulting in synthesis of the bacterial β -galactosidase. The β -galactosidase enzyme normally uses galactose as the substrate. The *lacZ* gene encoded β -galactosidase acts on the yellow-colored chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) to produce indigo-blue colored product. X-Gal is converted by the β -galactosidase into its indolyl derivative which in turn is oxidized to the blue colored product, the 5,5'-dibromo-4,4'-dichloro-indigo. Potassium ferricyanide is used as a catalyst for oxidation of the indolyl product. Potassium ferrocyanide is also added in equal proportion to prevent over-oxidation of the indolyl derivative to a colorless compound. Without the added oxidizing agent, the initial products of β -galactosidase reaction, the indole monomers, may diffuse out of the cell before being oxidized and dimerized.

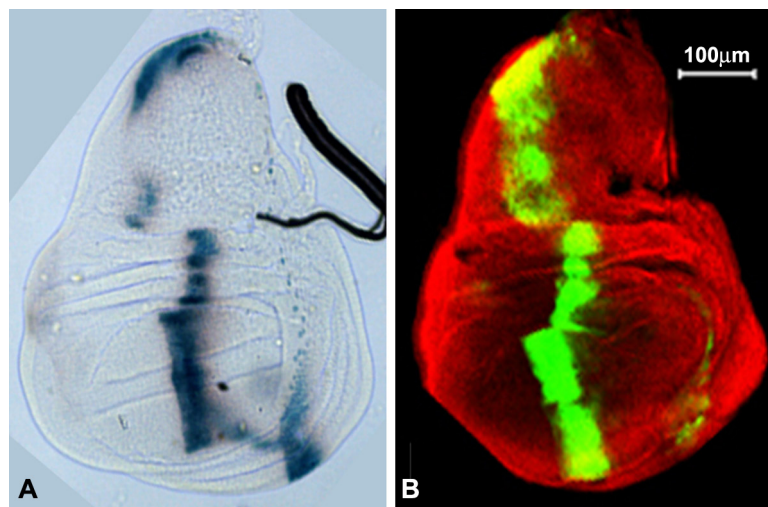


Fig. 5. Expression domain detected by the *dpp-GAL4>UAS-lacZ* binary system faithfully reflect the *dpp* promoter's normal activity. **A.** X-Gal histochemical staining to localize the β -galactosidase activity in *dpp-GAL4* driven *UAS-lacZ* expressing late third instar larval wing imaginal disc: the deep blue color along the anterior–posterior axis represents the specific region in which the *dpp* gene is expressed. **B.** Pattern of β -galactosidase expression (green signal) following immuno-staining (see Chapter 58) with anti- β - galactosidase antibody in a *dpp-lacZ* late third instar larval wing imaginal disc (in this enhancer-reporter transgene construct, the *dpp* enhancer-promoter is placed upstream of the reporter *lacZ* gene and thus directly drives expression of the *lacZ* reporter). Note the near identity between the domain of *dpp* activity detected by the *dpp-Gal4>UAS-lacZ* binary system and the *dpp-lacZ* transgene. The red fluorescence signal in **B** indicates distribution of the FasIII protein as detected by the anti-FasIII antibody.

The X-GAL staining in the above examples provide an indirect readout of the expression of the *dpp* gene during normal development. The close match between the indirect readout in the binary *GAL4>UAS* system (Fig. 5A) and the direct readout in *dpp-lacZ* (Fig. 5B) validates the reliability of the *GAL4-UAS* system. The *GAL4*-driven reporter transgenes are widely used to map the developmental expression of specific genes. The *UAS-GFP* (or its variants like *UAS-RFP* or *UAS-YFP*) is another very popular reporter (see Chapter 40) since it permits high resolution in vivo imaging.

C. Induction of ectopic eyes in progeny expressing *dpp-GAL4* driven *UAS-ey.H* transgene

Using a stereo binocular microscope, examine mid- or late-stage (pharate) progeny pupae for presence of the normal pair of compound eyes and multiple ectopic eyes on antennae, thorax cuticle, wings and/or legs. The presence of normal and ectopic eyes is visible through the brown pupal case of mid- or late-stage (pharate) pupae (Fig. 6A, B). For greater clarity, the pupal case can be carefully removed with fine needles to examine the eye more closely on the body of developing pharate (Fig. 6C, D).

Note: The *dpp-GAL4>UAS-ey.H* individuals mostly die at late pupal stage due to multiple developmental abnormalities following the *dpp-GAL4* driven ectopic expression of the Eyeless transcription factor.



Fig. 6. Induction of ectopic eyes following *dpp-GAL4* driven expression of *UAS-ey.H* transgene. A. Side view of mid-stage wild type pupa with normal red eyes (white arrow). B-D. Side views of *dpp-GAL4>UAS-ey.H* mid-stage pupa (B) or pharates (C, D) dissected out of their pupal cases, showing multiple ectopic eyes (white stars) on head, thorax, wings etc, in addition to the normal eyes on head (white arrows).

Questions for self-study and interpretation

1. What are the advantages of studying gene expression using the binary system over studying the same through the promoter-reporter construct?
2. What is an ectopic expression? Can this be used to learn about functions of a gene?
3. Suppose a transgene with the mammalian Pax-6 encoding gene (homolog of the *Drosophila eyeless* gene) cloned downstream of the UAS promoter was expressed in *Drosophila melanogaster* using the *dpp-GAL4* driver. Would this result in the development of ectopic eyes? If yes, would the eyes be like those in mammals or like the compound eyes present in *Drosophila*?
4. A toxin producing gene, whose expression can kill a cell, is cloned downstream of the UAS promoter. How can you use the UAS-toxin transgene to understand functions of specific cells in which a given GAL4 driver is expressed?
5. Can *Drosophila* promoters and enhancers be functionally characterized using this binary system?
6. Can the GAL4-UAS binary system be used to generate loss of function phenotypes of gene using RNAi technique? How?

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Chapter 40

***GAL4-UAS* system for genetic labeling and visualization of specific regions of brain**

Shamprasad Varija Raghu (shamprasadvarijaraghu@gmail.com) and
Rajashekhar K. Patil* (patilsirmu@gmail.com)
Neurogenetics Lab, Dept of Applied Zoology, Mangalore University,
Karnataka 574199

* For correspondence

Recommended Level: UG/PG/Research

Sub-discipline: Gene Expression/Neurobiology

Background:

Cellular functions are regulated by controlled levels of gene expression and production of specific proteins. Different signaling pathways control the initiation and regulation of gene expression. The commencement of transcription, the process in which DNA sequences get transcribed into mRNA, is controlled by binding of transcription activator to enhancer sequences found in DNA. It then recruits transcription machinery to the site to induce gene expression. These cellular processes can be visualized or controlled using different genetic tools. *GAL4-UAS* binary system is one such transcription activation system that has been co-opted from yeast into *Drosophila* (Brand and Perrimon, 1993; Raghu *et al.*, 2018). In recent decades, this system has become a *Drosophila* geneticist's main workhorse and is widely used for gene manipulations (on/off or gene overexpression/down regulations, etc.).

The *GAL4-UAS* binary system involves crossing a *GAL4*-driver and the *UAS*-responder fly lines (**Fig. 1**). In the *GAL4* driver transgenic line, the promoter region of the gene of one's interest controls expression of coding region of the yeast transcriptional activator, *GAL4*. The *UAS*-responder transgenic line carries the *GAL4*-responsive upstream activating sequence (*UAS*) is placed upstream of the desired gene's transcribed region. In the progeny of such a cross, the responder transgene is expressed in cells in which the given promoter placed upstream of the *GAL4* coding region drives expression of the *GAL4* transcriptional activator. *GAL4* binds with the *UAS* and this leads to activation of the responder gene (**also see Chapter 39**). The *UAS-GFP* is commonly used as a responder line to identify specific cells in which the given promoter driving the *GAL4* synthesis is active. In the *UAS-GFP* transgene, the green fluorescent protein (*GFP*) coding region is cloned downstream of *UAS* region taken from yeast. Thus the *GFP* fluorescence acts as the reporter gene for the promoter driving expression of the *GAL4* transcription factor. Only in

those cells or neurons that express the *GAL4*, the transcription of the *GFP* reporter gene will be activated which can be visualized by recording the fluorescence. In different approaches, modulators/toxic proteins that can alter the activity of the targeted cell can also be used as *UAS*-reporter to manipulate expression of gene/s in target cells.

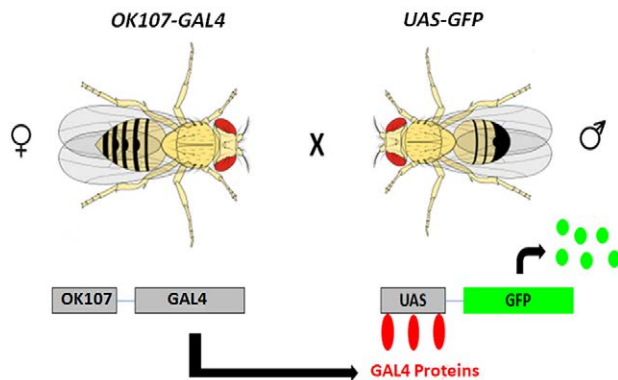


Fig. 1. The GAL4-UAS system to study expression of genes in targeted region in the brain. Here a transgenic line driving expression of *GAL4* in the Mushroom Body (MB) region (e.g. *OK107-GAL4*) is crossed with *UAS-GFP* line. In the F1 progeny it is expected to see GFP expression in MB region.

A number of *GAL4* driver lines that express the *GAL4* in different regions of the central nervous system are available (see **Table 1**). The *OK107-GAL4* driver expresses in the Mushroom body (MB) of the adult brain (**Fig. 1**). MB is the site of memory formation in *Drosophila* and other insects (see **Chapter 2**). A convenient way to monitor the expression of the given promoter is to cross the *GAL4* driver expressing line with a *UAS-GFP* transgenic line. In the present experiment, which uses progeny larvae from a cross between the *OK107-GAL4* and *UAS-GFP* parents, only the MB region in brain of F1 progeny flies will fluoresce green (due to the GFP expression).

Name of GAL4 driver	Domain of Expression
<i>Elav-GAL4</i>	Pan-neuronal expression
<i>GMR-GAL4</i>	Expression in photoreceptors
<i>GHI46-GAL4</i>	Expression in projections neurons
<i>OK107-GAL4</i>	Expression in Mushroom Body
<i>Cha-GAL4</i>	Expression in cholinergic neurons
<i>vGAT-GAL4</i>	Expression in GABAergic neurons
<i>vGlut-GAL4</i>	Expression in Glutamatergic neurons
<i>DDC-GAL4</i>	Expression dopaminergic and serotonergic neurons
<i>TH-GAL4</i>	Expression in Dopaminergic neurons
<i>TRH-GAL4</i>	Expression in Serotonergic neurons
<i>DB331-GAL4</i>	Expression in LPTCs

Objective:

To label a sub-population of neurons in *Drosophila* brain using the *GAL4-UAS* binary system.

Materials Required:

1. *GAL4* driver flies (*OK107-GAL4*)
2. *UAS*-reporter flies (*UAS-GFP*)
3. Phosphate Buffer Saline (PBS): To prepare 1000 mL of 1× PBS solution, dissolve 8 gm of NaCl, 0.2 gm of KCl, 1.44 gm of Na₂HPO₄ and 0.24 gm of KH₂PO₄ in 800 mL of H₂O. Adjust the pH to 7.4 with 1 N-HCl and then add H₂O to make 1000 mL final volume.
4. 1% PBT: Mix 10 μL Triton X-100 in 1 mL PBS (prepare fresh)
5. 4% Paraformaldehyde (4% PFA): Dissolve 4 gm of PFA in 100 mL of 1X PBS (the 4% PFA solution needs to be prepared fresh).
6. Vectashield (mounting media)
7. Slides and coverslips
8. Fine-tipped forceps for dissection
9. Stereo-binocular and fluorescence microscopes

Experimental Details:

1. Cross virgin females from the *OK107-GAL4* driver line with males from the *UAS-GFP* reporter line. Reverse cross (*UAS-GFP* virgin females and *OK107-GAL4* males can also be made.)
2. Grow the cultures at 24-25°C.
3. Remove parents to new food vials after 4-5 days to avoid mixing with their progeny.
4. Select F1 progeny female flies (3-5 days old) for dissection (larger in size of females than males makes it easier to dissect the brain).
5. The flies are immobilized by keeping them at 4°C for 30-60 min or may be anesthetized. The brains are removed under a stereo-microscope by carefully opening head region using two fine-tipped forceps and fixed in 4% paraformaldehyde for 30 min at room temperature.
6. Subsequently, the brains are washed for 45–60 min in 1% PBT.
7. The washed brains can be immunostained with anti-DLG primary antibody and corresponding secondary antibody (see [Chapter 58](#)) to visualize rest of the brain regions.
8. Mount the brain on slide in 1X PBS for microscopy.

Note: Anti-DLG antibody binds to Disc Large protein in the post-synaptic regions of all the neurons, and therefore, DLG staining highlights all the major anatomical regions in the brain. However, this staining procedure may be avoided to view only the GFP expression.

9. Observe the samples under fluorescent microscope (480 nm excitation for *GFP*).

Note: Confocal microscopy would give sharper and brighter images.

Results:

Fluorescence microscopic examination of the brain preparations, using 10X or 20X objective, reveals that Mushroom body (MB) region in the central nervous system (CNS) gives very prominent green fluorescence signal (**Fig. 2**) because the *OK107-GAL4* driver induces *UAS*-directed gene expression specifically in the MB region. The Green Fluorescent Proteins (*GFP*) under the control of the *UAS* region thus helps to visualize the Mushroom body in the entire brain. The *OK107-GAL4* driver, besides its predominant expression in the MB region, also expresses in some neurons in the antennal lobe and ocelli region (see **Chapter 2**), which also, therefore, show green fluorescence. The background anti-DLG staining is used for observing brain anatomy.

The *OK107-GAL4* driven expression is highly specific to MB region and, therefore, can be further used for gene manipulation experiments to understand the role of MB region/ neurons.

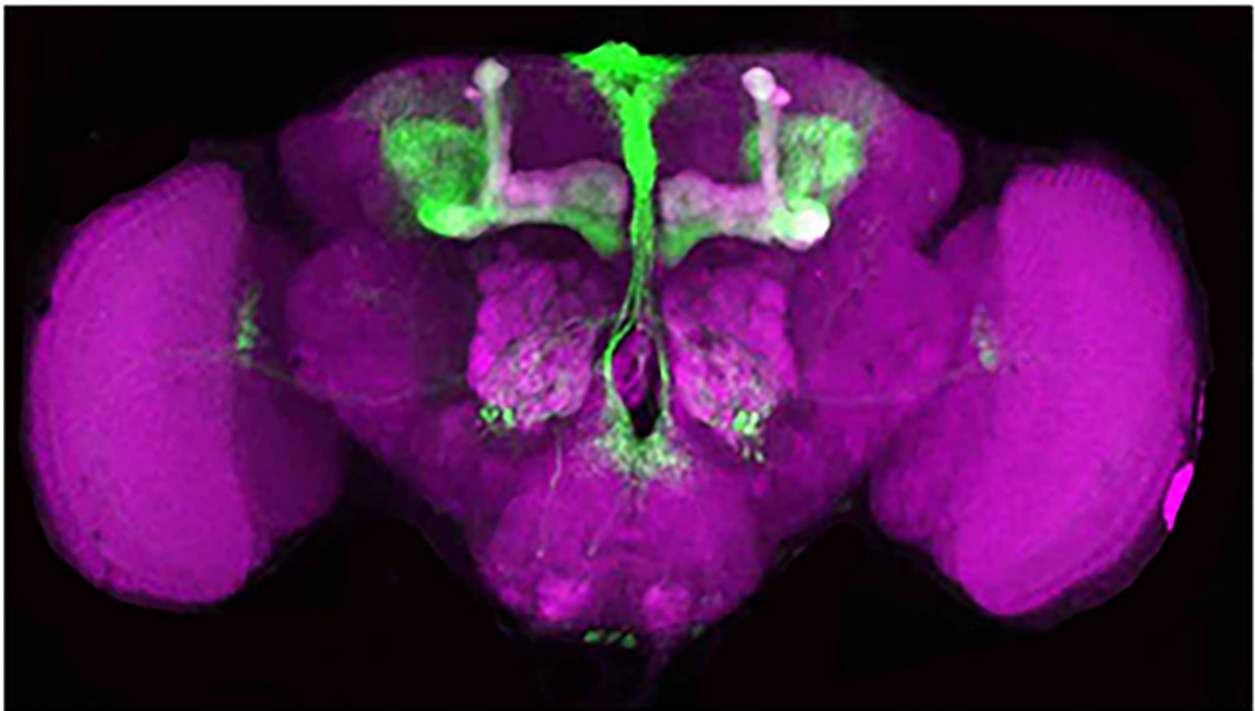


Fig. 2. The MB in CNS of adult flies shows strong *UAS-GFP* expression (green fluorescence) when driven by the *OK107-GAL4* driver. The anti-DLG immunostaining of the brain is seen in purple. The *OK107-GAL4* driver, besides its predominant expression in the MB region, also expresses in some neurons in the antennal lobe and ocelli region, which too, therefore, show *GFP* signal.

Questions for self-study and interpretation:

1. What makes each *GAL4* driver to be so specific?
2. How can the *GAL4-UAS* system be used to understand the functional properties of neuronal populations?

3. Can the *GAL4-UAS* system be used in other animal models like *Caenorhabditis*, mouse, zebra fish etc?

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Importance of Regulatory Mutations

From examination of instability of genic action at a number of known loci in maize, it is concluded that mutations need not express changes in genes, but may be the result of changes affecting the control of genic action.

(In: McClintock, B. 1953. Induction of instability at selected loci in maize. *Genetics*, 38: 579 <https://www.genetics.org/content/genetics/38/6/579.full.pdf>)

Chapter 41

Cell lineage analysis using GAL4-dependent G-TRACE patterns

Akanksha Pandey (akanksha.pandey4@bhu.ac.in), S. C. Lakhotia (lakhotia@bhu.ac.in) and Jagat Kumar Roy* (jagatkroy@gmail.com)

Cytogenetics Laboratory, Department of Zoology, Institute of Science, Banaras Hindu University, Varanasi-221005

* For correspondence

Recommended Level: Research

Sub-discipline: Cell Biology/Developmental Biology/Neurobiology

Background:

Our knowledge of the morphogenetic processes during the development of multicellular creatures has greatly benefited from studies on cell lineages in a tissue. Complex tissues are made up of diversity of cell types that develop from a smaller number of precursors. Determining the lineages that make up a given tissue can provide a better understanding of the developmental processes that lead to formation of a multicellular structure. Lineage tracing is the process of identifying a single cell and the cohort of its offspring in a tissue, which allows determination of the number, position and differentiation status of the founder cell's progeny. The first lineage-tracing studies using direct observation of living embryos were performed by C. O. Whitman and his followers at the end of the 19th century (Fox *et. al.*, 2008; Hsu, 2015; Kretzschmar and Watt, 2012). Subsequently, a variety of approaches, ranging from direct observations to the use of "vital dyes" and other reagents (e.g., radioactive molecules and intracellular tracers) have been used to physically or directly identify specific cells of interest in a developing tissue (Fox *et. al.*, 2008; Hsu, 2015; Kretzschmar and Watt, 2012).

In recent times, methods for lineage tracing have undergone a remarkable transformation, thanks to the rapid developments in genomic technologies since the 1990s. Many lineages have now been identified using genetic markers like fluorescent proteins or enzymes (beta-galactosidase and alkaline phosphatase). Targeted activation and maintenance of the expression of a reporter gene in the descendants of a cell in a tissue, provides a convenient approach to trace the cells' origins and their lineage-relations during tissue formation. It is now possible to activate transgenic reporters in the genome through several site-specific DNA recombination techniques using GAL4-UAS, FLP/FRT and other similar strategies. In addition, genetic approaches allow labelling of all cells descended from a single progenitor. Some of the recently developed lineage-tracing approaches are: Positively Marked Mosaic Lineage (PMML) method (Liu and Hou 2008), the GAL80-Based Mosaic Analysis with a Repressible Cell Marker (MARCM) technique (Liu and

Hou, 2008, [see Chapter 81](#)), the Twin Spot Generator (TSG, Lee 2009, [see Chapter 42](#)) and the GAL4 Technique for Real-time and Clonal Expression (G-TRACE, Evans *et. al.*, 2009; Ledru *et. al.*, 2022; Lee, 2009; Olson *et. al.*, 2019).

The G-TRACE system, created by the UCLA Undergraduate Research Consortium (Olson *et. al.*, 2019), is a *Drosophila* cell lineage screening tool using GAL4/UAS, FLP/FRT and fluorescent reporters in combination to create cell clones that offer spatial, temporal and genetic details regarding the genesis of distinct cohort of lineage-related cells in *Drosophila* (Casas-Tintó *et al.*, 2017; Evans *et. al.*, 2009; Ledru *et. al.*, 2022; Lee 2009; Olson *et. al.*, 2019). Unlike the MARCM and TSG methods for generation of marked clonal population of cells ([see Chapters 42, 81](#)), the G-TRACE system permits simultaneous identification of the progenitor stem cell and the cohort of differentiated progeny cells produced by the stem cell.

The GAL4/UAS ([see Chapters 39, 40](#)) and the FLP-FRT systems ([see Chapters 42, 81](#)), both derived from Yeast, are widely used tools in *Drosophila* research. *FLP* or *Flip* is a recombinase encoding gene, while *FRT* (Flip Recombination Target) is the DNA sequence on which the FLIP recombinase makes a cut and allows recombination between two FRT sites (Fisher *et. al.*, 2017; Voutev and Hubbard 2008). In the commonly used approach in *Drosophila*, the FLP is made to express under an inducible promoter so that the recombination event can be induced as and when desired. For inducing homologous recombination in somatic cells, the two FRT sites are required to be present at specific sites on the two homologs, so that the FLP-mediated recombination at FRT sites produces recombined and non-recombined sister chromatids. This system is commonly used for generating somatic clones (Fig. 1A, also [see Chapters 42, 81](#)). On the other hand, if the two FRT sites are present in similar orientation on the same chromosome, the FLP-mediated recombination event between these two sites will involve a looping of the intervening chromosome region, which will get removed or flipped out (Fig. 1B). This ‘flip-out’ strategy for the removal of stop codon or part of the exon within a gene (Fig.1B) is used in the G-TRACE system.

In the G-TRACE system (Fig. 2), the Red Fluorescent Protein (RFP) reporter encoding transgene is placed downstream of the UAS sequence and the desired cell specific GAL4 driver is brought in the progeny’s genome through appropriate genetic crossing, so that all cells which express the desired GAL4 driver, also produce the RFP reporter on a real time basis. The ‘real time’ refers to the developmental time when the desired specific GAL4 is/was active in the progenitor cell whose lineage is being traced. The same cell also carries the FLP/FRT system, with the FLP encoding gene under a UAS promoter, while the two FRT sites flank a stop codon within the Enhanced Green Fluorescent Protein (EGFP/GFP) coding gene placed under a ubiquitously expressing promoter like the *Ubi^{p63E}*. Thus, in the RFP expressing cells, the FLP (recombinase) will also be produced which causes flipping out the FRT-flanked stop codon within the GFP encoding region by recombination between the two FRT sites (Fig. 2). Consequently, these cells and their progeny will start and continue to express the *Ubi^{p63E}* promoter driven GFP expression but may stop expressing RFP transgene if the progeny cells acquire new developmental fate which silences the progenitor cell specific GAL4 driver (Fig. 2). The continuing GFP expression

permits easy tracing of lineage of these cells to the original RFP-producing cell. On the other hand, if the fate of the original RFP-expressing cell's progeny is not changed, they will produce both the RFP (under the cell specific GAL4) and GFP (under the ubiquitous GAL4) and therefore show yellow fluorescence (red + green).

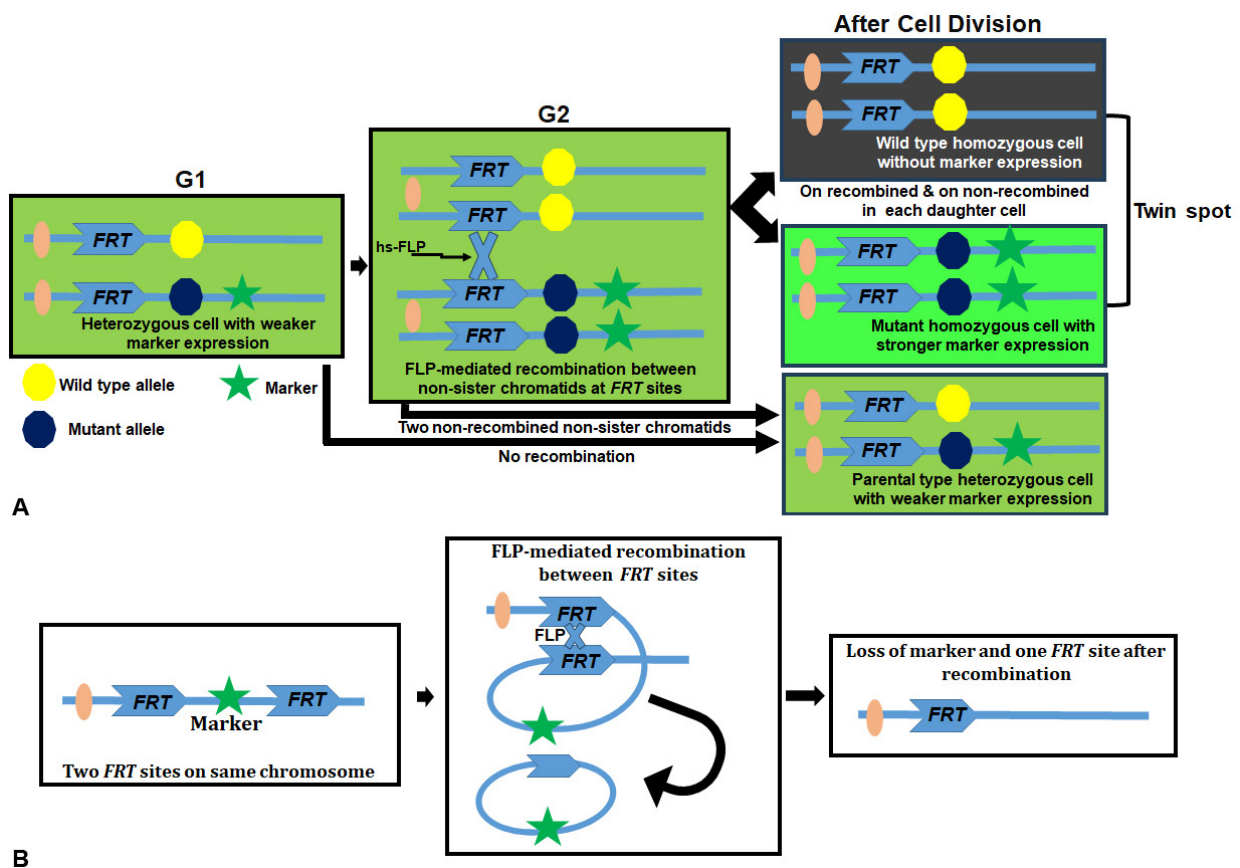
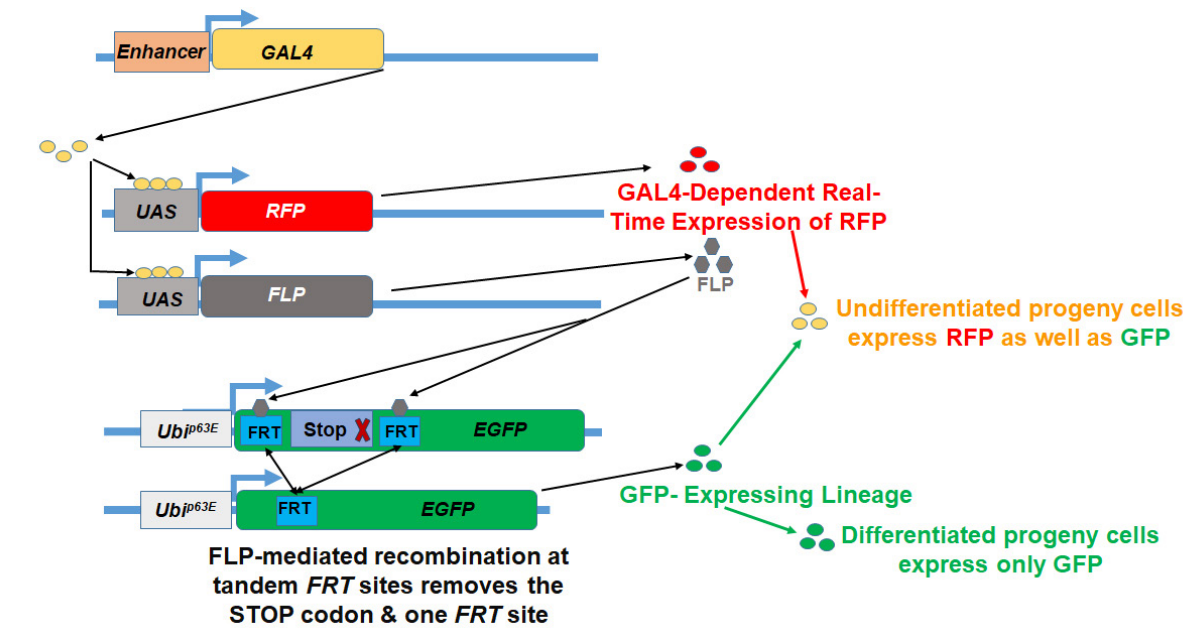


Fig. 1 : The FLP/FRT system for site-specific recombination in *Drosophila* to induce somatic recombination between homologous chromosomes to generate ‘twin spots’ (A) or for ‘flipping out’ intervening region between two tandem FRT sites (B).

The *Drosophila* gut has three major regions, viz., the foregut, midgut, and hindgut. The crop and Malpighian tubules emerge from the boundaries of the foregut/midgut and midgut/hindgut, respectively. The longest part of the gut is the midgut, which is classified into the anterior, middle, and posterior midgut regions based on differences in tissue constriction and pH (see Chapter 16 and Fig. 4). The adult *Drosophila* gut and its populations of stem cells originate from the gut progenitor cells present in larval gut. The adult midgut progenitors (AMPs) are present as small clusters on the outer surface of the larval midgut epithelium. The progenitor cells of adult hindgut are present as a ring in the hindgut proliferation zone (HPZ), a restricted area located at the anterior end of hindgut (Deng et al., 2019). In addition to the AMPs, larval midgut also contains large enterocytes (ECs) and enteroendocrine (EE) epithelial cells. The G-TRACE approach helps in tracing the division patterns of the AMPs and HPZ cells in the late larval gut.



A

G-TRACE Flow-Chart

The Stem Cell-specific-GAL4 driven G-TRACE to identify stem cells and their differentiated progeny cells

- A Stem Cell-specific promoter drives RFP expression : Red fluorescence in Stem Cells
- The Stem Cell-specific promoter driven Flippase expression leads the stop codon located upstream of GFP coding region in the *Ubi63-eGFP* transgene to 'flip-out' due to recombination between the flanking *FRT* sites – these cells show Red as well as Green fluorescence

AS DEVELOPMENT PROCEEDS

B

Stem cells do not change fate

- Show only Red fluorescence (stop codon in *Ubi63-eGFP* transgene not 'flipped out')
- OR
- Show Red as well as Green fluorescence

Stem cells or their progeny differentiate

- Stem Cell-specific promoter inactive: RFP expression lost
- Progeny cells show only GFP expression driven by the constitutive *Ubi63* promoter

Fig. 2 : The G-TRACE system. **A.** GAL4 expression under the desired tissue-specific enhancer/promoter, leads to UAS-driven expression of RFP (red) and FLP-recombinase. The FLP-expression by the same GAL4 driver leads to removal of the FRT-flanked STOP cassette located between the ubiquitously expressing *Ubi^{63E}* promoter and the EGFP encoding region, leading to expression of GFP protein inheritably in daughter cells irrespective of any change in their fate. **B.** A flow-chart indicating the sequence of events in the G-TRACE system, which permits developmental tracing of a cell's lineage.

In the example here, the *inscuteable-GAL4* (*insc-GAL4*) driver is used to illustrate lineage tracing of stem cells in the midgut region of 3rd instar larvae. Although the *insc-GAL4* driver is commonly known for its expression in neuroblasts and in the young neurons of the central and peripheral nervous systems (Casas-Tinto et al., 2017), its expression is also seen in mid- and hind-gut stem cells (Ferveur et al., 1997; Pandey and Roy,

2024). The *insc-GAL4* driver induces expression of *UAS-RFP* because of which these cells show red fluorescence; the *insc-GAL4* driven *UAS-FLP* transgene expression also induces ‘flip-out’ of the stop codon in the *Ubi^{65E}-EGFP* so that these cells begin to express green fluorescence. Since many large endo-replicated enterocytes in midgut show green fluorescence, their lineage can be traced to the *insc-GAL4* expressing AMPs.

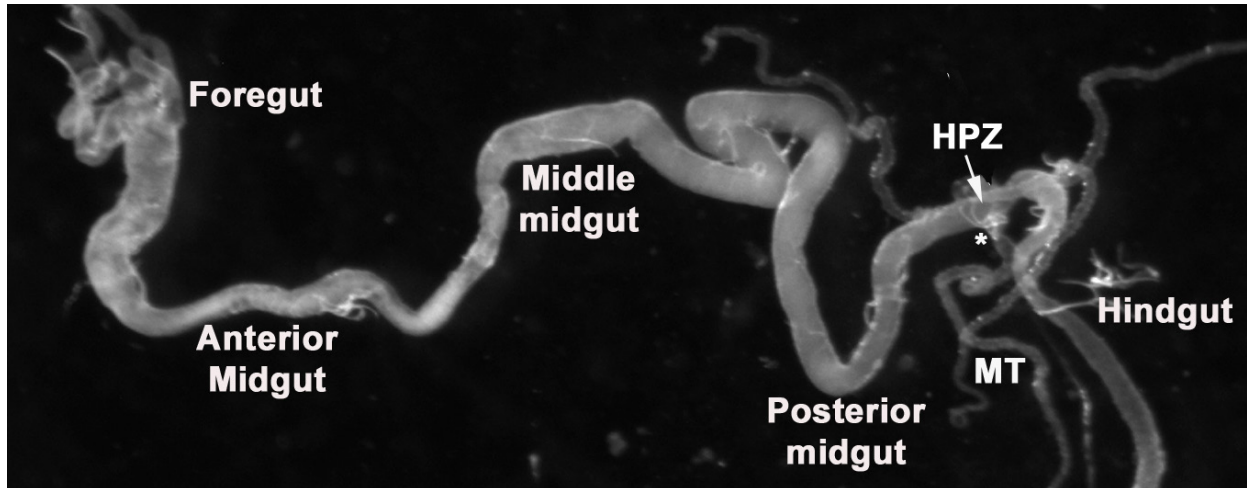


Fig. 3. Regions of larval gut in *Drosophila*. HPZ = Hindgut proliferating zone; MT= Malpighian tubules; * = Ureter opening in gut at the junction of mid- and hind-gut.

Objective: Analysis of the cell lineage in larval tissues using *inscuteable-GAL4* (*insc-GAL4*) dependent G-TRACE system

Materials Required:

A. Transgenic Fly lines

- i. $w^{1118}; insc-GAL4; +$. This stock carries the *inscuteable* promoter driven GAL4 expressing transgene on chromosome 2.
- ii. $w[*]; P\{w[+mC]=UAS-RedStinger\}4 P\{w[+mC]=UAS-FLP.D\}JD1, P\{w[+mC]=Ubi^{p63E}(FRT.STOP)Stinger\}9F6/CyO (G-TRACE/CyO)$. This stock carries the GAL4 inducible UAS-RedStinger (RFP, red fluorescence) and Flippase transgenes, and *Ubi^{p63E}* promoter driven ubiquitous expression of Stinger (GFP) which will produce green fluorescence only when the stop codon ahead of the GFP-coding region is flipped out by Flippase induced recombination at the FRT sites flanking the stop codon (Fig. 2).

B. Equipment and other materials:

- i. Stereo-binocular microscope and other fly handling materials
- ii. Fluorescence microscope (Confocal microscope preferred)
- iii. Fine needles for dissection
- iv. Maximov slides or cavity blocks
- v. Micropipettes (100/200 μ L and corresponding microtips)
- vi. Slides and coverslips

C. Reagents:

i. PBS: 1X Phosphate Buffer Saline (PBS), pH 7.4:

NaCl	8 gm
KCl	0.2 gm
Na ₂ HPO ₄	1.44 gm
KH ₂ PO ₄	0.24 gm

Serially dissolve the above in 800 mL distilled water; when fully dissolved, adjust pH to 7.4 with 1N HCl as required. Finally make the volume to 1000 mL with distilled water, autoclave the solution and store at room temperature.

ii. 1% PBST: Mix 10 μ L Triton X-100 in 1 mL PBS.

iii. 4% (w/v) Paraformaldehyde (PFA) fixative: dissolve 4g PFA powder in 100 mL of 1X PBS at 37°C for 60 min with periodic shaking. Cool and store at 4°C.

iv. DAPI (4',6-diamidino-2-phenylindole dihydrochloride): Use 1 μ g DAPI/mL 1X PBS.

v. DABCO (mounting media)

Experimental Details:

1. Set up a cross between *insc-GAL4* driver (or any other desired GAL4) carrying males with virgin *G-TRACE/CyO* females (Fig. 4) and keep the vials/bottles with flies at 24°C.
2. After egg laying, remove the parents from the vials/bottles.

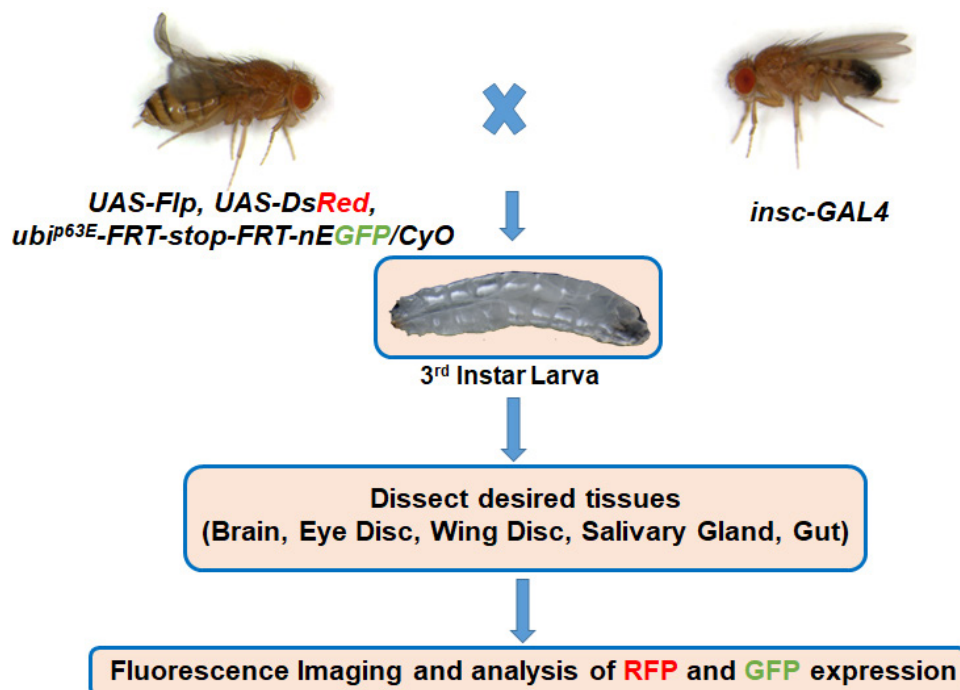


Fig. 4. Flow-chart of experimental steps for lineage tracing using the G-TRACE method

3. Collect late 3rd instar actively wandering F1-progeny larvae that show GFP and/or Ds-Red (RFP) fluorescence for dissection of the desired larval tissue (in the present case gut is used).
4. Using two needles dissect out the larval gut under a stereomicroscope.
5. Transfer the dissected guts from 4-5 larvae to a Maximov slide or Cavity block in small volume of PBS.
6. Remove the PBS and fix the tissues in 40 μ L of 4% paraformaldehyde for 30 min at room temperature.
7. After fixation, wash the gut tissues with 100 μ L of 0.1% PBST three times for 15 min each.
8. Stain the nuclei in gut tissue with 30 μ L of 1 μ g/mL DAPI for 20 min.
9. Wash the tissue three times with 100 μ L of 1X PBS for 10 min each to remove the excess stain.
10. Mount the tissue in DABCO for long-term storage.
Note: Unfixed tissue mounted on a slide in PBS can also be examined under a fluorescence microscope
10. Observe the patterns of RFP and GFP expression in different cells in mid and hind-gut regions under a fluorescence microscope using appropriate filters for GFP and RFP (use 40X objective for a better identification of the different cell types in gut that express RFP and/or GFP).

Note: If using a different GAL4 driver, examine the G-TRACE fluorescence pattern in the tissue in which that GAL4 driver is active.

Results:

Identify the patterns of *insc-GAL4* driven real-time expression of red and *Ubi^{p63E}* driven green fluorescence shown by the AMPs and ECs in mid- and hind-gut regions. The progeny cells derived from cells in which *insc-GAL4* continues to be active, because they did not change their fate, show both RFP and GFP fluorescence (appearing yellow when combined for both fluorescence patterns, ## and # marked cells in Fig. 5C) while the progeny cells in which the *insc-GAL4* was active earlier but having acquired a differentiated state do not express the *insc-GAL4* driven RFP, show only GFP fluorescence (e.g., most of the large EC cells in Fig. 5, some are ** marked in Fig. 5C). AMP cells in which the *insc-GAL4* is active but for some reason did not induce the flip-out, express only the RFP (red) fluorescence (* marked small AMP cells in Fig. 5C). A few of the GFP-positive large enterocytes also show RFP expression (appearing yellow in Fig. 5C), appear to be early enterocytes in which the parent cell derived RFP continues to be present.

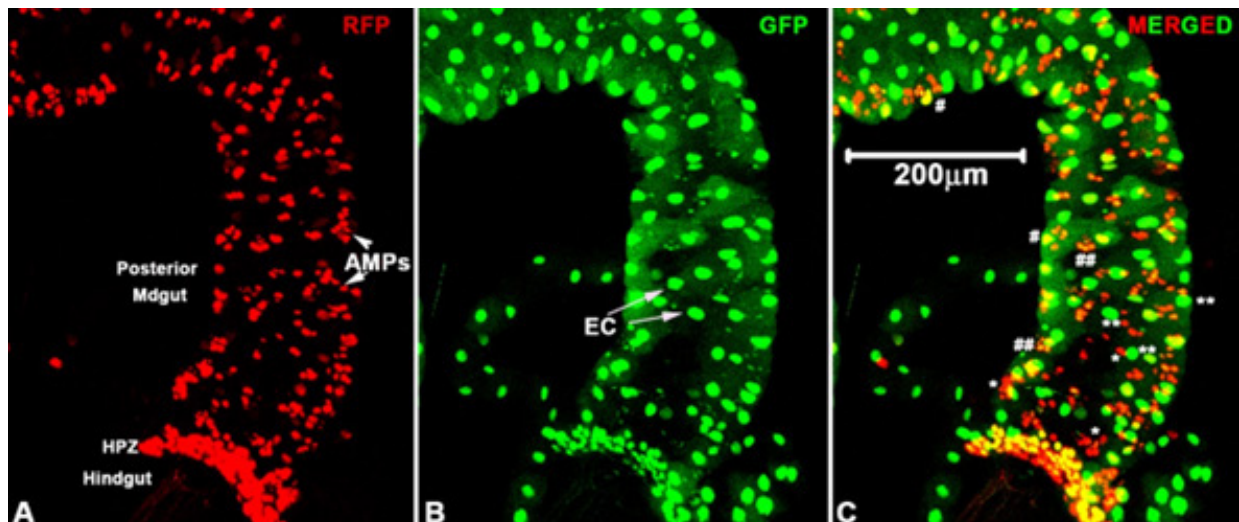


Fig. 5. Lineage tracing in third instar larval posterior midgut and the hindgut proliferating zone (HPZ). Confocal projection images of parts of posterior midgut and hindgut showing the *insc-GAL4* driven G-TRACE RFP (red, **A**), GFP (green, **B**) and merged (**C**) fluorescence patterns. **EC** = large Enterocytes; **AMPs** = clusters of Adult Midgut Progenitor cells; **HPZ**= Hindgut Proliferating Zone. **##** and **#** in **C** mark AMP and EC cells, respectively, expressing both RFP and GFP; ****** and ***** in **C** mark only GFP expressing EC and only RFP expressing AMP cells, respectively. The scale bar (200 μm) in **C** applies to **A-C**.

Questions for self-study and interpretation:

1. What is the advantage of inducing somatic recombination in developmental studies?
2. How does a study of cell lineage help in understanding development and differentiation?
3. How is the Real time expression and lineage expression different in the context of G-TRACE studies?

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Somatic Crossing Over & Segregation

The evidence presented in this paper shows that mosaic areas on the body of *Drosophila melanogaster* appear in a varying percentage of flies whenever they are heterozygous for genes whose homozygous effect is recognizable in small spots. Theories as to the causation of these spots have to be based on somewhat intricate deductions. The observable phenomenon is limited mainly to yellow or singed single spots, or yellow and singed twin spots, in different proportions and with a few other attributes such as sexual coloration or seta length. The test of any theory as to the mechanism of spot production has to consist in its application to a varied group of genic combinations. Such a test is able to exclude definitely theories which cannot account for the actual facts. But if it succeeds in giving a satisfactory basis for them, it cannot claim a final "proof." In our special case we can say that the theory of chromosome elimination has been shown definitely unsatisfactory. The validity of the theory of somatic crossing over and segregation rests on its faculty to explain the manifold results presented in this paper. Throughout the text the word "assumption" has been used freely in order to leave no doubt as to the procedure of deduction. But it should be noted that most of these assumptions are justified from other experience.

(In: Stern C. (1936). Somatic crossing over and segregation in *Drosophila melanogaster*. *Genetics*. 21: 625-730.
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Chapter 42

FLP/FRT mediated somatic recombination to generate somatic clones for studying gene function

Debleena Mandal (debleena@bhu.ac.in) and Bama Charan Mondal* (bamacharan@bhu.ac.in)

Cytogenetics Lab, Department of Zoology, Banaras Hindu University, Varanasi-221005, India

* For correspondence

Recommended level: Postgraduate/Research

Sub-discipline: Genetics/Developmental Biology/Cancer Biology/Neurobiology

Background:

Genetic mosaic individuals carry cells of two or more different genotypes in their body. Gynandromorphic *Drosophila* flies, first identified by T.H. Morgan in 1914, are mosaics for male and female cells which originate via the mitotic loss of one X chromosome during development of a female (XX) zygote. Later in 1926, Lilian Morgan observed a highly unstable ring-X chromosome in *Drosophila melanogaster*, which gets randomly lost during embryonic nuclear divisions, resulting in larger sized XO clones in XX organisms. The strategy of using such mosaics found extensive usage in studies directed towards tracing the developmental lineages of different cell types. Around the same period, H.J. Muller and Charlotte Auerbach discovered that besides mutations, X-rays as well as chemicals also generate mosaics in flies following induced somatic crossing over events (see Germani *et al.*, 2018).

The genetic mosaic techniques in *Drosophila* produce clones of a subset of genetically distinct somatic cells or tissues which offer multiple advantages that help to understand several developmental processes. A major advantage is that this technique provides a way for examining genetic changes that would be embryonic or early larval lethal when applied to an entire organism. The genetic mosaic technique circumvents this problem by permitting study of the mutant clones during later stages of development. During the past few decades, several approaches have been developed for the generation of genetic mosaics (see Blair, 2003). These include cell and nuclear transplantation (Geyer-Duszynska, 1967; Illmensee, 1968; Technau, 1986), inducing chromosomal loss (Ashburner, 1989), marking cells by using available tags like fluorescent protein and bacterial β -galactosidase (Blair, 1992) etc. The most widely used classical method for generating somatic mosaics involved usage of ionizing radiation (X-rays) to mediate mitotic recombination between homologous chromosomes by inducing chromosome breaks, which are sometimes

repaired in a way that promotes exchange between homologous chromosome arms. Consequently, patches of mutant clone are created in a wild-type background. However, two major limitations are associated with the use of X-ray irradiation for clone induction. Firstly, X-irradiation induces a low frequency of somatic recombination at random sites, and secondly, it causes a higher rate of cell lethality (Weasner *et al.*, 2017).

Limitations of earlier methods are overcome by new approaches that use FLP/FRT (Golic and Lindquist, 1989) mediated somatic recombination in conjunction with the Gal4/UAS system (Brand and Perrimon, 1993). Both these transgenic systems have been derived from yeast. The FLP/FRT system has been extensively used for somatic or *trans*-chromosomal recombination in the fly system (see Chapters 41, 81). The FLPase recombinase (FLP) enzyme is driven under the control of either *hsp70* heat shock promoter (*hs-FLP* transgene) or a tissue-specific promoter. The *hs-FLP* transgene is highly expressed when the organism is briefly exposed to temperatures between 33°C and 37°C. Although the heat shock promoter induces FLP throughout the body of an organism, clone induction occurs only in mitotically dividing cells. The frequency and clone size can be controlled, to some extent, by manipulating the duration of heat shock or the stage of development. A heat shock early in development when there are fewer cells leads to fewer but larger clones whereas a heat shock at a later stage of development when there are many dividing cells leads to numerous smaller clones. In the context of somatic crossing over induced somatic clones, the heat shock induced FLP mediates recombination during the G2 phase between 2 FRT sites inserted as transgenes at defined sites close to the centromere on homologous chromosomes (Fig. 1). One of the FRT sequence carrying chromosome also carries the *Ubi-GFP* transgene located on the same chromosome arm distal (with reference to the centromere) to the FRT site (Fig. 1) in which the fluorescent marker protein coding gene (e.g., the Green Fluorescent Protein, GFP) is placed under the control of a ubiquitously expressing *Ubiquitin (Ubi)* promoter so that all cells show green fluorescence under a fluorescence microscope. This chromosome carries a wild type allele for the desired gene X. The homologue of this chromosome carries the FRT sequence at corresponding site, and also a mutant allele (recessive lethal or visible mutation) of the gene of interest (say gene 'X') located on the same chromosome arm distal (with reference to the centromere) to the FRT insertion site. As shown in Fig. 1, following the FLP mediated somatic recombination between the two FRT sites on non-sister chromatids, and appropriate segregation of the sister and non-sister chromatids of the homologous chromosomes during anaphase, two recombinant daughter cells with different genotypes can be produced whose subsequent cell division products generate two types of daughter cell clones, one homozygous for the wild type allele and the other homozygous for the mutant allele. These clones generate the 'twin-spots', a term coined by Curt Stern (Stern, 1936). In the current protocol, twin spots comprise of two adjacent clones of cells in which one set has lost the GFP but is homozygous for the mutant allele of the gene X while the other neighboring sister clone of cells carry 2 copies of GFP (green) transgene and are homozygous for the wild type allele of the gene X. These two types of cells are surrounded by cells that did not undergo somatic recombination (Fig. 1). Examination of the non-GFP expressing clones permits analysis of the phenotypic consequence/s of the homozygosity of given mutant allele.

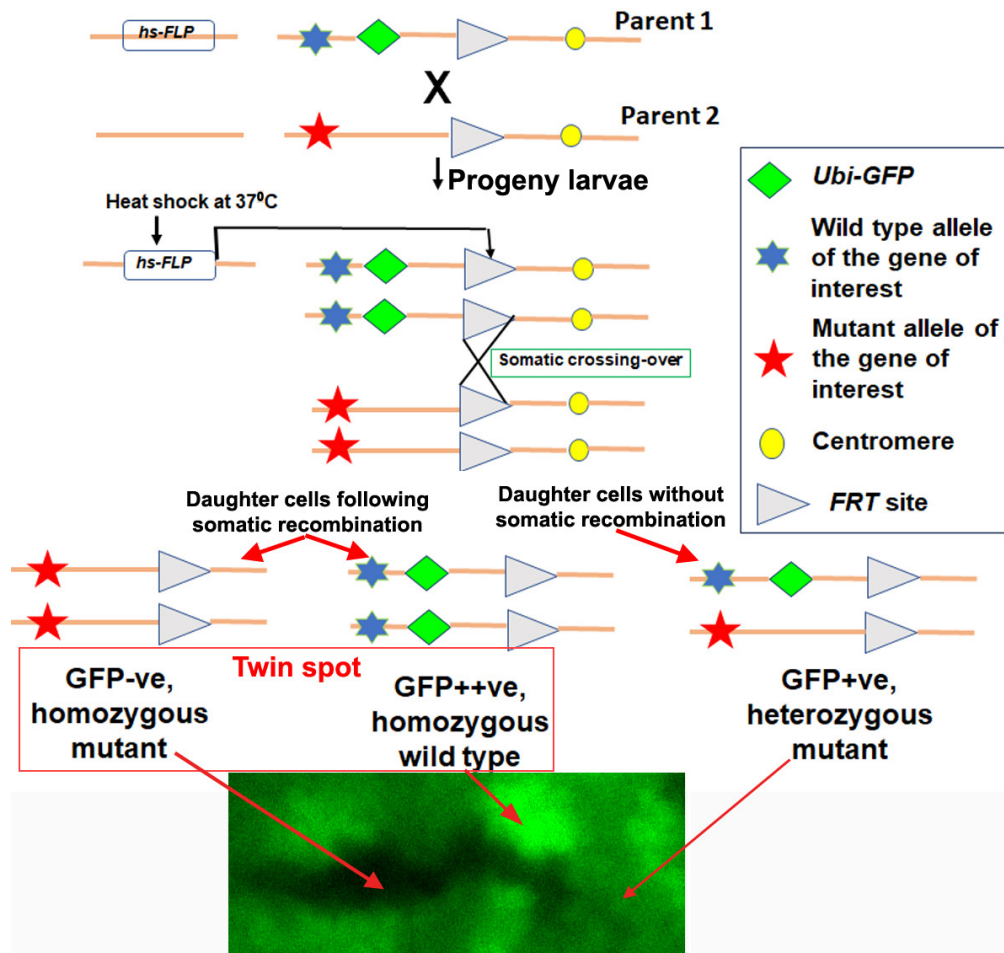


Fig. 1. Induction of twin spots through somatic crossing over at the FRT sites on the desired homologous chromosomes mediated by the FLPase enzyme activated by brief heat shock induced expression of the *hs-FLP* transgene. Following the somatic crossing over and a specific pattern of separation of sister chromatids, two daughter cells are produced with distinct genotypes: one cell is homozygous for the desired mutant allele of the gene X (red star) but lacks the *ubi-GFP* transgene (no GFP expression) while the other sister cell becomes homozygous for the wild type allele of the gene X (blue star) as well as for the *ubi-GFP* transgene (very high GFP fluorescence). The fluorescence microscope image at the bottom part shows a part of wing disc with ‘twin spot’ (no GFP and very intense GFP expressing areas, respectively) in background of non-recombinant cells that have one copy of *Ubi-GFP* transgene (less intense GFP fluorescence) and are heterozygous for the mutant allele.

Objective: To generate somatic mosaic clones (twin spot) using the FLP/FRT-mediated somatic recombination

Materials required:

A. Fly strains:

- i. $y w^* hsFLP; X^+ Ubi-GFP FRT40A/CyO-GFP; +/+$ (X^+ : wild type allele of the desired gene) as parent 1 (P1).
- ii. $w^*; X^m FRT40A/CyO-GFP; +/+$ (X^m : a mutant allele of the desired gene X) as parent 2 (P2).

Note: *Drosophila* stocks in which the *FRT* sequence is inserted close to the centromeric region of different chromosome arms are available from the Bloomington *Drosophila* Stock Centre (see Chapter 81). Depending upon location of the gene of interest, the mutant allele needs to be recombined (see Chapter 11) with the *FRT* transgene located near centromere on the same chromosome arm.

B. Equipment and other supplies:

- i. Stereo-binocular and fluorescence microscopes (optional: Confocal microscope).
- ii. BOD incubator maintained at 24°C for fly rearing.
- iii. Incubator/water bath maintained at 37°C for applying heat shock.
- iv. Fly handling materials.
- v. Maximov slides or cavity blocks.
- vi. Micropipettes (10 µL, 100 µL and 1000 µL) and corresponding micro-tips.
- vii. 1.5 mL microfuge tubes.
- viii. Slides and coverslips (22mm²).
- ix. Fine needles and forceps.

C. Reagents:

- i. PBS: 1X Phosphate Buffer Saline (PBS), pH 7.4:

NaCl	8 gm
KCl	0.2 gm
Na ₂ HPO ₄	1.44 gm
KH ₂ PO ₄	0.24 gm

Serially dissolve the above in 800 mL distilled water; when fully dissolved, adjust pH to 7.4 with 1N HCl or 0.4 N NaOH, as required. Finally make the volume to 1000 mL with distilled water, autoclave the solution and store at room temperature.

- ii. 4% (w/v) Paraformaldehyde (PFA) fixative: dissolve 4g PFA powder in 100ml 1X PBS at 37°C for 60 min with periodic shaking. Cool and store at 4°C.
- iii. PBST 0.1% (v/v): Add 10 µL of stock TritonX-100 in 10 mL of 1X PBS.
- iv. DABCO (1,4-diazabicyclo[2.2.2]octane) mounting medium: Dissolve 1.25gm of DABCO powder in 15 mL of 1X PBS. Add 35 mL of ultrapure glycerol, mix gently for 2 hours and store at -20°C.

Experimental details:

1. Cross virgin P1 females (carrying *hsFLP* transgene, and *FRT* site and *Ubi-GFP* transgenes on the same chromosome arm that also carries a wild type allele of the gene *X*) and P2 males (carrying the *FRT* site and a mutant allele of the desired gene *X* on homologous chromosome, see Fig. 1).

2. Collect eggs using an egg-laying chamber (see Chapter 70). Select first instar progeny larvae that hatch during an interval of 1 hr (synchronized larvae, see Chapter 15) and transfer them to normal food vials for growth at 24°C until required for application of the heat shock to induce FLP recombinase expression.
3. At the developmental time desired for induction of somatic clones (late second instar larvae in the present case), place the vials with larvae in a 37°C water bath for heat shock for the desired duration (**Note:** The duration of heat shock can be varied as required in specific cases, e.g., shorter heat shock like for 5 minutes is used for generating discrete somatic clones in epithelial tissues like wing or eye disc or 1 hour for mesodermal tissue like lymph gland. Shorter periods generate fewer and discrete clones while longer duration generates many clones which may fuse with each other during development. Cell division rate in the given tissue also affects the size and numbers of induced clones.)
4. Following the heat shock, transfer the larvae back to 24°C and let them grow to wandering late third instar stage.
5. Wash the larvae in distilled water to remove the adhering food particles and dissect the imaginal discs or other tissue of interest (for details see Chapter 18, 19 and 30)

Observation:

Observe the tissue (eye discs, wing discs) under a fluorescence microscope (or Confocal microscope, if available), using filters for blue excitation and green emission (to detect GFP fluorescence). Identify and draw the 3 different kinds of GFP expression patterns (moderate GFP, high GFP and no GFP signal) in same tissue (see Fig. 2).

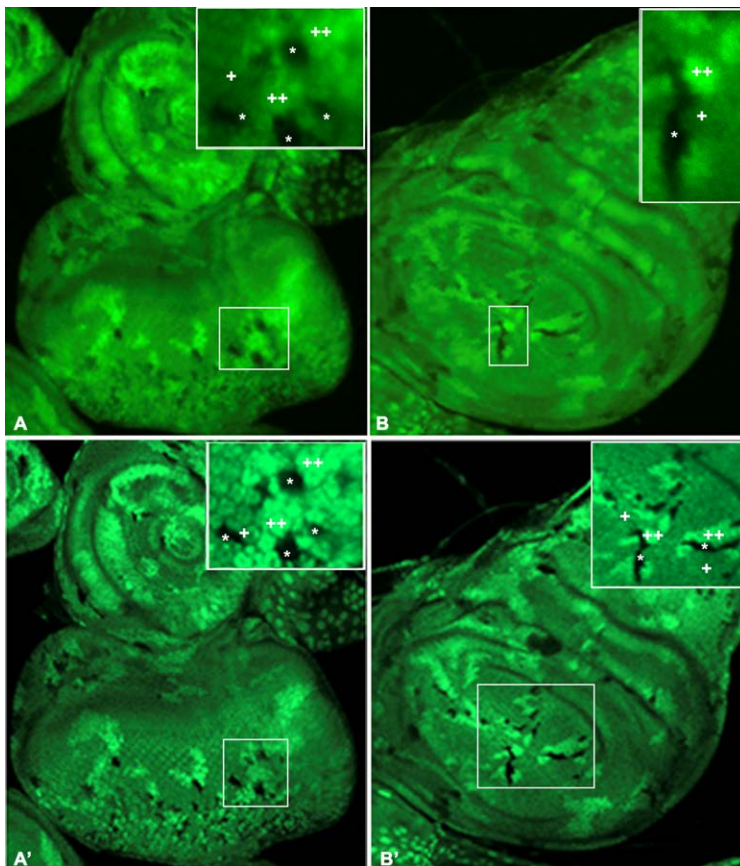


Fig. 2. Twin spots in eye (A, A') and wing discs (B, B') seen under fluorescence (A, B) or confocal (A', B') microscope. The inset in each shows the boxed area in the corresponding panel at higher magnification. * = GFP-ve, mutant homozygous clones (no GFP fluorescence); ++ = GFP and wild type allele homozygous clones (more intense GFP fluorescence); + = GFP and mutant allele heterozygous clones (less intense GFP fluorescence).

Questions for self-study and interpretation:

1. Why are different levels of GFP fluorescence seen in neighboring clones of cells?
2. Why are the high GFP expressing cells always adjacent to, and of nearly similar size as the non-GFP expressing groups of cells?
3. Draw the different patterns of separation of sister chromatids at anaphase following the induction of somatic crossing over and identify the pattern that gives rise to 'twin spot'.
4. How does FLP mediate recombination between the two FRTs?
5. How does varying the duration of heat shock affect the efficiency of formation of somatic clones in a tissue?
6. How does induction of somatic clones help in tracing developmental fates of different cells in a given tissue?

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Lilian V. (Mrs. T. H.) Morgan's Essence of Joy

No account of the formative days of *Drosophila* genetics can be deemed complete without acknowledging the contribution of Lilian V. (Mrs. T. H.) Morgan. As a wife and mother, she nurtured a son and three daughters. As a superb *Drosophila* geneticist, she discovered and characterized the first attached-X female whose genotype was later employed to maintain without selection sex-linked female sterile mutations. Attached- X females were also used in deducing that meiotic crossing over occurs between chromatids and not chromosomes. She also found the first ring-X chromosome which was used to produce genetic mosaics for studying development. Hers was a fulfilling career, at home and in the lab. This is the essence of joy!

(In: Green M. M. (2010). A century of *Drosophila* genetics through the prism of the *white* gene. *Genetics*. 184:3-7. <https://doi.org/10.1534/genetics.109.110015>.)

Chapter 43

Quantification of wing and body tergite pigmentation in *Drosophila*

Harshita Bhati (harshita.b@ahduni.edu.in) and Subhash Rajpurohit* (subhash.rajpurohit@ahduni.edu.in)

Division of Biological and Life Sciences, School of Arts and Sciences, Ahmedabad 380009, Gujarat, India

* For correspondence

Recommended Level: UG/PG/Research

Sub-discipline: Ecology/Evolutionary Biology/Taxonomy

Background:

Melanin plays a significant role in camouflaging, thermoregulation, and mate choice in a range of organisms (Kronforst *et al.*, 2012). Pigmentation pattern is diverse in insect species (True, 2003), and *Drosophila* species showcase variations in wing spot pigmentation patterns (Koshikawa *et al.*, 2020) as well as abdominal melanisation (Rajpurohit *et al.*, 2008). Melanin variations are involved in different aspects of ecology in ectothermic insect species. Molecular mechanisms of melanin formation have also been studied at length. Changes in the cis-regulatory DNA sequences that regulate gene expression of pigmentation genes contribute to the morphological trait variation between closely related species (Massey and Wittkopp, 2016). Geneticists have suggested that pigmentation variation is caused by adaptive changes in multiple genes like *yellow*, *ebony*, *tan*, *bric-a-brac* in *D. melanogaster*, which are involved in the pigment synthesis pathway (Wittkopp *et al.*, 2003). Melanin synthesis pathways are conserved in insects, making *Drosophilid* flies an ideal model system for understanding the function of and genetic basis for differences in pigmentation.

The pigmentation of abdominal tergites and mesothorax differs predictably with latitude and altitude in the cosmopolitan *D. melanogaster* (Pool and Aquadro, 2007; Rajpurohit *et al.*, 2008). Generally, insect pigmentation shows a response to temperature, i.e. insects inhabiting colder or higher latitudes are usually darker than those inhabiting warmer or lower latitudes. This holds true under the laboratory conditions as well where insects reared at lower temperatures develop darker pigmentation than those growing at higher temperature. The most common type of sexual dimorphism in abdominal pigmentation involves increased melanisation of posterior segments in males but not in females. This pattern has evolved independently in many distantly related clades. In convergent evolution, some species have lost spatial patterning and became uniformly dark or light.

There is substantial heterogeneity in the genetic basis of convergent pigmentation. The genetic basis of colour pattern variation in *Drosophila* is quite variable, sometimes involving the same genes but often times not (Kronforst *et al.*, 2012).

This exercise provides methods for quantification of pigmentation in some of the most variably pigmented regions of fly body which makes it possible to find correlation between environment and phenotypes.

Objectives:

To quantitate wing and body tergite pigmentation in *Drosophila* species.

Materials Required:

1. Flies (lab populations and/or wild-caught populations).
2. Equipment
 - a. Stereo-binocular microscope with attached camera
 - b. Reference scale and ocular micrometer
 - c. PC/Laptop with the Image J software (publicly available)
3. Other items
 - a. Cleaned glass slides and coverslips
 - b. Forceps and needles for dissection
 - c. Fine hair brush for fly handling
 - d. 70% ethanol

Experimental Details:

A. Wing spot pigmentation

- i. Specimen mounting
 - a. Fruit-flies can be collected from a local fruit market/garden by placing traps (plastic bottles containing apple and banana baits) overnight. Alternatively, an aspirator can be used to collect flies (see [Chapter 108](#)).
 - b. Bring the flies to the lab and mildly anaesthetize them with ether or a cold shock (10-15 min on an ice-cool plate).
 - c. Transfer the flies in a petri dish containing 70% alcohol, which helps in keeping the organs intact.
 - d. Place the fly in a drop of 70% ethanol on a glass slide under the stereo-binocular microscope. Carefully remove intact wings from the body with help of fine forceps and a needle. Ensure to remove any traces of extra tissue from thorax.
 - e. Transfer the wing on a clean glass slide, with its dorsal side up, and apply a drop of 70% ethanol to cover the entire wing.
 - f. Position the wing on the slide with the help of a needle and place a coverslip over it, allowing the coverslip to fall gently in the posterior to anterior direction, avoiding any air bubble between the slide and coverslip.

g. Remove excess alcohol from the edges of the coverslip with help of a tissue paper and seal all the edges of coverslip with a transparent tape or nail polish and label the slide.

ii. Specimen imaging

a. Adjust the position of the slide under the stereo-binocular microscope so that the entire wing is visible under the microscope without any obstruction or shadows/reflection.

b. Collect images using camera attached to a stereo-binocular microscope.

Note: The light intensity, the optical and digital magnification, and the exposure time must be kept constant for all the images that are captured.

iii. Image Analysis

a. Image analysis can be carried out using any image analysis software. The ‘Image J’ software, a publicly available image analysis software (<http://rsbweb.nih.gov/ij/>), can be conveniently used for the present purpose. A variety of Plugin files for this software are also available at the website, which can be installed and also updated from the menu> help> Update. With the help of this software various types of pixel scores and areas can be measured (**Fig 1**).

b. Before proceeding with imaging of samples of interest, the camera needs calibration with the image capturing software. This can be done by the use of a calibration slide and ocular micrometer. A known distance on the calibration slide could be calibrated to the image analysis software (for details read the Help at the Image J software).



Fig. 1: Image J working window showing an area of selection (yellow line) on the image of *Drosophila biarmipes* wing. The gray score for the entire wing area is in the red circle (A) and the wing spot area is in yellow box (B).

c. Open the wing image to be analysed. Convert the coloured image to black and white for the gray scale analysis by clicking on “image”> “type” > “8-bit”.

d. Set the scale by clicking “Analyze”> “Set Scale”> “distance in pixels” (provide the pixel value obtained from selecting the reference scale obtained at step ii above)> “known distance”, “pixel aspect ratio”, “unit of length” in mm, select “global” and click “OK”. Set these measurements for all the images to be analysed.

e. Start selecting the wing area according to your research interest by clicking on the “polygon selection” from the toolbar (which offers a range of functions to be selected).

f. Now click “Analyze”> “measure”. Mean gray score will be shown in the drop down

box (**Fig. 1A**). When the area is selected in the image, the software simultaneously calculates the actual area and gray score.

g. Copy the mean gray core and save it in an excel sheet for further analysis.

iv. Data processing and Analysis: The distribution trait variation within members of a species can be found from the data. Different populations can be compared for geographical differences in pigmentation. Simple statistical analysis can be performed using MS-Excel.

B. Body tergite melanisation

i. Specimen mounting

a. Following the steps i-iii for Wing-spot pigmentation study, a fly is kept in a drop of 70% ethanol on a clean slide under the stereo-binocular microscope and using fine forceps and a needle both the wings are removed and then the head and thorax are detached from abdomen.

b. Place the abdomen on a clean glass slide, dorsal side up, and apply a drop of 70% ethanol covering the entire abdomen.

c. Place a glass coverslip over the abdomen, allowing the coverslip to fall in the posterior end of the abdomen first and applying pressure towards anterior direction.

d. Press abdomen from posterior to anterior direction until all the viscera is squeezed out through the open abdominal cavity and all the segments spread smoothly parallel to the glass slide. Seal the coverslip on all sides with transparent cello tape or nail polish

Note: Multiple abdomen samples can be placed on a single slide.

ii. Specimen imaging

a. Once the slides are dry they are placed on the stereo-binocular microscope for imaging.

b. Adjust the position of the slide so that the abdominal segments are visible under the microscope without any obstruction or shadows/reflection.

c. Collect images of the abdomen using a camera attached to a stereo-binocular microscope (**Fig. 2**).

Note: The light intensity, the optical and digital magnification, and the exposure time etc., must be kept constant for all the images that are captured.

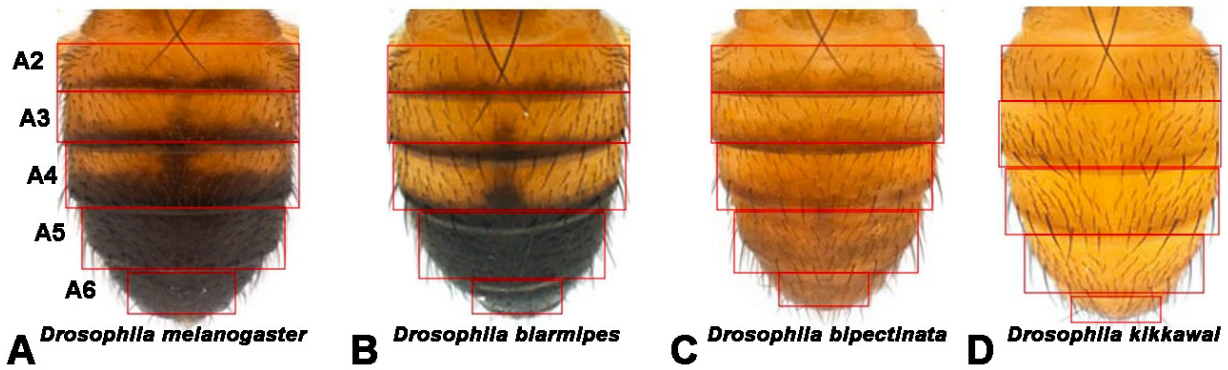


Fig. 2: Variations in pigmentation of abdominal tergites of males of (A) *Drosophila melanogaster*, (B) *Drosophila biarmipes*, (C) *Drosophila bipectinata*, and (D) *Drosophila kikkawai*. Abdominal segments labelled A2-A6 are indicated by red rectangles. The actual area to be selected for pigmentation of A5 is shown in Fig. 3 (Image source: <http://gompel.org/images-2/drosophilidae>, reproduced with permission of Prof. Dr. Nicolas Gompel).

iii. Image Analysis:

- a. Steps a-g are same as in the wing-spot image analysis above. The only difference is that instead of the wing area, the abdominal tergites are selected as shown in Figs. 2 and 3.

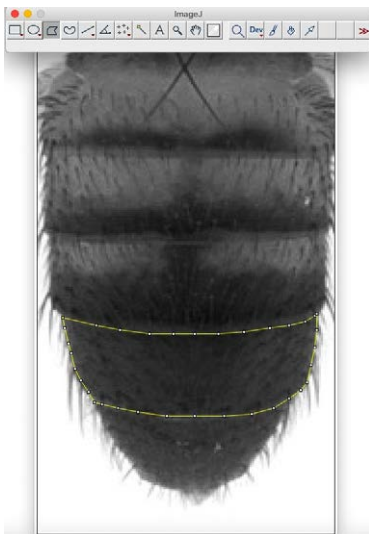


Fig. 3: Image J working window showing an area of selection for 5th abdominal segment of *D. melanogaster* male. Selected area is bordered by yellow lines.

- iv. Data processing and Analysis: Statistical analysis can be performed using single factor ANOVA or ANCOVA (in case of covariates) using appropriate software.

Results/Observations:

1. Examine and record variations in wing spot pigmentation and abdominal melanisation from at least five wings/abdomen and correlate with environmental (e.g. temperature/season when collected from field) or genetic factors (in known mutant flies).

Note: The abdomen comprises of six distinctly visible segments in *Drosophila melanogaster* males, and each body tergite segment has a black melanin strip, as well as complete melanisation in tergites A5 and A6. Melanization patterns of abdominal tergites are different in different species (Fig. 2).

2. Record the sample mean gray scale value for the wing area and wing spot (e.g. in individuals of *Drosophila biarmipes male*) as in **Table 1**. Analyse the data to determine which area has higher pigmentation (lower the gray score, higher the pigmentation).

3. Compare the mean gray scale values for the abdominal tergite pigmentation for males (segments A2-A6) and females (segments A2-A7) of different species (e.g., *Drosophila melanogaster*, *Drosophila biarmipes*, *Drosophila bipectinata*, and *Drosophila kikkawai*) or populations of a species (make a record in **Table 2**). Determine, which abdominal segment has higher pigmentation (lower the gray score, higher the pigmentation).

Table 1. Data on wing area and wing spot pigmentation for males using Image J. Scale of x pixels/mm (x = value as determined by calibration in specific cases) shown (known distance 0.1 mm or as set in the software).

Species/Population	S. No.	Parameter	Entire Wing	Wing spot
		Gray Score (pixels/mm)		
		Area (mm ²)		

Table 2. Data on abdominal pigmentation for *D. melanogaster*, *Drosophila biarmipes*, *Drosophila bipectinata*, and *Drosophila kikkawai* male using Image J. Scale of x pixels/mm (x = value as determined by calibration in specific cases) shown (known distance 0.1 mm or as set in the software). GS = Gray Score (pixels/mm); A = Area (mm²).

Species/Population	S. No.	Segment	Parameter	Entire Segment
<i>Drosophila melanogaster</i>		A2	GS A	
		A3	GS A	
		A4	GS A	
		A5	GS A	
		A6	GS A	

Questions for self-study and interpretation:

1. What is the ecological significance of *Drosophila* pigmentation?
2. Can environmental variables (for example, temperature) trigger changes in pigmentation?
3. Are flies darker in colour more fit in terms of survival?
4. Do other *Drosophila* species have other varying patterns of their wing and abdominal tergite pigmentation?
5. What could be the underlying genetic basis for the pigmentation variation within and among the *Drosophila* species?

References and further reading:

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***Drosophila* survives Academic Fund Crunch**

D. melanogaster's natural history in orchards and cider mills, I have argued, matched the natural history of academic departments. Its seasonal demography matched the seasonal cycle of student projects and student labor; cheap and lowly, *Drosophila* was not shut out by the poverty of academic research funding.

(In: Kohler, R. E. 1994. Lords of the fly: *Drosophila* genetics and the experimental life. University of Chicago Press)

Chapter 44

Study of sex-comb variations in *Drosophila* species

Kopal Singhal (kopalsinghal7@gmail.com) and Sujata Mohanty* (sujata.mohanty@jiit.ac.in)

Drosophila Genetics Lab, Department of Biotechnology, Jaypee Institute of Information Technology, Noida, 201309.

* For correspondence

Recommended Level: UG/PG/Research

Sub-discipline: Taxonomy/Ecology/Evolution

Background:

Natural populations showcase phenotypically diverse individuals exhibiting variations in different traits. Studying intra-and inter-specific variations in morphological traits among different populations of a species and understanding the factors underlying these variations is of great interest from an evolutionary perspective. There are various models and theories to understand the mechanism of these trait variations and their evolutionary significance (Bolnick *et al.*, 2011). The fruit fly *Drosophila* has emerged as a popular model organism for exploring such trait variations due to its widespread availability in nature and having an anatomically relatively simple and morphologically well-characterized body. *Drosophila* species often exhibit latitudinal clines for morphological traits, which may reflect adaptations to local climatic conditions (Negoua *et al.*, 2018).

The sex comb, one of the most rapidly evolving male-specific traits, consists of an array of modified thick black bristles (teeth) restricted to the proximal tarsal segments of the forelegs of male *Drosophila* (see **Figs. 1 and 2**). It is an attractive model to study morphological trait variation and the mechanism underlying its evolution. Sex comb is known to help in grasping the female's abdomen and genitalia during copulation. Previous studies on sex comb shows that this trait evolves rapidly and exhibits great divergence among different species of *Drosophila* (Kopp, 2011). Therefore, it is widely used in species identification (see **Chapter 108**) especially where other taxonomic differences are less prominent.

Objectives:

Observation of variations in the pattern and bristle number in sex comb in *Drosophila* species.

Materials Required:

A. *Drosophila* flies:

Male flies of different *Drosophila* species (laboratory bred or freshly collected from nature)

B. Equipment and other fly handling materials:

- i. Stereo-binocular microscope
- ii. A fine hair brush
- iii. A pair of sharp needles
- iv. A pair of fine forceps
- v. Anaesthetic ether, etherizer
- vi. *Drosophila* food vials
- vii. Glass slides and coverslips (22 mm²)

C. Media/Reagents/Solutions:

- i. Insect physiological saline (0.65% NaCl)
- ii. Glycerol (70%)

Experimental Details:

1. Collect wild type *Drosophila* species from different geographical locations or use laboratory established *Drosophila* stocks, if available.
2. If using flies from fresh field-collection, establish isofemale lines by placing individual nature-inseminated females in freshly prepared food vials. Use the F₁ flies obtained from each vial for species identification by examining their morphological and anatomical features.
3. Culture the established lines in replicates to get sufficient numbers of flies.
4. Anesthetize flies using ether or by cooling.
5. Separate 4-6 days old males and females under microscope based on their size (females are larger in size), sex specific features such as body pigmentation and genital features using a fine brush.
6. Keep an anaesthetized male on a slide under the stereo-binocular microscope, add a few drops of insect saline onto it and cut off its both forelegs using forceps and/or needles.
7. Remove the fly carcass and observe the forelegs under higher magnification.
8. Examine the presence or absence of the sex combs on the tarsal segments of front legs.
Note: In some species, sex comb may be absent or poorly developed.
9. For making a temporary preparation of forelegs, transfer the left and right forelegs of an individual to another clean glass slide, in that order, with the help of a brush and put 2-3 drops of 70% glycerol onto the leg and carefully cover with a coverslip without trapping air bubbles.
10. Observe and record the pattern and number of thick bristles or teeth in the sex-comb on left and right forelegs of 25-30 males from each population of a species. Draw or capture the microscope images of forelegs.

11. Calculate the mean number of teeth (bristles) in the sex-comb of left and right forelegs to determine if the sex comb shows an asymmetric arrangement.
12. Calculate the Mean number of teeth (bristles) present per species per population.
13. Analyze the data for finding the inter- and intra-specific variations among *Drosophila* species populations and also calculate their statistical significance.

Results/Observations:

The front leg consists of coxa (the attachment site of leg with the thorax), trochanter, femur, tibia, five tarsal segments and a claw (Fig. 1). Sex comb is found on the tarsi (first and/or second tarsus) of front legs in males (Fig. 1).

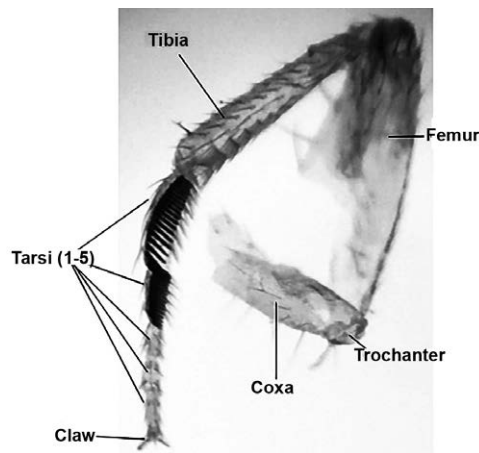


Fig. 1. First leg of male *Drosophila jambulina* showing various segments and the sex-comb (on Tarsus 1 and Tarsus 2)

1. Prepare an observation table to record presence/absence, position, pattern and teeth number in the sex comb in the examined individuals of a species.
2. Compare your data with respect to the information available for different species and different geographical locations (examples of sex comb variation in two species are shown in Fig. 2)

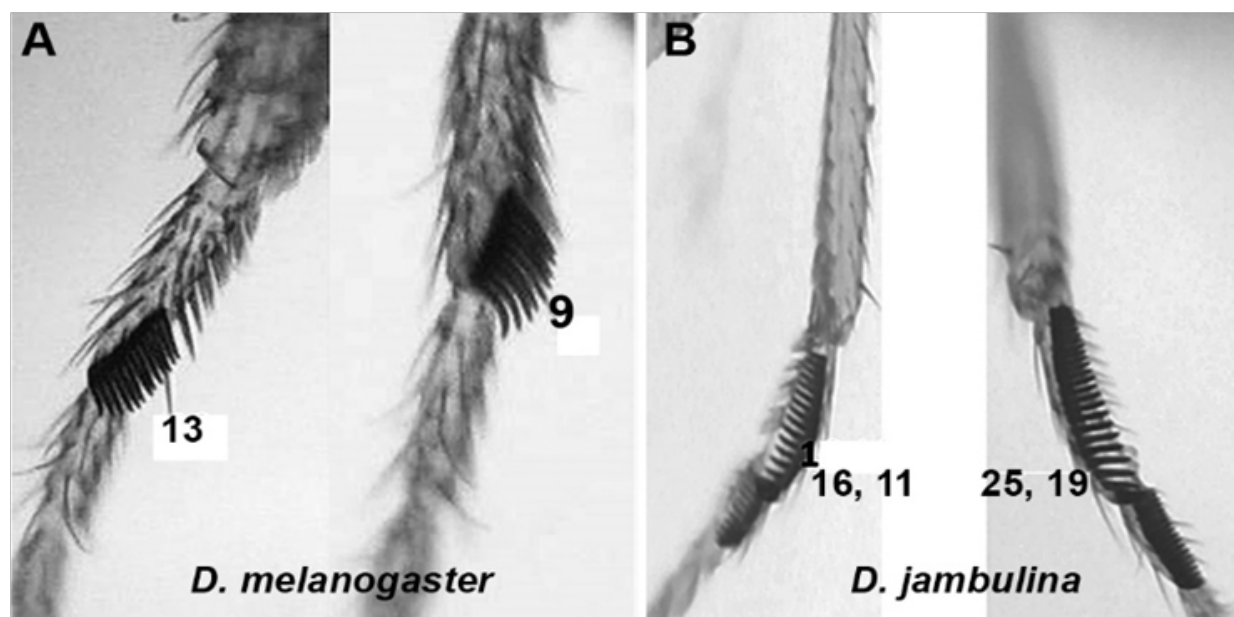


Fig. 2. Variations in pattern and numbers of sex-comb teeth in two *Drosophila* species

Questions for self-study and interpretation:

1. Is sex comb pattern species-specific?
2. Can intra-specific sex comb variations be correlated with ecological and/or geographic variations?
3. Are there similarities in sex comb pattern between phylogenetically related species of *Drosophila*?
4. Is sex-comb a quantitative trait (see **Chapter 12**)?
5. Would you expect populations with increased intra-specific trait variations to be more adaptive to new environments?

Note: Further selection experiments can be carried out by selecting males with highest and lowest sex comb bristles number. Mating experiments can be performed with flies from control (baselines) and selection lines to examine inheritance of the sex-comb pattern.

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Negoua, H., Chakir, M., David, J. R. and Capy, P. (2019) Climatic adaptation in *Drosophila* : phenotypic plasticity of morphological traits along a seasonal cycle, *Annales Société Entomologique France (N.S.)*. 55: 48-60. DOI: 10.1080/00379271.2018.1540281.

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Chapter 45

Preparation and study of male external genitalia of *Drosophila*

B. V. Shyamala (shyamalabv@yahoo.com)

Developmental Genetics Lab, *Drosophila* Stock Centre, Department of Studies in Zoology, University of Mysore, Manasagangotri, Mysore-570006

Recommended Level: PG/Research

Sub-discipline: Taxonomy/Phylogenetics/Evolutionary Biology/Anatomy

Background:

The genitalia of male *Drosophila* correspond to a set of external and internal structures in the distal part of the male abdomen involving the tergites of segments 8-10. The male genitalia is divided into two parts: 1) Phallic structures which comprise mainly the structures which are inserted into the female genital tract during copulation, and 2) Periphallallic organ which comprises the external structures which are secondarily connected and surround the phallic organ (Rice *et al.*, 2019). Periphallallic organ, commonly referred to as Genital Plate is a chitinous structure with discrete ornamentation of bristles and chitinous teeth. Genital plate exhibits extraordinary interspecific phenotypic variations in its structure, especially with respect to its shape, ornamentation pattern and nature of bristles. They have been found to diverge quickly following speciation, which makes them a highly divergent trait among closely related species than are the forms of other appendages such as legs, antennae and eye. This property of rapid evolution and species specificity makes it a taxonomically and evolutionarily important trait, which can be used to discriminate between closely related species (Bock and Wheeler, 1972; Eberhard, 2010). Studies in different groups of *Drosophila* have shown that even the sibling species, which are reproductively isolated but morphologically indistinguishable, do show distinct and consistent differences in the details of genital plate structure (Burla *et al.*, 1949; Coyne, 1983).

Experiments have demonstrated adaptive function of the spines on the genital plate. Experimentally shortened spines through laser microsurgery have been shown to impede with successful mating and fertility of the male *Drosophila* species. These structures have been demonstrated to serve as grasping and hold fast devices during mating (Grieshop and Polak, 2012; 2014; Rodriguez-Exposito *et al.*, 2019). Variations induced in the genital plate morphology by genetic manipulations, have been shown to affect several of the reproductive fitness parameters. Interestingly, it is also seen that isolated populations

within species often display substantial variation in the morphology of their genital plate, and may thus contribute to the reinforcement of reproductive isolation upon secondary contact (Frazee and Masly, 2015). Thus the study of structure and variations in genital plate offers a useful parameter to ask, and answer interesting questions in the field of Taxonomy, Phylogenetics and Evolutionary Biology. Presented here is a simple procedure for the preparation and study of the genital plate of *Drosophila* species.

Objectives:

Dissection and study of Periphalllic organ/Genital plate of *Drosophila*

Materials Required:

A. Fly Stocks: Stocks of different species of *Drosophila* or freshly collected from the wild.

B. Equipment and other fly handling materials:

- i. Stereo-binocular and Compound microscopes
- ii. Cavity slides, slides and coverslip
- iii. Glass plate, fine needles/forceps, brushes, etheriser etc.

C. Chemicals and other supplies:

- i. Insect Physiological Saline (0.7% Sodium Chloride solution in Water)
- ii. Potassium Hydroxide solution (4% in Water)
- iii. Creosote oil (used as a clearing agent)
- iv. Anaesthetic ether

Experimental Details:

1. Anaesthetize flies with ether or CO₂. Select and take a few male flies on a clean plain slide in a drop of saline solution.
2. Using fine needles cut and separate out, under a stereo-binocular microscope, the terminal abdominal segments (8-10) along with the genital plate (**Fig. 1A**).
3. Transfer the cut part into a cavity slide containing 4% Potassium hydroxide solution. Incubate for about 15-20 min at room temperature to soften the extra tissues attached to the chitinous genital plate.

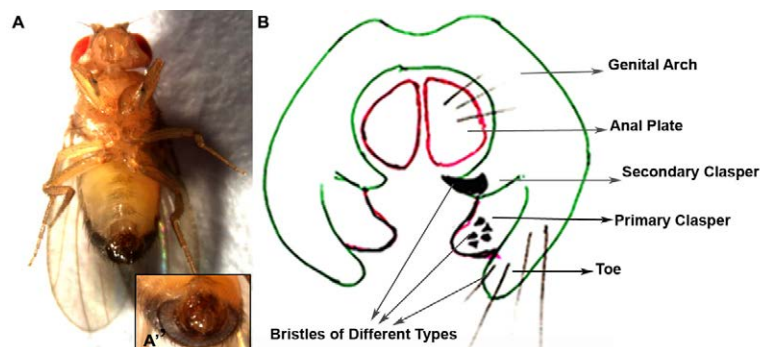


Fig.1 A. Ventral view of male *Drosophila melanogaster* showing the genital plate at the abdomen tip; inset A' shows an enlarged view of the genital plate region. B. Schematic diagram of a typical genital plate to show the different structures and bristle types.

- Carefully remove the attached soft tissue from the chitinous genital plate (Periphallic organ) with the help of fine needles/forceps. Separate out and remove the phallic organ, which appears as a rod shaped chitinous structure attached to the concave (dorsal) side of the plate.
- Transfer the intact genital plate into a fresh cavity slide containing about 200 μ L of Creosote. Leave the plate for 5-10 min during which time, fine pieces of soft tissues that may be still attached to the plate, get cleared off.
- Keep the genital plate with its convex (ventral) side up in Creosote in the cavity slide, and cover with a coverslip avoiding trapping of any air bubble.
- The preparation is observed under a compound microscope.

Observations:

Note the following important structures of the genital plate (**Fig. 1B**):

- Inverted U shaped Genital Arch
- A pair of Oval or triangular shaped Anal Plates
- Tip of the Genital Arch referred to as Toe region
- A pair of Primary Claspers
- A pair of Secondary Claspers

Each of these regions presents a characteristic shape, pigmentation and bristle pattern in different species (**Fig. 2**).

When examining genital plates of different species, note and draw the numbers and locations of different types of bristles on the genital plate, namely, i). Thin, long and tapering, ii) short chitinized stout peg like, and iii) very dark, highly chitinized teeth/spine-like. Enumerate the variations seen with respect to the structure of the plate, nature, number and arrangement of these bristles between different species.

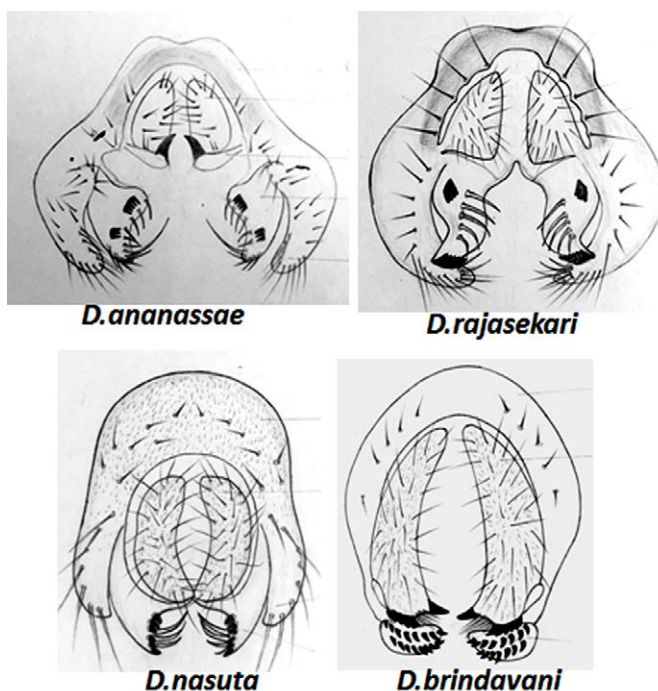


Fig. 2. Drawings showing the structure of genital plates in four different species of *Drosophila*.

Questions for self-study and interpretation:

1. How can the structure of the genital plates be used to identify different *Drosophila* species?
2. What features of the genital plate differ more frequently between species?
3. Can you group the examined species into evolutionarily closely related or distantly related clusters based on the genital plate structure?
4. Which are the features of genital plate structure that show greater differences between evolutionarily / taxonomically closely related species? What does this indicate?
5. What are the functional roles of the different components of the genital plate and how do they aid in reproductive isolation?

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Chapter 46

Demonstration of heat shock induced gene activity in *Drosophila*

S.C. Lakhotia (lakhotia@bhu.ac.in)

Cytogenetics Laboratory, Department of Zoology, Banaras Hindu University, Varanasi
221005

Recommended Level: UG/PG/Research

Sub-discipline: Genetics/Cell Biology

Background:

Sudden increase in surrounding temperature induces a cellular response, termed as Heat Shock Response (Arya *et al.*, 2007). Exposure of *Drosophila* larvae, adults or their tissues to temperature above 30°C for a short period (about 5 min or longer) causes activation of a specific set of genes, the **heat shock genes**, whose transcripts are rapidly translated to make the **heat shock proteins** (HSPs). The heat shock response is highly conserved across all domains of life. The major induced HSPs have been grouped in several highly conserved families like the HSP90, HSP70, HSP60 and small HSPs, on basis of the consensus sizes (in kilodaltons) of the induced proteins. Several other cellular stresses, like heavy metals, anoxia, salinity etc also induce some or all of the heat shock genes, in combination with some other stress-specific genes. The HSPs appear to protect cellular functions from thermal and other cell stress induced damages mainly through their chaperoning functions (Arya *et al.*, 2007). The selective induction of this set of heat shock genes is due to the presence of one or more conserved palindromic sequence (5'CnGAAnnTTCnG3'), the Heat Shock Element (HSE), upstream of the transcription start point of the stress inducible genes. Heat shock and other cell stresses activate the Heat Shock transcription Factor (HSF), which binds to the HSE sequences and induces rapid transcription of these genes. (Arya *et al.*, 2007).

Historically, the heat shock induced gene activity was first seen by Ritossa (1962) as induced puffs on polytene chromosomes since these chromosomes permit a direct microscopic visualization of active genes in the form of localized expanded chromosome regions known as puffs (see [Chapter 31](#)). With the advent of recombinant DNA techniques, gene constructs have been prepared to visualize activity of heat shock genes in any cell. This approach takes advantage of the specific interaction between the HSF and the HSE.

to make any gene heat-inducible by cloning the HSE upstream of the transcribed part of the desired gene. If the product of gene cloned downstream of the HSE can be easily visualized, the transgene can be used as a 'reporter'. Such 'reporter' product permits monitoring of activation of the HSF, which leads to transcription of the reporter via the HSE in the promoter of the transgene. Several reporter genes, viz., *ADH* gene (coding for alcohol dehydrogenase) of *D. melanogaster*, the *lacZ* gene (coding for β -galactosidase) of *E. coli*, the *luciferin* gene of fire-fly or the *GFP* (Green Fluorescent Protein) have been used. Since a variety of conditions, including environmental pollutants and pathological conditions induce cell stress (Arya *et al.*, 2007), assessment of activity levels of heat shock genes through the reporter transgenes is a useful parameter (Mukhopadhyay *et al.*, 2003).

Two different approaches are described here to study the heat shock response in *Drosophila* cells. In the first, polytene chromosomes are used to directly see the heat shock induced puffs while in the second, a transgenic line (*Bg9*, for details, see O'Kane and Gehring, 1984) carrying a reporter gene construct, in which the HSE of the *hsp70* gene of *Drosophila melanogaster* is placed upstream of the *lacZ* gene of *E. coli*, is used. The heat shock induced expression of the *lacZ* gene encoded β -galactosidase is detected histochemically using X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside), a chromogenic substrate for β -galactosidase (see [Chapter 39](#)). Cellular localization of the blue colored product thus provides information or 'report' about the spatial and temporal expression of the specific promoter (HSE-carrying *hsp70* gene promoter in the present case) being studied.

Objective:

To demonstrate heat shock induced gene activity in *D melanogaster* using (a) polytene chromosomes and (b) a *heat shock promoter-lacZ* fusion reporter gene.

Materials Required:

A. For examining heat shock induced puffing in polytene chromosomes

- i. **Larvae:** Healthy late third instar wild type *Drosophila melanogaster* larvae grown, without crowding, on food with additional yeast-supplement at 24°C.
- ii. **Equipment and other supplies:**
 - a. Stereo-binocular and Bright-field microscope (optional: phase-contrast optics)
 - b. Fly-handling items
 - c. Incubator/water bath maintained at 37°C
 - d. Slides and coverslips (22 mm²): To obtain good squash preparations, it is essential that the slides and coverslips are totally free of any dust-particles, fibers and greasy material. A simple way to achieve this is to store the fresh (or soap-cleaned, if desired) slides and coverslips in 90% ethanol in suitable containers and wipe them dry, immediately before use, with a clean soft silken cloth. These are stored in a covered dust-free box till used

- e. Fine-tipped forceps, fine dissection needles and soft brush for handling larvae
- f. Filter/blotting paper
- g. Microfuge tubes
- h. Plastic/glass droppers or Micropipette and tips

iii. Solutions:

a. Poels' Salt Solution (PSS, pH 6.8)

- NaCl 86 mg
- KCl 313 mg
- CaCl₂·2H₂O 116 mg
- NaH₂PO₄·2H₂O 88 mg
- KHCO₂ 18 mg
- MgSO₄·7H₂O 513 mg
- Dist. H₂O 100 mL

Adjust pH to 6.8 with 1M NaOH and filter.

b. Aceto-Methanol 1:3 (freshly prepared)

c. 50% Glacial acetic acid

d. Aceto-Orcein (2%) stain: Dissolve 2 gm Orcein in 100 mL of 50% Acetic acid by boiling for 30 min with a reflux condenser. Filter when cool. The prepared stain can be used for many months but it should be periodically filtered before use to avoid stain particles.

e. Aceto-Carmine (2%) stain: Dissolve 2 gm Carmine powder in 100 mL of 50% Acetic acid by boiling for 2 Hr under a reflux condenser. Filter when cool. The prepared stain can be used for many months but it should be periodically filtered before use to avoid stain particles.

f. Lacto-Aceto-Orcein (2%) (optional): Dissolve 2 gm Orcein powder in a solution containing 51 mL glacial acetic acid, 34 mL distilled water and 15 mL of 85% lactic acid by boiling for 2 hr on a very low flame in a flask fitted with a reflux condenser. Filter when cool. The prepared stain can be used for many months but it should be periodically filtered before use to avoid stain particles.

Note: Though these stains are also commercially available, those properly prepared in the laboratory seem to work better.

g. Transparent nail polish.

B. For examining β -galactosidase reporter activity in transgenic strain carrying *hsp70-lacZ* fusion gene

i. Fly strains:

Bg9 transgenic stock with the *hsp70 promoter-lacZ* fusion construct inserted at the chromosome region 9B on X-chromosome (Lis *et al.*, 1983).

ii. **Equipment and other supplies:** as listed under the heat shock induced puffing part above

iii. Solutions:

a. Poels' Salt Solution (PSS, pH 6.8)

b. 1M Phosphate Buffer (pH 8.0)

- 1M Na₂HPO₄ 93.2 mL
- 1M NaH₂PO₄ 6.8 mL

c. Wash Buffer (50 mM Phosphate Buffer pH 8.0)

- 1M Phosphate Buffer 5 mL
- Dist. Water 95 mL

d. 4% paraformaldehyde: freshly prepared in PBS by dissolving 40 mg of Paraformaldehyde in 1mL PBS by heating at 65°C with rapid stirring till completely dissolved

e. X-Gal Activity stain

- 5% X-Gal (in Dimethylformamide) 60 µL
- 100 mM K₃[Fe(CN)₆] 20 µL (2 mM)
- 100 mM K₄[Fe(CN)₆] 20 µL (2 mM)
- 1M Phosphate buffer (pH 8.0) 50 µL
- Dist. Water 850 µL

Note: It is important to ensure that the activity stain has a pH of 8.0 since, unlike the *D. melanogaster* β-galactosidase, the *E. coli* enzyme is active at alkaline pH.

f. 50% Glycerol

- Glycerol 5 mL
- Dist. Water 5 mL

Experimental Details:**A. For examining heat shock induced puffing in polytene chromosomes**

1. Wash healthy late third instar wild type larvae (grown under uncrowded conditions at 20°C-24°C) in water to free them of adhering food, etc., and transfer some larvae to a small Petri dish lined with a moist filter paper and cover with its lid. Place the closed Petri dish with larvae in an incubator maintained at 37°C for heat shock for 30 min. The Petri dish should be pre-warmed to 37°C before transferring larvae for heat shock. Parallel control larvae are similarly kept at 24°C for the same period. Alternatively, dissect salivary glands from 4-5 larvae in PSS (see [Chapter 31](#)) and transfer to a 1.5 mL microfuge tube containing 50 µL PSS. Place the microfuge tube in a 37°C water bath for 30 min for heat shock. Parallel control glands are similarly kept in PSS at 24°C for the same period.

2. Following the 30 min heat shock, remove the larvae or salivary glands from 37°C incubator/water bath. If the larvae were heat shocked, rapidly dissect out salivary

glands from heat shocked and control larvae in PSS at 24°C.

3. Fix salivary glands with freshly prepared aceto-methanol fixative for 1 min and proceed for staining and squashing of salivary glands as described in steps 3-7 of Experimental Details in [Chapter 31](#).

B. For examining β -galactosidase reporter activity in transgenic strain carrying *hsp70-lacZ* fusion gene

1. Wash healthy late 3rd instar *Bg9* larvae (grown under uncrowded conditions at 20°C -24°C) in water to free them of adhering food, etc., and heat shock them as in step 1 above. A parallel set of *Bg9* larvae is kept at 24°C as control.

2. Rapidly dissect out gut (or any other desired tissue) from heat shocked and control larvae in PSS at 24°C (see [Chapters 16, 18](#)). Transfer the dissected control and heat shocked tissues to separate and appropriately marked Maximov or cavity slide, each containing 50 μ L PSS.

3. Remove the PSS from each and fix the dissected tissues with 100 μ L of freshly prepared 4% paraformaldehyde. Cover the Maximov slide and keep for 10 min at 24°C.

4. Remove the fixative and wash 2 times with wash buffer.

5. Remove the wash buffer and add 50 μ L of the X-Gal staining solution (see [Chapter 39](#)). Cover the Maximov slide and keep overnight in a moist chamber in dark at 37°C.

6. Remove the X-Gal stain, wash the tissues with wash buffer.

7. Transfer tissues to a fresh slide in a drop of 50% glycerol; spread the tissues as desired and carefully mount with a clean coverslip. Seal the edges of coverslip with nail polish.

OBSERVATIONS:

A. Heat shock induced puffing in polytene chromosomes:

Examine well-spread polytene nuclei in squash preparations from control and heat shocked salivary glands to identify the puffs that are either larger or present only in polytene chromosome spreads from heat shocked glands. Using the images in [Fig. 1A](#) and [B](#) as references, identify the major heat shock puff loci in chromosome spreads in control and heat shocked samples.

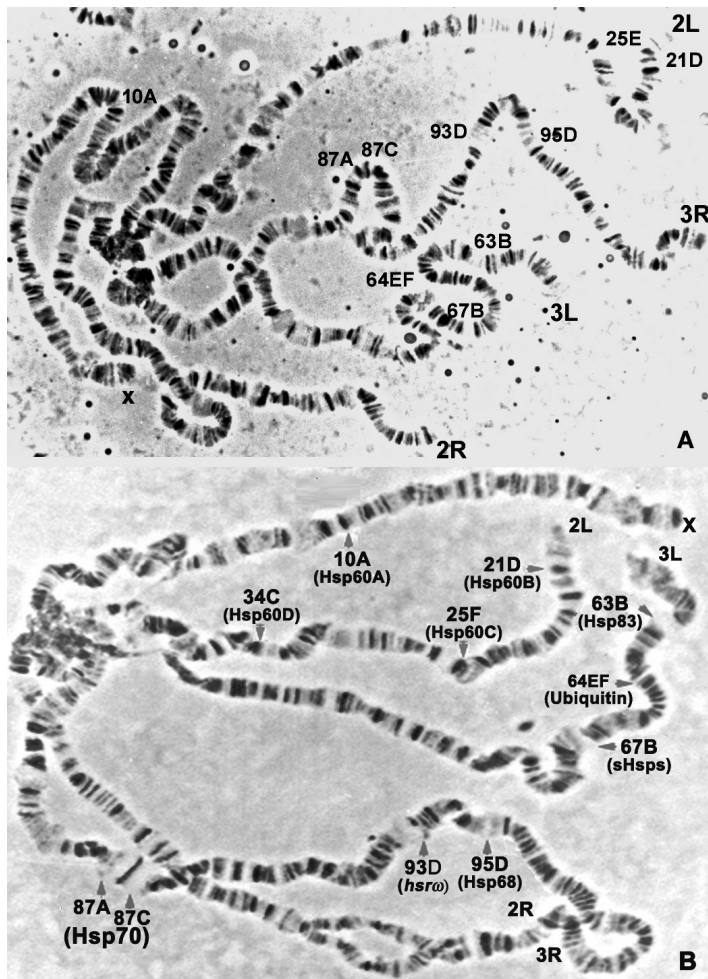


Fig. 1. Heat shock induces several major new puffs (e.g., 64EF, 87A, 87C, 95D) and enhances activity of some developmental puffs (e.g., 63B, 67B, 93D), which are active in control glands also. **A.** Polytene chromosome spread from a salivary gland maintained at 24°C. **B.** Polytene chromosome spread from a salivary gland heat shocked at 37°C for 30 min. The different long chromosome arms (X, 2L, 2R, 3L and 3R) and major heat shock gene sites are marked. The protein/RNA products of the major heat shock puffs are noted in parentheses near the identified heat shock loci in **B**. The *hsp60* gene loci on X (10A) and 2L (21D, 25F and 34C) do not develop heat shock induced puffs in larval salivary glands.

C. Heat shock induced β -galactosidase reporter activity in transgenic strain carrying *hsp70-lacZ* fusion gene

Examine the difference in the bluish-black X-Gal staining in tissues from control and heat shocked larvae.

Fig. 2 shows X-Gal stained gut (see [Chapter 12, 20](#)) from control and heat shocked *Bg9* larvae. The light bluish color seen in control gut is mostly background. Gut tissues from heat shocked *Bg9* larvae shows intense blue-black colour because of high levels of β -galactosidase. The larval Malpighian tubules, however, do not show induced β -galactosidase activity since, unlike any other larval or adult tissues, the larval Malpighian tubules do not show induction of *HSP70* (and other common members of heat shock protein families) following heat shock (Lakhotia and Singh, 1989).

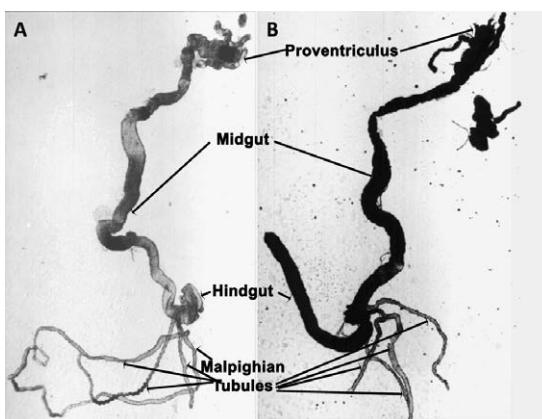


Fig. 2. Heat shock induces β -galactosidase activity as revealed by X-gal staining of gut of late third instar *Bg9* larvae, transgenic for the *hsp70-lacZ* fusion gene. **A.** Gut from control larva maintained at 24°C. **B.** Gut from larva heat shocked at 37°C for 60 min.

Questions for self-study and interpretation:

1. Why are heat shock genes specifically induced in stressed cells while most other genes become inactive?
2. Would you see the induced heat shock puffs in salivary gland if they were maintained at 24°C for 1 or 2 hr after the 30 min heat shock?
3. *Drosophila* cells express their own β -galactosidase? Would that not interfere in assaying the activation of the *hsp70-lacZ* transgene in *Bg9* larvae?
4. What are functions of different heat shock proteins?
5. One of the genes (*hsr ω* or *hsr ω*) induced by heat shock produces multiple long non-coding RNAs. Do you think this is a selfish gene?

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Heat Shock Puffs – Unique Way to Analyze Gene Function

The discovery of the induction of a unique set of puffs by heat shock (Ritossa, 1962) has led the way to an analysis of gene function and structure in *Drosophila* that is, so far, unique.

(In: Ashburner, M. & Bonner, J. J. 1979. The induction of gene activity in *Drosophila* by heat shock. *Cell* 17: 241-254).

Chapter 47

Assaying the effect of ambient temperature on lifespan of *Drosophila*

Chitrang Dani (chitrang@jncasr.ac.in) and Vasu Sheeba* (sheeba@jncasr.ac.in)
Chronobiology and Behavioural Neurogenetics Laboratory, Neuroscience Unit, Jawaharlal
Nehru Centre for Advanced Scientific Research, Bengaluru

* For correspondence

Recommended Level: UG/PG

Sub-discipline: Physiology /Behavioural Biology /Ecology

Background:

According to the ‘rate of living’ theory originally proposed by Alpatov and Pearl in 1929, the duration of life for an organism is dependent both on genetically determined metabolic energy and the rate at which this energy is spent (Grotewie *et al.*, 2005). Since *Drosophila* has been a model organism for aging related research for more than a century, assays to estimate adult lifespan (the duration of life span following eclosion from pupa till death) have been routinely carried out by many researchers. Lifespan is a polygenic trait i.e., several genetic factors contribute to this phenotype. In addition, several environmental factors as well as interactions between genotype and environment influence lifespan. Some non-genetic factors that are known to influence lifespan are temperature (Helfand and Rogina, 2003), humidity, light levels, food quality (Joshi and Mueller, 1997) and population density (Le Rohellec and Le Bourg, 2009). The length of lifespan has been found to be inversely proportional to the ambient temperature (Helfand and Rogina, 2003). In this simple experiment we aim to assess the effect of ambient temperature on lifespan of flies, attempting to keep all other factors similar.

Objective:

To evaluate effect of 25°C and 30°C ambient temperatures on lifespan of *Drosophila*.

Note: This is a long-term study and gives students an experience of carrying out a research project, rather than a single day study .

Materials Required:

1. Wild type fly cultures (Canton S, Berlin K, Oregon R, or any other locally caught and identified strains)

2. Vials with standard fly food medium

3. B.O.D. Incubators maintained at 25°C and 30°C (light and humidity controls are recommended but not essential)

Note: A room with an air conditioner, set at 25°C can be used in absence of incubators. In this case, you may choose to compare between two types of lighting conditions – constant lights ON or a 12:12 hr light:dark cycles.

Alternatively, lights may be turned ON or OFF at appropriate time manually or using a simple timer available at a nominal cost from local electrical shops or online, which can be fitted to the electrical points in the room or in the incubator.

Experimental Details:

1. Healthy wild type flies are fed on yeast paste supplemented medium for 3 days before collecting synchronously growing eggs. In order to ensure synchronous growth of eggs, flies are first transferred to fresh food for 1 hr for oviposition. These eggs are not used for experiment since the eggs laid in this first hr on fresh food are likely to be at various stages of development (asynchronous).

2. After 1 hr, the flies are transferred to fresh medium for oviposition for the next 1 hr. These timings need to be strictly followed to ensure that the eggs laid during this one hour develop synchronously.

3. With a soft moist brush, collect and dispense 50 eggs from the above step into a vial. Make 20 such replicate vials. These vials will be maintained a two different regimes of temperatures, viz., 25°C and 30°C (in order to avoid additional variables, it is desired that the growing larvae, pupae and adults are maintained under constant light conditions or under 12:12 hr light: dark cycles). Adults eclosing from these vials will be used for the lifespan assay.

4. Watch these vials closely around 8-9 days post egg-collection. Collect the freshly eclosed males and females and keep in food vials at a density of four males and four females per vial.

5. Set up 10 such vials each for maintenance at 25°C and, respectively.

6. Observe the vials everyday for any deaths. Verify deaths by tapping on vials to distinguish from immobility due to other reasons. Note in a record sheet (sample shown below).

7. Every alternate day gently transfer flies to fresh food vials. Note deaths in the same data record sheet.

8. The assay continues till the death of all flies which will typically last 8 – 10 weeks.

9. In case of flies escaping or accidental death due to crushing while transfer to fresh food vials, the same must be noted and must not be included in calculations.

Results/Observations:

1. Record the deaths of male and female flies every day. Estimate the age as days after emergence from pupal cases in the record sheet (sample as in **Table 1**). Continue to record these events till all the flies in all the vials are dead.

Lifespan assay data sheet: Number of dead flies on each day of the experiment (separate sheet to be prepared for each vial being examined).

Experimental conditions:		Temperature: 25°C/30°C (select as applicable)								
Lights: ON continuously										
Humidity: ~ 70%; Any other details:		Name of experimenter: _____								
Number of dead flies										
Date of observation (age in days)										
DD/MM/YY (1)	M									
	F									
DD/MM/YY (2)	M									
	F									
DD/MM/YY (3)	M									
	F									
DD/MM/YY (4)	M									
	F									
Continue till all the flies are dead	M									
	F									

2. Plot survivorship curves for:

- (a) each vial (males and females separately and pooled),
- (b) pooled across all vials (males, females, pooled) for each temperature regime. This is done by calculating percentage (or fraction) of the originally tested flies remaining alive on each day after the treatment and plotting the same on the y-axis, with the x-axis being age (in days).

3. Estimate the median lifespan for each of the above two maintenance temperatures.

4. Where do you see major points of inflection on the survivorship curves? How does that change across temperature?

5. For more detailed analysis, conduct a log-rank test to statistically compare survival curves

Questions for self-study and interpretation:

- 1. List factors in your assay that could impact the lifespan of the flies.
- 2. Is there a difference in lifespan across the temperature treatments?
- 3. Is there a sex specific difference in the lifespan of flies? Think of reasons why that might be the case.
- 4. How different would a protocol for assaying lifespan of virgin flies be? Would the results be different? In what ways?

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Chapter 48

Heat stress tolerance in *Drosophila*

Chitrang Dani (chitrang@jncasr.ac.in) and Vasu Sheeba (sheeba@jncasr.ac.in)*
Chronobiology and Behavioural Neurogenetics laboratory, Neuroscience Unit,
Jawaharlal Nehru Centre for Advanced Scientific Research, Bengaluru

* For correspondence

Recommended Level: UG/PG

Sub-discipline: Physiology/ Behavioural Biology/ Ecology

Background:

Heat shock occurs due to sudden, brief exposure to high temperature, usually 10 to 15°C above ambient conditions. The ability to survive physiological stress of high temperature is believed to be an important component of evolutionary fitness in insects and many other ectotherms/poikilotherms. Importantly, these stressful conditions have the potential to induce recombination or mutations leading to changes in genetic variance in the population residing in a habitat. This could lead to developmental changes that might lead to variation in thermal resistance of offspring (Hoffmann and Hercus, 2000). For *Drosophila melanogaster*, limits of thermal tolerance observed for egg-to-adult viability are usually in the range 10 to 32°C, while the range for male fertility is relatively narrower, from 12 to 30°C. Even a short thermal stress exposure can affect physiology of flies drastically and may render them susceptible to other stressors such as starvation (Klepsatel *et al.*, 2016). The quality of habitat is partly determined by how much exposure organisms get to extreme environmental factors, including thermal stress. The response of organisms to such stress can vary based on magnitude of the temperature rise above ambient conditions, length of exposure and sensitivity of organisms to stressor stimuli (Hoffmann, 2010). This laboratory exercise is intended to test the effect of varying magnitudes of thermal stress on survival of male and female *Drosophila melanogaster*.

Objectives:

To estimate tolerance to heat shock for 1 hour at different temperatures: 25°C (control), 30°C and 35°C for male and female flies.

Materials Required:

A. Fly cultures (wild type and/or mutant, as desired)

- B. Glass vials
- C. Cotton
- D. CO₂ or Ether for anaesthetising flies
- E. Fly food medium
- F. Temperature logger/thermometer
- G. Incubator or sand/water bath.

Experimental Details:

1. Use freshly emerged flies of the desired genotype (wild type in the present example), sex them and separate virgin males and females into different vials (75 of each sex is minimally required).
2. After collection is completed, transfer five flies of one sex to one food vial each to make 15 replicates for each sex. For assaying the effect of three different heat-stress temperatures (25°C, 30°C, and 35°C), a set of 5 vials per sex will be used for each temperature. Label the vials accordingly and keep them ready.
3. In the present example, the heat shock tolerance assay is to be conducted 3 days post-collection and separation of flies (4-5 days old). Until then, these vials are stored in the same conditions as the maintenance regime.

NOTE: This study can be modified to test the effect of ageing on heat shock tolerance by adding an additional set of flies of an older age group.

4. Immediately before exposing the flies to heat stress, transfer flies from each food vial to a correspondingly labelled empty vial plugged with cotton. If a water bath is used for administering the heat shock, care should be taken to ensure that the cotton plugs do not become wet and that water does not seep into the tube.
5. Heat shock for 60 min at 30°C, and 35°C (along with controls treated exactly the same, but kept in parallel at 25°C) should be given to 5 vials each of male and female flies. **NOTE:** Ideally, the time of day when the experiment is carried out should be restricted to a short window of 2 hr and kept uniform for all treatments. This is because most physiological processes vary across time of day. If unavoidable, the three temperature treatments can be carried out on separate days using similarly aged flies, but by ensuring that it is the same time window.
6. Immediately after administering the heat shock, transfer flies from the empty vials to appropriately labelled food vials for recovery.
7. Keep data recording sheet ready before starting the experiment. A sample is given in **Table 1**.

Note: It is generally good laboratory practice to note down the timing of lights ON and OFF in the laboratory or incubator or room where flies are reared and where the experiment is performed. It is also ideal to keep these conditions uniform throughout the study. For example ensure that lights are switched ON exactly at the same time of day and switched OFF at the same time of evening / night either manually or using a mechanical timer.

Furthermore, all experiments where one is comparing across treatments or genotypes must be performed within the same time window, even if it is spread across several days, since it is well known that almost all physiological and behavioural processes show changes across the time of day due to the presence of internal clocks and external cycling environmental factors.

Results/Observations:

1. Count the number of dead flies for each heat shock batch immediately after (0 hr) and 24 hr post-heat shock.

Note: The immobile flies at 0 Hr may be dead or simply knocked out by heat-stress.

2. Based on readings at 24 hr, post heat shock, calculate the percentage of surviving flies (based on dead flies) for males and females for each heat shock treatment after pooling the information from all the 5 vials.

Note: Students in a class can carry out replicate experiments either individually or in groups and obtain replicate values for percentage survival.

3. Compile this information for the entire class and finally, plot graphs for mean number (and standard deviation) of live flies for each treatment.

4. The following statistical comparisons are recommended:

- i. Testing for the effect of temperature of heat shock treatment and sex separately using Student's t-test.
- ii. Testing using a three-way ANOVA using temperature and sex (fixed factors) and trial (random factor).

Table 1: Heat stress tolerance assay: Number of flies surviving at different times after heat shock at different temperatures

Trial #: _____		Name of Experimenter: _____					
Lights ON: _____		Lights OFF: _____		Time of Heat Shock: _____			
SEX	VIAL NO.	Heat shock at					
		25°C (control)		30°C (treatment 1)		35°C (treatment 2)	
		Number of dead/immobile flies at different times after heat shock					
		0 hr	24 hr	0 hr	24 hr	0 hr	24 hr
Male	Vial 1						
Female	Vial 2						
Male	Vial 3						
Female	Vial 4						
Male	Vial 5						
Female	Vial 6						
Male	Vial 7						
Female	Vial 8						
Male	Vial 9						
Female	Vial 10						

Questions for self-study and interpretation:

1. Does heat shock tolerance of flies vary depending on the sex of the fly?
2. What is the trend in heat shock tolerance of males and females across different temperatures? Would the results be affected if males and females were subjected to the heat stress together?
3. Comment on the limitations/confounding factors in the experiment in assessing heat tolerance.
4. Does prior exposure to different temperatures change the heat tolerance ability of flies?
5. If these studies are done on young (5-10 days old) versus old (30-40 days old) flies, would you see any differences in heat shock tolerance?
6. If these studies were done on virgin versus mated flies, would you expect to see a difference?
7. Do flies sampled from different locations show variation in heat tolerance (e.g. compare across flies from the wild using baits or traps placed in outdoor markets, fruit orchards versus long-term laboratory strains)?

References and further reading:

- Hoffmann, A. A. and Hercus M. J. (2000) Environmental stress as an evolutionary force, *BioScience*, 50: 217–226.
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- Klepsatel, P., Gálíková, M., Xu, Y. and Kühnlein, R. P. (2016) Thermal stress depletes energy reserves in *Drosophila*, *Scientific Reports*. 6: 33667.

Chapter 49

Effect of environmental temperature on development time in *Drosophila*

Chitrang Dani (chitrang@jncasr.ac.in) and Vasu Sheeba* (sheeba@jncasr.ac.in)
Chronobiology and Behavioural Neurogenetics Laboratory, Neuroscience Unit,
Jawaharlal Nehru Centre for Advanced Scientific Research, Bengaluru

* For correspondence

Recommended Level: UG/PG/Research

Sub-discipline: Developmental Biology/ Physiology/ Behavioural Biology

Background:

Drosophila melanogaster is a holometabolous insect, which after the egg stage, passes through three larval instars and a pupal stage before emerging (or eclosing) as an adult. The time taken for these stages is strongly influenced by the environmental conditions experienced by each life stage. Under standard laboratory conditions (i.e. constant 24°-25°C and a 12:12 hr light:dark cycle), flies complete one lifecycle in ~10 days on nutritious food medium. Under these conditions, eggs hatch ~24 hr after being laid. Larvae are the major feeding life-stage, and the total duration of the three instars is about four days. The pupal duration is also about four-five days, after which adults emerge. The third-instar larvae must attain a critical size/weight before pupa formation (Robertson, 1963). The molting that leads to the next larval instar and conversion of third instar larva to pupal stage are correlated with pulses of the molting hormone, ecdysone (Warren *et al.*, 2006). Like several ectotherms, duration of development in *Drosophila* is sensitive to temperature and food quality. Furthermore, eclosion of adult flies from pupae is controlled by a circadian clock and the peak of eclosion occurs (i.e., the majority of flies eclose) shortly after the dark-to-light transition (Pittendrigh, 1954).

Objectives:

To estimate (A) egg to puparium formation and (B) egg to adult development times of *Drosophila* under different developmental temperatures, viz., standard laboratory temperature 24°-25°C, constant cool (20°C) or (C) constant warm (28°C) temperatures.

Materials Required:

A. Wild type fly culture

B. Fly food bottles, vials and other items required for fly handling

C. Stereo-binocular microscope

D. BOD incubators set at different temperatures (20°C, 25°C and 28°C; it is desirable that they also maintain 12 hr:12 hr light:dark cycles).

Experimental Details:

1. Three to five days old flies (~15 males and 20 females per bottle) are transferred to fresh culture bottles with yeast paste supplemented medium 3 days prior to the start of experiment.

2. Transfer the flies to egg collection chambers (see [Chapters 32, 34, 39, 70](#)) with fresh food plates for one hr after which the flies are transferred every hr to fresh food plates. Eggs laid in the first food plate are not used for experiments; this food plate helps in relieving the female flies of fertilized eggs that may have been retained in their body so that the next sets of hourly collections of eggs would develop more synchronously. The timings for egg collections in experimental plates must be noted to ensure accurate estimates of development time.

3. Under a stereo-microscope, using a soft wet brush, collect eggs from the hourly collection and dispense exactly 30 eggs into fresh food vials. Make 10 such replicate vials for each environmental regime that is to be examined.

4. (A) Pupariation time: After the first puparium is observed in a vial, check vials at regular intervals and count the number of pupae formed. Shorter intervals give more accurate estimates of development time, hence 2 hr intervals are ideal. If not possible, checks can be performed at longer intervals. Stop observations when no new pupariation event is seen for 24 hr.

5. (B) Pupation-to-adult emergence time: Fly emergence usually begins about 4 days after pupariation at 25°C, but may be shorter at higher temperature. Therefore, after about 48 hr of first recorded pupariation event, closely monitor fly emergence by checking vials at regular intervals to count the number of flies that emerge from the darkened pupae. Gently remove the emerged flies from the vial, anesthetise and note their sex. Take care not to damage or disturb the yet un-eclosed pupae. Terminate the assay when all pupae have emerged, or no emergence event is observed for 24 hr. It is ideal to observe every 2 Hr to obtain reasonably accurate estimates (see Paranjpe *et al.*, 2005).

Results/Observations:

1. Using the recorded observations (as in Tables **1A** and **1B**), estimate mean/median pupariation times and adult emergence times for the different developmental temperature regime used.

2. Calculate the percentage of flies that emerged from the total eggs dispensed (pre-adult survivorship) for each vial and average across vials for each regime.

3. Calculate average sex ratio from emergence data. Compare times for male and female flies from pupation to adult emergence.

4. Plot appropriate graphs for visualization and compare results statistically.

Table 1A. Format for recording observations on pupation times after egg collection at different developmental temperatures

Date and Time of egg collection:											
Experimental conditions: i) Growth temperature ii) Lights ON: 10:00 Lights OFF: 22:00 (optional)											
Days after egg laying	Hours after 1st pupation event	Number of pupation events on different days									
		Vial 1	Vial 2	Vial 3	Vial 4	Vial 5	Vial 6	Vial 7	Vial 8	Vial 9	Vial 10

Table 1B. Format for recording observations on adult emergence times after pupariation at different developmental temperatures

Date and Time of egg collection:													
Experimental conditions: i) Growth temperature ii) Lights ON: 10:00 Lights OFF: 22:00 (optional)													
Days after 1st pupation event	Hours after 1st eclosion event	Sex	Number of eclosion events on different days										
			Vial 1	Vial 2	Vial 3	Vial 4	Vial 5	Vial 6	Vial 7	Vial 8	Vial 9	Vial 10	
		M											
		F											
		M											
		F											
		M											
		F											
		M											
		F											
		M											
		F											
		M											
		F											

Questions for self-study and interpretation:

1. What are the possible factors that may have impacted pre-adult survivorship in your assay? Under real-world conditions for the fly, what factors may impact development time?
2. Can the assay results be expected to remain the same if the number of eggs per vial was increased to five times of what was used, i.e., 150 eggs/vial? Explain.
3. Would the weight/size of the flies emerging faster or slower due to different development temperatures be different? What may be implications of such a difference, if it indeed occurs, on physiological fitness of the flies?
4. Is a light regime based difference in development time expected? Why or why not?

References and further reading:

Pittendrigh, C. S. (1954) On temperature independence in the clock system controlling emergence time in *Drosophila*. Proc. Natl. Acad. Sci. USA. 40: 1018 – 1029

Paranjpe, D. A., Anitha, D., Chandrashekar, M., Joshi, A. and Sharma, V. K. (2005) Possible role of eclosion rhythm in mediating the effects of light-dark environments on pre-adult development in *Drosophila melanogaster*. BMC Developmental Biology. 5: 1-6.

Robertson, F. W. (1963) The ecological genetics of growth in *Drosophila* 6. The genetic correlation between the duration of the larval period and body size in relation to larval diet. Genetical Research. 4: 74–92.

Warren, J. T., Yerushalmi, Y., Shimell, M. J., O'Connor, M. B., Restifo, L. L. and Gilbert, L. I. (2006) Discrete pulses of molting hormone, 20-hydroxyecdysone, during late larval development of *Drosophila melanogaster*: correlations with changes in gene activity, Developmental Dynamics. 235: 315 – 326.

Chapter 50

Testing the ability of *Drosophila* to tolerate desiccation stress

Chitrang Dani (chitrang@jncasr.ac.in) and Vasu Sheeba* (sheeba@jncasr.ac.in)
Chronobiology and Behavioural Neurogenetics Laboratory, Neuroscience Unit,
Jawaharlal Nehru Centre for Advanced Scientific Research, Bengaluru

* For correspondence

Recommended Level: UG/PG

Sub-discipline: Physiology /Behavioural Biology /Ecology

Background:

Desiccation tolerance refers to the ability of the organism to tolerate low humidity. Insects have small sizes and high surface-area to volume ratio, thus rendering them highly vulnerable to water loss (Gibbs and Rajpurohit, 2010). *Drosophilid* species occupy a wide range of different ecosystems on this planet, among which, the availability of water may be limited in many habitats and therefore, desiccation is a significant challenge in such regions. Three primary mechanisms have been proposed to enhance tolerance to desiccation stress: reduced rates of water-loss through the cuticle, increased tolerance of water-loss or increased water storage (Gibbs *et al.*, 2003). All of these three mechanisms can contribute to variation in desiccation tolerance in different species of *Drosophila* or in different populations of the same species (Telonis-Scott *et al.*, 2006). In most laboratories, flies are reared in rooms with about 70% relative humidity (RH). However, it varies across the time of year (ranging from ~50 to ~80% RH), unless the air-conditioned rooms have strict humidity controls in place. In nature, the RH can vary greatly (<50% to >80%) across seasons and geographical regions.

Objectives:

To estimate desiccation tolerance of *Drosophila* using time of death due to desiccation stress as a proxy.

Materials Required:

A. Fly culture: Wildtype laboratory strains such as Canton S, Oregon R, Berlin K, or locally caught populations of *Drosophila melanogaster* from different habitats can be used.

B. Glass vials

C. Cotton

D. CO₂ or ether or ice cubes for anaesthetising flies (a mouth aspirator can also be used to handle flies).

E. Powdered CaCl₂ (laboratory grade)

F. Small plastic trays

G. BOD incubator

H. Humidity data logger (or manual recording using a hygrometer)

Note: A desiccant such as CaCl₂ is not required if an incubator is available in which humidity can be controlled.

Experimental Details:

1. On emergence of flies from the stock culture, sex them, separating virgin males and females into different vials (50 of each sex required).
2. After collection is completed, transfer five flies to one food vial (3 mL) each to make 10 replicates for each sex.
3. Desiccation tolerance assay is to be conducted 3 days post collection and separation of flies. Until then vials with flies should be stored in the same conditions as the maintenance regime.
4. For the experiment, place trays with CaCl₂ in the incubator with temperature set at 25°C and lights ON (optional). Check humidity and adjust till it is lowered to ~20% relative humidity (RH). Ideally, controls should be placed in an identical chamber without the CaCl₂ and assayed in parallel. If that is not possible, repeat the same procedure as experimental on another day in the same arena without CaCl₂, preferably within the same time window.
5. Transfer flies from each food vial to an empty cotton-plugged vial each and place inside incubator.
6. Monitor vials for dead flies every 2 hr (Record data as in sample **Table 1**). Add/replace CaCl₂ to maintain low humidity throughout the duration of experiment.
7. Assay continues till all flies are dead.

Table 1. Survival of flies exposed to desiccatio

Name of Sample		Date: _____		Experiment number: _____ Light conditions: _____ Temperature: 25°C Humidity: 20%; Any other details: _____									
				Number of dead flies t different intervals- Control (Ctrl) /Desiccation (Stress)									
Sex	Time	8:00		10:00		12:00		14:00		16:00		18:00	
	Vial	Ctrl	Stress	Ctrl	Stress	Ctrl	Stress	Ctrl	Stress	Ctrl	Stress	Ctrl	Stress
Male	1												
Male	2												
Male	3												
Male	4												
Male	5												
Female	6												
Female	7												
Female	8												
Female	9												
Female	10												

Results/Observations:

1. From the record of death of flies, plot a frequency distribution and estimate the mean and median time to death of male and female flies separately.
2. Compare all these measures between males and females. How do they differ? Do you see any difference in mean and median for these distributions? If so, why might that be the case?
3. Plot appropriate graphs for both sexes and their respective controls. Use a statistical test such as Student’s t-test to examine whether there are differences between male and female ability to tolerate desiccation.
4. What are the likely reasons for the difference if any among the sexes?
5. How does desiccation affect physiology of flies?

References and further reading:

Gibbs, A. G., Fukuzato, F. and Matzkin, L. M. (2003) Evolution of water conservation mechanisms in *Drosophila*. *J. Exp. Biol.* 206: 1183–1192.

Gibbs, A. G. and Rajpurohit, S. (2010) Cuticular lipids and water balance. *Insect Hydrocarbons: Biology, Biochemistry and Chemical Ecology*. 100-120.

Telonis-Scott, M., Guthridge, K. M. and Hoffmann, A. A. (2006) A new set of laboratory-selected *Drosophila melanogaster* lines for the analysis of desiccation resistance: response to selection, physiology and correlated responses, *J. Exp. Biol.* 209:1837–1847.

Chapter 51

Oxidative stress in *Drosophila*: Measurement of Trolox equivalent antioxidant capacity

Avinash, K. Kudva¹ (avinash.kudva@gmail.com) and Rajashekhar, K. Patil^{2*} (patilsirmu@gmail.com)

1. Department of Biochemistry, Mangalore University, Mangalagangothri -574199, Karnataka, India

2. Department of Applied Zoology, Mangalore University, Mangalagangothri -574199, Karnataka, India

* For correspondence

Recommended Level: UG/PG/Research

Sub-discipline: Biochemistry/Cell Biology/ Physiology

Background:

Oxidative stress is the general phenomenon of oxidant exposure and antioxidant depletion or oxidant–antioxidant balance. Oxidants, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), can generate free radicals that can cause severe oxidative damage to cellular lipids, membranes, proteins, and DNA. Antioxidants can scavenge these free radicals and prevent cellular oxidative stress by enzymatic and non-enzymatic mechanisms. Tocopherols, carotenes, vitamin A, and ubiquinols function as lipid-soluble antioxidants, whereas glutathione and ascorbate are water-soluble antioxidants. Total antioxidant capacity (TAC) is the measure of the amount of free radicals scavenged by a test solution, for example the antioxidant capacity of biological samples. Assays to measure TAC can be direct, which are based on the ability to inhibit the oxidation of a substance. One of the most commonly used direct assays is the Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) equivalent antioxidant capacity (TEAC) with modifications mainly based on period of time used for measurement, and radical formed (Erel, 2004; Miller *et al.*, 1993; Re *et al.*, 1999). This protocol is based on the ability of antioxidants in a sample to reduce or inhibit oxidized products generated in the assay. It is based on the principle that when ABTS [2, 2'-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid)] is incubated with an appropriate chemical, an ABTS radical (ABTS•+) is formed. The ABTS•+ has a blue-green color, with maximum absorptions at 650, 734 and 820 nm. Antioxidants in the sample reduce ABTS•+ production, and thus the absorbance to a degree that is proportional to the antioxidant's concentrations. Reaction rate is commonly calibrated with Trolox, a water-soluble analogue of vitamin E, and assay results are expressed in mM Trolox equivalent/mL.

Objectives:

To measure levels of non-enzymatic antioxidants present, in the tissue sample.

Materials Required:

A. Flies: 2-3 day old wild type male / female *Drosophila melanogaster*.

B. Equipments: UV-Visible spectrophotometer, Centrifuge, pH meter, micropestle homogeniser.

C. Glass and plasticwares: micro-volume pipettes and corresponding tips (10, 200, 1000 μ L), microcentrifuge tubes (1.5 mL), beakers.

D. Reagents:

i. Phosphate buffered saline (PBS)

a. NaCl- 8.0 g

b. KCl - 0.2 g

c. Na_2HPO_4 - 1.42 g

d. KH_2PO_2 - 0.272 g

Weigh the above chemicals and dissolve them step-by-step in 800 mL distilled water. Adjust the pH to 7.4 using dilute HCl or NaOH. Finally make up the volume to 1000 mL with distilled water. Autoclave and store at RT or 4°C.

ii. ABTS•+ Stock Reagent:

a. ABTS [2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid)]- 192 mg

b. Potassium persulfate - 32 mg

Dissolve 192 mg ABTS (7 mM) in 50 mL water. Similarly in another beaker dissolve 32 mg potassium persulfate (2.45 mM) in 50 mL water. Take equal volume of both the solutions and mix in a standard flask and allow it to stand in the dark at room temperature for 12-16 hr before use. A dark blue color should be developed.

iii. Trolox Stock solution (10 mM):

a. Trolox- 2.5 mg

b. Acetone -1 mL

Trolox (2.5 mg) is weighed and diluted in 1 mL of acetone (to be freshly prepared each time).

iv. Trolox working solution:

Serially dilute Trolox stock solution in acetone to get different concentrations between 0-500 μ M.

Experimental Details:

1. Treatment groups:

Control flies: 25 *Drosophila* flies fed on regular food for 3-5 days.

Treated flies 25 *Drosophila* flies fed on regular food along with any drug of choice (for example: Paracetamol (1 mg/gm food), Paraquat (2 mg/gm food) or Primaquine (2 mg/gm food, Curcumin (1 mg/gm food)) for 3-5 days prior to the assay.

2. Preparation of the fly tissue lysate

- i. Transfer the control and treated *Drosophila* flies in separate microcentrifuge tubes.
- ii. Add 0.5 mL PBS to each and homogenize with help of the homogenizer.
- iii. The samples are centrifuged at 10,000 rpm for 15 min; take the supernatant for further analysis.

A. Determination of TEAC:

Step 1: Trolox Standard curve:

1. Freshly prepare ABTS•+ Working solution by taking 50 mL of ABTS•+ stock reagent and diluting it with ethanol until the absorbance at 734 nm is 0.70 ± 0.02 .
2. Take 25 μ L of Trolox working solution in each microcentrifuge tubes and label them with appropriate concentration (0-500 μ M).
3. Add 1 mL ABTS•+ working solution into each tube.
4. Vortex these tubes and measure the absorbance at 734 nm. Measure the absorbance again after a lapse of 1 min.
5. The difference in absorbance between the two time points, ΔA_{734} (min^{-1}), indicates the rate of free radical scavenging activity of Trolox.

Step 2: Determination of the free radical scavenging activity of the sample:

1. Take 1 mL ABTS•+ working solution into a microcentrifuge tube.
 2. Add fly lysate (25 μ L) into individual microcentrifuge tubes.
 3. Vortex the tubes and then take an aliquot from each tube for absorbance measurement at 734 nm. After the lapse of 1 min again measure the absorbance.
 4. The difference in absorbance between the two time points, ΔA_{734} (min^{-1}), indicates the rate of free radical scavenging activity of the sample.
- Note:** Perform each of the two steps in triplicate.

Results/Observations:

1. Calculate the average absorbance values for every standard (Trolox), and sample.
2. Plot the average absorbance for the standards (y-axis) against the final concentration of the Trolox standards (x-axis) to determine the best linear curve (**Fig. 1**).
3. Determine the unknown antioxidant concentration in the sample by plotting the absorbance on the standard curve.
4. Only use values within the range of the standard curve.
5. Remember to account for dilution factors.
6. The result is expressed as μ M Trolox equivalents.

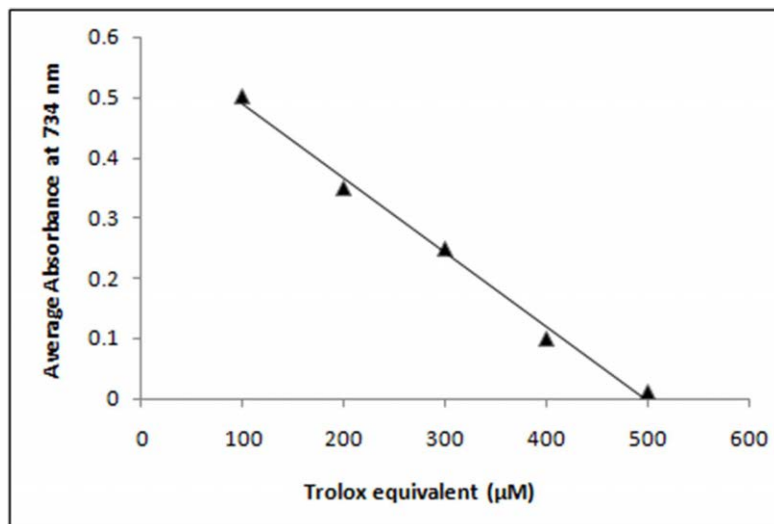


Fig. 1. The average absorbance for the standard (Y-axis) against the final concentration of the Trolox standards (X-axis) to determine the best linear curve

Questions for self-study and interpretation:

1. Why are the observed values that are within the range of standard curve only considered?
2. How are free radicals generated in the cell?
3. What are antioxidants?
4. How does antioxidant molecule concentration correlate with oxidative stress level?

References and Further Reading:

Erel, O. (2004) A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *ClinBiochem.* 37:277-285.

Miller, N. J., Rice-Evans, C., Davies, M. J., Gopinathanand, V., Milner, A. (1993) A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clin.Sci.*, Vol.84, pp.407-412.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. and Rice-Evans, C. (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free RadicBiol Med.* Vol.26, pp.1231–1237.

Chapter 52

Oxidative stress in *Drosophila*: Measuring activities of superoxide dismutase and catalase antioxidant enzymes

Avinash K. Kudva¹ (avinash.kudva@gmail.com) and Rajashekhar K. Patil^{2*} (patilsirmu@gmail.com)

1. Department of Biochemistry, Mangalore University, Mangalagangothri-574199, Karnataka, India.

2. Department of Applied Zoology, Mangalore University, Mangalagangothri-574199, Karnataka, India.

* For correspondence

Recommended Level: UG/PG/Research

Sub-discipline: Biochemistry/Cell Biology/ Physiology

Background:

Oxidation is a process that occurs naturally in the body when oxygen combines with reduced molecules, such as carbohydrates or fats, and provides energy. When there is decreased oxidation or decreased energy production, the cells can no longer function efficiently and therefore disease results. However, this normal process propagates short-lived intermediates known as free radicals, which are oxygen-containing molecules having uneven number of electrons because of which they easily react with other molecules and set up chain reaction. Thus, potentially harmful reactive oxygen or nitrogen species are produced as a consequence of biological metabolism and by exposure to environmental factors. Free radicals are then normally removed or inactivated by a group of natural antioxidants, which prevent these reactive species from causing excessive cellular damage. “Oxidative stress” is the general phenomenon of oxidant exposure and antioxidant depletion or oxidant–antioxidant imbalance. Many xenobiotics enhance oxidative stress and thus cause cellular damage. Status of the oxidative stress can be assayed by measuring the levels of free radicals in cells. An indirect measure of the oxidative status can also be ascertained by quantifying the cellular levels of anti-oxidants, which can scavenge free radicals and thus mitigate the oxidative stress. Redox homeostasis is regulated by two arms of antioxidant machineries: enzymatic components and non-enzymatic, low molecular compounds. The non-enzymatic antioxidants can be measured by Trolox Equivalent Antioxidant Capacity (see [Chapter 51](#)). Here we outline the protocol for estimation of the two key enzymes that play a vital role in redox homeostasis.

Superoxide dismutase (EC 1.15.1.1): is a family of antioxidant enzymes that regulate ROS levels by catalyzing the conversion of superoxide to hydrogen peroxide and molecular oxygen. The assay employs a reaction of pyrogallol autoxidation, which is an improved method to test the enzyme activity (Marklund and Marklund, 1974; Li, 2012). The activity of the enzyme is indicated as number of units. One unit is defined as the amount of SOD that inhibits 50% of pyrogallol autoxidation at 25°C.

Catalase (EC 1.11.1.6): is a common enzyme found in most living organisms exposed to oxygen (such as bacteria, plants, and animals). It catalyzes the decomposition of hydrogen peroxide to water and oxygen. One unit of catalase will decompose 1.0 μm of H_2O_2 per min at pH 7.0 at 25°C. The rate of disappearance of H_2O_2 is followed by observing the rate of decrease in the absorbance at 240 nm (Beers and Sizer, 1952; Stern, 1937).

Objectives:

To measure levels of antioxidant enzymes (Catalase and Superoxide dismutase), which help to counterbalance reactive oxygen species (ROS) generated in the cell.

Materials Required:

1. Flies: 2-3 days old wild type male or female *Drosophila melanogaster* (use only same sex flies in each sample to observe sex-dependent difference in these enzymes).

2. Equipment: UV-Visible spectrophotometer, Centrifuge, pH meter, micropestle homogeniser, micro-volume pipettes (10, 200, 1000 μL).

3. Glass and plasticwares: Micropipette tips (10, 200, 1000 μL), microcentrifuge tubes (1.5 mL), Beakers.

4. Reagents:

i. Phosphate buffered saline (PBS)

- a. NaCl 8 g
- b. KCl 0.2 g
- c. Na_2HPO_4 1.42 g
- d. KH_2PO_4 0.272 g

Weigh the above chemicals and dissolve them step-by-step in 800 mL distilled water. Adjust the pH to 7.4 using dilute HCl or NaOH. Finally makeup the volume to 1000 mL with distilled water. Autoclave and store at RT or 4°C.

ii. Tris-HCl buffer (0.1 M)

- a. Tris base 1.211 gm
- b. Disodium salt of EDTA 37.2 mg
- c. 0.1 M HCl 62.4 mL

Weigh the above chemicals and dissolve them in 62.4 mL of 0.1 M HCl. Adjust the pH to 8.20 by adding dilute HCl or NaOH, as required. Makeup the volume to 100 mL with distilled water.

iii. Pyrogallol solution

- a. Pyrogallol 56.7 mg
- b. HCl (37%) 42 μ L

Prepare 10 mM HCl solution by adding 42 μ L of HCl (37%) to 4.56 mL distilled water. Weigh 56.7 mg pyrogallol and dissolve in the 10 mM HCl. Make up the volume to 100 mL with distilled water.

iv. Hydrogen Peroxide Solution [0.036% (w/w)]

- a. Hydrogen peroxide (30% (w/w)) 100 μ L
- b. Phosphate buffered saline 99 mL

Add 100 μ L of Hydrogen peroxide to 99 mL of PBS. Determine the A₂₄₀ of this solution using PBS as a blank. The A₂₄₀ must be between 0.550 and 0.520 absorbance units. If necessary, add hydrogen peroxide to increase the absorbance or PBS to decrease the absorbance.

v. Distilled water

Experimental Details:**1. Treatment groups:**

- i. **Control flies** 25 *Drosophila* flies are fed with regular corn meal diet for 3-5 days prior to the assay.
- ii. **Treated flies** 25 *Drosophila* flies are fed with regular corn meal diet in which any oxidative stress inducing chemical (for example: Paracetamol (1 mg/gm food), Paraquat (2mg/gm food) or Primaquine (2mg/gm food) for 3-5 days prior to the assay.

2. Preparation of the fly tissue lysate

- i. Take 25 *Drosophila* flies in a microcentrifuge tube.
- ii. Add 0.5 mL PBS and homogenize with help of the homogenizer.
- iii. Centrifuge the homogenate at 10,000 rpm for 15 min; take the supernatant for further analysis.

A. Determination of Superoxide Dismutase (SOD) Activity:**Step 1:** Determination of the autoxidation rate (Blank):

1. Mix 2.35 mL Tris-HCl buffer and 2.00 mL distilled water in a 10 mL test tube.
2. Add pyrogallol solution (0.15 mL) into the test tube, and briefly vortex the tube immediately.
3. Take an aliquot from the test tube for absorbance measurement at 325 nm.
4. Take another aliquot after 1 min and measure the absorbance again.
5. The difference in absorbance between the two aliquots, ΔA_{325} (min^{-1}), indicates the rate of pyrogallol autoxidation. Note: ΔA_{325} should be ideally near 0.060.

Step 2: Determination of the SOD activity:

1. Mix 2.35 mL Tris-HCl buffer and 1.9 mL distilled water into a 10 mL test tube.
 2. Add fly tissue lysate (0.1 mL).
 3. Add pyrogallol solution (0.15 mL) into the test tube, and briefly vortex the tube immediately.
 4. Take an aliquot from the test tube and measure the absorbance at 325 nm.
 5. The tissue lysate should be properly diluted to give a 50% reduction in the autoxidation rate, i.e., ΔA_{325} should approximately be 0.030.
- Note:** Measure the SOD and Catalase activities in triplicate and determine the mean.

Results/Observations:

Record the ΔA_{325} BLANK and ΔA_{325} SAMPLE values and tabulate them into formula below to calculate the SOD activity in the sample.

$$\text{SOD activity (U/mL)} = [\{ (\Delta A_{325} \text{ BLANK} - \Delta A_{325} \text{ SAMPLE}) \times 100 / \Delta A_{325} \text{ BLANK} \} / 50] \times (4.5 \times 1/V \times D)$$

In the equation,

1. ΔA_{325} BLANK is the autoxidation rate determined in the blank test
2. ΔA_{325} SAMPLE is the autoxidation rate determined using a sample
3. V is the volume of sample in mL;
4. D is the dilution factor of the sample;
5. 4.5 is the total volume of the reaction mixture in mL

B. Determination of Catalase Activity:

1. Set the spectrophotometer to 240 nm.
 2. Add 3 mL Hydrogen peroxide solution [0.036% (w/w)] into a clean quartz cuvette and blank the absorbance.
 3. Discard the content of the cuvette and tap it dry.
 4. Take 2.95 mL fresh Hydrogen peroxide solution [0.036% (w/w)] in quartz cuvette.
 5. Add sample lysate (0.05 mL) to the cuvette and mix it well by inversion.
 6. Place the cuvette in the spectrophotometer and note the change in absorbance per second for ~180 sec.
 7. Record the time required for the A_{240} to decrease from 0.45 to 0.40 absorbance units.
- Note:** Perform the measurements in triplicate.

Results/Observations:

Note the time and dilution factor and use the values to determine Catalase activity using the following formula:

$$\text{Catalase Activity (U/mL)} = (3.45 \times \text{Dilution Factor}) / (\text{Time} \times 0.05)$$

In the equation,

1. 3.45 = corresponds to the decomposition of 3.45 μm of hydrogen peroxide in a 3.0 mL reaction mixture producing a decrease in the A240 from ~0.45 to ~0.40 absorbance units.
2. Time = minutes required for the A240 to decrease from ~0.45 to ~0.40 absorbance units.
3. 0.05 = mL of the sample lysate added to the cuvette

Questions for self-study and interpretation:

1. How are free radicals generated in the cell?
2. How do SOD and Catalase work in reducing oxidative stress?
3. How do activities of SOD and Catalase correlate with levels of oxidative stress?

References:

Beers, R. F. Jr. and Sizer, I. W. (1952) A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase, *J. Biol. Chem.* 195:133-140.

Li, Xican (2012) Improved pyrogallol autoxidation method: a reliable and cheap superoxide-scavenging assay suitable for all antioxidants. *J. Agric. Food Chem.* 60:6418-6424.

Marklund, S. and Marklund, G. (1974) Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase, *Eur. J. Biochem.* 47: 469-474.

Stern, K. G. (1937) On the absorption spectrum of catalase. *J. Biol. Chem.* 121: 561-572.

Free Radicals, Anti-oxidants and Disease

The antioxidants are said to have been overwhelmed by the free radicals. This happens when the production of free radicals exceeds the level which the body's natural antioxidant defense mechanisms can cope with; consequently creating a cellular oxidative environment which triggers the oxidation of essential biomolecules like DNA, protein and lipids, leading to multiple disease conditions. The interplay between the trio of free radicals, antioxidants and diseases is important in maintaining health, aging and age-related diseases.

(In: Ighodaro, O. M. and Akinloye, O. A. 2018. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid, Alexandria Journal Medicine. 54: 287-293. <https://www.sciencedirect.com/science/article/pii/S2090506817301550>)

Chapter 53

Visualization of nucleolus in the larval salivary gland polytene nuclei in *Drosophila melanogaster* using light microscopy

Bimalendu B. Nath^{1*} (bbnath@gmail.com) and Leena Thorat^{2*} (leenathorat@gmail.com).

1. Department of Zoology, S.P. Pune University, Pune-411007, India

2. Current address: Department of Biology, York University, Toronto, ON M3J 1P3, Canada

* For correspondence

Recommended Level: PG

Sub-discipline: Cytogenetics/Cell Biology

Background:

Nucleolus (NO) is a non-membrane bound structure found inside a eukaryotic cell nucleus, often also referred to as “an organelle inside an organelle”. NO is formed around the chromosomal region known as ‘Nucleolar Organizer Region’ (NOR). In the early 1930s, Barbara McClintock noticed this unusual structure associated with the maize chromosome and she called it “Organizer” of a small dense structure (now known as Nucleolus) inside the nucleus. The functional significance of both NO and NOR remained obscure till the 1960s when it was established that NO is the site of ribosomal biosynthesis and NORs are chromosomal loci for ribosomal RNA (rRNA) genes. In our routine cytological preparations, quite often nucleolus remains inconspicuous at the light microscopic level and one needs to use specialized histochemical and fluorescence methods (e.g. Fluorescence in situ hybridization technique using specific rDNA probes). Silver nitrate based staining protocol for NOR introduced by Matsui and Sasaki (1973) has remained one of the most preferred techniques. However, this silver nitrate based staining technique did not work satisfactory for many insect chromosomes, especially for polytene nuclei of dipteran insects like *Drosophila*. The protocol described here is based on the visualization of a lightly stained polytene chromosome squash preparations under phase contrast microscope. The choice of stain, its concentration, incubation period and mild treatment with acid to reduce background chromaticity are very crucial to obtain a satisfactory result. The difference between the refractive indices of membrane-less colloidal content of NO and its surrounding milieu will render easy identification of NO and associated chromosomal region, i.e. NOR.

Objective:

To observe nucleolus in polytene nuclei of larval salivary gland cells of *Drosophila melanogaster* using phase contrast microscopy.

Materials Required:

- A. Stereo-binocular microscope
- B. Bright-field and phase-contrast optical microscope
- C. 3rd instar larvae of *Drosophila melanogaster*
- D. Ringer's saline solution (0.67% NaCl)
- E. Fixative (Glacial acetic acid and Methanol in 1:3 ratio; to be freshly prepared)
- F. 1N HCl
- G. 1% Aceto-carmine stain
- H. 45% Acetic acid
- I. Microscope slides and coverslips (22 mm²)
- J. Filter or tissue paper

Experimental Details:

1. Place a third instar larva on a slide in a drop of Ringer's saline solution and dissect out the salivary glands [for dissection of larval salivary glands (see [Chapter 31](#) for dissection of salivary glands) in this collection].
2. Drain out the Ringer's solution and place a few drops of fixative on the tissues and leave for ~30 sec followed by addition of equal numbers of drops of 1N HCl for another ~30 sec.
3. Drain out the fixative, and immediately put a few drops of 1% aceto-carmine stain for 10-15 min (the duration may vary from one batch of stain to the other). Carefully remove the stain by tilting the slide and collecting the sliding stain by soaking on filter or tissue paper.
4. Put one or two drops of 45% Acetic acid on the tissue and hold in place for ~ 1 min.
5. Place a clear coverslip onto the glands and blot off the excess fluid using tissue/ filter paper and gently tap the surface of the coverslip with any blunt-end object. Now place the slide on a square piece of filter paper (~10 cm²). Position the slide towards one end of the filter paper sheet such that the remaining part of the paper can cover the top of the slide. With the slide placed firmly between the sheets. Holding the coverslip in place by firmly keeping thumb and index finger of one hand on edges of the coverslip, apply pressure by placing your other thumb over the coverslip. This squashing technique breaks open the cells and nuclei, and flattens the released chromosomes. While squashing with the thumb, avoid horizontal movement of the coverslip as this would break the chromosomes. Finally, seal all four sides of the coverslip with nail polish to prevent the preparation from drying.
6. Place the slide under a low-power objective lens (bright field) of a microscope. Select a polytene chromosome region with well spread arms and switch over to phase-contrast

objective lens along with corresponding phase ring (annulus) in the condenser system. Now carefully scan the same field of view and try to locate the nucleolus (as shown in the Fig.1b).

Observations:

Nucleolus will appear highly refractile relative to the surrounding region in the polytene nucleus when observed under a phase contrast microscope. In *Drosophila melanogaster*, nucleolus is associated with the X-chromosome (near centromeric region). If carefully squashed and if the nucleolus is not mechanically dissociated from the chromosome arm during squashing, one can also see the NOR as the fibrillary region in the nucleolus connected to the chromocenter (Fig. 1 a, b).

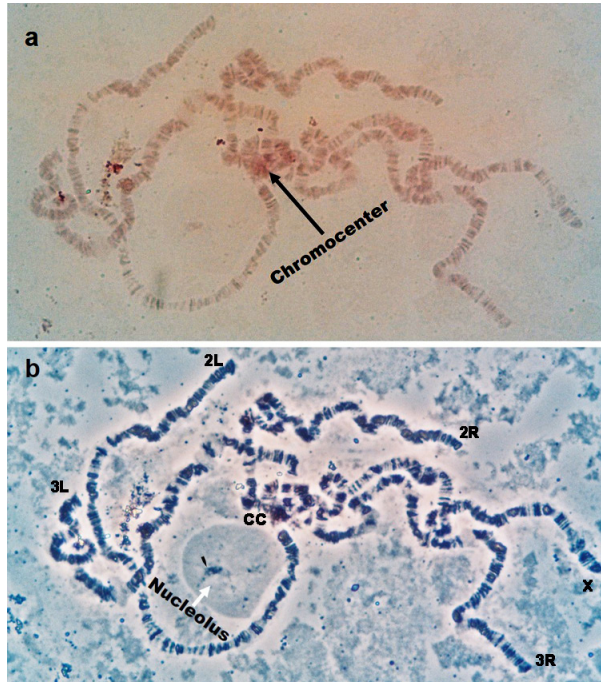


Fig. 1. Photomicrographs of squashed larval salivary gland polytene nuclei of *Drosophila melanogaster* as visualized under (a) bright field and (b) phase contrast optics. Nucleolus (white arrow) can be seen near the chromocenter region (CC). The small black pointer within nucleolus indicates the fibrillar component of NO. The five long chromosome arms (X = X-chromosome; 2L = left arm of chromosome 2; 2R = right arm of chromosome 2; 3L = left arm of chromosome 3 and 3R = right arm of chromosome 3) are also marked.

Questions for self-study and interpretation:

1. In one experiment, ^3H -uridine incorporation in different chromosomal regions of the polytene chromosome was evaluated to monitor transcriptional activities. The results revealed intense ^3H -uridine incorporation in ‘puffs’, NOR and nucleolus. When α -amanitin (an inhibitor of RNA polymerase II) was administered, no ^3H -uridine uptake could be observed in the puff regions but NOR and nucleolus appeared to be labelled. Why?
2. Why is the secondary constriction of metaphase chromosomes known as the nucleolar organizer?
3. In the literature, one can find a remarkable diversity of NOR-bearing chromosomes and chromosomal sites of NORs among different species. Can NOR be used as an additional cytogenetic marker for phylogenetic studies?

References and Further reading:

Lam, Y. W., Trinkle-Mulcahy, L. and Lamond, A. I. (2005) The Nucleolus. *J. Cell Science* 118: 1335-1337. <https://jcs.biologists.org/content/joces/118/7/1335.full.pdf>

Matsui, S. and Sasaki, M. (1973) Differential staining of nucleolus organizers in mammalian chromosomes. *Nature*. 246: 148-150.

Olson, M. O. J. (2011) *The Nucleolus*. Springer, Berlin.

Nucleolous – Cytological Manifestation of Gene Activity

The nucleolus was formally described between 1835 and 1839, but it was another century before it was discovered to be associated with a specific chromosomal locus, thus defining it as a cytogenetic entity. Nucleoli were first isolated in the 1950s, from starfish oocytes. Then, in the early 1960s, a boomlet of studies led to one of the epochal discoveries in the modern era of genetics and cell biology: that the nucleolus is the site of ribosomal RNA synthesis and nascent ribosome assembly. This epistemologically repositioned the nucleolus as not merely an aspect of nuclear anatomy but rather as a cytological manifestation of gene action—a major heuristic advance.

(In: Pederson T. 2011. The nucleolus. *Cold Spring Harbor Perspectives Biology*, 3:, a000638. <https://doi.org/10.1101/cshperspect.a000638>)

Chapter 54

Visualizing actin filaments in *Drosophila* tissues

Sonia Narwal, Shreyas Iyer and Meghana Tare* (meghana.tare@pilani.bits-pilani.ac.in)

Department of Biological Sciences, BITS Pilani, Pilani

* For correspondence

Recommended Level: PG/Research

Sub-discipline: Cell Biology/Developmental Biology

Background:

A cell contains several different kinds of organelles that perform various functions to keep the cell working. Besides the organelles, a cell requires systems for transport or movement within the cell and for maintenance of its size and shape. These later set of functions are carried out by the cytoskeletal system, which consists of 3 main elements: microfilaments (Actin and Myosin), intermediate filaments (Keratin) and microtubules (Tubulin). The microfilaments play an important role in cytokinesis, cytoplasmic streaming, cell motility and muscle contractions. Intermediary elements provide shape and support to cell elements to resist any stress and strain. Microtubules work in increasing surface area as cilia and assist in intra-cellular transport and chromatid separations in cells. The morphology and activity of cytoskeletal elements also affect cell's health. For example, these have been found to be affected in early onset and progression of neurodegenerative disorders and in metastasizing cancers (Parisiadou and Huaibin, 2010; Huynh, *et al.*, 2000; Izdebska, *et al.*, 2018). Using different methods, it is easy to visualize these structures in *Drosophila melanogaster*. The protocol described here utilizes fluorescence labelling of Filamentous-actin (F-Actin) as described earlier (Tare and Singh, 2009).

Fluorescence microscopy: Fluorescence microscopy (Sanderson, *et al.*, 2014) involves use of fluorophores, group of compounds which when excited by a specific wavelength of light, emit a different (higher) wavelength of light that can be detected. For more details, many resource websites (e.g., (<http://www.olympusmicro.com>, <http://www.microscopyu.com>, and <http://zeiss-campus.magnet.fsu.edu/index.html>) can be referred. Some examples of commonly used fluorophores/tags are Rhodamine, Alexa Fluor (group), Cy3, DAPI (4', 6-diamidino-2-phenylindole dichloride) and Green Fluorescence Protein.

F-Actin in the cell's cyto skeleton is commonly examined using a fluorescent dye, Phalloidin, which specifically binds with F-actin and can be visualized under fluorescence microscope.

Objective:

To examine the intracellular localization of F-Actin filaments (micro-filaments)

Materials Required:

- A. Wild-type (Canton-S/Oregon-R) fly stocks; Wild-type larvae.
- B. Stereo-binocular microscope
- C. Fluorescence microscope (with Green/Red/Blue filters)
- D. Dissecting forceps/tweezers and needles
- E. Glass Slides/Coverslips
- F. Mounting medium: Prepare 1 mL of 70% Glycerol in 1X PBST
- G. Phosphate Buffered Saline (1X PBS): : Add Sodium Chloride (1.37 M) 8.0 g, Potassium Chloride (27 mM) 0.2 g, Disodium hydrogen phosphate (100 mM) 1.42 g and Potassium dihydrogen phosphate (20 mM) 0.272 g in 800 ml distilled water, and adjust pH to 7.4 by adding 1N-HCl, as required. Finally add distilled water to make the final volume 1000 mL. Autoclave and store at 4°C.
- H. 4% formaldehyde fixative solution (freshly dilute 37% formaldehyde solution in chilled 1 X PBS to make 4% formaldehyde solution and add 0.1 mL of Triton X-100 to 100 mL of 4% formaldehyde)
- I. PBST: Phosphate Buffered Saline with 0.1% Triton X100 (PBST)
- J. Phalloidin stain: Prepare 20 μ L of 1:400 dilution of Phalloidin stock solution in 1 X PBS (this is enough for staining 4-5 pairs of salivary glands)

Experimental Details:

1. Collect the wandering late third instar larvae of the desired genotype.
2. Put a larva on dissection plate in a drop of cold PBS to avoid any deterioration while dissection.
3. Dissection of salivary glands: larva is immobilized by softly holding its abdomen with forceps while another sharp forceps are used to hold its mouth parts; pull the forceps in opposite directions so that internal organs come out.
4. The pulled out tissue would include brain, imaginal discs, salivary glands, fat body and gut etc.
5. Remove fat bodies, imaginal discs and gut parts while retaining salivary glands with some associated fat bodies. Care should be taken to ensure that salivary glands do not get damaged during this step. Repeat the steps 3-5 with some more larvae to collect 4-5 pairs of intact salivary glands.
6. Transfer the salivary glands to a 1.5 mL microfuge tube and add 100 μ L of 4% formaldehyde

(with 0.1% Triton-X) solution to fix the tissues at room temperature for 30 min.

7. Following fixation, slowly remove the fixative and give 3 consecutive washes to the sample for 10 min each with PBST (1 X PBS containing 0.1% Triton X-100) at room temperature.

8. Remove PBST and add 20 μ L of Phalloidin stain, prepared to a concentration of 1:400 in 1X PBST, and incubate at room temperature for 40 min in dark.

9. Remove Phalloidin and wash the tissue 3 times with 1X PBST for 10 min each and then transfer the tissues on a slide in 1X PBST

10. Clean the salivary glands from other adhering tissues and transfer them to fresh slide in a drop of the mounting medium (70% of Glycerol in 1X PBST); place a 22 mm² with coverslip and seal with transparent nail polish.

11. Observe the slide under fluorescence microscope using green excitation and red emission filters.

Note: Visualization of tissue can be further improved by simultaneously staining nuclei with 1 μ g/mL DAPI (blue fluorescence) in the same staining solution as Phalloidin.

Results/Observations:

The F-actin in cells, more prominent near cell borders, shows red fluorescence (see **Fig. 1**).

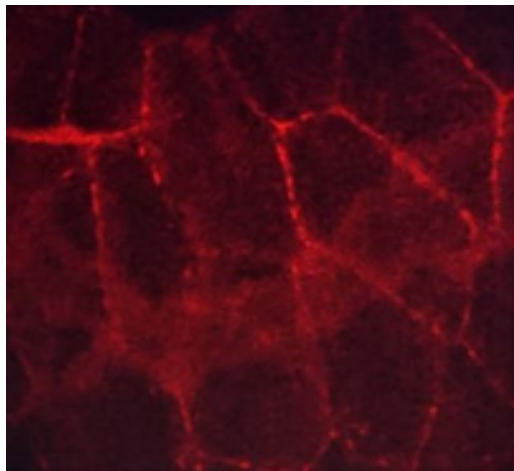


Fig. 1. Actin filaments (red) in salivary gland cell of third instar *Drosophila* larva. Image was captured using Zeiss Apotome microscope.

Questions for self-study and interpretation:

1. What are the roles of PBS/PBST and formaldehyde in the procedure?
2. What is the principle of fluorescence microscopy?
3. Why is the F-actin more abundant near cell membrane?
4. How can you study the distribution of microtubules in a cell using fluorescence microscopy?

References and further reading:

Huynh, D. P. *et al.*, (2000) Parkin is associated with actin filaments in neuronal and non neuronal cells. *Annals Neurology*. 48: 737-744.

Izdebska, M. *et al.*, (2018) The role of actin dynamics and actin-binding proteins expression in epithelial-to-mesenchymal transition and its association with cancer progression and evaluation of possible therapeutic targets. *BioMed Research International*. 2018: 4578373. doi:10.1155/2018/4578373.

Parisiadou, L. and Huaibin, C. (2010) LRRK2 function on actin and microtubule dynamics in Parkinson disease. *Communicative Integrative Biology*. 3: 396-400. doi:10.4161/cib.3.5.12286

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Chapter 55

Study of immune response in *Drosophila melanogaster*

Priyasi Jaiswal (shrutijaiswal128128@gmail.com), Debleena Mandal (debleena@bhu.ac.in), Jukta Biswas (juktabiswasjukta333@gmail.com), Gayatri Rai (gayatrirai2496@gmail.com), S. C. Lakhotia (lakhotia@bhu.ac.in) and Bama Charan Mondal* (bamacharan@bhu.ac.in)

Cytogenetics Lab, Department of Zoology, Banaras Hindu University, Varanasi-221005, India

* For correspondence

Recommended level: UG/PG/Research

Sub-discipline: Developmental Biology

Background:

Innate immunity is evolutionary conserved and serves as the first line of defense in all metazoans (Iwaski et al., 2015). In addition to the innate immunity, vertebrates also exhibit adaptive immunity which depends upon memory of a previous infection (Medzhitov and Janeway Jr., 2002). Like other invertebrates, *Drosophila melanogaster* exhibits only innate immunity which is activated in response to microbial (viruses/bacteria/fungi) or other parasite infection (see **Chapter 22**) or injury. The innate immune reaction in *Drosophila* can be categorized into cellular, systemic humoral and epithelial immune response. The cellular immune response is centered on the action of hemocytes, especially the phagocytic plasmatocytes. The humoral systemic immune response to microbial infections is characterized by the synthesis of a diverse variety of immune effector molecules, the antimicrobial peptides (AMPs), which are synthesized primarily in the fat body, functional equivalent of mammalian liver, and Malpighian tubules, functional equivalent of mammalian kidney (see **Chapters 20, 23**), and released into hemolymph for systemic action (Lemaitre and Hoffmann, 2007). Epithelial immunity fights against invading microorganisms at the level of the epithelial lining of gut and trachea through synthesis of AMPs and production of reactive oxygen species (ROS) (Davis and Engström, 2012).

Macrophages, the large blood cells with a diameter of ~21 µm in humans, are important innate immune cells known for their phagocytic activity. They are present in all organs with roles in tissue growth, homeostasis, and regeneration; their morphology varies according to their activity level (Hoeksema and De Winther, 2016). During early embryonic development, macrophages colonize all body tissues and adapt to their surroundings. The resident tissue macrophages of embryonic origin differ from the adult bone marrow-derived monocytes in that they self-maintain in specific tissues rather than circulate throughout the body. The tissue macrophages move like amoebae and play a defensive role through phagocytosis of parasites and microbes. Macrophages can

also remove cellular debris generated during tissue remodeling, and clear apoptotic cells (Wynn et al., 2013; Hoeksema and De Winther, 2016). Thus, the multifunctional tissue resident macrophages, which include microglia, Kupffer cells, Langerhans cells, and intestinal, cardiac, and tumor-associated macrophages, play roles in development, immunity, and homeostasis.

Drosophila melanogaster carries a variety of immune cells to combat pathogens. *Drosophila's* immune cells, known as hemocytes, act as barrier against pathogenic microbes, while also controlling damage-inducing signals and inflammation (Vlisidou and Wood, 2015). The fly hemocytes are classified as plasmatocytes, crystal cells, and lamellocytes based on their shapes and functions (Williams, 2007). A majority of third-instar larval circulating hemocytes (90-95%) are plasmatocytes, which are similar to the mammalian phagocytic macrophages (Melcarne et al., 2019). These cells, like vertebrate macrophages, can self-renew in the microenvironment and colonize tissues (Gold and Bruckner, 2015). Plasmatocytes develop from prohemocyte progenitors in *Drosophila* embryo's procephalic (head) mesoderm (Lan et al., 2020). Plasmatocytes behave like the vertebrate self-renewing tissue macrophages, remaining dormant until the end of embryogenesis. The lymph gland (*Drosophila's* larval hematopoietic organ, see Chapter 22) is located in the embryo's cardiogenic mesoderm and produces phagocytic cells (Banerjee et al., 2019). Like the mammalian macrophages, phagocytic activity of plasmatocytes contributes to the removal of apoptotic cells and foreign agents, such as bacteria.

Injury also induces immune response in the form of melanization at the site of injury. The crystal cells, which make up about 5% of larval hemocytes, cause melanization at the site of injury (equivalent to clotting on mammalian blood). The very large lamellocytes, which are generally nearly absent in healthy larval hemolymph, encapsulate the pathogen while plasmatocytes secrete antimicrobial peptides to curb the infection (Williams 2007).

Seven distinct inducible antimicrobial peptides (or peptide families) have been identified in *Drosophila* (Imler and Bulet, 2005, Lemaitre and Hoffmann, 2007). Their activity spectra are directed either against fungi (Drosomycins, Metchnikowin) or Gram-positive (Defensin) or Gram-negative bacteria (Attacins, Cecropins, Drosocin and Diptericins) (Lemaitre and Hoffmann, 2007).

The present chapter provides three different approaches to study the innate immune response using the fly model. In the first approach, a method to examine live hemocytes, especially the plasmatocytes, from the larval hemolymph is described. The second approach examines the immune response following an injury-induced breach of the epidermis that leads to the activation of crystal cells that produce melanization around the injury site and thus seal the wound (analogous to the vertebrate blood clotting response). Injury also triggers AMP gene activation resulting in a systemic antimicrobial response. The systemic activation of AMPs following injury is examined in the third approach through transgenic strains in which a particular AMP gene is tagged with reporter genes like *LacZ* or *GFP* (see Chapter 23), whose expression provides a suitable assay of the innate immune response (see Chapter 39, 40).

Objectives:

1. To study the third instar larval circulating blood cells (hemocytes)
2. To observe injury-induced melanization
3. To examine AMP expression following injury using transgenic flies carrying *LacZ* and/or *GFP* reporter gene/s.

Materials required:

A. Fly strains:

- a. For examining larval hemocytes: Wild type or *Hml^A-Gal4 UAS-2xEGFP* (these organisms express *Hml* promoter driven Gal4 which leads to GFP fluorescence in hemocytes; Hemolectin (Hml) is a large multidomain protein produced by hemocytes and is commonly used to identify these cells).
- b. For examining injury-induced melanization: Wild type or *P{ry[+t7.2]=Dipt2.2-lacZ}1, P{w[+mC]=Drs-GFP.JM804}1, y[1] w[*]*. The latter carries a transgene in which the *Drosomyacin* and *Diptericin* encoding genes are tagged with *GFP* and *LacZ* genes, respectively. The transgene is inserted on chromosome 1 and is referred to as *Diptericin-LacZ, Drosomyacin-GFP*.
- c. For examining injury-induced AMP expression: *Diptericin-LacZ, Drosomyacin-GFP*. Several other transgenic reporter stocks of AMPs are available from Bloomington *Drosophila* Stock Centre.

B. Equipment and other supplies:

- i. Stereo-binocular and microscope with phase-contrast or differential interference contrast (DIC) and fluorescence optics.
- ii. BOD incubator maintained at 24° C for fly rearing.
- iii. Fly handling materials.
- iv. Glass slides and coverslips.
- v. Bridge slides: Bridge slides are prepared by fixing two halves of a 22 mm² coverslip on a clean slide with DPX (or transparent nail polish) at a suitable distance (~1 cm) from each other to provide a space for mounting of the desired tissue. The DPX/nail polish must be completely dried before these slides are used. When covered with the coverslip, the tissue placed in this free space does not get vertically compressed by the coverslip which now rests on the two fixed coverslip pieces.
- vi. Micropipette (20 µL) with corresponding micro-tips or pasteur pipette.
- vii. Moist chamber is prepared by placing a wet tissue or filter paper in a petri dish.
- viii. Fine needles for infecting injury to adult fly.

C. Reagents:

- i. PBS: 1X Phosphate Buffer Saline (PBS), pH 7.4:

NaCl	8 gm
KCl	0.2 gm
Na ₂ HPO ₄	1.44 gm
KH ₂ PO ₄	0.24 gm

Serially dissolve the above in 800 mL distilled water; when fully dissolved, adjust pH to 7.4 with 1N HCl or 0.4N NaOH, as required. Finally, make the volume to 1000 mL with distilled water, autoclave the solution, and store it at room temperature.

Experimental details:

A. For examining hemocytes:

- i. Collect wandering third instar wild type or *Hml^Δ-Gal4 UAS-2xEGFP* larvae (Fig. 1) and clean with distilled water to wash out food particles.
- ii. Take a cleaned larva in a drop of 1X PBS (20 μL) on a clean bridge slide.
- iii. Open the larval cuticle with the help of a pair of fine forceps so that hemolymph comes out into the PBS. Remove all the larval body parts from the liquid drop.
- iv. Keep the slide in a humid chamber for 20 minutes to allow the plasmatocytes to spread and attach to the glass slide (optional step).
- v. Place a clean 22 mm² coverslip on the hemolymph drop and examine under phase-contrast/fluorescence microscope.

B. For examining injury-induced melanization and AMP expression:

- i. Anesthetize a few *Drosomyacin-GFP* adult flies.

Note: For examining injury-induced melanization, wild type flies can also be used.

- i. Use a fine needle to prick the thorax of the wild type or *Drosomyacin-GFP* fly on one of the sides (Fig. 3) to induce an immune challenge. Keep the fly at 24°C for 1 hr for the immune response to be elicited. Keep uninjured flies (wildtype or *Drosomyacin-GFP*, as required) as control.
- ii. Observe the fly under a stereo-binocular for melanization at the site of injury or under a stereo-binocular microscope with attached GFP fluorescence system (using appropriate filters for blue excitation and green emission to detect GFP fluorescence).

Observations:

A. Hemocytes:

- i. If using a preparation from *Hml^Δ-Gal4 UAS-2xEGFP* larva, use a stereo-binocular microscope equipped with GFP fluorescence attachment to view the GFP expressing

Lymph gland and plasmatocytes (Fig. 1) and a fluorescence microscope to view the GFP expressing hemocytes under low, high or oil immersion objectives.

- ii. Examine the preparation of live hemocytes from wild type (*Hml^Δ-Gal4 UAS-2xEGFP* cells can also be examined) under phase-contrast and/or DIC optics using a 40X dry or 100X oil immersion objective to identify the large plasmatocytes with spike-like filopodial extensions (Fig. 2).

Note: Addition of bacteria or small particles to the drop in which the live hemocytes are placed on the slide, stimulates the phagocytic activity of plasmatocytes. This can be examined under phase-contrast optics or under fluorescence microscope if fluorescently tagged bacteria are used.

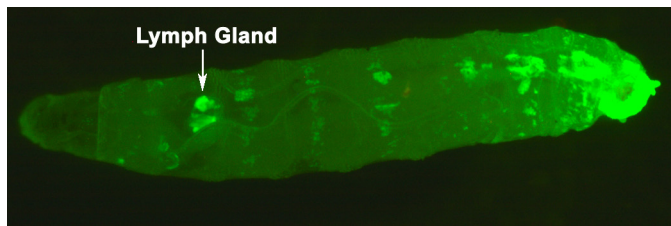


Fig. 1. *Hml^Δ > 2xEGFP* third instar showing GFP (green) expression in the lymph gland (white arrow) and hemocytes in the hemolymph, which show a higher abundance at the larval segment junctions.

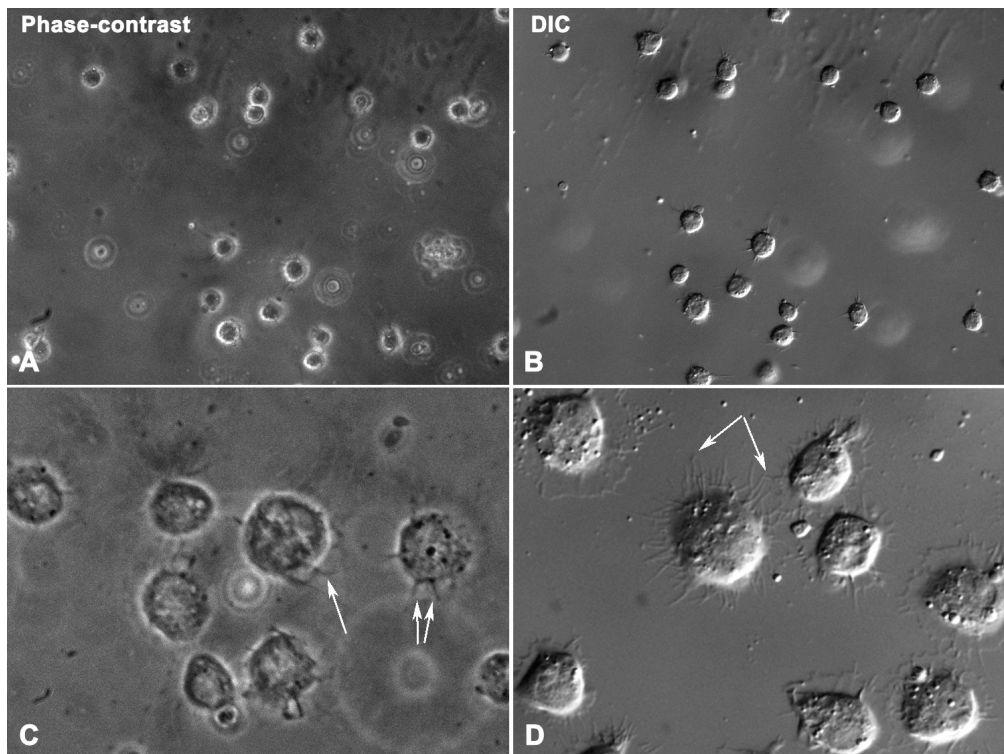


Fig. 2. Live plasmatocytes from late third instar larva imaged under phase-contrast (A, C), or DIC (B, D) optics using 40X (A-B) or 100X (C-D) objectives. White arrows in C and D point the spike-like filopodia extended by the plasmatocytes.

B. Injury-induced melanization and AMP expression:

- i. Examine the injured adult flies under a stereo-binocular microscope for melanization of the scar at the site of needle injury (Fig. 3A-D).
- ii. Examine the pre-injury and injured *Drosomycin-GFP* flies under a stereo-binocular microscope equipped with GFP-fluorescence attachment for detecting the

Drosomycin-GFP expression. A stronger GFP reporter expression in fat bodies near the abdominal tip in injured (Fig. 3F) than in uninjured (Fig. 3E) flies indicates a systemic immune response to the localized needle injury at thorax.

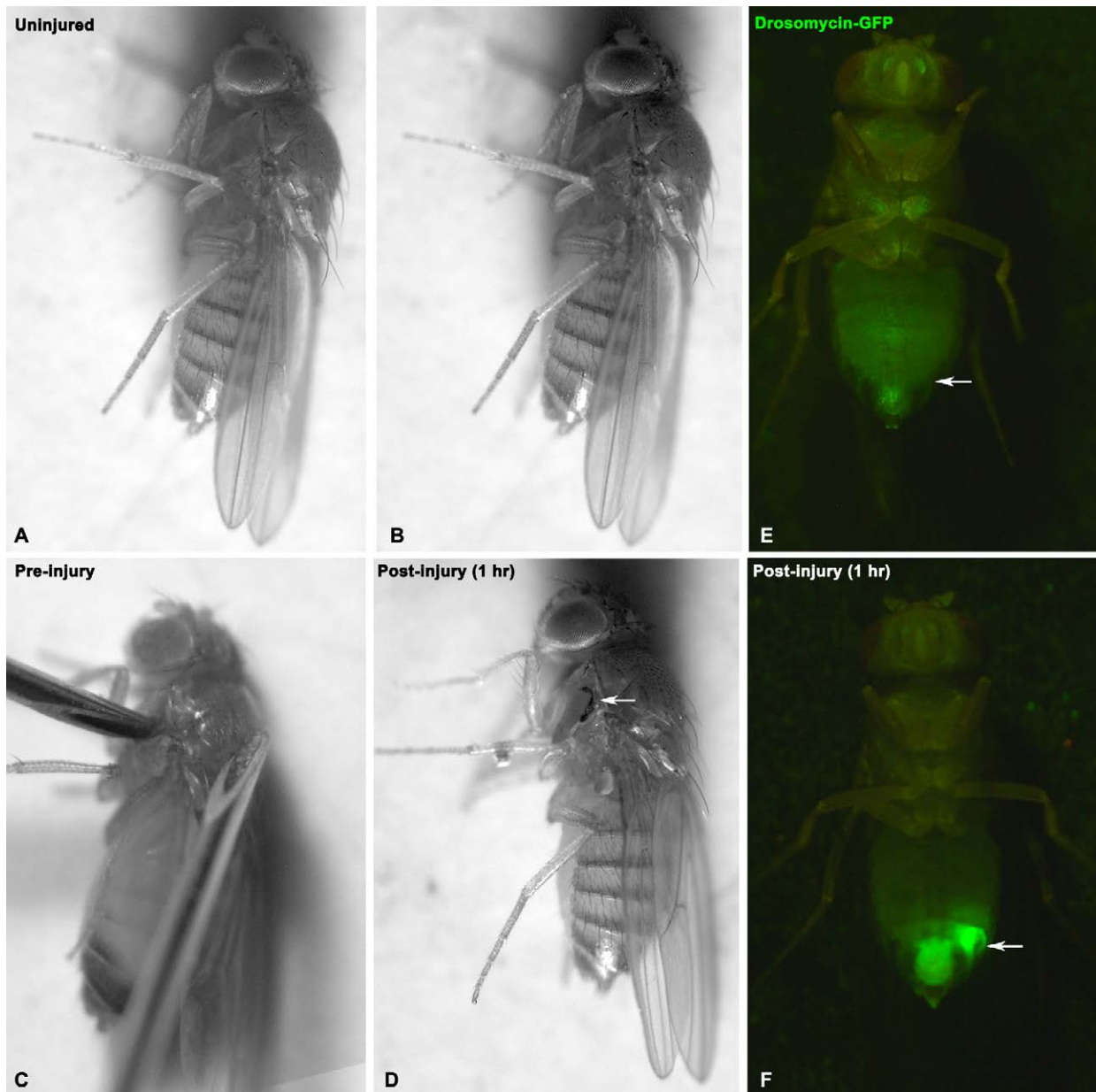


Fig. 3. Injury induces localized melanization and systemic innate immune response. A-B show control uninjured adult flies, without any melanization mark on thorax. C-D are experimental flies before the injury (pre-injury C) and 1 hr after injury (D); the needle used to inflict injury on thorax is seen in C while the white arrow in D points to the melanized scar formed at the site of injury. E-F show ventral views of a Drosomycin-GFP expressing (green fluorescence) fly before injury (E) and 1 hr after the needle injury at thorax (F); white arrow in F points to the elevated Drosomycin-GFP expression in abdomen after the injury.

Questions for self-study and interpretation:

1. Would administration of a toxic chemical (e.g., pesticide) or stress (heat or cold shock) to larvae affect the number of hemocytes and their phagocytic activity?

2. Would infection of larvae with bacteria change the plasmatocyte numbers in hemolymph?
3. Why does melanization occur at the site of injury?
4. How does the elevated AMP expression affect survival of the fly after injury?

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Variability in the Ancient Innate Immune Modules in Diverse Taxa

The various modules of the innate immune system evolved at different stages of phylogeny in response to specific challenges imposed by different classes of pathogen. The appearance of distinct innate host-defence modules during evolution also reflects changes in anatomy and physiology as animals evolved. In addition, a given module is not the same in different animals and might have been expanded or contracted during evolution in response to specific needs. The composition of the innate host-defence modules in any given animal species is therefore one of many possible configurations, presumably the optimal one for affording maximum protection in the specific physiological context. Thus, the innate immune system, although ancient in origin, is not equivalent in different animal phyla or classes or even between different species of the same class. For example, the number, expression and regulation of the antimicrobial molecules known as defensins varies between mammalian species.

(In: Medzhitov, R. Recognition of microorganisms and activation of the immune response. *Nature* 449, 819–826 (2007). <https://doi.org/10.1038/nature06246>).

Chapter 56

Localization of DNA sequence on polytene chromosomes of *Drosophila melanogaster* by *in situ* hybridization

J. K. Roy (jagatkroy@gmail.com) and S. C. Lakhotia* (lakhotia@bhu.ac.in)

Cytogenetics Laboratory, Department of Zoology, Banaras Hindu University, Varanasi
221005, India

*For correspondence

Recommended Level: PG/Research

Sub-discipline: Genetics/Cell Biology/Developmental Biology/Molecular Biology

Background:

Single stranded DNA and/or RNA molecules, independent of their biological origins can anneal through the process of nucleic acid hybridization to generate double stranded structures if sequences of the two nucleic acid strands are complementary. This provides a very powerful means of evaluating homology between DNA and/or RNA sequences of diverse origins. An understanding of this process has been a key catalyst for the recombinant DNA revolution. When a polynucleotide (DNA or RNA) sequence carrying a distinguishing label (e.g., a radioactive or fluorescent or a chemical tag) hybridizes with the target DNA/RNA sequence, presence of the tagged sequence, termed as 'probe', in the hybrid can be detected. When the tagged 'probe' is allowed to hybridize with its nucleic acid target in a cell *in situ*, the process is referred to as '*in situ* hybridization'. When a defined DNA or RNA sequence is used as probe to hybridize with cellular DNA as the target, it is called DNA: DNA or RNA: DNA *in situ* hybridization but when DNA or RNA probe is hybridized with cellular RNA as the target, it is called, respectively, DNA:RNA or RNA:RNA *in situ* hybridization (RNA *in situ* hybridization or RISH, see [Chapter 57](#)). This technique, which can be applied to single cells or tissues or even whole organism, provides very valuable information about spatial location of the probed nucleic acid sequence within a cell. In addition, it can also provide information about quantity of the probed sequence in a cell. When the target for the probe is an RNA sequence, very valuable information about gene expression and the spatial location and quantity of the given transcript in a cell can be obtained. The DNA: DNA *in situ* hybridization technique provided key information about physical mapping of genes on chromosomes and thus facilitated the assembly of the human genome and genomes of several model organisms.

The kinetics of nucleic acid hybridization is influenced by the numbers of copies of the complementary sequences in a given volume. Therefore, polytene chromosomes of

Drosophila (and other Dipteran) larvae (see [Chapter 31](#)), with their uniquely large size and well-defined morphological banding pattern across their length due to high copy numbers of sister chromatids as a result of endoreplication cycles, provide an unique system to detect the presence and specific chromosomal location of a given DNA sequence by DNA: DNA *in situ* hybridization. Therefore, polytene chromosomes of *Drosophila* (and other dipterans) have been very useful in physical mapping of genes by DNA: DNA *in situ* hybridization.

The RNA: RNA or DNA: RNA *in situ* hybridization method (RISH) that targets cellular RNA has, on the other hand, elucidated remarkably valuable information about spatial and temporal patterns of gene expression, RNA localization and its turnover at a sub-cellular level (see [Chapter 57](#)).

The following describes steps involved in localization of a specific gene sequence (the stress inducible *hsp70* genes are taken as example here) on polytene chromosomes of *Drosophila melanogaster* by DNA:DNA *in situ* hybridization, using signal detection by chromogenic method. If hybridization of the probe is detected by fluorescent signal (using a fluorescent-tag on the probe or a secondary antibody tagged with a fluorescent moiety), the method is named as 'Fluorescence *in situ* hybridization' (FISH). FISH is more sensitive than the chromogenic detection method described here.

Objectives:

To localize *hsp70* gene on polytene chromosomes by DNA: DNA *in situ* hybridization.

Materials Required:

1. Healthy late third instar wild type larvae
2. Other items required for preparation of polytene chromosomes squashes (see [Chapter 31](#)).
3. *Moist chamber for treatment/hybridization steps*: Clean filter papers of appropriate size are placed in the base of a 6 inch dia Petri dish and wetted with distilled water. Clean slides at appropriate distance are placed in pairs to provide support for placing the experimental slides so that the latter do not touch the wet filter paper. This provides a moist chamber for the experimental slides so that the small volume of solution underneath the coverslip does not dry up during the prolonged treatment. Keep the moist chamber, without the experimental slides at the desired temperature at least 30 min prior to its use so that the temperature inside the chamber is as desired.
4. **Solutions**
 - i. 20X Saline sodium citrate or SSC (3 M Sodium Chloride, 0.3 M Sodium Citrate)
 - ii. 0.1% freshly prepared gelatin solution
 - iii. 3 M sodium acetate (pH 5.2, adjusted with glacial acetic acid)
 - iv. RNase 10 mg/mL (stock solution)

- v. 70%, 90% and absolute ethanol grades
- vi. 1 M Tris pH 7.5, 1M Tris pH 8.0, 1M Tris pH 9.5
- vii. 5 M sodium chloride
- viii. 0.5 M EDTA
- ix. Tris-EDTA or TE solution (10 mM Tris, pH 8.0; 1 mM EDTA)
- x. Digoxigenin-dUTP labeled DNA probe
- xi. Salmon sperm DNA 10 mg/mL
- xii. Hybridization mix
- xiii. Color detection buffers
 - a. Buffer I (100 mM Tris, pH 7.5; 150 mM NaCl)
 - b. Buffer II (0.5% w/v Blocking reagent in Buffer I)
 - c. Buffer III (100 mM Tris, pH 9.5; 100 mM NaCl, 50 mM MgCl₂)
 - d. Buffer IV (TE)
- xiv. Color Developing Solution (to be prepared fresh just before use)
 - a. Nitroblue Tetrazolium (NBT, 75 mg/mL in Dimethyl Formamide) 4.5 μL
 - b. 5-Bromo-4-Chloro-3-Indolyl Phosphate or BCIP soln
(50 mg/mL in Dimethyl Formamide) 3.5μL
 - c. Buffer III to make 1 mL
- xv. Safranin stain

Safranin	100 mg
Dist. Water	100 mL

(Dissolve Safranin powder in water at room temperature: the prepared stain is stable for long time and can be used repeatedly)
- xvi. Entellan mountant (E. Merck),
- xvii. Immersion oil

5. Siliconization of coverslips (this *optional* step helps prevent adhesion of squashed chromosomes to the coverslip)

- a. Put fresh and clean coverslips in a Petri dish containing absolute ethanol and wipe them dry with a clean silken cloth.
- b. Dip the alcohol cleaned coverslips in 1% siliconizing fluid.
- c. Wash in running water for 1-2 hr. Rinse in distilled water and dry the slides in oven.

Experimental Details:

1. Labeling of probe DNA with digoxigenin dUTP (Dig-dUTP): A double-stranded radioactive or non-radioactive labelled DNA probe, which is ideal for DNA:DNA *in situ* hybridization, can be prepared by a variety of methods (e.g. nick-translation, random priming, polymerase-chain reaction (PCR) etc, see Sambrook *et al.*, 1989). Non-radioactive probes, which are safer and provide better sensitivity and resolution, can be prepared with

Dig or biotin or fluorescently labelled dUTP (deoxyuridine triphosphate). In the following example, a Dig-labelled DNA probe, prepared by random-priming method is used for *in situ* hybridization,

- a. Denature 1-3 μg of cloned double stranded *hsp70* gene sequence linear DNA in 1-5 μL water by heating in boiling water for 10 min.
- b. Quickly chill the tube on ice.
- c. Sequentially add the following to the same microfuge tube
 - i. Hexanucleotide mix 2 μL
 - ii. (Dig) labeled dNTP mix 2 μL
 - iii. Distilled water to make 19 μL
 - iv. Klenow enzyme (3-5 units) 1 μL
 - v. Add deionized dist water to make 20 μL
- d. Incubate at 37°C for 1 hr.
- e. Stop reaction by adding 0.8 μL of 0.5M EDTA (Final concentration 20 mM).
- f. Precipitate labeled DNA by adding the following:
 - i. 2.0 μL of salmon sperm DNA (10 mg/mL)
 - ii. 2.5 μL of 4 M Lithium Chloride
 - iii. 75 μL of pre chilled (-20°C) ethanol
 - iv. Leave at -70°C for 2 hr.
- g. Centrifuge at 12,000 rpm for 30 min at 4°C.
- h. Decant supernatant and wash the pellet with 70% ethanol.
- i. Dry at room temperature or lyophilize.
- j. Dissolve in required amount of TE. The labeled probe can be stored at -20°C and used for at least 2 years.

2. Checking the efficiency of DIG-labelin

- a. Take a small piece of nylon membrane, wet it with 2X SSC and dot blot (under vacuum) 10 pg, 1 pg and 0.1 pg of the prepared probe.
- b. Dry at room temperature (~30 min) following which cross-link the probe DNA with the membrane either by 3-4 min exposure to UV on a trans illuminator or by heating the filter at 70°C for 2 hr.
- c. Wash the membrane briefly in Buffer I and Incubate in Buffer II (blocking reagent) for 30 min at room temp (to block the membrane surface for non-specific binding of the antibody used in next step).
- d. Briefly rinse in Buffer I and incubate the membrane in Anti-DIG Antibody-Enzyme conjugate (1 μL in 4 mL of Buffer I) for 30 min at room temp
- e. Wash twice at 15 min interval in Buffer I.
- f. Briefly rinse in Buffer III.
- g. Put the blot in a small polythene bag and, working in dim light, add color developing

solution (4.5 μ L of NBT and 3.5 μ L of BCIP in 1 mL of Buffer III) and seal the bag.

h. Incubate the sealed bag in dark (e.g., by wrapping with aluminium foil) and periodically check in dim light till a colored signal is distinctly visible in the dot blot. When adequate signal is obtained, remove the blot from the bag and put in Buffer IV to stop the reaction. 0.1 pg of a good labelled probe produces a detectable signal within 30 min. The blot can be stored in Buffer IV or in dry condition.

3. Preparation of polytene chromosome squashes

a. Prepare polytene chromosomes squashes of salivary glands from healthy third instar larvae as described in **Chapter 31** except that the aceto-methanol fixed glands are directly transferred to 50% acetic acid (the Aceto-Orcein/Aceto-Carmine staining step is skipped) and squashed (optionally, siliconized coverslips may be used for making the squashes since siliconization prevents chromosomes from sticking to coverslips when the latter is flipped off from the slide in the next step).

b. Transfer the slides with coverslip to a slide box which has been pre-chilled and immediately store the slide box at -70°C . Keep at -70°C for a few hours or overnight.

c. After the desired period of freezing, flip off the coverslip using a sharp razor-blade, Pass the slides through 50%, 70%, 90%, absolute alcohol, and air-dry. **Alternatively**, transfer the slides after making the squash preparation to liquid nitrogen for ultra-fast freezing, and immediately thereafter, flip off the coverslip with a sharp razor blade. After flipping off the coverslip, quickly place the slide in 90% ethanol for 3-5 min and air dry.

d. Observe the dried slides under phase-contrast microscope (bright-field microscope can also be used if the microscope illumination light is dimmed and the sub-stage diaphragm is closed). *Proceed further only with slides that carry many polytene nuclei whose chromosomes are well-spread and flattened*

4. Pre-hybridization processing of prepared polytene chromosome slides

a. *Blocking of slides*: In order to prevent non-specific binding of the labelled probe on slide surface, a blocking step is carried out. Dip the prepared slides (with the desired cytological preparation) for about 5 sec in a freshly prepared 0.1% solution of Gelatin (100 mg Gelatin dissolved in 100 mL dist. water at 70°C for 1 hr). Let the slides air dry (the gelatin coating prevents background binding of the probe and also helps keep chromosomes/cells better preserved on the slide, Lakhotia *et al.*, 1993).

b. Arrange the slides in a moist chamber containing filter papers soaked in 2X SSC. Place 100 μ L of RNase (100 μ g/mL in 2X SSC) over the preparation on each slide and cover with 22 mm² coverslip (no air bubbles should be trapped) to remove RNA from preparations and incubate at room temperature for 2 hr.

Note: Unless the RNase used is known to be DNase-free, the RNase stock solution should be boiled/autoclaved to 'kill' any nuclease or protease contamination (RNase itself is insensitive to boiling)

c. Remove the coverslips by gently dipping slides in a beaker containing 2X SSC. Coverslips will fall in solution.

d. Wash slides in 2X SSC (3 times 5 min each), in 70% ethanol (2 times 10 min each) and in 95% ethanol for 5 min. Air dry. Slides can be stored at this stage, if required (**Note:** in certain cases when not much RNA is expected to be available for hybridization in the preparation, the RNase and subsequent washing (steps 2-4), may be omitted).

e. Denaturation of chromosomal DNA: Place slides in 0.07 N NaOH for exactly 3 min to denature chromosomal DNA (**Note:** optimal denaturation of chromosomal DNA is essential for the hybridization but longer alkali treatment can damage chromosome morphology). Wash slides in 3 changes of 70% ethanol (10 min each) and 2 changes of 95% ethanol (5 min each). Air dry. Slides are now ready for hybridization.

f. **Alternatively**, the alkali denaturation step is skipped and chromosomal DNA is heat-denatured after the hybridization mixture is added to the RNase treated slides (after step iii under the hybridization step below) by keeping the moist chamber with experimental slides at 70°C for 30 min before transferring to 37°C (or other desired temperature for DNA:DNA hybridization) for the hybridization to proceed.

5. Hybridization

a. Denature labeled probe DNA by placing the tube in boiling water-bath for 10 min. Add the desired amount of denatured probe to the hybridization mix. Alternatively, step f above (under the prehybridization step) can be used, after the step iii below, to simultaneously denature the chromosomal DNA and the probe.

b. Preparation of hybridization mix

- | | |
|--|--------------|
| i. Formamide | 500 μ L |
| ii. 20X SSC | 250 μ L |
| iii. DIG labeled probe (~20 ng/slide) | as required |
| iv. H ₂ O to make TOTAL volume to | 1000 μ L |

Note: The total volume of hybridization mix depends upon the number of slides being processed (15-20 μ L is enough for a slide when using a 22 mm² coverslip).

c. Add 20 μ L of hybridization mixture containing ~20 ng of labeled probe. Place a coverslips over the hybridization mixture and seal the edges with nail polish. No air bubbles should be trapped.

d. Incubate slides at 37°C (or different hybridization temperature as required for specific probe sequence) in a closed moist chamber. Allow hybridization to proceed for 12-14 hr (overnight).

6. Washing

a. Peel off the nail polish sealing with the help of forceps. Remove coverslips by dipping slides in 2X SSC.

b. Wash slides in 1X SSC, (3 times 15 min each) at 60°C.

7. Color detection

a. Rinse slides for 1 min in buffer I (100 mM Tris pH7.5, 150 mM NaCl).

b. Place them in buffer II (0.5% W/V Blocking reagent in Buffer I) and leave for 30 min.

c. Wash again in buffer I for 1 min.

d. Incubate in anti-Digoxigenin antibody alkaline phosphatase conjugate (diluted as recommended by the manufacturer in buffer I) for 30 min.

e. Wash in buffer I (2 times 20 min each).

f. Rinse in buffer III (Tris pH 9.5 100 mM, NaCl 100 mM, MgCl₂ 50 mM).

g. Prepare fresh color reaction reagent. Put 20-30 µL of color reaction reagent on the slide, cover with a coverslip, seal with nail polish and leave the slide in a dark chamber at room temperature for 1-12 hr, depending upon the time required (periodically check the slide under microscope) for optimal signal development.

h. Stop reaction in Buffer IV (10 mM Tris pH 8.0, 1 mM EDTA) after observing slides under microscope.

i. Air dry and counter-stain with Safranin by dipping the slides in the staining solution for 5 to 10 sec followed by 2-3 washes in clean distilled water. Air dry. (*Alternatively*, stain the slides with 2% aceto-orcein for 5-6 min (filter the aceto-orcein stain immediately before use to avoid ugly stain marks on the preparation) by applying 2-3 drops of the filtered stain on the chromosome areas and covering with a coverglass for 5- 6 min.

j. Following the staining, quickly rinse slides in two changes of 70% alcohol and air dry). Safranin staining gives better chromosome morphology and contrast so that the purplish-blue hybridization signal is more distinctly detectable.

k. Mount dried slides with Entellan (E.Merck). *Alternatively*, mount the slides temporarily using the stop buffer or the immersion oil; after examination the coverslip is removed and slides cleaned by rinsing in distilled water if mounted with the stop buffer or with xylene if immersion oil was used. Mounting with alcohol-based mountant like. DPX causes fading of color.

Observations:

Hybridization of the probe results in appearance of purplish-blue color deposit at the site of hybridization. The specific chromosome region that shows the hybridization signal can be identified by referring to standard polytene chromosome maps available at the Flybase (<https://flybase.org/maps/chromosomes/maps>). In the example shown in **Fig. 1**, *in situ* hybridization of the *hsp70* probe on the right arm of chromosome 3 is shown. Using the chromosome maps (Camera Lucida drawing of Bridges and photomap of Lefevre, see **Chapter 24**) given on top of the image of polytene chromosomes in **Fig. 1**, identify the two chromosome regions (marked by arrows) that show the hybridization signal.

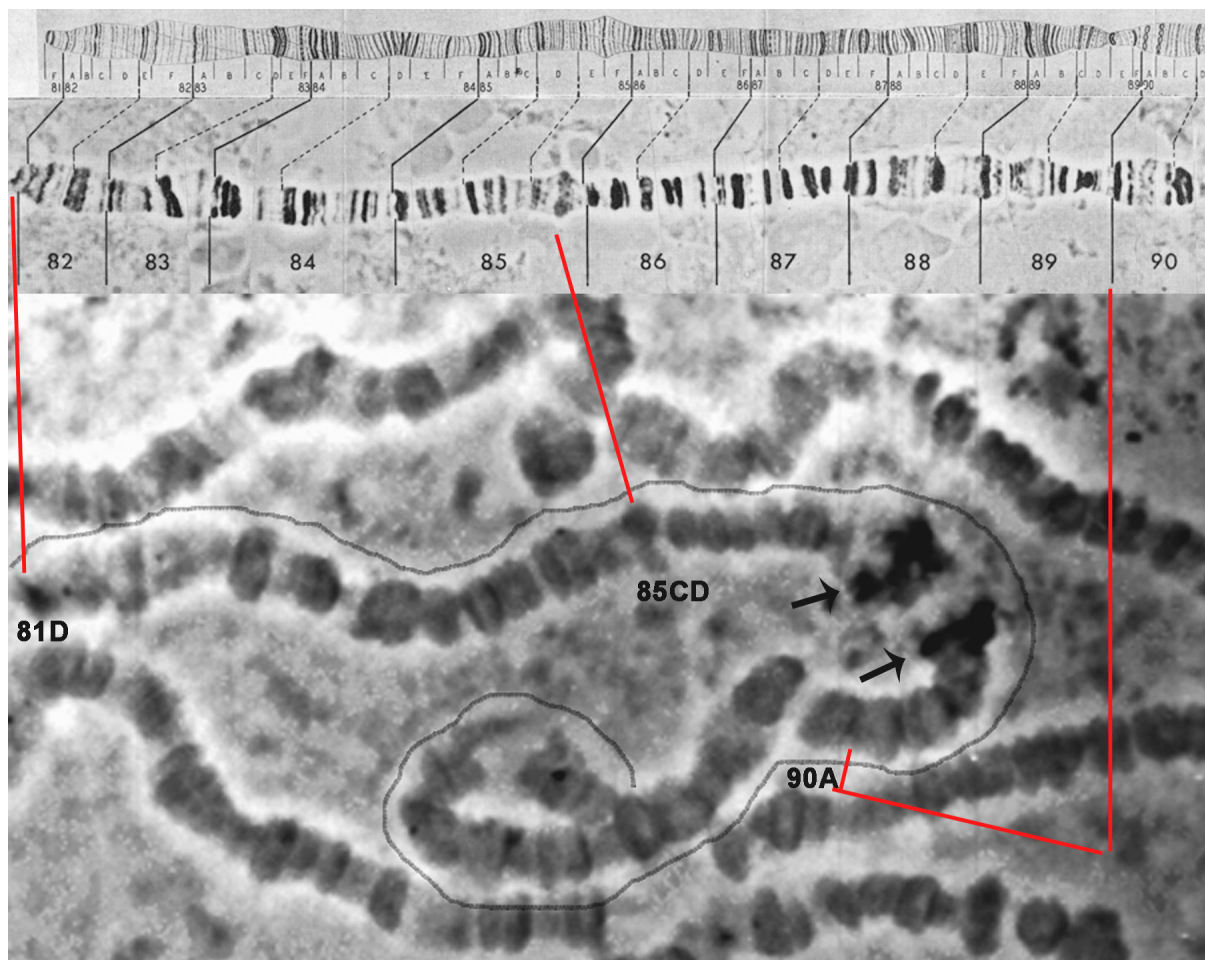


Fig. 1. In situ hybridization of a digoxigenin-labeled probe for *hsp70* gene to polytene chromosomes of *Drosophila melanogaster* (only a part of the nucleus is shown). The two arrows indicate the *hsp70* probe's hybridization with two regions, which are puffed due to prior heat shock to the dissected salivary glands. The top two panels show the standard maps (Bridge's Camera Lucida drawing and Lefevre's photomap, respectively) of the proximal part of right arm of chromosome 3 while a thin line is drawn along the corresponding region in polytene spread in the lower image; the red connecting lines identify the bands 81D (base of 3R), 85CD and 90A in the maps and the polytene chromosome arm.

Precautions and trouble-shooting:

Well spread and flattened preparations with good chromosome morphology are essential for a strong hybridization signal.

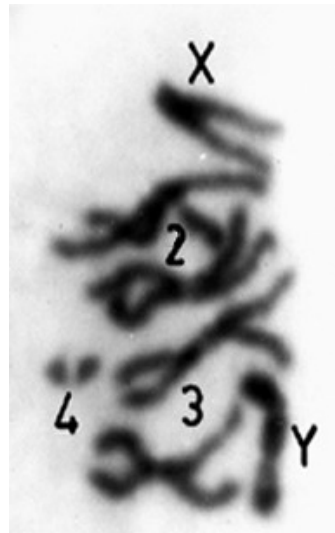
Denaturation of chromosomes must be precisely controlled since too long treatments would destroy chromosome morphology while too short a treatment would not denature the chromosomal DNA sufficiently for hybridization with the probe. The probe must also be denatured just before application.

Washing after the hybridization and after antibody binding must be adequate so that all the excess probe and the antibody are removed. Incomplete washing at any step would generate undesirable background.

Staining of chromosomes must be controlled so that the hybridization signal is not masked. Care must be taken to avoid trapping of air bubbles while mounting coverslips at the various steps since any trapped bubble would not permit the reaction in the local region and thereby prevent detectability of the hybridization signal.

Questions:

1. Why is the probe DNA labelled prior to hybridization?
2. Why did we denature the probe DNA as well as the chromosomal DNA before hybridization?
3. Why are 50% Formamide and SSC included in the hybridization mix? What may happen if any of these components are omitted in the hybridization mix?
4. Is there any significance of specific temperature or salt concentration in washing step?
5. Why do we go for a blocking step while detecting the hybridization?
6. The mitotic chromosome complement ($2N=8$) of *Drosophila melanogaster* is shown below. How many hybridization signals (2/4/8?) would you expect to see if the *hsp70* probe was hybridized to such mitotic chromosomes? Which chromosomes would show the signal?



Mitotic chromosomes of *D. melanogaster*

7. Does the presence of two different regions that show DNA: DNA *in situ* hybridization signals with the *hsp70* probe in the example here indicate gene duplication which may result in more than one copy of the same gene? Read more about the organization of stress-inducible *hsp70* genes in *Drosophila melanogaster* at <https://Flybase.org>.

References and further reading:

Lakhotia, S. C., Sharma, A., Mutsuddi, M. and Tapadia, M. G. (1993) Gelatin as a blocking agent in southern blot and *in situ* hybridizations. *Trends Genetics*. 9: 261.

[https://www.cell.com/trends/genetics/abstract/0168-9525\(93\)90002-Y](https://www.cell.com/trends/genetics/abstract/0168-9525(93)90002-Y).

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Polytene Chromosomes – Dream Come True for Geneticists & Cytologists

Ever since the gene hypothesis was generally accepted, geneticists and cytologists have dreamed of the day when it would be possible to see actual genes, instead of having to be satisfied with studying their “shadows”, which were reflected in the morphological development of generations of organisms. Giant salivary gland chromosomes of the fruit fly are proving to be a new genetic tool of the utmost importance. Just what these bands are, and just where the actual genes are located, is still not determined, but some of them have been “bracketed” in very small regions – close to, if not actually on, some of the dark bands

(In: Editor’s (Robert C. Cook) Frontispiece in in Journal of Heredity to introduce T. S. Painter’s paper on ‘Polytene chromosomes and the attack on gene’ in December 1934 issue of J. Heredity. <https://academic.oup.com/jhered/article-abstract/25/12/465/807076?redirectedFrom=fulltext>)

Chapter 57

RNA *in situ* hybridization (RISH) to visualize the cellular distribution and abundance of RNA

Anand K. Singh^{1,2*} (anandksingh@bhu.ac.in) and S. C. Lakhotia^{3*} (lakhotia@bhu.ac.in)

1. Interdisciplinary School of Life Sciences, Banaras Hindu University, Varanasi, 221005, India

2. Current address: Department of Biology, Indian Institute of Science Education & Research, Tirupati, 517507, India

3. Cytogenetics Laboratory, Department of Zoology, Banaras Hindu University, Varanasi, 221005, India

* For correspondence* For correspondence

Recommended Level: PG/Research

Sub-discipline: Genetics/Cell Biology/Molecular Biology/Developmental Biology

Background:

RNA *in situ* hybridization (RISH) is a cytological technique to visualize the subcellular distribution and abundance of specific RNA molecules in cultured-cells or tissues. The ability to ‘see’ the localization of specific RNA species and quantify the same in different cell types has contributed immensely to our understanding of gene expression and its regulation as a function of cell differentiation. RISH technique is based on the exclusive binding of the probe, a short stretch of single stranded polynucleotide, with its complementary sequence on cellular RNA to form stable double stranded hybrids (Gall and Pardue, 1969). The probes are specifically tagged with radioisotopes (³H or ³⁵S), chemicals (biotin, digoxigenin) or fluorochromes (e.g., fluorescein, rhodamine) to detect their presence within the cell and thus visualize the RNA with which they have hybridized on the basis of their sequence complementarity. The most sensitive and efficient RISH is fluorescence *in situ* hybridization (RNA-FISH) that uses fluorochrome tagged probes, which can be detected under fluorescence or confocal microscope. RNA-FISH technique has potential to detect multiple species of RNAs in the same cell by using a mixture of probes, against the desired cellular RNAs, with each probe being tagged with different fluorochrome that has distinct fluorescence excitation and emission spectra. RNA-FISH can be combined with immunostaining to understand *in vivo* RNA-protein interactions. Here we present a protocol for basic RNA-FISH technique by using digoxigenin (DIG) labelled riboprobe against 280 bp repeat region of the *hsr ω -n* long-non-coding (lnc) RNA and along with immunostaining for Hrp36 hnRNP to visualize the localization of these interacting molecules in *D. melanogaster*.

Objective:

To visualize *hsr ω -n* lncRNA and Hrp36 hnRNP by a combined RNA *in situ* hybridization and immunostaining method in larval tissues of *D. melanogaster*.

Materials Required:

1. **Flies:** Actively wandering late third instar wild type larvae of *D. melanogaster*.
2. **Antibodies:** The antibodies used in present study are anti-DIG rhodamine conjugated antibody (1:200 dilution), mouse anti-Hrp36 antibody (1:20 dilution) and anti-mouse AF488 conjugated antibody (1:200 dilution).
3. **Equipment and other materials:**
 - i. Stereo-binocular microscope
 - ii. Fluorescence/Confocal microscope
 - iii. Incubator set at 42°C
 - iv. Dissecting needles, forceps, fine hair brush and other fly handling items
 - v. Slides and coverslips (22 mm²)
 - vi. Moist chamber (large size Petri dish lined with moist filter papers)
 - vii. Coplin Jars
 - viii. Measuring cylinder
 - ix. Micro-pipettes and their tips
 - x. Microfuge tubes

4. Solutions and Reagents:

- i. 10X PBS (pH 7.4):

Reagent	Concentration	Amount
NaCl	1.37 M	80 gm
KCl	27 mM	2 gm
Na ₂ HPO ₄	100 mM	14.4 gm
KH ₂ PO ₄	18 mM	2.45 gm

Take 800 mL of distilled water (dH₂O) and dissolve all the four components. Adjust the pH to 7.0 and make up the volume to 1 L with dH₂O. Autoclave and store the 10X PBS at room temperature (RT). Dilute the 10X PBS with dH₂O in 1:9 ratio to make the 1X PBS as working solution and store the same at 4°C.

- ii. 20X SSC (pH 7.0):

Reagent	Concentration	Amount
NaCl	3 M	175.3 gm
Sodium Citrate	0.3 M	77.4 gm

Take 800 mL of dH₂O and dissolve both the components, adjust to pH 7.0 with HCl and make up the volume to 1 L with dH₂O. Autoclave and store at RT.

- iii. **4% Paraformaldehyde (PFA):** PFA is a polymer with a wide range of monomeric and oligomeric units of formaldehyde. It dissolves in water and depolymerises in monomeric formaldehyde form. Formaldehyde can fix the cellular constituents by crosslinking proteins, especially those with lysine residues. Formaldehyde is a toxic chemical and therefore, one should always wear mask and gloves during handling PFA. Dissolve 4.0 gm PFA in 75 mL 1X PBS at 60°C with constant stirring for 10 min. Slowly add 5 N NaOH to the cloudy mixture until a clear solution is formed. Filter the solution with a Whatman filter paper and adjust the

pH to 7.0 with HCl or NaOH, as required. Make up the volume to 100 mL with 1X PBS and store in small aliquots at -20°C. Defrost an aliquot and consume it on the same day of experiment. It is, however, preferred that small volumes of PFA solution are prepared fresh, as required. A long storage of formaldehyde leads to its polymerisation in paraformaldehyde form and thus reduces its fixative properties.

iv. **Diethyl Pyrocarbonate (DEPEC):** DEPEC irreversibly inactivates RNase contamination that may be present in dH₂O. It is carcinogenic and, therefore, needs to be handled carefully. Mix 0.1 mL DEPEC in 100 mL dH₂O (0.1% DEPEC) and autoclave after 4 hr incubation at 37°C.

Note: DEPEC treatment is a very effective way to make the solutions RNase-free but buffers like Tris or HEPES cannot be treated with DEPEC as they absorb DEPEC and make it unavailable for RNase. To prepare these buffers, DEPEC treated dH₂O should be used to dissolve the RNase free reagent powders.

v. **Triton X-100:** Triton X-100 is a non-ionic mild detergent which solubilizes proteins and is commonly used to permeabilize the cell membrane. Dissolve 0.03 mL Triton X-100 in 10 mL of 1X PBS to make 0.3% PBST, which is used as washing buffer in RISH.

vi. **RNase Inhibitor:** RNase inhibitors directly binds with RNases in non-competitive mode and thus block their activity. They have been widely used to stop the activities of RNases that are present within the cell. Presence of 1U of RNase inhibitor/μL of solution can block most of the RNase activity during RISH.

vii. **Riboprobe:** This is a DIG labelled RNA probe with a complementary sequence to a particular RNA. About 50-100 ng riboprobes are used for each RISH experiment. A detailed description of probe preparation, validation and detection are discussed below in Experimental Details section (also see [Chapter 56](#)).

viii. **Formamide:** Formamide interferes with hydrogen bonding in double stranded polynucleotides and thus destabilizes double strand formation. Presence of formamide in hybridization buffer, therefore, reduces the melting and annealing temperature which keeps the tissue morphology relatively intact due to low incubation temperature. The working concentration of formamide in hybridization buffer varies from 10% to 50%, depending upon the probe length and its GC content.

ix. **50% Dextran sulfate:** Dextran sulfate is highly hydrated polysaccharide that acts as effective volume exclusion agent. It affects the probe concentration by preventing their solubilization in water and thus gives higher hybridization rates in aqueous solutions. Dissolve 20 gm Dextran sulfate powder, little by little, in 30 mL DEPEC treated dH₂O and leave the tube overnight on the rotator. Make up the volume to 40 mL with dH₂O and store at -20°C in small aliquots. 50% Dextran sulfate is very viscous and special care should be taken during pipetting.

x. **100 mg/mL Yeast tRNA:** Yeast tRNA is used as a nonspecific blocking agent; it also protects the probe and cellular RNA. Dissolve 25 mg tRNA powder in 250 μL DEPEC treated dH₂O and store at -20°C.

xi. **1 µg/mL 4',6-diamidino-2-phenylindole (DAPI):** DAPI is a fluorescent stain that binds at AT rich region of DNA, with excitation at 405 nm and emission spectrum between 420-480 nm (blue colour). The fluorescence of DAPI is enhanced several times following its binding with DNA. Dissolve 1 mg DAPI in 1 mL dH₂O and store the stock (1 mg/mL) solution at -20°C. Dilute the DAPI stock solution in 1X PBS (1:1000) to make the 1µg/mL working DAPI solution, which can be stored at 4°C for several weeks.

xii. **Antifade mounting media:** These are commercially available glycerol based mounting media supplemented with antifading agent that protects the fluorescent signal intensity.

Experimental details:

1. Probe designing and synthesis:

i. The probe used for RISH is a single-stranded and non-self complementary short stretch of polynucleotides (20 to ~1500 nt long DNA or RNA) which has sequence complementary to the target RNA. Riboprobes are commonly synthesized with the bacteriophage RNA polymerases, like SP6, T7 or T3, by *in vitro* transcription on *pSPT* transcription-vector family which has respective promoter at the upstream of the probe sequence. A PCR primer with SP6/T7/T3 promoter sequence as 5' extension also can be used as DNA template for *in vitro* riboprobe synthesis. Riboprobes are synthesized using *in vitro* transcription kit with appropriate DNA templates and polymerases following the manufacturer's protocol.

ii. In the present example, a digoxigenin (DIG) conjugated uridine triphosphate (Dig-UTP) has been used to prepare the labelled riboprobe using commercially available DIG RNA labelling kit (for details, see [Chapter 56](#)). The *pDRM30*, a repeat specific clone of *hsrw* gene containing approximately 280 bp fragment in the *pGEM3* vector, was used to synthesize DIG labelled antisense *hsrw-n* riboprobe with T7 RNA polymerase. The riboprobe was detected using anti-DIG rhodamine tagged antibody.

2. Pre-treatment of tissue:

i. Collect the actively wandering third instar larvae and wash them with 1X PBS. Dissect out the desired internal tissues in 1X PBS with the help of fine forceps or dissecting needles. Fix the tissues with 4% paraformaldehyde at 4°C followed by room temperature for 15 min each in a microfuge tube or in Maximov cavity slide. **Note:** Fix the tissue immediately after dissection to minimise any stress to tissue. Make sure that tissue is properly submerged in the fixing solution and not sticking to the wall of container or floating on the surface.

ii. Remove the fixative and rinse the tissues with 100 µL of 0.3% Triton X-100 in 1X PBS (0.3% PBST) thrice for 10 min each.

Note: Concentration of Triton X-100 in the rinsing solution varies with tissue type, e.g. tissues with thick outer membrane/sheath (larval salivary gland and brain) need higher concentration (0.5%) while tissues with thin outer membrane (larval imaginal disc, Malpighian tubules, fat body etc.) need low (0.1%) concentration of the detergent.

iii. Incubate the tissue with permeabilization buffer (1% Triton X-100, 1% NP40 with 1 U/ μ L RNase-inhibitor) for 20 min at 4°C on rotator to permeabilize the cell and nuclear membrane.

Note: RNase-inhibitor like Vanadyl-ribonucleoside complex (VRC) with a final concentration of 10 mM can also be added.

iv. Rinse the tissue with 100 μ L of 0.3% PBST thrice for 10 min each. Rinse the tissue further with 2X SSC once for 10 min.

3. Probe hybridization:

i. For each sample, prepare ~30 μ L hybridization solution (50% Formamide, 2X SSC pH 7.0, 10% w/v Dextran sulfate, 1 mg/mL yeast tRNA and 5 ng/ μ L DIG labelled *hsr ω -n* riboprobe). Thoroughly mix all the components and incubate them at 37°C for 10 min.

ii. Denature the probe by incubating the hybridization solution at 90°C for 10 min followed by immediate chilling on ice.

iii. Incubate the tissue with hybridization solution in 500 μ L microfuge tube on slow a shaker at 42°C for 12-16 hr. Make sure that cap of the tube is properly closed and sealed with parafilm during incubation to prevent drying. **Note:** The composition of hybridization buffer and incubation temperature varies with probe length and its GC content. Smaller probes (~30 nt) need relatively lower concentration of formamide (10%) and lower incubation temperature (30°C-37°C) while longer probes (~500 nt) need higher concentration of formamide (50%) and higher incubation temperature (65°C). In the present example, 50% formamide was used at 42°C for *hsr ω -n* RISH.

iv. Rinse the tissue at 42°C with freshly prepared wash buffer (50% formamide in 2X SSC) thrice for 5 min each. Rinse the tissue further at 42°C with 2X SSC followed by 1X SSC thrice for 5 min each.

v. Rinse the tissue further with 1X PBS at RT thrice for 5 min each.

4. Co-immunostaining and Detection:

i. Localize the DIG labelled riboprobe, which is hybridized with *hsr ω -n* lncRNA, by incubating the tissue with anti-DIG Rhodamine antibody (dilution as recommended by the manufacturer) for 2 hr at RT.

ii. Process the tissue for immunostaining Hrp36 protein by incubating with anti-Hrp36 antibody (raised in mouse) for 2 hr at RT in dark (to avoid quenching of the Rhodamine fluorescence). Rinse the tissue with 0.3% PBST thrice for 5 min each and incubate with anti-mouse AF488 antibody (dilution as recommended by the manufacturer) for 2 hr at RT (for details see [Chapter 58](#)).

Note: Combined immunostaining can reduce the RISH signal intensity. This can be circumvented by using the two fluorochromes tagged secondary antibodies (e.g., anti-DIG Rhodamine and anti-mouse AF488 in this case) together after the immunostaining step (i.e., incubation with the anti-DIG Rhodamine antibody following the RISH is postponed till the immunostaining step).

iii. Counterstain nuclei with DAPI by incubating the tissue for 10 min at RT and mount the tissue in antifade mounting medium. Store the slides in dark at -20°C till further examination.

iv. Examine the fluorescence signals using appropriate excitation and emission filters in fluorescence or confocal microscope.

Note: The excitation and emission spectra of the fluorochromes used for tagging of the secondary antibodies should be sufficiently different for clear distinction of the two signals (to prevent any ‘bleeding’ effect).

Observations:

In the example used here, RNA *in situ* hybridization with DIG labelled *hsr ω -n* riboprobe shows that the *hsr ω -n* lncRNA is distributed in the nucleoplasm as speckles (arrow heads in **Fig. 1**) as well as in a diffuse form (**Fig. 1A, D**). Variations in sizes of these speckles, named as Omega speckles (Prasanth *et. al.*, 2000), indicates that each may contain different numbers of *hsr ω -n* lncRNA molecules. The very large block of Rhodamine fluorescence in the nucleus (arrows in **Fig. 1**) is the location of the *hsr ω* gene where these transcripts are being actively synthesized. A co-immunostaining for the Hrp36 hnRNP reveals that this protein co-localized with the *hsr ω -n* lncRNA in the Omega speckles as well as at the *hsr ω* gene locus (Singh and Lakhotia, 2015).

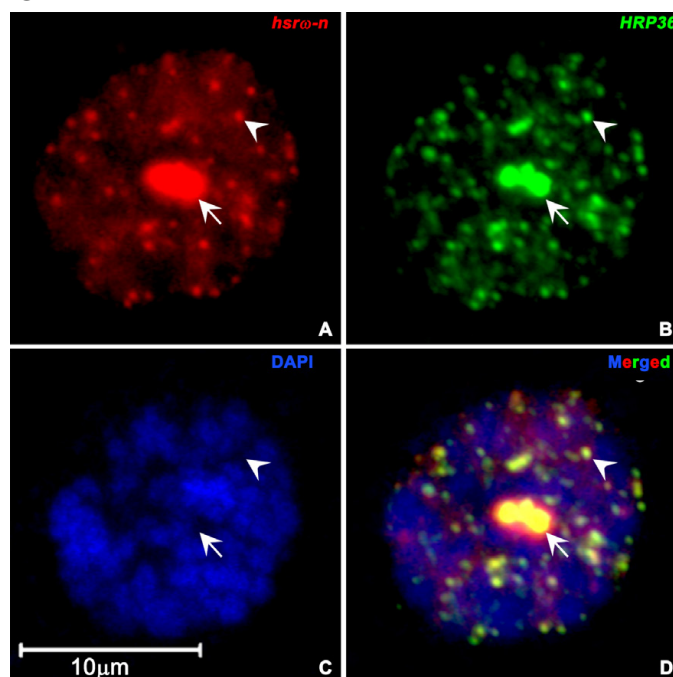


Fig. 1. Combined RNA in situ hybridization (RISH) and immunostaining to visualize the cellular distribution of *hsr ω -n* lncRNA and Hrp36 hnRNP in *D. melanogaster*: RISH with DIG labelled riboprobe (**A**) against repeat region of *hsr ω -n* lncRNA (red in **A, D**) and co-immunostaining (**B**) of hnRNP A1 homolog Hrp36 (green in **B, D**) in a principal cell nucleus of larval Malpighian tubules. Both *hsr ω -n* lncRNA and Hrp36 hnRNP colocalize at the Omega speckles, a subnuclear domain present in nucleoplasm (arrowheads). The Hrp36 hnRNP also binds with the newly transcribed *hsr ω -n* RNA at the transcription site (93D locus) to form a large aggregate (arrow). Nucleus is counterstained with DAPI (blue in **C, D**).

Precautions:

1. **Experimental controls for RISH:** In order to ensure that the observed RISH signal is genuine and not a false-positive result, positive and negative experimental controls should also be processed parallelly. The positive control could be a widely expressed and well-studied gene with known expression pattern like tubulin or G3PDH. RISH with probe against an mRNA expressing only in some specific cell types can also be used as a good control to confirm specificity of the observed signal. Likewise, a negative control could be probed with sense strand sequence of the same gene, which is expected to have no complementary sequence and, therefore, should not generate any hybridization signal. However, many genes express the antisense strand as well and therefore, can give positive signal with sense probe too. Another negative control can be a parallel sample treated with RNase, which must not produce any RISH signal. However, great care is needed since even a very small RNase contamination of the experimental sample can ruin the whole experiment.

2. **RNase contamination:** RNases are the natural enemy for an RNA molecule and blocking their activity is a big challenge in RISH. They can be present anywhere including reagents, solutions, instruments and most importantly on our body surface and therefore, special care is required in all experiments involving RNA. RNases are resistant even to autoclaving. Therefore, all the reagents should be kept in RNase free environment. The solutions and dH₂O used in the experiment should be treated with DEPEC. The working bench, pipettes and plasticwares should be wiped with 0.2 N NaOH and then with hot dH₂O. All the glasswares should be treated with 0.5% SDS and baked (dry heating at 150°C) after washing. The tubes and tips should be fresh, and stored in properly sealed containers. It is important to wear disposable plastic or latex glove to keep the sample safe from RNases present on our hand (finger-RNases). If the glove has touched RNase-contaminated lab equipment such as pipettes, microscopes, door handles etc. immediately change it as it would no longer be RNase free.

3. **Probe:** The sequence of the probe should be specific and complementary to the RNA one wants to localize. The concentration of probe in hybridization buffer must be in an adequate range as a very low or high concentration may lead either to no signal or to nonspecific signal, respectively. Keep the probe stock at -80°C and an aliquot of working concentration at -20°C as multiple freezing/thawing can degrade the probe.

Questions for self-study and interpretation:

1. What is T_m ? Is it same for DNA: DNA, DNA: RNA and RNA: RNA molecules?
2. Double stranded DNA can be denatured with mild alkali treatment (e.g., with NaOH). Can this treatment be also used for denaturing RNA to remove intra-molecular double-stranded regions?
3. Can co-localization of an RNA and protein or two proteins, as detected by fluorescence/confocal microscopy in RISH/immunostained preparations be taken as definitive evidence for their physical interaction in the cell?
4. What could be biological significance of the RNase enzyme being widely present in our body secretions and its being heat-resistant?

References and further reading:

Gall, J. G. and Pardue, M. L. (1969) Formation and detection of RNA-DNA hybrid molecules in cytological preparations. *Proc. Natl. Acad. Sci. USA.* 63: 378-383.

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Chapter 58

Spatial localization of a protein by immunostaining

S. C. Lakhotia* (lakhotia@bhu.ac.in), Olivia Majhi (olivia28majhi@gmail.com) and J. K. Roy* (jagatkroy@gmail.com)

Cytogenetics Laboratory, Department of Zoology, Banaras Hindu University, Varanasi 221005, India.

* For correspondence

Recommended Level: PG/Research

Sub-discipline: Genetics/Cell Biology/Developmental Biology/Molecular Biology

Background:

Immunostaining takes advantage of the specificity of antigen-antibody interaction and provides a sensitive method for in situ localization of a protein or other antigen at cellular and sub-cellular levels. To aid in visual detection, the antibodies are labelled with an enzyme or a fluorescent marker to detect the antigen-antibody complexes in cells or tissues. In the commonly used indirect immunostaining method, a primary antibody binds directly with the target antigen in cell/tissue and a secondary antibody, which carries the label, binds with the primary antibody-antigen complex. For the indirect detection method, a secondary antibody is raised against the primary antibody's constant region. For example, if an IgG primary antibody raised in rabbit is used as the primary antibody, the secondary antibody used would be an anti-rabbit IgG prepared in an organism other than rabbit. Generally, one antigen molecule may bind with several primary antibody molecules, each of which would further bind with several secondary antibody molecules. This leads to signal amplification for a better visualization. The commonly used enzyme tags for secondary antibodies are Alkaline Phosphatase or Horse-Radish Peroxidase, which can be detected histochemically using appropriate chromogenic substrates. With the increasing use of fluorescence/confocal microscopy, antibodies tagged with fluorochromes are more common now. A large variety of fluorescently tagged secondary antibodies are commercially available. Most of the fluorescent dyes used for tagging antibodies have low non-specific affinity for biological molecules but can be chemically coupled to purified antibodies (primary or secondary). Their presence in cells/tissues is detected under a fluorescence (or confocal) microscope. Immunostaining can be combined with in situ hybridization methods (see [Chapter 57](#)).

The examples described here are based on indirect immunostaining utilizing fluorescently labelled secondary antibody to localize a protein in cells. In one set of examples, we describe immunostaining of *Drosophila* tissues to visualize developmental expression of the Decapentaplegic (Dpp) signaling molecule in embryos and in late larval wing imaginal discs. Dpp is the fly homolog of vertebrate bone morphogenetic proteins (BMPs) and is

one of the vital secretory morphogens involved in growth and pattern formation in different tissues. The first mutant allele of this gene in *Drosophila* was named *decapentaplegic* (*decapenta* = fifteen and *plegic* = paralysis) because the mutation affected development of all the fifteen major larval imaginal discs (7 paired and 1 unpaired) that produce specific adult appendages and other external cuticular structures (see Chapters 2, 18).

The Phosphate Buffered Saline (PBS) is commonly used at various steps during immunostaining. In some cases, the blocking step, used during the immunostaining procedure to suppress non-specific binding, fails to completely prevent the non-specific sticking of antibody to the sample. An alternative method involves the use of PIPES (piperazine-N,N'-bis(2-ethanesulfonic acid)-EGTA (Ethylene glycol tetraacetic acid) buffer, which may reduce non-specific background signals during immunostaining. The PIPES-EGTA immunostaining protocol (Michel *et al.*, 2011) is also described here using adult testes as example. Depending upon the tissue and the specific antibody, this alternative method may be useful to reduce the background.

Objectives:

1. Immunostaining of a target protein expression using *dpp-lacZ* reporter in *w¹¹¹⁸*; *dpp-lacZ/CyO* stage 13 embryos and late third instar larval wing imaginal discs and immunostaining to localize the Patched protein in adult testes using the PIPES-EGTA buffer.

Materials Required:

A. Flies:

- i. *w¹¹¹⁸*; *dpp^{lacZ}/CyO*; +/+ (Bloomington Stock no. 8404). The *dpp^{lacZ}* or *dpp-lacZ* transgene is constructed by fusing the *dpp* enhancer/promoter with the *lacZ* reporter gene of *E. coli* so that the *lacZ* expression mimics normal developmental expression of the *dpp* gene. The *lacZ* expression can be detected either by X-Gal histochemical staining (see Chapter 39) or by immunostaining as described here.
- ii. Wild type flies.

B. Equipment and other supplies:

- i. Stereo-binocular microscope.
- ii. Fluorescence (or Confocal) microscope.
- iii. Egg collection chamber.
- iv. Egg/Larval handling and dissection instruments (soft fine hairbrush, fine needles, forceps).
- v. Cavity/Maximov slides.
- vi. Slides and coverslips (22 mm²).
- vii. Blotting/filter papers.

- viii. Micropipettes/droppers and micropipette tips.
- ix. Incubator set at 42°C.

C. Solutions/Reagents for PBS-based immunostaining:

- i. Poels' salt solution

NaCl	86 mg
KCl	313 mg
CaCl ₂ ·2H ₂ O	116 mg
NaH ₂ PO ₄	88 mg
KHCO ₃	18 mg
MgSO ₄ ·7H ₂ O	513 mg

Serially add the above salts in 80 mL distilled water, adjust to pH 6.8 and finally add distilled water to make the volume to 100 mL Autoclave and store at room temperature or at 4°C.

- ii. 1X Phosphate Buffered Saline (PBS, pH 7.4)

NaCl	8 gm
KCl	0.2 gm
Na ₂ HPO ₄	1.44 gm
KH ₂ PO ₄	0.24 gm

Serially dissolve the above in 800 mL distilled water; when fully dissolved, adjust pH to 7.4 with 1N HCl as required. Finally make the volume to 1000 mL with distilled water, autoclave the solution and store at room temperature or at 4°C.

- iii. Bleach solution: 5% Sodium hypochlorite or 5% commercial bleaching powder.
- iv. Fixative: n-heptane: 4% paraformaldehyde in 1X PBS in 1:1 ratio.
- v. PBST: 0.1% Triton X-100 in 1X PBS.
- vi. Blocking solution: 1% Bovine Serum Albumin in 0.1% PBST (0.1% Triton X-100 in 1X PBS).
- vii. DAPI (4',6-diamidino-2-phenylindole): Dissolve 1 mg DAPI in 1 mL deionized water (dH₂O) or dimethylformamide (DMF) and store the stock (1 mg/mL) solution at -20°C. Dilute the DAPI stock solution in 1X PBS or 1X PEM (1:1000) to make the 1µg/mL working DAPI solution, which can be stored at 4°C for several weeks.
- viii. DABCO: Dissolve 1.25 gm of DABCO powder in 15 mL of 1X PBS. Add 35 mL of ultrapure glycerol, mix gently for 2 hr and store at -20°C.

D. Solutions/Reagents for PIPES-EGTA based immunostaining:

- i. PIPES-EGTA-MgCl₂ (PEM) solution, pH 7.4 (for 100 ml):

80 mM Na-PIPES	2.68 g
5 mM EGTA	0.19 g

1 M MgCl₂ 100 μL

5M NaOH 7.5 mL

Dissolve in 80 mL dH₂O. Adjust pH to 7.4 with NaOH and make up the volume to 100 mL with dH₂O. Autoclave the solution and store it at 4°C.

Notes:

- a. Na-free PIPES can also be used (MW=302.37).
- b. EGTA has a higher affinity to calcium ions, whereas EDTA is more attracted to magnesium ions. Since the chelators in all the buffers are in large excess, EDTA can also work instead of EGTA.
- ii. PEMT: 0.2% Triton X-100 in 1X PEM: Dissolve 20 μL of Triton X-100 in 10 mL of 1X PBS to make 0.2% PEMT.
- iii. 1% Triton X-100 in 1X PEM: Dissolve .100 μL of Triton X-100 in 10 mL of 1X PBS to make 1% PEMT.
- iv. 4% Paraformaldehyde (PFA) in 1X PEM: Add 4.0 gm of PFA in 100 mL of 1X PEM to make PFA+PEM, heat at 60°C with constant stirring at regular intervals.
- v. 4% Paraformaldehyde (PFA) in 0.2% PEMT: Mix 4.0 gm of PFA in 100 mL of 0.2% PEMT to make PFA+PEMT at 60°C with constant stirring at regular intervals to dissolve PFA.
- vi. 1M NH₄Cl (Ammonium Chloride): Add 5.3 gm of NH₄Cl in 100 mL of distilled H₂O. To make the working solution of 50 mM NH₄Cl in 1X PEM, add 0.5 mL of 1M NH₄Cl in 9.5 mL of 1X PEM.
- vii. Blocking Solution: Dissolve 0.05 gm of Bovine Albumin Serum (BSA) in 10 mL of 0.2% PEMT.

E. Antibodies:

- i. Anti--β-galactosidase (Primary antibody raised in chicken in the present example; dilution as recommended by manufacturer).
- ii. Anti-Fasciclin-3 or Fas-3 (Primary antibody raised in mouse in the present example; dilution as recommended by manufacturer).
- iii. Anti-Patched (Ptc) (Primary antibody raised in mouse, dilution- 1:100).
- iv. Anti-chicken Alexa Fluor-488 (Secondary antibody against chicken IgG; dilution as recommended by manufacturer).
- v. Anti-mouse Alexa Fluor- 488 (secondary antibody dilution). as recommended by manufacturer).
- vi. Anti-mouse Alexa Fluor-546 (Secondary antibody against mouse IgG; dilution as recommended by manufacturer).

Note: Choice of the secondary antibody depends upon the animal host in which the primary antibody is raised.

Experimental Details:

A. Immunostaining of *Drosophila* embryos using PBS buffer

1. Embryo fixation:
 - i. Collect eggs from healthy 3-5 days old flies (wild type and/or desired genotype) over a 1-2 hr period using an egg-laying chamber (see Chapters 32, 34, 70).
 - ii. Remove eggs of the desired age from the Petri dish with a fine brush in an egg washing chamber (see Chapters 32, 34) and dechorionate, fix and devitelinize them as described in Chapters 32, 33, 39).

Note: Fixed embryos can be stored at this stage at -20°C till further processing for immunostaining.

1. Immunostaining:
 - i. Transfer the fixed embryos to the egg-washing chamber and rehydrate through 90%, 70%, 50%, 30%, 10% methanol diluted in 0.1% PBST at room temperature, keeping them for 5 min in each alcohol grade.
 - ii. Keep embryos in the blocking solution for 2 hr at room temperature.
 - iii. Remove the blocking solution and transfer eggs to a cavity/Maximov slide. Add the required amount (~30 μ L for 50 embryos) of the desired primary antibody solution, diluted in the blocking solution as recommended for immunostaining by the manufacturer. Cover with a coverslip, seal the edges with transparent nail polish and place in a moist chamber for 2-3 hr at room temperature or overnight at 4°C (this needs to be standardized depending upon the primary antibody's efficiency).
 - iv. Remove the antibody solution (this can be saved and stored at 4°C for future use since the primary antibody is re-usable for 2-3 times) and wash the embryos three times in 0.1% PBST for 15 min each. **Note:** If another primary antibody is to be used in sequential order, the last step of incubation with the desired dilution of the primary antibody may be repeated.
 - v. Replace the washing solution with the blocking solution and incubate for 1 hr at room temperature. **Note:** If two primary antibodies against two different antigens are to be used on the same tissue, care should be taken to avoid any potential cross-reaction. The two primary antibodies must be raised in two different host species (preferably phylogenetically distant hosts). In the present example, the antibody against Fas-3 is raised in mouse, while the antibody against β -Galactosidase is raised in chicken and, therefore, can be used together as a cocktail. When a cocktail of primary antibodies (as required) is added to the fixed tissue, the concentration of each antibody in the final mixture should be equal to its desired working dilution. For example, the working dilution required for the anti- β -galactosidase is 1:100 and that for anti-Fas-3 is 1:20. Therefore, for preparing 100 μ L of the primary antibody mixture, 1 μ L of anti β -galactosidase, and 5 μ L of anti-Fas-3 were added to 94 μ L of blocking solution.

- vi. Remove the blocking solution and add the secondary antibody diluted in blocking solution as recommended by the manufacturer. To avoid quenching of fluorescence by light, keep the tissue in moist chamber, protected from light.

Notes:

- a. If two or more different proteins/antigens are to be detected in the same cell/tissue, the corresponding secondary antibodies must be tagged with fluorophores that have non-overlapping excitation and emission spectra.
 - b. Some secondary antibodies may show variable degree of non-specific interactions with the target tissue. In such cases, it is desirable that the diluted secondary antibody is preadsorbed using freshly fixed and permeabilized wild type larval brain or embryos, which have not been exposed to any other antibody, for 1 hr at room temperature.
 - c. In some cases, the primary antibody may also need to be pre-absorbed if it detects some non-target molecules by incubating fixed tissues, that do not express the target antigen, for 1 hr. In the present example, the anti- β -galactosidase antibody shows high background signal in *w¹¹¹⁸; dpp^{lacZ}/CyO*; +/- samples but after preadsorption with wild type (which do not express the *E. coli* β -galactosidase) fixed tissues, the unwanted background is eliminated (see **Fig. 1**). This anti- β -galactosidase antibody seems to identify, besides its target *E. coli* β -galactosidase, the fly β -galactosidase as well. Such undesired binding is eliminated by the preadsorption with wild type tissues so that the fraction that recognizes the fly β -galactosidase is titrated out during pre-adsorption.
- vii. Remove the secondary antibody solution and wash the embryos at least thrice in 0.1% PBST for 15 min each (the used secondary antibody may be saved for reuse 2-3 times).
 - viii. Counterstain nuclei with DAPI (1 μ g/mL in 0.1% PBST) for 20 min followed by three washes in 0.1% PBST for 15 min each (optional step).
 - ix. Mount the stained embryos in DABCO/ Vectashield on bridge slides.

Notes:

- a. Bridge slides are prepared by fixing two halves of a 22 mm² coverslip on a clean slide with DPX at a suitable distance (~1 cm) from each other to provide a space for mounting of the desired tissue. The DPX must be completely dry before these slides are used. When covered with the coverslip, the tissue placed in this free space does not get vertically compressed by the coverslip which now rests on the two fixed coverslip pieces.
- b. If a secondary antibody tagged with an enzyme like alkaline phosphatase is used to detect the primary antibody binding, steps for chromogenic detection as described in **Chapter 39** can be followed and the stained tissues can be examined under a bright-field microscope with appropriate counter-staining of the tissue.

B. Immunostaining of larval (or adult) tissues using PBS buffer

- i. Dissect out the wing imaginal discs from healthy late third instar $w^{1118}; dpp^{lacZ}/CyO; +/+$ larvae in 1X PBS and transfer them to fresh PBS in a Maximov/cavity slide.
- ii. Remove the PBS and fix the wing discs in freshly prepared 4% paraformaldehyde for 10-15 min.
- iii. Following the fixation (detected by a reduced transparency of the tissue), rinse the tissue in 0.1% PBST thrice for 15 min each to completely remove the fixative.
- iv. Follow the subsequent steps **A. 2. ii-ix** for the embryo immunostaining above. **Note:** A visual of immunostaining procedure described by Spratford and Kumar (2014) can be seen at <https://www.jove.com/t/51792/dissection-immunostaining-imaginal-discs-from-Drosophila>.

C. Immunostaining of adult testis using PIPES-EGTA method (Michel *et al.*, 2011):

- i. Take 100 μ L of PEM on a clean glass slide and dissect the desired tissue (adult *Drosophila* testis is used here) in 1X PEM using fine forceps and/or dissecting needles (see **Chapter 107**). Carefully transfer the dissected tissue (adult testis in the present example) to a cavity block or Maximov slide containing PEM for further processing. **Note:** Complete the dissection within 30 min, while keeping the dissected tissues 1X PEM in a cavity block or Maximov slide on ice to prevent tissue damage.
- ii. Remove PEM and add 50 μ L of freshly prepared 4% PFA at room temperature, taking care that the tissues are completely immersed in the solution.
- iii. Remove the 4% PFA solution after 20 min and add 50 μ L 4% PFA prepared in 0.2% PEMT. Keep for 20 min. **Note:** The two-step fixation includes fixing without detergent to preserve the overall structure of the tissue while the 2nd fixing with detergent helps permeabilize cells and fixing internal structures.
- iv. Give 3 quick rinses (adding the buffer and immediately removing it) to the tissue with 100 μ L of 0.2% PEMT followed by two washes for 5 min each in 250 μ L PEMT.

Note: The general purpose of quick rinsing, before the actual washing, is to dilute out the previous solution (e.g., fixative, antibodies, etc. depending on the specific step). Add sufficient amount of 0.2% PEMT making sure that the tissues are properly dipped in the solution.

- v. Remove the PEMT and wash for 10 min in 250 μ L of 1X PEM + 50mM NH_4Cl (**Note:** Addition of NH_4Cl allows quenching of the aldehyde fixative as the NH_4^+ ions form Schiff bases linking carbon and nitrogen atoms).
- vi. Give three quick rinses with 0.2% PEMT followed by 1 hr in 250 μ L PEMT with 1% Triton X-100.

Note: This step generates a steep concentration gradient between the tissue inside and the solution outside to facilitate diffusion of antibodies, even when used at greater dilution. The duration of incubation and concentration of Triton-X can be adjusted, depending upon the tissue type.

vii. Rinse in 0.2% PEMT thrice and wash in 250 μ L of 0.2% PEMT for 10-20 min.

Note: Multiple washes and rinsing ensure permeabilization of the various basement membranes and other extracellular matrix (ECM).

vii. Block in 100 μ L of 0.2% PEMT + 0.5% BSA for 45 min to 1 hr at 24°C (RT).

viii. Incubate overnight with appropriately diluted primary antibody (Patched has been used in the example here) in 0.2% PEMT + 0.5% BSA at 4°C.

ix. Remove the antibody solution the next day (the removed antibody solution can be collected and stored at 4°C for 2-3 times future use).

x. Rinse in 0.2% PEMT three times, followed by three washes for 20 min each in 250 μ L PEMT.

xi. Incubate with secondary antibody in blocking solution for 2 hr at RT (from this step onwards, keep the tissues in dark to prevent quenching of fluorophore tagged with the secondary antibody).

xii. Rinse in 0.2% PEMT thrice, and wash for 20 mins each in 250 μ L of 0.2% PEMT thrice.

xiii. Wash for 10 min in 1 mL 1XPEM.

xiv. For counterstaining nuclei, add 50 μ L of DAPI made in 1XPEM and keep for 20 min.

xv. Wash for 15 min each in 250 μ L 0.2% PEMT thrice.

xvi. Mount using antifade mounting media DABCO.

Observations:

Examine the preparations under fluorescence (or confocal) microscope using appropriate excitation and emission filters as required for the fluorochrome tag on the secondary antibody. Identify the domains in embryo and in wing disc where Dpp expresses.

Note: Only the *dpp-lacZ* chromosome carrying embryos and larvae would express the *E. coli* β -galactosidase under the *dpp* enhancer/promoter and thus would show specific immunostaining with the anti- β -galactosidase antibody. The *CyO/CyO* homozygous embryos do not survive, see [Chapter 1](#)).

The examples in Fig. 1 are from *dpp-lacZ* expressing embryos and larval wing imaginal discs, immunostained using the PBS buffer. In these examples, the *lacZ* encoded β -galactosidase acts as a reporter for the *dpp* gene's promoter. The *dpp* expressing cells display green fluorescence following immunostaining with a primary antibody against *E. coli* β -galactosidase and a corresponding anti-chicken Alexa Fluor-488 tagged secondary antibody (excitation wavelength 488 nm). The red fluorescence in this example indicates presence of Fas-3 (Fasciclin-3) protein as these tissues were co-immunostained with anti-Fas-3 primary antibody and anti-mouse Alexa Fluor-546 secondary antibody detected with excitation at 546 nm (see Fig. 1A and B).

Note: A fluorescence microscope permits visualization of the two colours and their general spatial locations but does not provide sharp images due to thickness of the tissue samples. A confocal microscope permits collection of sharp images due to the feasibility of observing optical sections of the tissue.

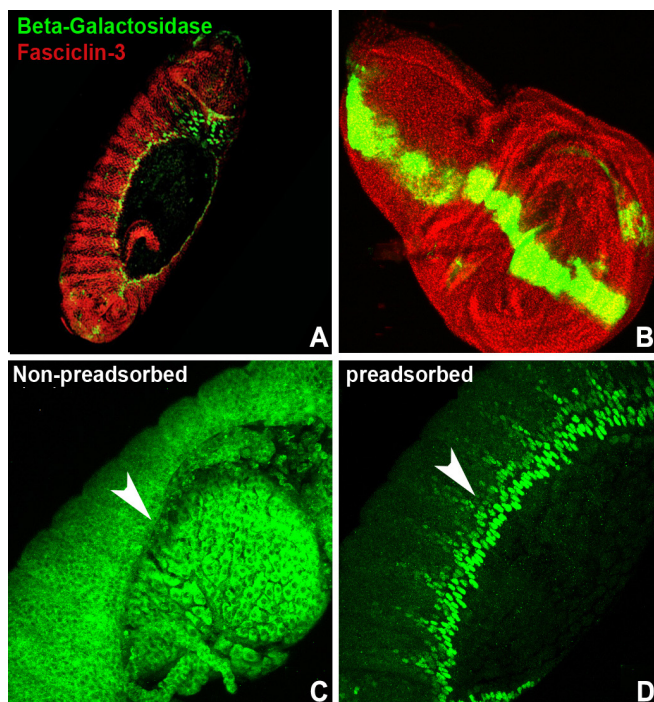


Fig. 1. Fluorescence immunostaining using the PBS buffer. Confocal projection images of stage 13 embryo (A) and third instar larval wing disc (B) expressing *dpp-lacZ* transgene and immunostained with anti- β -galactosidase (green) and anti-Fas-3 (red) antibodies. C-D. Immunostaining for β -galactosidase (green fluorescence) of stage 13 *dpp-lacZ* transgene expressing embryo without (C) or after (D) preadsorption of the primary anti- β -galactosidase antibody; the white arrowheads in C and D indicate the advancing edge of epithelial cells migrating for dorsal closure and which have high *dpp* expression. The examples in A and B were immunostained with preadsorbed anti- β -galactosidase antibody. Images not to same scale.

An example of immunostaining of the Patched (Ptc) protein, a receptor of the hedgehog pathway, in the apical region of the *Drosophila* testis (see Chapter 107) using the PIPES-EGTA buffer is shown in Fig. 2.

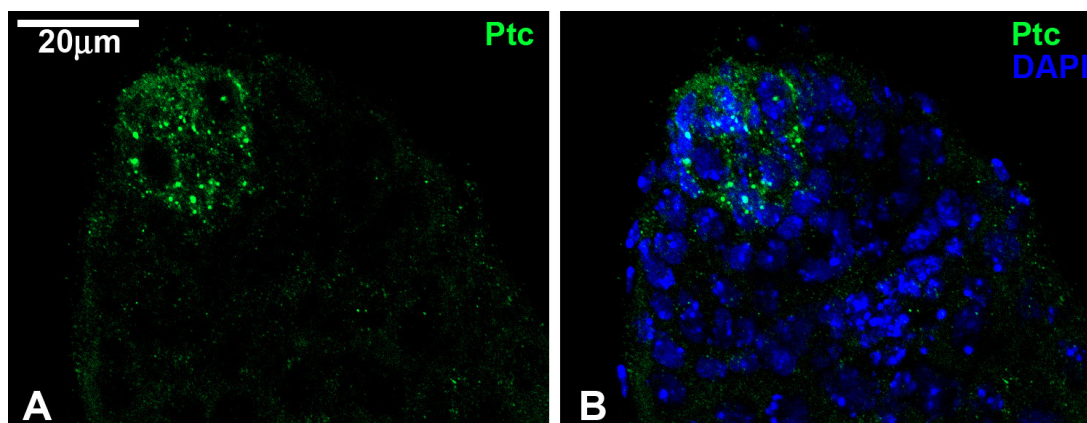


Fig. 2. Immunostaining using the PIPES-EGTA buffer. A-B. Confocal projection images of the tip (hub) region of the wild-type adult *Drosophila melanogaster* testis immunostained with Patched antibody (green) using the PIPES+EGTA protocol; B shows combined DAPI staining of nuclei (blue) and Patched (green) immunostaining.

Questions for self-study and interpretation:

1. Why is a tissue 'fixed' prior to staining?
2. Why is blocking solution used prior to incubation of the tissue in antibody solution?

3. What can be the limitation of using a labeled primary antibody for immunostaining?
4. What care is to be taken when we wish to simultaneously detect two different gene products in a biological sample using different primary antibodies?
5. What other methods can be used for studying the expression of a given gene at cell/tissue level?
6. Do you expect to see differences in immunostaining patterns in some mutants that affect the target gene's activity?

References and further reading:

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Spratford, C. M. and Kumar, J. P. (2014) Dissection and immunostaining of imaginal discs from *Drosophila melanogaster*. *J. Vis. Exp.* 91: e51792. <https://doi.org/10.3791/51792>.

Chapter 59

Comet assay for visualization of DNA damage in haemocytes

Prem Rajak¹ (prem.rjk@gmail.com) and Sumedha Roy^{2,3*} (sumedha.roy@ugent.be)

1. Department of Animal Science, Kazi Nazrul University, Asansol, West Bengal

Previous Address: 2. Toxicology Research Unit, Cytogenetics Laboratory, Department of Zoology, The University of Burdwan, Burdwan 713104

Current Address: 3. Department of Biomolecular Medicine, Faculty of Medicine and Health Sciences, Ghent University, Belgium

* For correspondence

Recommended Level: PG/Research

Sub-discipline: Cytogenetics/Cell Biology/Molecular Biology/Toxicology

Background:

Single-cell gel electrophoresis assay, popularly known as comet assay is a sensitive method for visualizing DNA breaks in single-cell suspension (Dhawan *et al.*, 2003). In this method, cells are first embedded in agarose gel layered on a conventional glass slide and are lysed with detergent or high salt to expose supercoiled loops of DNA. Electrophoresis at high pH helps the negatively charged DNA molecules to migrate through agarose pore towards the positive charge under the influence of constant current. Separation will depend upon mass by charge ratio of DNA fragments. Hence, smaller fragments of DNA will move faster than the larger pieces. Later cells are usually stained with Ethidium Bromide and observed under fluorescence microscope. Microscopic view of cells with smaller fragments of DNA look like “astronomical comets” each with a distinct head (with intact DNA) and a tail (containing damaged/broken pieces of DNA). Hence, the assay is popularly known as “Comet Assay”. The length and intensity of the comet tail signifies the degree of DNA breaks. Comet assay is suitable to assess the genotoxic potential of xenobiotics or any other chemical and ionizing radiation (Liao *et al.*, 2009; Rajak *et al.*, 2017). It can also be helpful to study DNA repair in response to several synthetic/natural agents.

Haemolymph of *Drosophila melanogaster* contains three different types of hemocytes viz., plasmatocytes, lamellocytes and crystal cells. Haemocytes encounter several toxins, xenobiotics and pathogens being absorbed across the alimentary canal. Such chemicals and/ biological agents can induce apoptosis which is the hallmark feature of DNA fragmentation in these cells. In addition, natural cell death also takes place due to aging phenomena. Hence these cells are better choice for comet assay to detect smaller pieces of DNA. Moreover, these immune cells are already available in single cell state enabling them less time consuming and more convenient for the assay.

Objectives:

1. To test the genotoxic potential of natural or synthetic chemicals.
2. To study DNA repair in response to a particular drug/neutraceutical.

Materials required:

1. Specimen: Third Instar larvae of *Drosophila melanogaster*

2. Glasswares/Plasticwares/papers: Blotting papers, two insect needles, conventional slides, coverslips (No. 1, 24 x 60 mm), microcentrifuge tubes (1.5 mL), pipettes (2-20 μ L, 20-200 μ L and 100-1000 μ L), micro tips, water bath, tray, conical flasks (50 mL), forceps, scissors, ice packs or powdered ice to provide flattened surface, paraffin strip (cut paraffin strip in size of conventional slides), small horizontal gel apparatus with power pack.

3. Solutions:

- i) 1.5 % Normal Melting Point Agar (NMPA)
- ii) 1 % Low Melting Point Agar (LMPA)
- iii) Phosphate-Buffered Saline (PBS) (pH 7.4)
- iv) Phosphate Lysis Buffer (pH 10)
- v) Electrophoretic Buffer (pH 13)
- vi) Neutralizing Buffer (pH 7.5) (0.4M Tris-HCl in distilled water)
- vii) Ethidium Bromide
- viii) Chilled methanol

4. Preparation of solutions:

i. 1.5 % Normal Melting Point Agar (NMPA): Dissolve 300 mg NMPA in 20 mL distilled water

ii. 1 % Low Melting Point Agar (LMPA): Dissolve 200 mg LMPA in 20 mL distilled water.

iii. Phosphate-Buffered saline (PBS; pH 7.4): Add Sodium Chloride 8.0 g, Potassium Chloride 0.2 g, Disodium hydrogen phosphate 1.42 g and Potassium dihydrogen phosphate 0.272 g in 800 ml distilled water, and adjust pH to 7.4 by adding 1N-HCl, as required. Finally add distilled water to make the final volume 1000 mL. Autoclave and store at 4°C.

iv. Phosphate Lysis Buffer (pH 10; store at 4°C)

2.5 M NaCl	14.61 gm
100 mM EDTA	3.722 gm
100 mM Tris	0.12114 gm
1% Triton X-100	1 mL
Distilled water	100 mL

v. Phosphate Electrophoretic Buffer (pH 13; store at 4°C temperature)

1 mM Na ₂ EDTA	37.224 mg
300 mM NaOH	1.199 gm
Distilled water	100 mL

vi. Neutralizing Buffer (pH 7.5; store at 4°C): 48.5 gm Tris added to ~800 mL distilled water, adjust pH to 7.5 with concentrated (>10 M) HCl: Store at room temperature.

Experimental Details:**1. Preparing grease-free slides**

- i. Slides are marked with diamond markers for their identification.
- ii. Slides are dipped into chilled methanol and then passed over a blue flame to remove oil and dust particles from the slides. Alternatively, they may be wiped dry with a soft clean cloth.
- iii. Slides are kept covered in a clean, dust-free and dry place.
- iv. Avoid touching slide surface with bare hands.

2. Preparation of Agarose bed for cell suspension

- i. Dip grease-free slides into hot and melted NMPA (1.5 %) with the help of forceps and then remove slowly. Chilled slides should be used to enhance the gelling of the agarose.
- ii. Wipe out the agarose layer from the underside of the slide and keep it upon a clean tray.
- iii. Air dry the slide.

Note: It is recommended to prepare agarose beds on slides at least one day before use. Store slides at room temperature and low humidity condition before use. Slides can be stored for 2-3 days.

1. Collection of hemolymph

- i. Third instar larvae are taken out of the *Drosophila* food vial and washed thoroughly with chilled PBS in a watch glass.
- ii. 50 µL of chilled PBS is taken in the middle part of a slide. Larvae are taken out one by one from the watch glass and kept near the chilled PBS.
- iii. Using two fine needles, rupture cuticular wall of each larva to drain hemolymph into the adjacent PBS solution on the slide. Please note that, internal organs should not be disturbed while rupturing the cuticle and draining the hemolymph.
- iv. Using the same procedure, collect hemolymph from 15-20 larvae. The process should be completed as early as possible.
- v. After bleeding hemolymph, PBS solution is collected through micropipette into a 1.5 mL microcentrifuge tube. The final volume should be made up to 300 µL by addition of extra chilled PBS.

4. Preparation of LMPA-hemolymph suspension

- i. LMPA is melted in a conical flask using a hot water bath or microwave oven.
- ii. 300 μL of LMPA is added and mixed with the 300 μL of hemolymph suspension in the 1.5 mL microcentrifuge tube .
- iii. The solution is mixed properly while avoiding bubble formation.

5. Coating LMPA-hemolymph suspension on previously prepared agarose bed (Fig. 1)

- i. Take 100 μL of the freshly prepared LMPA-hemolymph suspension with a micropipette and pour over the agarose bed on slide.
- ii. Immediately, place a paraffin strip over the solution to flatten the layer of LMPA-hemolymph suspension.
- iii. Place the slide over ice packs until the agarose layer hardens (usually 5-10 min).
- iv. Gently remove the paraffin strip and add a third layer of 100 μL of LMPA over the hardened LMPA-hemolymph suspension.
- v. Further, flatten the layer using a paraffin strip and place the slide over ice packs or ice for solidification.

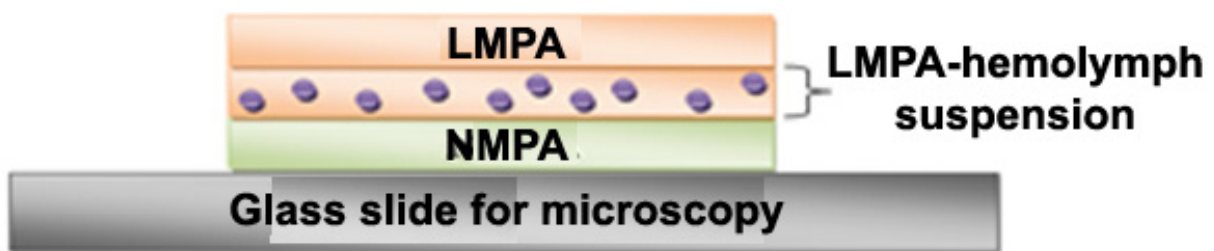


Fig. 1. A typical three-layered slide for Comet Assay

6. Electrophoresis (to be carried out at 4°C)

- i. After the gel containing cells has solidified, the slides are placed in a lysis solution for 2 hr. The lysis solution should be chilled prior to use.
- ii. Place slides in the small horizontal gel apparatus and fill the apparatus with chilled alkaline (pH > 13) electrophoresis buffer.
- iii. Prior to electrophoresis, the slides are incubated for 20 min in electrophoresis buffer to produce single-stranded DNA. Formation of single stranded DNA takes place because the solution of high pH is rich in hydroxide ions, and these negatively-charged ions can pull hydrogen ions off from base pairs in DNA. This process disrupts the hydrogen bonding that holds the two DNA strands together, causing them to separate.
- iv. After incubation, carry out electrophoresis out at 25 V and 300 mA for a period of 15 min. The electrophoresis chamber can be placed in a refrigerator to maintain its temperature at 4°C.
- v. The slide is shifted on a draining tray and is coated with the Neutralization Buffer in

a dropwise manner. Leave the slide with the overlaid buffer for at least 5 min, following which the neutral buffer is drained out. The slides are drained and the same process is repeated two more times. This process will help to neutralize the unwound DNA strands. During neutralization, the DNA strands separated by alkaline treatment in the comet head will readily renature due to their intact structure with supercoiled loops, while the DNA in the tail will remain single-stranded because the smaller pieces get entangled with agarose gel matrix and their complementary sequences are usually distantly placed.

vi. The slides are stained with 80 μ L Ethidium Bromide (20 μ g/mL) for 5-10 min in dark and then dipped in chilled distilled water to remove excess stain. Ethidium Bromide should be handled and disposed off safely since it has carcinogenic property.

vii. Finally a coverslip is placed over the slide.

Observations:

1. Observe the stained slides under 40 x objective of a fluorescence microscope using blue/UV excitation and red emission filters. Photographs of cells are taken for record and further analysis. Nuclei with little or no damaged DNA will appear intact (**Fig. 2a**) while those with damaged DNA will show a fluorescent ‘tail’ region (**Fig. 2b**). Area of the tail is proportional to the extent of DNA damage.

2. For quantitative analysis of DNA damage, a large number of cells in control and experimental sets should be randomly photographed. These digital images can be analyzed using suitable software such as Open Comet, Komet 5, Comet assay IV or Comet Score TM 2.0.

3. Three parameters viz. Tail length (TL, the distance to which the DNA migrated from boundary of the nuclear core), Tail intensity (TI, fluorescence intensity of the entire tail region corresponds to the amount of DNA present in the comet tail), and Tail Moment (TM, product of the tail length and fraction of DNA (i.e, ratio of DNA in nuclear core and tail region) found in the comet tail) are usually taken into consideration to evaluate the extent of DNA damage/fragmentation in the sample.

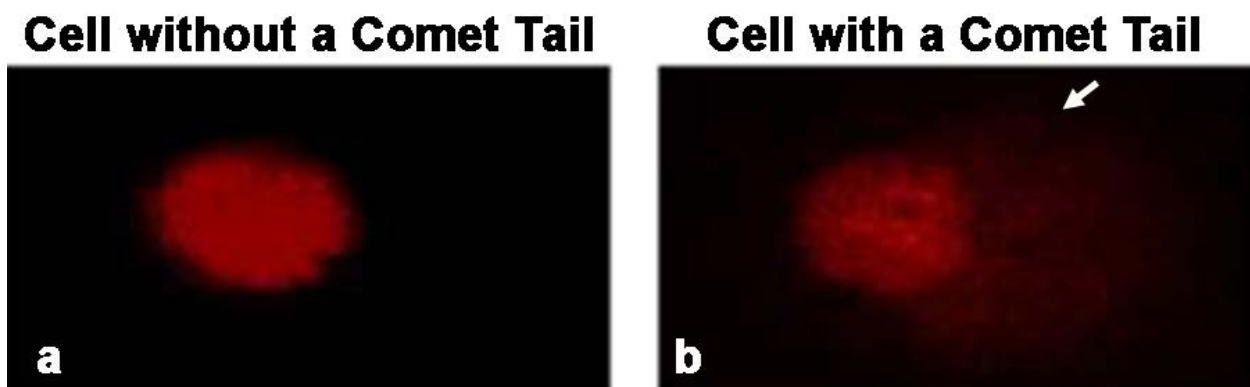


Fig. 2. Fluorescence images of Ethidium Bromide stained haemocytes after the Comet assay. White arrow in **b** indicates the comet tail observed in a cell with damaged DNA.

Questions for self-study and interpretation:

1. Why is comet assay so named?
2. Mention some uses of comet assay in research.
3. What is the role of lysis buffer in comet assay?
4. State the advantage of using hemocytes for the comet assay.
5. Why are slides incubated for 20 min in electrophoresis buffer prior to electrophoresis?
6. What other dyes can be used to visualize fragmented DNA under a fluorescence microscope?

Reference and Further reading:

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Chapter 60

Genomic DNA Isolation from *Drosophila*

Manish Pandey, Sakshi Bansal and Geetanjali Chawla* (gchawla@rcb.res.in)

Regional Centre for Biotechnology, Faridabad, Haryana.

*For correspondence

Recommended Level: Postgraduate/Research

Sub-discipline: Molecular Biology

Background:

Genomic DNA makes up the ‘genome’ of an organism, which stores all information required for the development and overall functioning of any living system. Since some of the basic and most crucial cellular processes like transcription and translation depend directly on instructions from the genomic DNA, methods for its isolation and purification become very important for studies directed to understanding of organization and functions of the genome. Genomic DNA isolation is often the first technique in molecular biology research involving DNA. Good quality genomic DNA is essential for advanced approaches like whole genome sequencing methods, polymerase chain reactions, chromatin immuno-precipitation experiments and to study polymorphisms and variations in a population.

There are multiple methods for genomic DNA isolation in *Drosophila* (Huang *et al.*, 2009). The one described here is an efficient way to purify good quality genomic DNA with only a few flies as starting material. This method is adapted from the Berkeley *Drosophila* genome project (<https://www.fruitfly.org/about/methods/inverse.pcr.html>). The method involves homogenizing flies in a buffer solution called buffer A, which consists of Tris-HCl, EDTA, NaCl and Sodium dodecyl sulphate (SDS). SDS is an anionic detergent that causes lysis of cells by disrupting the membrane and denaturing proteins (for example histones attached to DNA). NaCl also helps in the removal of proteins and neutralizes the charge on DNA (which later aids in precipitation). Tris-HCl is the primary buffering agent that maintains a stable pH and EDTA helps chelate divalent cations like Mg^{2+} to keep DNAses inactive (Mg^{2+} ions are co-factors for DNAses). After lysis, a solution of lithium chloride and potassium acetate is added to the reaction to enable precipitation of high molecular weight RNA without using ethanol. Since, ethanol precipitation would precipitate genomic DNA, RNA and protein, selectively using lithium chloride aids in separation of RNA (in pellet) from genomic DNA, which remains in aqueous solution. The precipitated RNA is discarded and the genomic

DNA in the supernatant is precipitated with isopropanol. The quantity and purity of the isolated genomic DNA can be assessed using a spectrophotometer and/or by agarose gel electrophoresis (for isolating DNA from a single fly, see [Chapters 61, 62, 63](#)).

Objectives:

To isolate genomic DNA from adult *Drosophila melanogaster* flies and to check the concentration, purity and integrity of the isolated genomic DNA using spectrophotometer and agarose gel electrophoresis.

Materials Required:

A. Flies:

Wild type *Drosophila melanogaster* flies (wild type and/or other desired genotype)

B. Equipment

- i. Bench top centrifuge
- ii. Heating block or Incubator or boiling water bath
- iii. Autoclave
- iv. -20°C freezer
- v. Spectrophotometer (models that measure very small liquid volumes, like 1 or 2 μL , are preferred)
- vi. Horizontal Agarose gel electrophoresis assembly
- vii. Homogenizer



Fig. 1. Typical laboratory setup for the isolation of genomic DNA.

C. Chemicals and reagents (most chemicals should be better than ‘laboratory’ grade)

- i. Buffer A- consists of 100 mM Tris-HCl (pH 7.5), 100 mM EDTA, 100 mM NaCl and 0.5% SDS. For preparing the buffer, following stock solutions are first prepared (the actual quantity of stock solutions to be prepared can be scaled down or up, as per requirement):
 - 1 M Tris-HCl, pH 7.5: Dissolve 121.1 gm of Tris base in 800 mL of milliQ or deionized water on a stirrer; when fully dissolved, adjust the pH to 7.5 using

Hydrochloric acid (HCl). After adjusting the pH make up the volume to 1 L with milliQ/deionised water and autoclave.

- 0.5 M EDTA: Dissolve 18.61 gm of disodium EDTA. 2H₂O in 80 mL of milliQ water on a stirrer. Adjust the pH to 8 using Sodium hydroxide (NaOH) (EDTA only dissolves at a pH of around 8), make up to 100 mL and autoclave.
- 5 M NaCl: Dissolve 292 gm of NaCl in 800 mL of milliQ/de-ionized water on a stirrer. When dissolved, adjust the volume to 1 L and autoclave.
- 10% SDS: Dissolve 10 gm of SDS in 80 mL autoclaved water and keep on a stirrer. Finally adjust the volume to 100 mL and filter sterilize.

The Buffer A is prepared by mixing 5 mL of 1M Tris-HCl (pH 7.5), 10 mL of 0.5 M EDTA, 1 mL of 5 M NaCl and 2.5 mL of 10% SDS. Make up the final volume to 50 mL and filter sterilize.

ii. Lithium Chloride/Potassium Acetate solution - This solution consists of -1 part 5 M potassium acetate stock and 2.5 parts 6 M lithium chloride stock. Prepare the stock solutions as follows-

- 6 M LiCl: Dissolve 12.717 g of LiCl in 30 mL of autoclaved water. Adjust the final volume to 50 mL.
- 5 M Potassium Acetate: Dissolve 24.53 gm of potassium acetate in 30 mL of autoclaved water. Adjust the final volume to 50 mL. After preparing these two stock solutions, mix 1 part of KOAc (14 mL) and 2.5 parts of LiCl (35 mL) to a total volume of 49 mL.

Note: It is desirable to filter sterilize the solution by passing it through a filter (of pore size 0.22 µm) to remove impurities and microorganisms like bacteria. Filter sterilization is preferred over autoclaving since the latter may cause degradation/decomposition of important components as a result of high temperature.

iii. Isopropanol

iv. 70% ethanol: Prepare by mixing 35 mL of 100% ethanol and autoclaved water to a final volume of 50 mL.

v. Tris-EDTA buffer: This buffer consists of Tris HCl (pH 8) and EDTA at a final concentration of 10 mM and 1mM respectively.

Note: 1 M Tris HCl, pH 8 is prepared as described above for Buffer A but with the pH adjusted to 8; (0.5 M EDTA stock can also be used from the above preparation).

To make TE buffer, mix 1 mL of 1 M Tris HCl , pH 8 and 200 µL of 0.5 M EDTA to a final volume of 100 mL with de-ionized water and autoclave.

D. Glassware/Plastic ware

i. MicroPipettes

ii. MicroPipette tips

iii. Polypropylene pestles

iv. 1.5 mL microcentrifuge tubes (DNase-RNase free)

- v. Microcentrifuge tube stand
- vi. Falcon tubes (50 mL)
- vii. Glass bottles (1 L)

E. Other supplies

- i. Disposable gloves
- ii. Ice bucket
- iii. Lab coat
- iv. Marker

Experimental Details:

This quick method for genomic DNA isolation from fruit flies will take only around 2 hr for completion (see **Fig. 2**).

1. Anesthetize 10 adult flies on a fly pad, transfer to 1.5 mL microcentrifuge tube and immediately homogenize in Buffer A. Alternatively, the flies can be frozen in liquid nitrogen or at $-70^{\circ}/80^{\circ}\text{C}$ for preparation of genomic DNA at a later time.
2. When ready to start with the DNA isolation, transfer these tubes to an ice bucket. Add 70 μL of Buffer A and grind the flies using polypropylene pestle. Add an additional 70 μL of Buffer A and grind till only the fly cuticles are left.
3. Incubate at 65°C on a heating block/incubator/water bath for 30 min.
4. Add 300 μL of LiCl/KOAc solution and incubate the samples on ice for 10-15 min.
5. Spin the samples at 12000 rpm for 15 min at room temperature.
6. At this stage the samples will have a white fluffy pellet and clear supernatant. Transfer 440 μL of the supernatant to a fresh microfuge tube. Make sure no floating piece is transferred; if it does, re-spin and transfer again.
7. Add 200 μL of isopropanol to the supernatant, mix by inverting, and spin at 12000 rpm for 15 min (room temperature). This will precipitate the genomic DNA and a white pellet will appear.
8. Aspirate the supernatant and wash the pellet with 350 μL of 70 % ethanol. Air dry the pellet and resuspend in 30-50 μL of TE buffer.
Note: In case the genomic DNA is to be utilized for PCR, it can be dissolved in 30-50 μL of 0.1 X TE. Reducing EDTA concentration would minimize chelation of Magnesium ions that are required for optimum performance of the Taq DNA Polymerase and thus help in higher yields of the PCR product.
9. The integrity and purity of the re-suspended genomic DNA can be verified by running a small aliquot of the sample (described in the section below on Quantification of Genomic DNA) on a 1% agarose gel. In case, RNA contamination is detected, the sample should be treated with RNase A (described in the Troubleshooting section).

10. Check the concentration and purity of the DNA samples on a sensitive spectrophotometer. The DNA sample may need to be appropriately diluted before measurement or running on gel, with the dilution factor having been recorded.

Note: As an alternative, the DNA concentration in the sample can also be visually assessed by comparing the Ethidium bromide fluorescence of the test sample by spotting its known volume on agarose gel or a small piece of parafilm with that of serial dilutions of known concentration of lambda DNA as a standard. This would provide only an approximation.

11. Keep the DNA samples at -20°C for longer storage.

Important precautions:

The following points should always be kept in mind during the genomic DNA isolation:

1. Genomic DNA is a long molecule and extremely susceptible to shearing forces. Avoid vortexing or vigorous pipetting at any step of the experiment to prevent shearing of genomic DNA molecules.
2. Carry out all the centrifugation steps at room temperature for this experiment.
3. Always record all observations and readings in the lab notebook.

Troubleshooting:

1. RNA contamination in the samples: Genomic DNA samples are often contaminated with RNA that precipitates along with the DNA. To remove the RNA, these samples can be treated with DNase-free RNase A to selectively digest RNA.

2. Low 260/280 and 260/230 ratio: If these ratios are less than 1.8 and 2 respectively it usually indicates contamination with proteins and salts. To improve these ratios, avoid any floating debris while transferring the supernatant after LiCl/KOAc precipitation step and ensure proper washing of the pellet with 70% ethanol.

3. Shearing and degradation of DNA: If the DNA samples look smeary on an agarose gel, it most likely indicates mechanical shearing or degradation by DNases. Avoid any vortexing or vigorous pipetting during the isolation. Also wear gloves and keep everything clean to keep DNases at bay.

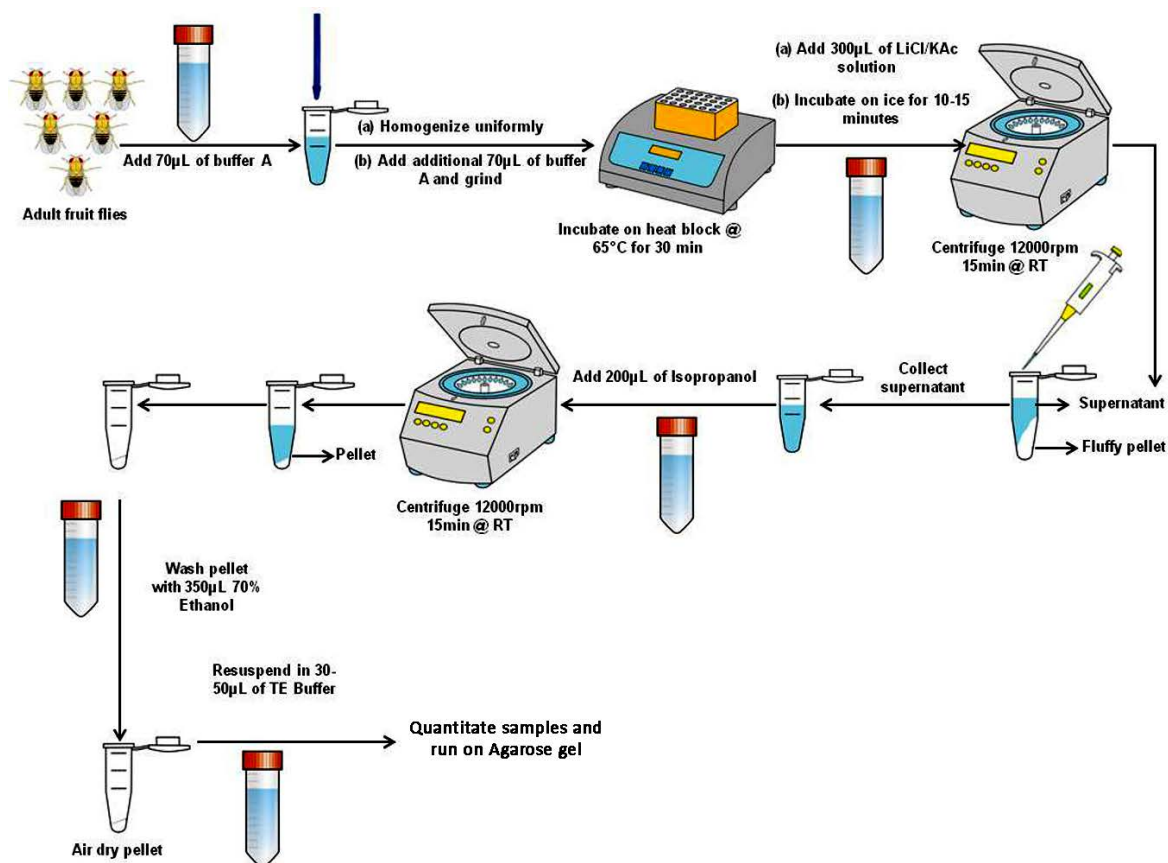


Fig 2. Schematic representation of the steps involved in isolation of genomic DNA from *Drosophila*.

Results/Observations:

After the isolation is complete, genomic DNA samples must be quantitated and data should be recorded as follows:

Quantification of genomic DNA by Spectrophotometry and quality check by agarose gel electrophoresis:

Once the genomic DNA is resuspended in TE buffer it can be quantified using UV spectrophotometry (see **Chapters 64, 65** for the principles underlying UV-visible spectrophotometry). Record the spectrophotometric data as below:

S. No.	Name of the sample	Dilution of the sample for reading	Absorbance at (nm)			260/280 absorbance ratio	260/230 absorbance ratio	Concentration ($\mu\text{g}/\mu\text{L}$)* of DNA	Name of the sample
			230	260	280				

Note: Spectrophotometry provides information about the quantity and purity of a given sample but not about the integrity and intactness of genomic DNA. For this the genomic DNA samples are usually resolved on an agarose gel and are visualized, after staining with Ethidium bromide (or other DNA fluorescent stains) under UV-light on a trans-illuminator. Agarose gel electrophoresis resolves DNA molecules depending on their molecular weight. Since DNA molecules are negatively charged they migrate from the

cathode (negative pole) towards the anode (positive pole). The bigger molecules migrate slowly while the smaller ones migrate fast leading to separation on the agarose gel. A lane with DNA fragments of defined sizes (DNA molecular size markers) is run in parallel (**Fig. 3**).

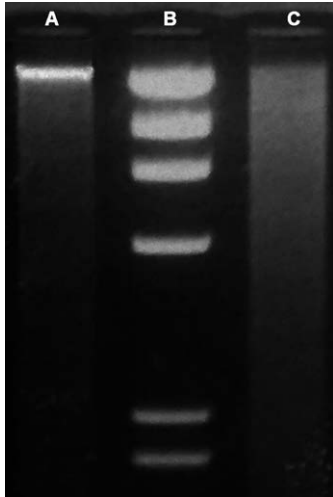


Fig. 3. Ethidium bromide stained *Drosophila* Genomic DNA samples resolved on 1% Agarose gel. Lane A shows good quality high molecular weight single DNA band close to the well; lane B is DNA molecular size markers (Lambda DNA cut with HindIII restriction endonuclease) while the lane C shows sheared genomic DNA showing a smear along the lane (Gel image contributed by Prof. S. C. Lakhotia).

If the genomic DNA is of good quality, the DNA will appear as sharp and clear single band, close to the well (see **Fig. 3**, lane A). Degraded DNA (**Fig. 3**, lane C) usually appears as a smear across the length of lane (due to random fragmentation of originally intact long chromosomal DNA molecules). The samples can also have RNA contamination, which will usually appear at the bottom of the gel. RNase A treatment can remove the contaminating RNA.

Questions for self-study and interpretation:

1. Would RNA contamination in the isolated genomic DNA samples be a cause of concern?
2. Why do we need to run genomic DNA samples on an agarose gel if we already have the spectrophotometric readings about quantity and quality of the isolated DNA?
3. The freshly isolated genomic DNA was dissolved in TE buffer. However, when this DNA was used for PCR, the reaction failed, while a positive control with a different DNA sample prepared in same manner, but re-suspended in autoclaved and de-ionised water, worked fine. What could be possible explanation for these varying results?
4. What could be possible uses of genomic DNA isolated from *Drosophila*?
5. One step in the experiment involves heating the samples to 65°C for 30 min. What could be the underlying principle for this step. Would it not lead to DNA degradation?

References and further reading:

Huang, A. M., Rehm, E. J. and Rubin, G. M. (2009) Quick preparation of genomic DNA from *Drosophila*. Cold Spring Harbor Protocols, 2009 (4): pdb.prot5198. doi: 10.1101/pdb.prot5198.

Muller's Prediction of Molecular Biology in 1922

D'Herelle found in 1917 that the presence of dysentery bacilli in the body caused the production there of a filterable substance, emitted in the stools, which had a lethal and in fact dissolving action on the corresponding type of bacteria, if a drop of it were applied to a colony of the bacteria that were under cultivation. That two distinct kinds of substances - the d'Herelle substances and the genes - should both possess this most remarkable property of heritable variation or "mutability," each working by a totally different mechanism, is quite conceivable, considering the complexity of protoplasm, yet it would seem a curious coincidence indeed. It would open up the possibility of two totally different kinds of life, working by different mechanisms. If these d'Hérelle bodies were really genes, fundamentally like our chromosome genes, they would give us an utterly new angle from which to attack the gene problem. They are filterable, to some extent isolable, can be handled in test-tubes, and their properties, as shown by their effects on the bacteria, can then be studied after treatment. It would be very rash to call these bodies genes, and yet at present we must confess that there is no distinction known between the genes and them. Hence we cannot categorically deny that perhaps we may be able to grind genes in a mortar and cook them in a beaker after all. Must we geneticists become bacteriologists, physiological chemists and physicists, simultaneously with being zoologists and botanists? Let us hope so.

(In: Muller, H. J. 1922. Variation due to change in the individual gene, *The American Naturalist*. 56: 32-50 <https://www.journals.uchicago.edu/doi/pdf/10.1086/279846>)

Chapter 61

Extraction of Genomic DNA from single *Drosophila* fly

Limamanen Phom (lim_angh@yahoo.co.in), Mohamad Ayajuddin (md.ayaj@gmail.com), Bovito Achumi (bovitoachumi@ymail.com), Imsusosang Longkumer (sosongl2@gmail.com), Zevelou Koza (zevi7koza@gmail.com), Priyanka Modi (pmodi82@gmail.com) & Sarat Chandra Yeniseti* (yschandrays@rediffmail.co)

1. *Drosophila* Neurobiology laboratory, Department of Zoology, Nagaland University (Central), Lumami-798627, Nagaland, India

2. Present affiliation National Research Center on Mithun (ICAR-NRCM), Dimapur, Nagaland, India

* For correspondence

Recommended Level: PG/Research

Sub-discipline: Molecular Biology

Background:

The extraction of genomic deoxyribonucleic acid (DNA) is the process by which DNA is isolated from other macro molecules and components of the cell. DNA extraction is an important part of modern molecular biology works since it is used for a variety of downstream experimental procedures like gene cloning, Southern hybridization, polymerase chain reaction (PCR), sequencing, editing, etc. The quality and integrity of the DNA obtained directly affects the reliability of all subsequent experiments (Cseke *et al.*, 2011). Different organisms and tissue types present unique challenges for extracting DNA. The extraction of DNA generally follows three basic steps: (a) lysis of cells, (b) separation of DNA from the other cell components and (c) isolation of pure DNA.

Extraction of pure DNA from a single fly involves some differences from the process followed for extraction of genomic DNA from many pooled flies (see [Chapter 60](#)) DNA from single fly is of primary importance for studying genotype diversity (DNA barcoding) at individual level (Cseke *et al.*, 2011). DNA from single fly is also used for identifying the presence of a certain allele or transgene in a given parent or progenys (also see [Chapters 62, 63](#)). It is also used in molecular phylogenetics in order to study the evolutionary relationships between individuals, populations, species, or higher taxa, using a combination of molecular data and statistical techniques (Fitch and Margoliash, 1967). This following optimized method has been used for DNA barcoding and confirming the status of a new *Drosophila* species, *Drosophila hegdii* from Nagaland, India (Achumi and Yeniseti, 2020).

Objective: Extraction of DNA from single fly.

Materials Required:

1. Equipment:

- i. Refrigerated centrifuge (4°C)
- ii. Spectrophotometer (for small volumes)
- iii. Dry block
- iv. Microcentrifuge tubes
- v. Homogenizer/crusher
- vi. Micropipettes
- vii. Heating block or incubator at 65°C

2. Chemicals:

- i. 8 M potassium acetate
- ii. 100% ethanol
- iii. 70% ethanol
- iv. Extraction buffer: 5% sucrose; 80 mM NaCl; 100 mM Tris, pH 8.5
- v. 0.5% Sodium dodecyl sulfate (SDS)
- vi. 50 mM EDTA

3. Other items:

- i. Ice
- ii. 1.5 mL microcentrifuge tubes
- iii. Micropipette tips
- iv. Filter and/or tissue paper

Experimental Details:

1. Procedure:

- i. Gently anesthetize flies on ice and transfer a single *Drosophila* to a 1.5 mL microcentrifuge tube using a brush.
- ii. Homogenize in 100 μ L of extraction buffer.
- iii. Rinse the crusher gently with additional 100 μ L of extraction buffer in order not to miss any part of the crushed fly.
- iv. Mix the contents thoroughly by swiftly tapping the bottom of the microcentrifuge tube and chill the contents by keeping on ice for 5 min.
- v. Incubate the tube at 65°C in incubator or heating block for 45 min to ensure complete lysis of cells and to degrade proteins and lipids.
- vi. Chill on ice for 5 min.
- vii. Add 30 μ L of 8 M potassium acetate and mix gently. Place the tube on ice for 45 min to precipitate the proteins and lipids.
- viii. Centrifuge at 10000 rpm at 4°C for 15 min to pellet out the precipitates.
- ix. Transfer the supernatant to a fresh microcentrifuge tube.
- x. Add 500 μ L of 100% chilled ethanol and mix gently and thoroughly which will enhance the level of DNA precipitation and yield.

- xi. Keep the tubes for 4-5 hr at room temperature or at 4°C for overnight to ensure maximum DNA precipitation.
- xii. Centrifuge at 10000 rpm at 4°C for 15 min to pellet the DNA: for a single fly, one may not see the pellet because of the small amount of genomic DNA.
- xiii. Wash the tube with 0.4 mL of 70% ethanol to remove salts that come from the buffer.
- xiv. Remove the ethanol as much as possible using a 10 or 20 µL micropipette and air dry the pellet by leaving the mouth of the tube open and gently placing the tube upside down on a clean filter/tissue paper for 1 hr. A translucent appearance of the microcentrifuge tube indicates that the precipitate is dried.
- xv. Re-suspend the precipitate in 15-20 µL of DNase free water. In order to ensure complete recovery of the isolated DNA, walls of the tube may be gently and patiently scrapped while re-suspending the DNA.
- xvi. Dissolve the precipitate at 55-60°C for 1-3 hr with intermittent gentle mixing. Finally, briefly spin the tube in microcentrifuge to bring a down the droplets from its walls.
- xvii. The sample is ready for further processes or store at 4°C for later use. For long term storage store either at -20°C or -70/-80°C.
- xviii. Quantify the DNA yield using UV spectrophotometer; its purity can be assessed by the ratio of OD at 260/280. Pure DNA should give a 260/280 ratio around 1.8-2.0

Results/Observations:

For a single fly, concentration of ~50 ng DNA/µL is considered a good yield.

The single fly genomic DNA has multiple applications.

Rationale and chemistry underlying the different steps in DNA isolation:

Cell lysis and DNA precipitation: Extraction of genomic DNA is based on the principle of lysis of the cell by a solution containing Tris, ethylene diamine tetra-acetic acid (EDTA) and sodium dodecyl sulphate (SDS). Tris buffers the DNA solution by interacting with lipopolysaccharide (LPS) in the membrane while EDTA binds to divalent cations such as calcium and magnesium to destabilize the membrane and also protects the DNA from degradation by DNAase. SDS, being a detergent, lyses the cells. Phenol-chloroform extracts lipids and proteins away from the DNA. DNA, being a polar molecule, attracts water molecules that suppress the binding of positively charged ions to the DNA. Ethanol disrupts the hydration shells of DNA and allows the unshielded phosphate residues to form ionic bonds with cations in the solvent. When the concentration of ethanol approaches 70% in the presence of Na⁺ ions, the repulsive forces between the polynucleotide chains are reduced to a point where the DNA precipitates. Ethanol precipitation can therefore only occur if cations are available in sufficient quantity to neutralize the charge on the exposed phosphate residues. The

most commonly used cations are ammonium acetate, lithium chloride, sodium chloride or sodium acetate. To suppress salt precipitates during extended periods of storage at low temperature, ammonium acetate rather than sodium acetate should be used as the counterion (Zeugin and Hartley, 1982).

Quantification & purity of isolated DNA: Getting good quality pure DNA from just one fly is important. Since different molecules in a sample have different light absorbability at a specific wavelengths, the quantity and purity of a sample can be assessed by absorbance in a spectrophotometer. Since nucleotides like RNA, ssDNA, and dsDNA have maximal absorbance at 260 nm, they together contribute to the total absorbance of the sample at 260 nm. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA samples. A DNA sample giving 260 nm/ 280 nm absorbance ratio between ~1.8-2.0 is generally considered as “pure”. The 260/280 ratio indicates level of protein contamination in DNA sample. If the ratio is appreciably lower than 1.8, it indicates the presence of protein or other contaminants that absorb strongly at or near 280 nm. Another secondary measure of nucleic acid purity is 260/230 ratio. The 260/230 values for “pure” nucleic acid are often higher than the respective 260/280 values. Expected 260/230 values are commonly in the range of 2.0-2.2. If the ratio is appreciably lower than expected, it indicates the presence of contaminants like EDTA, carbohydrates and phenol, which absorb at 230 nm.

Questions for self-study and interpretation:

1. Compared to isolation of genomic DNA from a pool of flies, what are the limitations in extracting DNA from single fly?
2. How can the genomic DNA isolated from a single fly be used for genotyping/DNA barcoding?
3. What are the possible experimental uses of DNA isolated from single fly?

References and further reading:

1. Achumi, B. and Yeniseti, S. C. (2020) *Drosophila hegdii* (Diptera: Drosophilidae), a new species from Lumami, Nagaland (a sub-Himalayan hilly state, India) Its molecular phylogeny. Journal Entomological Research. 44: 15-22.
2. Cseke, L. J., Kirakosyan, A., Kaufman, P. B. and Westfall, M. V. (2011) Handbook of molecular and cellular methods in biology and medicine, Eds. (3rd ed.), CRC Press pp. 3–28.
3. Fitch, W. M. and Margoliash, E. (1967) Construction of phylogenetic trees. Science. 155: 279-284.
4. Zeugin, J. A. and Hartley, J. L. (1982) DNA precipitation in the presence of ammonium acetate. Focus. 4: 12.

Chapter 62

Rapid extraction of genomic DNA from a single fly

Anjali Muraleedharan (anjali.kny@gmail.com), Vanshika Kaushik (vanshikakaushik0@gmail.com) and S. C. Lakhotia* (lakhotia@bhu.ac.in)

Cytogenetics Laboratory, Department of Zoology, Institute of Science, Banaras Hindu University, Varanasi 221005

* For correspondence

Recommended level: UG/PG/Research

Sub Discipline: Molecular Biology

Background:

Extraction of genomic DNA is a common point for beginning of many molecular genetic studies. Depending upon the quality and quantity of the genomic DNA required for specific studies, several methods have been developed (see **Chapters 60, 61**). Several genetic studies require DNA from a single fly for its genotyping through PCR amplification of specific regions. The procedure described here is a modification of the method developed by Gloor & Engles (1992). Unlike the method for extraction of pure DNA from single fly described in **Chapter 60**, the present method for isolation of crude genomic DNA takes less than an hour since this procedure does not involve a DNA precipitation step. Although the DNA obtained through this rapid method may not be of very high purity, it can be used for routine PCR and enzyme digestion studies.

The procedure involves homogenizing the fly in a solution of Tris-HCl (pH 8.2), EDTA, and NaCl, where Tris-HCl maintains the pH, EDTA chelates divalent ions like Mg^{2+} , which leads to inactivation of cellular DNAses, while NaCl dissociates proteins bound to DNA in the chromatin. The pH 8 is optimum for DNA stability, solubilization of EDTA, and for promoting proteinase K activity (<https://geniticeducation.co.in/importance-of-tris-edta-te-buffer-in-dna-extraction/>). Proteinase K is added after homogenization of the fly for degradation of proteins and enzymes. Following the separation of DNA molecules from proteins, proteinase K is inactivated by brief boiling or heating at 95°C. The liberated DNA can be quantified spectrophotometrically (the quantification is approximate because of the presence of some RNA and nucleotides), can be examined through gel electrophoresis, and can be used directly for PCR or other analysis.

Objective: To obtain genomic DNA from a single fly in less than an hour.

Materials Required

- A. Flies:** The desired single male or virgin female fly.
- B. Equipment and other supplies**

- i. Stereo binocular microscope and other fly-handling materials.
- ii. Glass homogenizer to fit the 1.5 mL microfuge tubes (can also be fabricated, Fig. 1A).
Note: if a glass homogenizer pestle is not available, a regular micropipette tip (200 μ L) can also be used for crushing/squishing the fly in a 1.5 mL microfuge tube).
- iii. Water baths: one set at 37°C, and another at 95°C.
- iv. UV-spectrophotometer that can measure micro-volumes (optional).
- v. Microcentrifuge or spinner.
- vi. Microcentrifuge tubes and stand.
- vii. Micropipettes and corresponding tips.
- viii. Horizontal Gel electrophoresis set up with power supply.
- ix. Thermocol/Foam floaters with holes for MCT and PCR tubes – prepared by pushing the required tube through small pieces of 1 cm thick Thermocol/Foam sheets.
- x. Disposable gloves.
- xi. Ice for keeping the tube with extracted homogenate.
- xii. Marker pen.

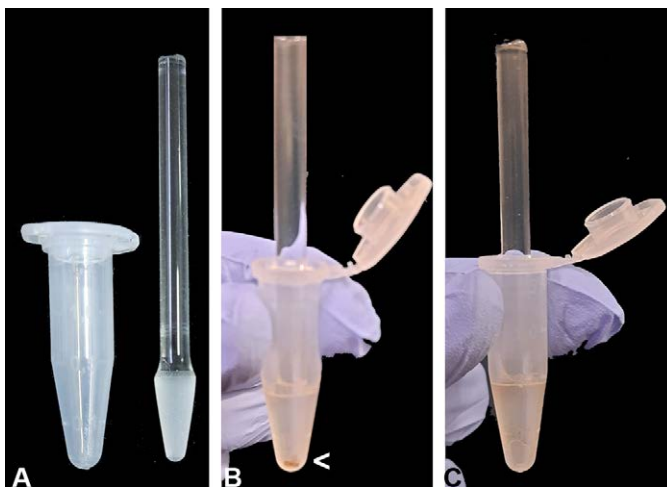


Fig. 1. A. 1.5 mL microfuge tube and glass homogenizer. B - C. Single fly (white arrowhead) in the tube with squishing buffer and homogenizer, before (B) and after homogenization (C).

C. Reagents

1. Stock solutions

- i. 1 M Tris-HCl, pH 8.2: Dissolve 12.11 gm of Tris base in 80 mL of deionized or distilled water on a stirrer; when fully dissolved, adjust the pH to 8 using 1N-HCl. After adjusting the pH make up the volume to 100 mL with deionised/distilled water and autoclave.
- ii. 0.5 M EDTA: Dissolve 1.861 gm of disodium EDTA. 2H₂O in 8 mL of deionised/distilled water on a stirrer. Adjust the pH to 8 using 1N-NaOH (EDTA only dissolves at a pH of around 8), make up the volume to 10 mL, and autoclave.

- iii. 5 M NaCl: Dissolve 29.2 gm of NaCl in 80 mL of deionized/distilled water on a stirrer. When dissolved, add distilled water to make the volume to 100 mL. Autoclave the solution and store at 4°C.

Note: Stock solutions can be stored for 2-3 months at room temperature.

2. Squishing buffer (working solution): Prepare 10 ml of squishing buffer from the above stock solutions (the working solution can be stored at 4°C for 2-3 months):

Stock solution	Quantity added	Final concentration
i. 1M Tris-Cl	0.1 mL	10mM
ii. 0.5M EDTA	0.02 mL	1mM
iii. 5M NaCl	0.05 mL	25mM
iv. Distilled water	9.83 mL	

3. Proteinase K 10mg/mL in distilled water (stock solution).

Experimental Details

1. Collect a male or virgin female fly of the desired genotype and anesthetize it.
2. Transfer the fly to a microcentrifuge tube in 30 μ L of squishing buffer and homogenize the fly using the glass homogenizer (Fig. 1). Quick spin the tube in a microcentrifuge or spinner to bring down the homogenate adhering to wall.
3. Add 1 μ L Proteinase K (10 μ g) to the homogenate and make up the volume to 50 μ L by adding 19 μ L of squishing buffer.
4. Incubate the mixture at 37°C for 30 min.
5. Heat the homogenate at 95°C in a water bath for 2 min to inactivate Proteinase K.

Note: Steps 4 and 5 can also be carried out on the PCR machine following programming for the temperatures and durations.

1. Use 1 μ L of the homogenate for estimating DNA concentration (see Chapter 60) using a UV-visible spectrophotometer that can measure μ L volumes (optional).
2. Check the quality of DNA through agarose gel electrophoresis (Fig. 2A) following the standard method (see Chapters 59, 60, 111) or use the homogenate directly for amplification of desired genomic sequence through PCR (Fig. 2B) using the specific primers and annealing temperature (see Chapters 37).

Note: The extracted DNA can be stored at 4°C for up to 1-2 weeks (see Oosting *et al.*, 2020).

Precaution: Avoid vigorous pipetting to prevent shearing of genomic DNA.

Observations:

Record the quantity of the extracted genomic DNA spectrophotometrically and check its quality through agarose gel electrophoresis.

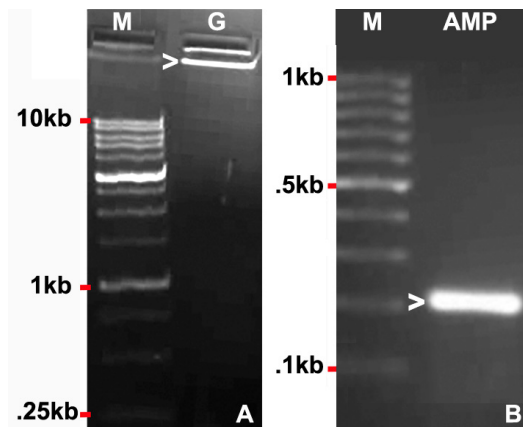


Fig. 2. Ethidium bromide stained agarose gels to show the high molecular weight DNA (**A**) and PCR amplicon (**B**) indicated by > marks. M indicates the DNA size marker lane (DNA sizes in kilobase pairs (kb) mentioned on the left of each gel).

Questions for self-study

1. Why do we need to inactivate Proteinase K before proceeding for PCR?
2. Why is the DNA isolated by this method not pure as obtained with the standard methods described in **Chapters 60 and 61**?
3. Can the extracted DNA be stored at 4°C for long term, why or why not?
4. If taking a female fly for DNA extraction, why only virgin fly should be taken?

5. **References and further readings:**

Gloor, G., and Engels, W. R. (1992) Single-fly DNA preps for PCR. *Dros Info.Ser* 71: 148-149. (also see http://ftp.flybase.net/flybase/associated_files/DIN_compilation.htm#dinvoll)

Oosting, T., Hilario, E., Wellenreuther, M., and Ritchie, P. A. (2020). DNA degradation in fish: Practical solutions and guidelines to improve DNA preservation for genomic research. *Ecology Evolution*, 10: 8643-8651. <https://doi.org/10.1002/ece3.6558>.

Chapter 63

Single step DNA genotyping of live flies

Anjali Muraleedharan (anjali.kny@gmail.com), Sanchari Gorai (sancharigorai1997@gmail.com) and S. C. Lakhotia* (lakhotia@bhu.ac.in)

Cytogenetics Laboratory, Department of Zoology, Institute of Science, Banaras Hindu University, Varanasi 221005

* For correspondence

Recommended level: PG/ Research

Sub Discipline: Developmental Biology/Molecular Biology

Background

Determination of specific genotype of an organism is essential in many genetic studies. Advances in DNA technologies permit genotyping at single base level. Consequently, compared to the conventional genetic approach to determine the genotype through phenotypic effects of mutant gene/s, it is now possible to determine the DNA genotype through either DNA sequencing, DNA fingerprinting, or through PCR amplification of specific DNA segments in the genome using appropriate primers. The usual process of DNA isolation, especially from smaller organisms like *Drosophila*, is associated with death of the organism which precludes any further live study on that particular individual (see Chapter 61). In this context, a method of isolating DNA for determining the presence or absence of a given allele/transgene from an individual without causing its death has many advantages in genetic studies, especially under conditions where identification of progeny carrying the desired genotype in which two or more mutants/transgenes that do not show a visible phenotype have been recombined. In conventional genetic approach, mutant alleles/transgenes located on a given chromosome can be brought together on the same chromosome only by recombination. However, since homologous recombination is a stochastic process, the conventional method for identification of recombinant progeny involves setting up a large number of pair-mating fly crosses and characterizing the progeny of each cross for presence of the desired recombined mutants/transgenes (see Chapter 11). DNA genotyping of live fly helps in pre-selecting the parent, which greatly reduces the strenuous effort and time otherwise required to screen a large number of pair-mating crosses for selecting the progeny carrying the desired DNA genotype.

This chapter describes a simple and reliable non-lethal method, developed originally by Carvalho et al (2009), for extracting DNA of a live fly for PCR-based DNA genotyping (see Chapter 118). Body part of the live male fly, like wings or 2nd pair of legs, whose removal does not affect the mating behaviour, is used for extracting DNA for downstream processes like PCR to determine the presence of specific DNA segment in the male fly before using it as one of the parents. This step provides information about the presence of the mutant alleles/transgenes of the desired type at least one generation earlier than classical method.

Objective:

To extract DNA from body parts for genotyping a live fly by PCR.

Materials Required

A. Flies: Live male fly whose DNA is required to be analyzed.

Note: Male fly is preferred firstly because the absence of homologous recombination in male *Drosophila melanogaster* ensures that all the sperms carry the same genetic combination, and secondly, because a single male can also be used to inseminate different females. In principle, however, this method can be used to genotype a live female's legs or wings as well.

B. Equipment and other supplies:

- i. Stereozoom-binocular microscope and other fly handling materials.
- ii. Thermal cycler.
- iii. UV-spectrophotometer with microcuvettes (optional).
- iv. Horizontal gel electrophoresis set up with power supply.
- v. 0.2 mL PCR tubes.
- vi. Micropipettes (20-200 μL , 2-20 μL , 0.5-10 μL) and tips.
- vii. Fine needles.
- viii. Forceps.
- ix. Surgical blade.
- x. Tissue paper.
- xi. Water bath set at 95°C.

C. Reagents

i. Squishing buffer (10mM Tris-Cl pH 8.2, 1mM EDTA, 25mM NaCl):

a. Stock solutions:

- 1M Tris-Cl (pH 8.2): Dissolve 12.11 gm Tris base in 80 mL distilled water. Adjust the pH with HCl and make up the volume to 100 mL with distilled water. Autoclave and store.
- 0.5M EDTA: Dissolve 1.861 gm in 8 mL autoclaved distilled water. Make up the volume to 10 mL with distilled water.
- 5M NaCl: Dissolve 29.2 gm NaCl in 80 mL distilled water. Make up the volume to 100 mL with distilled water and autoclave.

b. Working solution:

1M Tris-Cl	0.1 mL
0.5M EDTA	0.02 mL
5M NaCl	0.05 mL
Distilled water	9.83 mL

- ii. Proteinase K (20mg/mL): Dissolve 0.02gm of Proteinase K in 1 mL distilled water.
- iii. 70% Ethanol.
- iv. Diethyl ether.
- v. Distilled Water.
- vi. 1X TAE buffer:
 - a. **50X Stock solution:** Mix 24.2g tris base, 5.71 mL glacial acetic acid and 10 mL 0.5M EDTA. Dissolve it in 100mL of distilled water.
 - b. **1X working solution:** Dilute the 50X stock solution with distilled water as per requirement.
- vii. PCR reagents (10 μ L PCR reaction mixture):
 - 10X Taq buffer A: 1 μ L
 - 10mM dNTP mix: 0.1 μ L
 - Taq polymerase (5U/ μ L): 0.1 μ L (0.5 unit)
 - 5 μ M (5 pM/ μ L) primer sets (forward and reverse primers designed according to the gene of interest): Forward: 0.5 μ L; Reverse: 0.5 μ L.
 - Extracted DNA (as template): 6 μ L (~100 ng)
 - Make up the volume to 10 μ L with distilled water
- viii. PCR primers: Forward and reverse primers for amplifying the desired DNA region by PCR.
- ix. Ethidium Bromide: Dissolve 10 mg Ethidium bromide in 1 mL of distilled water completely. After dissolving, store it in an appropriate container and wrap the container with aluminium foil to prevent exposure of the fluorescent dye to light.

Note: Ethidium bromide is carcinogenic. Therefore, it needs to be handled with care. Solutions containing Ethidium bromide (<5mg/L) can be discarded in the sink with plenty of running water).
- x. Agarose (1% w/v Agarose) Gel in 1X TAE buffer: Mix 1g Agarose in 100 mL 1X TAE buffer and heat until the agarose powder is completely dissolved. Allow it to cool down to ~55 $^{\circ}$ C (it should not get solidified), add 10 μ L Ethidium bromide solution, mix well and pour into the horizontal gel cast, with combs in place. Let it solidify. Once solidified keep the gel in an appropriate size electrophoresis tank. Pour enough 1X TAE buffer to keep the gel fully submerged.

Note: The concentration of agarose in the gel and the specific buffer used to prepare and run the agarose gel can vary in relation to specific requirements.
- xi. 6X loading dye (0.125% Bromophenol blue, 0.125% Xylene Cyanol, 80% Glycerol): Dissolve 12.5 mg Bromophenol blue and 12.5 mg Xylene Cyanol in 7 ml of distilled water. Finally, add 3 mL glycerol and mix well.

D. Experimental Details

1. DNA Extraction
 - i. Anesthetize the desired fly.
 - ii. Cut wings or the second pair of legs using fine needles or surgical blade.
 - iii. Transfer wings or legs to 0.2 mL PCR tube containing 9.75 μ L of the squishing buffer and 0.25 μ L 20mg/mL Proteinase K.
 - iv. Incubate at 37^oC for 55 minutes.
 - v. Incubate again at 95^oC for 1.5 minutes to inactivate proteinase K.
2. Polymerase chain reaction and Agarose gel electrophoresis
 - i. Carry out PCR (take 6 μ L of the extracted DNA sample for a 10 μ L PCR reaction) using the desired primers.
 - ii. Following completion of PCR, mix 10 μ L of the PCR product with 2 μ L 6x Loading dye and load the sample and DNA size markers in separate wells of agarose gel, and run (typically at 100 Volts for small size gels) as described in [Chapters 59, 60, 111](#).
 - iii. After the gel run is complete, examine the gel under a UV-transilluminator to determine size/s of the amplicon/s.

Note: Keep a negative control, load the PCR sample without any DNA added to ensure absence of any extraneous DNA in the experimental sample. As a positive control for the expected amplicon size, extract DNA from whole fly of the desired genotype by the standard method (see [Chapters 60, 61](#)) and load, as above, in a parallel well in the gel.

Notes:

1. DNA may be extracted from single wing or leg of the fly, but pair of wings or legs gives better yield.
2. The body part must remain completely submerged in the squishing buffer during incubation.

Results and observations:

Examine if the PCR product/s is/are of the desired sizes. An example is shown in Fig. 1.

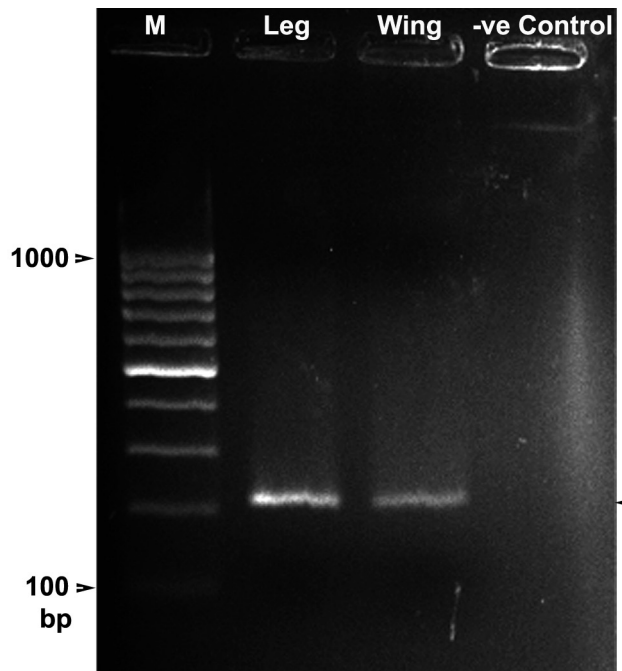


Fig 1. Agarose gel image of PCR products with G3PDH (a housekeeping gene) primers (Forward primer: 5'CCACTGCCGAGGAGGTCAACTA3'; Reverse primer: 5'GCTCAGGGTGATTGCGTATGCA3'; Annealing temp: 56⁰C); Amplicon size: 212 bps showing amplicons (arrowhead on right) generated with DNA extracted from a pair of second legs (lane 2) or wings (lane 3) of live fly; lane 4 is -ve Control without any DNA. Lane M shows 100 bp DNA ladder.

Questions for self-study and interpretation:

1. Why are the first pair of legs not used for extracting DNA from male flies?
2. Why is it necessary to inactivate Proteinase K?
3. What can be other applications of DNA genotyping of live organisms?

References and further readings:

Carvalho, G. B., Ja, W. W., & Benzer, S. (2009). Non-lethal PCR genotyping of single *Drosophila*. *BioTechniques*, 46: 312–314. <https://doi.org/10.2144/000113088>

Genomic Data & Conservation

Genomic data will become increasingly common for species of conservation and management concern. For the first time, these data will enable researchers to understand patterns of adaptive variation in rare and endangered species for which it is impossible to test for adaptation using traditional approaches such as reciprocal transplant experiments. As such, genomic data will play a crucial role in informing management and policy for species of conservation concern. Now is the time to consider carefully how best to take advantage of these data to delineate conservation units and characterize adaptive variation and then apply this information to improve conservation decision making.

(In: Funk, W. C. et al (2012). Harnessing genomics for delineating conservation units. *Trends in Ecology & Evolution*, 27: 489-496.
<https://doi.org/10.1016/j.tree.2012.05.012>).

Chapter 64

Total RNA isolation from *Drosophila* using TRIzol based reagent

Manish Pandey (manish.pandey@rcb.res.in) and Geetanjali Chawla* (gchawla@rcb.res.in)
Regional Centre for Biotechnology, Faridabad, Haryana.

* For correspondence

Recommended Level: Post-graduate/Research

Sub-discipline: Molecular Biology

Background:

RNA or ribonucleic acid is a polymer of ribonucleotides and is synthesized in the cell through transcription. In addition to playing a crucial role in protein synthesis, RNA molecules serve many critical regulatory functions. The extraction methods that are widely utilized for deoxyribonucleic acids (DNA) cannot be directly applied for isolation of RNA as the structure of these two nucleic acids varies considerably. RNA isolation is more demanding due to the abundance of Ribonucleases (RNAses-enzymes that degrade RNA) in the environment. These extremely stable enzymes are abundantly present on hands and work surfaces. Thus, cautious handling and the use of RNase-free solutions, pipet tips and glassware is critical for the isolation of intact RNA.

One of the most popular and well-established methods of total RNA isolation is the use of guanidium thiocyanate-phenol-chloroform extraction method. This method was originally developed by Dr. Chomczynski and colleagues in 1987 and has been successfully used to isolate a high yield of intact RNA in a short time from multiple sources (Chomczynski and Sacchi, 2006). Though the original method involved using the three principal components, namely, guanidium thiocyanate, phenol and chloroform. Separately, these are now combined in commercially available single reagent, often called TRIzol or TRI reagent (Simms *et al.*, 1993, Macedo *et al.*, 2014). This reagent can be used selectively for the extraction of DNA, RNA or proteins (hence the 'TRI' in its name), which primarily depends upon the pH of the solution. Chemically, TRIzol is a monophasic mixture of guanidium thiocyanate, phenol and chloroform with acidic pH. These chemicals maintain the integrity of RNA and cause phase separation resulting in selective isolation of RNA devoid of proteins and DNA.

Isolation of pure and high-quality RNA is a pre-requisite for many molecular applications such as cDNA synthesis and real time-PCR (**Chapter 65**), northern blotting, RNA sequencing, cDNA library construction and nuclease protection assays (Heidary and Kakhki, 2014).

Objectives:

To isolate total RNA from *Drosophila melanogaster* using TRIzol reagent and determine its concentration and purity using a spectrophotometer. The starting material for RNA extraction could be embryos, larvae, pupae or adult flies or dissected tissues from any stage.

Materials Required (Fig. 1):

A. Fruit fly (Wild type or of any other genotype as desired)

B. Equipment

- i. Bench-top centrifuge.
- ii. Vortex-mixer.
- iii. -70/-80°C freezer.
- iv. Autoclave (or Pressure Cooker).
- v. Homogenizer.
- vi. Spectrophotometer.

C. Chemicals and reagents

- i. TRIzol/RNA Iso/Tri reagent.
- ii. Chloroform.
- iii. Isopropanol.
- iv. Absolute ethanol.
- v. Glycogen [acts as a co-precipitant (Optional)].
- vi. RNase-free DNase I and DNase I Buffer.
- vii. Diethyl Pyrocarbonate (DEPC).
- viii. DEPC treated water: In order to make the water RNase-free, it is treated with Diethyl Pyrocarbonate (DEPC). To prepare 0.1% DEPC water, add 1 mL of DEPC to 1 litre water and keep on a stirrer for 12-16 hr followed by autoclaving.
- ix. 75% ethanol: To make 50 mL of 75% ethanol, take 37.5 mL of absolute (100%) ethanol and add DEPC treated water to make up the volume to 50 mL.
- x. Phenol-chloroform mix: Mix phenol and chloroform in 5:1 ratio (pH 4.3-4.7): Acidic pH of the solution is critical for selective partitioning of RNA in the aqueous phase.

xi. 3 M sodium acetate (pH 5.3): Dissolve 246.1 gm of sodium acetate in around 600 mL of DEPC water and adjust the pH to 5.3 using glacial acetic acid. Keep the solution overnight at room temperature and let it cool. Adjust the pH again to 5.3 and make up the volume to 1 L. Filter-sterilize the solution by passing it through a filter of 0.22 μm pore size to remove impurities and microorganisms. Since autoclaving this solution will cause degradation/decomposition of important components, it is sterilized through filtration. This solution is used to neutralize charge on the phosphodiester backbone of RNA which facilitates its precipitation by ethanol.

D. Glassware/Plasticware

- i. Pipettes.
- ii. Barrier tips (tips containing aerosol filters to prevent cross-contamination).
- iii. Polypropylene pestles.
- iv. Microcentrifuge tube stand.
- v. 1.5 mL microcentrifuge tubes (RNase-DNase free).
- vi. Falcon tubes (50 mL).
- vii. Glass bottles (1 L).

DI. Other supplies

- i. Disposable gloves.
- ii. Ice bucket.
- iii. Lab coat.
- iv. Marker Pen.

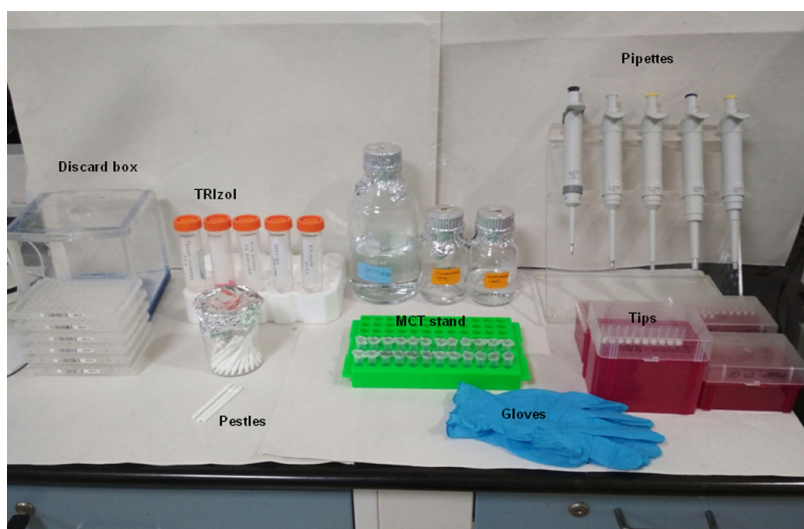


Fig 1. Typical set up for an RNA isolation working bench.

Experimental Details:

This experiment will require a maximum of two days for completion. Day 1 will involve lysis of cells/tissues, phase separation and precipitation, DNase I treatment and phenol chloroform extraction. At this stage, samples can be stored at -80°C or can be processed further (see **Fig. 2** for the flowchart of activities). In case they are stored, the second day will involve precipitation of RNA, washing and re-suspension.

The following **precautions** should always be taken during total RNA isolation.

1. Always wear gloves before starting the experiment and do not touch anything with bare hands. Also, clean all the surfaces, pipettes, microfuge stands with 70% ethanol. RNA is highly susceptible to degradation by RNAses and all these surfaces are full of them. RNAses are very abundant on the skin and in secretions like sweat/skin oils. These are quite stable enzymes and autoclaving is not sufficient to inhibit their activity. Upon cooling they can re-nature and hence degrade the RNA. Because common surfaces are full of RNAses, avoid touching them even while wearing gloves as the RNAses may adhere to the gloves and compromise the quality of RNA being handled with the gloved hands.
2. Handle Phenol carefully since it is corrosive. Phenol fumes are also toxic, more so to flies. Therefore, such solutions should never be handled in the room where live flies are maintained.
3. Label everything beforehand to prevent the mixing of samples and to avoid any confusion later.
4. TRIzol and phenol: chloroform mix are toxic and highly corrosive and thus should be handled very carefully.
5. Before starting, the bench top centrifuge should be set to 4°C .
6. Any organic waste left should be discarded according to proper biosafety guidelines.
7. RNA samples should always be kept on ice. For later usage, samples should be stored at $-70/ -80^{\circ}\text{C}$.

DAY 1:

1. Collect samples (5-10 whole larvae or adult fruit flies) in a 1.5 mL microcentrifuge tube.
2. Add 200 μL of TRIzol reagent to sample and homogenize with Polypropylene pestles.
3. Add 800 μL of TRIzol reagent and vigorously vortex the tube at room temperature for 10 min.
4. Add 200 μL of chloroform and briefly vortex the sample. Spin the sample for 15 min at 12,000 rpm at 4°C .
5. Carefully transfer the aqueous layer by pipetting to a new microcentrifuge tube and

add 500 μ L of isopropanol. Add 1.5 μ L Glycogen co-precipitant (optional) and mix by inverting the tube. Incubate the sample at room temperature for 15 min.

Note: Glycogen is a co-precipitant aids in the recovery of RNA from limited tissue samples by precipitating with the RNA).

6. Spin the samples for 15 min at 12,000 rpm at 4°C.

7. Remove the supernatant and wash the RNA pellet with 500 μ L of 70% ethanol. (**Optional:** The sample can be stored at -70/-80°C for overnight at this step).

8. Centrifuge the sample at 12,000 rpm for 5 min at 4°C. Remove the 75% ethanol by pipetting and air-dry the RNA pellet.

9. Re-suspend the dry pellet in 90 μ L of DEPC-treated water, and vortex for 10 min to dissolve the RNA. Set up DNase I digestion by adding 10 μ L of 10X DNase I buffer and 1 μ L DNase I enzyme. Incubate the sample at 37°C for 45 min.

10. For phenol: chloroform extraction, add 170 μ L of DEPC treated water and 30 μ L of 3 M sodium acetate (pH 5.3) and 250 μ L of phenol: chloroform (pH 4.3-4.7). Vortex and spin for 5 min at RT at 12,000 rpm. Carefully remove only the top aqueous layer into a new tube (RNase-free) and add 3 volumes (~900 μ L) of absolute ethanol. Incubate at -70 or -80°C for 2 hr or overnight.

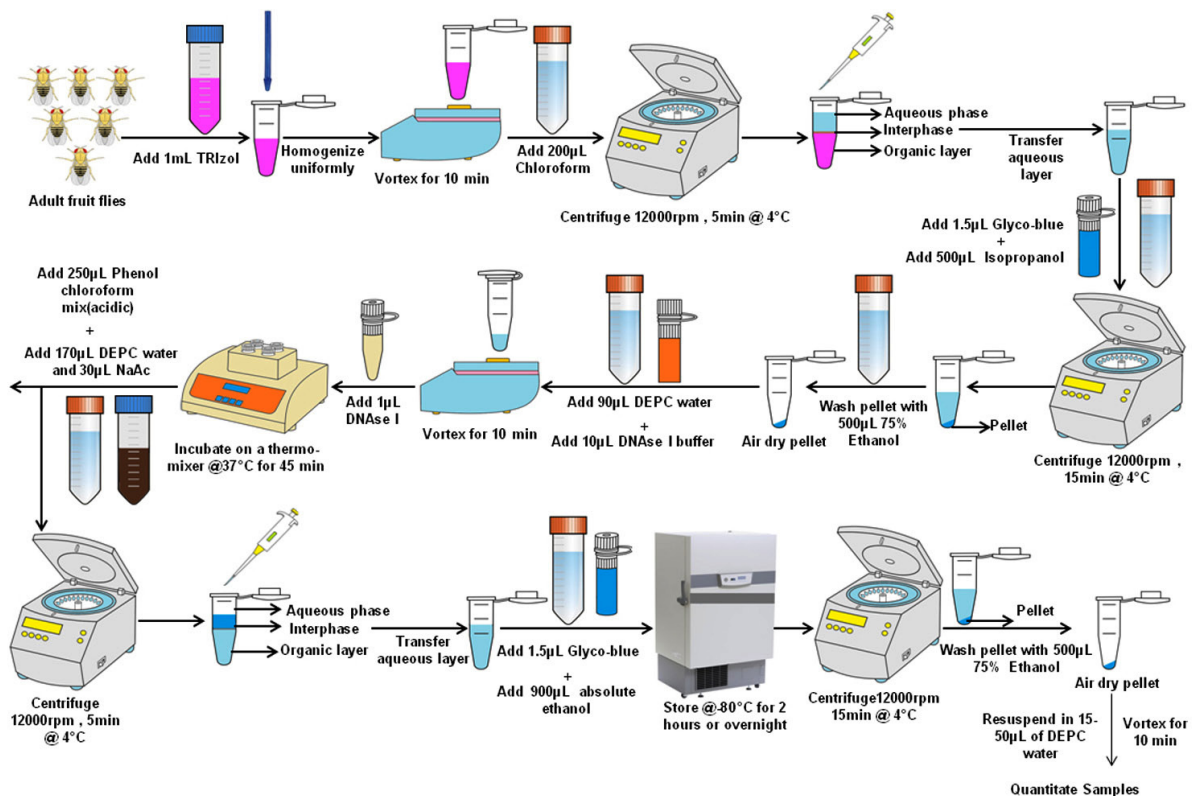


Fig 2. Schematic of steps involved in total RNA isolation from adult fruit flies.

DAY 2:

1. Centrifuge at 4°C for 15 min at 12,000 rpm. Remove supernatant and add 500 μ L of 75% ethanol to dissolve and remove any salt from the solution. Spin at 12,000 rpm for 5 min.

2. Remove ethanol and air-dry the pellet.
3. Resuspend the pellet in 15-50 μL (depending on the amount of starting material) of DEPC water.
4. Quantitate RNA by measuring absorbance at 230, 260 and 280 nm in a spectrophotometer.

Rationale and chemistry underlying the different steps in RNA isolation:

A. Cell lysis and phase separation:

1. The first step is the lysis of cells/tissues from which RNA is to be isolated. Homogenization of the desired tissues in TRIzol reagent lyses the cells while helping in inhibition of RNAses by the presence of guanidium thiocyanate. The guanidium salt, being a chaotropic agent disrupts the nucleoprotein complexes and denatures proteins, including RNAses. Phenol solubilizes proteins by flipping their hydrophobic residues outside so that the phenol soluble proteins end up in the interphase upon centrifugation. Chloroform helps in dissolving lipids.

2. The separation of RNA from DNA is primarily dependent on the pH and their structural differences. RNA isolation requires acidic pH (4-4.5). It is believed that phosphate diesters in DNA and RNA being negatively charged at neutral pH they remain soluble in aqueous phase. However, at a low pH (~ 4), most of the phosphate groups in DNA are neutralized with H^+ ions so that the uncharged DNA ends up in the interphase with denatured proteins. RNA being usually more polar due to the 2'-OH (hydroxyl) group on the ribose sugar mostly remains in the aqueous phase.

3. Phenol and chloroform are essential for the separation of the homogenates in three distinct phases. chloroform is highly miscible with phenol but because of its much higher density, pulls the phenol away from water in the samples upon centrifugation so that the aqueous and organic phases get separated with an interphase in between which contains the denatured proteins and DNA.

B. RNA precipitation:

1. The RNA in the aqueous phase is precipitated with isopropanol through the salting out principle. Isopropanol weakens interactions between water and RNA molecules because water molecules tend to interact more strongly with isopropanol than with RNA. Consequently, RNA precipitates out of the solution. At this stage, addition of glycogen aids the precipitation and improves the yield of total RNA, especially when the starting material is less. Washing of the precipitated RNA with 70-75% ethanol removes any salts, which may interfere with subsequent steps.

2. Even trace amounts of contaminating genomic DNA during aspiration of the RNA-enriched aqueous phase aspiration can lead to serious issues, especially in quantitative PCR reactions ([Chapter 65](#)). The contaminating genomic DNA is removed by using

RNAse-free DNase I enzyme. The re-extraction of pure RNA in acidic buffer saturated phenol: chloroform mixture removes the DNase I enzyme. Upon centrifugation, DNA and proteins move to the interphase while pure RNA remains in the aqueous phase.

C. Re-suspension and Quantification of RNA

1. RNA pellets are usually re-suspended in DEPC water or TE buffer. DEPC reacts with the catalytic site of RNAses and inhibits them irreversibly. The DEPC-treated water is RNAse-free. DEPC itself gets decomposed into ethanol and carbondioxide upon autoclaving.

2. After re-suspension, total RNA can be quantified using different methods, the most common being UV-visible spectrophotometry (Simms *et al.*, 1993). Some fluorescence-based methods are also used for quantification using fluorescent dyes like Ribo-Green. Spectrophotometry is based on the Beer-Lambert law which relates the concentration of a solute in the solvent to the amount of the specific wavelength of light absorbed by the solution.

3. The extinction co-efficients of DNA and RNA are constant at a particular path length and wavelength so that their concentration can be calculated if the absorbance is known. Both DNA and RNA strongly absorb UV-visible light at 260 nm and an absorbance of 1 at this wavelength has been conventionally related to the following concentrations:

Type of nucleic acid sample	Concentration of nucleic acid for an absorbance of 1 at 260 nm
dsDNA	50 µg/mL
ssDNA	33 µg/mL
RNA	40 µg/mL

Based on the above, **RNA concentration = 40 µg/mL x Absorbance at 260 nm x dilution factor** (if the original sample was diluted).

The conventional UV-spectrophotometers usually require a larger sample volume for measurement of absorbance. However, with small starting tissue material, the final RNA yield can be very small, which may be difficult to be measured. Spectrophotometers like NanoDrop spectrophotometer require very small sample volume (around 1 µL) and provide a direct readout of the concentration for a given sample. It also provides the absorbance for a sample at different wavelengths (230, 260 and 280 nm) which eventually are projected as the 260/280 and 260/230 ratios, which determine the purity of RNA samples. RNA absorbs UV- light most strongly at 260 nm but guanidium salt/phenol/ sugars absorb maximally at 230 nm and proteins at 280 nm. Thus, high 260/280 and 260/230 ratios indicate that the sample is largely RNA while low ratios indicate strong absorbance from contaminating salts and proteins. For pure RNA both these ratios tend to be around 2.

Spectrophotometry does not reveal anything about the integrity of RNA molecules or contaminating genomic DNA. This is assessed by agarose gel electrophoresis, which

separates nucleic acid molecules on the basis of their molecular size since shorter fragments migrate faster while longer or high molecular weight fragments migrate slowly in the electrical field. RNA (and DNA), being negatively charged, migrate from the cathode towards the anode in an agarose gel. Total RNA is a complex mixture of various mRNAs, tRNAs, rRNAs and other types of RNA molecules which get separated on the agarose gel on the basis of their size. Normally only the rRNA bands are visible the agarose gel because of their high molecular weights and very high abundance in cells. rRNA bands on the gel are visualized with the help of ethidium bromide (EtBr) which when bound to nucleic acids shows strong bright orange fluorescence on a UV trans-illuminator upon exposure to ultraviolet light (Fig. 3).

Another method of determining the integrity of RNA samples is the use of an instrument called the bioanalyzer. It requires very less volume of the RNA sample (around 1 μ L) and can provide the RNA Integrity number (RIN) depending on how intact the RNA is. A high RIN number indicates high quality RNA.

Results/Observations:

The isolated RNA should be quantitated spectrophotometrically and data tabulated as below:

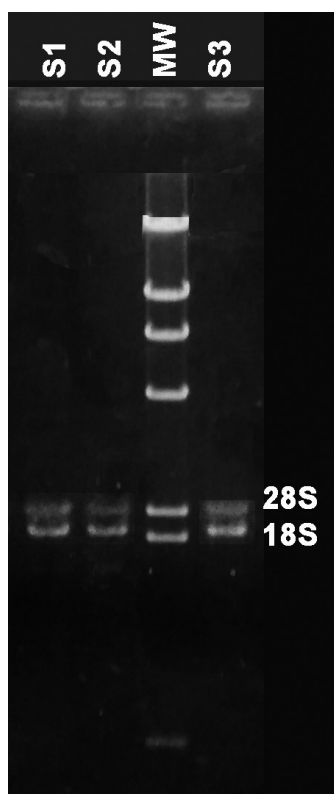


Fig. 3. Ethidium bromide stained total RNA from *Drosophila* larvae resolved on 1% Agarose gel. Lanes S1, S2 and S3 show good quality RNA as reflected by the distinct 28S and 18S rRNA bands. The MW lane shows the molecular size markers (Lambda DNA cut with Hind III restriction endonuclease) (Gel image contributed by Prof. S. C. Lakhotia).

S. No.	Name of the sample	Dilution of the sample for reading	Absorbance at (nm)			260/280 absorbance ratio	260/230 absorbance ratio	Concentration (μ g/ μ L)* of RNA	Total RNA (μ g) isolated
			230	260	280				

Problems encountered in RNA isolation and Troubleshooting:

Following table lists some of the most common problems encountered in total RNA isolation, their probable causes and possible solutions.

PROBLEMS ENCOUNTERED	DIAGNOSIS	PROBABLE CAUSE	TROUBLE SHOOTING
1. Genomic DNA contamination	1. High molecular weight smearing on agarose gel. 2. Amplification from negative RT-PCR controls.	1. Accidental aspiration of the interphase. 2. Some genomic DNA is always precipitated with RNA.	1. Always do a DNase I treatment to digest the DNA. 2. Remove the aqueous phase very carefully without aspirating the interphase.
2. Degradation of RNA or RNA with low integrity	The rRNA bands appear smeary on the gel.	1. RNase contamination. 2. Improper storage before or after isolation.	1. Always wear gloves and ensure that all solutions and surfaces are RNase-free. 2. Always keep the samples on ice and store only at $-70/-80^{\circ}\text{C}$. 3. Avoid repeated freezing and thawing of samples.
3. Inhibitors or contaminants present in the RNA	Very low 260/280 and 260/230 ratios.	Presence of guanidium salts phenol, proteins or other contaminants.	1. Give more washes with 75% ethanol and check for its quality. 2. Avoid using too much of the starting material.
4. Low yield of RNA	The concentration in $\text{ng}/\mu\text{L}$ is too low.	1. Less amount of starting material. 2. The starting material is enough but is not efficiently homogenized.	1. Start with enough material to get a reasonable yield. 2. Make sure the sample is completely homogenized.

Questions for self-study and interpretation:

1. What will happen if the pH of phenol-chloroform mix used for RNA isolation is near neutral?
2. Between ethanol and isopropanol, which should be preferred for RNA precipitation?
3. A sample of RNA was diluted 10 times. The diluted sample gave an absorbance of 0.54 at 260 nm. Calculate the concentration of RNA in the original sample?
4. Why a low 260/280 or 260/230 ratio for an RNA sample should be a cause of concern?

5. You isolated RNA from fruit flies and re-suspended it in 30 μL of DEPC water. You then diluted 10 μL of it in 100 μL of DEPC water. The following readings for absorbance were obtained at 230 nm, 260 nm and 280 nm: $A_{230}= 0.182$, $A_{260}= 0.450$, $A_{280}=0.220$.

- What is the RNA concentration of the 30 μL sample?
- How much total RNA (in μg) was isolated in the experiment?
- Comment on the purity of the RNA sample with reasons?

6. A solution of RNA has a concentration of 80 picomoles/ μL . If the absorbance of this solution at 260 nm is 0.48, find out the molar extinction coefficient in $\text{M}^{-1}\text{cm}^{-1}$ for a path length of 1 cm?

References and further reading:

Barbas, C. F., Burton, D. R., Scott, J. K., Silverman, G. J *et. al.*, (2007) Quantitation of DNA and RNA. Cold Spring Harbor Protocols (Cold Spring Harb Protoc). 1: 123-456.10.1101

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Macedo, N. J. and Ferreira, T. L. (2014). Maximizing total RNA yield from TRIzol® reagent protocol: A feasibility study. ASEE 2014 Zone I Conference.

Simms, D., Cizdziel, P. E. and Chomczynski, P. (1993). TRIzol: A new reagent for optimal single step isolation of RNA. Focus. 15: 532-535.

RNA isolation video based on the method described here

<https://www.youtube.com/watch?v=F251jkQJ4PA&feature=youtube>

Chapter 65

Quantitative real time PCR (qRT-PCR) for gene expression analysis

Abhik Das (dasabhik91@gmail.com), Rahul Chaurasia (Rahulneuro12@gmail.com),
Limamanen Phom (lim_angh@yahoo.co.in), Priyanka Modi (pmodi82@gmail.com) and
Sarat Chandra Yeniseti* (yschandrays@rediffmail.com)

*Drosophila Neurobiology Laboratory, Department of Zoology, Nagaland University
(Central), Lumami, 798627, Nagaland, India.*

*For correspondence

Recommended Level: PG/Research

Sub-discipline: Gene Expression/Molecular Biology

Background:

Alterations in gene-environment interactions leading to changes in biological pathways underlie molecular pathology of many human diseases. Although alterations in biological pathways are generally understood in terms of post-transcriptional changes, the variations in protein structure and state are mirrored in alterations in the transcriptional network (transcription rate, transcript stability and splice variation etc). Therefore, gene expression analyses are integrative approaches to understand cell differentiation, and function and the molecular pathology of a disease. Expression analysis can be carried out with qRT-PCR, which, unlike the conventional PCR (see [Chapter 118](#)), enables simultaneous detection and quantification of amplicons with each cycle in a real time manner and thus provides reliable quantitative data about the quantity of a given transcript in a sample. This is possible due to incorporation of a reporter dye conjugated nucleotide precursor, which when incorporated in the growing amplicon, produces fluorescence signal that is captured by detectors in the thermal cycler unit. The quantum of the fluorescence emitted during amplification is directly proportional to the amount of the reporter nucleotide incorporated, which in turn reflects the amount of amplified nucleic acid. A higher initial number of nucleic acid molecules in a sample will result in faster increment of fluorescence signal. At a certain amplification cycle, detectable and stable fluorescent signal above the baseline will generate a cycle threshold value or C_t value (discussed later). The obtained C_t value is utilized for absolute as well as relative quantification of the gene expression. For gene expression analysis using qRT-PCR, the isolated RNA is first converted to cDNA using reverse transcriptase, since DNA is more robust to withstand the extreme thermal changes during PCR cycles. Specific primers are then used to quantitatively amplify the desired cDNA and thus assess abundance of the target RNA in the isolated sample (see [Chapter 64](#)).

Principle: mRNAs are very unstable, fragile and present a risk of getting degraded by the omnipresent RNase and high temperature during the PCR step. A way to overcome this problem is to convert RNA into cDNA (complementary DNA) by reverse transcription, using reverse transcriptase (RNA dependent DNA polymerase), of all or the desired RNA

species in the total RNA isolate. The resultant DNA may be single or double stranded. Since most eukaryotic mRNAs possess a poly-A tail as a post-transcriptional modification, the reverse transcription is carried out using oligo (dT) primers for synthesis of the cDNA strand (**Fig. 1**).

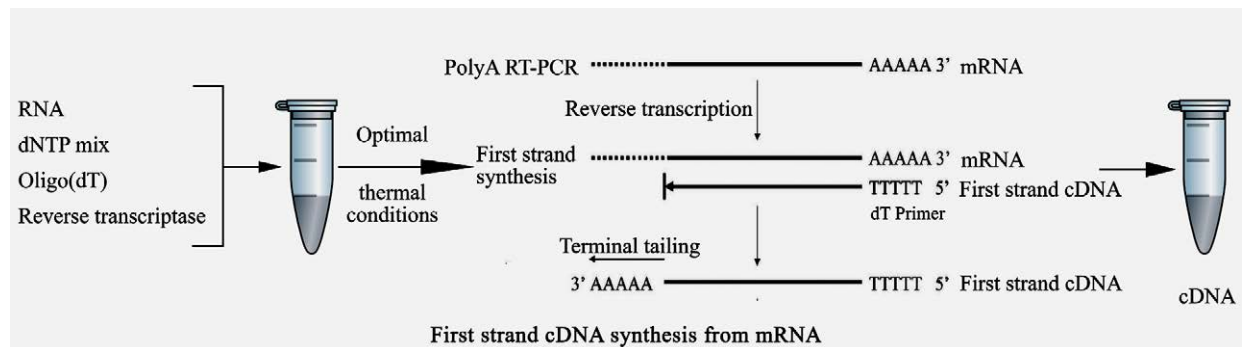


Fig. 1. A simplified workflow and polymerization dynamics of first strand cDNA using reverse transcriptase.

Much like a PCR based method, the reverse transcription generates cDNA strands from mRNA after thermal cycling through proper temperatures. All viral reverse transcriptase possess RNase H activity which recognizes RNA-DNA duplex and degrades the template RNA and helps in its disassociation from the DNA strand. Although the polymerization activity of the viral reverse transcriptase is higher than its RNase H activity, it has to be fine-tuned for optimal production of cDNA if used in *in-vitro* reverse transcription. Commercially available reverse transcriptases are highly thermostable and accurate MMLV (Moloney Murine Leukemia Virus) reverse transcriptase derivative with suppressed RNase H activity.

Material Required:

A. Total RNA isolated (see **Chapter 64**) from the desired tissue for synthesis of cDNA.

B. Equipment:

- i. Refrigerated micro-centrifuge and plate centrifuge.
- ii. Dry heating block.
- iii. Water bath.
- iv. Real-Time PCR System with 96 well plate compatibility.

C. Reagents, chemicals and other supplies:

- i. 70% ethanol.
- ii. RNase inhibitor (commercially available).
- iii. DEPC treated water.
- iv. DNase and DNase buffer (commercially available).
- v. EDTA
- vi. Oligo (dT) 12-18 primer (500 µg/mL).
- vii. dNTP mix containing 10 mM of each dNTP in nuclease free water (pH 7.5)(commercially available).
- viii. Reverse transcriptase with first strand buffer and 0.1 M DTT.

- ix. SYBR[®] Green master mix.
- x. Forward and Reverse primers for *RP49* (Internal Control).
- xi. Forward and Reverse primers for the gene whose expression is to be analysed across different cDNA samples (Gene target X).
- xii. 96 reaction well plate.
- xiii. Nuclease free, sterilized micro-centrifuge tubes.
- xiv. Nuclease free, sterilized pipettes and microtips.
- xv. Parafilm
- xvi. Ice bucket with flaky ice.
- xvii. Disposable nitrile powder free gloves.

Precautions:

1. Always wear nitrile powder free gloves and clean lab coats.
2. Clean equipment and work platform with 70% ethanol and RNase inhibitor solution.
3. Autoclave micro-tips and micro-centrifuge tubes a day before experiment or use commercially available nuclease free tips and tubes.
4. All other glassware and plasticware should be autoclaved a day before experiment and should be kept isolated.
5. Avoid touching containers, tubes etc to exposed skin while performing the experiments.
6. Always use fresh micro tip for pipetting of different reagents and samples.
7. Check the temperature of dry heat block and water bath with thermometer for stability.

Experimental Details:

For optimum reverse transcription and downstream expression study, the concentration of total RNA isolated from tissue should be at least 125 -130 $\mu\text{g}/\mu\text{L}$ or above as measured with spectrophotometer (see [Chapter 64](#)).

A. DNase treatment of RNA:

DNase treatment of the extracted RNA isolate is a necessary step to remove any carry over genomic DNA contamination that might have occurred during phase separation process of RNA extraction (see [Chapter 64](#)). The genomic DNA contamination may become a problem when performing qRT-PCR with SYBR[®] Green dye as it is a double stranded DNA binding dye and thus could result in false positive fluorescence signal during PCR cycle. Even though care is always taken to separate RNA containing aqueous layer, it is a good laboratory practice to go for DNase treatment of the isolated total RNA as a precaution.

- i. Take required volume of the isolated RNA solution which contains 1 μg of total RNA in a 1.5 mL micro-centrifuge tube.
- ii. Add 1 μL of DNase buffer.
- iii. Add 1 μL of DNase (Gently rinse the pipette tip by taking up the solution and disposing the whole again in the tube).
- iv. Make up the volume to 10 μL with RNase free or DEPC treated water if needed.
- v. Incubate for 15 min at room temperature.

- vi. Inactivate DNase with 1 μ L of EDTA.
- vii. Incubate at 65°C on a dry block for 10 min.
- viii. Quick-spin in micro-centrifuge for 5 sec at 2°C to precipitate the vapour residue on wall of the tube.

B. Performing reverse transcription:

- i. Take the DNase treated RNA solution and add 1 μ L of Oligo (dT) and 1 μ L of dNTP mix.
- ii. Heat to 65°C on a dry block for 5 min.
- iii. Quick chill on ice.
- iv. Centrifuge for a few seconds at 2°C to precipitate the vapour residue on wall of the tube.
- v. Add 4 μ L of First strand buffer.
- vi. Add 2 μ L of 0.1 M DTT.
- vii. Mix gently.
- viii. Incubate at 42°C for 2 min in a water bath.
- ix. Add 1 μ L of Reverse Transcriptase and mix gently.
- x. Seal the top of the tube externally by parafilm and incubate at 42°C for 50 min.
- xi. After incubation inactivate the reaction at 70°C for 15 min on a dry block.
- xii. Quick spin in micro-centrifuge for 5 sec at 2°C to precipitate the vapour residue on wall of the tube.
- xiii. The produced cDNA can be stored at -20°C for long term storage until further use.

C. qRT-PCR using SYBR® Green master mix:

A variety of techniques exist for expression analysis through RNA. However, Higuchi and colleagues (Higuchi *et al.*, 1992, 1993) pioneered the method of analysing PCR kinetics, where PCR reaction mix would contain ethidium bromide that detects and binds to the amplicons at each cycle of PCR reaction. The increasing fluorescence intensity in real time manner would be detected by an UV detector giving an insight on the amplification level of precursor DNA molecule. Quickly this concept developed into commercial qRT-PCR which eliminated the conventional post-PCR analysis. The SYBR® Green dye, which is an asymmetric cyanine dye with two positive charges, is widely used as a DNA binding reporter dye because of its high affinity for double stranded DNA (dsDNA) produced during qRT-PCR amplification. SYBR® Green generates very low fluorescence in the free state but when bound to dsDNA (at its minor groove), it emits high fluorescence, which is directly proportional to the amount of DNA.

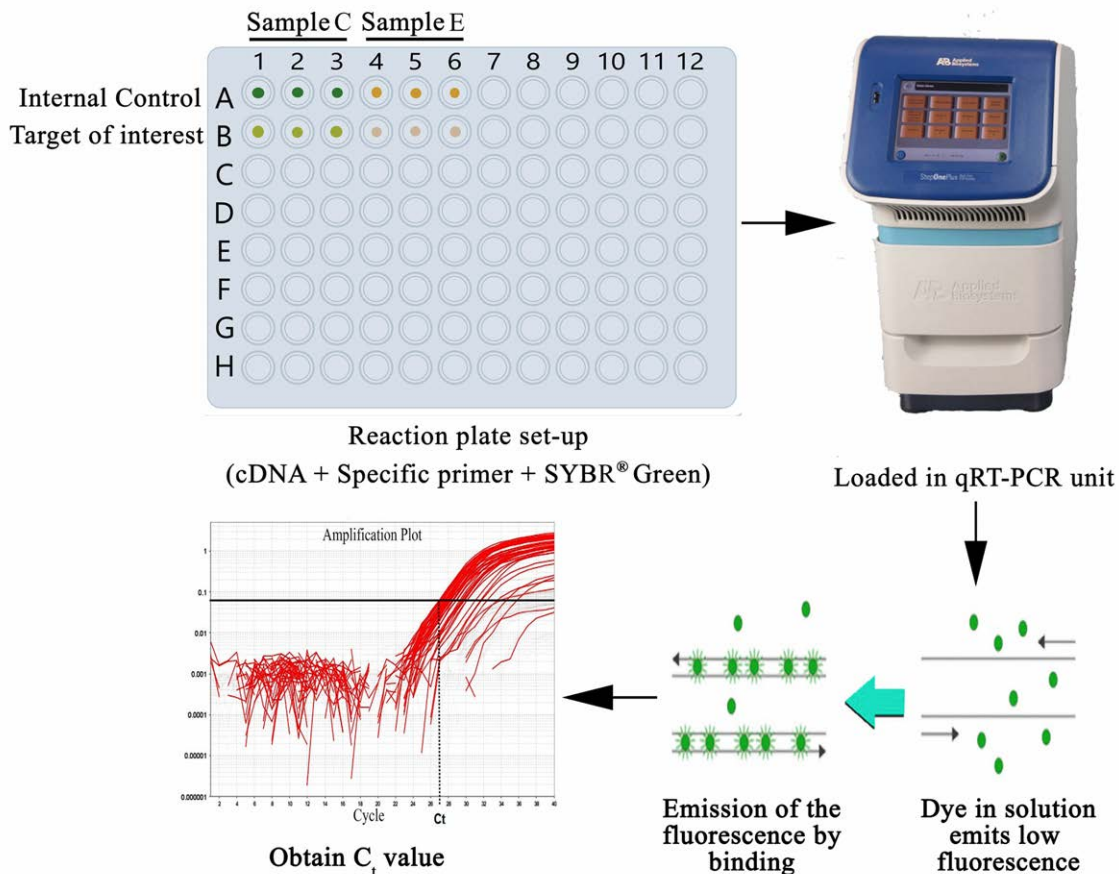


Fig. 2. A simplified workflow and reaction kinetics of qRT-PCR using SYBR® Green master mix.

The amplification of cDNA leads to formation of DNA-SYBR® Green conjugate which generates higher fluorescence of the dye at each amplification cycle (Fig. 2). The fluorescence signal reported by the system is the fluorescence signal of the base dye (SYBR® Green) normalized with the signal of a passive dye. ROX (Carboxyrhodamine) is a common passive dye added with SYBR® Green master mix. Passive dyes are inert fluorescent molecules, i.e. unlike base dye their signal intensity is not influenced by the qRT-PCR reaction kinetics (amount or amplification of template). Passive dyes are added to the reaction to compensate for the minute differences (e.g., volume or air bubble formation) between biological replicates. These minute differences in replicates also change the passive dye specific signal intensity which is used as normalization factor for the signal of base dye respective to the biological replicate. The normalization is obtained by dividing base dye signal with the signal intensity of passive dye. The obtained normalized signal value is termed R_n , while the term ΔR_n denotes R_n minus the baseline which consists of fluorescent signals that are noisy or are not exponentially increasing with amplification cycle. The software plots ΔR_n at 'Y' axis against the amplification cycle at 'X' axis generating a graph upon which an amplification curve will be produced with each qRT-PCR cycle. Before an amplification curve is evaluated a "Threshold line" is required for identification of a threshold value of fluorescent signal. The threshold line is a line drawn on graph perpendicular to the 'Y' axis correlating to a certain ΔR_n value which would be considered as the threshold fluorescence signal intensity for detection and analysis. The threshold line can be user defined or software default; anything below threshold line will be termed as background.

i. Preparation of reaction mixes

The total volume of the reaction master mix to be prepared is calculated on the basis that 10

μL is needed for each well used in the 96 well plate of the PCR machine. Triplicate reactions are carried out for each sample. In the example here, two genes i.e. internal control *RP49* and a gene target “X” in fly brain specific cDNAs prepared from two different samples/treatment groups (Control – C, Experimental -E) are considered. (**Fig. 2**).

- a. Gently thaw the cDNA samples and dilute with nuclease free/DEPC treated water by a factor of 5 (thus 20 μL of cDNA will be diluted with 80 μL of DEPC water).
- b. For cDNA sample “C”, take 30 μL of the Master Mix in a 200 μL micro-centrifuge tube and add 21.6 μL of DEPC treated water. Mix gently with pipette.
- c. Divide the solution into two equal parts of 25.8 μL .
- d. In one part add 0.6 μL each of *RP49* forward and reverse primers.
- e. In another part add 0.6 μL each of gene target X forward and reverse primers.
- f. Repeat steps a-e for cDNA sample “E”.
- g. Fix the well plate on the flaky ice firmly.
- h. The 96 well plate has 12 columns of wells marked 1-12 and 8 rows of wells marked A-H. Hence the numbering of the wells by each row will go like A1; A2...A12, B1; B2... B12 and so forth (**Fig. 2**).
- i. Fill up each of A1-A3 wells with 9 μL of *RP49* reaction master mix for sample ‘C’. Next fill up each of the A4-A6 wells with 9 μL *RP49* reaction master mix for sample ‘E’.
- j. Fill up each of B1-B3 wells with 9 μL of genetic target X reaction master mix for sample ‘C’. and each of the B4-B6 wells with 9 μL genetic target X reaction master mix for sample ‘E’.
- k. Pipette out 1 μL of sample ‘C’ cDNA in A1-A3 wells and 1 μL of sample ‘E’ cDNA in A4-A6 wells.
- l. Pipette out 1 μL of sample ‘C’ cDNA in each of the B1-B3 wells and 1 μL of sample ‘E’ cDNA in each of the B4-B6 wells.
- m. Seal the well plate with the transparent plastic film provided with the reaction well plate.
- n. Place the reaction plate in a plate centrifuge and quick-spin for 5 sec at 2°C – 4°C to bring down any droplet on the wall of the wells.
- o. Keep the plate carefully over the ice, while setting up the thermal cycler for the reaction.

ii. Setting up the reaction parameters:

- a. Turn the qRT-PCR unit on. Open the software interface to program the reaction parameters.
- b. At the Experiment properties section:
 - Provide the name of the experiment.
 - Select the Plate set up of the experiment (In our case 96 well plate).
 - Select the type of experiment as “Quantitation”.
- c. At the Methods & Materials section:
 - Select the quantitation method as “Cooperative CT”.
 - Select the reagent as “SYBR Green reagent”.
 - Select ramp speed as “standard of 2 hours”.
 - Select the template as “cDNA/Complementary DNA”.

d. At the target section:

- Define how many targets to be quantified during reaction (in this protocol 2 targets – *RP49* and genetic target X)
- Provide the name of the targets and assign them with colour.
- Select the reporter as “SYBR” and quencher as “NONE”.

e. At the sample section:

- Always keep plate arrangement by “Rows”.
- Define the number of samples (here in this protocol 2 samples).
- Specify replicates as 3.
- Define the number of negative controls if it is applicable.
- Assign sample name and colour.
- Setup sample or target reaction for all.

f. At the run method section:

- Set up the reaction volume to 10 μ L.
- The amplification parameters are as follows:
Holding stage - 95°C for 10 min.
Amp. Cycle – 95°C for 15 sec followed by 60°C for 1 min) X 40 times.
Melting Curve analysis – can be provided from the software standard set up if necessary.
- Save the run method.

g. Load the reaction plate in the thermal cycler and start the run.

Results/Observation:

Once amplification is initiated, fluorescence signal will be captured and an amplification curve will start taking shape in the software user interface. The curve shows 3 phases as follows:

i. Baseline Phase – Unstable noisy fluorescent signal or signals which are not exponentially increasing with amplification cycle i.e. this phase of this curve is horizontal to the ‘X’ axis.

ii. Exponential Phase – The signal intensity will gradually increase from the baseline with each amplification cycle and will reach and intersect the threshold line.

iii. Plateau phase – The exponential increase will be halted as the reaction runs out of raw materials such as dNTPs. This phase will be horizontal to the ‘X’ axis.

Note: During the ‘Exponential phase’, the point where the curve intersects with the threshold line is termed as **Cycle threshold** value or **C_t value**. The C_t value indicates the number of cycles it took to detect a significant signal from the sample specific to the target gene. Thus, the C_t value is a relative measure of the concentration. Higher expression of a certain gene will generate more of that specific cDNA. Hence, the target specific amplification will result in faster or slower increment of fluorescence signalling leading to detection of threshold signal at earlier or later amplification cycle. Hence, the C_t value is inversely proportional to the template concentration. The C_t values obtained from the software after the end of the experiment can be further analysed to produce a report on the relative expression of target gene(s) between two or more samples. Relative expression of a target gene is calculated following the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001) as explained below.

Example:

The expression of target gene X will be quantified in mutant fly brain denoted as “Experimental” (E) relative to normal wild type fly brain denoted as “Control” (C). Hence,

1. Internal control specific C_t values of samples ‘C’ & ‘E’ are RP_C and RP_E , respectively.
 2. Genetic target X specific C_t values of sample ‘C’ & ‘E’ are X_C and X_E , respectively.
- Each sample has triplicate C_t values for respective internal control and Target gene X. Accordingly, the following are calculated:-

Table 1: Calculations for quantifying relative expression of the target gene.

RP_{C1}	RP_{C2}	RP_{C3}	Average	RP_{E1}	RP_{E2}	RP_{E3}	Average
18.2	18.1	17.9	18	18.3	18.2	18.1	18.2
X_{C1}	X_{C2}	X_{C3}	Average	X_{E1}	X_{E2}	X_{E3}	Average
22	22.1	22.2	22.1	24.5	24.3	24.3	24.36
ΔX_C			$22.1 - 18 = 4.1$	ΔX_E			$24.36 - 18.2 = 6.16$
$\Delta\Delta XE = 6.16 - 4.1 = 2.06$							

Accordingly, the relative expression of target gene X in experimental sample brain is $= 2^{-(2.06)} = 0.23$ compared to control brain whose expression for the X gene will be 1. Expression value for the sample which is to be considered as the relative point for the expression study of the other sample(s) with this method is always taken as 1.

The inference can be drawn that the relative expression of X gene is downregulated by 77% in the mutant brain with respect to wild type control brain.

Troubleshooting:

ISSUE	PROBABLE REASON	SOLUTION
RNA yield is too low	Not appropriate amount of TRIzol to tissue has been applied.	Increase TRIzol amount. For 50-100 mg of tissue, at least 1 mL of Trizol is required.
RNA sample is impure	DNA, protein contamination at chloroform precipitation stage. Salt and phenol contamination at ethanol wash step.	Avoid touching any other layer while taking out aqueous phase. If not confident, repeat the chloroform precipitation step again. Repeat ethanol wash step thrice rather than twice.
RNA degraded when quality checked	Prolonged homogenization.	Homogenize in short pulses on ice bucket.

	RNA not stored in cold condition.	For a prolonged storage, RNA should be kept in a -80°C deep freezer.
C _t Values between three triplicates belonging to same sample and same target is not matching or the difference is very high	Pipetting Error.	Practice pipetting on expandable reaction plate with chilled water.
	Inadequate primer specificity.	Run conventional PCR with the primer on genomic DNA and gel check for the accuracy of the primer.

Question for self-study and interpretation:

1. What is the biological significance of spatio-temporal regulation of gene expression?
2. What are the different gene regulatory mechanisms that operate at transcriptional and translational levels?
3. What methods other than RT-PCR can be used for assessing levels of transcripts in a cell/tissue?
4. What were the classical or traditional cytological methods for studying gene expression at the level of RNA?

References and further reading:

Higuchi, R., Dollinger, G., Walsh, P. S. and Griffith, R. (1992) Simultaneous amplification and detection of specific DNA sequences. *Bio/Technology*. 10:413–417. DOI: 10.1038/nbt0492-413.

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RNA - Versatile Molecule

Recent years have witnessed an increasing understanding of the biological significance of heterochromatin in view of which the biological relevance of the so-called “selfish” or “junk” DNA is debatable. It is to be hoped that an increasing awareness of genetic functions through structural motifs as well, rather than through the primary base sequence alone, will stimulate an appreciation and understanding of this interesting class of genes. With RNA being the first “living molecule”, it is but to be expected that even today biological systems continue to utilize this versatile molecule directly. Progress in modelling the structure of RNA and RNP molecules will be of considerable help in this direction.

(In: Lakhota, S. C. 1996. RNA polymerase II dependent genes that do not code for protein. Indian Journal of Biochemistry and Biophysics, 33, 93-102
https://www.researchgate.net/publication/14447278_RNA_polymerase_II_dependent_genes_that_do_not_code_for_protein)

Chapter 66

Demonstration of genetic diversity by protein and isozyme analysis

S. R. Ramesh^{1*} (rameshuom@gmail.com) and K. Ravi Ram² (raviram@iitr.res.in)

1. Department of Studies in Zoology, University of Mysore, Manasagangothri, Mysuru 570006;
Current address: Chief Scientist, Department of Life Sciences, Pooja Bhagavat Memorial Mahajana Education Centre,, K. R. S. Road, Mysuru 570016.

2. Environmental Toxicology Group, CSIR-Indian Institute of Toxicology Research, Vishvgyan Bhavan, 31 MG Marg, Lucknow 226001

* For correspondence

Recommended Level: PG/Research

Sub-discipline: Genetics/Ecology/Evolutionary biology

Background:

Genetic diversity between individuals in a population of a species (intra-specific) or between closely related species (inter-specific) can be studied or demonstrated in different ways. Simple methods to study genetic diversity in *Drosophila* include the analysis of i) structural variations such as deletions, duplications, inversions and translocations using polytene chromosomes (see [Chapter 114](#)) and ii) polymorphism for isoenzymes/isozymes and proteins.

Isozymes or isoenzymes are multiple molecular forms, encoded by different genes, of the same enzyme. They catalyze same chemical reaction but may differ in some of their kinetic parameters and/or regulatory properties. Presence of isozymes in an organism is attributed to gene duplication or polyploidization (Engel *et al.*, 1973).

During the course of evolution, if one of the duplicated genes acquires mutation and if the mutation does not affect the active site, it would result in a variant with same catalytic function but with altered electrophoretic mobility, which may or may not affect kinetic properties and or its regulation. Point mutations or insertion and deletion (indel) events in a gene coding for an enzyme produce alleles. If these events do not affect the enzyme function, such genetic variants can persist in a population. Products of different alleles of the same enzyme coding gene, termed as Allozymes (see [Chapter 113](#)), can be demonstrated by gel electrophoretic technique (Singh *et al.*, 1982).

Over the past several decades, electrophoretic analysis of proteins and/or enzymes of *Drosophila* have enabled geneticists and biochemists to understand the nature and extent of genetic diversity/variation in natural populations. This biochemical technique can be conveniently used because it is simple, versatile and reproducible.

Peptides and proteins in a solution of a given pH have a net negative or net positive charge because of acidic (COOH) and basic (NH) groups of the constituent amino acids. When present in a n electric field, the positively charged molecules migrate

towards the cathode (-) while the negatively charged molecules migrate towards anode (+). The migration of charged molecules in solution under the influence of an externally applied electric field is known as Electrophoresis. Electrophoresis of biological macromolecules is generally carried out on a supporting medium like Paper, Cellulose Acetate, Agarose gel (see **Chapter 82**), Starch gel, Agar gel and Polyacrylamide gel.

Polyacrylamide gel is prepared by mixing and polymerizing two monomeric solutions namely, Acrylamide (coarse acrylamide) and N,N'-Methylenebisacrylamide (fine acrylamide). The polymerized gel is a cross-linking mesh-like matrix that serves as a sieve through which the large molecules like proteins migrate in an electric field applied on the aqueous solution (buffer) trapped in the matrix of the gel. The polymerization reaction is initiated by adding ammonium persulfate and a catalyst N,N,N',N'-tetramethylethylenediamine (TEMED) in presence of diffused sunlight or fluorescent light. During polymerization, methylenebisacrylamide forms cross-links between chains of acrylamide (**Fig. 1**). By varying the proportion of acrylamide solution while preparing the gel, the size of pores in the gel can be varied for getting optimum resolution.

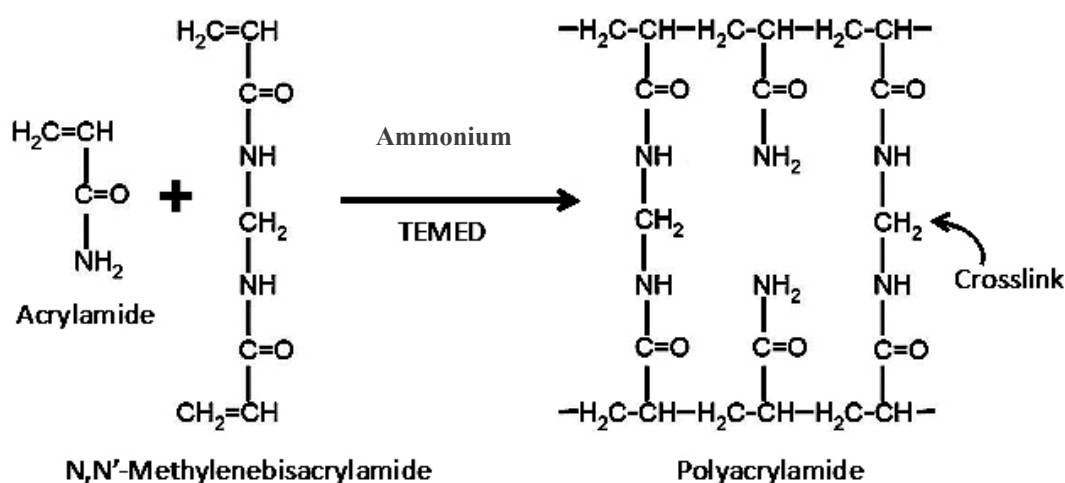


Fig. 1. Formation of Polyacrylamide from monomers of Acrylamide and Bisacrylamide

Polyacrylamide gel offers following advantages:

- i. It is chemically inert
- ii. The gel is transparent
- iii. Ease of handling the gel
- iv. Possibility to alter the pore size of the gel.

A 10% native gel (i.e., a gel prepared without addition of a protein denaturing agent such as SDS) is generally used for analysis of isozymes using whole body homogenates of *Drosophila*. Present chapter provides description of different steps involved in carrying out polyacrylamide gel electrophoresis for demonstrating polymorphism of a few representative examples of isoenzymes using single fly whole body homogenates and easily available reagents.

Objective:

To examine genetic variations in population by analyzing some isoenzymes through native polyacrylamide gel electrophoresis.

Materials Required:

A. *Drosophila* Stocks:

Different strains of *Drosophila* species either maintained in the laboratory (laboratory population) or first generation of isofemale lines of natural populations of wild species (e.g. *D. n. nasuta*, *D. s. neonasuta*, *D. bipectinata*, *D. montium*, *D. malerkotliana* etc.), or domestic species (e.g. *D. melanogaster*, *D. ananassae*).

B. **Fly-handling items** like Etherizer, Small paint brush; Soap solution (fly morgue), Fly food bottles and vials etc; stereo-binocular microscope.

C. Plastic and glass-wares:

- i. Micro tissue homogenizer
- ii. 1.5 mL Micro-centrifuge tubes
- iii. Micropipettes and tips (20 μ L, 200 μ L, 1 mL)
- iv. Pipettes
- v. Beakers
- vi. Conical flasks
- vii. Dilution flasks
- viii. Ice bucket
- ix. Medium/large Petri plates (depending upon the gel size)

D. Chemicals:

- i. Acrylamide
- ii. Ammonium persulfate
- iii. Bisacrylamide
- iv. Ethylenediaminetetraacetic acid (EDTA)
- v. Tetramethylethylenediamine (TEMED)
- vi. Tris.
- vii. Boric Acid
- viii. Bromophenol Blue
- ix. Coomassie Brilliant Blue R-250
- x. Fast Blue RR Salt
- xi. Sodium chloride
- xii. Sodium hydroxide
- xiii. Glacial Acetic Acid
- xiv. Glycerol
- xv. Glycine
- xvi. α -Glycerophosphate
- xvii. Magnesium chloride
- xviii. Manganese chloride
- xix. Methanol

- xx. β -NAD
- xxi. α -Naphthyl acetate
- xxii. β -Naphthyl acetate
- xxiii. Nitro Blue Tetrazolium (NBT)
- xxiv. Phenazine methosulphate (PMS)
- xxv. Polyvinyl pyrrolidone
- xxvi. Sucrose

E. Equipment required for electrophoresis:

- i. Electrophoretic chamber: includes two buffer tanks with platinum electrode.
- ii. Electrophoresis power supply: provides desired voltage/electric current.
- iii. Glass plates: one rectangular and one notched glass plates of same size.
- iv. Spacers: made of Teflon for casting polyacrylamide gel of desired thickness (gels of 1 mm thickness are preferred).
- v. Teflon Comb (of same thickness as the spacers): to generate slots in the upper gel into which samples are loaded.

Experimental Procedure:

A. **Stock Solutions:** Prepare following stock solutions using glass double distilled water/ Type II or I water:

- i. 40% Sucrose: Dissolve 4 gm sucrose in 6 mL water and subsequently make up the volume to 10 mL, to which add a few drops of 0.1% Bromophenol Blue.
- ii. 30% Acrylamide/Bisacrylamide: Dissolve 29.2 gm of coarse acrylamide and 0.8 gm bis-acrylamide in 60 mL distilled water and make up the volume to 100 mL with distilled water. Filter using Whatmann filter paper (Grade 1) and store at 4°C. This stock solution can be used for 3-4 weeks.
- iii. 1.5 M Tris-HCl buffer (Lower/Resolving gel buffer): Dissolve 18.15 gm Tris in 80 mL distilled water, adjust the pH to 8.8 using 6N HCl and make up the volume to 100 mL with distilled water.
- iv. 0.5M Tris-HCl buffer: (Upper/Stacking gel buffer): Dissolve 6 gm Tris in 80 mL distilled water, adjust pH to 6.8 using 6N HCl and make up the volume to 100 mL with distilled water.
- v. Ammonium persulfate: 100 mg/ mL (To be prepared just before mixing gel solutions).
- vi. Tray buffer (Buffer to fill upper and lower electrophoretic chambers) for localizing proteins: 0.05 M Tris, 0.384 M Glycine buffer (pH 8.65): Dissolve 6 gm Tris, 28.8 gm Glycine in 500 mL distilled water and made up to 1 liter.
- vii. Tray buffer (Buffer to fill upper and lower electrophoretic chambers) for localizing isoenzymes:
 - a. 0.1 M Boric acid Sodium hydroxide buffer (pH 8.65): Separately dissolve 6.183 gm Boric acid in 500 mL distilled water and 4.0 gm Sodium hydroxide in 500 mL distilled water. Add the Sodium hydroxide solution to the Boric acid solution till pH is 8.65; make the volume to 1 L with distilled water.

- b. Tris Borate EDTA buffer (87 mM Tris (hydroxymethyl) methylamine, 8.7 mM boric acid, 1 mM EDTA and 1mM NAD, pH 9.0): Dissolve 10.527 gm Tris in 500 mL distilled water and, add Boric acid solution (6.1 gm in 100 mL water) till pH is set to 9.0. Add 292 mg EDTA and 663 mg NAD to this solution and finally make the volume to 1 liter with distilled water.
- viii. Staining solution (for proteins): Mix 100 mL glacial acetic acid with 1 L 50% methanol. Dissolve 1.75 gm Coomassie Brilliant Blue R-250 in this mixture. Filter before use.
- ix. Destaining solution (for proteins): Mix 750 mL methanol and 190 mL glacial acetic acid and make the volume to 1500 mL with distilled water.
- x. Gel fixing solution (for isozyme analysis): 7% Acetic acid.

B. Preparation of gel:

- i. Wash the rectangular and notched glass plates thoroughly with a detergent followed by cleaning in absolute alcohol to make the glass plates grease free.
- ii. Set up the washed and dried glass plates with suitable spacers for gel casting (**Fig. 2**). Securely seal gap at the bottom between the two glass plates with a cellophane tape.
- iii. Mix the specified quantities (**see Table 1**) of different stock solutions to prepare gels of the desired acrylamide concentration (higher the concentration, smaller the pore size) in the resolving gel. 10% native gel is typically used for whole body homogenates of *Drosophila* for isozyme/protein analysis. For 1 mm thick gels of 8 X 10 cm size, 20 mL of gel solution is sufficient. Add TEMED after mixing all solutions and just before pouring the solution in the gel cast.

Table 1. Preparation of different concentrations of Polyacrylamide gels

Gel %	30% Acrylamide/ bisacrylamide (mL)	1.5 M Tris-HCl (mL)	Distilled Water (mL)	Ammonium persulfate (μL)	TEMED (μL)
8	5.33	5.0	9.47	200	11
10	6.66	5.0	8.14	200	8
12	8.0	5.0	6.8	200	8
15	10	5.0	4.8	200	8

- iv. Pour the prepared gel solution between the two glass plates to a desired height. Add a few drops of water very slowly to generate a layer of water above the lower gel solution. Expose this set up to the fluorescent light or to diffuse day light to facilitate polymerization of the small pore gel. Keep the polymerizing gel at a vibration free place.

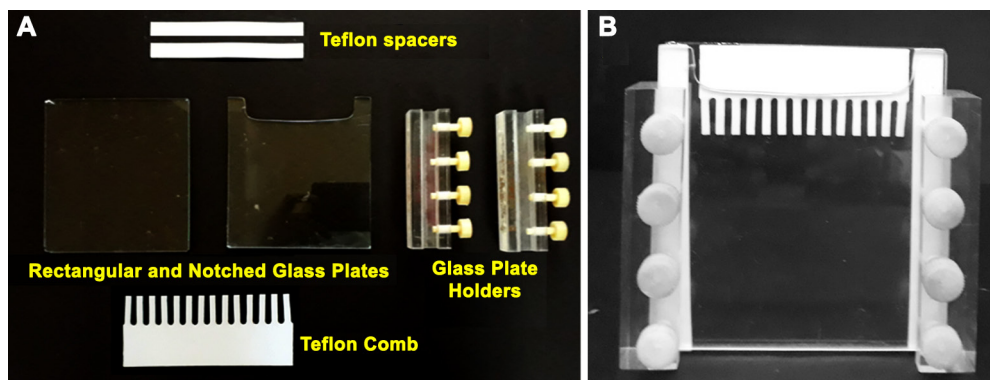


Fig. 2. A. Different components needed for casting vertical slab gel, **B.** Assembled gel cast with the comb inserted in upper gel for generating sample slots.

v. Completely remove/blot the water layer from top of the polymerized lower small pore gel.

vi. Prepare 10 mL 4% stacking gel (large pore gel) solution (this is the usual concentration for resolving gels of different percentage) by mixing the following stock solutions .

Acrylamide	1.70 mL
0.5 M Tris-HCl buffer	1.25 mL
Distilled water	6.89 mL
Ammonium persulfate	100 μ L
TEMED	10 μ L

Add and mix TEMED at the end, just before pouring the gel in the cast.

vii. Pour this mixture above the polymerized resolving gel and insert the comb (**Fig. 2B**). Expose this set up once again to diffuse day light or fluorescent light for polymerization of large pore gel.

viii. After polymerization of large pore gel, carefully lift the comb and wash the wells/ slots with distilled water.

At this stage, the gel can be stored overnight in a refrigerator, with enough quantities of water poured into the sample wells or slots to prevent desiccation and shrinkage of gel. This water in the wells has to be removed completely before loading of samples.

C. Preparation of sample:

i. For analysis of whole body proteins, homogenize 8 or 10 day old single fly (single fly whole body homogenate) in 100 μ L double distilled water using a micro tissue homogenizer.

ii. For analysis of isozymes, homogenization of single fly is done with the homogenizer placed in ice to prevent denaturation of enzymes due to heat generated during homogenization. Briefly centrifuge the samples for 5 min at 500 rpm and use immediately. Alternatively, they may be kept frozen for use within 24 hr.

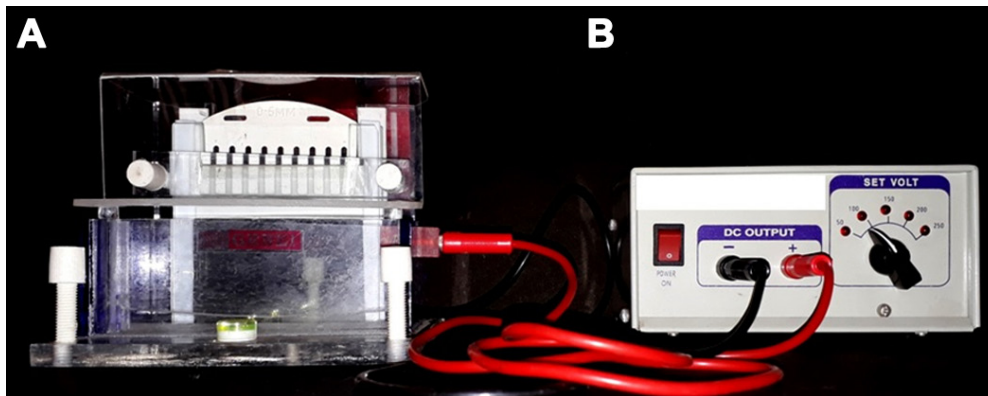


Fig. 3. A. Electrophoretic chamber fitted with polyacrylamide gel. B. Power supply

D. Electrophoresis:

- i. Remove the cellophane tape from the bottom of the glass plates and water from the wells/slots. Fix the gel assembly in the electrophoresis chamber.
 - ii. Mix 100 μL of 40% sucrose to each homogenate and load the desired quantity of sample (50 μL in the present case) into the sample wells using a micro pipette. Carefully record details of species/population/strain from which samples were prepared and loaded in which sample well for extrapolating the isozyme/protein patterns after staining of the gel.
 - iii. Layer the tray buffer carefully over samples in each well. Pour the appropriate tray buffer into upper and lower buffer chambers such that the gel on either side is in contact with the buffer and no air-bubbles are trapped at lower end or in the wells. The lower tank should be nearly completely filled with the buffer, without any overflow.
 - iv. Connect the terminals of electrophoretic chamber to the power supply (**Fig. 3**).
 - v. Carry out electrophoresis at constant voltage (80 V for Alkaline phosphatases, Acid phosphatases, α -esterase and β -esterase) and 100 V for α -Glycerophosphate dehydrogenase and Tetrazolium oxidase) for a period specified under the Assay and staining section or until the Bromophenol blue tracking dye migrates to the end of the lower gel.
For total body proteins in whole body homogenates, apply 100-120 V until Bromophenol blue reaches the end of small pore gel.
- Note:** For isozyme analysis, electrophoresis has to be carried out at 4°C; however, for analysis of protein patterns using whole body homogenates, electrophoresis can be carried out at room temperature.
- vi. Switch off the power supply at the end of electrophoresis and remove the glass plates from the chamber.
 - vii. Carefully release the glass plates in order to take out the gel and punch the left bottom corner of the resolving gel to facilitate identification of the order of samples loaded for analysis.
 - viii. Briefly wash the gel in distilled water.

ix. *For localizing proteins*, the gel is immersed and kept overnight in the Coomassie Brilliant Blue R-250 protein staining solution in an appropriate sized Petri dish. Keeping the Petri dish on a rotary shaker improves staining. Destain in the destaining solution, and store the gel in 7% acetic acid.

x. *For localizing isozymes*, the gel is incubated at 37°C in appropriate staining solution (see below), preferably on a rotary shaker. After the appearance of enzyme bands, the gel is fixed in 7% acetic acid.

E. Assay and Staining procedures for isozymes:

i. **Alkaline phosphatase (Aph):** Tray buffer: 0.1 M Boric acid Sodium hydroxide buffer (pH 8.65), electrophoresis with 80 V for 2 hr.

a. Prepare staining solution by mixing:

- 500 mg of Polyvinyl pyrrolidone
- 100 mg of α -Naphthyl phosphate
- 60 mg of Magnesium chloride
- 60 mg of Manganese chloride
- 2 gm of Sodium chloride
- 100 mg of fast blue RR salt
- 100 mL of 0.5 M Tris HCl buffer (pH 8.5)

b. After electrophoresis, place the gel in the staining solution at 37°C until enzyme bands appear.

ii. **Acid phosphatase (Acph):** Tray buffer: 0.1 M Boric acid Sodium hydroxide buffer (pH 8.65), electrophoresis with 80 V for 2 hr.

a. Prepare staining solution as above for Alkaline Phosphatase except that the 0.5 M Tris HCl buffer (pH 5.0) is used in this case.

b. After electrophoresis, place the gel in the staining solution at 37°C until enzyme bands appear.

Note: The Alkaline and Acid phosphatases are localized on gels due to their ability to hydrolyze α -naphthyl phosphate to liberate α -Naphthyl at pH 8.5 and at pH 5.0, respectively. The released α -naphthol is coupled with the diazonium salt (Fast Blue salt RR) which forms a coloured product at the sites of enzyme activity on the gel.

iii. **α -Glycerophosphate dehydrogenase (α -Gpdh):** Tray buffer: Tris Borate EDTA buffer (pH 9.0), electrophoresis with 100 V for 2 hr and 30 min.

a. Prepare staining solution by mixing:

- 800 mg Sodium α -Glycerophosphate
- 25 mg β -NAD
- 20 mg Nitro Blue Tetrazolium
- 5 mg N-methyl phenazine methosulphate
- 100 mL 0.05 M Tris HCl buffer (pH 8.5)

b. After electrophoresis, place the gel in the staining solution at 37°C in dark until purple colored enzyme bands appear.

Note: This staining solution for α -Glycerophosphate dehydrogenase contains the oxidized form of its coenzyme (NAD) and the substrate viz., α -Glycerophosphate. The reduced NAD formed during the reaction passes electrons to a tetrazolium compound (Nitro Blue Tetrazolium) through an intermediate electron carrier namely Phenazine methosulphate. These reactions result in the formation of an insoluble purple diformazan dye at the sites of enzyme activity.

a. **Tetrazolium oxidase (To):** Tray buffer: Tris Borate EDTA buffer (pH 9.0), electrophoresis with 100 V for 2 hr and 30 min.

a. Prepare staining solution by mixing

- 25 mg β -NAD
- 20 mg Nitro Blue Tetrazolium
- 5 mg N-methyl phenazine methosulphate
- 100 mL of 0.05 M Tris HCl buffer (pH 8.5)

b. After electrophoresis the gel is immersed in staining solution and exposed to light until white enzyme bands appear on a blue background. **Note:** The Tetrazolium oxidase enzyme is localized by its ability to oxidize Nitro Blue Tetrazolium, which is used as a substrate in the presence of Phenazine methosulphate. This enzyme is visualized as a white band against a blue background.

b. **α -Esterase (α -Est):** Tray buffer: 0.1 M Boric acid Sodium hydroxide buffer (pH 8.65), electrophoresis with 80 V for 2 hr.

a. Prepare staining solution by dissolving 100 mg α -Naphthyl acetate, 2 mL acetone, to which 2 mL glass double distilled water is added and mixed with 50 mL 0.1 M phosphate buffer (pH 5.9) followed by addition of 100 mg Fast blue RR salt. Finally, 50 mL 0.1 M phosphate buffer (pH 6.5) is added again and mixed.

b. After electrophoresis the gel is incubated in the staining solution at 37°C until grey colored bands appear.

c. **β -Esterase (β -Est):** Tray buffer: 0.1 M Boric acid Sodium hydroxide buffer (pH 8.65), electrophoresis with 80 V for 2 hr.

i. Prepare the staining solution as for α -Esterase above except that β -naphthyl acetate is used in place of α -Naphthyl acetate.

ii. After electrophoresis, incubate the gel in the staining solution at 37°C until insoluble grey or maroon/pink colored bands appear.

Note: The α - and β -Esterase enzymes are localized by trapping the enzymatically released naphthol or a naphthol derivative from substrates (α - and β -Naphthyl acetate, respectively) with Fast Blue RR Salt (diazonium salt). This reaction results in an insoluble maroon/pink product at the regions of enzymatic activity.

Results/observations:

Examine the stained gel and record the patterns and mobility of proteins or the isozymes in different species/populations/strains. The patterns of staining can be recoded photographically using a phone or other camera after placing the stained gel on a ground

glass plate illuminated with fluorescent light from below or using a gel-documenting system, if available. Variations in patterns and frequencies of each pattern should be documented.

Protein/isozyme variations in natural populations are ubiquitous and have been documented not only in *Drosophila* but also in a variety of plants and animals. Studies on isozyme variations in different tissues and populations provide useful information about the extent of genetic diversity. Correlative studies of the isozyme variations with ecological factors shed light on the adaptive significance of such genetic diversity.

Questions for self-study and interpretation:

1. What are isozymes and allozymes?
2. What determines the kinetics of enzyme-substrate interactions?
3. What is the basis of variations in electrophoretic mobility on native polyacrylamide gels?
4. Why is SDS-polyacrylamide gel electrophoresis is not generally used for isozyme studies?
5. What happens to genetic heterogeneity (isozyme/protein variability) when isofemale lines are inbred for many generations?
6. Whether isozyme/protein patterns change during aging or at different stages of development?
7. Is there any correlation between variation in isozyme/protein patterns and photo period or sex or population density?
8. Whether isozyme/protein patterns are different in individuals of a species collected from isolated geographic areas?
9. Whether isozyme/protein patterns are different in individuals of a species collected from same geographic area during different seasons or different altitudes?

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Triantaphyllidis, C. D., Scouras, Z. G., Panourgias, J. N. and Loannidis, G. C. (1982). Allozyme variation in Greek wild populations of *Drosophila melanogaster* and *D. simulans* along a north-south gradient. *Genetica*. 58: 129–136.

Unbiased Analysis of Genetic Variability in Populations

The back-to-back papers of Hubby and Lewontin (1966) and Lewontin and Hubby (1966) on genetic variability in *Drosophila pseudoobscura* represent a landmark in the study of variation in natural populations. The authors introduced the concept of studying variability across the genome, unbiased by prior knowledge of variability at the loci in question. Their experimental technique was gel electrophoresis of enzymes and soluble proteins, which detects most charge change variants. Electrophoresis had already been used to study within-species variation at specific loci

(In: Charlesworth, B., Charlesworth, D., Coyne, J. A., & Langley, C. H. 2016. Hubby and Lewontin on protein variation in natural populations: when molecular genetics came to the rescue of population genetics. *Genetics*. 203: 1497–1503. <https://doi.org/10.1534/genetics.115.185975>)

Chapter 67

Detection of proteins in wing imaginal discs of *Drosophila* larvae by western blotting using damage-induced p-Jun as an example

Chaitali Khan¹ (chaitalikhan@gmail.com, chaitali.khan@einsteinmed.org) and
Basuthkar J Rao^{2*} (bjrao1@gmail.com)

1. Department of Genetics, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, USA

2. Indian Institute of Science Education and Research (IISER) Tirupati, Transit Campus: Sree Rama Engineering College, Tirupati, India

* For correspondence

Recommended Level: PG/Research

Sub-discipline: Developmental Biology/Molecular Biology

Background:

Drosophila larval wing imaginal disc is a mono-layer epithelial tissue, which gives rise to adult wing and parts of thorax during the pupal metamorphosis. Wing disc starts to develop from ~50 cells during the 1st instar larval period and reaches ~50,000 cells by the end of the third instar period. During development, the wing disc cells proliferate leading to increase in size of the disc. At the same time, programmed activation of different signaling pathways organizes wing disc into compartments with specific developmental fates. Because of their proliferative nature, wing discs also exhibit high regenerative potential and has been a favorite tool to study, besides the mechanisms underlying development and differentiation, the processes of tissue regeneration, wound healing and tissue homeostasis (Khodaverdian and McVey, 2017).

This protocol describes the use of western blotting to study activation of Jun N-terminal kinase (JNK) signaling. JNK is a member of the mitogen-activated protein kinase family and is one of the prominent signaling pathways that gets activated during tissue response (La Marca and Richardson, 2020). The example here uses the *UAS-Gal4* system (see [Chapters 31, 32](#)) to induce tissue injury by targeted over-expression of a pro-apoptotic gene *hid*. It is known that localized over-expression of *hid* leads to tissue ablation. The resulting tissue injury activates signalling pathways, including JNK, which is implicated in tissue repair. *Drosophila* has a single JNK protein, encoded by the gene basket (*bsk*). The *Bsk* (JNK) protein is a substrate for the Hemipterous kinase (JNKK), which in turn is phosphorylated by other upstream kinases (JNKKK). The activation of Bsk leads to the phosphorylation of the transcription factors Jun and Fos that regulate the activity of genes responsible for the various cellular functions associated with JNK activity (Stronach, 2005). The activation of the JNK signalling can be quantitatively assayed through western

blotting by detecting phosphorylated Jun (p-Jun) protein as a read out of JNK activity in control (no *hid*) versus *hid* over-expression wing imaginal discs. The principle underlying western blotting is briefly described in [Chapter 68](#).

Objective:

Detection of phosphorylated Jun (p-Jun) in *Drosophila* larval wing imaginal discs by western blotting.

Materials Required:

A. Fly strains:

- i. *salm-Gal4* (Bl. 80573)
- ii. *UAS-hid* (Bl. 65403)
- iii. *UAS-LacZ* (Bl. 3955)

Note: Ubiquitous over-expression of *hid* is lethal but expression of the *UAS-hid* by the *salm-Gal4* driver, which is expressed only in the pouch region of wing disc during early 3rd instar larval period, does not cause lethality. The *salm-Gal4* driven *UAS-lacZ* expression does not affect the wing discs and, therefore, serves as control.

B. Equipment and other items:

- i. Stereo-binocular microscope.
- iii. Fine-tipped forceps, needles and other items for fly handling.
- iv. Insulin stab needles (DispoVan)/fine dissection needles.
- v. Watch Glass.
- vi. Vertical Electrophoresis apparatus.
- vii. Mini Trans-Blot Apparatus.
- viii. Power supplies for electrophoresis and western transfer.

C. Reagents for protein extraction and polyacrylamide gel electrophoresis (PAGE):

- i. *Drosophila* insect medium (HiMedia IM005).
- ii. Heat inactivated fetal bovine serum (HiMedia RM9955-100 mL).
- iii. 2X Laemmli buffer:
 - 4% SDS (400 mg)
 - 20% Glycerol (2 mL)
 - 0.004% Bromophenol blue (4 mg)
 - 0.125M Tris-HCl, pH 6.8 (1.2 mL of 1M Tris-HCl)
 - 6.8 mL water to make up the final volume to 10 mL.

Store the Laemmli buffer at room temperature. Bromophenol blue dye, which runs ahead of the proteins in the sample, serves as the tracking dye and is used to follow the progress of electrophoresis.

- iv. 0.5 mM Dithiothreitol (DTT)
- v. Protein molecular weight markers for SDS-polyacrylamide gels
- vi. Dry heating Block or heating water bath
- vii. 10% Poly-Acrylamide Gel (PAGE) Gel for separation of proteins: Mix in the following order:
- | | |
|--|--------|
| • H ₂ O | 4.1 mL |
| • 30% Acrylamide/bis-acrylamide (37.5:1) | 3.3 mL |
| • Tris-HCl (1.5M, pH 8.8) | 2.5 mL |
| • 10% SDS | 100 µL |
| • <i>N, N, N', N'</i> -tetramethylethylene-diamine (TEMED) | 10 µL |
| • Ammonium persulfate (APS), 10% | 32 µL |
- viii. 4% stacking gel. Mix in the following order:
- | | |
|--|--------|
| • H ₂ O | 6.1 mL |
| • Acrylamide/bis (30% 37.5:1) | 1.3 mL |
| • Tris-HCl (1.5M, pH 8.8) | 2.5 mL |
| • SDS, 10% | 100 µL |
| • <i>N, N, N', N'</i> -tetramethylethylene-diamine (TEMED) | 10 µL |
| • Ammonium persulfate (APS), 10% | 100 µL |

Note: TEMED and Ammonium persulfate are catalysts for polymerization of acrylamide/bis-acrylamide. Therefore, these should be added just before pouring the gel solution into the gel cast. For detailed instructions about casting the gel, see the Cold spring Harbor Protocols at <http://cshprotocols.cshlp.org/content/2015/7/pdb.rec087908.full?rss=1>

For preparation of polyacrylamide gels, see **Chapter 7**.

- ix. 10X Running buffer: Dissolve 30.0 gm of Tris base, 144.0 gm of glycine, and 10.0 gm of SDS in 1000 mL of H₂O. The pH of the buffer should be 8.3 and no pH adjustment is required. Store the running buffer at room temperature and dilute to 1X before use.
- x. Nitrocellulose membrane
- xi. Ponceau dye for staining gels

D. Reagents for western blotting and signal detection:

- i. Bovine Serum Albumin
- ii. 1X Tris-Buffered Saline (TBS, 50 mM, Tris-Cl, pH 7.5, 150 mM NaCl): Dissolve 6.05 gm Tris and 8.76 gm NaCl in 800 mL of H₂O. Adjust pH to 7.5 with 1 M HCl and make volume up to 1 L with H₂O. TBS is stable at 4°C for 3 months.
- iii. Washing buffer (1X TBST): 1X TBS with 0.05% Tween
Prepare 10% Tween solution; add 500 µL of 10% tween to 100 mL of 1X TBS.
- iv. Blocking buffer [1X TBST + 1% Bovine Serum Albumin (BSA)]:
Add 5 gm BSA (w/v) to 100 mL of 1X TBST.
- v. Antibodies:
- Rabbit anti-p-Jun (Cell Signaling 3270S)

- Mouse anti-Lamin (DSHB ADL 84.12)
 - Appropriate Secondary antibodies conjugated with Horse Radish Peroxidase (HRP)
- vi. X-ray film and developing system
- vii. Enhanced chemiluminescence (ECL) detection reagents

Experimental Details:

A. Protein extraction:

- Set up crosses using ~ 15 virgin females from *salm-Gal4* stock and males from *UAS-LacZ* or *UAS-hid* stock in separate food vials and keep at 24°/25°C. During the next 2-3 days, transfer the adult flies to fresh food vial every 24 hr. Keep all the vials. Add a small dab of yeast paste to the vials with eggs/larvae for healthy growth.
- Select well-fed wandering third instar larvae from the control (*salm-Gal4*>*UAS-LacZ*) and experimental cross (*salm-Gal4*>*UAS-hid*) in separate watch glasses/Petri dishes and wash with cold 1X PBS to remove adhering food particles.
- Transfer 5 larvae to the dissecting medium (*Drosophila* S2 cell medium supplemented with 10% FBS or in 1 X PBS) for dissection.
- Remove the two-third posterior part of larvae with the help of pair of fine forceps and invert the remaining anterior part inside out slowly with the help of insulin stab or dissection needles.
- Using insulin stab or dissection needles, carefully take out the wing imaginal discs (opaque white bag like structures, see **Fig. 1**; also see **Chapters 2, 18**) from larval body and keep them in the dissecting medium. Be careful not to damage the wing discs while removing them.
- Collect 10 wing discs from 5 larvae of each genotype.

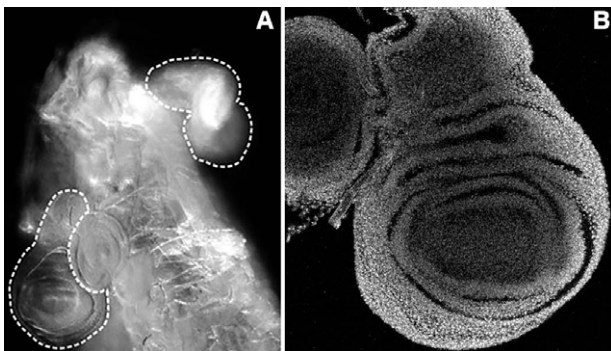


Fig. 1. Larval wing imaginal discs. **A.** Third instar larval wing imaginal discs attached to the body wall (reproduced with permission from, Khodaverdian and McVey, 2017). **B.** Confocal microscope image of third instar larval wing imaginal disc stained with a nuclear stain.

- Using a 10 μ L micro-pipet, transfer the wing discs to a fresh 1.5 mL microfuge tube. Spin the tube in a microfuge and remove the extra medium by pipet (pipetting in and out 1 X PBST through the micro-tip a few times before actually transferring the imaginal discs to the tube prevents sticking of wing discs in the tip).
- Add 5 μ L 2 X Laemmli buffer, 5 μ L 1 X PBS and 0.2 μ L of 0.5 mM DTT to the tube containing wing discs. Keep the tube in boiling water for 10 min (make sure to tightly close the lid; to further reduce any chance leakage, wrap the tube cap with

parafilm). Boiling in the SDS containing Laemmli buffer lyses wing disc cells and releases the de-natured proteins into the buffer.

ix. After cell lysis in the Laemmli buffer, spin in a microfuge for 5 min at 6000 rpm to settle the debris. Keep the supernatant (protein extract in Laemmli buffer) on ice for immediate use or store at -20°C for use within a week or at -80°C for longer storage.

B. Western blotting:

i. Cast and set up 10% Poly Acrylamide Gel for electrophoresis (PAGE) in vertical electrophoresis unit following instructions in **Chapter 66** or at <http://cshprotocols.cshlp.org/content/2015/7/pdb.rec087908.full?rss=1>

Fill the tank with the 1X Running buffer.

ii. Load a protein molecular weight marker (commercially available pre-stained protein molecular weight ladder can be convenient) in the first well, followed by 10 µL of the above prepared wing imaginal disc protein samples in other wells. **Note:** The adjacent wells may be left empty if samples are few but add 10 µL of 1 X Laemmli Buffer to the empty wells.

iii. Run the gel at 80 Volts, allow the samples get stacked and enter the resolving gel. After that change the voltage to 120 Volts, run the gel until dye front reaches the end of the gel (specific voltage to be applied for electrophoresis may vary in relation to the gel size).

iv. Set up the transfer apparatus (see **Chapter 68**), such that gel is placed facing the negative (black) electrode in the order: Negative electrode: Whatman Filter paper: Gel: Nitrocellulose membrane: Whatman Filter paper: Positive electrode. The Whatman filter papers and the nitro-cellulose membrane are soaked in the transfer buffer prior to their assembly in the transfer buffer. Allow the transfer to occur for 90 min at 90 Volts at 4°C (in the cold room). **Note:** Be careful about the orientation of electrodes while running the transfer.

v. Stop the transfer and remove the nitro-cellulose membrane, stain the membrane with Ponceau dye for approximately 2 min to check the transfer. Using the molecular weight markers, cut the membrane horizontally between around 55 kDa to separate the filter in two halves, the lower half containing p-Jun (48 kDa) whereas the upper half containing Lamin a/c (71 kDa) protein bands, respectively (the Lamin protein is used in the present example as a control reference protein whose levels should not be different between the control (*salm-Gal4>UAS-lacZ*) and experimental discs (*salm-Gal4>UAS-hid*). Gently rinse the membrane strips with autoclaved water to remove Ponceau stain. Now membrane is ready for the antibody probing.

C. Probing the blot with desired antibody and developing the western blot:

i. After the western transfer, place the membrane in a clean box, add 10 mL of blocking buffer, keep it on a horizontal shaker at low speed at room temperature for 1 hr. **Note:** For phosphorylated antigens (p-Jun in this case), all the washing and blocking buffer should be made in 1 X TBS instead of 1 X PBS, to prevent cross-reactivity from phospho- group in PBS.

- ii. Discard the blocking buffer and add primary antibody diluted in blocking buffer (1 X TBST+1% BSA). p-Jun (dilution as recommended by the antibody manufacture), Lamin (dilution as recommended by the antibody manufacture) on to the respective membrane strips in separate boxes. Incubate the membrane over night at 4°C with gentle shaking.
 - iii. Discard the used antibody solution and replace it with washing buffer (1 X TBST), give 3 washes 15 min each at room temperature.
 - iv. Add the required secondary antibody (dilution as recommended by the antibody manufacture) to each of the membranes, incubate for 1 hr at room temperature.
 - v. Finally, wash the membrane with 1 X TBS, store it at 4°C or proceed with detection of the signal.
 - vi. Mix ECL solution in 1:1 ratio as directed by the manufacturer. Place the membrane on a dry plastic surface or lid of a box, add ECL solution with 1 mL pipet, allow the membrane to soak in ECL for 1 min.
 - vii. Transfer the membrane to the X-ray film cassette, expose the X-ray film for different time periods (e.g., 10 sec, 30 sec and 1 min).
 - viii. Develop and fix the film with photographic developer and fixer in a photographic dark room, check for the bands at the size 48 kDa (corresponding to p-Jun) and 71 kDa (corresponding to Lamin), respectively in the X-ray films exposed to the two blots, respectively.
- Note:** The exposure time for the X-ray film has to be appropriate so that it is neither over nor under; this may need multiple trial exposures for standardization.

Observations:

1. Identify the band at around 48 kDa for p-Jun and 70 kDa for Lamin. Use the protein marker lane to determine the size of the bands. The expectation is that (*salm-Gal4>UAS-hid*) lane will have higher amount of p-Jun compared to the control (*salm-Gal4>UAS-LacZ*) lane.
2. Compare the relative intensities of the bands observed at 48 kDa (p-Jun) and 70 kDa (Lamin) in the control (*salm-Gal4>UAS-LacZ*) lane and the experimental (*salm-Gal4>UAS-hid*) lane. The Lamin band's intensity (expected to be similar in the control and experimental sets) serves as reference for any variations in the total protein content in the two sets of imaginal discs.

Note: Intensity of the bands can also be quantified (e.g., using the publicly available ImageJ software).

Questions for self-study and interpretation:

1. What is the use of detecting Lamin bands in the same experiment?
2. Do you think un-phosphorylated form of Jun can serve as control?

3. Can you express any other pro-apoptotic protein in place of *Hid* to activate p-Jun in wing discs?
4. What are the other ways to detect levels of p-Jun in wing imaginal discs?

References and further reading:

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Ed Southern & Evolution of Blotting Technique Names

Obviously the Southern blot was named after Edwin Southern. Hence, it is appropriate to capitalize the name “Southern,” as a proper noun, when referring to the corresponding blot. Subsequently, scientists developed the “northern blot” for detecting RNA (also the “reverse northern blot” using an RNA probe to detect DNA), followed by the “western blot” for detecting particular proteins. Without going into the history of these names, I think it is fair to say that they were inspired by the “Southern” blot.

Apparently finding this “direction”-oriented naming system irresistible, scientists have since come up with the “eastern blot” as a method to detect post-translational modifications (an extension of the western blot), the “far-eastern blot” for lipid analysis, the “far-western blot” (also called a “west-western blot”), which is a variation on the western blot that uses a protein other than an antibody to detect the protein of interest, the “middle eastern blot” for analysis of mRNA, the “northwestern blot” to detect RNA-protein interactions, and the “southwestern blot” that can be used to identify DNA-binding proteins.

(In: Klionsky D. J. 2017. Blame it on Southern, but it’s a western blot. *Autophagy*. 13: 1–2. <https://doi.org/10.1080/15548627.2016.1255382>)

Chapter 68

Extraction and processing of fly brain proteins for western blotting

Priyanka Modi (pmodi82@gmail.com), Abuno Thepa (abunzthep@gmail.com), Nukshimenla Jamir (nukshimenlajamir@gmail.com), Abhik Das (dasabhik91@gmail.com) and Sarat Chandra Yeniseti* (yschandrays@rediffmail.com)

Drosophila Neurobiology Laboratory, Department of Zoology, Nagaland University (Central), Lumami 798627 Nagaland, India.

*For correspondence

Recommended Level: UG/PG/Research

Sub-discipline: Molecular biology/ Neurobiology

Background:

Proteins are essential for all cell processes, including proliferation, differentiation, and death. Differential expression patterns or altered states of multiple proteins involved in signalling pathways, energy metabolism, oxidative stress, neurotransmission, etc. are essential for maintenance of homeostasis of cell/tissue/organ. Alterations in the homeostatic levels of proteins result in abnormal cellular functions which can also lead to a disease condition (Bass *et al.*, 2017). Quantification of the expression changes affecting known or novel genes and proteins can provide valuable information about the gene regulation/cell function and the molecular mechanisms involved in diseases/disorders. Western blotting, also known as immuno blotting, is one of the widely used informative techniques for qualitative and quantitative analysis of expression of a given protein in the desired tissue (Kurien, 2011). This technique involves separation of different proteins from the given biological samples by gel electrophoresis, transfer of the separated proteins from the gel to a protein-binding membrane, incubation of the membrane with antibodies specific for the proteins of interest and finally detection of the bound antibody (Jensen, 2007; Kurien, 2006). Once detected, the target protein is visualized as a band on a blotting membrane, X-ray film, or an imaging system (Fig. 1).

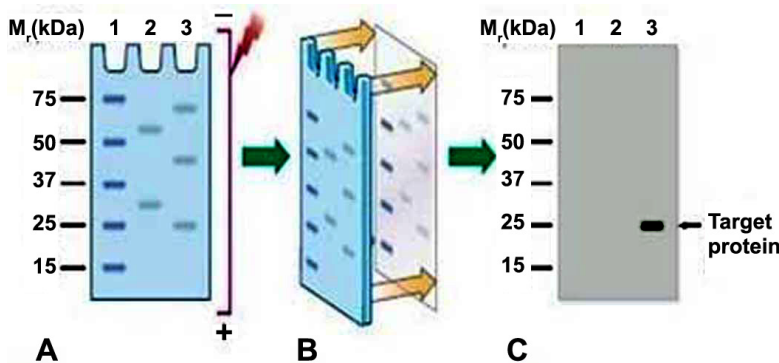


Fig. 1. Steps involved in Western blotting. A. Separation of proteins by electrophoresis on a gel matrix. B. Transfer ('blotting') of proteins from the gel matrix to a blotting membrane. C. detection of target proteins by immuno-detection.

(Adapted from <https://www.bio-rad-antibodies.com/western-blotting-immunoblotting-introduction.html>)

The protocol described here takes example of proteins from adult fly brains. This protocol can be adapted to any tissue of interest from larval, pupal or adult *Drosophila*.

Objectives:

Extraction and processing of protein from fly brain for western blotting and detection.

Material Required:

A. **Flies:** wild type or mutant *Drosophila* lines (as required).

B. **Equipment and other supplies:**

- i. Stereo-binocular microscope and other routine items for fly handling.
- ii. Vertical gel electrophoresis system with power supply.
- iii. Homogenizer.
- iv. Sonicator.
- v. Microfuge tubes, Pipettes and corresponding tips.
- vi. Cold centrifuge.
- vii. Ice tray.

C. **Chemicals and reagents:**

- i. Radiomunoprecipitation assay (RIPA) buffer (the buffer should be prepared fresh and chilled before use)
 - 50 mM Tris
 - 1% Triton-X 100
 - 0.5% Sodium Deoxycholate
 - 150 mM NaCl
 - 0.1% Sodium dodecyl sulfate (SDS)
 - 2 mM Ethylenediaminetetraacetic acid (EDTA)
 - Protease Inhibitor Cocktail (PIC)
- ii. SDS page gel: for 8.6 X 6.8 cm size

Resolving gel (for 10 mL)

Acrylamide (40%)	2.5 mL
1.5 M Tris pH 8.8	2.5 mL
10% SDS	100µL
10% Aammonium persulfate (APS)	50 µL
Tetramethylethylenediamine (TEMED)	5 µL
Autoclaved dist. water	4.845 mL

Stacking gel (for 10 mL)

Acrylamide (40%)	1 mL
0.5M Tris pH 6.8	2.5 mL
10% SDS	100 µL
10% Aammonium persulfate (APS)	50 µL
TEMED	5 µL
Autoclaved dist. water	4.845 mL

iii. Sample dye (1X) (for 10 ml)

0.5 M Tris pH 6.8	2.5 mL
10% SDS	2 mL
Glycerol	1 mL
0.1% Bromophenol blue	1 mL
β -Mercaptoethanol	0.2 mL
Autoclaved dist. Water	4.845 mL

iv. Protein molecular weight marker: molecular weight range from 10 kda to 250 kda, to monitor protein separation during electrophoresis.

v. Running buffer (10X)

Tris	30 gm
Glycine	144 gm
SDS	10 gm

Make up the volume to 1 L with autoclaved distilled water.

For 1X running buffer, take 100 mL of 10X stock and make up the volume to 1000 mL with distilled water.

vi. Transfer buffer (10X)

Tris	30 gm
Glycine	144 gm

Make up the volume to 1 L with distilled water.

To make 1X transfer buffer, add 100 mL of the 10X solution to 700 mL of autoclaved distilled water. Add 200 mL of Methanol and mix well to make the final volume to 1000 mL

vii. Blocking buffer: TBS-T (5X)

Tris HCl (1 M) pH 7.6	10 mL
NaCl	4 gm

Make up the volume to 100 mL with autoclaved distilled water.

To make 1X buffer: take 20 mL of 5X stock + 80 mL of autoclaved dist. water. To this add 500 μ L of Tween 20 and mix thoroughly with the help of magnetic stirrer.

Bovine serum albumin (BSA) or non-fat dried milk in TBST buffer is commonly used as blocking agent. To make 100 ml of blocking buffer, dissolve 5 gm of BSA or non-fat dried milk.

viii. Chemiluminescent solution

Chemiluminescent detection method depends on incubation of the western blot with a substrate that will luminesce when exposed to the reporter on the secondary antibody.

The light is then detected and image captured by CCD camera or by a photographic film. These are to be used following recommendations of the manufacturer.

Experimental Details:

1. Collect flies of the desired genotype, age and sex.
2. Snap freeze the flies by transferring them into a tube kept on ice without anaesthetization.
3. Transfer flies on a slide kept on ice in a Petri dish under the stereo binocular microscope and using a sharp needle or scalpel separate the heads from bodies. Collect 50 heads into a cooled labelled microfuge tube. Work on ice to keep the flies frozen.
4. Add 160 μ L of freshly prepared chilled RIPA buffer to the microfuge tube.

5. Homogenize on ice. First crush the heads in the tube initially by hand using a pestle (push down any head that may stick to walls of the tube into the buffer) and then homogenize well with homogenizer.
6. Sonicate for 20 sec on ice (2 pulses each of 10 sec at 30% amplitude).
7. Centrifuge at 13000 rpm for 5 min at 4°C.
8. Transfer supernatant into a fresh microfuge tube.
9. Centrifuge again at 13000 rpm for 5 min at 4°C.
10. Collect the supernatant into a fresh labelled microfuge tube and store the sample at -70°C or -80°C.
11. Estimate total protein concentration by spectrophotometry using bovine serum albumin (BSA) as a standard to plot the standard curve, against which the concentration of the protein sample is calculated.
12. The sample is mixed with sample dye and boiled for 5 min Sample is now ready for electrophoresis.
13. Assembly of Gel Apparatus:
 - i. Wash the glass plates, spacers, combs (1.5 mm width) under running water. Wipe clean with soft tissue paper, without leaving any fibre on any surface.
 - ii. Allow to dry and wipe with absolute alcohol.
 - iii. Clip together the glass plate and spacer plate and fit into the casting apparatus. Check the seal with water. Place the gel caster on a vibration-free table.
 - iv. Mix the gel components for running gel of desired Acrylamide concentration (see **Chapter 66**).
 - v. Add mix to the gel caster, approximately filling till $\sim\frac{3}{4}$ of its height. No air-bubble should be trapped in the solution between the glass plates. Any trapped bubble can be removed either by gentle tapping on the glass plate or with a thin (clean) metal wire. Save the remaining gel solution to assess its polymerising properties (in terms of the time taken by the gel solution to polymerise so that disturbing the resolving gel before it is polymerizes is avoided).
 - vi. Overlay the gel with isopropanol (approx. 500 μ L).
 - vii. When set (about 20 min) remove isopropanol and wash 3 times with autoclaved water.
 - viii. Mix the components for stacking gel solution and carefully pour the stacking gel solution over the polymerized resolving gel.
 - ix. Insert the comb in the stacking gel solution to appropriate depth and allow the solution to polymerize (about 20 min).
 - x. Assemble the polymerised gel assembly into the gel tank and fill the lower tank with \sim 1 L filtered 1X running buffer.
 - xi. Gently remove the comb from the upper/stacking gel and thoroughly wash the wells with 1X running buffer to remove any debris. Fill the upper tank with the 1X running buffer.
14. Load 40 μ L of the sample into each well. Take care that the samples should not mix between the wells while loading. Load 5 μ L of the pre-stained protein molecular ladder in one of the wells. Run the gel at 20 mAmp constant current at room temperature.

15. Stop the power when the Bromophenol Blue dye front reaches bottom of the gel and remove the glass plates holding the gel from the electrophoresis tank.
16. Remove the gel from glass plates and transfer it into a tray containing the transfer buffer.
17. Cut a piece of appropriate size of the transfer membrane (Polyvinylidene difluoride (PVDF) or nitrocellulose). If a PVDF membrane is used for transfer, soak it in methanol for 30 sec to activate the membrane. However, if using a Nitrocellulose membrane, methanol soaking is not required.
18. Set up the transfer apparatus (**Fig. 2**) as follows:
 - i. Soak the transfer pads/sponge, membrane and filter stacks in the transfer buffer in separate containers.
 - ii. Place the transfer cassette in the buffer tray.
 - iii. Place one of the transfer pads/sponge on the –ve pole side of the cassette (**Fig. 2**).
 - iv. Using a roller, gently remove air bubbles below and in the sponge. Roll in one direction only.
 - v. Place one set of filter stacks (of same size as the cassette platform) on the pad. Remove air bubbles by rolling the roller pin over the filter stack in one direction gently.
 - vi. Gently place the gel on the filter stack, without trapping any bubbles underneath the gel.
 - vii. Gently place the activated PVDF or the nitrocellulose membrane over the gel. Mark orientation notches on both the gel and membrane in same orientation and side (i.e., start of the loading order). Once the membrane is on the gel do not disturb it. Gently remove air bubbles using roller in one direction. Take care that the membrane should cover the gel completely, without any overhang on any side.
 - viii. Place the upper filter stack on the membrane. Remove air bubbles.
 - ix. Place the upper transfer pad over the filter stacks to complete the transfer sandwich (**Fig. 2**). The transfer pads, gel, membrane and the filter stacks should all be within the cassette with no edges hanging out of the cassette.
 - x. Close the cassette and place inside the transfer chamber with the membrane side of the cassette facing the + ve electrode (**Fig. 2**).

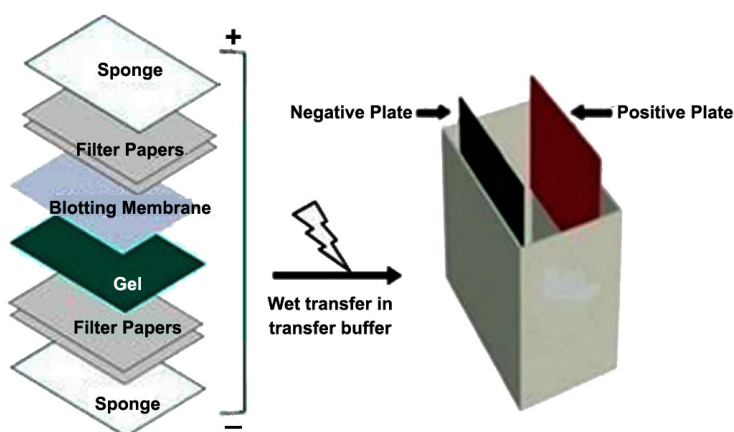


Fig. 2. Assembly of sandwich for western blotting. The membrane is placed between the gel surface and the positive electrode in a sandwich (shown on left) so that in an electric field perpendicular to the surface of the gel, proteins move out of the gel and get trapped onto the blotting membrane.

- xi. Fill the tank with chilled 1X transfer buffer. Place the apparatus in an ice bucket and pack the bucket with ice so that it completely surrounds the transfer tank. Take care that the electrode point connecting to the power supply do not get wet with ice.

- xii. Turn on the power supply at 90 V for 90 min (for an overnight transfer, apply 10 V).
- xiii. After the transfer, disassemble the transfer cassette and place the membrane in blocking buffer (blocking prevents non-specific binding of antibodies to the membrane, which has high affinity for proteins, including antibodies; thus, blocking reduces the background signal).
- xiv. Incubate the membrane in blocking buffer at room temperature for 1 hr.
- xv. Incubate the membrane overnight at 4°C in 10 mL primary antibody dilution buffer containing the desired primary antibody (at appropriate dilution as recommended in the product datasheet) with gentle agitation.
- xvi. After the antibody binding, wash the membrane three times for 5 min. each with TBST.
- xvii. Incubate the membrane with HRP conjugated secondary antibody (at the appropriate dilution as recommended in the product datasheet) in 10 mL of blocking buffer with gentle agitation for 1 hr at room temperature.
- xviii. Wash three times for 5 min each with of TBST.
- xix. Signal detection: After washing, place the membrane inside a clean and transparent poly bag pouch and add the chemiluminescent solution and seal the three sides of the pouch. Remove excess substrate and place in a geldoc imaging system for detection of signal. Alternatively, chromogenic substrates can be used for easy detection. HRP (horseradish peroxidase) catalyses conversion of a chromogenic substrate like 3,3',5,5'-tetramethylbenzidine (TMB), or 2,2'-azino-di-[3-ethylbenzthiazoline-6-sulfonic acid] (ABTS), or 3,3'-diaminobenzidine (DAB) to a coloured product which can be visualized/photographed.
- xx. Relative band densities of the detected signal can be quantified using a suitable software.

Observation:

Observe the band/s that show detectable signal in the sample lanes. Identify the molecular size of the positive band using the molecular sizes in the Marker lane as reference and confirm that this matches the expected size, in case of a known protein. Compare the intensity of signals for the same protein/polypeptide in different samples.

Troubleshooting:

Some of the commonly encountered issues in western blotting and their possible resolution steps are as follows:

A. Smiling of bands: this arises due to problem in migration of protein bands down the gel. This artefact can arise due to one of the following reasons: a) air bubbles in the gel, b) heat generated during running of the gel due to too high voltage or current.

- i. Air bubbles within the gel distort the migration of bands by distorting the flow of current through the gel matrix. Ensure that no air-bubbles are trapped while pouring the gel solution in the mould. If any is present, the same can be quickly removed by gentle tapping on outside of the glass surface.
- ii. A high voltage at the start of electrophoresis will force proteins of different sizes to enter and move through the gel matrix together for some time and thus cause nonlinear migration.

iii. Resistance in the gel, and therefore, heat generation is directly proportional to voltage/current. Therefore, a high voltage increases the temperature in the electrophoresis tank and distorts movement of proteins through the gel. Maintain appropriate voltage, keeping the gel dimensions in view. Keeping the gel apparatus in cold at 4°C in refrigerator or a cold chamber reduces heat induced smearing of bands.

iv. A large volume of loading samples can cause spill over into other lanes which would skew bands in those lanes.

B. Absence of bands: If the detection system shows no signal across all lanes except the ladder, the most common factor responsible is either poor transfer efficiency or poor probing.

i. The membrane, after transfer can be stained with Ponceau S to see all the protein bands. If bands are well defined on the membrane, the transfer efficiency is not an issue. If there are no bands, transfer settings need to be checked. If the transfer sandwich or the transfer cassette is wrongly oriented during the transfer process, proteins would move away from the blotting membrane and thus get lost.

ii. To determine the efficiency of transfer, the gel can also be stained after the transfer. Continued visibility of bands on the gel would indicate poor transfer.

iii. A washout of proteins can also occur wherein proteins from the membrane migrate to the filter paper. This is due to increased transfer time. By reducing voltage and/or transfer time, washout of bands can be prevented.

iv. Rechecking quality of transfer buffer, increasing transfer settings, and ensuring proper contact of gel and membrane can resolve the issue of poor transfer.

v. If a positive control lane also has no band/s, problem may be with the detection reagents, poor antibody, or an inappropriate antibody dilution.

C. Multiple Bands: Presence of multiple and/or unexpected bands in the blot may suggest a non-specific binding of antibodies. Washing may need to be more stringent. In addition, titration experiments should be performed to optimize the dilution/detection parameters.

D. High background with or without splotches: Poor membrane blocking, excessive antibody concentration, and a dry membrane can result in high background signal.

i. Increasing the period for blocking or changing the blocking agent in blocking buffer may resolve this.

ii. Insufficient washing can result in high background signal as well and are a major cause for splotches on the membrane.

iii. Membrane must be maintained wet throughout the experiment since a dry membrane can give high background signals.

Questions for Self-Study and Interpretation:

1. What are the differences between the Western, Southern and Northern blotting methods and their usages?

2. What are the various detection methods used for quantification of proteins in western blots?

3. What are the roles of APS and TEMED in SDS-PAGE?

4. How is western blotting useful in testing for diseases e.g., HIV, BSE (Bovine Spongiform Encephalopathy or mad cow disease), Hepatitis B infection etc?

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Chapter 69

Analysis of male fertility in *Drosophila*

Tushna Kapoor (tushna27@gmail.com) and Krishanu Ray (krishanu@tifr.res.in)*
Department of Biological Sciences, Tata Institute of Fundamental Research, Mumbai.

*For correspondence

Recommended Level: PG/Research

Sub-discipline: Genetics/ Developmental Biology/ Cell Biology/Molecular Biology/
Physiology/ Reproductive Biology

Background:

The reproductive success of an organism is defined as fertility. It refers to the output or success of reproduction. On the other hand, fecundity refers to the ability to reproduce. Similarly, infertility refers to the inability to retain a reproductive success, whereas sterility is the inability to reproduce. Several factors affect both the fertility and fecundity of an organism. These include, genetic, physical, hormonal, environmental and lifestyle, among others .

The ability of a male to be fertile or fecund depends on the continuous production of healthy sperms. Spermatogenesis involves the development of mature sperms from germline stem cells. It is regulated at multiple levels. Perturbations at any of the stages can be detrimental to fecundity. Stem cells undergo multiple rounds of mitosis. The number of mitotic divisions is species-specific, for example, spermatogonia in rats/ mice undergo ten rounds of mitosis, whereas *Drosophila* spermatogonia undergo four rounds of mitosis. After these mitotic divisions, all spermatogonial progeny go through one round of meiosis. Post meiotic spermatids have a haploid content of chromosomes, i.e. 'n', as opposed to '2n' in all other cell types. Spermatids go through a series of maturation events, such as the formation of a sperm tail, nuclear shaping, chromatin condensation, acrosome formation and extrusion of excess cytoplasm, before being released for storage in the specialised organs - the epididymis in mammals, and the seminal vesicle in *Drosophila* testes (**Fig. 1**, also see **Chapter 107**). Sperm cells have a genome that is quite different from that of the body cells. Therefore, the immune system considers them as 'foreign'. A set of specialised cells in the testes called the Sertoli cells in mammals, and somatic cyst cells in *Drosophila* provide a unique micro environment and nutrition to the developing spermatids and keep away the elements of body fluids such as blood cells and the immune system (**Fig. 1**). The enclosure formed by the Sertoli cells and somatic cyst cells also regulate the secretion

of growth factors and hormones and provide structural support. Regulation of hormonal factors, cell signalling, specialised sub-cellular cytoskeletal structures and dynamic cell-cell adhesion are all essential for successful sperm development and maturation. Cellular or molecular disruption to any of the intermediate steps can block or perturb the sperm development process. The underlying genetic conditions or environmental factors, such as diet, pollutants (such as dioxin), exposure to heavy metals (such as cadmium and lead), temperature and sedentary lifestyle are known to alter the fertility.

Drosophila is a good model organism to study the effect of genetic and environmental effects on sperm development since the process is very similar to that of the mammalian system. Fertility can be assayed easily by counting the number of progeny from a mating event. This is usually performed by keeping the male and virgin female flies together for a fixed period of time and counting the fertilised embryos/larvae. The ease of performing genetic, molecular and cell biological manipulations is an excellent advantage in this system.

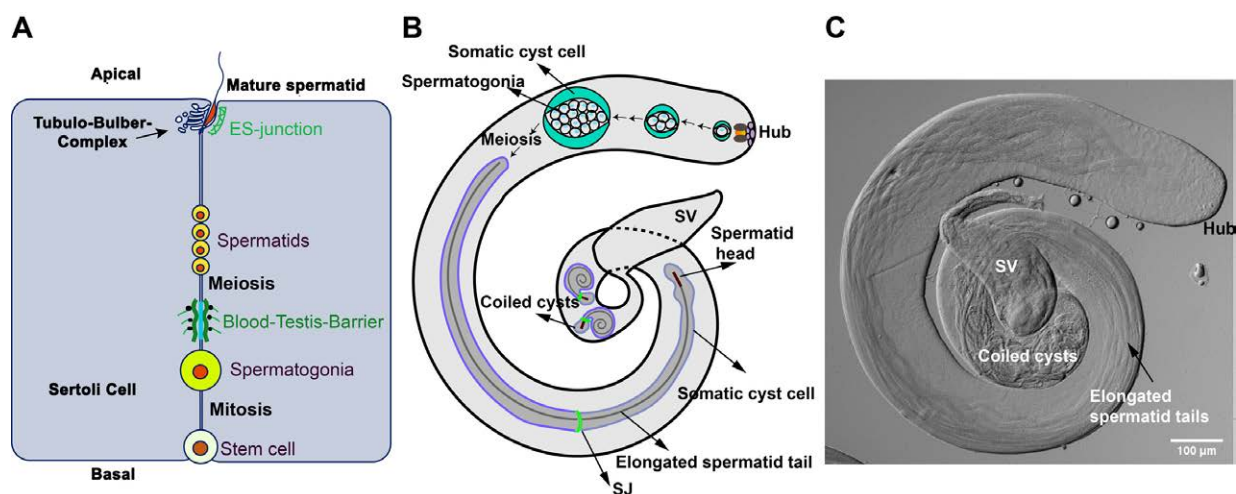


Fig. 1. Sperm development in mammals and *Drosophila*. **A.** Schematic of the progression of spermatids as they develop from the basal to the apical side of the enclosure formed by Sertoli cells in mammalian testis. **B.** Schematic of spermatid development in *Drosophila* testis. **C.** Image of a *Drosophila* testis (under DIC optics). SV = seminal vesicle; SJ – septate junction (a tight junction that forms between the somatic cyst cells); Hub indicates the stem cell niche.

A highly prolific organism, each healthy young (0-6 days old) *Drosophila* female, after one mating with a healthy young (0-10 days old) male, lays large number of eggs during its peak oviposition period (between days 4-7 after emergence). The actual numbers of eggs laid per day varies over a wide range, being dependent upon diverse factors, like composition of the food, the amount of male ejaculate transferred during each mating, the composition of accessory gland peptides in the male ejaculate, the sperm activity and various other conditions. These properties make it a suitable model to identify and study the effects of environmental factors, genetic pathways, age, diet, etc., on the rate of sperm production and fecundity/fertility.

In the following sections, we discuss the strategy for measuring male fertility index and its usefulness. This assay provides an estimate of the male fertility and correlate it to the factors causing the defect or change. A successful adaptation of this procedure will allow

the student to find out whether the presence of certain chemicals such as heavy metals or natural herbal products in the food could reduce or enhance fertility. Similarly, the student could also determine whether a mutation in a gene affects fertility and use this parameter to map the location of the mutation in the *Drosophila* genome. Besides, this assay has the potential to monitor environmental factors affecting male fertility. For this purpose, one may collect wild fruit flies from different locations outside the lab and study their fertility index and compare them

Objectives:

To measure the fertility of male *Drosophila* in different genetic and environmental conditions

Materials Required:

With the lab adapted strains of *Drosophila Melanogaster*.

A. Fly Strain Required:

- i. Wild-type flies (Canton S, Oregon R)
- ii. Mutant flies (as per experimental requirement). Some male sterile stocks, available from the Bloomington Stock Centre are: BL-25156- *ms(2)35Eb*; BL-11773- *P{PZ}Rcd-1r07717CG1310207717cn1/CyO*; *ry506*

B. Equipment and other fly handling items:

- i. Stereo-binocular microscope
- ii. Incubator
- iii. Vials containing fresh fly food
- iv. Anesthetization apparatus
- v. Cotton
- vi. Petri-dish
- vii. Nylon mesh/strainer (mesh size 0.2 mm)
- viii. Soft brush for handling flies
- ix. Sterilised distilled water
- x. Tap water
- xi. Marker pen

Experimental Details:

1. Collect male flies, which have just emerged from the pupal case (freshly eclosed, naive), of the desired genotype(s)/ isolate(s) to be assessed. These flies can be identified by the dark green spot in their abdomen, called meconium (**Fig. 2**), after anaesthetisation.

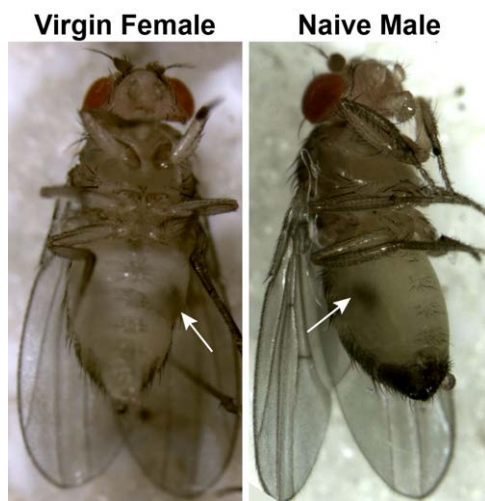


Fig. 2. Virgin female and naïve male within 6 hr of emergence from the pupal case. Arrows point to the meconium in the abdomen. The presence of meconium defines the fly's age to be less than 6 hr. They become competent to mate after shedding the meconium.

2. Keep each naïve young male fly in a separate vial and label the vials as per the genotype/isolate. Use at least ten such vials containing solitary males for each genotype/isolate/treatment.

Note: Steps 3-6 below are required only when effects of factors like temperature or age or diet or other environmental perturbation on the male fecundity are to be examined. These steps allow the clearing out of sperms that were produced before the males are subjected to any desired perturbation. If an experimental perturbation or genetic background is being examined for affecting male fertility, appropriate control (males not exposed to the experimental perturbation or wild type males, respectively, should be used as Control. *Proceed directly to step 7 if assaying the fertility of male flies without any perturbation*

3. In each vial, add three wild-type (Canton S or Oregon R) 2-3 days virgin females.

4. Allow the flies to mate for 48-72 hr as per the requirement of the experiment (see Note below), at the desired temperature, to clear out the accumulated sperms.

5. Transfer the flies daily into fresh food vials to facilitate females to lay eggs on media with freshly fermented yeast.

6. After 48-72 hr, discard the female flies.

7. Transfer each male fly to fresh vial (appropriately labelled with the genotypes) with three wild-type young (not more than 2-day old) virgin female flies.

8. Note the time, and keep the vials at an appropriate temperature with 12-hr alternate light-dark cycles or natural light, undisturbed for 24 hr. Lower the cotton plug, so as to confine the flies in a smaller space (**Fig. 3**).

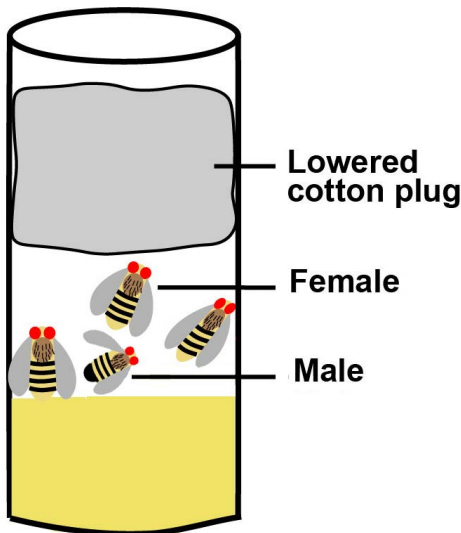


Fig. 3. One (test) male and three wild type virgin female *Drosophila* flies incubated for 24 Hr in a vial, with the cotton plug lowered.

9. After 24 Hr, discard/ transfer all the flies, and keep the vials back at an appropriate temperature, as required for the experiment.

10. Check the vials daily. Once the larvae start to crawl out of the food, add a few drops of sterilised distilled water if the food appears dry.

11. After 7-8 days, once all the larvae have crawled out and pupated on wall of the vial, count the number of pupae in each vial. Using a marker pen, label 1,2,3... next to each pupa as you count them. Later, count all the flies (males and females separately) that emerge in the vial.

Note: Certain conditions/ genotypes could cause lethality either at the embryo, larval or pupal stages in the F1. In such cases, count the number of eggs/larvae in the food within

24-48 hr. For extracting the embryos and larvae, add approximately 1 mL of tap/distilled water onto the media, gently agitate the water on the media using a soft fine brush, or just by gently shaking the vial. Collect the wash off into a petri-dish. Repeat the process 2-3 times to ensure extraction of all the eggs/larvae. Then strain the wash off using a nylon strainer (less than 0.2 mm mesh size), freeze them on an ice-salt mixture for 10 min, and transfer them onto a 0.8 mm thick glass slide using a wet soft brush. Take precaution to ensure that there are no larvae/embryos stuck on the mesh/ paint-brush. Arrange the eggs/larvae in ordered rows using the wet paint-brush, allow them to briefly dry, apply a tiny drop (~50 μ L) of halocarbon/paraffin oil, and mount a 22x40 mm coverslip (Fig. 4). Then count the number of eggs/larvae from each vial under a microscope (preferably under phase-contrast optics) with a low power (4X/10X) objective. To verify that the eggs are fertilised, the images in Campos-Ortega *et al.*, (1997) can be referred to. The embryos can also be examined when mounted in water.

Results/Observations:

The number of progeny derived from each male is counted, and the average number is calculated for each genotype/ condition. Statistical tests can be performed to calculate the significance between average values of control and test samples. Results can be represented in the form of bar graphs.

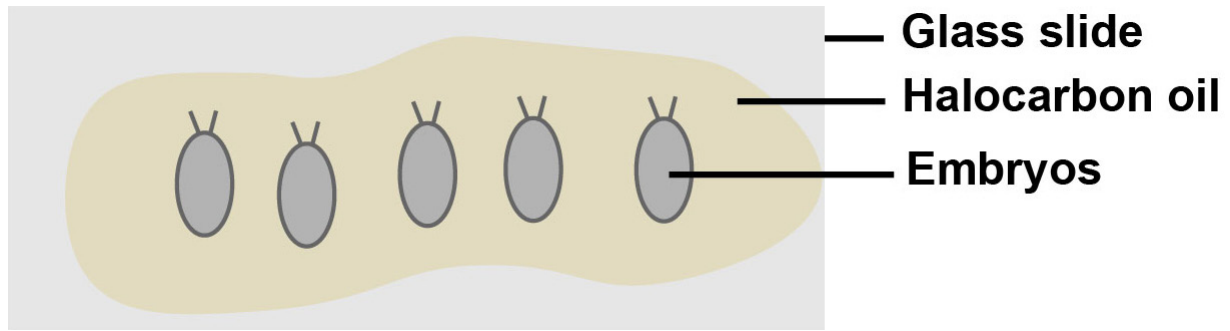


Fig. 4. Embryos aligned on halocarbon oil, on a glass slide, for analysis under the microscope. Schematic not to scale.

Further experiments and questions for self-study:

1. Can fertility vary with the age of the male fly – how would you test this?
2. Would the male fertility vary with rearing temperature? How can this be tested?
3. Would different kinds of food or different proportions of yeast extract/ sucrose, affect male fertility?
4. Would it affect the outcome of the experiment if the females were not virgins when introduced in the test vials? Why or why not?
5. Why is testis an immune-privileged organ?

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Chapter 70

Fecundity assay as a readout for xenobiotic toxicity and for genetic factors that affect female reproduction

Amartya Mukherjee (amartyam@iisc.ac.in) and Upendra Nongthomba* (upendra@iisc.ac.in)

Department of Molecular Reproduction, Development and Genetics, Indian Institute of Science, Bengaluru 560012.

* For correspondence

Recommended Level: PG/Research

Sub-discipline: Developmental Biology/ Physiology/ Behavioural Biology/ Ecology/ Toxicology

Background:

Fecundity of female *Drosophila* may be assayed by the number of eggs laid per day (or any other fixed period of time), under given environmental conditions. As such, this core life-history trait is closely tied to the reproductive success of an individual, and, on an evolutionary timescale, its potential to perpetuate its own species (Flatt, 2011). A host of dietary, physiological, genetic, and signalling pathways typically converge to modulate egg production (Partridge, *et al.*, 2005). Although the proximate bases of fecundity remain largely unexplored, the rate of egg production appears to be orchestrated by the secretion of specific neurohormones, such as, octopamine, corazonin, and dopamine (Nässel and Zandawala, 2019). Akin to many tissue types, there occurs an age-dependant decline in the productivity of the *Drosophila* female ovary as well (Miller, *et al.*, 2014). Moreover, fecundity is also affected by myriad other factors, including biotic ones, such as, male accessory gland contents, parasitic infection, endosymbiont persistence, etc., and abiotic ones, such as, photoperiod, light intensity, laying temperature and relative humidity, substrate hardness, etc. (see [Chapter 69](#) for how to analyse male fertility, and [Chapter 88](#) for more on rhythmicity in egg-laying). Interestingly, culture condition is one of the most important parameters governing fecundity, as shown by the studies on the effects of crowding and adult body size. Thus, the fecundity assay provides an elegant, scalable readout for assessing variation in fitness, and identifying the various correlates, biological or otherwise, of reproduction in *Drosophila*. Moreover, the assay can be used to address the adverse effects of environmental contaminants, xenobiotics, food adulterants, etc. on the female reproductive health, and to test the efficacy of alternative medicine, novel compounds, etc., which may aid in enhancing fertility.

For using this assay for toxicity testing, the following issues are to be considered: (1) identification of the chemical toxicant/xenobiotic to be used (for classroom study use

any of the chemicals like Cypermethrin, Cadmium, Ethylparaben, Lepidiline, etc. or other desired chemical/toxicant may be used); (2) mode of delivery of the chemical to the organism, and (3) the developmental stage for the treatment. Larvae and adult flies are readily exposed to chemical compounds by mixing in the fly food at the desired concentrations and letting larvae/adult feed on the supplemented food for the desired duration. Adult flies may also be exposed to vapours and aerosols of volatile organic solvents, such as, Benzene, Toluene, etc., in a controlled environment (Rand, 2010). The method described here makes use of a chemical that can be fed to larvae and/or adults after mixing in food.

This method can also be used to identify mutations that affect oogenesis and thus fecundity.

Objectives:

To use the *Drosophila* female fecundity as a quantitative read-out for physiological and metabolic changes due to food and environmental factors, or the genetic determinants of reproduction.

Materials Required:

A. Fly Strains: Wild-type flies (Canton-S, or Oregon-R) reared on normal or xenobiotic (e.g. Cypermethrin) supplemented food

B. Equipment and other materials:

- i. Stereo-binocular microscope and other items for culturing and handling flies/larvae
- ii. Reusable 100 mm polystyrene Petri dishes
- iii. Egg-laying chambers (see **Fig. 1** and description below)
- iv. Active dry yeast (granules/powder/tablets)

Experimental Details:

1. Treatment of larvae/flies with toxicant:

- i. Identify the test chemical to be used for the assay. Prepare *Drosophila* food medium (total quantity depends upon the number of vials to be prepared) and add the desired quantity of identified chemical in the warm food while it is still fluid and mix well (a concentration series of the chemical supplemented food plates are to be prepared). Pour the partially cooled but fluid food in vials for a height of ~ 1 inch. Control food vials, without the chemical added, are also prepared in parallel. Carefully, label the vials to indicate experiment number and control/treatment, etc., as required.
- ii. To expose to the identified chemical from the beginning of larval development, fifty first instar larvae of the desired genotype are transferred to each control and experimental (with medium containing the toxin of interest) vial. Vials seeded with larvae are placed in a BOD incubator (preferably with at ~60% RH) at 25°C. Development to pupal stages and emergence of flies from pupae is examined (see **Chapter 49**).

Exposure of larvae of some genotypes to some chemicals during the larval period may severely affect development so that very few adults emerge. In some cases, the experimenter may want to examine effects of feeding adults only. In such cases, feeding can be restricted to adult stage by transferring freshly emerged flies, which were reared during the larval period on normal control food, to vials containing food supplemented with varying concentrations of the test chemical for at least 3-5 days prior to mating and egg collection.

2. Preparation of the egg-laying chamber:

- i. Take a transparent (or semi-transparent) 500 mL plastic Erlenmeyer flask and cut its base out. Wipe the inside surface of the flasks with the base cut off with 70% ethyl alcohol and close its mouth with a cotton plug, tight enough to prevent entry of undesired fly or any other contamination, but loose enough to offer gaseous exchange.
- ii. Replace the lid of a food agar Petri dish with plugged (cotton plug) cut bottom plastic flask.
- iii. Place this set-up on top of the inverted lid of the Petri dish, and secure the configuration in place by sealing the junction of the lid, plate and flask's cut margin using an adhesive tape (e.g., electrical adhesive tape). The egg-laying apparatus is ready for use (**Fig. 1**).

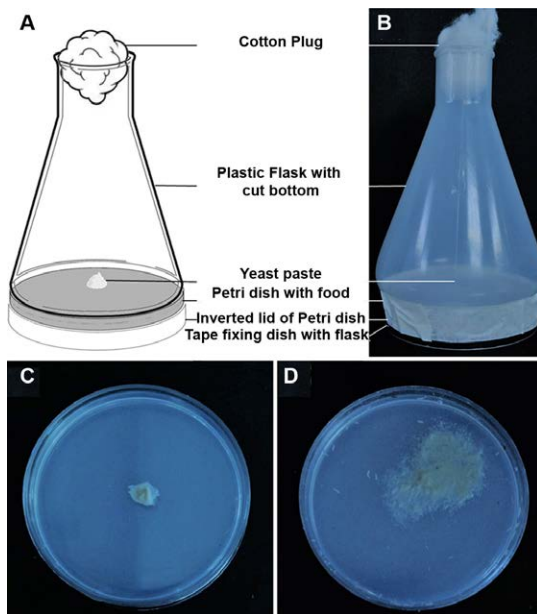


Fig. 1. A. Diagrammatic and actual B. image of the egg-laying chamber. C and D show the agar plate before and after egg-collection, respectively.

3. Preparation of Petri dishes for egg collection:

Prepare *Drosophila* food medium (total quantity depends upon the number of Petri dishes to be prepared) and pour the partially cooled but fluid food in the lower half of polypropylene Petri dish until it covers the bottom (~ 30 mL). Immediately cover the Petri dish with its cover and let the food cool and solidify. This medium is without the test chemical.

Alternative egg collection medium based on agar plates: For preparation of agar medium for egg collection Petri dishes, mix 15 gm Agar (regular bacteriological grade) and 5 gm sugar in 360 mL of water and boil till agar is completely dissolves; let the food cool till it

is fluid but safe to touch (50°-60°C). Add 1 mL propionic acid, mix well, pour in cleaned 100 polypropylene Petri dishes and let the agar solidify.

Mix active dry yeast and some water with a brush in a dish to prepare some fresh yeast paste. Adjust the ratio of yeast and water until the consistency of a cake frosting is obtained. Apply a dab of the prepared yeast paste to the centre of each food/agar plate using a brush (**Fig. 1C**). Recap each plate.

4. Setting the egg-laying:

- i. For each test, 15–20 freshly-eclosed females, reared during the larval period on control and experimental food, respectively, should be collected and mated with males of same genotype reared on normal control food for 2–3 days, prior to egg collection. If the females were exposed to the test chemical only during the adult stage, they are mated, 3-5 days after feeding on the chemical-supplemented food with males of same genotype reared on normal control food.
- ii. For starting the egg collection, remove the cotton plug of the egg-laying chamber and gently tap the control/experimental flies (50 flies in each case) into respectively marked chambers without anaesthesia to preclude anaesthesia-induced artefacts.
- iii. Allow flies to lay eggs undisturbed for a period of 12 (or less) hr at 24°-25°C.

5. Removing the flies after egg-laying:

- i. After the egg-collection period is completed, tap the egg-chamber so that all flies fall to its lower part, quickly open the cotton plug of the flask and transfer flies to a fresh bottle. These flies may be reserved for further experiments or discarded, as required.
- ii. Strip away the adhesive tape holding the egg-collection apparatus together.
- ii. Remove the cut-bottom flask from on top of the plate and replace the Petri dish lid on its bottom half containing the eggs.

Results/Observations:

1. Egg collection should be carried out with at least five biologically independent sets of flies for control and each experimental condition, respectively. Egg-laying conditions (number of flies, temperature, duration of egg collection, etc.) must be kept similar in all cases.
2. Count the number of eggs laid on each of the Petri dishes.
3. Calculate the **mean fecundity (MF)** using the formula, **MF = number of total eggs laid / number of females in the chamber** and tabulate data for control and different experimental sets.
4. Graphically plot the average number of eggs laid per female over a period of certain numbers of days for each genotype or treatment regimen.
5. Appropriate statistical analysis, such as a Student's t-test, or ANOVA, may be performed between the two or more data sets, respectively, to ascertain whether the difference in mean fecundity is statistically significant.

Note: The above assay may be extended to measure fertility, i.e. to determine how many of the laid eggs were fertile and developed to hatch as larvae. For this purpose, after counting the number of eggs on each plate, it may be further incubated at 24°/25°C and checked 24-30 hr later to count the number of eggs which actually hatched. Fertility should be plotted as percent eggs hatched out of the total eggs laid.

The fecundity assay can also be used to identify mutants that affect oogenesis or other aspects of female reproduction (Kairamkonda and Nongthomba, 2014).

Questions for self-study and interpretation:

1. Do you expect the composition and condition of the egg-laying medium to affect fecundity?
2. Discuss the differences you would expect between the fecundity of:
 - a) A virgin and a mated female
 - b) A young and an aged female
3. Do you think that fecundity may change if you were to increase or decrease the number of control flies in the egg-laying chamber or if you kept flies in a crowded culture?
4. Which categories of genes may be affected by the xenobiotics that reduce fecundity?

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Female Fecundity & Senescence

Presently, there exist several viable gene candidates influencing female fertility later in life. These genes are involved in varied processes including stress resistance, metabolism, insulin signaling, as well as novel functions and highlight the complicated nature of reproductive homeostasis. Because several studies show that reproduction and longevity can be uncoupled or are prolonged in tandem, the 2 do not appear to be fixed in a direct trade-off.

(In: Miller, P. B., Obrik-Uloho, O. T., Phan, M. H., Medrano, C. L., Renier, J. S., Thayer, J. L., Wiessner, G., & Bloch Qazi, M. C. (2014). The song of the old mother: reproductive senescence in female *Drosophila*. *Fly* 8: 127–139.
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Chapter 71

Estimation of total proteins in *Drosophila* using the Bradford method

Raifa Abdul Aziz¹ (raifaazeez@gmail.com), Deepa Mugudthi Venugopal¹ (deepavenugopalbvr@gmail.com), Ramesha Hanumanthappa² (rameshkoose18@gmail.com), Manvitha Kadandelu³ (manvithakashyap@gmail.com), Shamitha Shetty¹ (shettyshamitha1911@gmail.com), Avinash Kudva⁴ (avinash.kudva@gmail.com), Rajashekhar K Patil⁵ (patilsirmu@gmail.com), Kuramkote Shivanna Devaraju² * (ksdevaraju@kud.ac.in) and Shamprasad Varija Raghu^{1,3} * (shamprasadr@yenepoya.edu.in)

1. Neurogenetics Lab, Department of Applied Zoology, Mangalore University, Mangalagangothri, Karnataka 574199
2. Neurochemistry lab, Department of Biochemistry, Karnatak University, Dharwad, Karnataka 580003
3. Division of Neuroscience, Yenepoya Research Centre (YRC), Yenepoya (Deemed to be University), Deralakatte, Mangalore, Karnataka 575018
4. Department of Pediatrics, Division of Hematology/Oncology, College of Medicine, The Pennsylvania State University, Hershey, PA 17033, USA
5. Department of Zoology, Bangalore City University, Bengaluru, Karnataka 560001

* For Correspondence

Recommended Level: UG/PG/Research

Sub-Discipline: Biochemistry/Physiology

Background:

The concentration of total protein in *Drosophila* samples can be estimated using the Bradford protein assay. The assay is based on the principle that proteins bind to the Coomassie Brilliant Blue G-250 dye under acidic conditions, causing the dye to change color from brown to blue (Bradford, 1976). Coomassie dye is a negatively charged dye that binds to positively charged amino acids or proteins. In acidic conditions, the dye is protonated, which makes it more positively charged. This increased positive charge allows the dye to bind more tightly to proteins, resulting in a color change. The amount of color change is proportional to the amount of protein present, so the assay can be used to quantify protein concentration (Bradford, 1976).

However, it is important to note that the assay is not specific for proteins, and other substances that contain positively charged amino acids, such as peptides and nucleic acids, can also bind to the dye and cause a color change. Therefore, it is important to use a protein standard that is free of these contaminants when preparing the standard curve. Bovine serum albumin (BSA) is a protein that is often used as a standard in the Bradford protein assay (Zaguri et al., 2021). This is because BSA is a relatively pure protein that is available in a variety of concentrations. The absorbance of a BSA standard curve is measured at 595

nm; because the absorbance of the blue anionic form of Coomassie dye, which is formed when the dye binds to protein, is maximal at this wavelength (Chial et al., 1993). The absorbance of the standard curve will increase linearly with the concentration of protein in the sample, so it can be used to calculate the protein concentration of an unknown sample. By comparing the absorbance of the unknown sample with the absorbance of the BSA standard curve, the concentration of protein in the sample is estimated.

Objective: To quantify the total protein content in *Drosophila* using the Bradford assay.

Materials required:

A. **Flies:** Wild-type *Drosophila melanogaster* flies

B. **Equipment and other items:**

- i. Hand homogenizer (homogenize samples up to 1 to 2 ml)
- ii. Microcentrifuge
- iii. pH meter
- iv. UV-visible spectrophotometer.
- v. Micropipette and micro tips (10, 200, 1000 μ L)
- vi. Microcentrifuge tubes (1.5 mL)
- vii. Beakers (50 and 100 ml)
- viii. Whatman filter paper

C. **Reagents:**

i. **0.1M Phosphate buffer (PB) pH 7.4:**

Sodium phosphate dibasic heptahydrate 2.021 gm

Sodium phosphate monobasic monohydrate 0.3394 gm

Dissolve in 80 mL of distilled water, adjust the pH to 7.4 using diluted HCl or NaOH. Make up the volume to 100 mL with distilled water.

ii. **Bovine Serum Albumin (BSA) (1mg/ mL):** 1 mg of BSA dissolved in 1 mL of distilled water.

iii. **Bradford reagent:**

Coomassie Brilliant Blue G-250 (CBB) dye 10 mg

95% Ethanol 5 mL

85% Phosphoric Acid 10 mL

First mix Ethanol and Phosphoric Acid, add CBB and mix thoroughly until the dye is completely dissolved.

Filter the solution through Whatman filter paper No. 1.

Bring the filtered solution to a final volume of 100 mL with distilled water. The reagent can be stored at 4°C for up to 6 months.

Experimental details:

- i. **Starving the flies:** Transfer freshly emerged females or 4-5 days old fertilized females, maintained on normal food under uncrowded conditions, into vials containing filter paper soaked in distilled water for 24-hour starvation. The wet condition is to avoid dehydration and the mortality of the flies in different conditions. Keep parallel control flies in normal food vials.
- ii. **Preparation of fly homogenate:** Take 25 flies (for each rearing condition and age) in a microcentrifuge tube. Add 0.25 mL of 0.1 M PB and homogenize the sample using a hand homogenizer that fits the microcentrifuge tube.

Centrifuge the homogenate at 10,000 rpm for 15 minutes, and then transfer the supernatant to a fresh microcentrifuge tube for further analysis.

iii. **Determination of total protein concentration:**

a. Standard curve preparation:

- i. Prepare a dilution series of solutions with known BSA concentrations by taking 0, 2, 4, 6, 8 and 10 μL of 1mg/ mL BSA in separate microfuge tubes
- ii. Make up the BSA volume to 0.1 mL using 0.1M PB in each tube.
- iii. Add 1mL of Bradford reagent to each microfuge tube, close it and mix well by inversion.
- iv. Incubate at room temperature (30-35°C) for 30 minutes.
- v. Transfer the solution to spectrophotometer cuvette and measure the absorbance of each solution at 595 nm using a spectrophotometer.
- vi. Measure the protein concentration of blank and BSA samples in triplicate and determine the mean. Tabulate the observations as in Table 1.

Table 1. Determination of Standard curve for protein estimation

Sl. No.	Standard BSA (μL)	Phosphate buffe (PB) 0.1M (μL)	Concentration of BSA	Bradford reagent (mL)	Incubation at room temperature for 30 minutes	Mean absorbance at 595 nm
1	0	100	0	1 ml in each		
2	2	98	2			
3	4	96	4			
4	6	94	6			
5	8	92	8			
6	10	90	10			

b. Determination of protein concentration in fly samples:

- i. Take 5 μL of the desired sample homogenate in a micro centrifuge tube.
- ii. Add 95 μL of 0.1M PB pH 7.4 to the tube.

- iii. Add 1mL of Bradford reagent, close the tube and mix well by inversion. Do not vortex.
- ix. Incubate at room temperature for 30 minutes.
- x. Transfer the solution in the tube to cuvette and record the absorbance at 595nm using spectrophotometer.
- xi. Measure the absorbances of fly samples in triplicate, calculate the mean, and Tabulate the data as in Table 2

Table 2. Estimation of protein concentration in different fly samples

Sl.No.	Sample homogenate (µL)	Phosphate buffe (PB) 0.1M (µL)	Bradford reagent (mL)	Incubation at room temperature for 30 minutes	Mean absorbance at 595 nm
Control (Normal fed flies)	5	95	1		
Starved flies	5	95	1		

Results and Observations:

1. Plot the standard curve of absorbance values (Y-axis) against BSA concentrations (X-axis)
2. Using the standard curve, determine the concentration (as µg/5 µL) of total protein in samples of control and starved flies.

Questions for self-study and interpretation:

1. Is there a difference in total protein concentration between starved and normally fed flies? Why or why not?
2. Would the age of normally fed and starved flies affect protein concentration?
3. Would the treatment of flies with chemicals/drugs affect the protein content?

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Chapter 72

Determination of protein carbonyl content (PCC) by 2, 4-Dinitrophenylhydrazine method in *Drosophila melanogaster*

Ramesha Hanumanthappa¹ (rameshkoose18@gmail.com), Raifa Abdul Aziz² (raifaazeez@gmail.com), Deepa Mugudthi Venugopal² (deepavenugopalbvr@gmail.com), Avinash Kudva³ (avinash.kudva@gmail.com), Shamprasad Varija Raghunath^{2,4} * (shamprasadr@yenepoya.edu.in) and Kuramkote Shivanna Devaraju¹ * (ksdevaraju@kud.ac.in)

1. Neurochemistry lab, Department of Biochemistry, Karnatak University, Dharwad, Karnataka 580003
2. Neurogenetics Lab, Department of Applied Zoology, Mangalore University, Mangalagangothri, Karnataka 574199
3. Department of Pediatrics, Division of Hematology/Oncology, College of Medicine, The Pennsylvania State University, Hershey, PA 17033, USA
4. Division of Neuroscience, Yenepoya Research Centre (YRC), Yenepoya (Deemed to be University), Deralakatte, Mangalore, Karnataka 575018

* For Correspondence

Recommended Level: UG/PG/Research

Sub-Discipline: Biochemistry/Cell Biology/Neurobiology/Stress Biology

Background:

Proteins are oxidatively modified by a large number of reactive species, such as reactive oxygen species, lipid peroxidation-derived aldehydes, and reducing sugars (Colombo et al., 2016, Abe and Matsuki, 2000). The introduction of carbonyl groups such as aldehyde, ketone, and lactam into the amino acid side chains of proteins, as part of oxidative modifications, is a major hallmark for oxidative damage to proteins (Soglia et al., 2016). In Alzheimer's Disease (AD) and Parkinson's Disease (PD), protein carbonyls are a marker of oxidative stress in the brain. Their elevation in serum, plasma and tissues is proportional to the severity of the cognitive decline in AD and PD, and chronic stress. Oxidative stress can also damage DNA, RNA and lipids, leading to neuronal degeneration and impaired cognitive function (Luo and Wehr, 2009).

The present chapter describes a method for measurement of Protein Carbonyl Content (PCC) using the 2, 4-dinitrophenylhydrazine (DNPH) in *Drosophila* brain (Buss, et al., 1997). Carbonyl content is determined by the derivatisation of protein carbonyl groups reacting with DNPH and leading to the formation of stable dinitrophenyl (DNP) hydrazone adducts, which can be detected spectrophotometrically at 375 nm. Oxidative stress can be induced by feeding flies on Rotenone (for Parkinson's Disease) or Colchicine (for Alzheimer's disease).

Objective: To estimate the Protein Carbonyl Content (PCC) by 2, 4-dinitrophenylhydrazine method in *Drosophila* in the control, diseased and treatment conditions.

Materials required:

A. **Flies:** 2-3 days old wild type male or female flies

B. **Equipment and other items:**

- i. Stereo-binocular microscope and other fly handling items
- ii. UV-Visible spectrophotometer
- iii. Refrigerated high-speed centrifuge
- iv. pH Meter
- v. Refrigerator
- vi. Hand Homogeniser (to homogenize samples up to 1.5 to 2 mL)
- vii. Micro-volume pipettes (10, 100 and 1000 μ l)
- viii. Microfuge tube (1.5 and 2.0 mL)

C. **Reagents:**

- i. Yeast paste food: Immediately before starting the experiment, layer 0.5mL of 0.5% Yeast solution (5 g Yeast paste or granules mixed in 10mL Distilled water) on 1% agarose bed (mix 1 g Agar powder in 100 mL Distilled water by boiling, cool and pour in standard fly food vials to about 1 cm height) and plug the vials.
- ii. 20% Trichloroacetic acid: Dissolve 200 mg of Trichloroacetic acid in 10 mL of distilled water and it can be stored at room temperature.
- iii. 0.1 M Potassium Phosphate buffer (pH 7.4): Dissolve 12.119 gm of Potassium phosphate dibasic (K_2HPO_4) and 4.14 gm of Potassium phosphate monobasic (KH_2PO_4) in 800 mL distilled water. Adjust the pH to 7.4 using 0.1N HCl or 0.1N NaOH and make up the volume to 1000 mL with distilled water.
- iv. 2, 4-dinitrophenylhydrazine
- v. 6M guanidine hydrochloride: Dissolve 5.732 gm of Guanidine hydrochloride in 10 mL of distilled water and store at 4°C
- vi. Ethanol: ethyl acetate mixture: Mix 25 mL of Ethanol and 25 mL of ethyl acetate and store at 4°C.
- vii. Distilled water.

Experimental details:

- i. Collect 3-4 days-old adult male or female flies ($n = 60$) and divide them into two groups (control and treated group) of 30 male or female flies.
- ii. Feed the control flies on 0.5 mL of 0.5% Yeast solution for 5 days.

- iii. Feed the treatment group with the desired chemical (Rotenone/aluminium chloride/scopolamine/Colchicine that can induce oxidative stress) on 0.5 mL of 0.5% Yeast solution mixed with 10 mg of the desired chemical for 5 days.
- iv. After 5 days, anaesthetize and decapitate 20 flies each from the control and treatment groups, and immediately homogenize the heads in 100 μ L of 0.1M Potassium Phosphate buffer (pH 7.4) in hand homogenizer and centrifuge the homogenate at 10,000 RPM for 15 min at 4°C.
- v. Collect the supernatant in a fresh tube for further analysis.
- vi. Take 10 μ L of homogenate in a 2 mL microfuge tube. Add 250 μ L of 2,4-Dinitrophenyl hydrazine and mix them by vortexing at 500 rpm for 1 min. Incubate the mixture in the dark for 20 min.
- vii. Add 125 μ L of 20% TCA solution and incubate the mixture at -20°C for 15 min.
- viii. Centrifuge the reaction mixture at 4°C for 10 min at 9000 RPM.
- ix. Remove the supernatant and wash the pellet with ice-cold ethanol and ethyl acetate mixture (2-3 times).
- x. Resuspend the pellet in 1000 μ L 6M guanidine hydrochloride and take it in a 1 mL cuvette. Mix them well and measure protein carbonyl content by recording absorbance at 375 nm using a spectrophotometer.

Results and Observations:

- i. The amount of protein carbonyl content in the samples (control and treated) is calculated using the formula:

$$\text{nmol / mL} = \frac{\text{Reading of the sample}}{22} \times 1000$$

Note: Here, **22** is the Millimolar extinction coefficient of 2,4-dinitrophenylhydrazine (DNPH).

- ii. Final protein carbonyl content is expressed as nmol carbonyl per mg of protein, and can be calculated as follows:

$$\text{nmole Carbonyl per mg protein} = \frac{\text{nmole Carbonyl}}{\text{Protein content } (\mu\text{g})} \times 1000$$

- iii. The total protein content in the brain samples is calculated based on the method described in (**Chapter 71**).
- iv. Compare the protein carbonyl content in the control and chemically treated flies.

Questions for self-study and interpretation:

1. Why is protein carbonyl content expected to be different in control and chemically treated flies?
2. How is protein carbonyl generated in a living organism?

3. How is protein carbonyl generation regulated in the biological systems?
4. Does protein carbonyl formation affect all proteins in cells?

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Chapter 73

Determination of acetylcholinesterase (AChE) activity in *Drosophila melanogaster* using Ellman's method

Ramesha Hanumanthappa¹ (rameshkoose18@gmail.com), Raifa Abdul Aziz² (raifaazeez@gmail.com), Deepa Mugudthi Venugopal² (deepavenugopalbvr@gmail.com), Avinash Kudva³ (avinash.kudva@gmail.com), Shamprasad Varija Raghu^{2,4} * (shamprasadvr@yenepoya.edu.in) and Kuramkote Shivanna Devaraju¹ * (ksdevaraju@kud.ac.in)

1. Neurochemistry lab, Department of Biochemistry, Karnatak University, Dharwad, Karnataka 580003
2. Neurogenetics Lab, Department of Applied Zoology, Mangalore University, Mangalagangothri, Karnataka 574199
3. Department of Pediatrics, Division of Hematology/Oncology, College of Medicine, The Pennsylvania State University, Hershey, PA 17033, USA
4. Division of Neuroscience, Yenepoya Research Centre (YRC), Yenepoya (Deemed to be University), Deralakatte, Mangalore, Karnataka 575018

* For Correspondence

Recommended Level: UG/PG/Research

Sub-Discipline: Biochemistry/Neurochemistry/Cell Biology

Background:

The acetylcholinesterase (AChE) enzyme is responsible for the breakdown of the neurotransmitter acetylcholine (ACh) into choline and acetic acid, providing an endpoint for cholinergic neurotransmission (Rienda et al., 2023). The enzyme is distributed in a specific pattern in the brain and thus exhibits differential activity in accordance with the cholinergic synapses (Akinade et al., 2023). ACh is a major excitatory neurotransmitter in the central nervous system (CNS) of *Drosophila melanogaster*. Most of the neurons using ACh for communication with neighbouring neurons are characterized in the *Drosophila* brain (Raghu et al., 2011). The changes in acetylcholine transmission due to the effects of different toxic chemicals or neuromodulators can be analyzed and evaluated by measuring AChE enzyme activity in the *Drosophila* brain (Anupama et al., 2022).

Several methods have been developed to estimate the AChE activity using colorimetric, spectrophotometric, fluorometric and electrochemical approaches. Ellman's method is a reliable procedure for estimating the AChE activity (Ellman, et al., 1969). In this method, the substrate acetylthiocholine is hydrolyzed by AChE to generate thiocholine, which reacts with the 5,5'-dithio-bis-2-nitrobenzoic acid to generate a yellow-coloured complex (Williamson, et al., 2013). The amount of the produced yellow-coloured complex is assayed spectrophotometrically at 412 nm.

Objective:

To estimate the level of acetylcholinesterase (AChE) activity in the brain of control and chemically treated *Drosophila melanogaster* using Ellman's reagent.

Materials required:

A. Flies: 2-3 days old wild type adult male or female *Drosophila melanogaster*.

B. Equipment and other items:

- i. Stereo-binocular microscope.
- ii. UV-visible spectrophotometer.
- iii. Refrigerated microcentrifuge.
- iv. pH Meter.
- v. Hand homogeniser (to homogenize samples up to 1.5 to 2 ml).
- vi. Micro-volume pipettes and corresponding tips (10, 100 and 1000 μ L).
- vii. Microfuge tube (1.5 and 2.0 mL).

C. Reagents:

- i. Yeast paste food: Immediately before the start of the experiment, layer 0.5ml of 0.5% Yeast solution (5 g Yeast paste or granules mixed in 10ml Distilled water) on 1% agarose bed (mix 1 g Agar powder in 100 ml Distilled water by boiling, cool and pour in standard fly food vials till about 1 cm height) and plug the vials.
- ii. 0.1 M Potassium Phosphate buffer (pH 7.4): Dissolve 12.119 gm of Potassium phosphate dibasic (K_2HPO_4) and 4.14 gm of Potassium phosphate monobasic (KH_2PO_4) in 800 mL distilled water. Adjust the pH to 7.4 using 0.1N HCl or 0.1N NaOH and make up the volume to 1000 ml with distilled water.
- iii. 10 mM Potassium Phosphate buffer (pH 7.4): Dissolve 1.212 gm Potassium Phosphate Dibasic (K_2HPO_4) and 414 mg Potassium Phosphate Monobasic (KH_2PO_4) in 50 mL of distilled water. Adjust the pH to 7.4 using 0.1N HCl or 0.1N NaOH and make up the volume to 100 ml with distilled water.
- iv. 10 mM Ellman's reagent (5,5'-dithio-bis-2-nitrobenzoic acid): Dissolve 39.63 mg of 5,5'-dithio-bis- 2-nitrobenzoic acid in 10 ml of 0.1 M potassium phosphate buffer (pH 7.4).
- v. 8 mM of Acetylthiocholine: Dissolve 23.13 mg of Acetylthiocholine in 10 ml of 0.1 M potassium phosphate buffer (pH 7.4).
- vi. Rotenone or Pentylentetrazol in Yeast paste: mix 10 mg of Rotenone or Pentylentetrazole in in 0.5 mL of 0.5% Yeast solution. These chemicals interfere with neurotransmission (Coulom and Birman, 2004; Mohammad et al., 2009)

Experimental details:

- i. Collect 3 days old wild-type flies of any one sex (n = 60) and divide them into two groups (control and treated group) of male (or female) flies.
- ii. Feed the control flies with 0.5% Yeast solution for 20 days.

- iii. Feed the treatment group with Rotenone or Pentylentetrazol mixed yeast paste (10 mg of the desired chemical in 0.5 ml of 0.5% Yeast solution) for 20 days.
- iv. After 20 days, anaesthetize and decapitate 20 flies from the control and treatment groups. Homogenize the heads in 100 μ L of 0.1M Potassium Phosphate buffer (pH 7.4) in hand homogenizer and centrifuge the homogenate at 10,000 RPM for 15 min at 4°C.
- v. Collect the supernatant in the fresh tube for further analysis.
- vi. Add 660 μ L of distilled water in 1 mL of microfuge tube. Add 100 μ L of 10 mM Potassium Phosphate buffer (pH 7.4), 100 μ L of 10 mM Ellman's reagent and 100 μ L 8 mM of Acetylthiocholine. Mix them well using a vortex mixer.
- vii. Add the brain homogenate and dilute it in a ratio of 1:5 using 10 mM Potassium Phosphate buffer (pH 7.4) (10 μ L of homogenate and 40 μ L 10 mM Potassium Phosphate buffer). Add these diluted samples to the reaction mixture prepared from step vi.
- viii. Mix the tissue samples and reaction mixture. Take readings at 412 nm over a period of 5 min at 15-second intervals.

Note: 50 μ L of 10 mM potassium phosphate buffer (pH 7.4) is used as blank.

Table 1: Sample table for recording AChE activity at 412 nm

	Reaction Mixture	Brain homogenate	10 mM potassium phosphate buffe	Time points in seconds	Absorbance at 412 nm
Blank	960 μ L	0.0 μ L	50 μ L	300	
Control/Treated	960 μ L	50 μ L		15	
				30	
				45	
				60	
				75	
				90	
				105	
				120	
				135	
				150	
				165	
				180	
				195	
				210	
				225	
				240	
				255	
				270	
				285	
				300	

Results/Observations:

1. Observe the colour change from light yellow to dark yellow in control and treated samples.
2. Record the absorbance at 412 nm for every 15 seconds till 300 seconds (Table 1). The control and treated samples need to be recorded separately. The absorbance should increase with time.
3. Calculate the AChE activity using the following formula:

$$\mu\text{mole} / \text{min} / \text{mg enzyme} = \frac{\text{OD Sample} - \text{OD Blank} \times 1.01 (\text{Sample volume}) \times 5 (\text{DF})}{5 (\text{Time}) \times 13600 \times 0.05}$$

Notes:

1. Here, **1.01** is the total volume of the reaction mixture, **5** (DF) is the dilution factor, **5** (Time) is the total reaction time in min, **13,600** is the Millimolar extinction coefficient of Ellman's reagent (5,5'-dithio-bis-2-nitrobenzoic acid) and **0.05** is the volume of homogenate in the reaction mixture.
2. For the total activity, all the values are added together (15 seconds to 300 seconds) and averaged.
3. Total AChE activity is expressed as $\mu\text{mole}/\text{min}/\text{mg}$ of protein and calculated using the following formula:

$$\mu\text{mole} / \text{min} / \text{mg of protein} = \frac{\mu\text{mole} / \text{min} / \text{ml enzyme}}{\text{mg of protein} / \text{mL enzyme}} \times 100$$

4. The total protein content in the brain samples is calculated based on the method described in ([Chapter 71](#)).

Questions for self-study and interpretation:

1. What is the role of 5,5'-dithio-bis-2-nitrobenzoic acid in the determination of AChE activity?
2. How can estimation of acetylcholinesterase (AChE) activity help in diagnosing disease conditions?
3. How can Acetylcholinesterase activity be determined in different parts of the brain?

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Non-neuronal functions of Acetylcholinesterase

The classical role of AChE is to terminate cholinergic neuro-transmission by hydrolysis of ACh. However, the biological role of AChE is not limited to cholinergic transmission. The first evidence for additional functions for the AChE protein was based on the high levels of AChE observed in non-neuronal tissues such as blood cells, notably erythrocytes and mega-karyocytes, developing avian cartilage, developing oocytes and sperm, where a role for AChE in cholinergic transmission is difficult to imagine.

(In: Grisaru, D. et al. (1999). Structural roles of acetylcholinesterase variants in biology and pathology. *European Journal of Biochemistry*. 264: 672-686.
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Chapter 74

Estimation of Glutathione-s-Transferase (GST) in *Drosophila melanogaster* using 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate

Ramesha Hanumanthappa¹ (rameshkoose18@gmail.com), Deepa Mugudthi Venugopal² (deepavenugopalbvr@gmail.com), Raifa Abdul Aziz² (raifaazeez@gmail.com), Avinash Kudva³ (avinash.kudva@gmail.com), Shamprasad Varija Raghu^{2,4} * (shamprasadvr@yenepoya.edu.in) and Kuramkote Shivanna Devaraju¹ * (ksdevaraju@kud.ac.in)

1. Neurochemistry lab, Department of Biochemistry, Karnatak University, Dharwad, Karnataka 580003
2. Neurogenetics Lab, Department of Applied Zoology, Mangalore University, Mangalagangothri, Karnataka 574199
3. Department of Pediatrics, Division of Hematology/Oncology, College of Medicine, The Pennsylvania State University, Hershey, PA 17033, USA
4. Division of Neuroscience, Yenepoya Research Centre (YRC), Yenepoya (Deemed to be University), Deralakatte, Mangalore, Karnataka 575018

* For Correspondence

Recommended Level: UG/PG/Research

Sub-Discipline: Biochemistry/Cell Biology

Background:

Glutathione-s-transferase (GST), previously known as ligandins, is a major phase-II detoxification enzyme family in both eukaryotes and prokaryotes. This enzyme family catalyses conjugation of the reduced form of glutathione to the xenobiotic substrate for detoxification (Singh and Reindl, 2021). The multigene GST family includes eight dimeric enzymes like alpha(A), kappa (K), mu (M), omega (O), pi (P), sigma (S), theta (T) and zeta (Z). Depending on their subcellular locations (Habig et al., 1974), GST is grouped as cytoplasmic (A, P, M, S, T, Z) or mitochondrial (M). Glutathione is used to scavenge potentially toxic compounds, including those produced as a result of oxidative stress and is part of the defence mechanism neutralizing the mutagenic, carcinogenic and toxic effects of such compounds (Akinade et al., 2022).

The colorimetric method for determination of GST activity is based upon the GST-catalysed reaction between glutathione (GSH) and the GST substrate, CDNB (1-chloro-2,4-dinitrobenzene) (Pour et al., 2014), which has the broadest range of isozyme detectability. The interaction between glutathione and CDNB is entirely dependent on the presence of active GST (Strange et al., 2001). The GST-catalysed formation of the GS-DNB complex (Fig. 1) can be assayed spectrophotometrically at 340 nm.

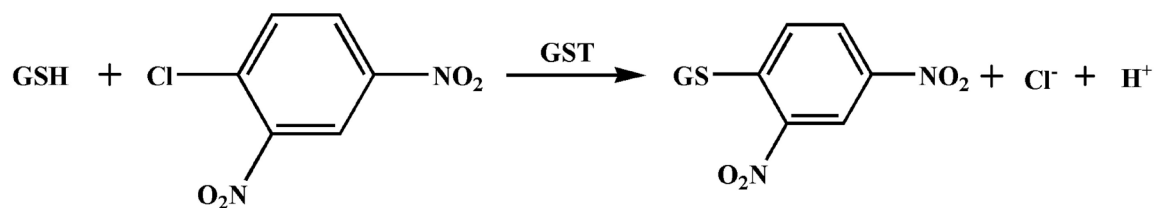


Fig. 1. Glutathione S-transferase (GST) enzyme catalyses the reaction of glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) to form a yellow-coloured conjugated GS-DNB complex.

Objective: To measure the glutathione-s-transferase (GST) activity in *Drosophila melanogaster* using 1-chloro-2, 4-dinitrobenzene (CBD) as a substrate.

Materials Required:

A. Flies: 2-3 days old wild type adult male or female *Drosophila melanogaster*

B. Equipment and other items:

- i. Stereo-binocular microscope and other fly handling materials.
- ii. UV-Visible spectrophotometer.
- iii. Refrigerated microcentrifuge.
- iv. pH meter.
- v. Hand homogeniser (to homogenize samples up to 1 or 2 mL).
- vi. Micro-volume pipettes (1000 μ L, 100 μ L and 10 μ L).
- vii. Micropipette tips (1000 μ L, 200 μ L and 10 μ L).
- viii. Microfuge tube (1.5mL and 2 mL).
- ix. Beakers (50 mL and 100 mL).

C. Reagents:

- i. Yeast paste food: Immediately before starting the experiment, layer 0.5 mL of 0.5% Yeast solution (5 g Yeast paste or granules mixed in 10 mL Distilled water) on 1% agarose bed (mix 1 g Agar powder in 100 mL Distilled water by boiling, cool, and pour in standard fly food vials to about 1 cm height) and plug the vials.
- ii. 0.1 M Potassium Phosphate buffer (pH 7.4): Dissolve 12.119 gm of Potassium phosphate dibasic (K_2HPO_4) and 4.14 g of Potassium phosphate monobasic (KH_2PO_4) in 800 mL distilled water. Adjust the pH to 7.4 using 0.1N HCl or 0.1N NaOH and make up the volume to 1000 mL with distilled water.
- iii. 100 mM Potassium Phosphate Buffer with 1.0 mM EDTA (pH 6.5): Dissolve 414 mg of Potassium Phosphate Monobasic (KH_2PO_4), 1.212 g of Potassium

phosphate dibasic (K_2HPO_4) and 10.87 mg of Ethylenediaminetetraacetic Acid in 80 mL of distilled water. Adjust the pH to 6.5 using 0.1N HCl or 0.1N NaOH and make up the volume to 100 mL with distilled water.

- iv. 75 mM Glutathione, Reduced Solution: Dissolve 0.23 mg of reduced glutathione in 10 mL of distilled water and store it at 4°C.
- v. 30 mM 1-Chloro-2,4-Dinitrobenzene Solution (CDNB): Dissolve 607 mg of 1-Chloro-2,4-Dinitrobenzene (CDNB) in 95% Ethanol. The mixture is slightly warmed till it forms a clear solution.
- vi. 95% Ethanol.
- vii. Distilled water.

Experimental Details:

- i. Collect 3 days old wild-type *Drosophila* male or female flies (n = 60) and divide them into two groups (control and treated group). Each group can be of 30 male or female flies.
- ii. Feed the control flies with 0.5% Yeast solution for 5 days.
- iii. Feed the treatment group with the desired chemicals (Rotenone/Aluminium Chloride/Scopolamine/Colchicine that can induce oxidative stress) for 5 days on 0.5 mL of 0.5% Yeast solution containing 10 mg of the desired chemical.
- iv. Anaesthetize and decapitate 20 flies from the control and treatment groups. Homogenize separately the heads of control and treated flies in 100 μ L of 0.1M Potassium Phosphate buffer (pH 7.4) in a hand homogenizer and centrifuge the homogenate at 10,000 RPM for 15 min at 4°C.
- v. Collect the supernatants in fresh tubes for further analysis.
- vi. Add 2.8 mL (for blank) and 2.7 mL (control/treated sample) of 100 mM potassium phosphate buffer (pH 6.5) in separate clean tubes. Add 0.1 mL of 75 mM Glutathione and 0.1 mL of 30 mM CDNB Solution to both tubes.
- vii. Add 0.1mL of homogenate to the tube assigned as control/treated.
- viii. Mix and record the reading at 340 nm in a UV-spectrophotometer for the control and treatment group samples separately. Record the absorbance readings for 5 minutes at 15-second intervals.
- ix. Measure the total protein content in the samples using the method described in (Chapter 71).

Table 1. Sample Table for recording the absorbance values at 340 nm (prepare separate tables for Control and treated samples).

	100 mM Potassium Phosphate Buffer (pH 6.5)	75 mM Glutathione	Volume of homogenate	25 mM CDNB	Absorbance time points (seconds)	Absorbance at 340 nm
Blank	2.8 mL	0.1 mL	-	0.1 mL	300	
Control/	2.7 mL	0.1 mL	0.1 mL	0.1 mL	15	
					30	
					45	
					60	
					75	
					90	
					105	
					120	
					135	
					150	
					165	
					180	
					195	
					210	
					235	
					250	
					265	
					270	
					285	
					300	

Results and Observations:

1. Record the absorbance at 340 nm at 15 seconds intervals till 300 seconds (5 min) in Table 1. The control and treated samples need to be recorded separately.
2. Calculate the GST enzyme activity using the following formula.

$$\mu\text{mole} / \text{min} / \text{ml enzyme} = \frac{OD \text{ test} - OD \text{ blank} \times 3.0 \text{ (total volume)} \times 5 \text{ (DF)}}{5 \text{ (Time)} \times 9.6 \times 0.033}$$

Notes:

1. Here **3** is the total volume of the reaction mixture, **5** (DF) is the dilution factor, **9.6** is Millimolar extinction coefficient of CDNB and **0.033** is the percent volume of homogenate in the reaction mixture.

2. For the total GST activity, all the values are added together (15 seconds to 300 seconds) and averaged.
3. Total GST activity is expressed as $\mu\text{mole}/\text{min}/\text{mg}$ of protein and calculated using the following formula:

$$\mu\text{mole} / \text{min} / \text{mg of protein} = \frac{\mu\text{mole} / \text{min} / \text{ml enzyme}}{\text{mg of protein} / \text{ml enzyme}} \times 100$$

Note: The total protein content in the brain samples is calculated based on the method described in **Chapter 71**.

Questions for self-study and interpretation:

1. Why do living systems need enzymes like GST?
2. Why is GST a multienzyme complex family?
3. How is the estimation of blood GST level helpful in certain clinical conditions?

References and Further Readings:

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Glutathione-s-transferase, a Good Samaritan

Considering the functions of GSTs in detoxification and the roles of NO in cell signaling and cytotoxic effector functions, the interactions of GSTs with multiple effector proteins highlight an innovative role of GSTs in NO metabolism. Future studies examining these GST interactions and their impact on the function of NO will be valuable in understanding the potentially expanding roles of GSTP1 as a good Samaritan.

(In: Russell, T. M., & Richardson, D. R. (2023). The good Samaritan glutathione-S-transferase P1: An evolving relationship in nitric oxide metabolism mediated by the direct interactions between multiple effector molecules. *Redox Biology*. 59: 102568. <https://doi.org/10.1016/j.redox.2022.102568>)

Chapter 75

Measuring glucose content in *Drosophila* hemolymph

Raifa Abdul Aziz¹ (raifaazeez@gmail.com), Deepa Mugudthi Venugopal¹ (deepavenugopalbvr@gmail.com), Ramesha Hanumanthappa² (rameshkoose18@gmail.com), Manvitha Kadandelu³ (manvithakashyap@gmail.com), Avinash Kudva⁴ (avinash.kudva@gmail.com), Rajashekhar K Patil⁵ (patilsirmu@gmail.com), Kuramkote Shivanna Devaraju² * (ksdevaraju@kud.ac.in) and Shamprasad Varija Raghul^{1,3} * (shamprasadr@yeneponya.edu.in)

1. Neurogenetics Lab, Department of Applied Zoology, Mangalore University, Mangalagangothri, Karnataka 574199
2. Neurochemistry lab, Department of Biochemistry, Karnatak University, Dharwad, Karnataka 580003
3. Division of Neuroscience, Yenepoya Research Centre (YRC), Yenepoya (Deemed to be University), Deralakatte, Mangalore, Karnataka 575018
4. Department of Pediatrics, Division of Hematology/Oncology, College of Medicine, The Pennsylvania State University, Hershey, PA 17033, USA
5. Department of Zoology, Bangalore City University, Bengaluru, Karnataka 560001

* For Correspondence

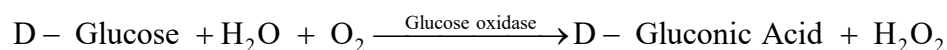
Recommended Level: UG/PG/Research

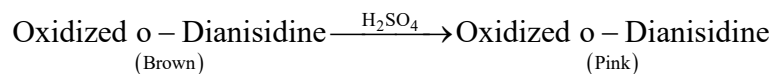
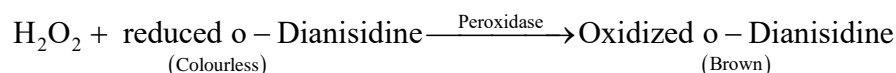
Sub-Discipline: Physiology/Biochemistry

Background:

Glucose is a sugar-constructed nutrient present in cells and is used to generate the energy necessary for different physiological functions in our body. Insulin, a protein hormone produced by the pancreas, regulates the glucose levels in the body fluids. A low level of insulin or its activity in the body causes excess glucose levels in the blood, which causes diabetes. The blood acts as a carrier to supply glucose to different organs and tissues in the body. In insects, the body fluid or the haemolymph carries glucose to different parts of the body (Wyatt et al., 1957, Oram et al., 2014). *Drosophila melanogaster* has now been developed as a good model for diabetes (Murillo-Maldonado & Riesgo-Escovar, 2017).

Glucose level can be measured through a colorimetric-based enzymatic assay. This assay employs the Glucose Oxidase (GO) enzyme to catalyze the oxidation of glucose to hydrogen peroxide and gluconic acid (Bergmeyer, 2012). The generated hydrogen peroxide reacts, in the presence of peroxidase, with o-dianisidine, an added compound, to produce an orange-coloured oxidized form of o-dianisidine. Sulfuric acid is then added to quench the reaction and convert the colour to a pink hue (see the reactions below). The absorbance of the pink hue can then be measured at 490 nm using a multi-plate reader.





Analyzing whole animal homogenate is a high-throughput way to measure the total glucose content in the animal. However, this method does not differentiate between circulating and stored sugars and is also affected by the food in the gut. Therefore, to assay the circulating levels of glucose in the organism, glucose content in hemolymph is determined (Piyankarage et al., 2012).

Objectives: To collect hemolymph from adult *Drosophila melanogaster* and measure the glucose content.

Materials required:

- A. **Flies:** 2-3 days old wild-type *Drosophila melanogaster* male or female flies maintained on a white cream-agar medium at 20-25°C with a relative humidity of 65-70%.
- B. **Equipment and items required:**
- i. Stereo-binocular microscope.
 - ii. Hand homogenizer (homogenize samples up to 1 to 2 ml).
 - iii. Refrigerated microcentrifuge.
 - iv. Micro-volume pipettes (10, 200, 1000 µL) and corresponding micropipette tips.
 - v. Microplate reader (96 wells).
 - vi. Micropipette tips (10, 200, 1000µL).
 - vii. Microfuge tubes (1.5 mL).
 - viii. 0.5 mL microfuge tubes with a small hole made at the bottom with a hot fine needle.
 - ix. Beakers (100 ml).
 - x. Measuring cylinders (100 ml and 500 ml).
 - xi. 96-well microplate.
- C. **Reagents:**
- i. **Phosphate buffered saline (PBS, pH-7.4):** Dissolve 8 gm of NaCl, 0.2 gm KCl, 1.44 gm Na₂HPO₄ and 0.24 gm KH₂PO₂ in 800 mL of distilled water. Adjust the pH to 7.4 using 0.1N HCl or 0.1N NaOH and make up the volume to 1000 ml with distilled water.
 - ii. **o-Dianisidine**

- iii. **Glucose oxidase-peroxidase reagent (GOD reagent):** Prepare GOD reagent freshly by adding o-Dianisidine to Glucose oxidase in the ratio of 0.04: 1.96 (in mL). The total amount of GOD reagent needed for each set of experiments is about 1 mL.
- iv. **1mg/1 mL Glucose standard:** The total amount of Glucose standard required for each set of experiments is about 50 μ L.
- v. **6M H₂SO₄:** Take 50 mL distilled water in a volumetric flask and slowly add 16.6 mL of conc. H₂SO₄ with gentle stirring till the acid is completely mixed. Finally, make the volume 100 mL by adding 34.4 mL distilled water.
- vi. Distilled water

Experimental details:

A. Extraction of fl haemolymph:

- i. Collect 2-3 days old 20 flies (of same sex) in the blank vial, plug the vial and place in a refrigerator (4°C) for 10 min for anesthetising the flies by cold.
- ii. Transfer the flies on a clean slide under a stereomicroscope and puncture their abdomen using a fine sterile needle.
- iii. Immediately transfer the punctured flies to the 0.5 mL microfuge tube with a hole at the base.
- iv. Keep the 0.5 mL microfuge tube along with the abdomen-punctured flies in a larger volume microfuge tube (1.5 mL). Centrifuge the 1.5mL tube at 12,000 rpm for 10 minutes so that the hemolymph gets collected in the 1.5 mL microfuge tube. Discard the 0.5 mL microfuge tube with the carcass of flies.
- v. Dilute the hemolymph collected in the 1.5 mL microfuge tube with 100 μ L PBS and store at -20° C until further analysis (Samples can be stored for up to one month).

B. Glucose Standard curve preparation:

- i. Add 0, 2, 4, 6, 8, and 10 μ L of glucose standard solution (1 mg/mL glucose) in a 96-well microplate. Make up the volume to 50 μ L in each well using PBS.
- ii. Add 100 μ L of GOD reagent to each well and incubate for 30 minutes at room temperature.
- iii. Stop the reaction by adding 6 M H₂SO₄ at the end of 30 minutes. Carefully mix the content in each well by gentle tapping.
- iv. Take the absorbance reading at 490 nm using a multi-plate reader and record the values as in Table 1.

Notes:

- i. A standard curve is used to measure the concentration of the given molecule in the sample. The absorbance signal (Y-axis) from the test sample is plotted on the standard

curve and the corresponding value on the X-axis corresponds to the quantity of the molecule in the sample.

- ii. The glucose concentration in the blank and all samples must be estimated in triplicates and for the final calculation, average of triplicate readings is considered.

Table 1: Sample Table for recording the glucose standard value at 490 nm

S. No.	Glucose standard (µL)	PBS(µL)	GOD reagent (µL)	Incubation at room temperature for 30 minutes	6M H ₂ SO ₄ (µL)	Absorbance at 490 nm (Mean of triplicate readings)
1	0	50	100		100	
2	2	48				
3	4	46				
4	6	44				
5	8	42				
6	10	40				

C. Determination of glucose concentration in the sample:

- i. Add 5 µL diluted hemolymph sample and 45 µL of PBS to the micro-well plate.
- ii. Add 100 µL of GOD reagent and mix it well by gentle tapping of the plate. Incubate the mixture at room temperature for 30 minutes.
- iii. Stop the reaction by adding 6M H₂SO₄ and measure the absorbance at 490 nm using the multi-plate reader. Record the measurements as in Table 2.
- iv. Measure the concentration of protein in the hemolymph sample as in (Chapter 71). and express the final result as µg glucose/mg protein.

Table 2: Sample table for recording glucose content at 490 nm

Sl. No.	Sample	PBS	GOD reagent	Incubation at room temperature for 30 minutes	6M H ₂ SO ₄	Absorbance at 490 nm (Mean of triplicate readings)
Test	5 µL	45 µL	100 µL		100 µL	

Results/Observations:

Plot the standard curve (as in Fig. 1) for the absorbance of glucose using the average absorbance values (Y-axis) for the standard glucose dilution series (X-axis). Using the standard curve, determine the concentration of glucose in the test sample and express the value as µg glucose/mg protein.

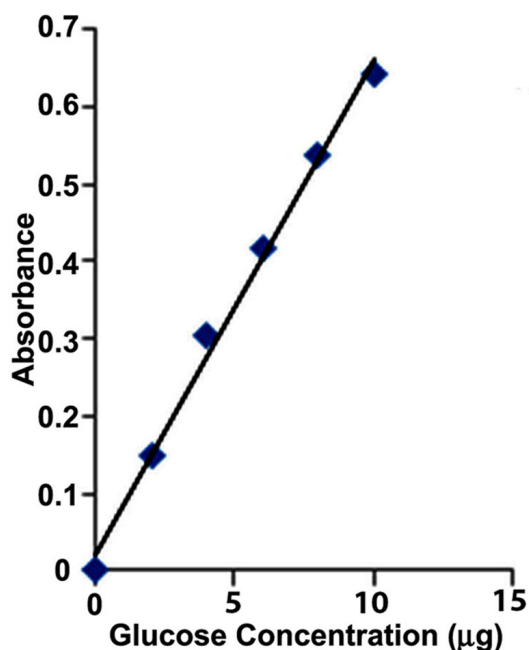


Fig. 1. A sample Standard Absorbance (Y-axis) Curve for different glucose concentrations (X-axis).

Questions for self-study and interpretation:

1. Does the glucose content in hemolymph vary between male and female flies?
2. Would the glucose content in *Drosophila* hemolymph vary in relation to the nature of diet?
3. Would age of the fly (young vs old) affect glucose content in hemolymph?
4. How does H_2SO_4 help in stopping the reaction?
5. Would glucose levels in hemolymph be altered in insulin receptor mutants?
6. Can this method be used for screening novel anti-diabetic drugs?

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Diabetes - Prevention better than Cure

The road towards treating diabetes was not then and will not be smooth and straight in the future, but it is certainly worth travelling, since it is the only route towards scientific progress and human well-being. A better understanding of the pathophysiology of diabetes will certainly lead to better therapeutic options in the future. However, we should not forget that the main focus of the policymakers and all of us should be a focus on prevention, including physical activity and a healthy diet, which are the keys to disrupt the increasing trends in epidemics of diabetes.'

(In: Polyzos, S. A., & Mantzoros, C. S. (2021). Diabetes mellitus: 100 years since the discovery of insulin. *Metabolism*, 118, 154737. <https://doi.org/10.1016/j.metabol.2021.154737>)

Chapter 76

Estimation of triglycerides content using *Drosophila* hemolymph

Deepa Mugudthi Venugopal¹ (deepavenugopalbvr@gmail.com), Vishnu Shreekara (vishnushreekara@gmail.com), Keith Davin Netto (bernardstain461@gmail.com), Raifa Abdul Aziz¹ (raifaazeez@gmail.com), Ramesha Hanumanthappa² (rameshkoose18@gmail.com), Shripriya Bhat¹ (shripriyabhat05@gmail.com), Manvitha Kadandelu³ (manvithakashyap@gmail.com), Avinash Kudva⁴ (avinash.kudva@gmail.com), Rajashekhar K Patil⁵ (patilsirmu@gmail.com), Kuramkote Shivanna Devaraju^{2*} (ksdevaraju@kud.ac.in) and Shamprasad Varija Raghu^{1,3*} (shamprasadvr@yenepoya.edu.in)

1. Neurogenetics Lab, Department of Applied Zoology, Mangalore University, Mangalagangothri, Karnataka 574199
2. Neurochemistry lab, Department of Biochemistry, Karnatak University, Dharwad, Karnataka 580003
3. Division of Neuroscience, Yenepoya Research Centre (YRC), Yenepoya (Deemed to be University), Deralakatte, Mangalore, Karnataka 575018
4. Department of Pediatrics, Division of Hematology/Oncology, College of Medicine, The Pennsylvania State University, Hershey, PA 17033, USA
5. Department of Zoology, Bangalore City University, Bengaluru, Karnataka 560001

* For Correspondence

Recommended Level: UG/PG/Research

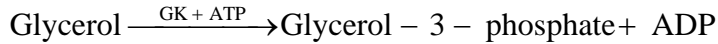
Sub-Discipline: Physiology/Biochemistry

Background:

Triglycerides are the most common type of lipids. They are obtained from food sources such as dairy, oils, and other fats. These are often referred to as extra calories as they are not readily involved in energy production but are stored in animals as fats. Triglycerides are broken down during the lipolysis process and are liberated as free fatty acids whenever needed (Buckley et al., 1966). The abundance of carbohydrates and saturated fats in a diet raises the body's level of triglycerides. An abnormal level of triglycerides is a biomarker of metabolic syndrome, liver dysfunction, cardiovascular diseases, stroke, etc. The *Drosophila* model has been found to be effective in understanding the pathophysiology of human metabolic diseases like obesity and diabetic conditions (Hildebrandt et al., 2011).

The biochemical assay for triglycerides makes use of the following reactions. The lipoprotein lipase (LPL) converts triglycerides in the given sample to glycerol and free fatty acids. Glycerol kinase (GK) in the presence of ATP transforms glycerol into glycerol-3-phosphate (G3P) and adenosine 5-diphosphate (ADP). Glycerol phosphate dehydrogenase (GPO) then transforms glycerol-3-phosphate (G3P) into dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H₂O₂). In the final step, the generated hydrogen peroxide

(H₂O₂) interacts with 4-aminophenazone (4-AP) (antipyrine) and p-chlorophenol in the presence of peroxidase (POD) to produce Quinoneimines, which is red-colored (also see Chapter 78).



The intensity of the red colour is proportional to the triglyceride concentration in the sample. This can be spectro-photometrically measured at 490 nm.

Objective: To estimate the total triglyceride content in *Drosophila* haemolymph.

Materials Required:

- A. **Flies:** 3-4 days old wild type Canton S (CS)
- B. **Equipment and items required:**
 - i. Stereobinocular microscope and other fly handling materials.
 - ii. Refrigerated microcentrifuge.
 - iii. Microplate reader (96 wells).
 - iv. Benchtop vortex.
 - v. Micropipettes (10, 200, 1000 μL) and corresponding micropipette tips.
 - vi. 0.5 mL microfuge tubes with a small hole made at the bottom with a hot fine needle.
 - vii. 96 well microplate.
 - viii. Ice bucket with ice.
 - ix. Sterile needle.
 - x. Microcentrifuge tubes (0.5mL and 1.5mL).
- C. **Reagents:**

Commercially available Triglyceride estimation kit.

Experimental procedures:

- A. **Extraction of fly haemolymph**
 - i. Collect 2-3 days old 20 flies (of same sex) in blank vial, plug the vial and place in a refrigerator (4°C) for 10 min for anesthetising the flies by cold.
 - ii. Transfer the flies on a clean slide under a stereomicroscope and puncture their abdomen using a fine sterile needle.
 - iii. Immediately transfer the punctured flies in the 0.5 mL microfuge tube with hole at the base.

- iv. Keep the 0.5 mL microfuge tube along with the abdomen-punctured flies in a larger volume microfuge tube (1.5 mL). Centrifuge the 1.5mL tube at 12,000 rpm for 10 minutes to collect hemolymph in the 1.5 mL microfuge tube. Discard the 0.5 mL microfuge tube with carcass of flies.
- v. Dilute the hemolymph collected in the 1.5 mL microfuge tube with 100 μ L PBS and store at -20° C until further analysis (Samples can be stored for up to one month).

B. Standard curve preparation:

- i. A standard curve is used for measuring the concentration of triglycerides in the given sample. Since the signal from the test sample may not directly indicate the triglyceride concentration in the sample, a standard curve is used to determine the concentration.
- ii. Add 0, 2, 4, 6, 8, and 10 μ l of standard triglyceride solution in different wells in the 96-well microplate. Make up the volume to 100 μ L in each with Triglyceride reagent.
Note: The standard Triglyceride and Triglyceride reagents are available in the kit and are to be used following instructions in the kit.
- iii. Mix the standard and Triglyceride reagents thoroughly by tapping the microwell plate. Incubate the mixture at room temperature for 10 min.
- iv. Take the absorbance reading at 490 nm using a microplate reader and record your observations as in the sample Table 1.

Notes:

- i. A standard curve is used to measure the concentration of the given molecule in the sample. The absorbance signal (Y-axis) from the test sample is plotted on the standard curve and the corresponding value on the X-axis corresponds to the quantity of the molecule in the sample.
- ii. The triglyceride concentration in the blank and all samples must be estimated in triplicates and for the final calculation, the average of triplicate readings is considered.

Table 1: Sample table for recording absorbance of the standard triglyceride dilution series at 490 nm.

	Triglyceride reagent (μ L)	Standard triglyceride (μ L)	Triglyceride Concentration (μ g)	Incubate for 10 min at room temperature	Absorbance at 490 nm (Mean of triplicate readings)
Blank	100	-	0		
S1	98	2	2		
S2	96	4	4		
S3	94	6	6		
S4	92	8	8		
S5	90	10	10		

C. Estimation of total triglycerides in the sample:

- i. Add 10 µL of diluted haemolymph sample and 90 µL of triglyceride reagent to the microwell plate.
- ii. Mix thoroughly by tapping the microwell plate and incubate the mixture at room temperature for 10 min.
- iii. Take the absorbance reading at 490 nm (in triplicate) using a microplate reader.

Table 2: Sample table for recording absorbance for triglyceride content in the hemolymph.

		Triglyceride reagent (µL)	Haemolymph sample (µL)	Incubate for 10 min at room temperature	Absorbance at 490 nm (Mean of triplicate readings)
Blank		100	-		
Test sample		90	10		

- iv. Calculate the triglyceride concentration in the hemolymph of each control and test sample using the formula below:

$$\text{Triglyceride mg/dL} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times 20$$

Note: Triglyceride concentration in the kit is 200 mg/dL. 100µL of the sample corresponds to 20mg/dL and hence the multiplication factor of 20.

Result/ Observations:

Plot the average absorbance values for every standard (y-axis) against the concentration of triglyceride (x-axis) in the solution (see Chapter 9 for preparation of Standard Curve). Using the standard curve, determine the concentration of triglyceride in the test sample and express it as mg triglyceride/dL sample.

Questions for self-study and interpretation:

1. What conditions can affect the level of triglycerides in hemolymph/blood, and what may be the consequence
2. Do male and female individuals have similar levels of triglycerides in their hemolymph/blood? Why?
3. Does the triglyceride level in hemolymph change with age of the flies?

References and Further Readings:

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Triglycerides and Heart Disease

Although hypertriglyceridemia is a common biochemical abnormality in humans, except for pancreatitis risk, its relationship to human disease is far from certain. Is hypertriglyceridemia merely a marker of other metabolic abnormalities, or does it initiate or accelerate progression of vascular disease? Does this only occur in specific genetic or environmental settings? Despite the plethora of data showing atherogenic-like effects of lipids or lipolysis products on cultured cells, animal models to allow the study of the effects of hypertriglyceridemia on arteries in vivo are needed. Perhaps it is not the triglycerides themselves but VLDL particle number or changes in HDL or LDL that are most important for vascular biology.

(In: Goldberg, I. J., Eckel, R. H., & McPherson, R. (2011). Triglycerides and heart disease: still a hypothesis?. *Arteriosclerosis Thrombosis Vascular Biology*, 31: 1716-1725. <https://www.ahajournals.org/doi/10.1161/ATVBAHA.111.226100>)

Chapter 77

Staining of lipids in larval fat body of *Drosophila melanogaster*

Khushboo Sharma (khushboo.zoology@bhu.ac.in) and Madhu G. Tapadia* (madhu@bhu.ac.in)

Cytogenetics Laboratory, Department of Zoology, Institute of Science, Banaras Hindu University, Varanasi, 221005, Uttar Pradesh, India.

* For correspondence

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Sub-discipline: Cell Biology/Developmental Biology/Physiology

Background

Drosophila melanogaster offers a wealth of paradigms to study the regulation and physiological effects of fat accumulation (Butterworth *et al.*, 1965). Obese flies accumulate triacylglycerols in the fat body, an organ like the mammalian adipose tissue, which specializes in lipid storage and catabolism. One of the characteristic features of Type 2 diabetes, which can be induced in *Drosophila* by high sugar diet, is obesity (Musselman *et al.*, 2018). The obese flies exhibit pathophysiological complications, including hyperglycaemia, hyperinsulinemia, reduced lifespan and cardiovascular function, like those seen in Type 2 diabetic humans and thus serve as good model for type 2 diabetes (Ecker *et al.*, 2017; Musselman *et al.*, 2018; Lindsey *et al.*, 2021).

Level of fat accumulation in the fat body can be assayed by staining with Nile red (NR), 9-diethylamino-5H-benzo[alpha]phenoxazine-5-one, which is an excellent lipophilic fluorescent stain for the detection of intracellular lipid droplets. NR exhibits strong fluorescence in presence of hydrophobic environment. The spectral range for short excitation wavelength (450-500nm)/ yellow-gold emission ($\leq 580\text{nm}$) favours detection of neutral lipids (Triacyl glycerides, TAGs) while longer excitation wavelength (515-560nm)/ red emission ($\geq 590\text{ nm}$) favours general fluorescence for most of the polar lipids (Greenspan 1985). In this chapter, we describe a protocol for staining of third instar larval fat body to assay levels of fat accumulation.

Objective:

To study changes in the levels of lipid droplets in fat body of high-sugar diet induced Type 2 diabetic model larvae of *Drosophila melanogaster* using Nile red stain.

Materials Required:

A. Flies: Wild type flies reared at $24\pm 1^\circ\text{C}$.

B. Equipment and other supplies:

- i. Stereo-binocular microscope and other fly handling materials.
- ii. BOD incubator at $24\pm 1^{\circ}\text{C}$.
- iii. Pasteur pipettes, fine forceps and dissecting needles.
- iv. Ice box/Thermocol box and ice.
- v. Wash bottle, Tissue paper, cavity blocks or Maximov slides.
- vi. Microscope slides and coverslips (22 mm^2).
- vii. Fluorescence microscope.

C. Control and High Sugar Diet:

- i. **Control Diet:** Standard food media (see Chapter 1 Appendix I) which has 0.1M sucrose concentration)
- ii. **High Sugar Diet:** Add 123.12g Sugar (e.g., Sucrose) to 360 mL of standard food while cooking to make the sucrose concentration as 1M.

D. Reagents:

- i. 1X Phosphate Buffered Saline (PBS), pH 7.4:

Sodium Chloride (1.37 M)	8.0 g
Potassium Chloride (27 mM)	0.2 g
Disodium hydrogen phosphate (100 mM)	1.42 g
Potassium dihydrogen phosphate (20 mM)	0.272 g

Dissolve the salts in 800 mL distilled water, and adjust pH to 7.4 adding 1N-HCl, as required. Finally add distilled water to make the final volume 1000 mL. Autoclave and store at 4°C .

- ii. Nile Red (NR) stain: Dissolve 1 mg NR in 1mL DMSO to prepare the stock solution. To prepare the working solution (1:4000 dilution), take 1 μL of the NR stock solution and add 1X PBS to make 4 mL. Store both solutions in dark.
- iii. Paraformaldehyde 4% (w/v). To prepare the PFA, mix 2g paraformaldehyde (stored at 4°C in Aluminium foil covered or amber colour bottle) for 50 mL of PBS and heat at 65°C for 15 min with frequent shaking. The final solution is clear solution with the characteristic formalin odour and is ready to use. Store at 4°C for use within a week.
- iv. PBST (0.1%): Add 100 μL Triton X-100 in 10 mL 1X-PBS.
- v. Mounting medium: DABCO (1,4 Diazabicyclo- 2,2,2-octane).

Experimental Details:

1. Collect eggs from healthy *Drosophila melanogaster* flies and transfer freshly hatched 10-15 first instar larvae each into food vials with control or high sugar food media. Prepare 2-3 replicates for each feeding condition.

2. Let the larvae grow up to third instar stage (approximately 96 hrs at 24°C±1).
3. Take the third instar wandering larvae from the vials and wash them in Petri dish with 1X PBS to remove the food particles adhering on their body.
4. Take larva of a given diet condition on a clean slide with a small drop of 1X PBS.
5. Dissect the larva by keeping tip of one needle/forceps onto the anterior end near the mouth hook and of another at the posterior end and pulling the two needles in opposite directions to tear the larval cuticle at the anterior end. The larval body parts including the fat body tissue flow out of the larval cuticle (see Chapter 21 and 31).
6. Use fine needles to pick and transfer the dissected fat body tissues to a small drop of 1X PBS on a fresh slide, kept on ice to keep the tissue cool and avoid any metabolic changes during the preparative steps.
7. Repeat steps 1-6 above for high sugar-diet fed larvae.
8. Collect fat bodies from 5 larvae of each group in PBS on two separate slides on ice.
9. Remove the slides from ice and keep on table on a filter paper to soak the water from underside of the slide. Carefully remove PBS from control and high-sugar diet fat body tissues slides and immediately add 100 µL of 4% Paraformaldehyde fixative for fixing the tissue. Keep for 20 minutes.
10. Wash the fixative with PBST (0.1%), for 15 minutes. Repeat this step thrice.
11. Add 100 uL of Nile Red working stain (1:4000) for 30 minutes. Keep the slides in a box to avoid exposure to light.
12. Wash the samples with PBST (0.1%) thrice with a duration of 15 minutes each (keep in dark).
13. Finally mount the tissue using DABCO mountant.
14. Observe the sample under fluorescence microscope, using 510-560 nm (green) excitation filter and >590 nm (red) emission filter to detect polar lipids.

Observations/Results:

Examine the numbers of lipid droplets, their sizes and fluorescence intensity in fat bodies from larvae reared on standard and high sugar containing food under a fluorescence microscope using green excitation and red emission filter combination (Fig. 1).

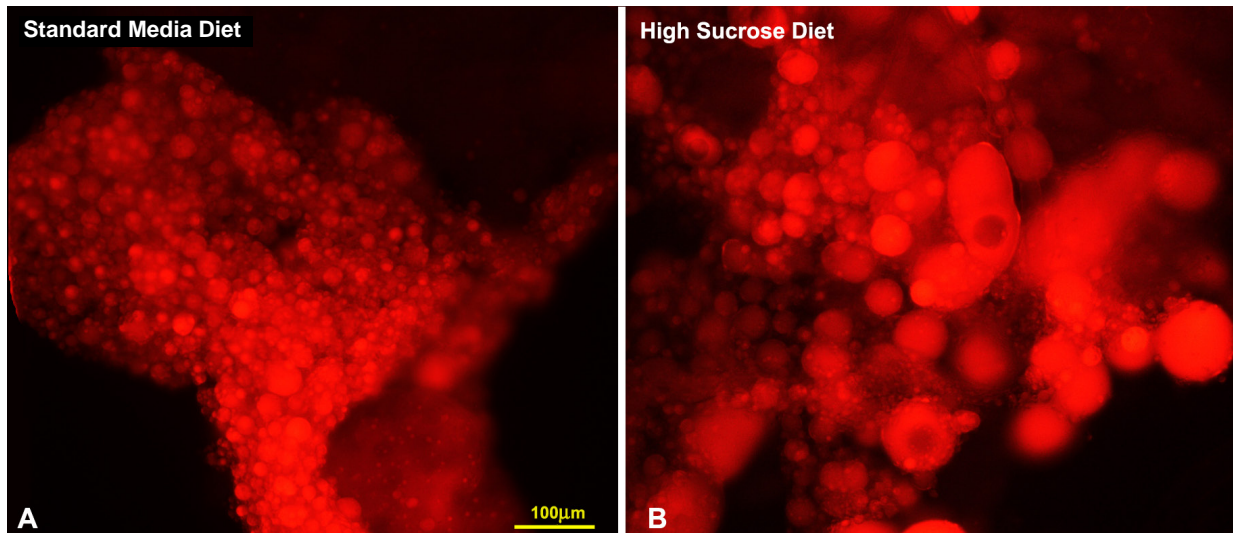


Fig. 1. Nile Red staining of larval fat body of *Drosophila melanogaster*. Fluorescence microscope images of fat body from larvae reared on under standard media diet (control, **A**) and high sugar diet (**B**). The scale bar in **A** represents 100 μm and applies to **B** also.

Questions for self-study and interpretation:

1. Why high sugar diet increases levels of fat in the fat body tissue?
2. What is the role of fat body in metabolism related activities and how their study can be helpful in understanding obesity related diseases?
3. How does fat body serve as energy reservoir during the pupal stage of metamorphosis?

References and further reading:

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Chapter 78

Assaying the effect of a high-fat diet on triglyceride levels in *Drosophila melanogaster*

Megha (megha@tdu.edu.in)

Centre for Ayurveda Biology and Holistic Nutrition, The University of Trans-Disciplinary Health Sciences and Technology, Bangalore

Recommended Level: UG/PG

Sub-discipline: Physiology

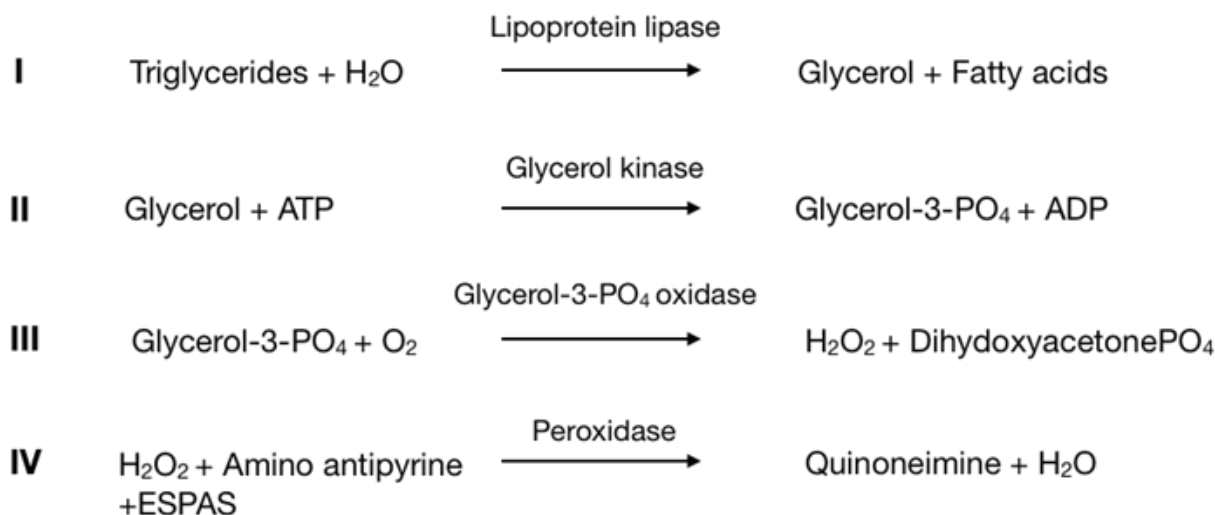
Background:

The role of diets in public health has become an increasingly important topic, as cases of obesity and diabetes keep rising. This is especially problematic in India, which suffers from the triple burden of malnutrition: under-nourishment, micronutrient deficiency and obesity (Wells *et al.*, 2019). Consumption of diets high in fats, sugars and salt have been correlated with the increasing prevalence of metabolic disorders. However, not all fats, sugars and salts are bad for human health; in addition to quantity, parameters such as composition, quality and method of preparation can influence their effects on metabolism. *Drosophila melanogaster* provides a simple but efficient model to analyze how different food ingredients interact with metabolism, and to understand impacts of dietary intake at the systemic, tissue, cellular and molecular levels.

The present chapter describes the use of *Drosophila* to assess the impact of a high-fat diet on lipid metabolism. Triacylglycerols (TAGs) are the major lipid storage molecules in animals and an increase in their levels serves as a biomarker that reflects fat accumulation and increased lipid anabolism, features which are observed in individuals classified as obese. Much like in humans, consumption of a high-fat diet has been shown to increase the level of TAGs in flies (Birse *et al.*, 2010). Lipid metabolism is strongly conserved between flies and mammals, and this has been exploited to not only understand the genetic basis of obesity (Musselman and Kuhnlein, 2018) but also physiological processes under-pinning TAG metabolism (Heier and Kuhnlein, 2018). Birse *et al.*, (2010) introduced the use of coconut oil to create a high-fat diet for flies and demonstrated a proportional increase in systemic (whole-body) TAG levels with increasing concentrations of coconut oil (up to 30%). High-fat and sugar diets also increase circulating levels of TAGs as measured in the hemolymph (Musselman *et al.*, 2011). At the tissue level, high-fat diets cause accumulation of lipid droplets in fat body, which can be visualized by Nile-red staining (Subramanian *et al.*, 2013). At the molecular level, expression of genes

involved in lipid metabolism, such as *brummer* and *lip3*, can be monitored (Gronke *et al.*, 2007).

There are many ways to measure TAGs, ranging from sophisticated mass spectrometry (Carvalho *et al.*, 2012) to simpler techniques like thin-layer chromatography and enzyme-based colorimetric assay (Hildebrandt *et al.*, 2011, also see [Chapter 76](#)). The protocol here describes the enzyme-based colorimetric method, adapted from earlier published works (Hildebrandt *et al.*, 2011; Tennessen *et al.*, 2014; Megha and Hasan, 2017). Read more (Al-Anzi and Zinn, 2010; Hildebrandt *et al.*, 2011) to learn about limitations of the colorimetric method. The Serum TAG quantification kit, which is used in this protocol, involves the following reactions:



Source: BeneSphera GPO/ESPAS kit instruction booklet

Objective:

To measure the amount of total triglycerides in adult flies exposed to a high-fat diet.

Materials Required:

A. Flies

- i. Wild type fly strains
- ii. Number of flies needed for each experiment: Assuming 3 flies/sample and 3 samples for each experiment, a minimum of 9 age-matched and sexed flies per condition would be needed. To account for handling errors, it is advised to place 12 males and females per vial per feeding condition. Therefore, for two conditions, viz., normal and high-fat diet, 24 females and 24 males would be required per experiment.

B. Reagents and chemicals

- i. Double-distilled water
- ii. Yeast extract
- iii. D-Glucose (dextrose)
- iv. Agar

- v. Propionic acid
- vi. p-hydroxy benzoate (0.45 mg/mL dissolved in ethanol).

vii. Virgin coconut oil.

Note: Quality of coconut oil is critical since flies are extremely sensitive to the quality of oil. We use Arya Farm organic cold-pressed virgin coconut oil. If experimenting with a different brand, set up pilot experiments to test if flies survive on the oil-supplemented food.

viii. Tween-20.

ix. Homogenizing buffer (HB): 0.05% Tween-20 in water.

Note : Pipetting small amounts of viscous solutions is not recommended. Make a 10% stock by dissolving 10 mL of Tween-20 in 90 mL of water. Dilute this fresh each time to get 0.05% Tween-20 solution.

x. Serum TAG quantification kit with TAG standard included (e.g., Benesphera GPO/ESPAS Catalog # 15000774, TransAsia/ Erba, Tulip Diagnostics).

C. Equipment and other materials

- i. Stereo-binocular microscope and other fly culture and handling materials.
- ii. Spectrophotometer with microplate reader accessory to measure absorbance at 545 nm.
- iii. Bench-top vortexer.
- iv. Centrifuge (Room temperature, bench-top also works).
- v. Water bath or Dry bath set at 70°C.
- vi. 96-well flat-well microplate (can be re-used after washing with dilute nitric-acid).
- vii. 1.5 mL microfuge tubes.
- viii. Microfuge tube stand.
- ix. PTFE (Polytetrafluoroethylene) pestle for 1.5 mL microfuge tubes.
- x. 50 mL polypropylene tubes.
- xi. Pipettes (20 μ L, 200 μ L and 1000 μ L) with corresponding tips.
- xii. Ice bucket.

Experimental Details:

A. Preparation of high-fat supplemented food: Calculate the amount of food required for the experiment. Preparation of 50 mL of HFD and ND is described below.

- i. Keep a water bath at 60°C to provide a ready supply of hot water and to warm the fly food if it starts to thicken.
- ii. **Solution A:** Weigh and add 4.5 gm active dry yeast plus 10 gm glucose in a 50 mL tube. Add 20 mL of warm (50-60°C) sterile double distilled H₂O. Let it sit for a minute, followed by mixing with vortexing. Ensure sugar is completely dissolved.

iii. **Solution B:** In a separate beaker, add 0.8 gm agar to 20 mL hot sterile double distilled H₂O. Dissolve the agar completely by boiling on a heater or in microwave oven (60-120 sec). Watch the mixture carefully as it tends to bubble up and spill if heated continuously.

iv. **Solution C:** Pour solution B into solution A and mix. Add 1 mL of methyl parahydroxy benzoate (dissolved in ethanol, 0.45 g/mL) and 0.4 mL propionic acid to this mix. Make up the solution to 50 mL with hot water. Mix thoroughly by gentle rocking. This is 2X strength food with proportions of different ingredients as listed the table below.

v. Divide Solution C into two beakers with 25 mL each; label them as C1 and C2

vi. Add 25 mL hot water to the C1 beaker. Mix thoroughly and pour into culture vials (~5 mL/ vial). This is ND (normal diet; control).

vii. In a separate 100 mL beaker, fully dissolve 3.1g granulated agar in ~10 mL of water by pulse heating. Add this solution to the C2 beaker. Mix in 10 mL coconut oil and make up the solution in C2 beaker to 50 mL with hot water. Mix thoroughly and pour into vials (~5 mL/vial). This is HFD (high-fat diet; experimental).

viii. Allow to cool and set. The food-vials can be stored at 4°C and used for up to a week. The presence of oil makes the food sticky when cold. Therefore, it is important to bring the vial to room temperature before using. **Note:** The final concentrations of different ingredients in the ND and HFD, respectively, are as in the table below.

Type of diet	Active dry yeast	D-glucose	Agar	Coconut oil	p-hydroxy benzoate	Propionic acid
Normal (ND)	4.5%	10%	0.8%	X	0.1%	0.4%
High Fat (HFD)	4.5%	10%	3.5%	20%	0.1%	0.4%

B. Feeding flies a high-fat diet

i. Take 7-10 day old flies, with males and females housed together and reared on standard food under normal laboratory conditions.

ii. Consider the day on which feeding of high-fat media starts as Day 0. Feed flies on ND (control) or HFD (experimental) for 3, 5 or 7 days. Since the HFD food is sticky, it is recommended to transfer the flies into fresh ND or HFD media after every 48 hours, regardless of the number of days of feeding.

Note: Take care while placing flies in HFD vials: i) ensure that vials are at room temperature; ii) keep the vials horizontal after placing the anaesthetized flies in the vial so that the flies recover on vial wall rather than on the food and later on also always keep the vials horizontal. Insert a cone made with small dry filter paper (3 cm X 1 cm) into the food in each vial to increase dry surface area. For subsequent transfer of flies to new food vial, gently tap the side of vials to bring flies to the bottom and invert the fresh vial with its plug removed over the old vial and allow flies to crawl up into the fresh vial. Once all the flies have moved up, carefully plug both the vials.

C. Assaying flies for total glycerides using a biochemical assay:

- i. Pre-label 1.5 mL microfuge tubes in which flies will be collected. For the 3 replicates, labels may be: ND1, ND2, ND3 and HFD1, HFD2, HFD3. Prefix with “M” or “F” depending on the sex being tested.
- ii. Anaesthetize flies, sort by sex and place 3 flies of same sex per vial. Keep the microfuge tubes on ice.
- iii. Add 200 μ L of homogenisation buffer (HB) to each microfuge tube.
- iv. Crush the flies with constant motion of the pestle with at least 30 strokes of the PTFE pestle.
- v. Place the tube back on ice and wash the pestle with 200 μ L of HB, collecting the wash into the same tube. Thus effectively the lysate from 3 adult flies will be in 400 μ L of HB.
- vi. Serially complete homogenizing all the samples, ensuring that all the other tubes remain in ice for the entire duration. Thoroughly wash the pestle with water after homogenizing each sample.
- vii. Place the tubes in a dry or wet bath at 70°C for 10 min to heat inactivate the lysate.
- viii. Spin at 2000 gm (~5000 RPM depending on centrifuge and rotor model) for 3 min. Take 350 μ L of the supernatant into a fresh tube, avoiding capture of tissue debris that would have accumulated at bottom of the tube. This fly lysate will be used for testing.
Note: Samples can be frozen at this time to be assayed at a later time point. If possible, quick freeze in liquid nitrogen (if dry ice available, an equal mix of dry ice and acetone can provide extremely low temperature for freezing) and place in -80°C or -20°C. Stored samples should be processed within ~2 weeks.
- ix. Bring the serum TAG quantification kit to room temperature.
- x. Observe the 96-well plate carefully. Columns (1-12) and rows (A-H) are indicated on the plate. Use this notation (A1, B1...) to record the well number for setting up the experiment. Ideally each sample should be tested twice (technical replicate). Hence, 12 wells are needed for the standard and 2 each for every lysate being tested. In this protocol, 6 X 2 = 12 wells will be used for ND1, ND2, ND3 and HFD1, HFD2, HFD3, respectively.
- xi. Generate the TAG standard curve by dispensing 1, 2, 3, 4, 5 μ L of the 1 mg/mL stock solution, respectively, in the designated wells. Make up the volume to 50 μ L final volume in each well with the homogenizing buffer.
- xii. In two wells (blank), add only 50 μ L homogenizing buffer.
- xiii. Dispense 50 μ L of each of the fly lysates into the designated wells.
- xiv. Read the instructions in the kit regarding reagent preparation for detection (some kits require mixing of solutions, some can be used direct).
- xv. Add 150 μ L of the assay detection reagent per well. Pipette gently to avoid generation of bubbles.

xvi. Mix gently by rotating the plate. Leave at RT for 10 min followed by reading the absorbance at 545 nm for each well.

Results/Observations:

1. Note the absorbance values for all the wells. Average the technical replicates of each well.
2. Subtract the blank absorbance value from each recording.
3. Plot the absorbance values for obtaining the standard curve, i.e., values for the TAG standard, by plotting concentration on the X-axis and respective absorbance on the Y-axis. Draw a line through the points. This should be a linear line. Calculate the slope of this line. The R2 should be as close to 0.99 as possible.
4. Plot the absorbance values of the experimental samples (ND1, HFD1 etc.) on the standard curve to compute the amount of total glycerides in fly lysates. Report the value as mg/mL/fly (Recall that the lysate tested contains homogenate from 3 flies in 400 μ L).

Questions for self-study and interpretation:

1. Why is detergent used to extract TAGs? Learn about the different types of detergents commonly used in lysate preparations (SDS, Triton X-100 etc.) and utility of each.
2. In the described method, detergent-soluble lipids are measured. Is this the best way to extract lipids? Are there other chemicals that can better extract TAGs?
3. The assay assumes that all the fly samples being tested are of equal size and that homogenization is uniform. Is this a fair assumption? What internal controls or measurements can be made to normalize for size or homogenization?
4. Is the read-out of the lipase assay measuring TAGs only?
5. There are advantages as well as disadvantages to using *Drosophila* as a research model for lipid metabolism. Can you think of human diseases or conditions where this research model may not be appropriate?

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Fly Model for Obesity & Metabolic Diseases

Drosophila is a particularly useful model for obesity and metabolic disease for a number of reasons. First, flies contain tissues, organs and systems analogous to all those involved in human obesity and associated metabolic diseases. In addition, *Drosophila* develop obesity and its associated complications during caloric overload, similar to humans. Moreover, most genes and gene families known to function in metabolic disease are conserved between flies and humans.

(In: Musselman, L. P., and Kühnlein, R. P. 2018. *Drosophila* as a model to study obesity and metabolic disease. J. Exp. Biol. 221, jeb163881. doi:10.1242/jeb.163881 https://jeb.biologists.org/content/221/Suppl_1/jeb163881)

Chapter 79

Intestinal inflammation in *Drosophila*

Raifa Abdul Aziz¹ (raifaazeez@gmail.com), Deepa Mugudthi Venugopal¹ (deepavenugopalbvr@gmail.com), Manvitha Kadandelu² (manvithakashyap@gmail.com), Shamitha Shetty¹ (shettyshamitha1911@gmail.com), Shripriya Bhat¹ (shripriyabhat05@gmail.com), Avinash Kudva³ (avinash.kudva@gmail.com), Chandrashekhar G Joshi⁴ (josheejoshee@gmail.com), Rajashekhar K Patil⁵ (patilsirmu@gmail.com) and Shamprasad Varija Raghu^{1,2*} (shamprasadvr@yeneponya.edu.in)

1. Neurogenetics Lab, Department of Applied Zoology, Mangalore University, Mangalagangothri, Karnataka 574199
2. Division of Neuroscience, Yenepoya Research Centre (YRC), Yenepoya (Deemed to be University), Deralakatte, Mangalore, Karnataka 575018
3. Department of Pediatrics, Division of Hematology/Oncology, College of Medicine, The Pennsylvania State University, Hershey, PA 17033, USA
4. Department of Biochemistry, Mangalore University, Mangalagangothri, Karnataka 574199
5. Department of Zoology, Bangalore City University, Bengaluru, Karnataka 560001

* For Correspondence

Recommended Level: UG/PG/Research

Sub-Discipline: Anatomy/Physiology

Background:

Inflammatory bowel diseases (IBD) are chronic and lifelong diseases in humans characterized by gastrointestinal inflammation. The two types of IBD, Ulcerative Colitis (UC) and Crohn's Disease (CD), share many similar symptoms and significant overlapping genetic components (Yeschi et al., 2020). Globally, around 6.8 million cases were reported in 2017, and over the past few decades, IBD has been the most dominant disease in the Western world. However, in recent times, IBD incidence has shown consistent rising trend in many Asian countries (Yeschi et al. 2020). The exact cellular and molecular mechanism of IBD has not yet been fully understood. Absence of adequate animal models has also hampered understanding of the pathogenesis of IBD. The most widely used animal models of IBD are induced by administering toxic chemicals such as Dextran Sulfate Sodium (DSS) (Kiesler et al., 2015; Elson et al., 1995). DSS causes gut inflammation that mimics similar features of ulcerative colitis and is widely perceived as a good model for experimental colitis (Gaudio et al., 1999; Strober et al., 2002).

Drosophila melanogaster and humans have many conserved features of digestion and absorption (Lemaitre and Miguel-Aliaga, 2013), which can make *Drosophila* a good animal model system to study DSS-induced colitis (Keshava et al., 2022). Smurfing or dye leakage assay is widely used for evaluating intestinal wall damage in different animal

models. The present chapter describes the Smurf assay to show toxic impact of DSS on the permeability of the gastro-intestinal tract (Keshava et al., 2022).

Objectives: To test the intestinal inflammation or damage in the *Drosophila* intestinal colitis model using dye leaking or smurfing assay.

Materials Required:

- A. Flies: Age synchronized (One day after eclosion) male/female flies.
- B. Equipment and fly handling materials:
 - i. Stereo binocular microscope and other fly handling materials.
 - ii. Permanent Marker.
 - iii. Capillary tubes (75 mm long and 0.28 mm diameter).
- C. Reagents:
 - i. Dextran Sodium Sulfate (DSS) – prepare a 3% DSS solution in 5% sucrose solution or in 5% yeast paste.
 - ii. Bromophenol Blue.

Experimental Details:

- i. One-day-old flies are fed with 3% DSS in 5% sucrose solution using a capillary or 3% DSS in 5% yeast paste for 5 days to induce intestinal inflammation.
 - a. **Capillary feeding:** A capillary tube is filled with 3% DSS in 5% Sucrose solution and introduced into a food-free vial containing 15-20 flies (flies) so that flies feed on the solution by sucking from the free end. The capillaries are refilled as and when the solution content is nearing exhaustion. The feeding continued for 5 days without any breaks. Feed the control flies with only 5% Sucrose in capillaries.
 - b. **Yeast paste:** Thoroughly mix 3gm DSS in 100 ml 5% yeast paste (5gm yeast granules dissolved in 100 ml Distilled water) and add ~10ml of the mixture to a food-free vial. Carefully transfer 15-20 flies to the tube and allow them to continuously feed on the DSS-Yeast mixture for 5 days. Feed the control flies on only 5% yeast.
- ii. After 5 days, transfer the control and DSS-fed flies to normal food vials and examine them after 5 days.
- iii. On the tenth day, both DSS-fed and control flies are capillary fed with 5% sucrose solution containing 0.2% Bromophenol Blue dye for 30 minutes.
- iv. Both sets of flies are transferred to normal food for the next 4 hours.
- v. The flies are anesthetized and examined under a binocular microscope.

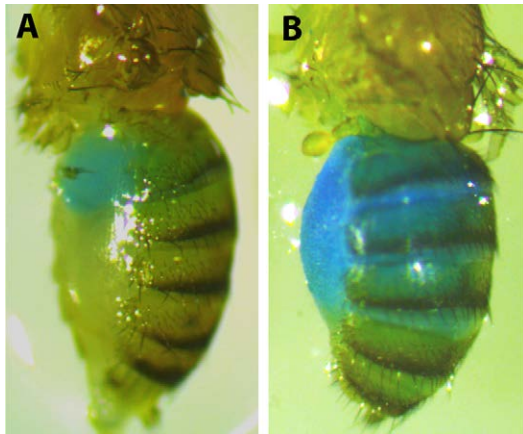
Observations:

Fig. 1. Smurfing in *Drosophila*. Control (A) and DSS-fed (B) flies showing leakage of the blue dye in hemolymph in B (Images reproduced from Keshava et al., 2022, with permission)

The control flies retain the dye within the intestinal wall (Fig. 1A) but the DSS-fed flies show leakage of Bromophenol Blue dye into the abdominal cavity (Fig. 1B).

Questions for self-study and interpretation:

1. What does the appearance of dye in abdominal cavity in DSS-fed flies indicate?
2. Can this assay be used for other toxic chemicals to test their effect on gut wall?
3. Do male and female flies show any difference in the intestinal inflammation caused by DSS?
4. Can DSS be used for modeling intestinal colitis in other animal models?
5. Do aged flies show dye leakage through the gut wall without any treatment?

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Chapter 80

Screening of novel potential anti-cancer agents using *Drosophila* tumour models

Sushree Sulava (sushree.sulava@niser.ac.in) and Debasmita Pankaj Alone* (debasmita@niser.ac.in)
School of Biological Sciences, National Institute of Science Education and Research (NISER)
Bhubaneswar, HBNI, P.O. Jatni, Khurda, Odisha 752050.

* For correspondence

Recommended Level: PG/Research

Sub-discipline: Genetics, Cell biology, Molecular biology, Cancer biology

Background:

About 75% of the disease associated genes in humans have functionally conserved counterparts in *Drosophila*, thereby making the fly an useful model organism to generate tumours through various mutations. This provides an effective genetic background to delineate the molecular hierarchy to the multi-faceted web of factors/signaling pathways responsible for triggering tumourigenesis (Ugur, 2016). The loss of function of many tumour suppressor genes like *dlg* (*discs large*), *lgl* (*lethal giant larva*), *l(3)tb* (*lethal (3) tumourous brain*), is associated with disruption of cytoarchitecture and neoplastic growth (Bilder 2004 and Gateff, 1989; Mishra *et al.*, 2020). Mutation in *HUGL-1*, one of the *lgl* human homologs has been associated with hepatocellular carcinoma and colorectal cancer (Cao, 2015). Several contemporary recombinant DNA based technologies like CRISPR-Cas9, TALENs (Transcription Activator like Effector Nucleases), etc. can be used in *Drosophila* to mutate specific genes to identify their tumourigenic potential (Ren, 2017). The fly tumour models, based on established tumourigenic gene mutations, on the other hand, permit screening of novel therapeutic agents. The example presented here for screening of novel therapeutic agents for cancer makes use of established fly strains generated by forward screening of mutations in tumour suppressor genes. These approaches can also be utilized with tumourous conditions generated by other gene mutations.

Lethal (2) giant larvae (l(2)gl) gene encodes the protein isoforms p127 and p78, which are involved in cellular proliferation and possess tumour suppressive function. Thus, a loss of function of this gene can result in robust over-proliferation and eventual neoplastic transformation. The *l(2)gl/CyO* strain of *Drosophila* acts as a brain tumour model in which the homozygous *l(2)gl/l(2)gl* mutants bear brain tumour and exhibit lethality in the

late third instar stage or pupal stage. Hence, only the heterozygous mutants complete their life cycle to emerge as adults (Woodhouse, *et al.*, 1998; Das, *et al.*, 2014).

Lethal (3) tumorous brain [l(3)tb] is another tumour suppressor which when mutated results in disruption of cellular homeostasis and oncogenic transformation of cells with the *l(3)tb* homozygous larvae developing enlarged brain and ultimately dying without undergoing pupal development. The *l(3)tb* is an allele of *DCP2* (De-capping Protein 2) which encodes the m7G (5') pppN diphosphatase and has a myriad of roles starting from cellular organization and developmental functions to stress response mechanism. The *l(3)tb* is a loss of function allele of *DCP2* which results in tumorigenesis. This third chromosomal recessive lethal mutation is maintained against the *TM6B, Tb* balancer so that the *l(3)tb* homozygous larvae show a non-tubby phenotype and exhibit larval/pupal lethality. Thus, non-tubby adult flies homozygous for the *l(3)tb* mutant allele do not emerge (Mishra, *et al.*, 2020).

The homozygous mutants from both the strains display neoplasia in the brain lobes and imaginal discs. The *l(2)gl* homozygous mutants also have been reported to show locomotory defects. Only the heterozygous larvae in each case complete their cycle to emerge as adults (Das, *et al.*, 2014 and Mishra, *et al.*, 2020). The experimental approach presented here makes use of these phenotypes to screen potential anti-tumour activities of synthetic compounds or natural products provided with food (oral route). Any agent that has anti-tumour activity and suppresses tumour growth would also suppress these tumour-associated phenotypes normally displayed by the *l(2)gl* or *l(3)tb* homozygous larvae (Das, *et al.*, 2014).

Objectives:

To utilize two established (*l(2)gl* and *l(3)tb*) *Drosophila melanogaster* tumour models for in vivo screening of potential drugs for their anti-cancer properties.

Materials & methods:

A. Fly strains:

- i. *Canton-S* or *Oregon-R* (wild type)
- ii. $y^l w^{67}c^{23}, l(2)gl^4 / CyO, y^+$
- iii. $y w; +/+; l(3)tb / TM6B, Tb^l Hu e^l$

B. Fly food and egg collection media: (see [Chapter 1](#)) for preparation of these media.

C. Chemicals and Reagents:

- i. Neelicol brilliant blue FCF dye (Neelicol food dye) solution in dist. water (0.125 mg/mL) (15.625 mg of the FCF dye is added to 125 mL of distilled water with thorough mixing).
- ii. 0.1% Agarose (0.02 gm Agarose in 20 mL water) – dissolve by boiling, mix a few drops of the Neelicol food dye solution, and pour in plastic Petri plates (60 mm x 15 mm) to solidify.

- iii. 1X PBS: Dissolve 8 gm NaCl, 0.2 gm KCl, 1.42 gm Na₂HPO₄ and 0.272 gm KH₂PO₄ in 800 mL distilled water, adjust pH to 7.4 and make up the final volume to 1 L with dist. water. Autoclave and store at 4⁰C.
- iv. 70% Ethanol
- v. Diethyl ether

D. Equipment and other supplies:

- i. Stereo-binocular microscope with imaging facility
- ii. Polystyrene vials (24.5 x 95 mm)
- iii. Petri plates (60 mm X 15 mm)
- iv. Brushes (size 0 and 1)
- v. Fine dissecting needles and forceps
- vi. Non-absorbable cotton
- vii. Cellotape
- viii. Plain slides and cover slips
- ix. Pasteur pipettes

Experimental Details:

A. Food preparation:

The standard fly food is prepared as control. The desired chemical compound/natural product, which is to be tested for potential anti-cancer activities, is added at the desired concentrations and thoroughly mixed when the cooked food (see [Chapter 1](#)) is still fluid at about 50-55°C. A few drops of the Neelicol brilliant blue FCF dye solution are also added to the drug in fused food medium and mixed well prior to pouring into vials/Petri plates. When the larvae ingest the food, the blue colour is observed in their gut, which confirms the intake of the test compound by the larvae.

Note: The concentration of the test drug in the food depends either upon results of any previous study or is to be determined by testing various wide range of concentrations in pilot studies to determine the desired concentration (Das, *et al.*, 2014).

B. Collection of synchronized larvae:

Transfer 5-6 days old mated female flies of the desired genotype [*l(2)gl/CyO* and/or *l(3)tb/TM6B*] in the present example] from their normal food bottles to empty vials for 3 hr and then transfer for 2 hr in vials containing normal food medium to which a drop of additional yeast paste is added to facilitate shedding of eggs that may have been not laid earlier (Bilder, 2004). The flies are then transferred to agar-sugar-yeast food for egg collection (see [Chapters 32, 33, 34, 70](#)) for 2 hr to collect synchronously developing embryos. The *l(2)gl* homozygous third instar larvae can be distinguished from *l(2)gl/CyO* heterozygotes since the former have colorless Malpighian tubules (being homozygous for the X-linked *w* allele) while Malpighian tubules of the *l(2)gl/CyO*

heterozygotes appear yellow (heterozygous for w/w^+) gene (see **Chapter 20**). The Malpighian tubules are visible through the larval cuticle (**Fig. 1A-D**). In the other case, the $l(3)tb$ homozygous larvae are non-tubby while the $l(3)tb/TM6B$ heterozygotes larvae display short and stout tubby phenotype (see **Chapter 1**) and thus can be easily identified (**Fig. 1E, F**).

C. Larval and Pupal Lethality Assay:

The $l(2)gl$ as well as $l(3)tb$ homozygotes are known to show significant larval/ pupal lethality due to the tumourous growth. (Woodhouse, 1998; Mishra, *et al.*, 2020). Therefore, comparison of survival of different genotypes on normal and drug-supplemented food provides information about any potential beneficial or harmful effects of the test compound.

- i. Collect eggs from flies of the desired genotype on an embryo collection medium in Petri plates at 2 hr intervals using an embryo collection chamber.
 - a. Transfer 100 eggs (in 3 replicates), collected over a 2 hr period, for each genotype (Wild type, $l(2)gl$ and/or $l(3)tb$) to normal food (control) or the test-drug supplemented food (experimental) in Petri plates.
 - b. Monitor the larval development for any developmental issues.
 - c. Count and record the number of live third instar larvae pupating by the sixth day of egg laying in each Petri plate (see **Table 1**).

- ii. Count the number of $l(2)gl$ and/or $l(3)tb$ homozygous larvae that pupate in each Petri plate and gently transfer them to fresh vials with help of a fine brush.

- iii. Count and record the numbers of adults that eclose in each vial (different genotypes and control and treated).

Note: If the test compound has any anti-tumour activity, some or all of the $l(2)gl$ or $l(3)tb$ homozygous larvae would survive, pupate and emerge as adults. The difference in the numbers of eggs transferred and live larvae seen on day 5/6 provide an estimate of larval death and that between the numbers of pupae and the emerging adults provide an estimate of the pupal death. The difference in the survival of wild type eggs/pupae on control and experimental food also provides information about any toxicity effect of the test compound.

- iv. Plot the mean percentages of these values for different genotypes and experimental conditions to determine if the treatment had any positive (tumour suppression), or detrimental effect (greater larval/pupal death in wild type) or no effect.

D. Brain and wing disc size measurement:

The $l(2)gl$ as well as $l(3)tb$ homozygotes are known to show enlarged larval brain due to tumourous growth. (Woodhouse, 1998; Mishra, *et al.*, 2020). Therefore, a comparison of the brain size at late third instar larvae of different genotypes on control and test food provides information about the extent of tumourous growth.

- i. Dissect 20 to 30 late third instar larvae of each genotype and treatment condition (control and test-chemical supplemented food) in 1X PBS to separate their brain ganglia and transfer the same in a drop of 1X PBS on a plain slide using needles or forceps.
 - ii. Image all the dissected brains immediately under identical conditions of magnification, illumination, exposure time, etc (**Fig. 1G, H**).
 - iii. The mean areas (\pm S.E.) of brain hemispheres for different genotypes and treatment regimen are calculated for comparison between the treated and untreated mutant and the wild type larvae are plotted.
- Note:** Same steps may be followed for dissection and measurement of sizes of wing discs of larvae.

E. Locomotion assay:

- i. For this assay, individually transfer the 6-7 days old third instar homozygous mutant larvae from drug treated medium, 10 days old third instar homozygous mutant larvae from untreated medium and 7 days old wild type control and experimental larvae from the synchronous cultures to the Neelicon brilliant blue dye agarose plates.
- ii. Using smart phone camera, prepare 30 sec pulse video to record the crawling movement of larvae on the contrasting blue background of the medium (see **Chapters 1, 91**).
- iii. Plot the average distance (\pm S.E.) covered by the treated and untreated mutant and wild type larvae in 30 sec (Das, *et al.*, 2014).

F. Negative geotaxis assay:

If the test compound suppresses tumour development and thereby permits emergence of homozygous *l(2)gl* adult flies, the adults may be subjected to negative geotaxis assay. The emerging *l(2)gl* mutant flies can be identified by their normal wings (the *l(2)gl/CyO* heterozygous flies have curly wings). Likewise, any surviving *l(3)tb* homozygous fly can be distinguished from the *l(3)tb/TM6B* heterozygotes by their non-tubby phenotype. Test *l(2)gl/CyO*, *l(2)gl/l(2)gl*, *l(3)tb/TM6B* and *l(3)tb/l(3)tb* flies emerging after rearing on standard and drug containing food medium, respectively, for the negative geotaxis assay; wild type flies are used as control.

- i. Transfer 5 flies of a given genotype and treatment condition to an empty fresh 10 cm long polystyrene vial and, immediately invert another empty 10 cm long polystyrene vial over the open mouth of the first and seal the facing mouths of the two tubes with a transparent cellotape. A distance of 10 cm from the bottom of a vial is prominently marked on the outside of this assembly.
- ii. Gently tap the assembly so that all flies fall to the bottom. Keep the assembly upright without any jerks/vibrations.
- iii. Count how many flies climb up and reach the 10 cm distance marker in 10 sec.

iv. In a parallel assay, use a stop watch to measure the time taken by different flies to reach the distance marker.

Note: This assay can also be recorded as videos using a smart phone for subsequent analysis of the number of flies crossing the distance marker and the time taken by them to cross the mark. (also see [Chapters 92, 94, 96](#) for slightly different procedures for climbing assay)

Observations:

A. Survival assay:

Record the number of live larvae and pupae and the number of emerged flies for each case. For *l(2)gl* mutant progeny, the number of *CyO/ Non-CyO* flies is also recorded for experimental and control groups, whereas for *l(3)tb* mutant strain, the numbers of tubby and non-tubby larvae, pupae and flies are recorded for experimental and control groups (**Table 1**).

Table 1: Effect of the drug on survival of larvae and pupae in different genotypes

Parental Genotype	Treatment	No. of live 3rd instar larvae		No. of pupae formed		% larval death		No. of eclosed adults		% Adult survival		Phenotype of eclosed adults	
		Non-mutant	Mutant	Non-mutant	Mutant	Non-mutant	Mutant	Non-mutant	Mutant	Non-mutant	Mutant	Non-mutant	Mutant
Wild type	Control												
	Treated												
<i>l(2)gl/CyO</i>	Control												
	Treated												
<i>l(3)tb/TM6B</i>	Control												
	Treated												

B. Brain and Imaginal Disc sizes:

i. Manually trace the periphery of the dissected and mounted brain hemispheres to measure their area using appropriate software (**Fig. 11**). The mean values (\pm S.E.) for different genotypes and treatment conditions are recorded (**Table 2**) for comparison between control and experimental groups.

ii. Record the area of the wing imaginal discs for 30 larvae of respective groups as above for estimation of their sizes (as for brain ganglia) and record the mean values (\pm S.E.) for comparison between experimental and control samples (**Table 2**).

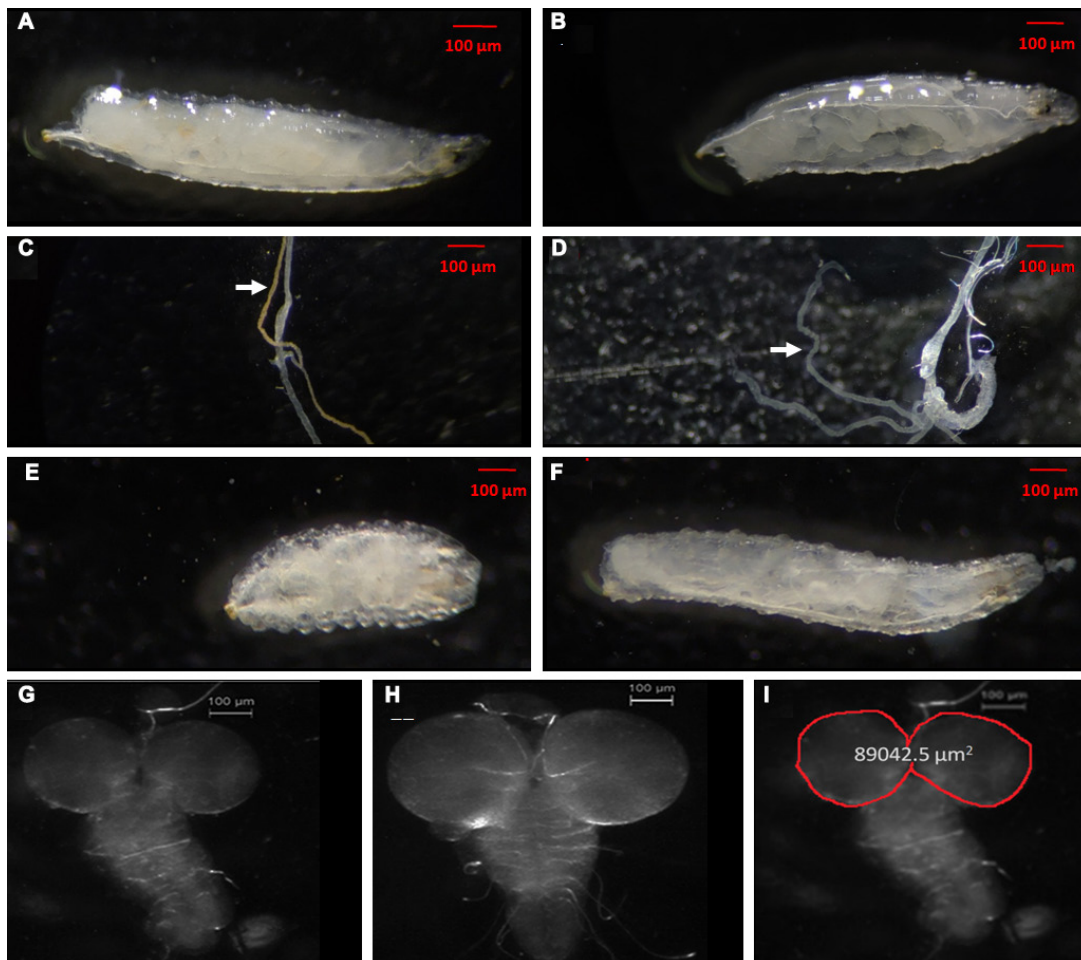


Fig. 1. Representative images of **A.** *l(2)gl/CyO* third instar larva with characteristic yellow Malpighian tubules; **B.** *l(2)gl/l(2)gl* third instar larva with colorless Malpighian tubules; **C.** Yellow Malpighian tubules (white arrow) of *l(2)gl/CyO* third instar larva. **D.** Colorless Malpighian tubules (white arrow) of *l(2)gl/l(2)gl* third instar larva; **E.** *l(3)tb/TM6B* third instar larva with tubby phenotype; **F.** *l(3)tb/l(3)tb* third instar larvae with non-tubby phenotype; **G-H.** Brain and ventral nerve cord of third instar wild type (**G**) and *l(2)gl/ l(2)gl* (**H**) larvae; **I.** Measurement of the area of the optic lobes of brain via software analysis tool (Das, *et al.*, 2014).

Table 2: Effect of the drug on larval brain size in different genotypes

Parental Genotype	Treatment	Mean Area of Brain	
		Non-Mutant	Mutant
Wild type	Control		
	Treated		
<i>l(2)gl/CyO</i>	Control		
	Treated		
<i>l(3)tb/TM6B</i>	Control		
	Treated		

C. Larval Locomotion Assay:

Record and estimate the distance covered by larvae of different genotypes and treatment conditions in 30 sec (N=30 for each group) (**Table 3**).

Table 3: Effect of drug treatment on locomotory behaviour of larvae of different genotypes.

Mean \pm S.E. distance (in cm) covered by the larvae in 30 s (N= 30)					
Wild type		<i>l(2)gl/CyO</i>		<i>l(3)tb/TM6B</i>	
Control	Treated	Control	Treated	Control	Treated

D. Negative geotaxis assay:

Record the mean numbers of flies of different genotypes and treatment conditions that cross the marked boundary within the given time and the mean time taken by flies of different genotypes and treatments to reach the defined boundary (**Table 4**). Since untreated tumour bearing *l(2)gl* or *l(3)tb* homozygous flies do not survive, the rescued mutant homozygous flies are compared with their heterozygous siblings and wild type flies.

Table 4: The negative geotaxis assay for adult flies of different genotypes and treatment groups

Genotype	Treatment	No. of flie	Mean No. flies reaching the boundary	Mean time taken to reach the boundary
Wild type	Control			
	Treated			
<i>l(2)gl/l(2)gl</i>	Control *			
	Treated			
<i>l(2)gl/CyO</i>	Control			
	Treated			
<i>l(3)tb/l(3)tb</i>	Control *			
	Treated			
<i>l(3)tb/TM6B</i>	Control			
	Treated			

* No flies of these genotypes are expected to be available under control conditions

Based on the above assay results, suggest if the test drug has a tumour-suppressive or tumour enhancing effect or has no effect.

Questions for self-study and interpretation:

1. What are tumour suppressor genes and proto-oncogenes? Categorize the mutants mentioned in the study.
2. What are the various signaling pathways discovered using the *Drosophila* model?
3. Why are balancers used?
4. What will be the genotypes of the progenies from the following crosses?
(A) *l(2)gl/CyO* X *l(2)gl/CyO*, (B) *l(3)tb/TM6B* X *l(3)tb/TM6B*, and (C) *l(2)gl/CyO* X *l(3)tb/TM6B*
5. What are the desirable features of an anti-cancer drug?
6. How would you proceed further if some test compound provides positive results from this screening?
7. What is the necessity to further test a drug in a higher animal model that has already been identified for its anti-cancer properties in a *Drosophila* model?

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Chapter 81

Generation of tumor cell clones in a normal tissue background by MARCM technique in *Drosophila melanogaster*

Abhijit Biswas (abhijitbiswas956@gmail.com) and S. C. Lakhotia* (lakhotia@bhu.ac.in)

Cytogenetics Laboratory, Department of Zoology, Banaras Hindu University, Varanasi 221005, India

* For correspondence

Recommended Level: PG/Research

Sub-discipline: Genetics/Developmental Biology/Cancer Biology

Background:

Functional analysis of a gene using its mutant alleles is a common approach in genetics. However, if homozygosity of a recessive mutant allele has severe developmental consequences (including death of the organism) at an early stage of development, the given gene's functions at later stages remain unresolved. In such situations, the mosaic analysis, where only some cells are homozygous for the mutant allele while all others remain heterozygous, is an effective method for understanding diverse functions of the gene under different developmental contexts and stages (see [Chapters 41, 42](#)). The mosaic analysis also permits cell-cell interactions so that cell-autonomous or non-cell-autonomous actions of the given mutant allele can be analyzed.

The use of genetic mosaic analysis approach in *Drosophila* has become more convenient following the development of FLP/FRT system for somatic recombination and efficient clone marking techniques (Golic and Lindquist, 1989; Xu and Rubin, 1993). According to the widely used cell marking method for generating FLP/FRT mediated 'twin-spot' mosaics (see [Chapter 42](#)), the homozygous mutant cells generated after somatic crossing lose the cell marker transgene, like *Ubi-GFP*, positioned distal (in the context of centromere location) to the FRT site, and in trans to the mutant gene of interest. Consequently, the generated clones of mutant cells lose the GFP cell marker while the surrounding cells show GFP (cell marker) expression. However, in many studies it is preferred to provide a cell marker (like GFP fluorescence) for the homozygous mutant clonal cells rather than the heterozygous parental or the homozygous wildtype sib cells.

Expression of a fluorescent cell marker for the homozygous mutant cell clone for live analysis is achieved by the Mosaic Analysis with a Repressible Cell Marker (MARCM) method (Fig. 1), which enables, following the mitotic recombination, expression of the cell marker in the homozygous mutant cells in the background of unlabeled heterozygous surrounding cells (Lee and Luo, 1999). Consequently, the MARCM method provides a more sensitive approach to identify the mutant cells and examine the impact of homozygous

mutations in specific cell/tissue types. The MARCM method uses a combination of the yeast GAL80 repressor protein, an inhibitor of the GAL4 transcription factor used in the GAL4-UAS binary expression system (see Chapter 39), and the FLP/FRT system to generate genetically marked clones (see Fig.1). In the MARCM system, the GAL80 and GAL4 are expressed by different transgenes, each under the ubiquitously active *Tubulin 1* (*Tub*) promoter. The *Tub* promoter driven GAL80 represses the activity of GAL4 protein, and thus globally prevents expression of any of the GAL4-responding UAS carrying transgenes present in the genome. The MARCM chromosome carries FRT-site, typically close to the centromere, on both homologs of the chromosome that carries the desired mutant allele. The desired mutant allele, which is required to become homozygous in cells following the induced homologous recombination at the FRT sites, is located distal (with reference to the centromere) to the FRT-site on one of the homologs. The other homolog carries the FRT site, wild type allele of the desired mutant, and the UAS-GAL80 transgene. As shown in Fig. 1, the conditional activation of the *UAS-FLP* (or *hs-FLP* in the example here) transgene, located on a different chromosome, which also carries the *UAS-GFP* transgene, causes transient expression of the FLP Recombinase (FLP) which causes somatic recombination between non-sister chromatids at the FRT-sites on the homologs with high efficiency. Depending upon the pattern of separation of sister and non-sister chromatids at the anaphase stage following the recombination event, three different types of daughter cells are produced: i) GFP-expressing cells homozygous for the mutant allele of the gene of interest that have lost the *Tub-GAL80* transgene because of somatic recombination, ii) GFP-negative wild-type cells (homozygous for the wild type allele of the gene of interest) carrying two copies of *Tub-GAL80*, and iii) GFP-negative parental type cells carrying one copy of the *Tub-GAL80* and one copy of the mutant allele of the gene of interest (Fig. 1). The loss of GAL80 releases the *Tub-GAL4* from repression so that expression of the GAL4-driven reporter gene (*UAS-GFP* in the present case) is seen only in the mutant clonal cells (see Fig.1).

In order to facilitate generation of somatic clones for genes located on different chromosome arms, *Drosophila* stocks have been generated in which the FRT sequence is inserted close to the centromeric region of different chromosome arms. The following stocks are available at the Bloomington *Drosophila* Stock Center (USA):

Stock number	MARCM component
5132	<i>FRT</i> and <i>TubP-GAL80</i> recombinant for X: FRT19A (1C2)
5191	<i>FRT</i> and <i>TubP-GAL80</i> recombinant for 3L: FRT80D (75E1)
5192	<i>FRT</i> and <i>TubP-GAL80</i> recombinant for 2L: FRT40A (31E3)
5140	<i>FRT</i> and <i>TubP-GAL80</i> recombinant for 2R: FRT42B (47A7)
5190	<i>FRT</i> and <i>TubP-GAL80</i> recombinant for 3L: FRT80B (75E1)
5135	<i>FRT</i> and <i>TubP-GAL80</i> recombinant for 3R: FRT82B
8862	<i>hs-FLP</i> on X-Chromosome (FLP driven by a heat-shock promoter)
5136	<i>UAS-mCD8::GFP</i> on X chromosome
28832	<i>hs-FLP</i> and <i>UAS-mCD8::GFP</i> on X chromosome
36289	<i>l(2)gl⁴ P{neoFRT}40A/CyO</i>

The example considered here generates tumorous clones in larval imaginal discs that become homozygous for a mutant allele of the *l(2)gl⁺* gene following FLP-FRT targeted somatic recombination. The *lethal (2) giant larvae (l(2)gl⁺)* is a tumor suppressor gene in *Drosophila*, whose product maintains the apico-basal cell polarity in epithelial cells (Cao, 2015). Larvae homozygous for its recessive loss of function allele (*l(2)gl^Δ*, referred to here as *lgl^Δ*) develop malignant tumors in imaginal discs and brain ganglia; they attain large size ('giant' larvae) and after a prolonged larval life die as larvae or pseudopupae. This scaffold protein has 60% sequence similarity with the Hugel-1, associated with human epithelial carcinomas (Cao, 2015). Using the MARCM technique, *lgl^Δ* tumorous clones can be generated in dividing cells of otherwise heterozygous wild type (*lgl⁺/lgl^Δ*) imaginal discs, brain etc. Although *lgl^Δ* homozygous larvae develop malignant tumors, the *lgl^Δ* homozygous clones in *lgl⁺/lgl^Δ* heterozygous background do not grow well as they fail to compete with the neighbouring wild type cells and, therefore, undergo apoptosis (Agrawal *et al.*, 1995). Therefore, to permit the survival and malignant growth of the *lgl^Δ* MARCM clones, a tumor helper mutant is also included in the genotype of the *lgl^Δ* homozygous clones. In the present example, overexpression of Yorkie, a Hippo signaling member that promotes growth and cell division, in the *lgl^Δ* MARCM clones is achieved by including the *UAS-yki (yki^{OE})* transgene linked to the *lgl^Δ* mutant allele, and which also gets driven by the *Tub-GAL4* activity in the MARCM mutant clones following loss of *Tub-GAL80* transgene due to somatic crossing over (Fig. 1).

Objective: To generate the MARCM clones (*lgl^Δ yki^{OE}* tumor clones in imaginal discs in the example here) in *Drosophila melanogaster*.

Materials Required:

A. Flies:

- i. *y w hs-FLP UAS-GFP Tub-GAL4/ y w hs-FLP UAS-GFP Tub-GAL4; FRT40A lgl⁺ Tub-GAL80/ CyO-GFP; +/+*
- ii. *w; FRT40A lgl^Δ UAS-yki/CyO-GFP; +/+*

Note: These stocks can be obtained from Cytogenetics Lab, Department of Zoology, BHU, Varanasi

B. Equipment and other supplies:

- i. Stereo-binocular microscope and other fly handling materials.
- ii. Fluorescence (or Confocal) microscope.
- iii. BOD set at 24°C.
- iv. Water bath set at 37°C.
- v. Vials with 2ml *Drosophila* food.
- vi. Cavity/Maximov slides.
- vii. Slides and coverslips (22 mm²).
- viii. Blotting/filter papers.
- ix. Micropipettes (1000 μL, 200 μL and corresponding micropipette tips).

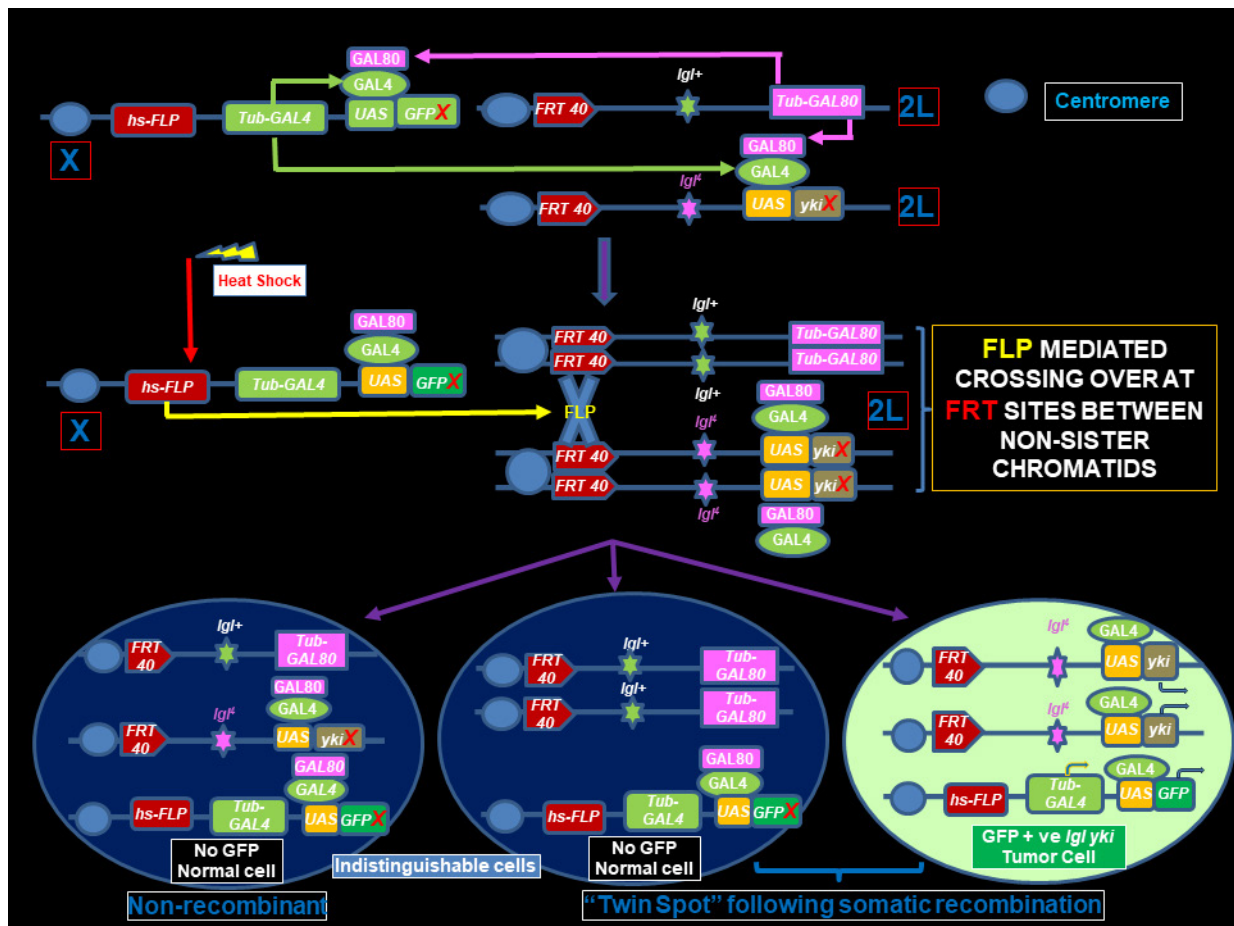


Fig. 1 : A schematic representation of the MARCM technique to generate *Igl^{fl} UAS-yki* (*Igl^{fl} yki^{OE}*) tumors. In the example here, the X-chromosome of the MARCM progeny carries *hs-FLP*, *Tub-GAL4* and *UAS-GFP*, while *FRT40*, *Igl⁺* and *Tub-GAL80* are present on left arm (2L) of one of the chromosome 2 homologs; the 2L of its homolog carries *FRT40*, *Igl^{fl}* and *yki^{OE}*. The GAL80 protein, ubiquitously expressed under the *Tub-GAL80* transgene, represses activity of the *Tub-GAL4* dependent GAL4 transcription factor so that the GAL4-dependent expression of *UAS-GFP* and *yki^{OE}* transgenes remains blocked in all cells (top). Heat shock drives *hs-FLP* transgene leading to transient synthesis of FLP recombinase, which induces site-specific mitotic recombination between FRT sites (middle). Depending upon the pattern of separation of somatically recombined non-sister chromatids at the ensuing Anaphase, daughter cells may retain the parental genotype (bottom left cell) or produce two kinds of daughter cells ('Twin spots') which are either homozygous for *Igl⁺* and *Tub-GAL80* (bottom mid cell) or for *Igl^{fl}* and *yki^{OE}* (bottom right cell), respectively. Each *Igl^{fl} yki^{OE}* cell generates a tumorous clone as it divides. Having lost the *Tub-GAL80* transgene because of somatic crossing over, only the *Igl^{fl} yki^{OE}* homozygous tumor cells express the *Tubulin* promoter driven GFP and thus only the induced clones show GFP-fluorescence.

C. Reagents:

- i. 1X Phosphate Buffer Saline (PBS pH 7.4)

NaCl	8 gm
KCl	0.2 gm
Na ₂ HPO ₄	1.44 gm
KH ₂ PO ₄	0.24 gm

Serially dissolve the above in 800 mL distilled water; when fully dissolved, adjust pH to 7.4 with HCl, as required. Finally make the volume to 1000 mL with distilled water, autoclave the solution and store at room temperature.

- ii. 4% Paraformaldehyde in 1X PBS: dissolve 4 gm paraformaldehyde powder in 100ml 1X PBS at 37°C for 60 min with periodic shaking. Cool and store at 40C, but use within a few days.
- iii. PBST: 0.1% Triton X-100 in 1X PBS.
- iv. DABCO as Mounting Media.

Experimental Details:

1. Cross *y w hs-FLP UAS-GFP Tub-GAL4/ y w hs-FLP UAS-GFP Tub-GAL4; FRT40A lgl⁺ Tub-GAL80/ CyO-GFP*; + virgin females and *w; FRT40A lgl⁺ UAS-yki/CyO-GFP*; + males in food bottles (see schematic crosses in Fig. 2).
2. Next day, collect eggs over a period of 1-3 hr.
3. Next day, collect 25 freshly hatched first instar larvae and transfer to vials with only 2ml food using soft brush. Prepare a set of four of such vials. Grow them at 24°C for 48 hr when they reach the late second instar stage.
4. Prewarm 1x PBS in 37°C water bath for applying heat shock to the 48 hr old progeny larvae by adding 200 µL pre-warmed 1x PBS in the above four food vials with 48 hr old larvae and immediately place the vials in the 37°C water bath using a vial stand/tray for 3 min. Add 200 µL of 1X PBS maintained at 24°C to the other two vials with larvae and keep them at 24°C to serve as control. (**Note:** Larvae growing in vials with only 2 mL of food experience uniform heat shock rapidly than when reared in vials with normal amount of 5-10 mL food. Duration of heat shock may be varied depending upon the tissue to be examined for the induced clones).
5. After the heat shock, return the vials with larvae to 24°C for 48 hr or 72 hr or 96 hr for examination of the induced clones at different times after heat shock mediated somatic crossing over.
6. At the desired time after the brief heat shock, wash the larvae from control and experimental vials with distilled water to remove adhering food particles and examine them under fluorescence stereo-binocular microscope or a fluorescence microscope (using a low power objective) for the pattern of GFP fluorescence to sort larvae that show (Fig. 3A, B) or do not show the *CyO-GFP* pattern of fluorescence (Fig.3C, D). Select the latter group of larvae as they are expected to carry the GFP expressing *lgl⁺ yki^{OE}* homozygous clones induced following the FLP-mediated somatic crossing over at FRT sites.

Note: The GFP fluorescence patterns differ between *CyO-GFP* chromosome carrying larvae (genotypes 1 and 2 in F1 progeny larvae in Fig. 2) and *l2gl⁺ yki^{OE}* homozygous clone bearing larvae (genotype 3 in F1 progeny larvae in Fig. 2). The GFP fluorescence visible through the larval cuticle is prominent in the proventriculus region of the gut in the *CyO-GFP* chromosome carrying larvae (Fig. 3A). The genotype 2 larvae in

the F1 progeny (Fig. 2) show *Cyo-GFP* as well as ubiquitous GFP fluorescence as they did not inherit the *Tub-GAL80* transgene bearing chromosome 2 (Fig. 3B). On the other hand, the MARCM genotype 3 larvae in the F1 progeny do not show proventricular or ubiquitous GFP fluorescence; instead, if the FLP/FRT-mediated somatic clones are induced, they display multiple small GFP-positive regions, located mostly in their anterior half (Fig. 3C). If the MARCM genotype 3 larvae are not exposed to heat shock or if for some reason the FLP/FRT-mediated clones do not develop, they will be completely devoid of GFP fluorescence (Fig. 3D).

7. Place a larva which shows the presence of *lgl⁴ yki^{OE}* GFP-positive clones (Fig. 3C) in a drop of 1X PBS on a clean slide under stereo-binocular microscope. To dissect out the desired tissue (imaginal disc or brain ganglia), hold the larva with a dissection needle at about 1/3rd the length from its anterior end; place another needle on the black mouth hooks and pull the two needles in opposite directions. Mouth hook along with the brain, salivary glands, and cluster of the eye-antennal, pro and mesothoracic leg imaginal discs will get separated from rest of the larval tissues; the metathoracic leg, haltere and wing discs will usually be found attached as two clusters to the inner side of the body wall of the larva (see Chapters 18, 30, 31).
8. Separate the imaginal discs from parts of gut, salivary glands, fat body etc, and transfer them along with the mouth hook and brain into freshly prepared 4% paraformaldehyde fixative in a glass cavity block. Keep the sample covered to minimize evaporation. Fix the tissues for 20 min at room temperature.
9. Following fixation, separate individual discs from the other attached tissues (mouth hook, brain lobes, body wall etc) using fine dissecting needles.

Notes:

- i. The GFP Fluorescence can be seen in unfixed tissues, but the fixed imaginal discs are less fragile and thus easier to separate from other tissues than the very delicate unfixed discs.
 - ii. Since exposure of the tissue to acidic pH quenches the GFP fluorescence, ensure that pH of any solution is not less than 7.0.
10. Carefully remove the fixative from the slide without losing the discs. Wash the discs once with 0.1% PBST for 15 min.
Note: If required, the discs at this stage can be processed for in situ hybridization and/or immunostaining and/or (see Chapters 57, 58).
 11. Transfer individual discs carefully into a drop of DABCO on a clean slide. Allow the discs to settle flat on the slide and slowly place a coverslip, avoiding trapping of air bubble.
 12. After mounting, observe the imaginal discs under a Fluorescence microscope using filter set for GFP fluorescence.
Note: Confocal microscopy provides more resolved and better images of the induced tumorous clones.

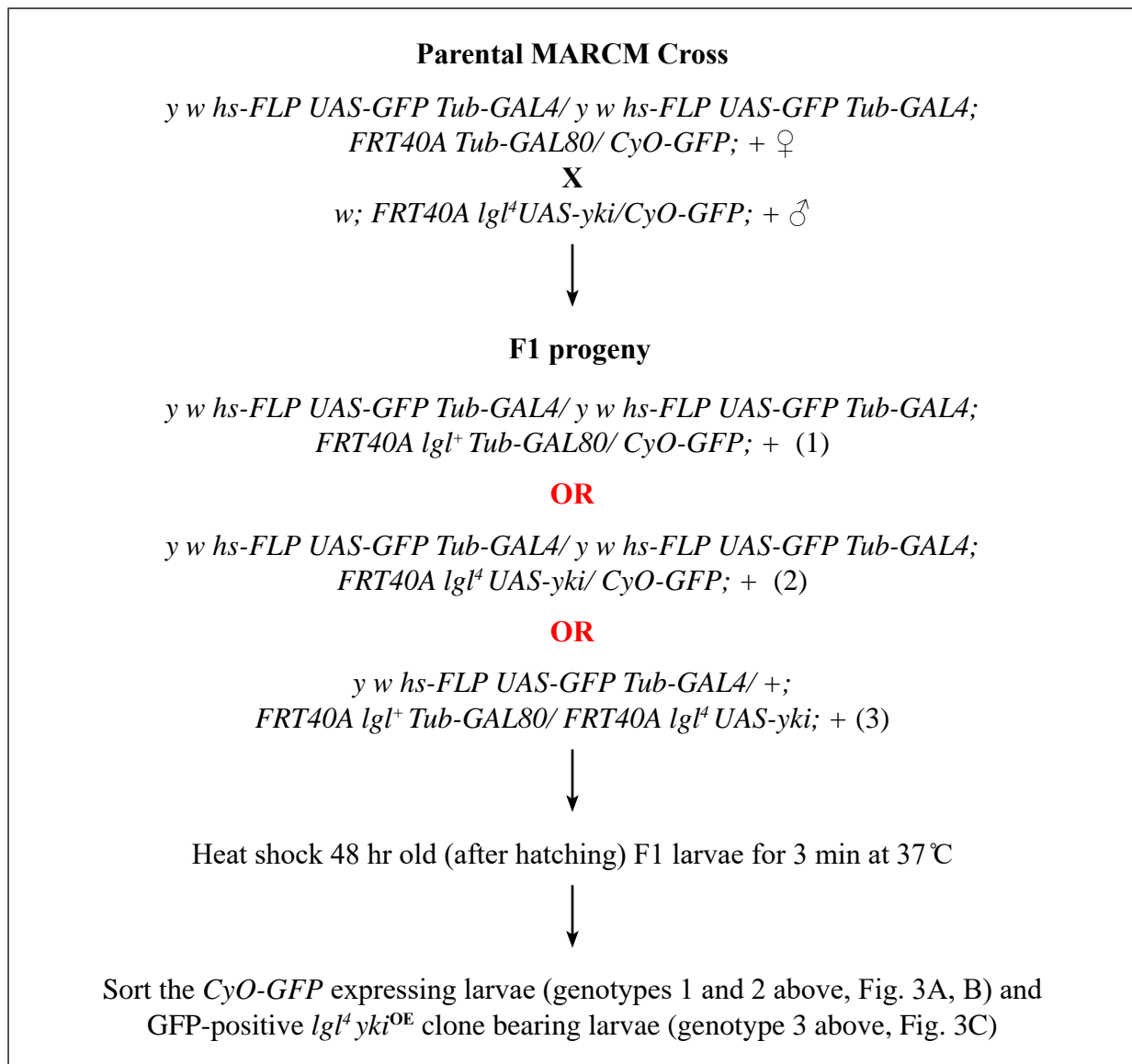


Fig. 2: Scheme of genetic crosses for induction of FLP-driven somatic crossing at FRT sites to generate *lgl⁴ yki^{OE}* tumorous somatic clones in mitotically dividing tissues of *Drosophila* larvae.

Observations:

1. Examine imaginal discs from larvae that have grown for different periods of time (48 hr, 72 hr or 96 hr) after the heat shock and note differences in size and numbers of GFP-positive tumorous clones (Fig. 4). Compare the GFP fluorescence patterns in the control larvae that were not exposed to the brief heat shock.
2. Follow the developing F1 progeny control and heat shocked larvae and determine if those carrying the *CyO-GFP* chromosome and those without or with the *lgl⁴ yki^{OE}* GFP-positive clones show differences in their pupation and emergence as adults.

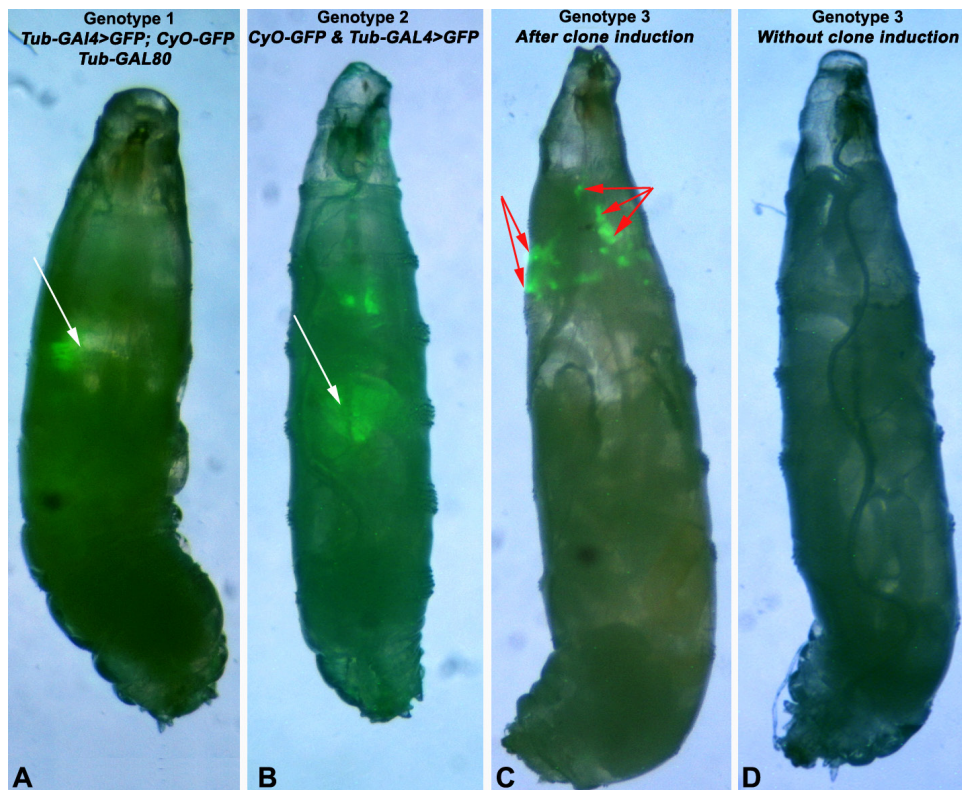


Fig. 3 : Fluorescence microscope images of the MARCM-cross progeny larvae showing green fluorescence in proventriculus region (white arrow) in larvae (A, B) that carry the *CyO-GFP* chromosome (F1 larval genotypes 1 and 2, respectively, in Fig. 2); C. MARCM genotype larva with GFP-positive *lgl⁴ yki^{OE}* tumorous clones (red arrows) following FLP/FRT-mediated somatic crossing over (F1 larval genotype 3 in Fig. 2); D. MARCM genotype larva (F1 larval genotype 3 in Fig. 2) without any GFP fluorescence in absence of any *lgl⁴ yki^{OE}* tumorous clones.

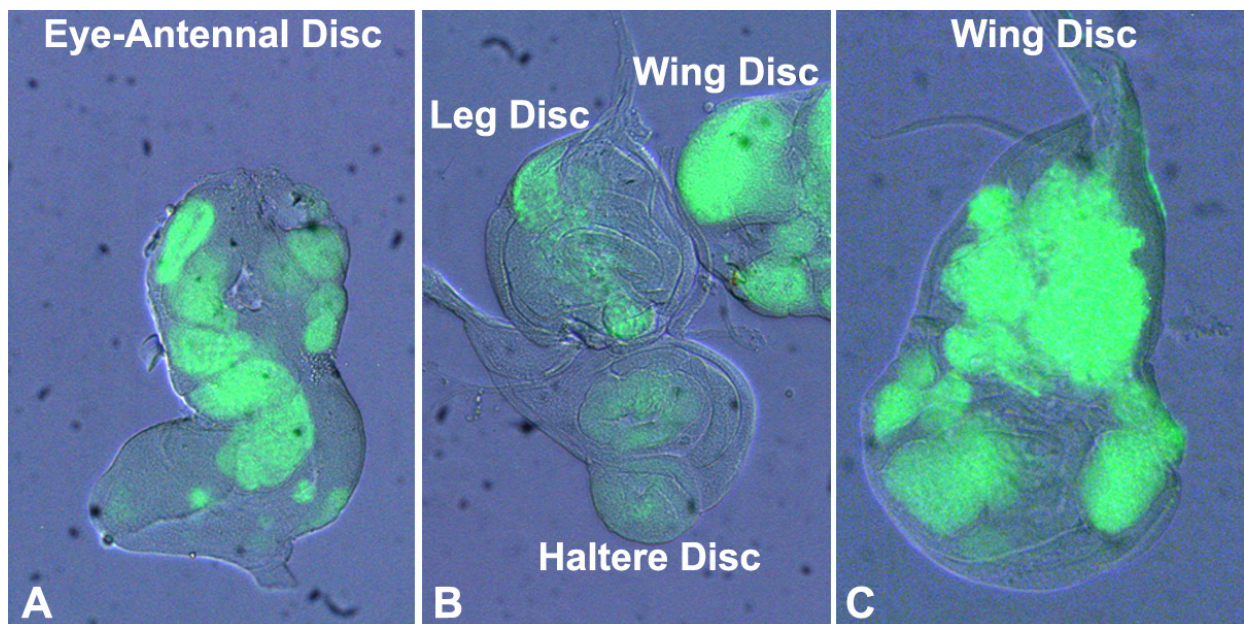


Fig. 4 : Combined DIC and GFP-fluorescence images showing MARCM *lgl⁴ yki^{OE}* tumorous clones (GFP (green) fluorescence) 96 hr after induction by heat shock. A. Eye-antennal disc; B. Wing (part only visible), leg and haltere discs; C. Wing imaginal disc.

Questions for self-study and interpretation:

1. How are the MARCM clones different from the FLP-FRT driven ‘twin spot’ mosaics?
2. How does GAL80 suppress the activity of GAL4 transcription factor? What is the advantage of using this the GAL80 expressing transgene expression in combination with GAL4 expression?
3. Besides the use of MARCM technique to generate tumorous clones, what may be other possible applications of this approach for generating mosaic individuals?

References and further reading:

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***Drosophila* - A Model of Promise in Cancer Research**

Drosophila is also offering a significant contribution to the investigation of organotypic cancers, since despite the evident differences at the macroscopic level, organ cells and functional units are usually well conserved at the biochemical and structural levels, respectively. This conservation allowed to develop thyroid, lung, prostate, gut, brain and blood cancer models starting from the most characteristic genetic lesions found in the same human cancers.

These models are greatly helping in dissecting the contribution of specific molecular pathways to the final cancer phenotype. Given the heterogeneous nature of mammalian solid cancers, new strategies are being developed to decipher cancers at single-cell resolution. The international *Drosophila* community has always been engaged in the development of novel, sophisticated genetic tools, which allowed in the last 30 years to revolutionate functional gene analysis. For this reason, we anticipate that the use of the fruit fly will move fast into the field of precision medicine, contributing to seminal findings in this new era of cancer research.

(In: Mirzoyan, Z. et al. (2019). *Drosophila melanogaster*: A model organism to study cancer. Front Genet. 10: 51. <https://doi.org/10.3389/fgene.2019.00051>).

Chapter 82

Visualizing neurons in *Drosophila* using classical staining

Rajashekhar K Patil (patilsirmu@gmail.com) and Shamprasad Varija Raghu* (shamprasadvarijaraghu@gmail.com)

Neurogenetics Lab, Dept of Applied Zoology, Mangalore University, Karnataka 574199

* For correspondence

Recommended Level: UG/PG/Research

Sub-discipline: Neurobiology and Anatomy

Background:

A knowledge of neuroanatomy permits deductions of the functioning of the nervous system. Camillo Golgi (1883) developed a method of staining neurons, popularly known as “Golgi silver technique”. Some of the fundamental principles of neural organization and the Neuron Doctrine were laid down by Santiago Ramon Y’ Cajal who revealed the detailed structure and splendor of neurons by using Golgi silver technique (Cajal, 1903). This technique was applied to insect nervous systems with considerable success (Rajashekhar and Shamprasad, 2004) to explore the neuroanatomy of the insect brain. The procedure involved incubating fixed brain tissues in potassium dichromate followed by silver nitrate. This however, is a cryptic and stochastic technique that reveals neurons at random. A recent review of Golgi techniques is provided by Kang *et al.*, (2017) that summarizes the variants of Golgi techniques.

The introduction of cobaltous chloride back filling of neurons was a non-random neuronal staining technique by Pitman *et al.*, (1972). The procedure involved placing cut-end of a neuron and exposing it to cobaltous chloride. Axoplasmic circulation carries CoCl_2 which would be precipitated by ammonium or hydrogen sulphide to reveal the arbors of the neuron. The success of this technique was enhanced by silver-intensification by Bacon and Altman (1977). The fly being a small-sized animal model makes it easy to carry out neuroanatomical studies on its brain. As neurons occupy three-dimensional space and may traverse long distances, the smaller size of fly brain provides an added advantage of visualizing complete neurons. The time needed for staining complete brain is also shorter in flies compared to that in other higher animal models. .

Objectives:

To stain neurons in the brain and nervous system of *Drosophila*.

Materials Required:

- A. Flies: Wild type or other desired genotype flies.
- B. Equipment and fly handling materials:
 - i. Oven
 - ii. Microtome
 - iii. Stereo binocular microscope
 - iv. Bright-field microscope
 - v. Needles, forceps, fine brushes, etherizer etc., for fly handling
- C. Reagents and solutions:
 - i. Golgi-rapid technique:
 - a. Karnovsky's fixative: To prepare 100 mL solution, mix 10 gm Paraformaldehyde, 35 mL distilled water and 25 mL 4 M sodium cacodylate. Dissolve at 65°C and add 4-5 drops of 1 N NaOH. Filter the solution with filter paper. Add 5 mL of 25% glutaraldehyde and make up to 100 mL with distilled water.
 - b. 2 M phosphate buffer (pH 7.0)
 - c. 4% Osmium tetroxide
 - d. 2.5% potassium dichromate
 - e. 0.1% silver nitrate
 - f. 0.75% silver nitrate
 - g. Grades of alcohol
 - h. paraffin
 - i. DPX
 - j. Xylene
 - ii. Cobaltous chloride backfilling of neuron:
 - a. Wild type flies
 - b. 3% Cobaltous chloride
 - c. 2% Ammonium sulphide or hydrogen sulphide (to be bubbled in 50 mL of saline for 10-15 min)
 - d. Modelling clay or Playdoh®
 - e. 1% hydroquinone
 - f. Formaldehyde
 - g. 10% Citric acid
 - h. 1% Silver nitrate
 - i. 30% Gum acacia
 - j. Xylene

Experimental Details:

A. Golgi-rapid technique:

- i. Immobilize the flies by placing them on ice.
- ii. Decapitate 25 (3-4 day old) flies under 500 μ L Karnovsky's fixative, cut the proboscis and place the head in fixative for 4 hr at room temperature.
- iii. Wash the fixed specimen with 2 M phosphate buffer thrice each wash lasting for 20 min.
- iv. Incubate the specimen in a mixture containing 25 μ L of 4% Osmium tetroxide in 1 mL of 2.5% Potassium dichromate in dark for two days.
- v. Quickly rinse the tissue in 500 μ L of 0.1% silver nitrate until no red precipitate is formed in the washings. Further incubate the samples in 500 μ L of 0.75% silver nitrate solution in the dark for two days and washed with distilled water 3-4 times.
- vi. Repeat steps iv-v once to enhance staining.
- vii. Dehydrate samples by keeping them for, 30 min each in 500 μ L of 70%, 80%, 90% and 100% alcohol, respectively, and finally embed in paraffin. Cut 20-30 μ m thick serial sections of the head with a microtome and mount on a clean slide. Deparaffinise the sections in Xylene, mount with DPX, and examine under a compound microscope.

B. Cobaltous chloride backfilling of neuron:

- i. Immobilize flies on ice.
- ii. Tether flies using modelling clay or playdoh® in a suitable position.
- iii. Place the cut end of an appendage (antenna, maxilla, legs, wings) or sensilla in 500 μ L of 3% cold (4-8°C) CoCl_2 for 2 hr. Cobaltous chloride may also be injected into a nerve using a capillary.
- iv. Dissect out the cuticle as much as possible and immerse the fly in 500 μ L of 2% yellow ammonium sulphide prepared in saline. Alternatively, the fly can be submerged in saline with hydrogen sulphide bubbled through it just before use.
- v. Carry out Silver intensification of CoCl_2 - filled neurons as below:
- vi. Fix the tissue in 500 μ L of FAA (100% Alcohol 0.8 mL, acetic acid 0.8 mL, formaline 1.2 mL) for 4 hr.
- vii. Hydrate fixed head to 30% alcohol.
- viii. Place all glassware and reagents in an incubator at 50°C.
- ix. Place tissue in solution containing 1 mL of 30% gum Arabic, 0.1 mL of 1% hydroquinone and 0.2 mL of 10% citric acid. Incubate for 20 min at 50°C.
 - x. Transfer the tissue to a solution containing 30% gum Arabic (10 mL), 10% hydroquinone. 50 μ L of 10% citric acid, and 50 μ L of 1% silver nitrates. Incubate in DARK at 50°C for 1 hr Observe for appearance of Khaki color and formation of silver mirror on the walls of glassware.

- xi. Transfer the tissue to 1% acetic acid in 30% alcohol.
- xii. Dehydrate in grades of alcohol, clear in xylene, embed in paraffin. Section at 20 micron thickness.

Results/Observations:

A. Golgi-rapid technique:

Neurons are stained dark brown or black at random against a yellow to orange background of unstained neuropil (**Fig. 1**).

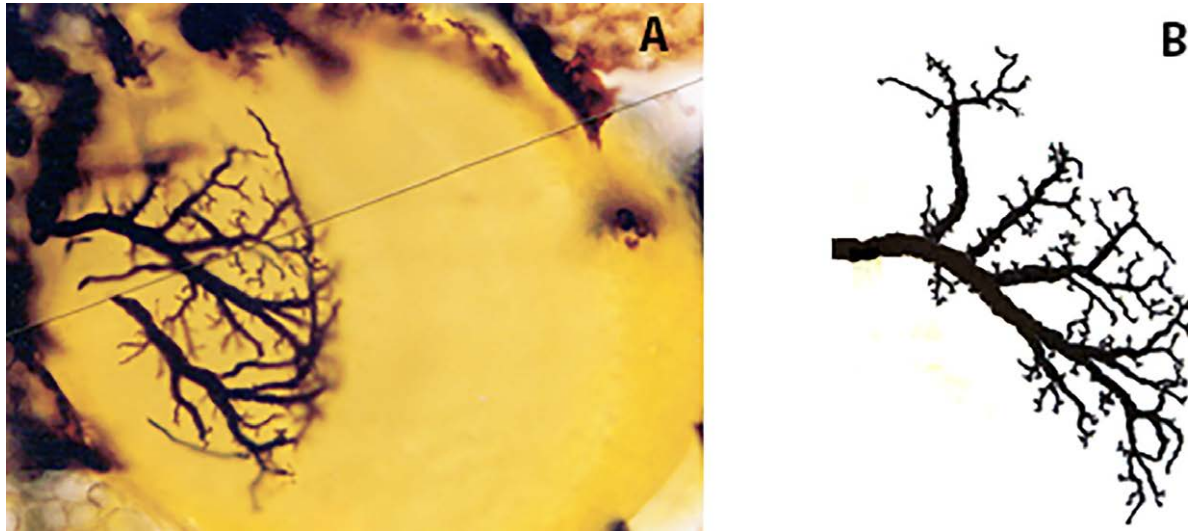


Fig. 1. Golgi-silver stained giant neurons in the optic lobe of *Drosophila melanogaster*. A. HS cell in the optic lobe and they are involved in visual motion detection in insects. B. Camera Lucida drawing of one of the HS cells reconstructed from serial sections.

B. Cobaltous chloride backfilling of neu on:

Neurons filled with CoCl_2 appear black in color.

Questions for self-study and interpretation:

1. How do motor-, inter-, and sensory neurons differ in anatomy?
2. What are ipsi- and contralateral projections?
3. Why sensory projections confine to a neuropil and how accurate is this?

References and further reading:

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Golgi and His “Black Reaction”

The Golgi silver impregnation technique is a simple histological procedure that reveals complete three-dimensional neuron morphology. This method is based in the formation of opaque intracellular deposits of silver chromate obtained by the reaction between potassium dichromate and silver nitrate (black reaction). Camillo Golgi, its discoverer, and Santiago Ramón y Cajal its main exponent, shared the Nobel Prize of Medicine and Physiology in 1906 for their contribution to the knowledge of the nervous system structure, Their successes were largely due to the application of the silver impregnation method. However, Golgi and Cajal had different views on the structure of nervous tissue. According to the Reticular Theory, defended by Golgi, the nervous system was formed by a network of cells connected via axons within a syncytium. In contrast, Cajal defended the Neuron Doctrine which maintained that the neurons were independent cells. In addition, Golgi had used a variant of his “black reaction” to discover the cellular organelle that became known as the Golgi apparatus. Electron microscopy studies confirmed the postulates of the Neuron Doctrine as well as the existence of the Golgi complex and contributed to a resurgence of use of the Golgi stain.

(In: Torres-Fernández O. The Golgi silver impregnation method: commemorating the centennial of the Nobel Prize in Medicine (1906) shared by Camillo Golgi and Santiago Ramón y Cajal. *Biomedica*. 2006:498-508. Spanish)

Chapter 83

A study of the distribution of campaniform sensilla on the wings of *Drosophila melanogaster*

Shamprasad Varija Raghu¹(shamprasadvarijaraghu@gmail.com), Avinash K. Kudva² (avinash.kudva@gmail.com) and Rajashekhar K. Patil^{1*} (patilsirmu@gmail.com)

1. Neurogenetics Lab, Department of Applied Zoology, Mangalore University, Karnataka 574199

2. Department of Biochemistry, Mangalore University, Karnataka 574199

* For correspondence

Recommended Level: UG/ PG/Research

Sub-discipline: Anatomy/Neurobiology

Background:

Insects have a chitinous exoskeleton. Any movement of the segments or appendages imparts cuticular stress and strain. Campaniform sensilla (CS) are mechano receptors found on the cuticular surface to detect such cuticular stress and strain. They detect mechanical load and resistance to contraction of muscles inserted into the cuticle (Dinges *et al.*, 2020). CS are therefore, comparable to the Golgi-tendon organs of mammals. *Drosophila* has a vast number of CS on the cuticle at various regions all over the body. An excellent scanning electron micrograph based atlas of these CS in *Drosophila* has been published recently by (Dinges *et al.*, 2020). In general, CS can be arranged in fields, groups, or as single sensilla. In *Drosophila melanogaster*, CS are located on the wings, halteres, leg segments and thorax (Dinges *et al.*, 2020). A good description of CS on the wings and a comparison of wild type and homeotic mutants (*abx*, *bx3*, *pbx*) of *Drosophila* wing CS was reported by Cole and Palka (1982). CS on the wings may provide a sensory feedback while flying. As a component of complex courtship, males produce courtship song by vibrating their wings. It plays a significant role in sexual selection and species recognition by female (Talyn and Dowse, 2004). A survey of CS can be carried out on the ventral and dorsal surfaces of the wing by preparing whole mounts. It is also easier to locate and identify CS on the wings.

Objective:

Localizing and identifying Campaniform sensilla on the wings of *Drosophila*.

Materials required:

1. 2-3 days old wild type flies.

2. Common items for fly handling (**Chapter 1**).
3. Grades of alcohol (70%, 80%, 90%, 95% and absolute alcohol).
4. Methyl salicylate.
5. DPX.
6. Cleaned slides and coverslips (22 mm²).
7. Micro-scissors or forceps and needles.
8. Light Microscope with a good quality condenser.
9. Stereo-binocular microscope.

Experimental details:

1. Anaesthetize flies (preferably by cold) and fix in 70% alcohol in a tube.
2. Dehydrate in grades of alcohol (80%, 90%, 95% and absolute alcohol), with 30 min in each in tubes.
3. The right wing of dehydrated flies is cut along with a small portion of the thorax.
Note: Since identifying the dorsal and ventral sides of the wing can be difficult after preparation of the slide, it is recommended to confine to the right wing. As CS are aggregated near the base of the wing, retaining a piece of thorax is useful for processing. It can later be detached while mounting.
It is advisable to cut the wing under alcohol to prevent entry of air into trachea of the wings, which would hinder visualization.
4. Clear the wings in methyl salicylate in a tube for 60 min at room temperature.
5. Transfer the wings to a clean slide with its dorsal side up in a drop of DPX and carefully place a coverslip.
6. Observe under microscope with proper adjustment of the condenser to get a clear view of the cuticular structures.

Results and Observations:

Examine veins and the base of the wings for isolated, dual and assemblies of CS. Compare the types of CS and their aggregation with the illustrations provided by Cole and Palka (1982), who have described six different morphological types of Campaniform sensilla in *Drosophila melanogaster*. These are: type 1 (Circular, high-profile with socket), type 2 (Circular, high profile without socket), type 3 (Circular, low-profile with socket), type 4 (Circular, low-profile without socket), type 5 (Elliptical, high-profile with socket) and type 6 (Elliptical, low-profile with socket) (Cole and Palka, 1982). Large numbers of CS are observed near the veins at the base of the wing (**Fig. 1A**). They look like small bubbles under the microscope (**Fig. 1B**). Examination under a 40 X objective provides clearer visualization.

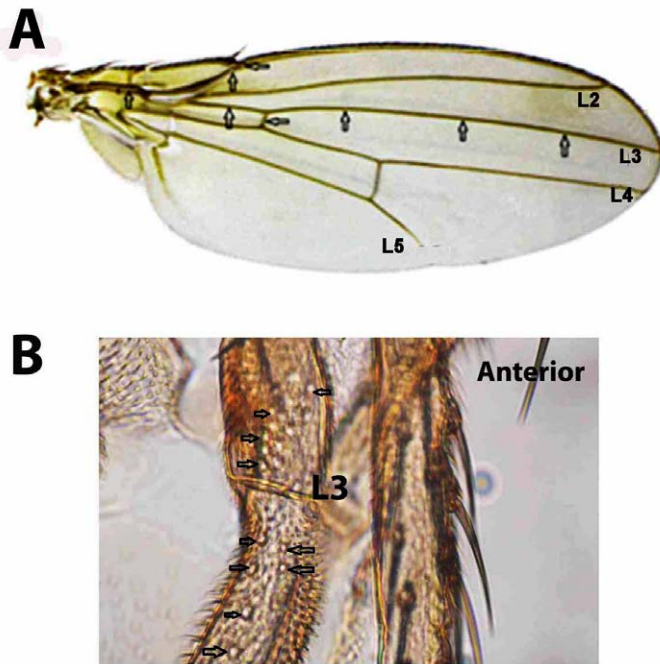


Fig. 1. A. Normal wing showing different veins on the surface (L2, L3, L4 and L5). Locations of Campaniform sensilla on wing is marked by arrows. B. Magnified view of proximal part of the wild type wing surface showing Campaniform sensilla (arrows).

Questions for self-study:

1. Do wings of female and male flies have similar CS types and distribution?
2. Do the CS types and distribution differ between *Drosophila* species?
3. What is the significance of the Campaniform sensilla being placed close to wing veins?

References:

Cole, E. S. and Palka, J. (1982) The pattern of campaniform sensilla on the wing and haltere of *Drosophila melanogaster* and several of its homeotic mutants. *Development*. 71: 41-61.

Dinges, G. F., Chockley, A. S., Bockemühl, T., Ito, K., Blanke, A. and Büschges, A. (2020) Location and arrangement of campaniform sensilla in *Drosophila melanogaster*. *J Comp. Neurol.* 1–21. <https://doi.org/10.1002/cne.24987>.

Talyn, B. C. and Dowse, H. B. (2004) The role of courtship song in sexual selection and species recognition by female *Drosophila melanogaster*. *Animal Behaviour*. 68:1165-1180.

If the Science is Good, You know it First in Your Gut

Seymour Benzer discontinued molecular biology experiments and began contemplating the genetics of behavior from watching his two daughters, Barbara and Martha: one was amazingly lively; one was amazingly calm. He took a sabbatical leave to Caltech to learn neurobiology with Roger Sperry; there, he also discovered a love for the fruit fly, *Drosophila melanogaster*, thanks to Ed Lewis.

“If the science is good, you know it first in your gut. Eventually, your brain will agree.” He (Benzer) would say: “We must always be on guard, so as not to do pedestrian science.”

(In: Tanouye, M. A. (2008) Seymour Benzer 1921–2007. *Nature Genetics*. 40: 121.
<https://www.nature.com/articles/ng0208-121>)

Chapter 84

Labeling and identification of chemo- and mechano-sensilla on the appendages of *Drosophila melanogaster*

Rajashekhar K. Patil (patilsirmu@gmail.com) and Shamprasad Varija Raghu* (shamprasadvarijaraghu@gmail.com)

Neurogenetics Lab, Dept of Applied Zoology, Mangalore University, Karnataka 574199

* For correspondence

Recommended Level: UG/PG/Research

Sub-discipline: Anatomy/Neurobiology

Background:

Sensilla (singular sensillum) are cuticular sense organ in Arthropods, which project as small hair or peg on the exoskeleton and carry sensory neurons inside to gather information about the environment. Insects are endowed with numerous and varied sensilla. Success of insects in diverse habitats is often attributed to their specialized and exquisitely sensitive sensilla (Keil and Steinbrecht, 1984; Couto *et al.*, 2005; Elgar *et. al.*, 2018; Zhao and McBride, 2020). The sensilla are present on appendages such as antenna, mouthparts, wings and legs and are categorized into chemo-sensory (olfactory and taste) and mechano-sensory sensilla (Stocker, 1994). Based on structure, the common types of sensilla are: *sensilla coeloconica*, *sensilla basiconica* and *sensilla trichoidea*. Several studies have elucidated roles of sensilla in the behavior and biology of *Drosophila* (Zhao and McBride, 2020). The method described here is adapted from Navasero and Elzen (1991) and works well to differentiate between the chemo-sensory and mechano-sensory sensilla in the fly. This method exploits the permeability of chemo-sensory hairs to the external medium. When placed in silver nitrate solution, the salt enters into hairs and can be visualized by reducing the silver nitrate into a black precipitate.

Objectives:

To identify sensilla in *Drosophila* and differentiate the chemo- and mechano-sensory sensilla.

Materials Required:

1. Adult *Drosophila* flies

2. Photographic developing solution: dissolve 1.88 gm of Metol (4-(methylamino) phenol sulfate) and 25.0 gm of anhydrous sodium sulphite in 100 mL of warm water. The metolis followed first, followed by sodium sulphite. (A commercial photographic developer can also be used)
3. 0.1 M Silver nitrate,
4. 10% Acetone,
5. 3% Acetic acid,
6. Methyl salicylate,
7. DPX,
8. Ethyl alcohol grades
9. 1.5 mL microfuge tubes
10. Slides and 22 mm² coverslips

Principle:

Chemosensory hairs are porous in nature and, therefore, are permeable to the external medium and substances. The insects are washed with dilute acetone to remove dust or debris on the pores of hairs to assist entry of silver nitrate. The cleaned insects are placed in silver nitrate solution. Silver nitrate enters these hairs where it gets precipitated following its interaction with the haemolymph. This precipitate is further processed using photographic developer to form a black precipitate that enhances their visibility. Such black precipitation and, therefore, black tipped hairs are not observed in the case of mechano-sensory hairs as their surface is not porous.

Experimental Details:

1. Ten live flies are washed in 10% acetone three times (30 sec each) in a small test tube to remove all the dust and dirt on the surface of hairs and open the pores.
2. The acetone solution is removed from the tube by carefully decanting. 2 mL of 0.1 M silver nitrate is added to the tube and left for 3-5 min. This will give enough time for silver nitrate to enter the pores on the surface of chemosensory hairs. (**Caution:** Avoid contact of the silver nitrate solution with skin since it can lead to temporary blackening of the skin).
3. Carefully remove the silver nitrate solution and wash the flies in tube with distilled water for 15 min.
4. Remove water and soak the flies in 2 mL of photographic developing solution for 5 min. The photographic developing solution reduces the silver nitrate to silver which becomes visible as black precipitate inside the chemosensory hairs.
5. Remove the developer solution and rinse flies in 3% acetic acid for one min to remove the photo developing solution from the surface of specimen.

6. Dehydrate the specimens in the tube using 70%, 80%, 90% and 100% alcohol (10 min each).
7. Transfer the dehydrated samples to a microscope slide in a drop of 100% methyl salicylate for 10 min. This process will make the cuticle transparent to permit visualization of the black precipitate.
8. Dissect the antenna/maxillary palp/proboscis/wings/legs.
9. Transfer the dissected organs to a different slide in a drop of DPX. Carefully cover with a coverslip, avoiding trapping of air bubbles.
10. Observe the samples under a microscope.

Results/Observations:

The tips of chemo-sensilla which are involved in chemoreception appear darkly stained (**Fig. 1**) while mechano-sensilla are not stained.

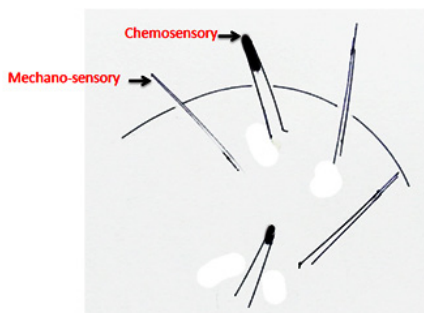


Fig. 1. Schematic diagram showing silver deposition in chemo-sensory hairs. The chemo-sensory hairs are stained black at the tips but the mechano-sensory hairs are not stained.

Questions for self-study and interpretation:

1. What are the different types of sensilla found in insects?
2. Why should chemo-sensory hairs be porous?
3. Where do they occur and why?
4. How do mutant flies differ from wild type in the distribution of sensilla?

References and further reading:

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- Stocker, R. F. The organization of the chemosensory system in *Drosophila melanogaster*: a review. *Cell Tissue Research*. 275:3-26, 1994.
- Zhao, Z. and McBride, C. S. Evolution of olfactory circuits in insects. *Journal Comparative Physiology. A*. 206:353–367, 2020.

Fly – Attractive Model for Olfaction

One additional factor that makes the fly so attractive for olfactory research is the uniquely comprehensive description of the sensory periphery, including complete molecular descriptions of the repertoire of identified olfactory receptor neurons, their projection into the brain and extensive data about their physiology. This provides an unmatched platform to investigate the logic of olfactory information processing in the brain.

(In: Masse, N. Y., Turner, G. C., and Jefferis, G. S.X.E 2009. Olfactory Information Processing in *Drosophila*. *Current Biology*. 19: R700-R713.
<https://doi.org/10.1016/j.cub.2009.06.026>)

Chapter 85

Quantification of dopamine and its metabolites in *Drosophila* brain using HPLC

Mohamad Ayajuddin (md.ayaj@gmail.com), Abhik Das (dasabhik91@gmail.com), Limamanen Phom (lim_angh@yahoo.co.in), Zevelou Koza (zevi7koza@gmail.com), Rahul Chaurasia (rahulneuro12@gmail.com) and Sarat Chandra Yeniseti* (yschandrays@rediffmail.com)
Drosophila Neurobiology Laboratory, Department of Zoology, Nagaland University (Central), Lumami-798627, Nagaland.

* For correspondence

Recommended Level: PG/Research

Sub-discipline: Neurobiology /Biochemistry

Background:

Catecholamines (CAs) are neurotransmitters in the central and peripheral nervous systems and also work as hormones in the endocrine system. CAs are slow-acting neurotransmitters that counteract against fast acting neurotransmitters. One of the major catecholamines that have been of interest in Parkinson's Disease (PD) research is Dopamine (DA). Depletion of DA results in the expression of motor and non-motor symptoms of PD. However, extensive research on aetiology of PD shows prevalence of multi hits in DA metabolism. This multi-hit phenomenon is known as "Autotoxicity" (when CA catabolism and catabolites become toxic) and "Allostatic Load" (when CA catabolite quantity increases beyond cell's capacity). It describes changes in DA metabolism through MAO (mitochondrial Monoamine oxidase) mediated pathway where DA is automatically oxidized in the cell and converted to downstream CAs like Dihydroxyphenyl acetic acid (DOPAC) and Homovanillic acid (HVA). The conversion of the DA generates ammonium ion, peroxides and free radicals. Under stressed condition, hyperactivated MAO results in excess of downstream catabolites of DA, which may be further metabolized to products like DOPAC and HVA. In combination with reactive ions, this condition results in autotoxicity and puts allostatic load on the cell leading to neurodegeneration. DA is naturally oxidized or reduced inside neuron at threshold level depending on physiological process. Stress sabotages this mechanism to contribute to the neuronal death. Hence, DA metabolism is an important component of research on PD.

High Performance Liquid Chromatography (HPLC) is a key platform for detection and separation of the CAs at high efficiency in a short time. Further, the Electro chemical detector (ECD) ensures replication of oxidative and reductive environment inside the HPLC platform like that in vivo. This ensures detection of an array of CAs even at trace amounts in the pico- or femtogram ranges. Modern research on understanding DA catabolism/ metabolism in models of Parkinson's disease has seen extensive use of HPLC-ECD system with various modifications on the experimental procedure to understand different aspects of CA recycling in neurons (Goldstein *et al.*, 2013; Filograna *et al.*, 2015).

The separation principle of HPLC is based on the distribution of the analyte (sample) between a mobile phase and a stationary phase. Depending on the chemical structure of

the sample and its affinity towards the stationary phase, the molecules are retarded while passing through the stationary phase. A combination of hydrophobic and Van der Waals type interactions between the target compound and both the stationary and mobile phases enables retention of these compounds. The specific intermolecular interactions between the molecules of a sample and the stationary phase define their retention times. Hence, different constituents of a sample are eluted at different retention times, which will help in separation of sample components. Detection of the eluted metabolites is best done through an electrochemical detector (ECD), which provides high sensitivity and efficiency. Neurotransmitters or any biological metabolites which remain dissolved in complex matrices (body fluids) often undergo voluntary oxidation and reduction according to the physiological need. The robustness and precision of ECD in mimicking such an oxidative and reductive environment inside the HPLC detector module helps to precisely identify and detect catecholamine in the body fluid extract.

The HPLC-ECD based method to quantify brain specific DA and its metabolites described here is a modified version of Yang and Beal (2011).

Objectives:

To quantify dopamine and its metabolites in whole brain of *Drosophila*.

Materials Required:

A. **Brain tissue from flies** (wild type and of the desired genotype) of different age groups.

B. Equipment:

- i. HPLC-ECD 3000 RS system (or other systems with Electro Chemical Detectors).
- ii. Stereo-binocular microscope and other fly handling items.

C. Chemicals and Reagents:

- i. Dopamine (DA)
- ii. Dihydroxyphenyl acetic acid (DOPAC)
- iii. Homovanillic acid (HVA)
- iv. Phosphate-buffered saline (PBS)
- v. Mobile phase
- vi. HPLC grade water
- vii. Acetonitrile
- viii. Methanol

D. Other items:

- i. Sterilized Eppendorf tubes.
- ii. Pipette (cleaned with 70% ethanol before and after use).
- iii. Sterilized pipette tips.

Experimental Details:

A. Preparation of Catecholamine Standards and Brain tissue extract:

i. Preparation of Standards:

Standards are prepared by dissolving 2 mg Catecholamines (procured commercially) in 2 mL PBS. It is further diluted to get varying concentrations as following:

Standard	PBS	Concentration	Stock Name
2 mg	2 mL	1000 µg/mL	S
100 µL of S	900 µL	100 µg/mL	S1
100 µL of S1	900 µL	10 µg/ mL	S2
100 µL of S2	900 µL	1000 ng/ mL	S3
200 µL of S3	800 µL	200 ng/ mL	S4
150 µL of S3	850 µL	150 ng/ mL	S5
100 µL of S3	900 µL	100 ng/ mL	S6

ii. Sample preparation:

- a. Take 15 heads of adult flies of the desired genotype/s in 300 µL 1X PBS (prepared in HPLC grade water).
- b. Homogenize and sonicate once for 20 sec with 5 sec intervals and 30% amplitude (always keep on ice during the process).
- c. Centrifuge at 6000 rpm for 10 min at 4°C.
- d. Collect supernatant.
- e. Take 200 µL of supernatant (leave the remaining for protein quantification) and add 200 µL of 5% TCA (before using, centrifuge the 5% TCA at 6000 rpm for 10 min at 4°C to remove any undissolved solute particles).
- f. Centrifuge two times at 5000 rpm for 10 min each at 4°C.
- g. Collect the supernatant for assay.

Precautions

- i. Homogenize and sonicate tissue on ice (to avoid heat generation and prevent degradation).
- ii. Keep the tissue extract and standard solution on ice between the steps to avoid any degradation of the standard molecules and components of the tissue extract.
- iii. Always use fresh pipette tips for preparing serial dilutions of the standard and for transferring tissue extract.
- iv. Prepare all the reagents in HPLC grade or Milli-Q water to avoid any contaminant molecules which may produce a false positive peak in the chromatogram.

B. Setting up the HPLC system (Fig. 1):**i. Solvent Reagents Required:**

Load, following the “Pre loading instructions” (see below), the solvent tubing ports of the HPLC-ECD system with

- a. 100% HPLC grade Methanol
- b. 80% Acetonitrile (Prepared in HPLC grade water)
- c. 20% Acetonitrile (Prepared in HPLC grade water)
- d. MDTM Mobile phase for solvent reagents

Instructions for preloading of solvent reagents

“Preloading of the solvent reagents” refers to the process of placing solvent reagent bottles on the HPLC solvent rack and attaching the respective tubings to the bottle. “Preloading Instructions” given below guide the handling of the solvent reagents and their containers while preparing solutions, placing the containers on the solvent rack and attaching to the tubing ports of the HPLC platform.

- Check all the reagent bottles that they are adequately filled (minimum 350 mL in each).
- When filling, tilt the bottle and pour the reagents slowly to avoid bubble formation.
- Mobile phase should be filtered with 0.22 micron filter paper and then poured onto the respective reagent bottle as mentioned on point 2 (Miscibility of the components of mobile phase is a concern when running through the column. Even the readymade mobile phases might have undissolved salt residues and suspended material due to minute level coagulation of organic component of the mobile phase. Filtering through 0.22 micron nylon membrane ensures separation of such residues which might otherwise choke the C18 column fitted with the HPLC-ECD system).
- Sonicate all the reagent bottles in bath sonicator at ultrasonic frequency of 40 KHz for 15 min at room temperature before attaching them in the HPLC system.

ii. System/ Column cleaning:

Columns and electrodes of the detectors may contain tissue debris from the previous experiment with the HPLC platform. Further, the components of HPLC platform such as column, ECD and tubing are filled and stored in 100% methanol after completion of an experiment to avoid any fungal growth. Therefore, it is important to clean the system when it is turned on with flow of mobile phase to discard any left-over tissue debris and methanol. The cleaning also ensures removal of any air bubble during the idle phase of the HPLC platform. The following steps are used for system/ column cleaning:

- a. Clean the system by purging (each solvent port is put into high rate of flow from the pump to outside the system) from all the ports for 5 min each to discard any trapped air bubble.
- b. After purging, close the purge knob (to divert the flow from pump to column) and give 100% flow with 20% acetonitrile for 30 min at 0.5 mL/min flow rate to start cleaning the column.
- c. Make a flow of 100% mobile phase through the column at the same flow rate for another 30 min after which mobile phase may be recycled (drainage pipe outlet from column will be wiped with tissue soaked in mobile phase and will be inserted back into the mobile phase container bottle).

iii. Setting up the HPLC parameters:

Detection of catecholamines through ECD is best when the oxidation potential is between the range of 340 mV (Yang and Beal, 2011). With the DIONEX ULTIMATE ECD 3000R system, we have found that the catecholamines are best detected at reduction and oxidation potential range of -175 mV and 225 mV respectively. The reduction potential provides an equal state for all the catecholamines inside the HPLC platform irrespective of their physiological redox state in the tissue. The optimum oxidation potential acts as a common threshold for the excitement of all concerned catecholamines inside the HPLC platform.

Set the following parameters for efficient detection and analysis of catecholamines

Oxidation potential	+225 mV
Reduction potential	-175 mV
Omni cell:	± 500 mV (for noise reduction)
Gain range:	1 μ A
Data collection rate:	5 Hz
Detection Filter:	2.0 (for all cells)
Column temperature:	Room temperature
Autosampler temperature:	4°C
Flow rate:	0.5 mL/min

iv. ECD priming:

- Once the mobile phase is put on recycle mode, start priming the ECD.
- After setting up the required parameters for ECD, keep the system on acquisition mode for at least 2 hr to check the status of the base line.
- Watch for the two lines denoted as ECDRS 1 & ECDRS 2 to run in parallel denoting equilibrium while fluctuation will be denoted by non-parallel line over time.
- The baseline is considered to be stabilized if the drifting is less than 2nA/Hr.

C. Standard and Sample loading:

Load 20 μ L of Standards first and then 50 μ L of the sample for analysis. 20 μ L of 150 ng/mL concentration of DOPAC and DA show optimal peak, but HVA shows clear peak at 10 μ g/mL.

Note: Standard metabolites are dissolved in PBS and the same PBS is used for sample preparation. A minimum amount of 300 μ L is kept in the vial for loading 20 μ L of the standard or sample, if the HPLC platform comes with an auto sampler.

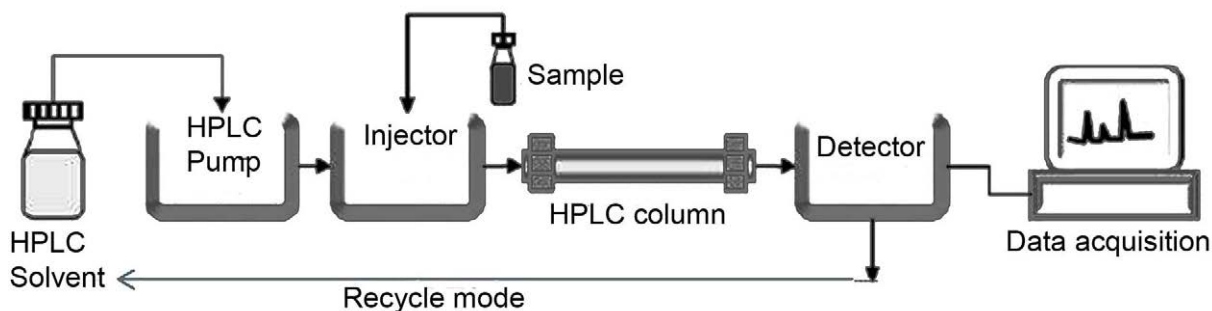


Fig. 1. Schematic of HPLC workflow (Adapted from Czaplicki, 2013)

D. Analysis

- Use the chromatogram acquired with ECDRS2 for analysis.
- Set inhibit integration range on the solvent front of the unknown sample chromatogram (Fig. 3).
- Overlay the chromatogram with ECDRS2 channel of the standard chromatogram (Fig. 2).
- Upon comparing the two chromatograms, one can deduce the peaks of the particular catecholamine in the sample.

- v. If two peaks are co-eluted or joined together as if a single peak, then split the peak to make it into two peaks.
- vi. Make the peaks more accurate by using software tools like automatic tool, delimiter tool, peak tool, baseline tool etc.
- vii. After the peak is accurately set, one can further process for quantitative analysis (Quantitative analysis is performed using Chromaleon Version 6.8 software (or other) provided along with the HPLC system).
- viii. Note the area of the peak which is compared with that of the standard for calculation.

Result/Observations:

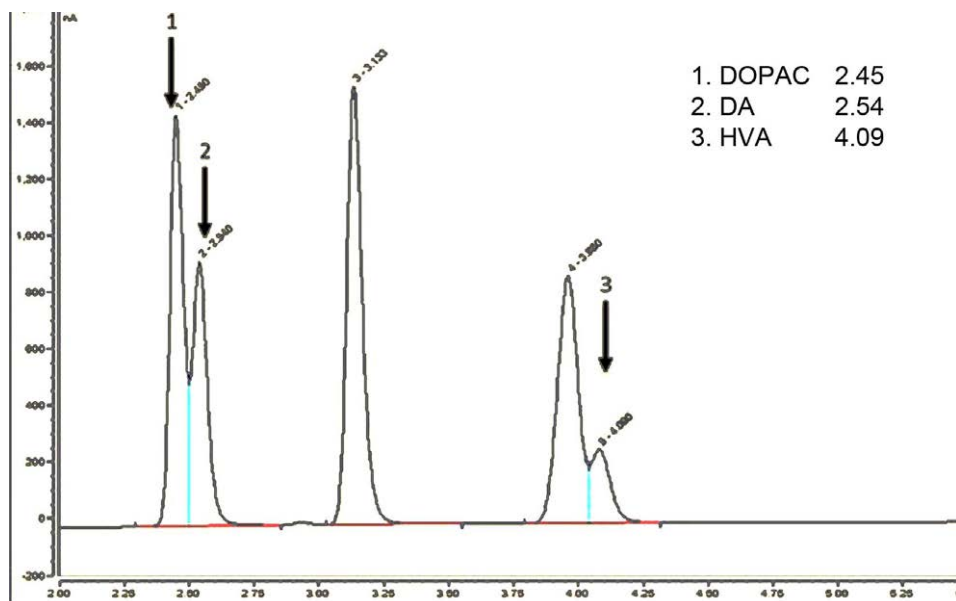


Fig. 2. Chromatogram of standards (dopamine, DOPAC and HVA) showing their retention time.

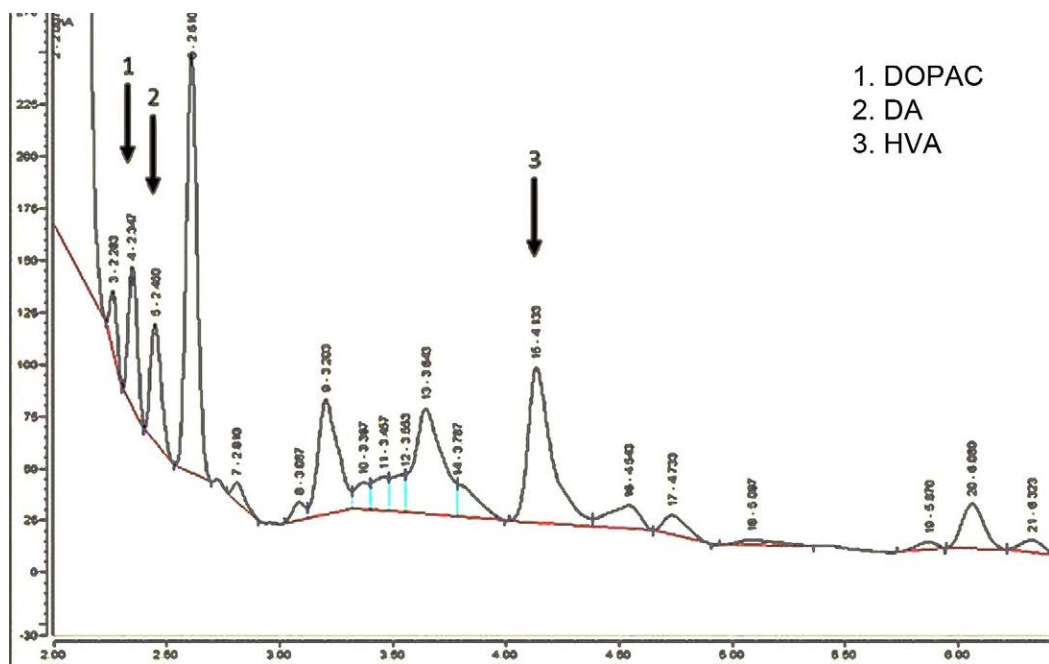


Fig. 3. A typical chromatogram of sample (fly brain extract) showing peaks for DA, DOPAC and HVA (in both the chromatograms, blue line indicates the splitting of the peak)

when there is co-elution or two peaks are joined together as if a single peak, while the red line represents the baseline; X-axis indicates the retention time; Y-axis denotes the voltage of redox reactions of the catecholamines).

1. The retention time of the catecholamine peaks is shifted to the right in chromatogram of the brain tissue extract. This is not uncommon since tissue extract has complex matrices and elements solubilized together with the catecholamines unlike the pure commercially available standards. The user has to figure out “which peak corresponds to which catecholamine” in tissue extract by comparing with the fingerprint pattern of standard mixture(s) of catecholamines. If confusion persists, one way to identify the peak of interest is by spiking (adding standard to sample) and comparing the peaks with chromatogram of standard.

2. Calculation of concentration of DA in sample:

i. Concentration of the standard DA that is used in HPLC assay is $C_{std} = 200 \text{ ng/mL}$.

ii. Injection volume of standard DA to the HPLC column is $I_{std} = 20 \text{ } \mu\text{L}$.

iii. Area of the Peak of the standard DA in chromatogram is $A_{std} = 100$.

iv. Injection volume of standard DA to the HPLC column is $I_{samp} = 50 \text{ } \mu\text{L}$.

v. Area of the Peak of DA in tissue sample chromatogram is $A_{samp} = 2$.

vi. The particular tissue extract which will be used for HPLC assay will also be quantified before for total protein which is $TP_{samp} = 0.12 \text{ } \mu\text{g/mL}$.

3. Calculation:

i. The concentration of standard DA in 20 μL of injection volume is -
 $(C_{std} \times I_{std})/1000 = (200 \times 20)/1000 = 4 \text{ ng/mL}$.

ii. The concentration of DA in brain tissue extract as deduced from the standard DA chromatogram is - $(A_{samp} \times 4) / A_{std} = (2 \times 4)/100 = 0.08 \text{ ng/mL}$.

iii. 50 μL (I_{samp}) of sample was injected to HPLC column so the total protein that was injected to HPLC column would be - $(TP_{samp} \times I_{samp}) = (50 \times 0.12) = 6 \text{ } \mu\text{g}$.

iv. Hence 6 μg of total protein from brain tissue extract has 0.08 ng of DA.

v. Then 1 mg of total protein should have $[0.08 \times (1000/6)] = 13.33 \text{ ng}$ of DA.

vi. As the tissue extract is diluted with TCA by a ratio of 1:1 before injecting into the HPLC column, the true concentration of DA will be $13.33/2 = 6.66 \text{ ng}$ in 1 mg of protein.

Questions for self-study and interpretation:

1. Are there any simpler and reliable biochemical or other assays for deciphering dopaminergic degeneration?

2. What are the advantages of HPLC quantification over in vivo neuronal visualisation using microscopy?

3. How would you interpret results if there is no depletion in DA levels in PD brain but the DA metabolite levels are altered?

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Chapter 86

Observing surface topography of *Drosophila* eye by scanning electron microscopy

Sonia Narwal (p20180001@pilani.bits-pilani.ac.in), Shreyas Iyer (p20200005@pilani.bits-pilani.ac.in), Arushi Rai (arushi.raii5@gmail.com), Meghana Tare (meghana.tare@pilani.bits-pilani.ac.in)*

Department of Biological Sciences, BITS Pilani, Pilani

* For correspondence

Recommended Level: UG/PG/Research

Sub-discipline: Developmental Biology, Neurodegeneration

Background:

The compound eyes of *Drosophila* have been widely used since the beginning of fly genetics because of convenience of identifying visible mutants that affect eye development and morphology (Hales *et al.*, 2015). Molecular genetic studies have provided detailed understanding of the diverse cascades of signaling pathways that bring about the remarkably organized arrays of ommatidia seen in adult eyes. Since nearly 75% protein-coding genes share high similarity between fly and human genomes (Pandey, 2011), fly has become a very good model for many human diseases, including the diverse neurodegenerative disorders. The *GAL4-UAS* binary system (see [Chapters 39, 40](#)) directed ectopic expression of transgenes, carrying mutant alleles causing human neurodegenerative disorders, in developing eyes of *Drosophila*, and examination of the adult eye surface as a morphological readout for neurodegeneration have been widely used to understand the events underlying neurodegeneration (Lu, 2009). Although, the eye phenotypes can be observed by conventional microscopy (see [Chapter 87](#)), imaging with Scanning Electron Microscope (SEM) provides a very high resolution so that even minor damage can also be discerned. SEM Imaging of other cuticular structures of fly also provides detailed information about anatomy and genetic variability. Here we describe a simple protocol for imaging eyes (Tare *et al.*, 2005) or other cuticular tissues of adult flies under the SEM to understand their phenotypes.

Scanning electron microscopy permits 3-dimensional visualization of surface features of biological tissue at nanometer scale resolution. Since the wavelength of electron beams is much less than that of the visible light, SEM provides a resolution between 0.5 nm to 4 nm. SEM produces images by a focused electron beam, generated from a metallic (usually from Tungsten) filament under high voltage, which probes and scans the sample across a defined area. Electrons in the beam interact with the atoms constituting the sample material and are scattered back, producing the back scattered electrons

or the secondary electrons. The signals of these back scattered electrons are collected by a detector to generate a visible image on a screen.

Principles of sample preparation for SEM: The SEM requires vacuum around the sample and the electron beam to avoid any interference in the electron beam path by other material which would generate unwanted diffractions. Obtaining SEM images with good ultrastructural preservation, therefore, needs careful sample preparation methods. Biological samples must be properly i) dehydrated, ii) dried completely, iii) mounted, and iv) sputter coated before proceeding for SEM analysis.

i. Dehydration: Dehydration is an important step to deprive the sample of its water content without rupturing its structure. This is achieved by keeping the desired tissue in ascending concentrations of Acetone (or ethanol) from 30% to 100% for defined durations so that the water is gradually and gently removed, without causing sample to shrink.

ii. Drying: This step is to allow the dehydrated sample to completely get rid of the water content for better imaging quality without any interference. Simply allowing the acetone or ethanol to evaporate from sample surface would create disruption in tissues due to relatively high surface tension of these solvents. Therefore, the dehydrated samples are incubated overnight with Hexa-Methyl-Di-Silazane (HMDS) and Acetone. HMDS is the most preferred chemical for air drying of the SEM samples as this requires minimal expertise and equipment. The other methods used for critical point drying need vacuum and liquid CO₂ for obtaining a critical temperature and pressure point at which the water/liquid content vaporizes.

iii. Mounting: Air dried samples are mounted on a metallic stub covered with a carbon tape to enhance adhesivity and hence stability of sample on the stub or the surface of mount, besides increasing the tissue's conductivity.

iv. Sputter Coating: This is an optional step required to enhance the conductivity of biological samples. When samples are not very conductive, the electron charge will accumulate within the sample and will create a charging effect causing the images to be blurred. The metal coating also enhances topographic contrast, secondary and back scattered electrons emission and hence better resolution of the sample. Gold is most commonly used for coating because of its high atomic number and yield good contrast in BSE and SE images (Suzuki, 2003); other materials like Palladium, Platinum, Iridium, Chromium, and Tungsten etc. are also used depending on the sample type. The coating is done using the Sputter-Coater apparatus. Low acceleration voltage should be used to reduce the charge effect if samples cannot be coated with a conductive coating.

There are newer methods which do not require sputter coating, if sample is already conductive, and low voltage is sufficient for imaging. More details about differences obtained with and without sputter coating the samples can be found here. More details about the process and working of SEM can be seen here.

Objective:

To examine surface features of eyes of adult flies by Scanning Electron Microscopy.

Materials Required:

- A. Flies: Desired fly stocks.
- B. Equipment and other items:
 - i. Stereo-binocular microscope
 - ii. General fly handling items
 - iii. 1.5 mL centrifuge tubes
 - iv. Pipettes (1 mL capacity)
 - v. Scanning Electron Microscope
 - vi. Metallic stub
 - vii. Carbon Tape
 - viii. Sputter-Coater
- C. Chemicals/Reagents:
 - i. Acetone grades (dilutions in dist. Water): 30%, 50%, 70%, 90%, and 100%
 - ii. HMDS (Hexa-Methyl-Di-Silazane)

Experiment Details:

1. Anaesthetize adult flies of desired age and genotype.
2. Transfer whole fly to a 1.5 mL microfuge tube containing 500 μ L of 30% acetone for 2 hr.
3. After 2 hr, completely remove 30% acetone and add 500 μ L of 50% acetone. Leave for 24 hr.
4. Repeat step 3 with 500 μ L (each) of 70%, 90% and 100% acetone, each for 24 hr.
5. Completely remove 100% acetone and add 500 μ L of 1:1 mixture of 100% Hexa-Methyl-Di-Silazane (HMDS) and Acetone. Leave the tissue for overnight incubation.
6. Replace the 1:1 mixture of 100% HMDS and Acetone with 250 μ L of 100% HMDS and leave for 24 hr.
7. Discard HMDS and leave the tube open to air dry the samples at room temperature. Take care to prevent any dust or other particles to fall on the samples. **Note:** The volumes mentioned here should be optimized; the idea is to completely immerse the flies into different concentrations of acetone, and/or HMDS. Since tubes are left to air dry after step 7, a lesser volume of HMDS is used compared to the earlier steps.
8. Mount the fly on carbon tape over the stub in such a direction that the area of interest (in this case the eye tissue) in the sample is perpendicular to the plane of the observation in the microscope.
9. Sputter coat the mounted samples in vacuum with an electrically conductive layer of gold (10 nm thick) at a pressure of 100 psi for 45 sec.
10. Image the preparation under SEM at a magnification of 600 X or as desired.

Observation:

Note the highly ordered array of 750-800-unit eyes, each called ommatidium (ommatidia for plural), in each of the compound eyes, with a short bristle protruding from one corner of each ommatidium (**Fig. 1A**). *GMR-Gal4* driven expression of *UAS-Parkin-RNAi* causes down regulation of the *Parkin* gene, which results in disruption of the ommatidial arrays due to degeneration of the photoreceptor rhabdomeres in each ommatidium, giving a rough eye phenotype because of the protruding bristles (**Fig. 1B**).



Fig. 1. Scanning electron micrographs of compound eyes of *Drosophila melanogaster* adults. **A.** Eye of wild type fly display the highly ordered lattice of 700-800 unit eyes or ommatidia. **B.** Eye showing rough eye phenotype because of the *GMR-Gal4* driven expression of *UAS-Parkin RNAi* transgene. The eye image in **A** appears smaller than in **B** because the magnification in **A** is smaller than in **B** (see the scale bar and other details of image collection on left side in each).

Questions for self-study and interpretation:

1. Highlight the importance of drying and sputter coating in sample preparation for SEM.
2. Why is vacuum important in SEM?
3. Draw a working model of SEM to illustrate its principle.
4. List the different samples which can and cannot be visualized using SEM.
5. What are the differences between images produced by Scanning and Transmission Electron Microscopes? What kind of information can each mode provide for Biological samples?

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<https://www.nanoscience.com/techniques/scanning-electron-microscopy/#how>

Physicists Continue to Invent New Techniques for Biologists

The book (*Science Is Beautiful: The Human Body Under the Microscope*) may interest physicists for at least two reasons. First, some of the technologies used to create the images, such as electron microscopy, confocal microscopy, and x-ray radiography, were invented and developed by physicists. And as last year's Nobel Prize in Chemistry evinced, physicists continue to invent new technologies for observing biological systems.

The second source of potential interest lies in what the images depict. Just as physicists continue work on new biophysical techniques—imaging and nonimaging—they also continue to apply their knowledge of physics to understand biological systems.

(In: Day, C. 2015. Electron microscopy is beautiful. *Physics Today*.
<https://physicstoday.scitation.org/doi/10.1063/PT.5.010305/full/>)

Chapter 87

Nail polish imprint of adult eyes

Richa Arya^{1*} (aryaricha@bhu.ac.in), S. C. Lakhotia¹ (lakhotia@bhu.ac.in), Akanksha Verma²

1. Cytogenetics Laboratory, Department of Zoology, Institute of Science, Banaras Hindu University, Varanasi 221005, India

2. Dr. B. R. Ambedkar Center for Biomedical Research (ACBR), University of Delhi, New Delhi, India

* For correspondence

Recommended Level: Post-graduate/Research

Sub-discipline: Neurobiology, Developmental Genetics

Background:

The adult *Drosophila* eye contains a highly ordered array of about 800 ommatidial units (Ready *et al.*, 1976). Each ommatidial unit comprises of eight photoreceptor cells surrounded by four cone and two primary pigment cells (see [Chapter 2](#)). There are twelve additional cells, which surround each ommatidium, which are also shared by the neighboring ommatidial clusters. The intricate series of cellular signaling events during larval and pupal stages which transform the single layered larval eye imaginal disc into a well-organized functional adult eye are well characterized (Ready and Benzer, 1978). In recent decades, *Drosophila melanogaster* has become a very informative model for many human diseases, including neurodegenerative disorders like those associated with polyglutamine-expansion (e.g. Huntington's Chorea and diverse Spino-cerebellar Ataxias or SCAs), Alzheimer's and Parkinson's (Warrick *et al.*, 1998). Since the eyes are not essential for survival of flies in the laboratory cultures, fly eye provides a good read out of neurodegeneration since the photoreceptors (neuronal cells) are amongst the first cells to be formed during the eye development so that the early defects in the photoreceptor neuron development result in easily identifiable deformed adult eye. Availability of the *GAL4-UAS* binary system for targeted expression of mutant human transgenes (Brand and Perrimon, 1993) in developing *Drosophila* eyes provides a convenient system of inducing and assessing neurodegeneration. Conventionally, the eye morphology is examined directly under a stereo-binocular microscope. However, the resolution provided by such examination is rather low. Therefore, Scanning Electron Microscope (SEM, see [Chapter 86](#)) is commonly used to provide a much better resolution of the disrupted organization of ommatidial arrays.

The nail-polish imprint technique developed by Arya and Lakhotia (2006) is a simple, rapid, inexpensive and widely used light microscope based method, that provides high quality images of the eye surface (**Fig. 1**), generally comparable to those obtained by the

conventional SEM method (see [Chapter 86](#)). It does not require any expensive facility and can be done in any laboratory with a good light microscope. The very little time required to prepare the eye imprints, facilitates examination and scoring a large number of samples in class room settings as well as in research labs. The protocol can be used for studying any mutation or pathological condition that affects the photo receptors during eye development.

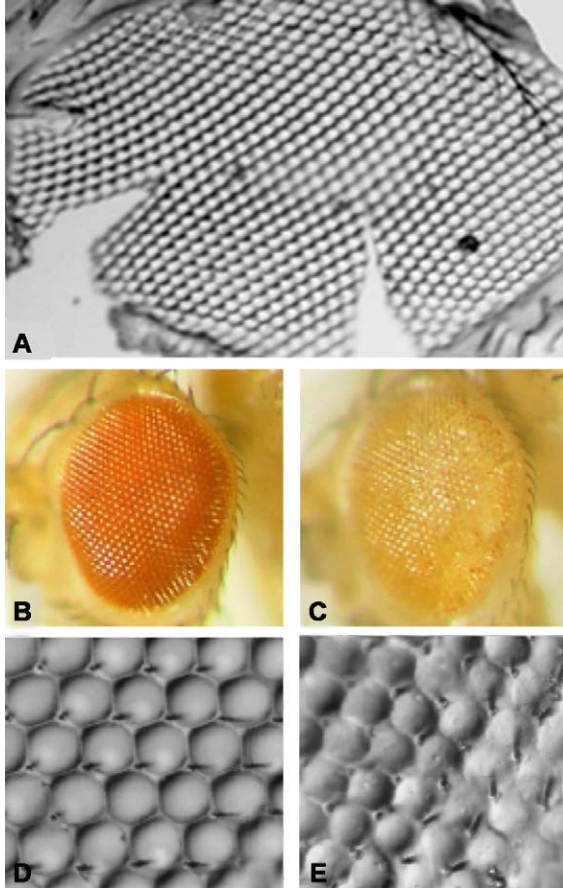


Fig. 1. Nail polish imprints of wild type and neuro-degenerated eyes. A. Nail polish imprint of wild type eye imaged with 10X objective using DIC optics. B-E show morphology (B, C) and nail polish imprints (D, E) of eyes of *GMR-GAL4>UASMJDtrQ27* (B, D) and *GMR-GAL4>UASMJDtrQ78* (C, E) adults, respectively. Images in D and E are under 40x objective and DIC optics. Note the distinctly identifiable mild degeneration in the ommatidial arrays in eyes of *GMR-GAL4>UAS-MJDtrQ78* (E) adult flies, which is not so distinct in the eye morphology image in C.

Objectives:

Examination of ommatidial organization in wild type and neurodegenerative disease model.

Materials required:

A. Flies:

- i. Wild type
- ii. *w*; *GMR-GAL4 UAS-MJDtrQ27/CyO* (control)
- iii. *w*; *GMR-GAL4 UAS-MJDtrQ78/CyO* (experimental)

Note: The *GMR-Gal4* driver (for details about the *Gal4-UAS* system, see [Chapters 39, 40](#)) causes expression of the responder *UAS-MJDtrQ27* or *UAS-MJDtrQ78* transgenes in developing eyes. The *UAS-MJDtrQ27* transgene carries 27 tandem glutamine residues of the truncated human Spinocerebellar ataxia type-3 protein. Since its expression does not cause any damage to ommatidial development, it serves as control. On the other hand, the *UAS-SCA3trQ78* contains 78 tandem glutamine residues in truncated mutant *SCA3* protein, serving as *Drosophila* model of human Spinocerebellar ataxia type-3 (Machado Joseph) disease. In these stocks, the *GMR-Gal4* and the *UAS-MJDtrQ27* or the *UAS-*

MJDtrQ78 have been recombined and maintained with the *CyO* balancer. Flies of any other genotype that affects eye development can also be used.

B. Equipment:

- i. Stereo-binocular microscope
- ii. Bright-field compound microscope (availability of DIC optics would improve the quality of images)

C. Other items:

- i. Common fly handling items
- ii. Fine forceps and dissecting needles
- iii. Transparent nail polish
- iv. Cleaned slides and coverslips (22 mm²)

Experimental Details:

1. Three to five days old flies of the desired genotype (e.g., wild type, *w*; *GMR-GAL4>UAS-MJDtrQ27* (control) and *w*; *GMR-GAL4>UAS-MJDtrQ78* (experimental) are anaesthetized, placed on a clean and dry slide.
2. The flies are decapitated by separating the head region at the junction of head and thorax with a pointed needle (or a sharp blade) under a stereo-binocular microscope.
3. A small drop of transparent nail polish is placed on another clean microscope slide and the decapitated head, held with forceps or needles, is quickly dipped in the still fluid drop of nail polish so that a thin layer of nail polish covers both the eyes (Fig. 2A, B).
4. The head, with a layer of nail polish, is transferred to a dry area of the same slide positioned with its ventral side facing the slide and the nail polish layer on the eyes is allowed to dry at room temperature (24°C) for 5–10 min.

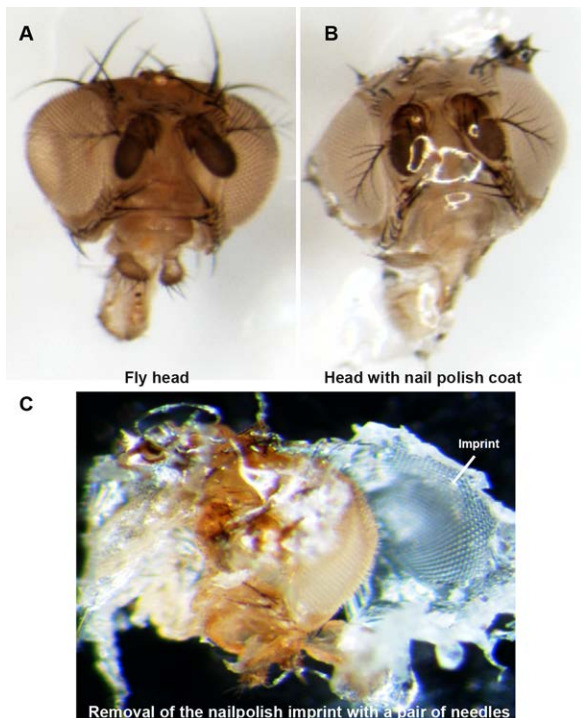


Fig. 2. Steps in preparation of a nail-polish imprint of eye of adult fl . A. Freshly separated head; **B.** Head covered with nail polish and awaiting drying in air; **C.** The dried nail polish imprint being removed from the fly's head.

5. The dried layer of nail polish is carefully peeled-off from the eye with help of fine dissecting needles/fine-tipped forceps (**Fig. 2C**). The separated peel, being an exact replica or imprint of the eye surface, assumes a goblet-shaped appearance.

6. This nail polish imprint is carefully placed on another clean glass slide, preferably with the nail polish surface that was in contact with the eye facing up. It can be now directly examined and photographed under a stereo binocular microscope or a low power objective of a compound microscope to provide a low magnification image of the eye.

7. For higher magnification and better-resolved image of the eye surface, the peel is covered with a drop of 50% glycerol and gently covered with a 22 mm² coverslip over it.

8. The eye imprint can be examined under a microscope using 10X, 20X or 40X objectives with reduced illumination, which can be achieved by adjusting the sub-stage iris diaphragm and condenser of the microscope. Availability of phase contrast or differential interference contrast (DIC) optics on the microscope would improve quality of the image.

Observation:

1. Observe the nail polish imprints of the ommatidial organization in eyes of adult flies of wild type, disease-models (control and experimental) from at least five individuals of each genotype to see the ordered (wild type and *GMR-GAL4>UAS-MJDtrQ27*) or disordered (*GMR-GAL4>UAS-MJDtrQ27*) arrays of ommatidia (**Fig. 1**).

Questions for self-study and interpretation:

1. Why does nail polish produce a replica of the eye surface? Can some other material be used in place of nail polish for preparing the imprint?

2. What are poly-glutamine (polyQ) expansion disorders in humans and why such expansion result in disease?

3. Why are only the developing eyes affected when the *UAS-MJDtrQ78* transgene is expressed using the *GMR-GAL4* driver?

4. Why are the eye phenotypes different in *GMR-GAL4>UAS-MJDtrQ27* and *GMR-GAL4>UAS-MJDtrQ78* flies?

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Fly Eye – Most Beautiful and Useful Organ for Developmental Biologists

The *Drosophila* eye is one of nature's most beautiful structures and one of its most useful. It has emerged as a favored model for understanding the processes that direct cell fate specification, patterning, and morphogenesis.

(In: Cagan, R.. 2009. Principles of *Drosophila* eye differentiation. Current Topics Developmental Biology. 89: 115–135.

[https://doi.org/10.1016/S0070-2153\(09\)89005-4](https://doi.org/10.1016/S0070-2153(09)89005-4))

Chapter 88

Do female *Drosophila melanogaster* flies show a preference to lay eggs at a particular time of the day?

Anuj Menon (anujmenon@jncasr.ac.in) and Vasu Sheeba* (sheeba@jncasr.ac.in)

Chronobiology and Behavioural Neurogenetics Laboratory, Jawaharlal Nehru Centre for Advanced Scientific Research, Bengaluru

* For correspondence

Recommended Level: PG/Research

Sub-discipline: Physiology/Behavioural Biology/Ecology

Background:

Fruit flies need to lay eggs (oviposit) on or near a food surface to ensure that the hatching larvae have ready access to food. This is a carefully considered action on part of the female fly - a phenomenon at the end of a complex series of behaviours, eventually resulting in oviposition. Female fruit flies have a preference for their physical environment such as level of light, temperature, humidity and even the smell, taste and texture of the substrate. Thus, it is likely that the time of day at which to lay eggs may also be a crucial factor that determines the survivability of the offspring. It is known that like many other behaviours of the fly *Drosophila melanogaster*, such as its locomotion, sleep and eclosion, the act of oviposition also exhibits an approximately 24 hr rhythm (Manjunatha *et al.*, 2008). In the experiment described here we can test whether flies, placed in an environment with a clear day-night cycle (such as a 12:12 hr light:dark regime), exhibit any preference in the timing of oviposition. In this exercise we describe a simple study to examine if the egg laying behaviour of flies maintained under standard laboratory condition ~ 25°C and 12:12 hr light:dark cycle, exhibits a daily rhythm. The ‘experimental regime’ of light:dark cycle regulation is achieved best in an incubator with in-built temperature and light controls, but can also be performed in a room with an Air-conditioner set at 25°C and the lights can be manually switched ON or OFF at pre-defined times.

Objective:

To study the time-course of egg-laying in laboratory under fixed environmental conditions and examine any rhythmicity in this behaviour.

Materials Required:

- A. **Flies:** Wild type fly culture.
- B. **Equipment and fly handling items**
 - i. Stereo-binocular microscope

- ii. Standard fly food preparation for rearing flies.
- iii. Egg collection medium (see **Chapter 1 Appendix I**) with additional charcoal added, if the food colour is too light to distinguish eggs present on surface.
- iv. Two sturdy metal/plastic racks for storing 20-30 glass vials in upright condition.
- v. A foam / Styrofoam / sponge pad ~6*6 inches (an old mouse pad also works) to tap down glass vials (optional).
- vi. CO₂ anaesthesia setup or alternative methods of anaesthesia (see **Chapter 1 Appendix II**).
- vii. Soft fine hair brushes for handling flies.
- viii. Red lamp for working in the dark (details below).

Experimental Details:

A. Pre-Assay Setup:

- i. Expand a healthy culture of wild-type flies to be tested at least 8-10 days in advance so that about 50 freshly emerging male and female flies can be obtained 1-2 days before the assay. Ideally, flies must be reared in the same room where the experiment is to be conducted.
- ii. Sampling frequency: Plan collection of eggs 6 times per day with sampling carried out at 4 hr intervals (see **Table 1**).
- iii. Sampling duration: About 5-7 days of data (with the above daily sampling frequency) should provide information about any pattern of the timing of the various components of the oviposition rhythm, such as its peak, onset of egg-laying, offset of egg-laying etc.
- iv. The food for the egg laying assay must be prepared a day before the assay is started. If 20 flies are being tested at 4 hr sampling interval, 120 vials would be needed per day. So the experimenter must prepare 150 vials of food each day or 300 vials every alternate day.

B. Assay Setup:

- i. Take 30 fresh food vials, plug them with non-absorbent cotton, and place them in a light but sturdy rack. Label this rack as the 'test' rack. 10 of these will be used as back-ups to replace flies in case of accidents during the setup; the remaining will be the actual experimental flies to be tested.
- ii. Under CO₂ anaesthesia (or using an alternative method), transfer a single gravid female to a fresh food vial, using a gentle brush, within 10-30 sec of CO₂ exposure. Continue till all 30 vials are occupied with single female each. Keep them serially in a test rack.
- iii. After loading the flies in the food vials, move them from the working fly station to the place where the assay will be conducted. This is the experimental regime/ area. Environmental conditions here should be fixed for the duration of the assay: maintenance of 12:12 hr light:dark cycles with constant 25°C temperature for 6-7 days. Give flies 24 hr to acclimatize to the new vial environment as well as the assay conditions. Ensure that there are no other physical / mechanical / acoustic disturbances in that area.
- iv. After ~22-23 hr ensure that all females, (including backups) are alive and laying eggs on the food and that these eggs are starting to hatch. Examine the vials carefully and discard vials in which the female is not laying eggs/laid unhatched eggs. Replace

discarded vials with vials from the backups to get ideally 20 vials which will be used for the experiment. Number the vial position in this rack in a consistent easy-to-track fashion.

v. Prepare an identical new rack with similar number of fresh plugged food vials and numbered labels and designate the rack as the ‘transfer’ rack (**Fig. 1**). Prepare the transfer rack prior to each scheduled transfer (4 hr interval).

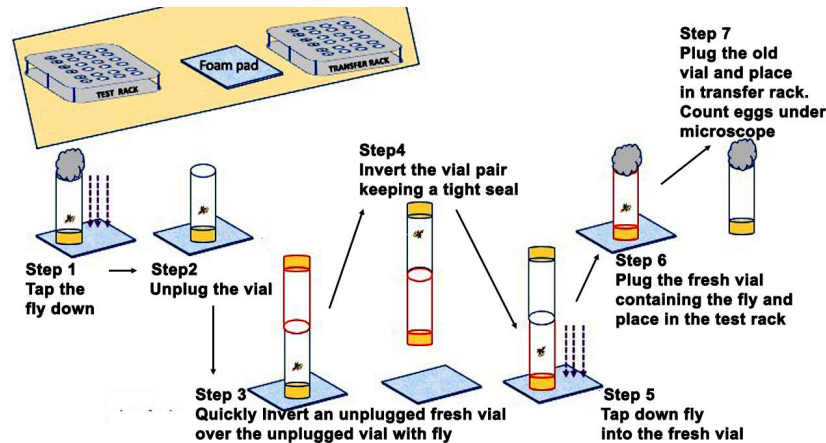


Fig. 1. Flow chart for transfer of flies from the test vial to fresh vial for oviposition.

1. To begin the assay, bring the transfer rack with fresh plugged food vials to the experimental regime /area. Find a level stable surface to start fly transfers. One by one, transfer females from the previous food vial into the correspondingly numbered new vial by first tapping down the old vial gently on foam pad, quickly removing the plug, rapidly placing the mouth of a fresh food vial onto the old unplugged food vial, with a tight hand seal in place (such that the new food vial is on top of your hand seal and the old one is at the bottom). Quickly invert the 2-vial column and tap down again, to dislodge the fly from the old vial on top to the fresh food vial (at the bottom) and quickly plug this new vial before the fly escapes. Ensure that the female is present (and uninjured) in this fresh food vial before placing it back in the same location on the test rack (**Fig. 1**).

2. Carefully note the position of the old vial taken from the test rack and put it in corresponding position in the transfer rack. Fly vials should NOT be accidentally placed in the transfer rack. Take the transfer rack to the fly lab for counting of eggs under a stereo-binocular microscope.

3. Count number of eggs laid in each vial and enter them in your data sheet (example data sheet. **Table 1**).

4. Discard the food vials after counting and recording the number of eggs in each of them (wash and reuse the discarded vials).

Observations:

Prepare an assay sheet, as exemplified in **Table 1**, in advance. Make a careful note of number of eggs present, in each vial and the time. Analyze the data for the rhythm analysis.

Results:

1. Plot number of eggs laid per time point across the seven days to get a broad overview of the time-series. For each day, mark out the times of day where you get a peak in egg laying and /or a sharp drop in eggs laid.

2. Calculate the distance between peaks or between drops from day to day. This gives an estimate of the period of the rhythm, if any. If the data is noisy, consider smoothing methods or fitting a cosine curve to get a reliable estimate of the period (for help, contact the authors of this chapter).

Table 1. A typical data sheet for recording the number of eggs laid in each vial at defined time points. In this example, sampling is done at 4 hr intervals during each 24 hr period.

Experiment number : _____																			Date: _____		
Lights ON time: _____						Lights OFF time: _____						Temperature: _____									
Time	Number of eggs in different vials (V1-V20) at different time intervals																				Sign
	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12	V13	V14	V15	V16	V17	V18	V19	V20	
10:00																					
14:00																					
18:00																					
22:00																					
02:00																					
06:00																					

General Precautions:

1. Minimise the time for CO₂ or ether anaesthesia to prevent negative impact on fecundity of either sex.
2. Practice vial to vial transfers ahead of the actual experiment, both under light and dark conditions to ensure that the experimental flies are not lost during the assay.
3. Under darkness, be sure to have a red lamp in the assay chamber before hand. Red light of wavelength greater than 650 nm does not impact the circadian clocks present in the fly and provide enough illumination required for fly transfers.
4. Make sure the rack is on a level surface with vials in upright condition.
5. Make sure that the food vials do not have any contaminating fly.
6. The transfer time and the environmental conditions should be coordinated. Avoid sampling in the middle or end or beginning of a change in the environment (for example shift from lights ON to lights OFF or reverse).
7. Divide the transfer times between workmates with randomised checks to ensure regular and precise transfer times. A missed transfer is a lost reading.
8. Females frequently prefer to lay eggs on the corners of the food where it meets the glass or on curved/vertical surfaces. Ensure that bubbles (if any present) in the food are not mistaken for egg. Some mutants can lay eggs sporadically on the glass surface near the food and they should be counted as well.

9. Check to see if eggs are present more on the periphery or the center of the food. If the eggs are laid in tight clusters or in a line, make a note of it.

Questions for self-study and interpretation:

1. Which environmental factors appear to be essential in triggering oviposition? Which factors appear to result in immediate cessation of egg laying?
2. Compare the estimated period calculated from peaks versus that obtained from troughs. Which one is more variable across the days? What is the value of the estimated period? What is the variation in oviposition period across vials?
3. What might be the consequence on this rhythm if multiple females are present within one vial?
4. What might be the consequence of carrying out this experiment in a semi-natural environment where flies can experience the natural cycles of light and darkness, cold and warmth? What if these experiments were carried out in different seasons – summer, winter, rainy or spring seasons?

References and further reading:

Manjunatha, T., Hari Dass, S. and Sharma, V. K. (2008) Egg-laying rhythm in *Drosophila melanogaster*. *J. Genet.* 87:495–504. <https://www.ias.ac.in/article/fulltext/jgen/087/05/0495-0504>

Genes and Circadian Rhythm

The modern era of ‘clock genetics’ began in 1971 with the discovery by Ron Konopka and Seymour Benzer of the *period (per)* locus in *Drosophila* (Konopka and Benzer, 1971). At the time, even the existence of single-gene mutations affecting a process as complex as circadian rhythms was met with great skepticism. The eminent phage geneticist, Max Delbruck, is purported to have said, “No, no, no. It is impossible,” when told of Konopka’s results. Benzer’s reply: “But Max, you don’t understand. They’ve already been found”

(In: Takahashi J. S. 2004. Finding new clock components: past and future. *Journal Biological Rhythms*, 19: 339–347. <https://doi.org/10.1177/0748730404269151>)

Chapter 89

Food searching behaviour in *Drosophila melanogaster*

Nisha Sharma¹ (nishasharma18phd@cup.ac.in) and Shahnaz Rahman Lone^{2*} (slone@gitam.edu; lonesr@gmail.com)

1. Department of Zoology, Central University of Punjab, Bathinda, 151001, Punjab, India.
2. Department of Biochemistry and Bioinformatics, The Gandhi Institute of Technology and Management (GITAM), (Deemed to be University), Visakhapatnam, Andhra Pradesh, 530045, India.

*For Correspondence

Recommended Level: PG/Research

Sub-discipline: Neurobiology /Behavioural Ecology

Background:

Food-seeking behaviour is a vital survival instinct present in all animals. It is influenced by internal cues like hunger and satiety, as well as external cues such as smell and visual stimuli. Different species have evolved unique strategies to find and capture their preferred food sources. This food-searching and capturing behaviour, like birds swooping to catch fish and bears foraging for berries, is an integral part of the living world. The fruit fly, *Drosophila melanogaster*, serves as a valuable model organism in neuroscience for studying food-seeking behaviour. Their efficient ability to detect and locate food sources provides insights into understanding this behaviour across different organisms.

Drosophila's remarkable sensory capabilities guide its foraging decisions. Olfactory cues are essential in locating food sources as fruit flies use their sensitive olfactory system to detect specific odors from ripe fruits and thus make precise food-seeking choices (Root et al., 2011). Their gustatory system assesses the food quality (Inagaki et al., 2012), while the dopamine-driven reward system influences motivation and persistence in seeking food (Pool et al., 2014). Genetic factors, like neuropeptide F (NPF) and its receptor, also regulate hunger-driven foraging choices in *D. melanogaster* (Wu et al., 2005). Understanding the interplay between sensory perception, neural circuits, and neuropeptide signaling is crucial for studying food-seeking behaviour in fruit flies.

Objective:

To study the food searching behaviour of *Drosophila melanogaster*.

Materials Required:

- A. Flies:** Canton-S or other desired wild type and mutant (e.g., Olfactory defective mutant *Orco*¹, Bloomington stock no. BDSC 23129) flies.

- B. Equipment and fly handling materials:
- i. Stereo-binocular microscope and other fly handling materials.
 - ii. B.O.D. incubator maintained at $24^{\circ}\text{C} \pm 1^{\circ}\text{C}$.
 - iii. Stopwatch/timer.
 - iv. Table lamp with red light (red night lamp).
 - v. CO_2 or alternative methods for anesthetizing flies.
 - vi. Petri-plates (95 mm X 15mm).
 - vii. Aspirator for transfer of flies.
 - viii. Vials with 1% Agar medium: dissolve 1 gm Agar powder in 100 mL water by boiling, cool and while still fluid, pour into empty vials. Plug the vials.
 - ix. Yeast paste: dissolve 400 mg yeast granules or powder in 10 mL water by mixing.

Experimental Details:

1. Collect 40-60 freshly eclosed males of the desired genotype and place them in groups of 20 males per food vial at $23^{\circ}/24^{\circ}\text{C}$ for 4-5 days.
Note: Males are preferred since female's food searching behaviour is affected by their virgin/mated state. If desired, freshly eclosed females can be used in parallel with freshly eclosed males for a comparison.
2. On day 5, transfer the flies to vials containing 1% agar medium for 24-hour starvation.
3. Perform following steps under red light to avoid visual cues about food
Note: *Drosophila* visual system is insensitive to far red light.
4. Take a fresh Petri-plate (95mm x 15mm) and place approximately 5 μL of yeast solution in its center.
5. Using an aspirator, gently introduce one male fly to the dish near the wall and close the Petri-plate.
6. Switch on timer/stopwatch while fly searches yeast by its smell.
7. Once the fly rests for at least 3 seconds on the yeast solution, consider that the fly has located the food source. Note the time (in seconds) the fly took from its introduction in the Petri-plate to it finding the food.
8. Repeat the process with 10 males.
Note: Maximum time given to a fly to find the food-source is 600 seconds (10 min).

Observations:

1. Record observations for 10 males of a given genotype as in the sample Table 1.
2. For calculating the *Food-seeking index*, subtract the food-probing time from the allotted time of 600 seconds (10 minutes) and calculate the food-seeking index for each fly as $\text{Food-seeking index} = (600 \text{ seconds} - \text{time taken to find food}) / 600 \text{ seconds}$.

3. Repeat the above procedure for other genotypes (e.g., *Orco*^l mutant flies).

Note: Based on the distribution of data, determine the type of statistics required for analysis: if data shows parametric distribution, perform t-test or ANOVA but if data distribution is non-parametric, perform Mann-Whitney-U-test or some other non-parametric test.

Table 1 : Sample data sheet for recording the food probing time.

Genotype: Fly no.	Food-probing time	Food-seeking index = 600 - Food probing time (sec)/600
1.		
2.		
3.		
4.		
5.		
6.		
7.		
8.		
9.		
10.		
Mean±S.E.		

Questions for self-study and interpretation:

1. Can environmental factors like temperature or humidity affect the food seeking behaviour of *Drosophila*?
2. Can social interactions within a group of flies influence their food-seeking behaviour?
3. Where are the olfactory receptors located in fly?
4. Are there sex-specific differences in the food seeking behaviour in *Drosophila*?
5. Can experience and learning influence the food seeking behaviour of *Drosophila*?
6. Do *Drosophila* larvae also show olfaction-based food-searching behaviour?

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Energy Budget of Foraging

When food density decreases, an animal has to spend progressively more time searching for food, and it may at times have difficulty finding enough time to cover its energy budget. If it were to continue with the same foraging technique it would eventually find it difficult even to make foraging gains catch up with energy losses due to foraging activities, let alone provide also the energy required for non-foraging periods.

(In: R. Ake Norberg (1977) An ecological theory on foraging time and energetics and choice of optimal food-searching method. *Journal of Animal Ecology*, 46: 511-529).

Chapter 90

Measurement of olfactory response of larvae of *Drosophila melanogaster* using larval plate assay

Jawaid Ahsan* (jahsan@cub.ac.in), Md. Zeeshan Ali (zeeshanali@cusb.ac.in) and Anushree (anushreebtn@cusb.ac.in)

Central University of South Bihar, Gaya, Bihar

* For correspondence

Recommended Level: High School/ UG/ PG/ MPhil/ Research (PhD)

Sub-discipline: Neurobiology/ Behavioral Biology/

Background:

The sense of smell is a vital sense and is important for all the organisms starting from bacteria to human beings. The fruit fly, *Drosophila melanogaster* can sense and distinguish wide variety of odours with high sensitivity and varied specificity. The fruit fly relies on olfaction for finding food, mates and oviposition sites. The fruit fly has been a model of choice for researchers to study olfaction, learning and memory (de Bruyne *et al.*, 2001) due to its anatomically and genetically simple olfactory system with remarkable similarity with that of human beings. The fruit fly smells using a pair of antenna and maxillary palp which possess morphologically different types of sensilla housing olfactory sensory neurons (OSNs). There are around 1200 OSNs in the antenna and 120 OSNs in the maxillary palp. The OSNs express odorant receptors (ORs) and ionotropic receptors (IRs) which sense the odours. The OSNs send their axons to the antennal lobe (AL) of the brain, which is the first processing centre of olfaction and contains around 43 spherical neuropils called glomeruli. The glomeruli have excitatory and inhibitory local interneurons (LINs or LNs). The output neurons from the AL, the projection neurons (PNs), innervate into the Kenyon cells (KCs) in Mushroom body (MB) and the lateral horn (LH), the higher centres of fly brain (see [Chapter 2](#)).

Compared to the adult fly's olfactory system (see [Chapters 2, 96](#)), the larval olfactory system is relatively simple (Gerber and Stocker, 2007) since unlike the adult fly which needs to navigate, the larvae reside in the medium itself. The larva can differentiate many different odours (Heimbeck *et al.*, 1999) through three sense organs, viz., dorsal organ (DO), terminal organ (TO), and ventral organ (VO) (Gendre *et al.*, 2004). The dorsal organ (DO), also called the central dome, has 21 OSNs which innervate 21 larval antennal lobe glomeruli.

There are several established olfactory behavioral paradigms for measuring the olfactory responses in fruit fly larvae. An easy to use and highly versatile method called 'larval plate assay' (Khurana *et al.*, 2003) is described here. This assay can measure the larval odour response qualitatively as well as quantitatively. This can be used to learn more about the attractants and repellants and to screen olfactory mutants.

Objective:

Examining olfactory response of third instar larvae.

Materials required:

A. Fly Strains:

- i. Wild type (any strain e.g., Canton Special Benzer i.e., *CsBz* flies)
- ii. Any mutant strain for comparison (e.g., *Orco*, *Olf C*, *chs B* mutants.)

B. Equipment and other items for fly handling

- i. Stereo-binocular microscope
- ii. Ringer's-Agar plate: A 1% solution Agar in Ringer's solution is prepared by heating 1 gm Agar in 100 mL Ringer's solution (the quantity of Agar and Ringer's solution can be scaled down or up depending upon how many plates are required) mixing slowly till bubbles stop coming out and agar is completely dissolved. 10 mL of this hot Ringer's-Agar solution is poured into each glass (or transparent plastic) Petri dish, spread evenly and allowed to cool down and solidify. This plate will be used as testing plate in the larval plate test.
- iii. Fly handling items: Needles, fine brushes, forceps, etherizer, re-etherizer etc for fly handling.

C. Reagents and solutions:

i. Ringer's solution (isotonic to larvae):

• Solution A:

Sodium chloride (NaCl)	7.48 gm
Potassium chloride (KCl)	0.35 gm
Calcium chloride (Dihydrate)(CaCl ₂ .2H ₂ O)	0.27 gm
Dissolve in 1 L distilled water and autoclave.	

• Solution B:

<i>di</i> -Sodium hydrogen <i>ortho</i> phosphate anhydrous (Na ₂ HPO ₄)	0.13 gm
Potassium dihydrogen <i>ortho</i> phosphate (KH ₂ PO ₄)	0.05 gm
Dissolve in 1 L distilled water and autoclave.	

Solution A and B are mixed together to form Ringer's solution.

ii. **PEG solution for separation of larvae (Optional):** 30 gm of the Polyethylene glycol (PEG 6000) powder is mixed with 100 mL distilled water on a stirrer. The solution is autoclaved and used for separation of large number of larvae from the food.

Note: Any other method for quick and gentle isolation of enough number of larvae from food can also be used.

iii. **Odour Preparation:** The required concentration (10^{-1} to 10^{-9}) of the odour compound to be tested is prepared using odourless high grade mineral oil or liquid paraffin as solvent. Ethyl acetate (EA) is one such odorant which has a sweet 'fruity' smell (similar to 'pear drops') which has been shown to be attractive for third instar larvae at lower concentrations. For testing an odorant of unknown response, one should begin with mid-range concentrations (e.g., 10^{-2} or 10^{-3} or 10^{-4}). Depending upon the

response, the full concentration profile can be tested. While working with more than one concentration at the same time, first test the lower concentration and then move to testing higher concentrations.

Experimental Details:

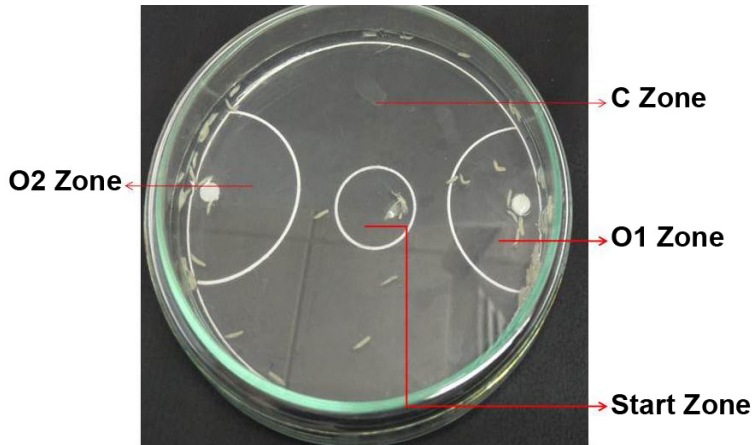


Fig. 1. Larval plate assay using a Petri dish. C Zone = Control zone, O1 and O2 Zones = zones for placing the odorant 1 and odorant 2, respectively; Start Zone = area where the test larvae are initially placed.

1. Enough number of third instar larvae, required for the experiment, are obtained by keeping about 200 well-fed and 3-5 days old flies of the desired genotype/s in food bottles and allowing them to lay eggs for 12 hr at 25°C. After 12 hr, the flies are removed to a fresh food bottle and the bottle with eggs is kept at 25°C for larval development till early third instar (4 to 5 days).
2. Prepare the Ringer's-Agar plates on the day of testing.
3. Collect early third instar larvae from the food bottle using soft hair brush and place in water (at 25°C) for manually washing away the adhering food. Alternatively, after taking out the larvae from the food bottle with soft brush and washing gently with water, transfer them to a tube containing 30% Polyethylene Glycol (PEG) solution. The larvae swim on the top of the PEG solution, from where they are easily collected in a sieve and washed again in gently flowing water.
4. Keep the separated larvae in ~1 mL Ringer's solution in a separate Petri-dish and soon start the test.
5. The testing Ringer's Agar plate is kept on a black surface (to provide good contrast for the whitish larvae) and using fine forceps two round filter discs are placed in the O1 and O2 odorant zones each (**Fig. 1**).
6. Take around 50 larvae with a soft brush and keep on the filter paper for soaking the extra Ringer's solution, following which the larvae are quickly transferred to the centre (Start zone) of the testing plates.
7. Dispense 20 μ L of the test odorant (e.g., Ethyl acetate) on each of the two filter discs in O1 and O2 zones, quickly close the lid of the dish, and immediately switch ON timer to mark start of the odour test.
Note: Since the larval response to an odour can be affected by its concentration, the odorant can be applied neat (undiluted) or diluted to varying concentration using odourless high grade mineral oil or liquid paraffin.
8. After two min, quickly count the numbers of larvae in the zones O1, O2 and C (control zone). Repeat the test with a given odorant three times with fresh larvae and fresh test dishes.

General Precautions:

1. Larvae should be handled with care using soft brush to avoid any mechanical stress.
2. All solutions used should be at room temperature prior to their use.
3. The odour is volatile, therefore, the solution should not be kept open for long time and different odorants should be handled carefully. Do not mix the odorants.
4. Immediately after the 2 min test period, the larvae in different zones of the plate should be counted as fast as possible.
5. The testing should be done at room temperature (25°C) with uniform light.

Observations/Results:

1. Count the number of larvae in the O1, O2 and C zones and calculate the olfactory response index (R.I.) using the formula:

$$R. I. = \frac{O1 + O2}{O1 + O2 + C} \times 100$$

(O1, O2 and C represent the numbers of larvae in corresponding zones)

2. Calculate the Mean (\pm S.E.) R.I. from 3-6 replicates for the tested odorant concentration. The data for different concentrations of the given odorant can be plotted in a table as shown in **Table 1**.

Table 1: The olfactory Response Index (R.I.) of wild type and mutant third instar larvae to different concentrations of the tested odour.

Log dilution of odour	Mean (\pm) R.I. of wild type	Mean (\pm) R.I. of mutant
10 ⁻¹		
10 ⁻²		
10 ⁻³		
10 ⁻⁴		
10 ⁻⁵		
10 ⁻⁶		
10 ⁻⁷		
10 ⁻⁸		
10 ⁻⁹		

3. Using data like that in **Table 1**, draw a graph showing relation between the **R. I.** (Y-axis) and the odorant concentration (X-axis) for wild type and mutant larvae.

4. A higher **R. I.** indicates the odorant to be attractive and a low value (more larvae in the C zone than in O1 abs O2) to be repulsive while nearly equal numbers in O1+ O2 and C zones, respectively indicates the odour to be neutral.

Note: This odour-response assay can also be used to calculate the preference index (**P. I.**) if two different odorants or different concentrations of the same odorant are used in the O1 and O2 zones, respectively, using the following formula:

$$\text{P. I.} = \frac{\text{O1} - \text{O2}}{\text{O1} + \text{O2} + \text{C}} \times 100$$

A positive **P. I.** value indicates that the odour in the O1 zone is preferred (i.e., more attractive) than the odour placed in the O2 zone. In a comparative study, it is advised to repeat the experiment by interchanging the places of the two odours to avoid any external bias, e.g., variable intensity of light on the two sides.

Questions for self-study and interpretation:

1. Why is odour sensing important for an organism?
2. Can different stages of life cycle of *Drosophila* have different odor response index?
3. What could be the functional significance of some odours being attractive and others repulsive to the fly?
4. Why higher concentrations of some odorants are less attractive?
5. What may be the basis for differences in olfactory responses of wild type when compared with some mutant larvae?
6. Why should some mutants lose their olfactory response to one odour but remain sensitive to another as wild type?

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Chapter 91

A rapid assessment of neuromuscular performance of *Drosophila* larvae

Prerna Aggarwal (aggarwal.prerna16@gmail.com) and Surajit Sarkar* (sarkar@south.du.ac.in) Department of Genetics, University of Delhi South Campus, Benito Juarez Road, New Delhi-110 021, India

*For correspondence

Recommended Level: Post-graduation/ Research

Sub-discipline: Genetics/Neurobiology/Behavioral biology

Background:

Locomotion is one of the complex physiological activities of animals. A well-coordinated locomotor activity can be attributed to the continuous relay of information (stimuli) between the central nervous system and the muscles, bridged by a dense network of peripheral neurons and neurotransmitters for molecular communication. It is increasingly evident now that several genetic and environmental factors play a cardinal role in proper reception of stimuli, generation of appropriate response, transportation and release of neurotransmitters at the axon endings. Despite such notable advancement, the factors that modulate neuronal and muscular activity are still elusive. The need to find these discrete molecular players become even more significant in conditions like neurodegenerative disorders (such as Parkinson's disease, Alzheimer's disease, Huntington's disease, etc.), and other neuropathological conditions that predispose an individual to develop neuromuscular disability. Neuromuscular impairments cause disoriented locomotion and such altered behavior can be utilized as phenotypic reads. *Drosophila* has emerged to be one of the most successful, tractable, and versatile model system to investigate and understand in depth the correlation of disoriented behavior with several aspects like human neurodegenerative disorders, locomotor disabilities and innate learning behavior (McGurk *et al.*, 2015; Bolus *et al.*, 2020). *Drosophila* wandering third instar larvae exhibit the appropriate amalgam of slow recordable speed, enabling to scrutinize various physical parameters such as distance travelled, the speed, body bends, and crawling pattern. These parameters can be utilized to examine the neuromuscular performance in an array of genotypic/environmental backgrounds. Comparative analysis of such parameters can offer an opportunity to correlate genes to behavior, resolving sensory to motor output at cellular and molecular levels (Kohsaka *et al.*, 2017). Therefore, the crawling behavior of larvae can be exploited to study effect of drugs, modulated gene expression and olfactory malfunctioning. Furthermore, studying such aspects at the larval stage can identify the precocious nature of a genetic or environmental change, which might be beneficial in

designing an early prognosis. The protocol outlines a rapid, semi-automated, reproducible method which can provide high throughput information. A large number of test subjects can be examined with ease and the data can be graphically represented and statistically validated. This strategy was originally designed to evaluate the crawling behavior in *Caenorhabditis elegans* (Nussbaum-Krammer *et al.*, 2015). The present protocol is a modified version for the assessment of the locomotor abilities/ disabilities in *Drosophila* larvae.

Objectives:

To investigate the impact of gene(s) and/or chemical compound(s) on neuro-muscular performance using locomotory behavior of *Drosophila* larvae.

Material required:

In order to carry out the experiment, the following stocks, chemicals and equipment are required:

A. Fly stocks: Healthy third instar larvae from any wild type and the desired mutant stock grown on suitable food (see **Chapter 1**) at $24\pm 1^\circ\text{C}$, and preferably under 12:12 hr light:dark cycle.

B. Equipment:

- i. Stereo-binocular microscope and other items for fly handling.
- ii. Camera: A good quality camera or a phone camera with video recording facility.
- iii. Computer: A desktop computer or laptop with at least 2 GB RAM and 500 GB storage, and running on: Windows (64 Bit), Linux or Mac OS X operating system. The following freely available softwares should be installed:
 - a. **MOV or MP4 to AVI converter:** The raw video file format is generally ‘.mov’ or ‘.mp4’ and should be converted to ‘.avi’ format, since ImageJ reads ‘.avi’ files. Based on the raw file format, a suitable converter can be downloaded from <http://www.pazera-software.com> or any other trusted website for conversion of the captured video files.
 - b. **ImageJ:** This software can be downloaded from <http://imagej.nih.gov/ij/>. This versatile software is used for a wide range of biological analysis. The software is utilized to process the video.avi into a readable input for wrMTrck plugin. (*Note: Occasionally, ImageJ shows memory issues. Hence, it is advised to keep the video size lower than 500 Mb and avoid high definition settings*)
 - c. **wrMTrck:** This software, available at <http://www.phage.dk/plugins/wrmtcrk.html>, functions as a plugin in ImageJ software. The webpage also offers details about the developer and other related information. Once installed, the plugin can be integrated with ImageJ software.

C. Other items:

- i. Soft fine brush, forceps, etc.
- ii. Petri plates with Agar-sugar medium (see **Chapter 1**).

- iii. Petri plates (8-10 cm diameter) with filter/blotting paper discs of the same size.
- iv. Black sheet to provide good dark background under the agar plate to provide good contrast for monitoring larval movements.
- v. 1X Phosphate Buffered Saline (PBS): Add 8.0 g NaCl, 0.2 g KCl, 1.42 g Na_2HPO_4 , 272 mg KH_2PO_4 in 800 mL dist. water; adjust the pH to 7.4 using HCl. Make up the volume to 1 L with dist. water. Autoclave and store at 4⁰C.

Experimental Details:

A. Videography of moving larvae:

- i. With the help of a wet brush, collect the age-matched third instar larvae from vials/ bottle carefully and wash them with 1X PBS, in order to remove traces of food sticking to the larvae (do not leave larvae for long in PBS, since larvae tend to get lethargic and this might affect their crawling efficiency).
- ii. Immediately after washing, transfer the larvae on a wet blotting paper soaked in 1X PBS or distilled water.
- iii. Place 3-5 wild type larvae in the center of the agar plate and wait for ~1 min for larvae to acclimatize. With the help of the brush draw the larvae back to the center.
- iv. Record the movement (30 frames/sec) of wild type larvae for the next 1 min with a good quality camera/ phone camera. An appropriate camera stand may be used while capturing the video. Alternatively, hold the phone/camera firmly in hand while recording the movement. Ensure that there is minimal reflection of light from surface of the plate.
- v. Collect tracks from at least 10 larvae following the step iv above. The larvae already tracked should be discarded to avoid mixing with the fresh set of larvae.
- vi. Repeat the procedure with larvae of other genotypes to be examined and compared with the wild type control.
- vii. Rename the recorded videos immediately to avoid any mix up in data. Capture sufficient numbers (at least 10 for each genotype) of videos for data stringency and application of statistical tools.

B. Processing of captured videos for the analysis of crawling behavior:

- i. **Converting mov or mp4 video files into avi format:** Run the downloaded converter and add the file(s) to be edited. Before ‘converting’, change the output file format/ video settings and select the video codec to ‘motion JPEG’, with 30 frames/sec.
- ii. Run the ImageJ software and open the video file (.avi) to be processed, one at a time.
 - a. **Video pruning:** Convert file .avi into grayscale through an ‘Avi reader box’. Further, trim excessive frames from the beginning and the end of the video. (**Note:** For more accuracy, unnecessary (~10-100) frames can be removed from either end of the video clip in which the larvae are not showing any locomotion). The total number of frames corresponds to the length of the video and can be adjusted accordingly.
 - b. **Adjusting threshold:** wrMTrck requires a threshold image to precisely analyze

the larval movements against the given background. If needed, the brightness contrast can be adjusted with the help of ImageJ software (Brooks *et al.*, 2016), or can be followed as suggested in <http://www.phage.dk/plugins/download/wrMTrck.pdf> such that the larvae stand out clearly from the background. The appropriately captured/ processed video can be used now to carry out downstream analysis using wrMTrck plugin.

c. **Running wrMTrck to monitor larval movements:** Go to plugins wrMTrck. The command is followed by a dialogue box. The values to be specified must be as per the earlier report (Brooks *et al.*, 2016), which have been optimized for *Drosophila* larvae.

Results/observations:

Following analysis of the captured videos, a substantial amount of information on various attributes of larval locomotion can be deduced. Based on manual as well as computerized data analysis, tabulate an array of the following physical parameters:

- i. Length (in mm) of trajectory.
- ii. Distance (in mm) between the start and the finish point (displacement).
- iii. Speed (in mm/sec) of the larval locomotion (this parameter accounts for the sluggish or brisk movement of the larvae).
- iv. Pattern of body bends (closely observe the videos and count the number of events when larvae change its direction).

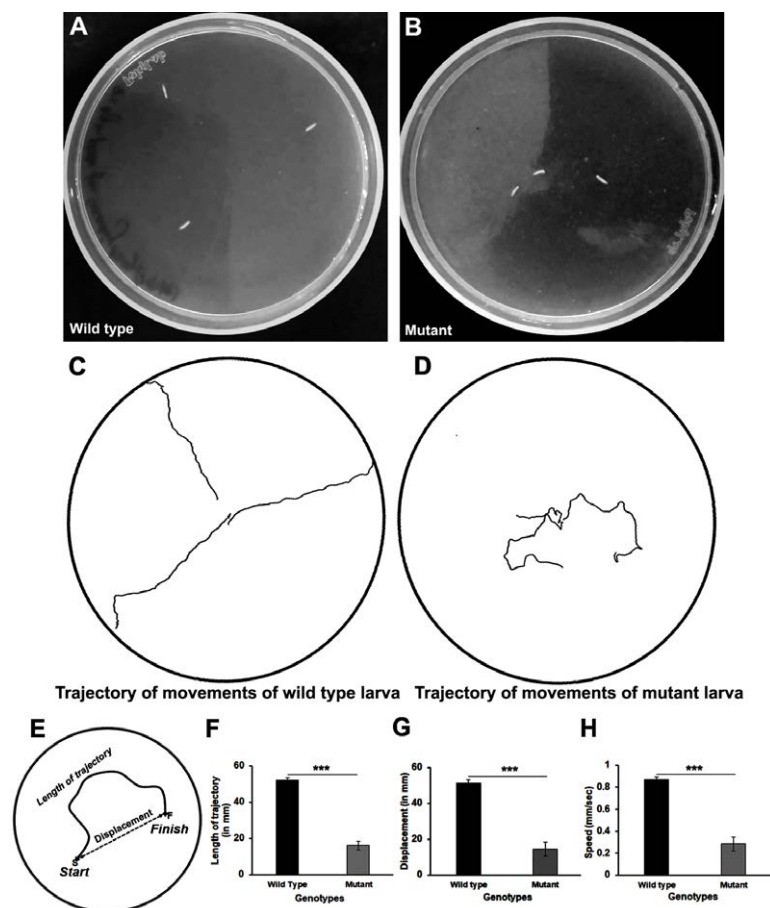


Fig. 1. Crawling behaviour of wandering third instar larvae of *Drosophila*. A. Track of a wild type larva; B. Track of a mutant larva with compromised neuro-locomotor activity; C and D. Trajectory of A and B, respectively generated by wrMTrck software; E. Calculated lengths of trajectories and displacements of tracks in C and D; F-H. Graphical representation of the various physical attributes of crawling behaviour of the wild type and mutant larvae as calculated in E.

Besides scorable quantities, wrMTrck constructs path trajectories as the larvae crawl during the experimental time duration reflecting the abrupt crawling nature of larvae which can be further utilized to quantify the percentage of abnormal crawling pattern manifested by a particular genotype (penetrance of the defect within the population). The generated numerical data from the tracks (**Fig. 1**) can be tabulated as in **Table 1** and appropriate statistical tools such as student's t-test or ANOVA can be applied to calculate the significance levels. The findings can also be represented in pictorial and graphical formats (**Fig. 1**).

Table 1. Sample table for recording data for larval locomotion from analysis of the captured videos.

S. No.	Wild type			Mutant		
	Length of trajectory (mm)	Displacement (mm)	Speed (mm/sec)	Length of trajectory (mm)	Displacement (mm)	Speed (mm/sec)
1.						
2.						
3.						
4.						
5.						
6.						
7.						
8.						
9.						
10.						
Average Standard deviation						
Student's t-test (p-value)						

Questions for self-study and interpretation:

1. Other than the locomotor abilities, can you use this experimental design for determination of any other physiological parameter in *Drosophila* larvae?
2. What is the importance of including controls in this experiment?
3. If you wish to study the effect of drugs on a genetically predetermined condition, how will your selection of suitable controls change?

4. Why are second instar and late third instar larvae not suitable to carry out this experiment?
5. How can you increase the stringency and reproducibility of your data?

References and further reading:

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Chapter 92

Negative geotaxis or climbing assay for assessment of locomotor function in adult *Drosophila*

Girish Chandran (changirish86@gmail.com)

Pooja Bhagavat Memorial Mahajana Education Centre, PG wing of SBRR Mahajana First Grade College, KRS Road, Metagalli, Mysuru, India

Recommended Level: UG/PG/Research

Sub-discipline: Neurobiology/ Behavioral Biology

Background:

Motor disorders, like Parkinson's and Huntington's diseases, are neurodegenerative disorders that are associated, among other malfunctions, with involuntary or uncontrolled movements or actions of the body. Several chemical induced or transgenic system based models have been developed in mice, *Drosophila* and other organisms to mimic the physiological and symptomatic traits of such neurodegenerative disorders. Rotenone is an inhibitor of mitochondrial complex I and is also known to induce Parkinsonian traits like walking disability in mice and rats (Coulom and Birman, 2004). Recent developments have paved way for using *Drosophila* to study neurological disorders. Here we discuss how *Drosophila* can be used to study the motor disorders using Rotenone as a chemical inducer. Wild type adult *Drosophila melanogaster* flies are exposed to Rotenone in the food medium for four days and monitored for their climbing efficiency in a negative geotaxis assay. This assay works on the fact that flies normally move against gravity (negative geotaxis) geotaxis (also see [Chapters 94, 96](#)). and stay near the cotton plug at top of the bottle/vial. Any damage to the dopaminergic motor neurons would induce motor disorder making the flies less efficient in negative geotaxis. This model can also be used to monitor neuroprotective drugs that reduce or ameliorate motor disorders (Girish and Muralidhara, 2012).

Objective:

To assess the locomotor activity of the adult *Drosophila* in relation to motor function.

Materials Required:

A. Flies: Age synchronized (preferably younger than 10 days after emergence) male flies of *Drosophila melanogaster* (wild type and/or mutant as desired. Although females can also be used for the assay, they will need frequent transfers to fresh food vials during the treatment period because of the eggs laid by them. Therefore, males are preferred.

B. Equipment and fly handling materials

- i. Stereo-binocular microscope.
- ii. Fly-handling items like ether, etherizer, fine soft brushes etc.
- iii. Foam pad for tapping the culture bottles and tubes.
- iv. Empty culture bottles and culture vials (2 cm diameter, 15 cm height).
- v. Glass rod of 5 mm diameter and 20 cm length.
- vi. Micropipettes 1 mL, 0.1 mL.
- vii. Micropipette tips 1 mL, 0.1 mL.
- viii. Cotton
- ix. Permanent marker
- x. White board: Vitrified tile or plastic board.
- xi. Micro-centrifuge tubes (1.5 - 2.0 mL volume)
- xii. Tissue paper
- xiii. Stopwatch or wrist watch with display for seconds.
- xiv 250 mL beaker
- xv. Negative Geotaxis assay tubes: glass or plastic test tubes of 2 cm diameter and at least 20 cm height. A ring should be marked on the vial using a dark permanent marker at 10 cm height from the bottom of the tube.

C. Reagents

- i. 40 mM Rotenone stock solution: Dissolve 15.7 mg in 1 mL Dimethyl sulphoxide (DMSO) in a micro-centrifuge tube (wear hand gloves).

Experimental Details:

A. Day 1: Preparation of culture tubes

- i. Prepare 100 mL fly food medium (see [Chapter 1](#)) in a 250 mL glass beaker.
- ii. Immediately pipette out 2 mL of the freshly prepared medium which is still in liquid form, into each of the 4 culture vials labeled as Control, five culture vials labeled as ROT 100, ROT 250, ROT 500, ROT 1000 and DMSO Control, respectively.

iii. Pipette out 5, 12.5, 25 and 50 μL of the 40 mM Rotenone stock soln, into food vials ROT 100, ROT 250, ROT 500, ROT 1000, respectively, and 40 μL DMSO into the DMSO Control vial while the food is still hot and liquid. Mix well and let the food solidify as it cools. During this period, keep the vials covered with a clean fine net or muslin cloth to prevent unwanted flies from entering the food vials.

iv. Plug the culture tubes and keep them upright at room temperature in a clean undisturbed area.

Day 2: Introduction of flies into culture tubes

i. Take the culture bottle containing age synchronized wild type *Drosophila* flies, tap it gently on the foam pad to let flies fall on the food, quickly remove the cotton plug, taking care not to allow any fly to escape and immediately invert this bottle onto the etherizer (in hour glass arrangement).

ii. Once the flies are properly anaesthetized, collect them on the white plate.

iii. Identify male flies by their dark abdominal tip and smaller body size (see [Chapters 1, 2, 44, 45](#)), and introduce 25 male flies into each of the above prepared Control and ROT food vials using the fine hair brush. Take care that flies do not touch the medium surface when they are unconscious.

iv. Replace the cotton plugs onto the culture vials containing flies, and keep them in horizontal condition until the flies regain consciousness (less than 20 min).

v. Once all the flies have started moving, turn the vials upright.

Note: Number of flies per tube can be increased or decreased as per requirement

Day 3: Look for any dead flies in all the tubes and record the same in the observation book.

Day 4: Climbing Assay

i. Introduce flies from the different food vials, one by one, into the negative geotaxis tube, using a small funnel to prevent escape of any fly, tap down the geotaxis tube on the foam pad, and plug the open end of the tube. Let the tube stand vertically and immediately start the stopwatch or start noting time in your wrist watch.

ii. Observe the flies climbing up the tube (see **Fig. 1**) and note the number of flies reaching the marked ring at 10 cm height within 10 sec. Calculate the % of flies, out of the total number of flies tested. that climb to 10 cm height in 10 sec.

iii. Repeat the observation by tapping down the flies. Make a minimum of three observations for each tube and calculate average % of flies that climb to the 10 cm height within 10 sec.

iv. Carry out steps i-iii for flies in each of the Control, ROT and DMSO Control tubes.

v. Calculate percent climbing activity in each tube using the formula.

$$\% \text{ Flies Climbed} = \frac{E}{T} \times 100$$

Where, T = Total number of live flies in the culture tube, and E = Number of flies reaching 10 cm mark in 10 sec

The data are averaged and tabulated as Mean \pm Standard deviation (**Table 1**).

vi. Plot a bar graph for the % Flies Climbed on Y axis and treatment condition on the X axis.

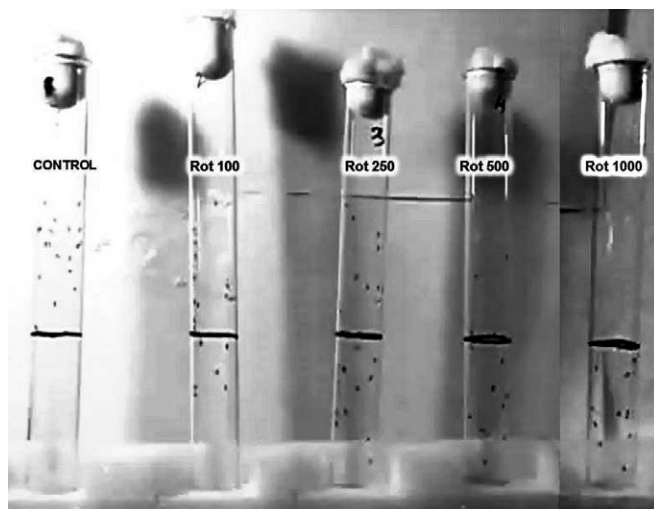


Fig. 1. Negative geotaxis assay showing the flies climbing up with different efficiency after feeding on food carrying different concentrations of Rotenone (indicated on each tube). The black ring on each tube corresponds to 10 cm height.

Precautions:

1. Wear gloves all the time while using Rotenone as it can be absorbed via skin.
2. Do not add Rotenone solution to very hot food medium.
3. Discard the food vials and other tubes/materials that may carry Rotenone in the labeled waste bin for appropriate disposal.

Results/Observations:

Calculate the Mean % Flies Climbed under different treatment conditions and record the data in a tabular form as suggested in **Table 1**.

Table 1. Climbing efficiency of differently treated flies in geotaxis assay

Name	Total number of live flie	Flies climbing to 10 cm height in 10 sec			Average	Percent Flies Climbed
		Trial I	Trial II	Trial III		
Control						
DMSO control						
Rot 100 μ M						
Rot 250 μ M						
Rot 500 μ M						
Rot 1000 μ M						

Questions for self-study and interpretation:

1. How does Rotenone affect neuromuscular activity?
2. Design an experimental screen to test various drugs/chemicals that may improve climbing activity of Rotenone treated flies for determining their therapeutic value in neurodegenerative disorders.
3. Suppose in your above test, one of the test compounds improves the climbing activity of Rotenone exposed adult flies. What are the possible mechanisms for the test compound's action?

References and further reading:

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Fruit Fly: Pioneer of Neurogenetics

Where do animal behaviours come from and are they controlled by genes? This is the fundamental question posed by the field of neurogenetics. Pioneering work from the 1960s in Seymour Benzer's laboratory demonstrated for the first time that *Drosophila melanogaster* fruit flies could be mutated to obtain animals with insomnia, learning disabilities and homosexual courtship behaviours.

(In: Vosshall, L. B. 2007. Into the mind of a fly. Nature. 450.193-197.
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Chapter 93

Is negative geotaxis in *Drosophila* a ‘group effect’?

Navya G¹ (navyagshetty@gmail.com), Rajashekhar K Patil^{1,*} (patilsirmu@gmail.com), Shamprasad Varija Raghu^{2,*} (shamprasadr@yenepoya.edu.in)

1. Department of Zoology, Bengaluru City University, Bengaluru, Karnataka 560001

2. Division of Neuroscience, Yenepoya Research Centre (YRC), Yenepoya (Deemed to be University), Deralakatte, Mangalore, Karnataka 575018

* For Correspondence

Recommended Level: UG/PG/Research

Sub-Discipline: Neurobiology/Behaviour

Background

Fruit fly, *Drosophila melanogaster*, is a good model organism for behavioral studies. The observations can be extended to understand the molecular basis of behavior, physiology and diseases. The genetic bases of different behaviors have been analyzed by several groups. Often such experiments and analyses of behavior are performed on groups of flies. For example, climbing behaviour (Gargano et al., 2005), olfactory conditioning, learning and memory assays (Davis, 2023) have been carried out on groups of flies. Some of the behavioral assays, such as taste conditioning (Masek et al., 2016) are carried out on single flies. Recent studies suggest that flies and/or larvae behave differently when in a group (Neiwalda et al., 2014) than when isolated. It is likely that interaction between flies causes a variation in their response to a given stimulus. ‘Bandwagon effect’ is an important concept in psychology (Schmitt-Beck., 2015) which has been used less often in studies on animal behavior. Individuals behave differently than a group since the behavior of an individual may be strongly influenced by other flies in the group. Sensing the activity of peers could be through tactile, olfactory or substrate-borne vibrations (Mckelvey et al., 2021).

Drosophila flies have been known to exhibit negative geotaxis. When placed in a vertically kept tube, wild-type flies climb up against gravity after being tapped down or when tubes are inverted (also see [Chapters 92, 94](#)). These observations are typically made when flies are in a group. Since flies are known to be influenced by social cues rather than by stimulus alone (Ramdya et al., 2014), it is possible that the negative geotaxis exhibited by wild-type flies may also be influenced by interactions between the flies in the group. The present experiment compares the geotaxis behavior of isolated (single) and grouped flies.

Objective: To test whether a single and group of flies show different geotaxis behavior.

Materials Required:

- A. Flies: Age synchronized (3 days after eclosion) segregated wild-type male and female flies.
- B. Equipment and other supplies:
 - a) *Drosophila* culture bottles with food and other fly handling materials.
 - b) Ice cubes and ice bucket (for cold anesthesia, see Chapter 1).
 - c) Permanent marker pen.
 - d) Stopwatch or Timer.
 - e) Negative geotaxis assay tubes – glass test tubes of 2 cm diameter and 15 cm height. Mark serial rings on the tube to form 4 quarters using a permanent marker pen at 3.5cm, 7cm, and 10.5cm, respectively, from the bottom of the tube (Figure 1).

Experimental Details:

1. Take three days old male or female flies separately, cold anesthetize them (see Chapter 1) and transfer them into separate test tubes.
Note: Keeping flies in tubes at 4°C in a refrigerator for 10 min can also be used for cold anesthesia
2. Place the differently grouped (single, and groups of 5, 10 and 25) male and female flies in separate and marked test tubes and close the mouth of the test tube with cotton plugs.
3. Place the tubes horizontally for 30 minutes and allow the flies to recover.
4. To observe geotaxis, tap the tubes on a Styrofoam pad to bring all the flies to the bottom of the tube. Keep the tubes vertical.
5. Set the stopwatch or timer for 10 seconds and let the fly/ flies move.

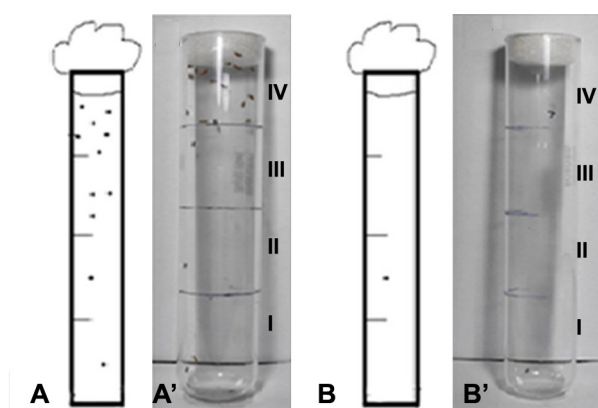


Fig. 1. Climbing assay for a group of flies or a single fl . A, B show a schematic of the setup (A with a group of flies and B with a single fly). A' and B' show images of tubes with a group of flies or a single fly, respectively (I-IV in A' and B' show marks for the fly-climbing quarters).

Observations:

A. Negative Geotaxis Climbing Assay for single fly

1. Observe and record the quarter of the tube where the fly is present after 10 seconds. Carry out 10 trials with different single flies and record your observations following the sample below (Table 1).

2. Based on the data, calculate the % times when the fly climbed to one of the quarters using the formula (calculate separately for each quarter (QI/II/III/IV) of the climbing tube):
3. % fly climbed to QI/II/III/IV = (Total No. of times a fly present in the quarter ×100)/10 (Total number of trials)

Table 1. Frequencies of single fly climbing to different heights

Trial no.	Fly climbed to			
	Q. I	Q.II	Q.III	Q.IV
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
Total for each quarter				

B. Negative Geotaxis Climbing Assay for flies in group:

1. Repeat the assay for the tubes containing 5, 10 or 25 flies. Carry out 10 trials for each group of flies and record observations as follows for each trial with groups of 5/10/20 flies:

Table 2. Frequencies of flies climbing to different heights in groups

Group of 5/10/25 flies:

Trial no.	Number of flies climbed to			
	Q. 1	Q.2	Q.3	Q.4
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
Total for each quarter				

2. Based on above data, calculate the % of flies climbing to different quarters of the tube after 10 seconds in each trial using the formula:

% fly climbed to Q1/2/3/4 = (Total No. of flies present in the quarter × 100)/ 10 (Total number of trials) (to be calculated separately for each quarter)

- Calculate the mean and standard deviation for each group and tabulate data as in following table and prepare a graph (sample in Fig. 2) to show the percentage of flies climbing on the y axis and number of quarters on the x axis in different samples.

Table 3: Summary of climbing assay for single fly and flies in a group of 5, 10 or 25

Quarter	I	II	III	IV
Mean % of single flies				
Mean % of group of 5 flies ±SD				
Mean % of group of 10 flies ±SD				
Mean % of group of 25 flies ±SD				

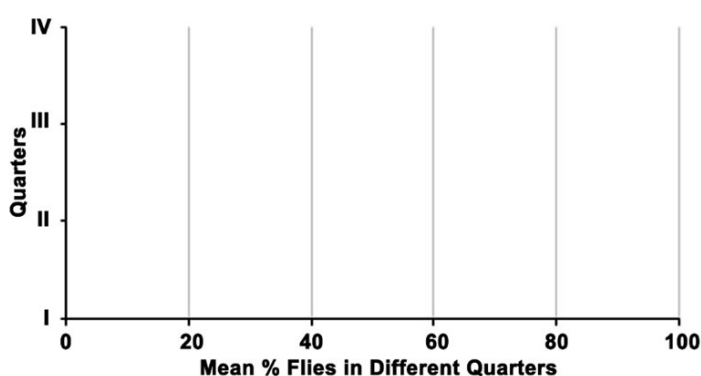


Fig. 2. Climbing assay of groups of flies and single fly (the bars for different groups of flies and single fly can be depicted in different colours).

Questions for self-study and Interpretation:

- Was there a difference in the climbing behavior of single and group flies?
- What could be the possible reasons for the difference if such a difference was observed?
- Why is ether anesthesia of flies avoided in behavioral studies?

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Group Behaviour

The phenomenon of individual units interacting with each other and exhibiting novel behaviours is common in physics, chemistry and biology.

In physical systems, matter above the level of elementary particles exhibits group-level properties at a series of different levels that extend to the properties of bulk matter familiar to us from everyday experience. A proper understanding of the latter requires the application of relativistic quantum field theory. It is not possible to derive the physics of daily experience by taking the so-called classical limit in which Planck's constant vanishes.

The gel transition is a simple example of collective behaviour in groups of molecules that can be triggered abruptly even though the properties of the system (e.g. concentration or temperature) change smoothly. There are interesting analogies between the behaviour of gels and the behaviour of social groups. In an inversion of the usual approach, a biological (epidemiological) model can clarify the dynamics of a sol-gel transition.

Living groups permit an additional mode of explanation that is based on their evolutionary history. Where natural selection is the appropriate explanation, one can ask whether variations in aspects of group behaviour can be correlated with genetic variations between individuals and, if so, whether they influence longevity or reproduction or both. The long-term differential survival of genes can be influenced by traits at the level of the chromosome, the individual or the group. At the same time, self-organization is a powerful principle in physics, chemistry and biology, and it remains an open question whether instances of group behaviour can be explained by self-organization too.

(In: Saçlıoğlu, C., Pekcan, Ö. & Nanjundiah, V. (2014). Group behaviour in physical, chemical and biological systems. *J Biosci.* 39: 177–189. <https://www.ias.ac.in/article/fulltext/jbsc/039/02/0177-0189>).

Chapter 94

A primary screening assay to characterize mobility defects in *Drosophila* model

Limamanen Phom (lim_angh@yahoo.co.in), Mohamad Ayajuddin (md.ayaj@gmail.com), Zewelou Koza (zevi7koza@gmail.com), Priyanka Modi (pmodi82@gmail.com), Nukshimenla Jamir (nukshimenlajamir@gmail.com) and Sarat Chandra Yeniseti* (yschandrays@rediffmail.com)

Drosophila Neurobiology Laboratory, Department of Zoology, Nagaland University (Central), Lumami 798627, Nagaland, India.

*For correspondence

Recommended Level: UG/PG/Research

Sub-discipline: Neurobiology/Behavioural Biology

Background:

Certain neurodegenerative disorders are frequently associated with a progressive loss of movement ability, reduced life span and age-dependent neurodegeneration. These disorders result due to exposure to environmental neurotoxins such as Paraquat, Rotenone, MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) or due to other gene-environment interaction leading to bradykinesia (slow movement), akinesia (loss of power of voluntary movement), learning and memory deficiencies, and reduced life span. The mobility defects generally are due to muscle- or neuro-degeneration. A simple and reproducible assay for mobility defects in model organisms such as *Drosophila* is useful for screening a large number of genotypes or drugs affecting locomotion of flies in a short period of time. By default, the fly shows negative geotaxis and positive phototaxis. Taking advantage of this stereotyped behaviour, it is possible to assess mobility defects. Many studies have characterized degeneration of the dopaminergic neurons by taking advantage of the negative geotaxis behaviour of *Drosophila* (Chen and Feany, 2005). Here, we describe a modified negative geotaxis assay (Botella *et al.*, 2004) in which rather than group of flies (see [Chapters 92, 96](#)), individual fly's mobility performance is assayed by giving three chances to each fly. This modification helps in quantification of mobility defects with an enhanced sensitivity, which helps in detection of mobility phenotype during early stages of disease onset.

Objectives:

Characterization of mobility defects in *Drosophila* model.

Materials Required:

A. **Flies:** Wild type and/or mutant *Drosophila* flies

B. Fly-handling materials:

- i. Whatman filter paper (No.1).
- ii. Glass vials.
- iii. Plastic tubes.
- iv. 10 mL graduated glass or plastic pipettes.
- v. Stopwatch.
- vi. Mouth aspirator.
- vii. Sponge pad (at least 2.5 cm height).
- viii. Test tube stand.

C. Chemicals:

- i. Sucrose.
- ii. **Paraquat or Rotenone solutions:** Paraquat solution is prepared by dissolving it directly in 5% sucrose solution, while Rotenone stock is prepared by dissolving in DMSO and further diluted with 5% sucrose to prepare a 500 μM Rotenone solution.

Experimental Details:

1. Collect bachelor male and female flies (see **Chapter 1**) and age for 4-5 days on normal food.
2. Place twenty-five flies in an empty food vial carrying a filter paper soaked in 250 μL of 10 mM Paraquat in 5% sucrose solution for 24 or 48 hr (Phom *et al.*, 2014) or 250 μL of 500 μM Rotenone in 5% sucrose for 5 days. Keep control flies in parallel on filter paper soaked in 5% sucrose solution.
3. For geotaxis assay, stand a graduated 10 mL pipette vertically on a sponge (**Fig. 1**) and gently drop a single control or experimental fly with an aspirator into the pipette. Quickly close the open end of the pipette with cotton.
4. Leave it undisturbed for 2 min.
5. After 2 min, gently tap the fly to base of the pipette.
6. Watch for next 12 sec and using the graduation marks on the pipette wall, record the distance each fly climbs up in 12 sec.
7. Repeat step 5 and 6 three times for each fly and determine the mean distance climbed up in 12 sec.
8. Repeat steps 3-7 for at least 15 flies for each group (control and experimental).

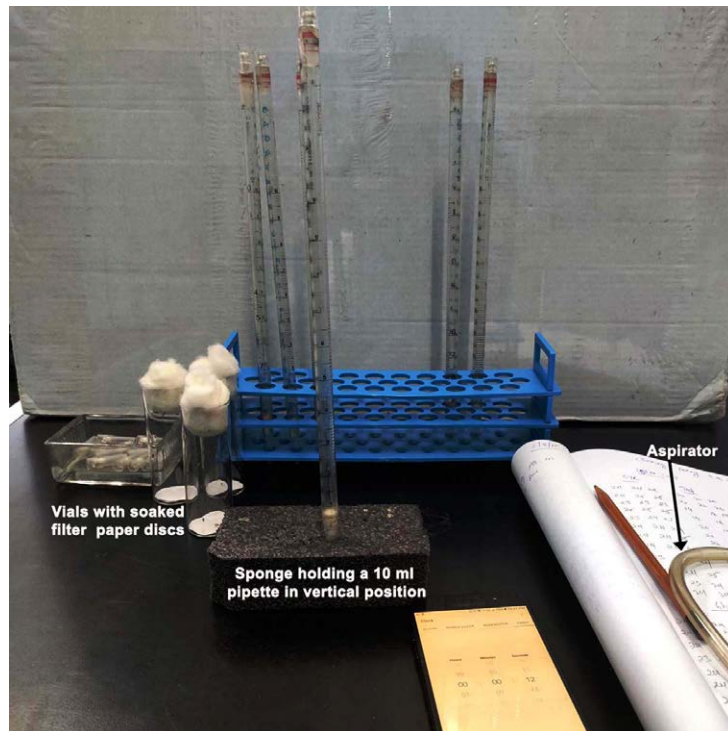


Fig. 1: Experimental set up for performing negative geotaxis assay.

Results/Observation:

Compare the mean distances climbed up by control and experimental flies to obtain information about control and treated flies' motor activity required for climbing. Is the climbing ability of flies exposed to Paraquat (or Rotenone) affected? Collect data for males and females separately. Is there any sex-dependent difference in the climbing performance?

Note: Flies with damage to dopaminergic neurons show impaired locomotive behaviour such as slowness of movement (distance/time - provides the speed of the fly's movement). They show circling movement instead of moving upwards. These are similar to bradykinesia and rigidity phenotypes seen in PD patients. The decrease in climbing speed correlates with dopaminergic neurodegeneration. It should be noted that the climbing assay provides only a primary screen for detection of degeneration of dopaminergic neurons. Further validation by other more specific methods to confirm such neurodegeneration is necessary.

Questions for self- study and Interpretation:

1. Why should defective functioning/degeneration of dopaminergic neurons lead to the mobility defects?
2. Does degeneration of neurons other than the dopaminergic ones also lead to mobility defects?
3. What other factors may cause the observed mobility defects in the experimental flies?
4. Are there other simple ways to demonstrate mobility defects in fly?
5. Can there be other behavioural phenotypes that can be used as markers for degeneration of dopaminergic neurons?

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Chapter 95

Phototaxis assay to study functionality of the visual system in *Drosophila*

Sachin G Swamy (sachings@bhu.ac.in)^{1,2}, Ankit Dhakad (ankit11.genetics@gmail.com)², Anand K Singh* (anandkumar@iisertirupati.ac.in)^{1,3}, S. C. Lakhotia* (lakhotia@bhu.ac.in)¹,

1. Cytogenetics Laboratory, Department of Zoology, Institute of Science, Banaras Hindu University, Varanasi - 221 005.
2. Interdisciplinary School of Life Sciences, Institute of Science, Banaras Hindu University, Varanasi - 221 005.
3. Present address: Department of Biology, Indian Institute of Science Education & Research (IISER), Tirupati - 517507.

* For correspondence

Recommended Level: UG/PG/Research

Sub-discipline: Genetics, Developmental Biology, Neurobiology, Behavioural Biology

Background:

The phototaxis behaviour, reflecting movement of organisms in response to light, has been widely studied in different species. Phototaxis is affected by the organism's ability to perceive the light through its photoreceptors. In *Drosophila*, the photoreceptors are primarily located in its compound eyes. The compound eye of *Drosophila* has a regular pattern of approximately 750 units called ommatidia (Cagan, 2009; also see Chapters 19, 86, 87). The phototaxis assay is based on the movement of an organism towards or against the light source, and like most insects, *Drosophila* flies also show a positive response to the light and, therefore, move towards the light source. Defects that affect the photoreceptors functions in eyes, therefore, affect the fly's phototaxis. Since, eye is a dispensable organ for survival of flies, several neurodegeneration disease models have exploited the photoreceptor optical neurons to understand the morphological and functional changes in these disorders (Mallik and Lakhotia, 2010; Nitta and Sugie, 2022). Structural defects in the photoreceptor neurons can be assessed through eye pigmentation, external eye morphology and visualization of deep pseudopupil (Arya and Lakhotia, 2007; Nitta and Sugie, 2022). However, investigations relating to functioning of photoreceptor neurons are more challenging. The two main approaches to study the functioning of photoreceptor neurons are electroretinography and the phototaxis assay. Electroretinography is an extracellular recording of a neural signal in the fly eye in response to a flash of light. This requires sensitive instruments and expertise to record the electroretinogram (Vilinsky and Johnson, 2012). Unlike electroretinography, the phototaxis assay is a rapid, cost effective, robust, and reproducible method to assay the functioning of photoreceptor neurons. Phototaxis assay helps to correlate the morphological differences in eye to the changes in functioning of optical neurons and, therefore, is frequently used for screening of genes involved in proper development or functioning of optical neurons, for examining functional damage to eyes in fly models of neurodegenerative disorders, and for therapeutic applications. This chapter describes the phototaxis assay in fruit flies.

Objective:

To study the phototaxis response of adult fruit fly.

Materials Required:

A. Fly stocks: Wild type and desired mutant flies (e.g., *GMR-GAL4* driven expanded polyQ transgene expressing flies in which the photoreceptor cells are degenerated (Jackson et al., 1998) of same age group, reared under standard conditions on the normal fly food (see Chapter 1).

B. Equipment and other materials:

- i. Stereo-binocular microscope and other common fly handling materials.
- ii. Y-maze: The Y-maze used in phototaxis assay is a Y-shaped glass tube in which all the three arms have 12 mm internal diameter; the base arm of the Y maze is ~13 cm long while each of the two arms are ~25 cm long. The base arm of the Y maze acts as the inlet for flies, while one of the longer arms serves as the light arm and other as the dark arm. The base arm of Y maze is tightly wrapped with black paper/tape to make it completely opaque to external light (Fig. 1A). One upper arm of the 'Y-maze' is prepared as dark arm by covering with a removable cylindrical opaque sleeve, which is prepared by rolling a thick A4 size paper into a cylinder (~1.5 cm internal diameter) and carefully sealing one end of the cylinder with tape, and finally tightly covering it with aluminium foil (Fig. 1B) to prevent light to pass through the paper cylinder. This one-end-open opaque sleeve is slipped down the dark-arm (Fig. 1C) during test. The other side arm is left uncovered (light arm). All the three openings of the Y-maze are closed with cotton plugs to prevent flies from escaping.
- iii. White light lamp (light source).
- iv. Soft foam piece for tapping the Y-maze.
- v. Funnel to transfer the flies in the basal arm of the Y-maze.
- vi. Dark area/ less illuminated room.
- vii. Timer.

Experimental details:

Day 1

1. Anesthetize flies of the desired genotype and collect 20 flies in individual food vials. Prepare at least five such vials for a given genotype.
2. Keep all the vials with flies at 24°C with 12 hr light and 12 hr dark periods for 24 hr before the phototaxis assay.

Note: Exposure of flies to Diethyl ether or CO₂ for anesthetization can affect their behaviour. Likewise, continuous exposure to light can also affect the sensitivity of photoreceptor neurons.

Day 2

1. Plug the three open ends of clean Y-maze with cotton plugs. Place the Y-maze flat on a table with a lamp placed close to the 'light' arm of the Y-maze. At this time, keep the lamp off.
2. Hold the Y-maze upright so that its darkened base arm faces upward with the plugged two side arms resting on a foam piece. Remove the cotton plug from the upward facing base arm and insert appropriate size funnel in the base arm through its open end.
3. Transfer the dark-conditioned flies from one vial into the base arm of the Y-maze through the funnel with gentle tapping of the Y-maze on the foam piece to move all the flies from the food vial into the base arm.
4. Remove the funnel and immediately close the base arm with cotton plug.
5. Turn the Y-maze to bring its base arm down on the foam sheet and tap gently to let all the flies fall to bottom in the base arm. Quickly cover one of the top arms with the light-proof cylindrical paper sleeve (Fig. 1B) to create the dark arm and place the Y-maze horizontal on the table with the light arm close to the light source (Fig. 1A).
6. Turn the light on immediately. Start the timer as soon as the light is turned on and wait for 30 seconds without disturbing the Y-maze.
7. After 30 seconds, count the number of flies in the light arm – these flies are positively phototactic. The flies remaining in the base and dark arms of the Y-maze are not phototactic.
8. Repeat the experiment with the other sets of flies (at least 5 replicates for a given genotype/rearing condition).

Note: The above steps on Day 2 should preferably be performed in a relatively less illuminated room so that the major source of light is only the lamp placed near the light-arm.



Fig. 1. The Y-maze for phototaxis assay. **A.** Y-maze glass tube with black tape tightly wrapped around the base arm; * = cotton plugs covering three open ends of Y-maze. **B.** Opaque paper cylindrical sleeve with one close (#) and one open end(##). **C.** Y-maze with one arm (dark arm) covered with the opaque paper sleeve.

Observations:

Calculate and compare the mean (\pm S.E.M.) percentage of the positively phototactic flies in wild type and any other genotype (e.g., neurodegeneration models in which eyes are affected) or different age groups of wild type and/or mutant flies to examine effect of mutation or age on phototactic response. Record the data as in Table 1.

Table 1. Phototactic response of flies

Sr. no.	Genotype	Replicate	Total Flies	Number of flies i Light arm	% Flies in Light arm	Mean (\pm S.E.) Phototactic flie	Mean (\pm S.E.) % Phototactic flie
1		i					
		ii					
		iii					
		iv					
		v					
Mean (\pm S. E.) of 5 replicates							
2		i					
		ii					
		iii					
		iv					
		v					
Mean (\pm S. E.) of 5 replicates							

Precautions:

1. Ensure that the Y-maze is clean and odour free.
2. Light source should be bright white light and unidirectional.
3. Enough recovery time (~24 hrs) should be provided for flies to recover from anesthetization.
4. Mutation or other conditions that affect the mobility or flight of the fly may impact their mobility and thus may influence phototactic movements.
5. The 30 seconds time for the phototaxis assay suggested here is based on the length of the base arm of the Y-maze. If the base arm of the Y-maze is longer, the test time may be appropriately increased based on pilot experiments with healthy wild type flies.

Questions for self-study and interpretation:

1. Do white eye mutant flies show a phototactic response?
2. Which specific cells in the eyes of *Drosophila* are responsible for perceiving light?
3. The *GMR-GAL4* transgene is commonly used to drive a neurodegeneration-causing transgene carrying the *UAS* promoter. Why is the phototactic response reduced or lost in these flies?

4. Would a reduced phototaxis response in *Drosophila* flies affect their mating behaviour?
5. *Drosophila* larvae do not have eyes. Do they show any phototactic response?

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Behavioral Responses to Light: Roles in Evolution

It appears then that light may have an effect on population structure and evolution not only by its influence on other behavioral systems but also by its action as a directing and orienting cue. It is clear that the elucidation of the underlying behavioral mechanisms is needed for a fuller understanding of the ecological and evolutionary aspects of this behavior. Research in each of these areas necessitates the common denominator of a usable definition, especially if the findings are to enhance one another.

(In: Rockwell, R. F., & Seiger, M. B. (1973) Phototaxis in *Drosophila*: A critical evaluation: Misunderstanding has arisen in interpretation of the measurement of phototaxis because the response is modulated by a number of factors. *American Scientist*, 61: 339-345.
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Chapter 96

Analysis of olfactory behaviour by ‘Jump-Test’ assay

Priya Dutta (priya.dutta@tifr.res.in) and Krishanu Ray* (krishanu@tifr.res.in)

Department of Biological Sciences, Tata Institute of Fundamental Research, Mumbai.

* For correspondence

Recommended Level: PG/Research

Sub-discipline: Genetics/ Neuroscience/Behavioural Biology

Background:

Sensing and discriminating different types of volatile chemicals, commonly categorized as odours, is one of the most important modalities of the animal brain and is essential for finding food and the mating partner. It is also critical for avoiding predators and harmful chemicals/gases. Thus, sensing odour is intimately linked with several essential behaviours such as fight or flight response, attraction to the opposite sex, and feeding (Chapters 90, 97). It is also associated with the learning and memory formation, which in turn helps the animal to navigate through the environment and survive. The sense of smell or olfaction is carried out through multiple steps. The association of odour molecules with the odour receptor (OR) displayed on the sensory cilia of a neuron generates an electrical response or action potential which is conveyed to the connecting neurons in the brain and then passed on through multiple other neurons to a set of motor neurons for action.

In *Drosophila melanogaster*, nearly 1200 Olfactory Sensory Neurons (OSNs) express about 62 different types of ORs that are displayed on the olfactory sensory cilia innervating inside the hollow cuticle shafts called sensilla. These are hair-like structures projecting from the surface of the third segment of the *Drosophila* antenna (Fig. 1A and B) (Shanbhag, *et al.*, 1999). Altogether, three different types of sensillae are found on the antenna - *sensilla basiconica* (senses food odours and CO₂), *sensilla trichodea* (senses pheromones) and *sensilla coeloconica* (senses food odours, water vapour, and ammonia) (Fig. 1C). Both mammalian and insect ORs are seven-pass transmembrane proteins; however, unlike the mammalian ORs which are G-protein coupled receptors, the insect ORs are ligand-gated ion channels. Functional insect ORs are heterodimeric complexes composed of a variable odour binding subunit of the ORs and a universal co-receptor, Orco or Or83b protein (Benton, *et al.*, 2006).

At the periphery, the *Drosophila* olfactory system (also see [Chapter 2](#)) consists of the OSNs, which project on the antennal lobe in the brain via the antennal nerve (AN). The antennal lobe (AL) is a bilateral structure analogous to the olfactory bulb present in the mammals (**Fig. 1D**). There are 56 neuropil regions, called glomeruli, in the AL (Twick, *et al.*, 2014). In the fly olfactory system, each OSN maps to a specific glomerulus following a 1 OR to 1 glomerulus rule. In the glomeruli, the OSNs synapse with the projection neurons (PN) and local interneurons (LNs). The LNs interconnect multiple glomeruli and the PNs, further project to the calyx region of the mushroom body (MB) and the lateral horn (LH) of the *Drosophila* brain (Wilson 2013; Twick, *et al.*, 2014). The MB and LH centres of the insect brain decide and correlate olfactory sense with that of memory and learning.

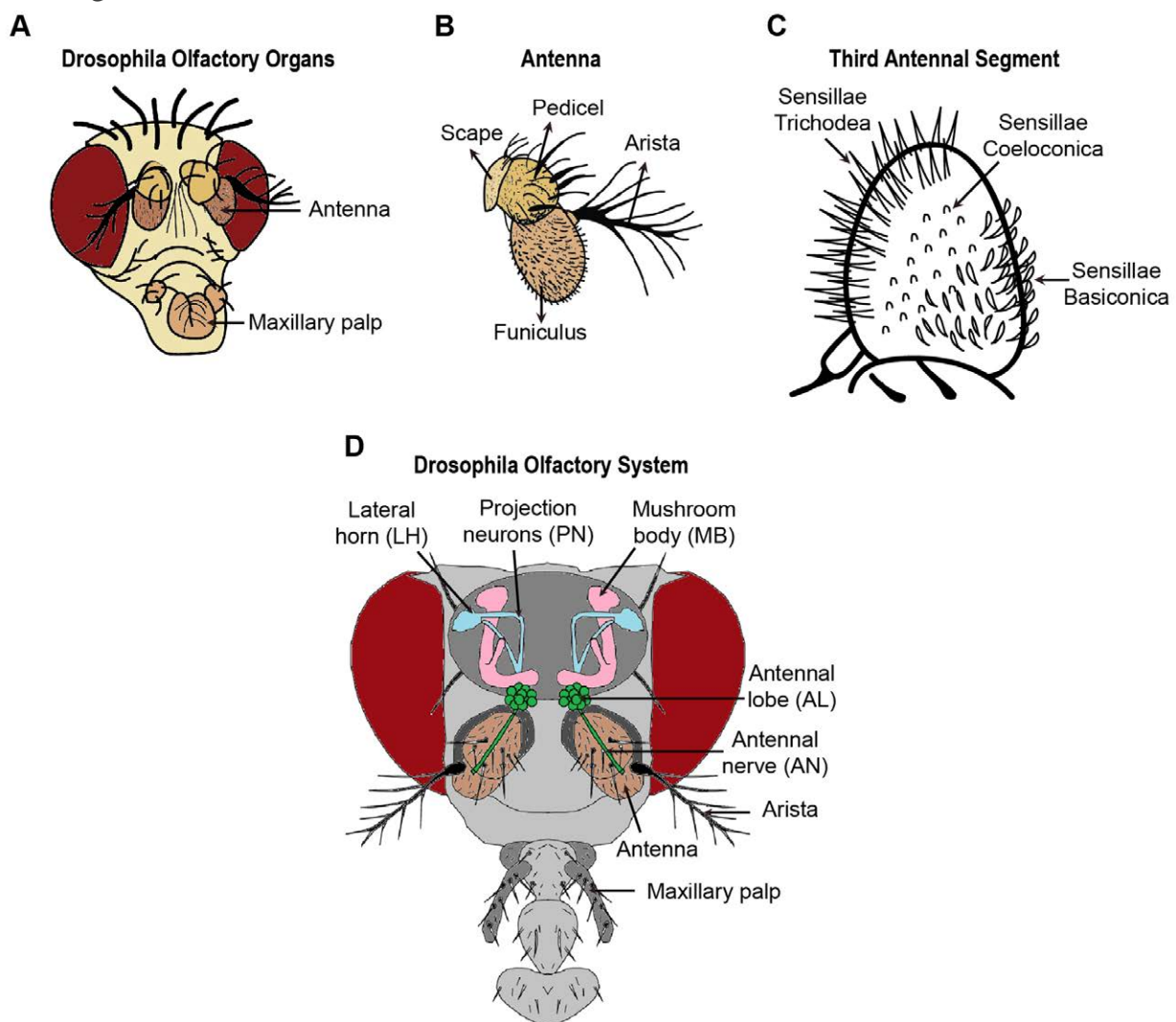


Fig. 1. Schematic of the *Drosophila melanogaster* olfactory system. **A.** Two olfactory organs, Antenna and Maxillary palp on the head of *Drosophila* adult. **B.** Four segments of antenna: Scape, attached to the head, Pedicel carrying the chordotonal/hearing organs, Funiculus housing the olfactory sensillae, and Arista. **C.** Three types of sensillae, viz. *S. Basiconica*, *S. Trichodea*, and *S. Coeloconica* found on the Funiculus or the third antennal segment. **D.** Components of the olfactory system in the adult brain.

Drosophila serves as a good model organism to study olfactory learning and memory formation because of the general similarities in anatomical arrangement and coding of the odour perception with the vertebrate olfactory system. A comparatively smaller number of ORs and OSNs present in the *Drosophila* olfactory system makes it less complex than the vertebrate system. Further, the *Drosophila* olfactory neural circuit connectome is well deciphered to study the effects of olfactory manipulation on learning and memory. Additionally, shorter life span, modest maintenance requirements and the most extensive genetic and genomic information available for *Drosophila* make it a favourable model for such studies.

One of the primary issues in understanding the olfactory system is figuring out whether *Drosophila* likes or dislikes an odour. Like other insects, *Drosophila* has a very keen sense of smell and can sense certain chemicals at very low concentrations. Some of the known chemical/food attractants for *Drosophila melanogaster* are glacial acetic acid, propionic acid, apple cider vinegar, balsamic vinegar, banana juice/powder, lemon juice, mango juice, and apple juice.

This chapter describes a simple experimental procedure, the jump test (Ayyub, *et al.*, 1990), for assaying the odour avoidance response of *Drosophila*. This assay can also be used to test the presence of repellent or 'unpleasant' chemicals in the environment. This assay, however, has some limitations. Firstly, since the 'jump' is an escape response, it can be used only to identify odours that are not liked by the fly. Secondly, defects in the motor neuron circuit and muscle function, independent of the olfactory response, also affect the jump response. Nevertheless, as the first test for odour sensing ability of the fly, the assay is effective.

Objective:

To assay the olfactory response of *Drosophila melanogaster* to potentially repellent odours.

Materials Required:

A. Fly Strain:

- i. Wild-type flies (Canton S, Oregon R)
- ii. Mutant flies (as per experimental requirement). Two of the olfactory mutants available at the Bloomington Stock Centre (USA) (<http://flystocks.bio.indiana.edu/>) are i) *BL-23129:w[*]; TI{w[+m*]=TI}Orco[1]*; ii) *BL-23130:w[*]; TI{w[+m*]=TI}Orco[2]*. Besides, stocks carrying mutant alleles for *rutagaba* and *dunce* also show altered olfactory response.

B. Chemicals:

- i. Repulsive odour- benzaldehyde or acetophenone
- ii. Attractive odour- iso-amyl acetate, ethyl acetate

iii. Diluent: mineral oil or liquid paraffin

C. Other supplies:

- i. The 'jump assay' apparatus (**Fig. 2**), which includes the jump tube (glass), aquarium pump, teflon tubing and cotton plug (adopted from Ayyub, *et al.*, 1990)
- ii. Vials containing fresh fly food
- iii. Nylon paint brush, No. 0, 1 and 2
- iv. Odour free detergent
- v. Sterilized distilled water
- vi. Tap water
- vii. Marker pen
- viii. B.O.D. incubator (kept at 25°C for rearing of flies).
- ix. Oven (for drying the glassware after washing).

Experimental Details:

1. Wash the jump assay tube with odour free detergent and dry them in a hot air oven at 25°C. Alternatively, the glassware can be heated on a flame to eliminate traces of odorants. Allow the tube to cool down before beginning the experiment.
2. Collect two to four days old flies of the desired genotype/s (wild-type or mutants) by anaesthetizing them on ice and place them in fresh fly food for 24 hr.
3. For inserting the flies into the glass jump tube, anaesthetize them on ice. Gently put a batch of five such anaesthetized flies in the jump tube using a #1 paintbrush and close the open end of the tube with a cotton plug (**Fig. 2**). Place the tubes vertically with the cotton plug at the bottom on a rack inside a humidified dark (or cardboard) box until the experiment. Leave the flies in the tube for 20-30 min to allow them to recover from cold anaesthesia. *Drosophila* being normally negatively geotactic, the flies would climb up the glass wall soon after 'waking up'.
4. To start the Jump-Test, place the jump tube with flies in a vertical position using a stand and holder as shown in **Fig. 2** with the cotton plug facing down and tapered end up.
5. Connect the tapered end of the jump tube to the exit/outflow end of the dispenser having the odour solution using a soft plastic/Teflon tube (**Fig. 2**). Connect the inflow end of the odour dispenser to an aquarium pump (**Fig. 2**).
6. To make the odour solution, prepare serial dilutions, as required, of the odour concentrate in mineral oil or liquid paraffin.
7. Tap the tube to make flies fall on the cotton plug and then climb up the walls of the jump tube. When the flies reach an approximately two-third length of the jump tube, switch on the aquarium pump for 5 sec only. The rate of airflow should be regulated to be 0.5 L/min.

8. The odour puff, if the odour is a repellent, will make the flies jump. The fraction of flies jumping would increase with the repelling quality of the odour. A few flies will remain on the wall of the glass tube even after the 5-sec puff (**Fig. 3**). Count the number of flies which hit the cotton plug 5 sec after turning on the airflow/pump.

Note:

i. If some flies hit the cotton plug before turning on the pump, then wait until they climb back onto the wall.

ii. Flies also respond to light flashes and jump spontaneously in the presence of light. Therefore, to avoid spurious jumps, the experiment must be conducted in a dark room with a red light from a red LED lamp emitting at 600-700 nm or a red lightbox used in a photographic darkroom.

iii. Keep the light source away from the table where the experiment is conducted or create a diffuse lighting condition.

9. Repeat the jump experiment with a given odour for four batches of flies. The jump score is calculated as an average of four such jump tests involving a total of 20 flies. The group score is defined as the percentage of the flies that jumped.

Note:

i. Use a fresh and clean jump tube for each batch of flies.

ii. The Teflon tubing must be cleaned before testing another odour.

iii. Always test a set of control flies (wild type) along with the experimental genotype/condition.

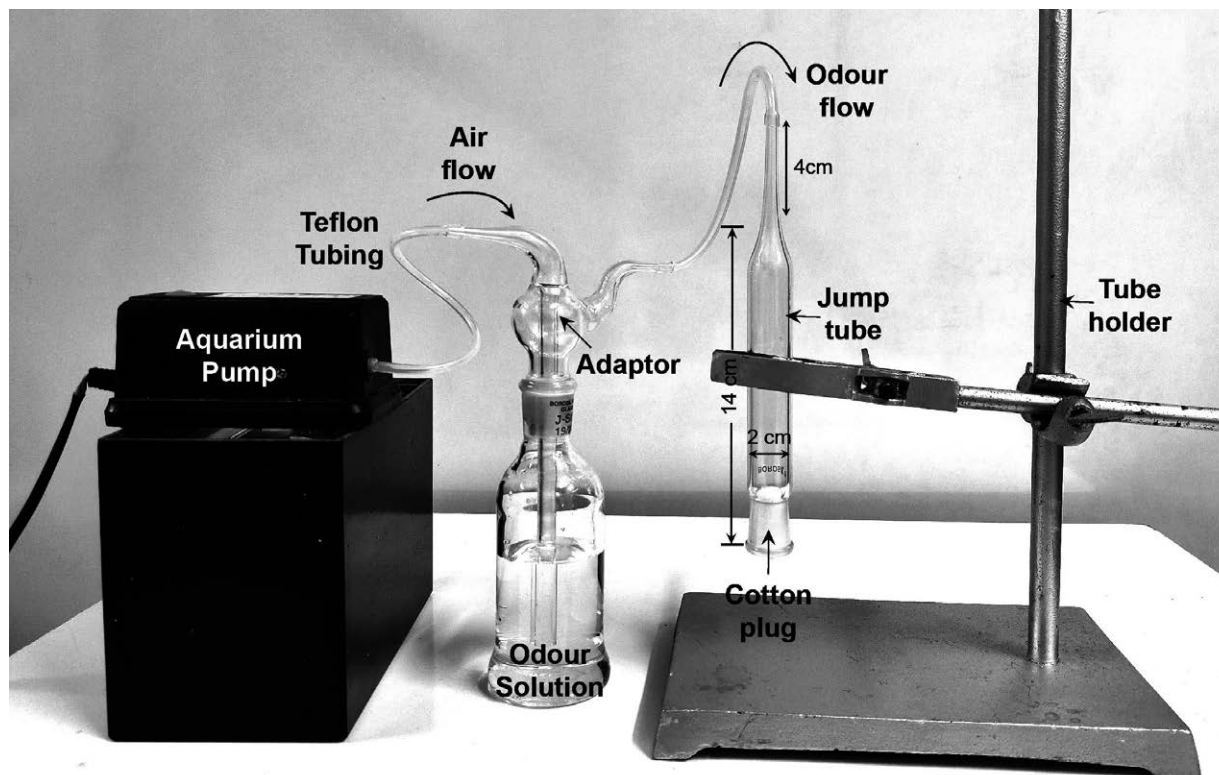


Fig. 2. The Jump-test set up. The jump tube should be 14 cm long with an inner diameter of 2 cm, while the tapered end should be 4 cm long with an inner diameter of 1 mm at the open tip.

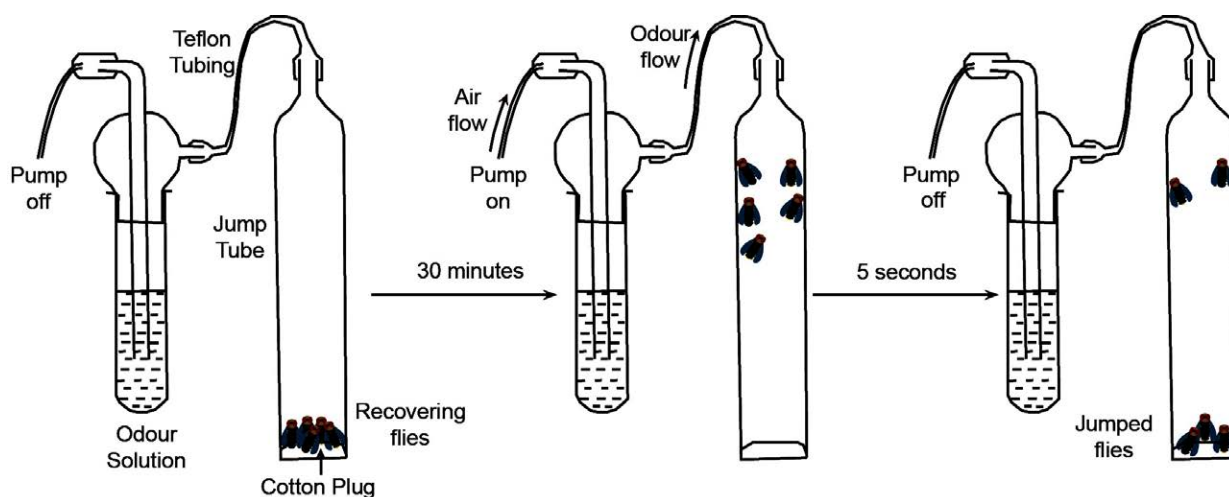


Fig. 3. The Jump-test workflow.

Results:

Count each fly which hits the cotton plug after jumping because of the 5-sec odour exposure, and indicate the total score as the **Average Percentage Response** (see the formula below) for the entire group consisting of 20 flies (4 batches of 5 flies each).

Percentage Olfactory Response = (Number of flies jumped X 100)/ (Total number of flies tested)

Collect at least four such jump data for every odour condition and calculate the statistical significance between the average values of the control/wild-type and experimental/mutant samples. Represent the result in the form of a bar graph or box plot with data spread. Some known repellent/attractant odours are listed in **Table 1**.

Table 1: List of repulsive or attractive odours and their percentage response (as published) at different concentrations for wild type *Drosophila*.

Odour	Odour Percentage Response (Mean + S.E.M)		Reference
	Undiluted	10 ⁻² dilution	
Benzaldehyde (Repulsive)	88.5 ± 3.7	81.0 ± 3.0	Ayyub <i>et. al.</i> , 1990 Helfand <i>et. al.</i> , 1989
Ethyl acetate (Attractive)	>85	67.0 ± 6.0	Helfand <i>et. al.</i> , 1989 McKenna <i>et. al.</i> , 1989
Iso-amyl acetate (Attractive)	84.0 ± 2.4		Ayyub <i>et. al.</i> , 1990
Ethanol	8.0 ± 2.0		McKenna <i>et. al.</i> , 1989
Air	5.0 ± 1.0		McKenna <i>et. al.</i> , 1989

Note: Attractive odours like ethyl acetate and iso-amyl acetate are repulsive at high concentrations.

Further questions for self-study:

1. Can olfactory response to odour stimulus vary with the age of fly?
2. Can olfactory response to odour stimulus vary between male and female flies?
3. Why does an attractive odour become repellent at higher concentration?
4. Does fasting or feeding cycles affect the olfactory response to odour stimulus?
5. Does circadian rhythm, that is the time of the day or night, affect the response to an odour stimulus?
6. Can this test be used to find if a particular volatile chemical or gas is attractant or neutral?
7. Does the response to a particular odour become less strong if the flies were exposed to the same odour again?

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Seymour Benzer's Remarkable 'Jumps' from Transistors to Phage to Flies

Like many of the pioneers of molecular biology, Benzer was originally a physicist. Soon after starting graduate school at Purdue in 1942, he entered into a secret war-related mission: improving semiconductors for use in radar. This work, done while he was still in his early 20s, also led to the development of the transistor. While working on his doctorate, Benzer (again like many renegade physicists) read Erwin Schrodinger's *What Is Life?* Schrodinger's book and a chance meeting with the geneticist Salvador Luria led the restless Benzer to Max Delbrück and bacteriophage genetics. Although such a dramatic change was risky (especially because neither Benzer nor his family had any money), Benzer felt compelled to follow his curiosity. He felt that "happiness is the pursuit of curiosity" and that "a fall from pure science is a fall from grace"

After a decade or so of phage molecular genetics, Benzer again became restless and spent 1965 on sabbatical leave in the lab of Roger Sperry, a Cal-tech neurobiologist. Benzer realized that if he wanted to unravel the genetics of behavior, he would need a simpler, more tractable model system than the vertebrates used by Sperry and his students. The intellectual remains of Thomas Hunt Morgan's fly lab and talks with Ed Lewis, who was using classical genetics to investigate how *Drosophila* developed, led Benzer to the fly. Benzer's choices of the fly as research organism and classical genetics as a tool were met with snickers from both Sperry's group and molecular biologists.

(In: Johnson, N. A. 2000. Am I not a fly like thee? Time, love, memory: A great biologist and his quest for the origins of behavior. *BioScience*, 50: 267–268
<https://academic.oup.com/bioscience/article/50/3/267/241515?login=true>)

Chapter 97

Study of food preference in *Drosophila* to test the palatability and discrimination of food

Shamprasad Varija Raghu (shamprasadvarijaraghu@gmail.com) and
Rajashekhhar K Patil* (patilsirmu@gmail.com)

Neurogenetics Lab, Dept of Applied Zoology, Mangalore University, Karnataka 574199

* For Correspondence

Recommended Level: UG/PG/Research

Sub-discipline: Genetics/Neurobiology/Physiology

Background:

Insects use their gustatory system to evaluate the palatability of food sources and infer nutritional quality. The system also helps insects to avoid harmful substances. Taste neurons are organized in sensilla that are distributed largely on the labellum at the distal tip of the proboscis and the distal tarsal segments of the legs. The *Drosophila* gustatory system provides an attractive model for the studies of taste discrimination. Similar to mammals, fruitfly *Drosophila* distinguishes different tastes and concentrations in a dose-dependent preference and avoidance behaviors. The underlying molecular mechanism shows similarities to mammalian taste detection (Freeman and Dahanukar, 2015; Chen and Dahanukar, 2020). In the current set of experiments, flies are used to demonstrate discrimination of different tastes based on their preference.

Objectives:

To study the feeding preference in *Drosophila*.

Materials Required:

1. Adult flies (wildtype and/or of the desired genotype)
2. 1% sucrose solution in water
3. 1% Asafoetida solution in water (or any other chemical that is bitter and not normally preferred by *Drosophila* for feeding)
4. Ether for anesthetizing flies,
5. Capillary tubes,

6. Brilliant blue food dye (2% in water)
7. Sunset Yellow FCF food dye (2% in water)

Experimental Details:

1. Collect 3-4 days old adult flies and transfer them to an empty vial and allow them to starve for 4-6 hr. Provide water by placing a wet filter paper at base of the empty vial.
2. Culture vials with normal fly food are prepared.
3. The blue coloured Brilliant blue food dye is mixed with 1% sucrose solution and the orange coloured Sunset Yellow FCF, 2 food dye is mixed with 1% Asafoetida solution (or any other repellent chemical) in water. One small capillary tube is filled to top with the blue coloured sucrose solution and another with orange coloured Asafoetida mixture.
4. The starved flies can be now introduced into the vial with fly food. The capillaries filled with two different mixtures are also carefully introduced into the vial from a side taking care that flies do not escape (**Fig. 1A**).
5. Allow the flies to feed on mixtures in the capillaries for about 20-30 min.
6. Anaesthetize the flies using ether and observe them under a stereo-binocular microscope.

Results/Observations:

The feeding preference of flies can be determined by examining their translucent abdomen. Flies feeding on sucrose will show blue coloration of abdomen while those feeding on Asafoetida will show orange coloured abdomen. In the current set of experiments, most of flies would show blue coloration (**Fig. 1B**) as they prefer to feed on sucrose but not on Asafoetida.

The experiment can be repeated using different types of chemicals in order to test the feeding preference of flies on different substances mixed with color codes. These experiments demonstrate preference for different types of food. The use of color in food is just to visually demonstrate the dietary preference without the need for dissecting the animals. This assay can also be used for detecting flies with abnormalities in taste perception.

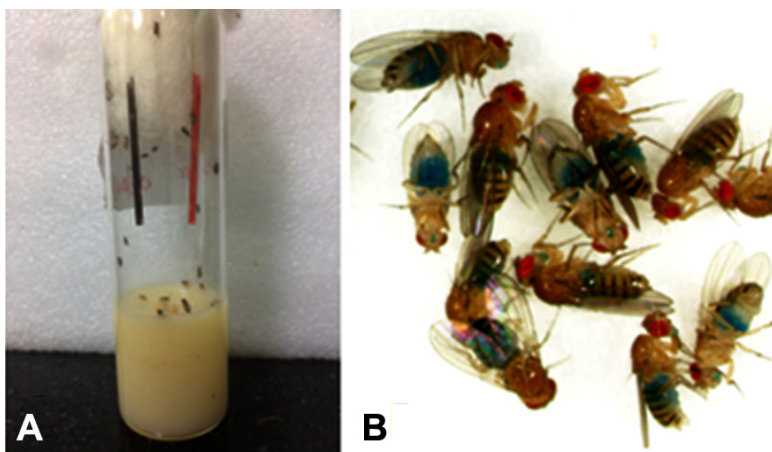


Fig. 1. A. Feeding preference setup. Capillaries filled with mixture of substance and food dyes are introduced into a culture vial along with flies. **B.** The abdominal region of flies shows blue coloration as they feed preferentially on sucrose (mixed with Brilliant blue food dye), but not on Asafoetida solution (mixed with Sunset yellow food dye).

Questions for Self-study:

1. Would the addition of color alone to food reveal the dietary preference?
2. How is the gustatory system organized in insects?
3. Is the gustatory system in insect anatomically and physiologically comparable to human/vertebrate gustatory system?
4. Do you expect to find some mutant flies that do not avoid food which is not liked by wild type flies. If yes, what could be the basis for loss of such preference?

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How any Work Got Done at all in the Fly Room?

Just a few months after the white-eye discovery—Bridges and I got desks in his laboratory, the so-called “Fly room” at Columbia, where the three of us worked together for the next 18 years. This was a room 16 × 23 feet in which there were eight desks. There was a place where we cooked Fly food, and there were usually at least five people working in there. Bridges and I practically lived in this room; we slept and ate outside, but that was all. And we talked and talked and we argued, most of the time. I’ve often wondered since how any work at all got done with the amount of talking that went on, but things popped.

(In: Sturtevant, A. H. 1957. *A History of Genetics* CSHL Press, 2001.
<http://www.esp.org/books/sturt/history>)

Chapter 98

Effect of organismal density on larval feeding rate and adult weight in *Drosophila melanogaster*

Khushboo Sharma* (khushboo.zoology@bhu.ac.in)^{1,2} and Mallikarjun N. Shakarad (beelab.ms@gmail.com)¹

ORCID id- 0000-0003-2278-3635 (KS) and 0000-0003-1134-3001(MS)

1 Evolutionary Biology Laboratory, Lab no. 205, Department of Zoology, University of Delhi 110007- Delhi

2 Cytogenetics Laboratory, Department of Zoology, Institute of Science, Banaras Hindu University, Varanasi- 221005, Uttar Pradesh

* For correspondence

Recommended level: UG/PG/Research

Sub-discipline: Behaviour Biology/Physiology

Background:

Drosophila melanogaster thrives in ephemeral habitats in nature. Many early adult life activities depend on resources gathered and stored during the larval stages. The larvae are semi-aquatic since they live, feed, and excrete within the food source. Consequently, both the quality and quantity of food depletes fast. Under these conditions, the larvae can ensure adequate nourishment through two processes: i) they can intake more food during their growth period or ii) they can evolve increased efficiency of converting the ingested food to biomass, or both. Selection of *Drosophila melanogaster* for survival at higher organismal densities favours adaptation through increased feeding rate (Joshi and Mueller, 1996) as well as improved food to biomass conversion efficiency as indicated by the increased weight of such flies at emergence than in unselected ones (Bierbaum *et al.*, 1989). Most tissues in adult flies do not have dividing cells. Larval nutrition, feeding rate and environmental conditions, therefore, affect body size/weight, longevity, and fecundity of adult flies (Robertson 1963; Handa *et al.*, 2014; Sharma and Shakarad, 2021). There is a direct correlation between the body size and larval feeding rate (Prasad *et al.*, 2001; Sharma *et al.*, 2020). Metamorphosis is dependent upon the energetic reserves accumulated during larval life and, therefore, larval stage can be an energetically vulnerable stage of development. The U-shaped metabolic curve for energy budgeting during the process of metamorphosis requires the organism to utilize the resources optimally (Merkey *et al.*, 2011).

The amount of food taken in during the active feeding stage of *Drosophila* larvae can be indirectly quantified by manually counting the number of cephalopharyngeal sclerite (black mouth hooks, see Fig. 1A) retraction movements made by the larvae per unit time

(Joshi and Mueller, 1996). Examination of the feeding behaviour helps understand the growing larvae's neuronal health regarding hunger and satiation responses (Lin *et al.*, 2019). Feeding rate can also be used to assess food choice, including the acceptability of new drug formulations.

The present method is adapted from Joshi and Mueller (1996) to estimate the feeding rate in larval stages of *Drosophila melanogaster*. It requires manual counting of the larval sclerite retractions (back and forth movement of the mouth region). Early third instar larvae are used for assessing feeding rate as they feed voraciously (Rajamani *et al.*, 2006). The adult body weight is used to find the biomass conversion efficiency.

Objective: To determine the feeding rate of *Drosophila* larvae by sclerite retraction and food to biomass conversion efficiency.

Materials required:

- A. **Flies stock:** Wild type *Drosophila melanogaster*.
- B. **Liquid food:** Prepare regular food (with maize powder, sucrose, yeast paste as in [Chapter 1](#)) but without adding Agar powder so that the food remains fluid even when cooled to room temperature. Dispense the liquid food in 9 cm and 5.5 cm diameter petri plates. Keep them covered till use.

Note: Liquid food helps in studying the sclerite retraction of larvae in petri plate (Fig. 1B)

C. Equipment and other supplies:

- i. Stereo-binocular and other fly handling materials
- ii. Microbalance (sensitivity to 1 or 0.1 mg)
- iii. Petri-plates (9cm and 5.5cm diameter) with liquid food media
- iv. Wash bottle, tissue paper
- v. Stopwatch/timer.

Experimental details:

A. Measuring sclerite retraction rate:

1. Collect 24 hrs old (late first instar stage) *Drosophila* larvae growing on regular food. Wash the 24 hr old larvae with distilled water to remove food particles and transfer them to Petri plates with liquid food media to develop further at following densities:
 - a. Low density (uncrowded): 30, late (22-24 hr after hatching) first instar larvae per 3 mL liquid media in 5.5 cm Petri plate.
 - b. High density (crowded): 200, late (22-24 hr hatching) first instar larvae per 3 mL liquid media in 5.5 cm Petri plate (Klepsatel *et al.*, 2018).
2. Incubate them at $24^{\circ}\pm 1^{\circ}\text{C}$ for 24 hrs.

Note: After 24 hrs, the larvae moult and begin the early third instar stage.

3. Wash the early third instar larvae from Low- and High-density Petri plates with distilled water, roll them on tissue towel and release them individually (one larva at a time) in a fresh liquid media Petri plate.

4. Place the Petri plate with the larva under a stereo-binocular microscope, allow the larva to acclimatize for 10 sec and then start manually recording the number of sclerite retractions during one minute.
5. Following the recording, return the larva to a fresh food vial (regular solid food) for further growth to the adult stage.
6. Repeat the acclimatization and sclerite retraction recording (steps A.4-5 above) for 30 larvae each grown under low or high density.
7. Repeat steps 1-7 above for three different sets of larvae (3 biological replicates) reared under low and high densities.
8. Calculate the average and standard deviation of each set.

B. Assessment of the food to biomass conversion efficienc

1. Collect freshly emerging flies from the food vials, prepared at the step A.5 above for crowded and uncrowded sets of larvae.
2. Sort males and females, and group them into batches of 5 unisex groups.
3. Etherize a group of 5 flies and carefully weigh the group on a microbalance
4. Weigh at least five groups of flies for each larval densi .
5. Estimate the mean and standard deviation of fly weight for each larval densit .

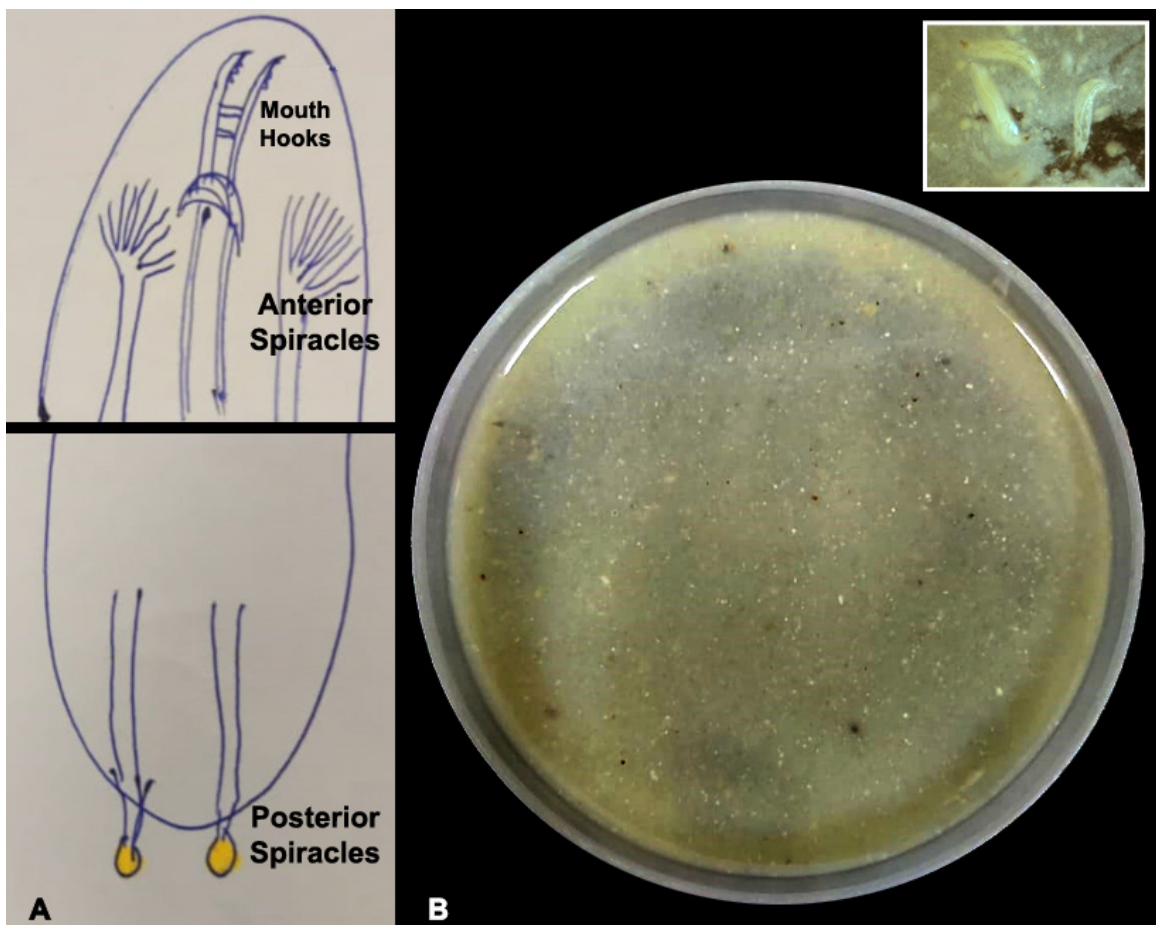


Fig. 1 : A. Schematic of anterior and posterior ends of late third instar larva to indicate the locations of black and hard chitinous sclerites or mouth hooks, anterior and posterior spiracles. B. Image of a Petri plate with liquid food medium through which larvae can be seen (inset in B).

Observation/Results:

Record and tabulate the observations on feeding rates and adult body weights as in the sample Tables 1 and 2, respectively.

Table 1 : Larval feeding rate under different organismal densities

Replicate I	Larva number	Cephalopharyngeal retractions/min			Mean±S. D
Uncrowded condition	1				
	2				
	3				
	Add more rows for recording 30 larvae				
Replicate II	Larva number	Cephalopharyngeal retractions/min			Mean±S. D
Uncrowded condition	1				
	2				
	3				
	Add more rows for recording 30 larvae				
Replicate III	Larva number	Cephalopharyngeal retractions/min			Mean±S. D
Uncrowded condition	1				
	2				
	3				
	Add more rows for recording 30 larvae				
Replicate 1		Cephalopharyngeal retractions/min			Mean±S. D
Crowded condition	1				
	2				
	3				
	Add more rows for recording 30 larvae				
Add rows for Replicates 2 and 3 for crowded condition					

Table 2. Weight of eclosed flies reared under different larval densities.

Set I	Replicate	Mean fresh weight of adult flies
Uncrowded condition	1	
	2	
	3	
	4	
	5	
Set II	Replicate	
Crowded condition	1	
	2	
	3	
	4	
	5	

Note: It is also possible to identify sex of the larvae (see Chapter 28) and assay the larval feeding rate and weight of adults on the basis of gender.

Determine if the feeding rates and fly weight vary between low-density and high-density populations of larvae and adults, respectively. Use z-test to determine, if the average larval feeding rates are different between the low and high density cultures. Use Mann-Whitney U-test to compare adult weights.

Determine if there is any correlation between i) larval density and feeding rate, ii) larval density and adult body weight and iii) larval feeding rate and adult body weight.

Question for self-study and interpretation-

1. How does crowding affect feeding behaviour
2. What effect the crowded growth condition has on fly weight and what does this indicate about the energy utilization?
3. Is the relation between larval feeding rates and adult weights different in the two sexes?

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Chapter 99

Measuring aggressive behavior in *Drosophila*

R. Sai Prathap Yadav (rachagolla.sai@learner.manipal.edu), Shreeraksha Upadhyaya (rakshu123upa@gmail.com), Pranava Swaroopa (pranava.swaroopa@learner.manipal.edu), Pavan Agrawal* (pavan.agrawal@manipal.edu)

Centre for Molecular Neurosciences, Kasturba Medical College, Manipal, Manipal Academy of Higher Education, Udupi-576104, Karnataka, India.

* For correspondence

Recommended Level: UG/PG/Research

Sub-discipline: Neurobiology/Behavioral Biology

Background:

Aggression is conserved across the animal kingdom and is crucial to ensure survival and reproduction of animals. Due to its powerful genetic toolkit, *Drosophila* has emerged as an attractive model in the last two decades to dissect neurons and genes involved in aggressive behaviors (Kravitz & Fernandez, 2015). Environmental stressors such as social isolation influence animal behavior. For example, social isolation increases aggression (Agrawal *et al.*, 2020; Kim *et al.*, 2018; Kravitz & Fernandez, 2015) in *Drosophila*, whereas, social enrichment suppresses such behaviors. Aggression is a complex behavior and its analysis requires use of sophisticated computational tools to carry out the assay in a high throughput manner. Here we present a step by step protocol to measure aggressive behaviors in *Drosophila*. The exercise described here is modified from a previously published assay (Dankert *et al.*, 2009; Dierick, 2007), and instead of using automated machine vision setup, it utilizes smartphones for recording, and freely available software for data analysis. This can be easily extended to study the effects of factors like dietary changes, starvation, access to mates etc. on complex decision making in *Drosophila*.

OBJECTIVES:

Identification of various aggressive behaviors in *Drosophila melanogaster* and quantification of male aggression under different social environments.

MATERIALS REQUIRED:

A. Equipment:

1. A 12 well aggression arena (16 mm diameter and 10 mm height for each well) with

lid, food tray and screws (**Fig. 1A - H**). The arena including top plates are fabricated as described (Dierick, 2007). As an alternative, commercially available transparent petri dishes (25 mm diameter) can be used by making a loading hole on each lid and covering it with transparent tape (**Fig. 2A**).

2. Stereo-binocular microscope.

3. CO₂ pad for anaesthetizing and sorting flies. Alternatively, flies maybe anaesthetized by keeping on a cold plate (see **Chapter 1** for methods of anaesthetizing flies by CO₂ or cold plate).

4. Weighing balance.

5. Microwave/heater to prepare apple juice agar.

6. Autoclave for fly food preparation and decontamination.

7. Timer or stopwatch.

8. Recording room with uniform light, controlled temperature (25°C) and humidity (60%).

9. Camera: Smartphone camera with stand, capable of recording at 30 frames per sec (fps).

10. Computer with at least 4 GB RAM.

11. White paper to serve as background for recording.

Note: after loading the test flies, the top lid is pushed gently to one side so that the loading holes (circles and arrows, **Fig. 1F, H**) are no longer open.

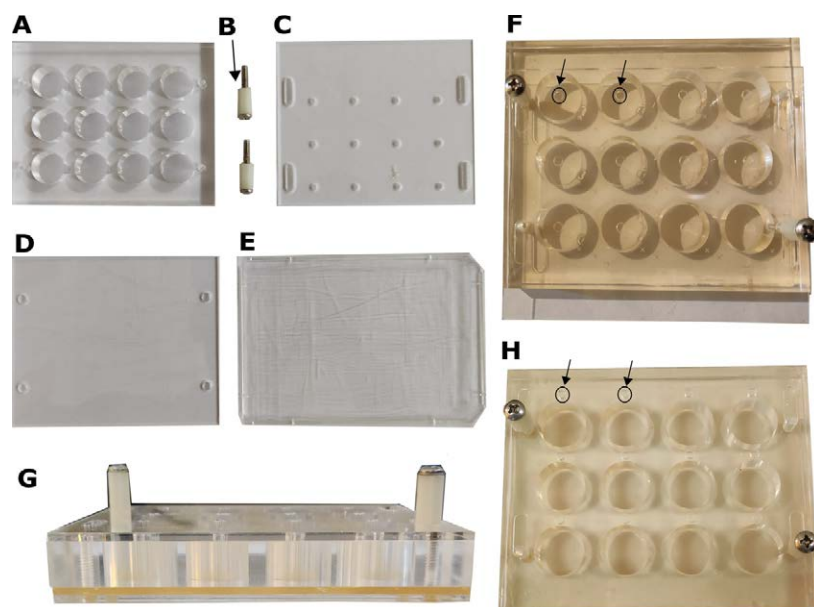


Fig. 1. The 12 well aggression arena used for assaying fly aggression. (A) Top view of the components of 12 well aggression arena, (B) screws with studs, (C) Top plate with fly loading holes, (D) bottom food plate and, (E) food holding tray, (F) Top view of the 12 well arena containing apple juice agar food (well diameter- 16 mm diameter and height- 10 mm) with arrows showing loading holes (circles) on top of each individual well, (G) Side view of the aggression arena. (H) Top view of aggression arena before recording.

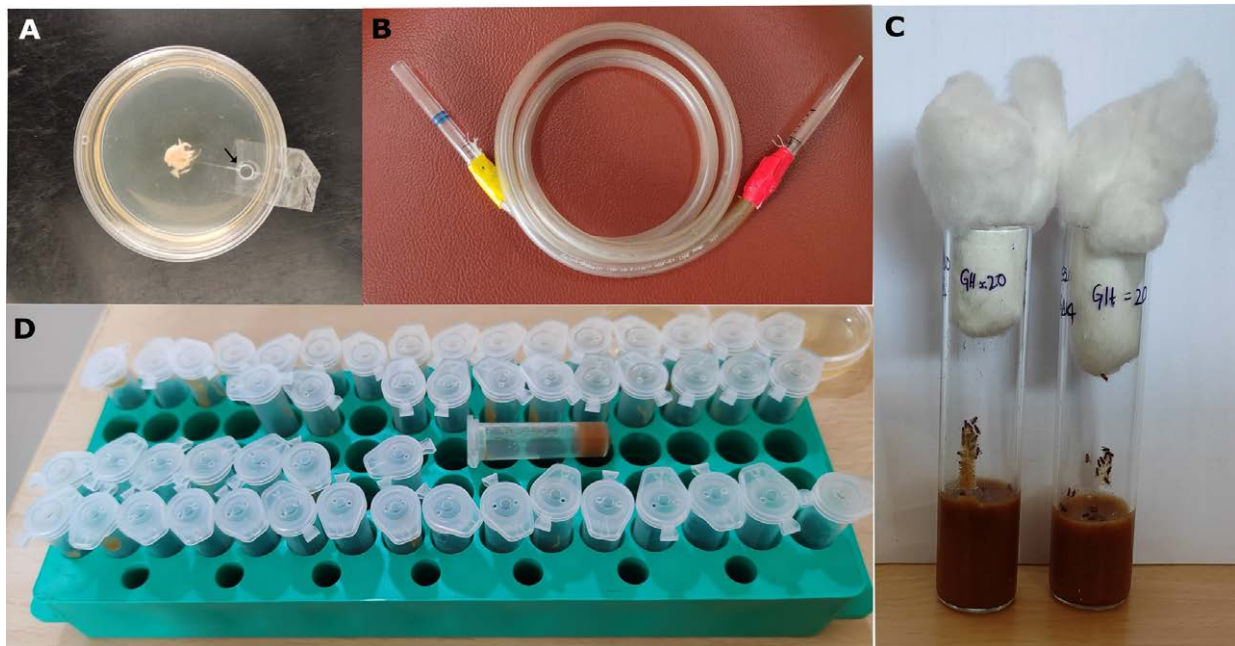


Fig. 2. Experimental setup for rearing and handling flies for aggression assay. (A) A transparent Petri dish (25 mm diameter) with apple juice agar and yeast paste at the insert a comma after bottom and a loading hole (arrow) drilled in its cap (several such Petri dishes can serve as alternative to the aggression arena described in Fig. 1), (B) Fly aspirator used for transferring flies into the arena: the rubber tube of the aspirator is covered at both ends using cheese cloth to prevent flies from getting sucked inside the tube and restraining them in the initial part of the tube, (C) Vials containing group-housed males (20 males/vial) with standard fly food, (D) 1.5 mL micro-centrifuge tubes with fine holes (to facilitate air-exchange) on top and food at the bottom are used to individually house male flies.

B. Other Materials & Reagents:

1. Sigmacote (Sigma: SL2-25 mL) to coat the lid of the 12 well arena to prevent the flies from walking on the arena ceiling.
2. Fluon/PTFE (Insect-a-slip; Bioquip or equivalent) to coat the walls of the 12 well aggression arena. Fluon makes the walls slippery and prevents flies from climbing.
3. Apple juice (without added sugars and preservatives).
4. Sugar (organic sulphurless sugar or equivalent).
5. Agar Powder, Bacteriological grade.
6. Micro-centrifuge tubes- 2 mL.
7. Micro-centrifuge tube rack.
8. 5 mL syringe needle (Dispovan or equivalent).
9. Autoclave and microwave safe gloves.

C. Flies:

1. Newly eclosed wild type *Drosophila melanogaster* males or mutants, as required.
2. Fly vials with cotton plugs (25 mm diameter X 90 mm height).

D. Software:

1. VLC (Windows/Mac/Linux compatible) for video file tagging to keep a record of identified behaviors.
2. Microsoft office excel/GraphPad Prism or equivalent for plotting and statistical analysis.
3. HitFilm Express (or equivalent software)- this is a suggested free video editing software to segment videos from 12 well aggression arena into single chamber videos.

EXPERIMENTAL DETAILS:

A. Fly stock rearing and handling:

Maintain *Drosophila melanogaster* (Canton-S) or wild caught strains under standard conditions, i.e., ~25°C and ~60% relative humidity with a 12-hr light/dark cycle on standard fly food. In the absence of a B.O.D. incubator with light cycle control, light/dark cycle can be achieved by keeping vials in a naturally lit environment and air conditioning can be used to provide constant 25°C environment. The room temperature must not rise beyond 30°C as this can reduce baseline aggression.

B. Manipulation of social experience:

- i. Create holes in the 1.5 mL microcentrifuge tubes with a syringe needle to allow air exchange to house single-housed flies.
- ii. Anesthetize 24-48 hr old flies on fly pad with CO₂ or on a metal pad cooled with ice and separate males.
- iii. Place individual male in 1.5 mL microcentrifuge tube containing 0.5 mL of standard food for a period of 4-5 days to generate single-housed.males. Group-housed flies are generated by keeping 20 males in standard sized fly vials with standard fly food for a period of 4-5 days (**Fig. 2, C,D**).

C. Aggression arena preparation:

- i. The aggression arena to record aggressive behaviors contains a desired resource, i.e. sugar and nutrient rich food which motivates flies to fight and defend their territory.
- ii. Prepare apple juice agar (2.5% w/v sucrose and 2.24% w/v agar in apple juice) as follows:

Part (A):

1. Apple juice 300 mL
2. Agar 9 gm

Part (B):

3. Apple juice 100 mL

4. Sugar 10 gm

Each part is separately heated until boiling in a microwave or on a heater to fully dissolve the contents, following which they are mixed together. Recipe makes 400 mL of food. Unused food is covered in aluminium foil and placed in refrigerator which can be reheated for reuse within the next 2-3 days.

Note: It takes longer to dissolve agar in microwave, Therefore, mix occasionally with a steel spatula and monitor continuously to prevent any spillage. Heat **Part B** for ~2 min. Allow it to cool down a bit and mix with **Part A**. Microwave the mixture again for 1 min and allow to cool down a bit before pouring into petri-dishes or the bottom food tray of the arena. Ensure that there are no air bubbles; if any are trapped, remove them with the help of a steel spatula.

iii. The bottom food tray of arena is filled with approximately 40 mL apple juice agar, covered with cheese cloth and allowed to dry at room temperature for a day. Alternatively, add approximately 4-5 mL of apple juice agar to 25 mm diameter Petri dishes.

iv. Remove the bottom plate from the food tray next day with the help of a spatula and clean any extra apple juice agar from the side using a blade. Put the 12 well arena with screwed lid on top of the food plate (**Fig. 1 B-D**).

D. Aggression Assay:

i. Place the fully assembled 12 well aggression arena (**Fig. 1D**) on a white sheet of paper to allow simultaneous recording from multiple arenas from their top view. Write identity of housing condition on paper to clearly distinguish between group- and single-housed flies in the arena. Tighten screws so that the top lid stays firmly in place.

ii. If the alternative small Petri-dishes (25 mm diameter) with loading hole on top plate covered with transparent tape are used (**Fig. 2, A**), they may also be labelled and arranged serially on a white paper sheet. Increased diameter of the Petri-dish will lead flies to move away from each other, which can decrease the frequency of aggressive encounters. Adding yeast paste in the center of arena on top of apple juice agar (**Fig. 2, A**) mitigates this effect to some extent.

iii. Gently introduce a pair of male flies (either from single- or group-housed flies) in each well of the aggression arena/Petri dish with the mouth aspirator (**Fig. 2B**).

iv. After introducing the pair of single- or group-housed flies into the aggression arena, close the loading holes by covering with pipette tips. In case of Petri dishes, quickly cover the loading holes with a transparent plastic sheet to prevent flies from escaping.

v. Flies are allowed to acclimatize to the arena for 5 min following which the video recording is started and continued for 20 min. Any smartphone or a digital camera fixed on a stand, and capable of 30 fps recording can be used. Camera view should cover entire arena from the top.

vi. Various aggressive behaviors are annotated and analysed manually by playing back the

videos on a computer. Follow the steps listed below to annotate the different aggressive behaviors using the VLC software (Windows/Mac/Linux compatible).

E. Analysis of aggressive behaviors:

The analysis of behavioral data is divided into three parts (i) video segmentation, (ii) manual annotation, and (iii) plotting and statistical analysis. These are described below:

(i) Video segmentation:

We recommend using Hit Film Express, a free video editing software, for segmenting videos into individual arena files, Follow these steps:

- a. Import the video into the software and use the ‘Composite Shot’ feature to resize. Position the video such that only one arena is visible in the frame.
- b. Apply ‘Black and White’ effect. This makes the video annotation part easy by allowing easier differentiation between the flies and the background.
- c. While exporting the video, ensure that the output videos retain the same frame rate as the original recording (30 fps) and the bit rate is sufficient i.e. not much lower than the input bit rate for the selected video dimensions. Lower bit rate will cause deterioration in the video quality.

(ii) Manual annotation:

Aggressive behaviors like lunge and chase should be precisely identified. Lunge is defined as a fly raising its forelegs and snapping downward and forward on its opponent (an example of a lunge in slow motion, can be seen at <https://www.cell.com/cms/10.1016/j.cub.2007.12.052/attachment/1dcb6cc9-2e8e-4566-866d-5326238e8509/mmc2.avi>; Hoyer et al., 2008). Chase is defined as one fly running after the other. During manual annotation segmented videos are played back on computer and annotated by the experimenter either manually or with help of a video tagging software. It is recommended that each annotated video is cross-checked by a second experimenter to minimize individual bias. Steps for using software to annotate video files are described below:

Video tagging using VLC

Download VLC extension “Time”(v3.2 as tested) from <https://addons.videolan.org/p/1154032/>.

- a. Install the extension to VLC and restart the application.
- b. Open the segmented video in VLC media player and open the extension menu from the “view” tab (**Fig. 3 A,B**). This may be configured to fit the conditions.
- c. Use an excel spreadsheet to simultaneously record your observations.
- d. The spreadsheet should list: time stamp of the start of an observation, behavior type (Lunge or Chase) and duration of the behavior in milliseconds.
- e. Use keypress ‘E’ to go ahead frame by frame.

Note: VLC does not support going back to the previous frame but manually moving the slider can achieve this.

(iii) Statistical analysis:

Data generated from aggressive encounters are usually non-normally distributed. Therefore, use non-parametric tests for statistical analysis with appropriate post-hoc correction e.g., Mann-Whitney U test. Statistical analysis of behavioral data can be performed using Prism 8 (GraphPad Software) or Microsoft excel. See (Agrawal *et al.*, 2020) for examples.

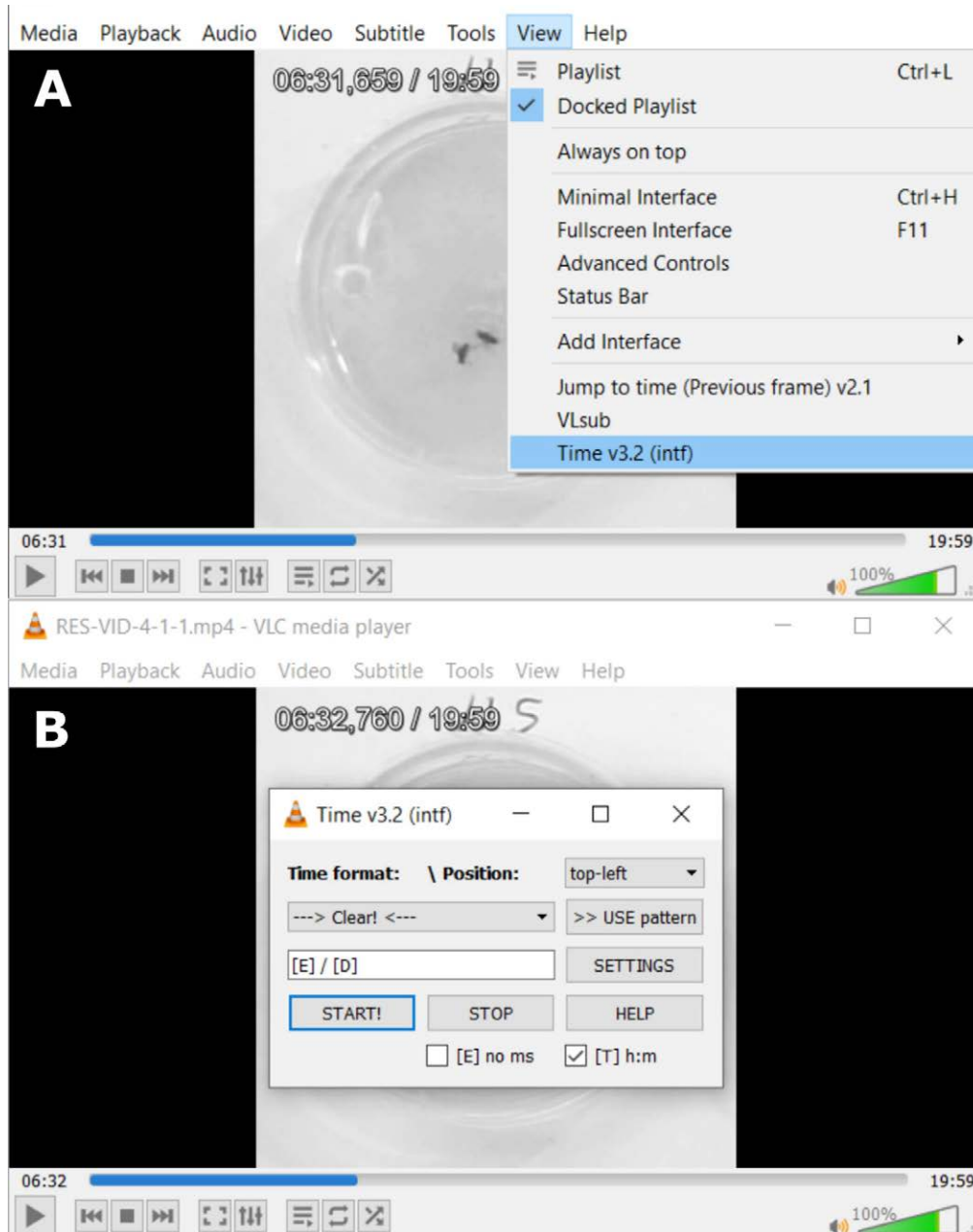


Fig. 3. VLC interface showing steps for video tagging to aid in manual annotation: (A) Configuration for selection of time extension, (B) VLC Interface showing suggested configuration.

QUESTIONS FOR SELF-STUDY AND INTERPRETATION:

1. Would aggressive behavior be different between male and female *Drosophila*?
2. Single housing (Fig. 2 D) for 4-6 days is known to increase aggression in males compared to

group housing (10-20 males/vial, **Fig. 2C**, also see reference (Agrawal *et al.*, 2020). What will be the effect of varying the duration of social isolation vs. social enrichment on aggression? Will longer duration of social isolation increase aggressive behaviors?

3. What will be the effects of varying diet components, e.g., starving flies for a few hr to a day by providing only agar lacking any sugar or nutrients before conducting aggression assay?

4. Would starvation increase aggression by increasing ‘motivation’ to fight for the coveted food resource? If yes, what duration of starvation will be sufficient to motivate the flies e.g., 6 hr, 12 hr, 24 hr etc?

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Chapter 100

Measure of flight motor activity as a function of ageing

Steffy Manjila (steffybm@gmail.com) and Gaiti Hasan* (gaiti@ncbs.res.in) National Centre for Biological Sciences, Bengaluru 560065

* For correspondence

Recommended Level: PG/Research

Sub-discipline: Neurobiology/Behavioural Biology

Background:

Motor control is essential for any mobile organism to survive and interact with the external environment. For flying insects, motor control is required for flying, walking, jumping, feeding and mating, apart from other more advanced behaviours such as grooming and aggression. Motor control requires the central nervous system to integrate different sensory inputs and convey this information to the relevant neural circuit for execution of motor function through motor neurons and muscles. Neural circuits that co-ordinate rhythmic motor actions like flight or walking are called central pattern generators. Many motor functions undergo degeneration with age. Measurement of *Drosophila* flight ability is an easy behavioural assay that can be performed once a week over three to six weeks. Appropriately aged fresh flies need to be used for each flight test. Measurement of flight time provides a direct experimental readout of how ageing can affect motor function. It can also help quantitate the process of muscle degeneration and neurodegeneration. (This experimental setup is adapted from Manjila and Hasan (2018).

Objectives:

To compare flight times of differently aged flies of the two sexes

Materials Required:

- A. A wild type fly strain
- B. Glass bottles and/or glass vials with fresh *Drosophila* medium available three times a week.
- C. Plastic tray of approximately 22 x 18 x 5 cm.

- D. Basal half of a Petri dish of ~9 cm diameter with its inside covered firmly with filter paper.
- E. Tethers (5-10) made of stainless steel rods (diameter 0.2 cm, length 6-8 cm) attached to one end of a stainless steel wire (diameter 0.01 cm, length 2-3 cm). Super glue or solder is used to fix the wire to the rod.
- F. Polystyrene foam of approximately 10 x 6 x 5 cm
- G. One glass slide
- H. Round hair brush, size 4 (Camlin, series 66, size 4)
- I. Ice
- J. Transparent nail polish

Experimental Details:

1. Place the Petri dish base with filter paper attached to its bottom on ice in a plastic tray so that the filter paper is facing towards the investigator. Make sure that the filter paper edges are not touching the ice. Keep the Petri dish on ice for 10 min before using it for anaesthetising the flies.
2. Add 5-6 either male or female flies of the desired age separately into an empty glass vial. Flies are transferred to the empty vial by tapping the vial with flies on a pounding pad so that flies are located away from the cotton plug and are at the bottom of the vial. Following the tapping, the plug is removed quickly and the vial is inverted rapidly onto a fresh empty vial, tapped hard so as to transfer all the flies into the empty vial which is closed immediately with another cotton plug to prevent flies from escaping. Flies in the empty vial are then cold-anaesthetized by keeping the vial in ice for 5 min. Do not use carbon-dioxide or ether for anaesthetizing flies since they affect the function of the nervous system and can thus alter the behaviour.
3. Gently tap the empty vial with flies on ice after 1 min and make sure that all flies are at the bottom of the vial. Once anaesthetized, all flies will be immobile and will remain at the bottom of the vial.
4. Once the flies are fully anaesthetised (within 5 min; it is important that flies are not kept on ice longer than 5 min), gently place the flies with a fine brush with their dorsal side up on filter paper in the cold Petri dish.
5. Add a tiny drop of nail polish (~10 μ L) onto a glass slide at room temperature, (nail polish dries up faster on a pre-cooled glass slide). Take a very small drop of it (~1-2 μ L) on the tip of the tether (0.7-1 mm thin wire) by gently dipping its tip in the drop of nail polish.
6. Using a very fine brush in your left hand, gently hold the fly above its wings on the abdomen and hold the tether in your right hand. Gently place the tether on the neck in such a way that the drop of nail polish touches the junction of head and thorax. This position of the tether is important to ensure that the body and head of the fly are not tilted. It is important that the nail polish drop is of the optimum size, as bigger drops

might cover the eyes or the sensory bristles on the thorax and thus affect flight durations. Tether a maximum of 5-10 flies as a batch. Once every fly is tethered, fix the tethers to a polystyrene foam in a row.

7. Keep the polystyrene foam with tethered flies in a place with even lighting and a temperature of 25-30° C. Once all flies recover from anaesthesia (~5 min), touch the legs gently using a brush to stop them flying spontaneously. Keep a stop watch ready, give a gentle mouth blown or automated air puff to all the flies (5-10) in a batch and immediately turn on the stop watch. Observe and record flight duration for all the flies in a batch. If tethered correctly, young flies (3-5 days) will fly (rapidly move their wings) continuously for 5-15 min or more. Since the flies are tethered on the steel wire, they cannot fly but would still move their wings as during real flight.

8. Note down the time at which each fly stops the rapid wing movements (i.e., stops 'flying'). This is the flight duration of a single fly. Some flies may continue flying beyond 15 min. Their flight time can be recorded as 15 min. The flight duration can be monitored either manually or by recording a video using, for example, a smart phone camera.

9. A minimum of 30 flies (of either sex) should be tested for each age group in batches of 5-10. Males can be mated or unmated because the physiology of mated and unmated males does not change discernably. However, in case of females it is important to separate unmated females from mated females and test them separately. It is known that mated females expend more energy towards laying eggs resulting in a trade-off between flight and reproduction.

Observations:

1. First optimize the ability to measure flight time in young (3-5 day old) flies. Record flight times of batches of at least 7-10 young flies and calculate the average flight time. Plot the data as average flight time per batch of 7-10 flies. The flight protocol is working well when the average flight time of the 3-5 day old wild type fly batches is at least around 6-7 min.

2. For the ageing experiment, tabulate age of flies, number of flies tested and the flight time of individual flies. Calculate average flight time and standard error of mean and plot the graph with age of the flies on the X-axis and average flight durations on the Y-axis.

3. The ageing experiment can be carried out separately with males and females to see if there is a difference in age and motor coordination between the two sexes.

Questions for self-study and interpretation:

1. Do other motor behaviours like walking or jumping show change with age?
2. Why do some flies fly for longer than others even though they are all of the same age?
3. Can nutritional inputs during larval development alter the average flight duration?
4. Does flight duration change with ambient temperature?
5. What happens to flight if sensory bristles on the thorax, legs or the wing are hindered?

6. Provide an explanation of your interpretations/findings.

References and further reading:

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Associated video: <https://bio-protocol.org/e2742>

Chapter 101

Drosophila courtship behaviour

Zevelou Koza¹ (zevi7koza@gmail.com), Abhik Das¹ (dasabhik91@gmail.com), Beyau M Konyak¹ (beyaukonyak45@gmail.com), Limamanen Phom¹ (lim_angh@yahoo.co.in), Mysuru Siddaiah Krishna² (malerkrish@yahoo.com), Shridhar Narayan Hegde² (malerhegde@yahoo.com), and Sarat Chandra Yeniseti^{1*} (yschandrays@rediffmail.com)

1. *Drosophila* Neurobiology Laboratory, Department of Zoology, Nagaland University (Central), Lumami 798627, Nagaland

2. Department of Studies in Zoology, Manasagangotri, Mysuru University, Mysuru 570006 Karnataka

*For correspondence

Recommended Level: UG/PG/Research

Sub-discipline: Behavioural Biology/Genetics

Background:

Courtship behaviour in *Drosophila* is an innate behaviour displayed upon encountering a suitable partner. Visual, auditory, and chemosensory signals/ pheromones play great role as courtship stimuli for the complex series of well characterized behaviours of males and females, culminating in their successful copulation. The courtship behaviour is easily quantifiable. It involves a sequential action of display allowing a female to evaluate the 'fitness' and conspecific status of the male partner. The courtship behaviour thus serves to strengthen the reproductive barriers between closely related *Drosophila* species. In the natural habitat, the male fly is believed to be primarily responsible for reproductive isolation and the female for sexual selection (Spieth, 1974). Courtship behaviour in *Drosophila* is governed by genes such as fruitless (*fru*) and double sex (*dsx*). Therefore, study of courtship behaviour has been widely used to explore the genetic basis and the role of environmental factors on fly behaviour, as several human diseases are modelled in *Drosophila*. The courtship behaviour study in flies can also help investigate sexual dysfunctions (SD) reported in several neurodegenerative disorders like Parkinson's disease (PD), Alzheimer's disease (AD) and other dementias. Taking advantage of the fly model, it would be possible to decipher genetic basis of SD, which can help in identification of interacting partners of disease causing genes and developing therapeutic strategies for human disorders.

Objectives:

1. Examining the courtship behaviour in *Drosophila* (these can be extended to study changes in courtship behaviour due to different stimuli like altered light and temperature conditions and neurodegenerative conditions).

Materials Required:

1. **Fly Strain:** Wild type flies (and mutant strains as desired)

2. **Reagents:**

- i. Ether
- ii. 70% alcohol
- iii. Fly culture media

3. **Equipment:**

- i. Mouth Aspirator
- ii. Mating chamber (4x4 cm glass cavity block with inner circular diameter of 3.3 cm or a small Petri dish with transparent cover)
- iii. Filter paper
- iv. Test tubes and Vials
- v. Timer/Stop watch
- vi. Camera or smart phone for video-recording (optional)

Experimental Details:

In *Drosophila* courtship behaviour (**Fig. 1**, also see **Chapter 104**), the male upon sensing a female, orients his body towards her (orientation), approaches her and taps her abdomen (tapping). While the female moves around, he follows and enthrals her with a species-specific love song, which involves extending out one or the other wing accompanied by vibration (wing vibration). When the female gets stationary, the male circles around her (circling). After a few seconds or minutes of the above activity, he extends his proboscis and licks the female's genitalia (licking) and attempts to copulate her by bending his abdomen (attempted copulation). If the female is receptive, she accepts his approaches by spreading the wings to accommodate the male and thus successful copulation takes place (Spieth, 1974). Courtship is an innate behaviour since male flies raised in complete isolation from egg to adult also perform the entire courtship act once they come across a virgin female (Greenspan and Ferveur, 2000). Several factors like visual, acoustic and pheromonal stimuli play major roles in triggering the courtship. Steps in setting up the experiment to observe and measure the courtship behaviour quantitatively are described here for understanding the sequential actions of display of fly courtship. This set up also allows one to analyse the effect of changed environmental, genetic and physical conditions on the behavioural parameters.

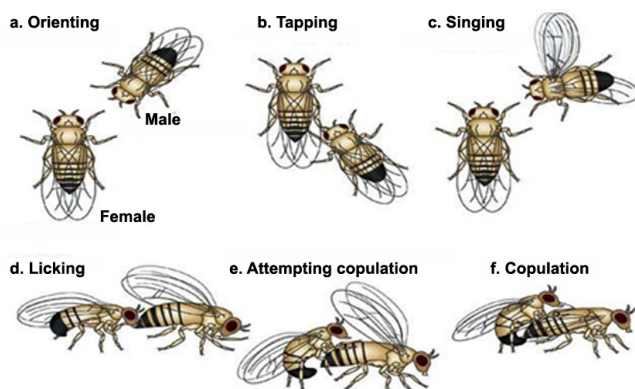


Fig. 1. Courtship behaviour of *Drosophila melanogaster*. (Adapted with permission from Sokolowski (2001).

Experimental Steps:

1. Collection of virgin and bachelor flies For collecting unmated male and female flies, existing flies in the culture vials are removed. The flies that eclose within the next 2 hr are collected and lightly anaesthetised for separating males and females. They are aged 5 days in same-sex groups of 25 in separate food vials.

2. Courtship Observation: After 5 days, transfer a single unmated male into a transparent cavity glass block or a small transparent (glass or plastic) Petri dish (this would serve as the mating chamber) with the help of a mouth aspirator and allow the male to acclimatize in the chamber for one minute. Flies are not anaesthetised since that would affect their behaviour. Next, carefully transfer an unmated female into the same mating chamber using mouth aspirator. Immediately, start the timer and continue to observe each pair for 10 min.

Result/Observations:

1. Observe and record the following courtship parameters for males and females in each pair in a tabular form (Tables 1 and 2) for quantifying the following behavioural parameters.

A. Parameters of Male courtship to be observed and noted (Spieth, 1974; Hegde and Krishnamurthy, 1979):

i. Non-sexual encounter: Male and female come cross each other but there is no sexual encounter. (Shaltiel-Karyo *et al.*, 2000).

ii. Courtship latency: Time between introduction of male and female together into mating chamber until orientation of male towards the female.

iii. Tapping: Male lifts, straightens foreleg, and strikes downward against the other individual; almost invariable behaviour at the start of courtship.

iv. Vibration: Male expands the wings laterally from the resting position and rapidly moves them up and down.

v. Scissoring: Male repeatedly and rapidly extends both wings horizontally outward and back to the resting position.

vi. Circling: Male periodically circles around the female, facing her as he moves, often from rear to front and back; sometimes completely around female.

vii. Licking: Male opens labellar lobes, extends proboscis, and licks female genitalia.

viii. Attempted copulation: The number of times the male attempts to copulate or mount the female.

ix. Chasing: The male fly chases the female for a few seconds or minutes until he is able to mount on the female.

x. Mating latency: Time between introduction of male and female into the mating chamber until initiation of copulation between them.

B. Parameters of female courtship to be observed and noted (Spieth, 1974; Hegde and Krishnamurthy, 1979):

i. Ignoring: when courted, the non-receptive female sometimes simply keeps on with whatever activity she has been engaged in and apparently ignores the male's actions.

ii. Extrusion/ Extruding: Female extends and elongates tip of abdomen, thus exposing articulating membranes around genital sclerites and simultaneously directs tip of her abdomen toward male face, usually causing male to turn quickly away and engage in cleaning behaviour.

iii. Decamping: Female breaks contact with courting male by running, jumping, or flying.

iv. Kicking: Female kicks vigorously backward with hind legs, striking face of the male when he courts at her rear.

v. Fluttering wings: Female rapidly flutters wings in movements of small amplitude; often occurs when male initiates tapping action.

Note:

a. Pairs which do not mate within the stipulated time can be recorded as not mated and replaced by a fresh pair.

b. After each observation, clean the mating chamber thoroughly with 70% alcohol and air dry prior to introduction of a fresh pair.

Table 1. Courtship behaviour displayed by male in each pair:

S. No. of mating pair	Non-sexual encounter	Clapping	Tapping	Scissoring	Vibration	Circling	Attempted copulation	Licking	Chasing	Mating Latency

Table 2. Courtship behaviour displayed by female in each pair:

Sl. No. of mating pair	Ignoring	Extruding	Decamping	Kicking	Wing flutterin

Questions for self-study and interpretation:

1. What factors influence courtship behaviour in *Drosophila*?
2. How is courtship behaviour different in male and female fly?
3. What genes govern/ regulate courtship behaviour in fly and how do they act?

4. Would dopaminergic neurodegeneration affect courtship behaviour?
5. How can study of courtship behaviour in flies be used to understand the courtship behavioural deficit reported in human diseases like autism spectrum disorder?
6. How would a male of one species behave when placed with female of a related species?

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Link to video on Courtship behaviour:

https://drive.google.com/file/d/11nWafOnNIVSUwh0qN3kTtl_cLO18aIP0/view?usp=drive_web

Courtship Behaviors evaluate Potential Mate

The behaviors prior to copulation convey visual, olfactory, gustatory, auditory, and tactile cues that allow males and females to recognize and evaluate potential mating targets and drive the progression of courtship from one behavior to the next. A male will not execute these courtship behaviors (especially the later steps) unless he senses a potential mate. Indeed, throughout the animal kingdom there is a strong linkage between the percept of a potential mate and the display of male sexual behavior. The nature of the coupling between these two processes is largely unknown.

(In: Yufeng, P., Robinett, C. C. and Baker. B. S. 2011. Turning males on: activation of male courtship behavior in *Drosophila melanogaster*. PLoS One. 6: e21144 <https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0021144>)

Chapter 102

Assaying mating rhythm in *Drosophila melanogaster*

Sukriti Mishra¹ (sukritimishra19phd@cup.ac.in) and Shahnaz Lone^{2*} (slone@gitam.edu; lonesr@gmail.com)

1. Department of Zoology, Central University of Punjab, Bhatinda 151001
2. Department of Biochemistry & Bioinformatics, The Gandhi Institute of Technology and Management (GITAM, Deemed to be University), Visakhapatnam. 530045

* For Correspondence

Recommended Level: PG/Research

Sub-discipline: Neurobiology/Behaviour

Background:

The mating behaviour of *Drosophila* is a complex process involving various courtship rituals. Male flies engage in behaviours such as tapping, contacting, singing species-specific courtship songs, and vibrating their wings, all aimed at achieving reproductive success (Villella and Hall, 2008). Females assess males through sensory cues, and virgin females can exhibit rejection behaviours to resist courtship attempts. Once a male is accepted, the female becomes receptive for copulation. Successful copulation renders mated females unreceptive to further attempts while increasing their rate of egg-laying (Kubli, 2003).

The copulation assay in *Drosophila* is a widely used method for studying reproductive behaviour and fertility. This assay involves observing and recording the mating behaviour of male and female flies, including parameters such as copulation latency, copulation duration, and mating frequency (see Chapters 101, 104). It provides valuable insights into the mechanisms underlying reproductive success and allows for investigations into the effects of genetic, and environmental manipulations on mating behaviour.

Reproductive fitness requires organisms to be aware of availability of the opposite sex and to segregate time windows for mating and other daily activities. Accordingly, the mating activity in *Drosophila melanogaster* displays peaks and troughs in mating rhythm (also see Chapter 88) to ensure optimal conditions for timing and frequency of female receptivity during the circadian cycle (Sakai et al., 2001). The mating rhythm is entrained to the light-dark cycle and can persist even under constant conditions such as constant darkness. However, like locomotor activity, constant light can lead to arrhythmic mating. Fruit flies exhibit a circadian mating pattern, with a peak in mating activity during the daytime, and a decrease during the evening, close to lights-off time. However, in clock mutants such as *per⁰* or *tim⁰*, the mating rhythm becomes arrhythmic. Additionally factors like environment, age, mating experience, genetic composition, and social interactions

also influence the mating behaviour of *Drosophila* (Jacobs, 1960, Hall, 1994; Krupp et al., 2008). Thus, the circadian clock intricately regulates fitness of the organism.

Unlike sophisticated locomotor recording apparatus, study of the mating rhythm does not require complex equipment, making it a simple assay for demonstrating circadian rhythm. To understand the role of the circadian clock in mating, it is recommended to conduct the mating assay under Light-Dark (LD) cycle (12:12 LD), where the lights-on phase begins at 8AM (ZT0) and lights off at 8PM (ZT12). Zeitgeber time or ZT refers to a standard 24-hour notation of the phase in an entrained state; accordingly, ZT0 indicates the beginning of day or light phase, while ZT12 refers to the beginning of night or the dark phase.

Objective: To study mating rhythm using *Drosophila melanogaster*.

Materials Required:

1. Wild type flies (Canton-S or any other wild type strain)
2. BOD incubator with light control
3. Fly food vials/bottles and other fly handling materials
4. Glass vials (preferably 95 x 25 mm)
5. Mouth aspirator for transferring flies
6. Timer/stopwatch

Experimental Details:

A. Fly cultures:

To maintain flies and to obtain healthy progeny for the experiment, place four female and three male flies together in each food vial and label. Transfer flies into the vial gently using a fine hairbrush to minimize stress or injury. Prepare 15-20 such vials to obtain large number of progeny flies. Alternatively, 5 food bottles with 10 female and 7-8 male flies in each can be prepared.

B. Preparation of flies for experiments:

1. Collect freshly eclosed 120-130 and sexually naive (i.e., flies without any previous sexual experience) male and female flies in separate food vials. The number of flies in each vial should not exceed 30 males or females. Use carbon dioxide or ice to anaesthetize the flies and separate males and females; alternatively male and female pupae can be identified and kept in separate vials/bottles as described in [Chapter 1](#).
2. Maintain flies under a light-dark (12:12 LD) cycle, with lights on from 8 AM and lights off at 8 PM at constant temperature (25°C) and steady humidity levels.
3. Age flies for 8 days for them to become sexually mature.

Notes:

- i. The light intensity during the light phase should be ~250-500 Lux, while the dark phase should have 0 Lux.
- ii. For maintaining humidity, place a 27 x 35 cm plastic tray (for a medium size BOD incubator; the tray size may be adjusted depending upon size of the incubator) filled halfway with water in the incubator.

C. Mating Rhythm Assay:

1. At ZT0 (8AM) on day 9 (when lights are turned on), aspirate 10 males and 10 females separately into an empty vial and keep for 10 min to allow them to acclimatize to their new environment. Make 5-10 such vials for a single experiment.
2. After 10 min, mix the males and females in a single vial (without food), and repeat this exercise with the other 5-10 vials prepared in step 1 to generate replicates of each time (ZT) point.
3. To facilitate easy visualization of the pairs, arrange the vials horizontally on a white paper. Record the number of copulating male and female pairs during the 30 min period. Calculate the Copulation Frequency (Observed number of copulating pairs/10) for each vial.
4. Calculate the Mean (+ S.E.M.) Copulation Frequency using the data generated from the above 5-10 replicates.
5. Steps 1-4 above are repeated with different sets of male and female flies at 4 hr intervals (i.e., at 12 noon (ZT4), 4 pm (ZT8) and 8 pm (ZT12) on day 9) during the light phase of LD cycle.

Notes:

- i. This experiment may be carried out by groups of 2 or more students so that for a given time point, 5-10 replicates (5-10 sets) of the above steps of LD flies can be examined in parallel.
- ii. This protocol is simplified for classroom experiments; for research purposes, copulation frequency should be measured at 2 hr intervals on LD/LL cycles of 24 hr.

D. Observations and Statistical Analysis:

1. Tabulate the Mean Copulation Frequencies from the above experiments (as in the template in Table 1).
2. Is there any evidence for copulation frequencies being different at some ZT time points?
3. Determine the statistical significance of difference between copulation frequencies of different time points through appropriate statistical test (either an analysis of variance (ANOVA) for normal distribution or Mann-Whitney test with Bonferroni corrections for non-parametric data).

Table 1 : Observed Copulation frequencies at different ZT time points in LD cycles.

Time points	Mean Copulation Frequencies
ZT0	
ZT4	
ZT8	
ZT12	

Questions for self-study and interpretation:

1. What factors can influence mating in *Drosophila*.
2. What is the importance of maintaining constant light and dark cycles for circadian experiments?
3. Would the copulation rhythm be affected if flies were maintained in continuous light or continuous dark condition?
4. Is the copulation rhythm of flies entrained in LD cycle but later changed to constant light or dark condition expected to change if the above assay was conducted on the first subjective day, i.e., the day predicted by the entrained clock?
5. What impact can mutations in clock genes have on copulation rhythm?

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Chapter 103

Study of courtship memory using *Drosophila melanogaster*

Nisha Sharma¹ (nishasharma18phd@cup.ac.in) and Shahnaz Rahman Lone^{2*} (slone@gitam.edu; lonesr@gmail.com)

1. Department of Zoology, Central University of Punjab, Bathinda, 151001, Punjab, India.
2. Department of Biochemistry & Bioinformatics, The Gandhi Institute of Technology and Management (GITAM), (Deemed to be University), Visakhapatnam, Andhra Pradesh, 530045, India.

*For Correspondence

Recommended Level: PG/Research

Sub-discipline: Neurobiology, Behavioral Biology

Background:

Social experiences exert a profound influence on the internal state of various organisms, including the fruit fly *Drosophila melanogaster*, leading to the modulation of several behaviors such as sleep, memory, ethanol consumption etc. (Lone et al., 2016; Shohat-Ophir et al., 2012; Chabaud et al., 2009). This behavioral modulation plays a critical role in the species' survival and reproductive fitness (Siegel and Hall, 1979; Svetec and Ferveur, 2005; Krupp et al., 2008). A notable example of social plasticity in *Drosophila* is observed in male flies, who possess the remarkable ability to remember past aversive courtship experiences and adjust their behavior accordingly when encountering potential mates (Griffith & Ejima, 2009). During courtship, male flies engage in a series of stereotyped behaviors, including wing extension, wing vibration, orientation, licking, and attempted copulation, to attract and court females (Pavlou and Goodwin, 2013; Siegel and Hall, 1979, also see Chapters 101 and 102 for details of courtship stages). Research suggests that the molecular and cellular mechanisms underlying courtship memory share similarities with olfactory memories, as both types of behavior rely on the sense of smell. However, courtship memory involves a more complex learning process that encompasses experiences of rejection in addition to pheromonal cues.

Understanding courtship memory involves analyses of different stages like conditioning, memory formation, and subsequent testing their courtship behavior. During conditioning we harness the male's capacity to avoid engaging with unreceptive females during courtship. This involves co-housing males with freshly mated females, either for a brief one-hour period to establish Short-Term Memory (STM) or extending to 8-9 hours for Long-Term Memory (LTM) formation. Following this conditioning, the courtship behavior of the conditioned and naive males towards a virgin female is carefully observed

and compared during the testing phase. Compared to naive males, the conditioned males typically exhibit diminished courtship behavior, even in the presence of virgin females, thus demonstrating male's ability to learn to avoid freshly mated females and adjust his subsequent behavior accordingly (Kamyshev et al., 1999; Koemans et al., 2017; Bray and Amrein, 2003).

Objective:

To assay the courtship memory of fruit fly *Drosophila melanogaster*.

Materials Required

1. **Flies:** Wild type or other desired genotype flies.
2. **Equipment and other materials:**
 - i. Stereo-binocular microscope, vials with standard fly food medium, and other fly handling materials.
 - ii. B.O.D. Incubator at $24^{\circ}\text{C} \pm 1^{\circ}\text{C}$.
 - iii. Aspirator.
 - iv. CO_2 or alternative methods of anaesthesia.
 - v. Clean glass tubes (65 x 7mm) open at both ends – 120 nos.
 - vi. Rubber bands (to hold a bunch of 30-40 7mm glass tubes).
 - vii. Mouth aspirator for transfer of live flies.
 - viii. Camera or mobile phone camera with good storage capacity.
 - ix. Courtship chamber(s): 12 mm (dia) x 10 mm (height): this can be specifically designed for *Drosophila melanogaster* experiments, such as a circular glass or plexi-glass chamber with suitable dimensions (diameter 12 mm, depth 10 mm) for testing process. Each courtship chamber consists of multiple circular compartments or arenas using transparent covers with a hole to introduce flies.

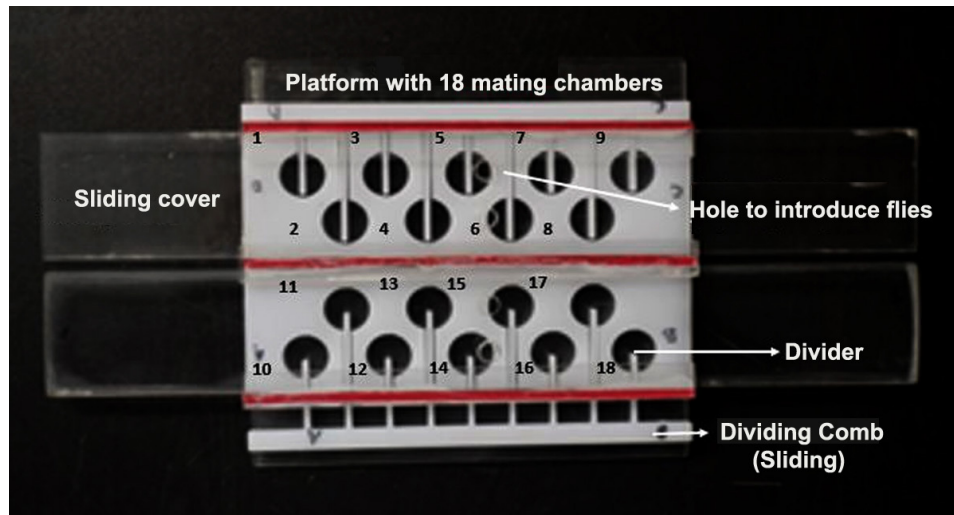


Fig. 1. Courtship chamber assembly (17 x 12 x 1.5cm (L x B x H)) with individual chambers of 12 mm (dia) x 10mm (H) in the platform. In total, there are 18 courtship chambers, divided into two groups, labelled as 1-9 and 10-18, respectively. Each group is equipped with channels on either side (red lines) for movement of a top sliding cover which serves as a shared lid for all the chambers; each of the sliding cover has two holes (one for each lane of courtship chambers) for inserting the desired flies into the desired chamber in the given lane. The added males and females initially remain separated by the movable dividing comb (as shown in Chambers 1-9, top row). Following their acclimatization period, the dividing comb is pulled out (shown in Chambers 10-18, lower row) to enable the male and female flies to interact.

Experimental Details:

1. Culturing flies for experiment:

- i. Create three bundles of 40 clean glass tubes with a 7 x 65 mm diameter, secured with rubber bands (total of 120), for collecting males, food change and conditioning. Fill a 90 x 10 mm Petri dish with hot fluid food and insert the bundle of 40 tubes for being filled with food by capillary action. Repeat this with each of the 3 bundles of 40 tubes. After solidification, rotate the bundles before their vertical removal. Clean the adhering food on outside of the tubes on the food end and wrap them with parafilm to prevent drying. Plug and place the food containing bundles of 40 tubes in a clean airtight bag and store at 4°C. Bring the tubes to room temperature before use.
- ii. Set 4-5 fly cultures in regular food vials with 4 virgins and 3 males in each vial for each genotype to be tested.
- iii. Collect 40 freshly eclosed males from the progeny of step 1.ii and keep one male in each of the 7mm food tubes (prepared in step 1.i above).
- iv. Divide them randomly into two groups of 20 tubes each, and Label one group as Conditioned group (C1-C20) and the other as Naive group (N1-N20).
- v. Collect another group of eclosed males and females (from step 1.ii above) and keep them in separate food vials, with each vial containing either 20-30 males or females. Label them as Mixed male or female flies (MF-M and MF-F, respectively).

- vi. Age the C, N and MF groups of flies for 4-5 days at $24^{\circ}\text{C}\pm 1^{\circ}\text{C}$ with transfer to corresponding fresh tubes/vials on alternate days.
- vii. On day 6, take flies of MF group and aspirate 7-8 males and 5 females to a single food vial to create a single heterosexual group. Prepare 10 such groups and label the 10 food vials with mixed flies as MF1-MF10. Keep them together for 48 hr to allow the flies to mate.
- viii. After 48 hr, separate the females of “MF” groups and use them immediately for conditioning of “C1-20” males in the next step; discard the males.

2. Conditioning:

- i. On day 8, transfer single male flies of C and N groups into fresh food tubes. Add single mated female of the MF group (step 1.vii above) into the ‘C’ group of tubes and co-house the single C male and MF female for 1 hr for STM or 8 to 9 hours for LTM (label each tube corresponding to the duration of conditioning).
- ii. Keep ‘N’ group of naive males individually (without females) during conditioning of ‘C’ males.
- iii. Both ‘C’ and ‘N’ group should be kept at the same place to receive similar micro-environment during conditioning.
- iv. After the conditioning/training period of 1 hr or 8-9 hr, separate the ‘C’ males from females by aspiration.
- v. Now transfer the ‘C’ and ‘N’ males to fresh food tubes, one male in each tube.

3. Recording the mating behavior through direct observation or videography:

- i. Transfer the single ‘C’ males into each courtship chamber and allow them to acclimate to the new environment for 1 hr under a low-intensity light (1-2 lux) to prevent excessive courtship initiation.
Note: The simpler courtship chamber described in [Chapters 101 and 104](#) can also be used.
- ii. After acclimatization introduce one virgin female fly through the hole in each courtship chamber and allow the flies to engage in courtship behavior.
Note: Both males and virgin females are acclimatized in the instrument where they can be kept them separate using dividers before bringing them together for testing, as shown in Fig. 1. However, when using a simple Courtship Arena (as in [Chapters 101 or 104](#)), males are first acclimatized, and female is added to the arena just before testing.
- iii. Observe the behavior of each pair manually or alternatively record the courtship behavior of ‘C’ male flies for 10 min using a video or mobile camera fixed about the courtship arena to record behavior of the flies as described in the next section.
- iv. Repeat the steps 3.i-iii with the ‘N’ males and virgin females to record their mating behavior.

4. Observations and Video Data Analysis and Statistics:

- i. Analyse each conditioned and naive male paired with the virgin female for the time of courtship interaction. The percentage of the 10 min time spent in courting (wing extension, wing vibration, orientation, licking, and attempted copulation) indicates the Courtship Index (**CI** = Total time in seconds spent in courting behavior during the 10 min period/600 seconds).

Notes:

- a. When recording manually, note the total time spent by male and female pair in courtship behavior.
- b. If recorded on a camera, analyse the video recording frame by frame: this will allow measuring the time spent in each part of the courtship behavior (wing extension, wing vibration, orientation, licking, and attempted/successful copulation). Calculate the total time in seconds for which the male courted the female per minute.
- ii. Finally, calculate the **Courtship Index (CI)** for each Conditioned and Naïve males using the formula: Total time in seconds spent in courting behavior during the 10 min period/600 seconds.
- iii. Tabulate the data (as in Sample Table 1 below) and perform statistical analysis according to the distribution of the data. The choice between parametric or non-parametric tests would depend on the data distribution.

Note: The ‘C’ males are expected to show lower CI compared to the ‘N’ males.

Table 1 : A Sample data sheet for recording the courtship behavior of Conditioned ‘C’/ Naive ‘N’ males (separate tables for ‘C’ and ‘N’ males).

Courtship Time interval (Minute)	Seconds spent in courtship behavior during each one minute interval										
	Pair 1	Pair 2	Pair 3	Pair 4	Pair 5	Pair 6	Pair 7	Pair 8	Pair 9	Pair 10	Total pairs
1.											
2.											
3.											
4.											
5.											
6.											
7.											
8.											
9.											
10.											
Sum (in seconds)											
Courtship Index (Sum/600)											

Questions for self-study and interpretation

1. What is the significance of courtship memory.
2. Can environmental or physiological factors modulate courtship memory formation?
3. How long does it take for courtship memory to become established?
4. Which part of the neural system is important for the courtship memory?

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Chapter 104

Effect of inhibition of stimulation of neurotransmission on behaviour in *Drosophila*

Hemant Singh (singhemant060@gmail.com), Prathamesh Dhamale (sunnydhamale21@gmail.com) and Amitabha Majumdar* (amitavamajumdar@gmail.com)

National Center for Cell Science, Pune

*For correspondence

Recommended Level: PG/Research

Sub-discipline: Neurobiology/Behavioural Biology

Background:

Nervous system is responsible for processing various sensory inputs and coordinating the behavioural outputs of an organism. The primary organ responsible for this is the brain, which contains neurons and glial cells. Neurons are responsible for the transmission of information, processing, and acting upon the stimulus, whereas the glial cells are responsible for support, nourishment, and maintenance of the brain tissue. Neurons transmit information by using action potentials, which are achieved by rapid changes in membrane polarization of the neuron.

Neurons have a resting potential of -70 mV, which is maintained by Na^+/K^+ channels. Neurotransmission starts when a neuron is stimulated by upstream neurons. This results in an increase of membrane potential by the influx of Na^+ ions causing the generation of action potential that travels through the axon. At the presynaptic end of the axon, neurotransmitters are packaged in vesicles, which are released into the synaptic cleft when an action potential reaches the axon terminal. The released neurotransmitters then bind to the appropriate receptors on postsynaptic neuron. The binding of neurotransmitters may either have an excitatory or inhibitory effect on the postsynaptic neuron.

In the experiments described here, we will use *GAL4-UAS* system to express proteins which can be used to directly inhibit or stimulate the release of neurotransmitters in the axons (for details about the *GAL4-UAS* system, see [Chapter 40](#)). The first set of experiments for assessment of paralysis takes advantage of the normal negative geotactic behaviour of flies because of which they climb up (away from gravity) in the vials (see [Chapters 92, 94](#)) but if neuro-transmission is blocked and the flies paralysed, they fall to the bottom of vials. For inhibiting the neurotransmitter release, a temperature sensitive mutant allele of *Shibire*, under a UAS promoter, is expressed in neurons using the pan-

neuronal *Elav-GAL4* driver. Shibire is the *Drosophila* homolog of Dynamin, a protein crucial for endocytosis. Several mutations in the Shibire gene show temperature sensitive paralysis. The *Shibire^{ts1}* mutant allele carries a G273D mutation at the boundary of the GTPase domain of the protein. The reason for temperature sensitive paralysis is most likely due to the loss in activity of this mutant Shibire protein, caused by conformational shift at higher temperature. Electron microscopy studies showed depletion of vesicles and blockage of endocytosis in nerve terminals of these flies (Poodry and Edgar, 1979). The temperature-sensitive *Shibire^{ts1}* is expressed pan-neuronally using the *GAL4-UAS* system (Kitamoto, 2001). The *Elav-GAL4; UAS-Shi^{ts1}* flies when shifted to the non-permissive temperature of 32°C, show temperature-induced paralysis caused by block in endocytosis and neurotransmission across the nervous system.

The Transient receptor potential cation channel A1 (*TrpA1*) is expressed in neurons. *TrpA1* is an evolutionary conserved thermo-sensitive cation channel which mediates thermosensation across animals ranging from *C. elegans*, *Drosophila*, Zebrafish, rattle snakes to humans. Expressing the *TrpA1* channels in neurons and subjecting them to a higher temperature will cause the channels to open and will result in Calcium influx in the neurons and cause its depolarization and firing (Hamada *et al.*, 2008, Berni *et al.*, 2010). In the *Elav-GAL4; UAS-TrpA1* flies such continuous firing of action potentials will further result in depletion of the vesicle pools and paralysis of the flies.

The *TrpA1* channel expression can also be used to demonstrate its use in studying neural circuits since different actions are controlled by organized neural circuits which when stimulated artificially will result in the animals showing the same actions. Male *Drosophila* performs specific courtship steps directed towards females before mating (see [Chapter 101](#)). Females assess male fitness based upon the display of these courtship behaviours. *Fruitless (fru)* expressing neurons have been identified as the master regulator of courtship behaviour and sexual orientation in male *Drosophila* (Ryner *et al.*, 1996). Here we will selectively express *TrpA1* in the *Drosophila* courtship circuit using *Fruitless GAL4* driver (Pan *et al.*, 2011). Exposing the *Fru-GAL4; UAS-TrpA1* flies to a higher temperature will lead to the stimulation of the courtship circuit and one can observe the courtship actions like male flies following other males, vibrating their wings to generate courtship song and attempted copulations. These behaviours are specifically shown by male flies and can be activated irrespective of presence of a female fly (Ryner *et al.*, 1996).

Goal of these experiments is to demonstrate the effect of silencing and stimulating neurons in *Drosophila*. These experiments will also introduce the concept of recycling of synaptic vesicles. The release of neurotransmitters at the synapse occurs through exocytosis, which involves fusion of the vesicles with the synaptic membrane. This is balanced by a continuous process of endocytosis which involves formation of vesicles from the presynaptic membrane which are refilled with neurotransmitters. The process of simultaneous exocytosis and endocytosis is called synaptic vesicle recycling. The temperature sensitive block with Shibire will show what happens when exocytosis is happening but endocytosis is blocked. Under this condition, the synapse, after some time, will be depleted of all vesicles, so the process of vesicle recycling stops and causes block in neurotransmission and consequent paralysis. In the *TrpA1* based stimulation, there will be continuous exocytosis of vesicles but as the endocytosis rate is slower, it would not be able to replenish the vesicles causing a block in the vesicle recycling process and paralysis.

Objective:

To examine effects of inhibition and stimulation of neurotransmission on the fly behaviour by targeted expression of *Shibire^{ts1}* or *TrpA1* proteins in neurons.

Materials Required:

A. Fly stocks:

- i. Wild type flies
- ii. $P\{GawB\} Elav[C155]-GAL4$ (BDSC_458): – The Elav protein is a pan-neuronally expressed protein. In this transgenic line, GAL4 is expressed under the Elav promoter and is thus expressed in all fly neurons. This line is referred to as *Elav-GAL4*.
- iii. w^* ; $P\{y+t7.7 w+mC=UAS-TrpA1(B).K\}attP16$ (BDSC_26263): The temperature sensitive *TrpA1* cation channel protein is expressed under the *UAS* promoter, and is used to activate neurons at temperature above 30°C. This line is referred to as *UAS-TrpA1*.
- iv. *PJFRC 99 20X UAS-IVS syn21- Shibire^{ts1} p10* (Rubin lab, Janelia) - a temperature sensitive dynamin mutant which is used to inhibit neurons at temperature above 30°C. This line is referred to as *UAS-Shi^{ts1}*.
- v. $P\{w+mW.hs=GawB\} FruNP0021-GAL4$ (BDSC_30027) - GAL4 is expressed under the *fruitless* promoter. This line is referred to as *Fru-GAL4*.

Note: These stocks can be obtained from author's lab.

B. Equipment and other fly-handling materials

- i. Stereo-binocular microscope
- ii. Small incubators that can be set up at different temperatures
- iii. Circular courtship arena (**Fig. 1**)
- iv. Dry bath with metallic platform
- v. Fly food vials
- vi. Empty tubes
- vii. Other fly-handling items (for anaesthetizing flies, forceps, fine hair brushes etc)

Experimental Details:

1. Grow stocks of *UAS-Shi^{ts1}*, *UAS-TrpA1*, *Elav-GAL4*, *Fruitless GAL4* (*Fru-GAL4*).
2. Once the adult flies start eclosing, collect *Elav-GAL4* and *Fru-GAL4* virgin females and *UAS-Shi^{ts1}* and *UAS-TrpA1* males.
3. After collecting 5-10 virgin females and males for each, set up the following crosses:
 - a. *Elav-GAL4* (females) X *UAS-Shi^{ts1}* (males)
 - b. *Elav-GAL4* (females) X *UAS-TrpA1* (males)
 - c. *Fru-GAL4* (females) X *UAS-TrpA1* (males)
4. Allow the flies to lay eggs at 22°C which is the permissive temperature. Transfer the

parents once every 3-4 days to fresh food vials to make sure that the parents do not mix up with the progeny. Temperature at which the progenies are grown should be at 22°C or below (**Note:** When grown at 24/25°C, these flies do not get paralyzed but become sluggish; therefore, growth at 22°C or below is preferred).

5. Once the progeny starts eclosing, transfer the eclosed F1 flies of *Elav-GAL4; UAS-Shi^{ts1}*, *Elav-GAL4; UAS-TrpA1* and *Fru-GAL4; UAS-TrpA1* genotypes to empty, and respectively marked vials. Transfer 10 flies into each tube. As the parents are homozygous, all the progeny of a given cross will be *Elav-GAL4; UAS-Shi^{ts1}* or *Elav-GAL4; UAS-TrpA1* or *Fru-GAL4; UAS-TrpA1*, respectively.

6. Place one vial carrying *Elav-GAL4; UAS-Shi^{ts1}* flies at 22° C and another at 30° C for 5 min.

7. For vials carrying *Elav-GAL4; UAS-TrpA1* flies, and keep one vial at 22° C and transfer another to 32° C for 5 min.

8. For examining effect of over-stimulation of the courtship circuit, transfer 2-4 male flies of genotype *Fru-GAL4; UAS-TrpA1* in the circular arena (**Fig. 1**). The circular arenas are fabricated from cut transparent acrylic sheets with the inner dimensions being 2.6 cm diameter and 0.4 cm thickness (**Fig. 1**). This size gives the flies enough space to walk on the floor but not for climbing on the walls. In place of such arenas, one can use caps of 30 mm tissue culture dishes. The temperature of the arena must not be more than 30° C since that would paralyze and kill the flies.

Note: Wild type flies are to be similarly treated and examined in parallel as Control.

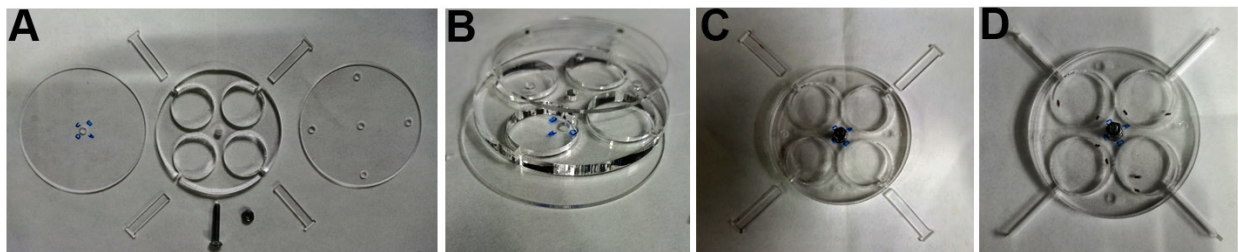


Fig. 1. Design of the courtship arena: **A.** Individual parts of the arena are shown, the three acrylic cut plates are fastened by screw placed in a 4 mm central hole. The diameter all three plates is 78 mm; thickness of central plate is 4 mm whereas, top and bottom plates are 2 mm thick each. The separator combs are of 4 mm height, 2 mm in breadth and 31 mm in length. The central plate has arena cutout of 26 mm diameter, and the top plate has 4 mm hatches to place flies inside the arena. **B.** Assembled stack of plates with the left one in **A** being at bottom, central one in middle and the right one in **A** on top. **C.** The stack fastened with screw through the central hole and with the separator combs placed through the sides of the arena. Flies can be introduced in the arena either through the gaps for the separator combs or through the top holes. If introduced through the top holes, the top sheet is rotated so that the holes move away from the arena. **D.** Arena with males and females placed through the top holes, and the separator combs opened to initiate interaction between the male and female in each arena.

Observations:

1. For flies of the genotype *Elav GAL4; UAS-Shi^{ts1}*: Observe and record differences in the behaviour of flies during the 5 min period at 22°C or at 30°C. Compare with wild type flies maintained at 22°C or at 30°C.

2. For flies of the genotype *Elav GAL4; UAS- TrpA1*: Observe and record differences in the behaviour of flies during the 5 min period when they are kept at 22°C or at 32°C. Compare with wild type flies maintained at 22°C or at 32°C.

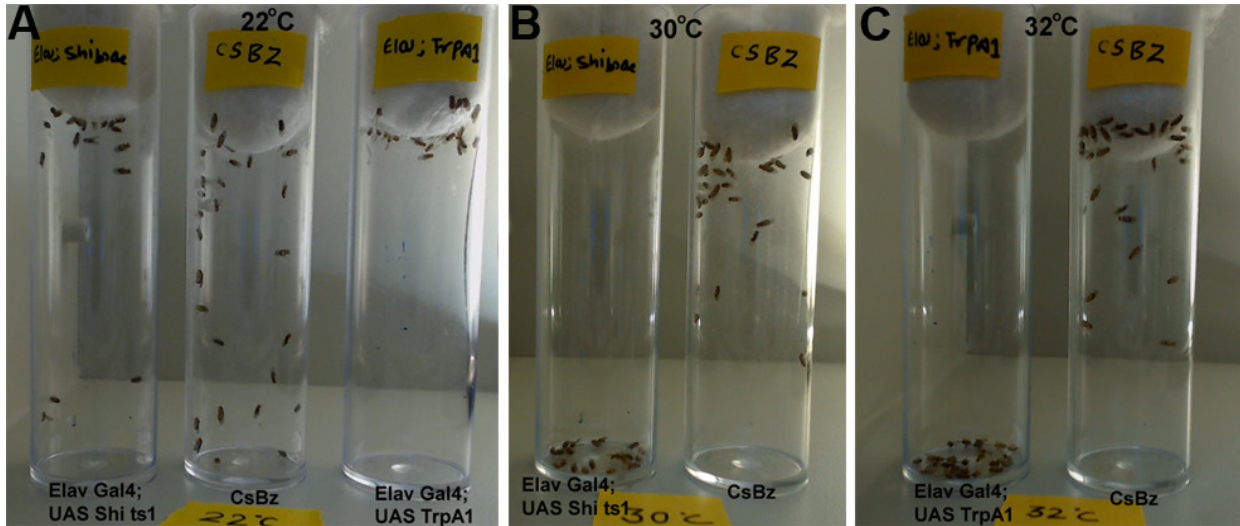


Fig. 2. Effect of temperature dependent inactivation and activation of neurons. A. *Elav GAL4; UAS Shi^{ts1}*, *Elav GAL4; UAS TrpA1* and wild type (CsBz) flies at 22°C. **B.** Inactivation of neurons by expression of *shibire^{ts1}* at 30°C causes *Elav GAL4; UAS-Shi^{ts1}* flies to get paralyzed and fall to bottom of the tube. **C.** Expression of *Elav GAL4>UAS TrpA1* at 32°C causes flies to get paralyzed and fall to the bottom of the tubes. The CsBz (wild type) flies in all cases show normal geotactic response.

3. For examining courtship behaviour of flies *Fru-GAL4; UAS-TrpA1*: Observe and record the differences in courtship behaviour of male and female flies during the 5 min period when they are kept at 22°C or at 30°C. First, observe the courtship behaviour at 22°C. After 30 min, transfer the circular arena on a metallic dry bath kept at 30°C. Observe the courtship behaviour shown by flies in the arena for the next 5 min. Compare with the courtship behaviour in wild type flies maintained at 22°C or at 32°C.

Note: The courtship behaviour components (see [Chapter 101](#)) to be examined are: (a) fly following another fly. (b) fly orienting at an angle with respect to another and vibrating its wings to generate courtship song. (c) attempt to lick the genitalia. and (d) bending its abdomen with an attempt to copulate (see [Fig. 3](#) for normal courtship behaviour displayed by wild type male flies).



Fig. 3. Normal courtship behaviour of wild type *Drosophila melanogaster* male fl . A. Orientation; B. Following a female; C. Male vibrating wings to generate courtship song; D. Licking; E. Bending abdomen with attempt to copulate.

Questions for self-study and interpretation:

1. Why does expression of *UAS-Shi^{ts1}* or *UAS-TrpA1* with *Elav-GAL4* driver at 30°C lead to paralysis in flies but *Fru-GAL4* driven expression of *UAS-TrpA1* does not lead to paralysis?
2. What are the normal functions of Shihire and TrpA1 proteins?
3. Why does expression of *UAS-TrpA1* with *Fru-GAL4* driver at 30°C lead to altered courtship behavior? Would *Fru-GAL4* driven expression of *UAS-Shi^{ts1}* at 30°C also affect courtship behavior?
4. How Shihire and TrpA1 channel can be used to identify neural circuits in memory, feeding, and locomotion?
5. What are action potential generation and neurotransmission. Are they related?

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Videos for these experiments at: <https://amitavamajumdar.wixsite.com/nccs/movies>.

Chapter 105

Investigating impact of traumatic brain injury (TBI) on climbing behavior in *Drosophila melanogaster*

Nisha Sharma¹ (nishasharma18phd@cup.ac.in), Shamprasad Varija Raghu² (shamprasadvr@yenepoya.edu.in), Sunil Kumar Birua¹ (sunilbirua309@gmail.com), Avinash K. Kudva³ (avinash.kudva@gmail.com), Chandrashekhar G. Joshi^{4*} (josheejoshee@gmail.com) and Shahnaz Rahman Lone^{5*} (slone@gitam.edu; lonesr@gmail.com)

1. Department of Zoology, Central University of Punjab, Bathinda, 151001, Punjab, India.
2. Division of Neuroscience, Yenepoya Research Centre (YRC), Yenepoya (Deemed to be University), Karnataka 575018
3. Department of Pediatrics, Division of Hematology/Oncology, College of Medicine, Pennsylvania State University, Hershey, PA 17033, USA.
4. Department of Biochemistry, Mangalore University, Mangalagangothri, Karnataka 574199.
5. Department of Biochemistry & Bioinformatics, The Gandhi Institute of Technology and Management (GITAM), (Deemed to be University), Visakhapatnam, Andhra Pradesh, 530045.

*For Correspondence

Recommended Level: UG/PG/Research

Sub-discipline: Anatomy/Neurobiology/Behaviour

Background:

Physical injuries arising from violence, vehicular accidents, falls, slips, and sports activities can have diverse impacts on an individual's well-being and quality of life. Unfortunately, some of these injuries can also result in long-lasting consequences, contributing to morbidity and mortality. Traumatic brain injury (TBI) encompasses a range of injuries resulting from head trauma (Kay et al., 1993). Mild TBIs cause temporary symptoms like confusion, headache, and memory issues, while moderate and severe TBIs lead to more profound effects such as seizures, paralysis, and cognitive impairment (Langlois et al., 2006; Tenovuo et al., 2021).

Fruit fly *Drosophila melanogaster* serves as a valuable model organism for TBI research due to many similarities of its genetic and physiological aspects with those of humans. Researchers can induce TBI-like conditions in fruit flies through controlled mechanical trauma for studying cellular and molecular responses to brain injury (Katzenberger et al., 2015; Katzenberger et al., 2013). Fruit fly's well-characterized nervous system allows for in-depth analysis of neuronal responses and synaptic changes, aiding in identifying therapeutic targets and treatments for TBI patients. The simple assay of the climbing

behavior (see [Chapter 92, 93, 94](#)) helps in understanding impact of Traumatic Brain Injury (TBI) on health.

Objective:

To study the impact of Traumatic Brain Injury (TBI) on the climbing behavior of *Drosophila melanogaster*.

Materials Required:

- A. **Flies:** Canton-S or other desired wild type or mutant flies.
- B. Equipment and fly handling materials:
 - i. Stereo-binocular and other fly handling materials.
 - ii. B.O.D. Incubator maintained at $24^{\circ}\pm 1^{\circ}\text{C}$.
 - iii. Empty vials and Cotton plugs
 - iv. CO_2 or alternative methods of anesthesia.
 - v. Pad made of hard polyurethane.
 - vi. Soft fine hairbrushes for handling flies.
 - vii. Marker pen.
 - viii. Climbing assay device (see [Chapters 92, 93, 94](#) for climbing assay)
 - ix. Stereo-bioncular microscope.
 - x. Stopwatch or timer.
 - xi. HIT (High Impact Trauma) device (Fig. 1)

Note: The HIT device, shown in Fig. 1 can be fabricated locally. Fix the spring on a wooden board ($23\text{ cm} \times 17\text{ cm} \times 2\text{ cm}$) using clamps and screws (Fig. 1A) so that it is parallel to the length of the board. The clamped end of the spring should be $\sim 3.2\text{ mm}$ from the edge of the board, and the free end should extend over the board by $\sim 19\text{ mm}$. Wrap the free end of the spring with adhesive strip of Velcro loops (Katzenberger et al., 2013) so that this end fits tightly within an empty vial.

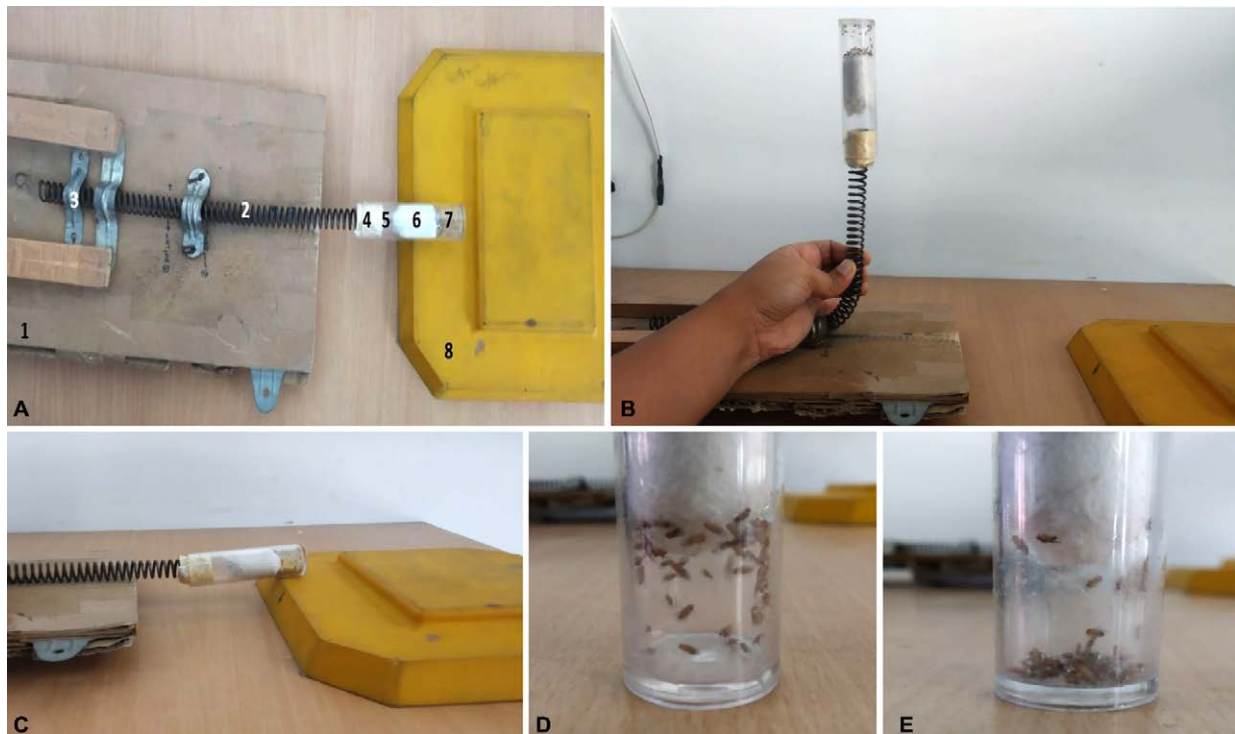


Figure 1 : HIT device. **A.** Top view of the “high-impact trauma” HIT device. Key parts: wooden board (1), spring (2), metal fasteners for fixing the spring on the wooden board (3), Velcro wrap around the spring (4) to ensure that this end tightly fits in the mouth of plastic vial (5), cotton plug (6) to restrict the flies within the bottom 1-2 cm (7) of the plastic vial, and the polyurethane pad (8) placed at appropriate distance from the wooden board so that when the vial is released, its bottom end with flies in it hits the pad. **B.** HIT device deflected to 90° before strike. **C.** HIT device immediately after the strike. Flies before (**D**) and after (**E**) TBI.

Experimental Details:

1. Maintain *Drosophila melanogaster* cultures in a suitable laboratory environment, following standard procedures for breeding and maintaining fly stocks as described in the [Chapter 1](#).

Notes:

- i) It is preferred to maintain flies in a light-dark (LD) cycle with light on from 8 AM and lights off at 8 PM. The light intensity during the light phase should be 250 -500 Lux, and the dark phase should have 0 Lux.
 - ii) Maintain constant humidity level within the incubator by placing a plastic tray (preferably 22 x 27 cm) half-filled with water in the incubator.
2. Collect freshly eclosed male and female flies separately and let them age for 5 days on normal food under uncrowded condition of 30 flies per food vial.
 3. Test the HIT device by inserting the free end of the spring with Velcro tape in the mouth of an empty plastic vial. Ensure that the Velcro tape end tightly fits in the plastic vial. Pull the vial with inserted spring upward (Fig. 1B) and release it at a 90° angle so that the lower end of the tube hits the hard polyurethane pad placed at appropriate position (Fig. 1C).

4. Transfer 40-50 male or female flies into the plastic vial using an aspirator, and quickly insert a tight-fitting cotton plug in the vial. Push the plug carefully towards the bottom of the tube to restrict flies to about 1-2 cm height within the tube.
5. Prepare 4 such tubes with male or female flies (as in step 4) and label them as TBI_M1, TBI_M2, TBI_M4, and TBI_M6 (for male flies) and TBI_F1, TBI_F2, TBI_F4, and TBI_F6 (for female flies), which are subjected to 1, 2, 4 or 6 trauma events (see below).
6. Prepare two other tubes with male or female flies, and label them as TBI M0 and TB F0, respectively. These are used as Control, without being subjected to the trauma injury.
7. Attach the TBI_M1 or TM_F1 vial tightly to the spring by inserting the Velcro wrap end in mouth of the vial. Ensure that the Velcro tape end tightly fits in the vial.
8. Pull the vial with spring upward at a 90° angle, with respect to the surface of the HIT device, and release the spring quickly so that because of the spring's tension, the bottom of the vial hits the polyurethane pad positioned appropriately to create the traumatic event. Record the time of the impact.
9. Repeat step 8 with TBI_M2, TBI_M4, and TBI_M6 and TBI_F2, TBI_F4, and TBI_F6 tubes.
10. After a 5-minute interval induce another event of trauma to the TBI_M4, and TBI_M6/ TBI_F4, and TBI_F6 (the tubes TBI_M1/TBI_F1 do not receive any more trauma).
11. Repeat the previous step (step 10) 2 and 4 times with TBI 4 and TBI 6 sets of vials, respectively, at 5 minute intervals (these flies thus experience a total of 4 or 6 TBI events, respectively). Record the time and date of TBI applied to each vial.
12. After causing trauma, carefully transfer the TBI and control flies into correspondingly marked fresh food vials and place them in the BOD incubator used for maintaining fly cultures.
13. After 48 hours, separate dead flies (those suffering injuries to wings and other body parts usually die within 24 hours) and perform Climbing assay with 10 of the surviving flies in each group.
14. Mark 95mm empty vial lengthwise, dividing it into 4 zones, each approximately at 20mm height. Label these zones using a marker, with the bottom zone marked as zone 1 and the top zone (plugged end) marked as zone 4, respectively (for slightly variant Climbing assays, [see Chapters 92, 93 and 94](#)).
15. Transfer the 10 selected flies from each treatment/control group into the marked 95mm vial, close the plug and let them acclimatize in the vial for 10 min.
16. After 10 min, gently tap the vial on a foam pad to bring all the flies to bottom. Leave the tube vertically on the table and watch the climbing of flies during the next 20 seconds to record the number of flies in each zone (Zones 1-4): a camera or mobile phone can also be used to take a photograph to record positions of flies in the tube

after 20 sec. Assign scores to the flies based on their location in the vial after 20 seconds. Flies in zone 4 receive a score of 4, while those in zones 3, 2, and 1 get respective scores of 3, 2 or 1.

17. Calculate the average score for the group (sum of individual scores/10) and repeat the climbing assay 3-5 times for each set of flies to obtain a mean score for each control/experimental group.
18. Plot the climbing scores of TBI series and Controls flies in a graph and perform appropriate statistical analysis to measure the impacts of single and multiple events of TBI.

Questions for self-study and interpretation:

1. Does TBI cause visible damages on the fly head?
2. Do the flies that suffered multiple TBI show greater head injury and/or early death?
3. Does TBI affect the climbing ability of flies? If yes, why?
4. Do males and females differ in their response to TBI?
5. Do TBI events affect the sleep of flies?
6. How long does the effect of TBI last?
7. What other behavioural tests can be performed to assay the TBI?
8. Can one examine and identify the actual sites in brain where TBI would have caused a damage? What possible approaches can be used for such studies?
9. What are possible long-term cognitive and behavioral consequences of recurrent TBIs, such as those experienced during contact sports or military combat?

References for further study:

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Chapter 106

Vortex assay to measure the epileptic behavior in *Drosophila*

Deepa Mugudthi Venugopal¹ (deepavenugopalbvr@gmail.com), Raifa Abdul Aziz¹ (raifaazeez@gmail.com), Ramesha Hanumanthappa² (rameshkoose18@gmail.com), Shripriya Bhat¹ (shripriyabhat05@gmail.com), Manvitha Kadandelu³ (manvithakashyap@gmail.com), Avinash Kudva⁴ (avinash.kudva@gmail.com), Rajashekhar K Patil⁵ (patilsirmu@gmail.com), Kuramkote Shivanna Devaraju² * (ksdevaraju@kud.ac.in) and Shamprasad Varija Raghu^{1,3} * (shamprasadr@yenepoya.edu.in)

1. Neurogenetics Lab, Department of Applied Zoology, Mangalore University, Mangalagangothri, Karnataka 574199
2. Neurochemistry lab, Department of Biochemistry, Karnatak University, Dharwad, Karnataka 580003
3. Division of Neuroscience, Yenepoya Research Centre (YRC), Yenepoya (Deemed to be University), Deralakatte, Mangalore, Karnataka 575018
4. Department of Pediatrics, Division of Hematology/Oncology, College of Medicine, The Pennsylvania State University, Hershey, PA 17033, USA
5. Department of Zoology, Bangalore City University, Bengaluru, Karnataka 560001

* For Correspondence

Sub-discipline: Neurobiology/Behavior/Physiology

Recommended level: UG/PG/ Research

Background:

Epilepsy has become a common neurological condition, globally impacting approximately 50 million people (Mituzait et al., 2021, Song and Tanouye, 2008). It is characterized by unprovoked reoccurring seizures. This excessive electrical activity in the brain can result in a variety of symptoms depending on which area of the brain is affected. Even though epilepsy treatment includes an armoury of antiepileptic drugs, one-third of the patients do not respond to them. This indicates the need for a better understanding of the mechanisms underlying epilepsy. In view of similarities in the expression of genes associated with epilepsy in humans, *Drosophila* has recently been employed as an invertebrate model for investigations on genetics and physiology of seizures. The *Drosophila* seizure model includes 11 seizure-susceptible mutants known as bang-sensitive (BS) paralytic mutants (Song and Tanouye, 2008). Among them, the mutant alleles of *bang senseless* (*bss*), also known as the *paralytic* (*para*) gene, are susceptible to mechanical shock. The *para* or *bss* gene encodes a voltage-gated Na channel Domain IV. When mechanically shocked, individuals carrying a mutant allele of this gene (e.g., *para*^{bss1} allele) display a behavioral seizure phenotype that includes leg tremors, abdominal muscular spasms, wing flapping, and proboscis extension. These behaviors are marked by an initial seizure, momentary paralysis, a phase that resembles a tonic-clonic episode (extreme muscle spasms and

jerking movements of the body), followed by a recovery seizure and recovery (Mituzaitte et al., 2021). Vortex assay is a method to apply mechanical shock to study seizure behavior and susceptibility in adult flies (Mituzaitte et al., 2021).

Objectives: To characterize the epileptic behavior in *Drosophila* by measuring seizure threshold and duration.

Materials Required:

A. **Flies:** Wild-type Canton S (CS) and *para^{bss1}* mutant flies.

Note: The *para^{bss1}/FM7* mutant fly stock can be procured from the National Centre for Biological Sciences, Bengaluru or from the Division of Neuroscience, Yenepoya Research Centre (YRC), Yenepoya (Deemed to be University), Mangalore). *FM7* is an X-chromosome balancer (see Chapter 1) used for maintaining the fly stock in a heterozygous condition.

B. **Equipment and other items required:**

- i. Stereo-binocular microscope.
- ii. Benchtop vortex.
- iii. Stopwatch.
- iv. Refrigerator.
- v. CO₂ or cold anaesthesia system.
- vi. Fly culture vials (plastic vials, 9 cm x 2.8 cm).
- vii. Double-sided adhesive tape.

Experimental details:

- i. Collect 3-4 days-old adult wild-type male or female flies (n = 10) and anaesthetize them by keeping the vial in a refrigerator (4°C) for 10 min (or by using CO₂ as described in Chapter 1).
- ii. Introduce 10 flies into a fresh food-free vial and keep them at 24/25°C for 30 minutes to let them recover from the anaesthesia stress.
- iii. Using a piece of double-sided adhesive tape, fix bottom of the vial with flies on a benchtop vortexer and vortex the vial at the maximum speed for 10 seconds to induce seizures (Fig. 1).
- iv. Observe the flies for characteristic seizure behavior, i.e., wing flapping, and proboscis extension, paralysis and tonic-clonic (body stiffness, leg twitching/jerking) phases.
- v. Seizure recovery is measured as the time required by each fly to regain its normal posture and agility.
- vi. Repeat steps i-v with *para^{bss1}* mutant male or female flies.

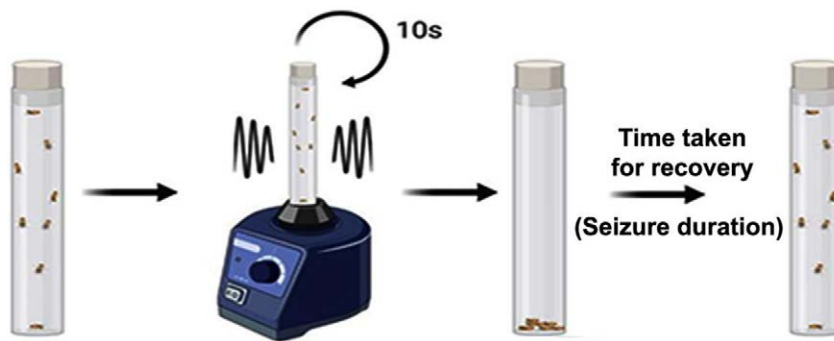


Fig. 1. Schematic of the vortex assay. Flies are vortexed for 10 seconds and the time required for recovery is recorded.

Table 1: Sample Table for recording the seizure recovery time in control and *para^{bss1}* mutant flies

Fly type	Seizure induction time (sec)	Seizure recovery time (sec)
Wild type	10	
<i>para^{bss1}</i> mutant		

Results and Observations:

Note the response of wild type and the *para^{bss1}* mutant flies to the vortexing stress.

Questions for self-study and interpretation:

1. Is there any difference in epileptic response between male and female flies?
2. How does the *para^{bss1}* mutant allele affect seizure behavior?
3. Can this assay be used for primary screening of new anti-epileptic drugs?
4. How can the epileptic behavior observed in the mutant flies be rescued?

References and Further Reading:

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***Drosophila* - Model for Seizure-suppressor Mutations**

.... an alternative approach that relies on *Drosophila* seizure-suppressor genes to identify potential AED targets. This approach has, thus far, proven to be surprisingly productive mainly due to the ease with which novel seizure suppressor mutations have been identified. Using this approach, a new, novel class of potentially effective AEDs may have been discovered, top1 inhibitors including CPT and related drugs. This is a promising first try using the power of the *Drosophila* seizure-suppressor gene approach. The hope is that continued identification of seizure-suppressor genes will lead to even more promising AED candidates. If further tests confirm the use of top1 inhibitors and other promising AED candidates in the neurology clinic, we suggest *Drosophila* will have a major impact on directly improving the human condition through advances of medical treatments in nervous system and other debilitating disorders.

(In: Song, J., & Tanouye, M. A. (2008) From bench to drug: human seizure modeling using *Drosophila*. *Progress in Neurobiology*, 84: 182-191. <https://doi.org/10.1016/j.pneurobio.2007.10.006>).

Chapter 107

Visualizing dynamic organization of nuclei and mitochondria during spermatogenesis in *Drosophila*

Anand K Singh* (anandkumar@iisertirupati.ac/in)^{1,2} and S C Lakhotia* (lakhotia@bhu.ac.in)³

1. Interdisciplinary School of Life Sciences, Institute of Science, Banaras Hindu University, Varanasi - 221 005.
2. Current address: Department of Biology, Indian Institute of Science Education & Research Tirupati - 517507.
3. Cytogenetics Laboratory, Department of Zoology, Institute of Science, Banaras Hindu University, Varanasi - 221 005.

* For correspondence

Recommended Level: UG/PG/Research

Sub-discipline: Cell Biology, Developmental Biology, Genetics

Background:

The reproductive system of adult *Drosophila melanogaster*, like other insects and higher organisms, comprises of a pair of testes, each of which is connected via a short vas deferens with the swollen seminal vesicle, which in turn opens into the common anterior ejaculatory duct. The initial part of the anterior ejaculatory duct is enlarged as an ampulla while its junction with the small posterior ejaculatory duct, which opens to exterior at the male genital plate, is marked by a pair of small bulbous ejaculatory bulbs. A pair of large accessory glands, which are functionally analogous to mammalian prostate glands (Wilson *et al*, 2017), open into the swollen ampulla of the anterior ejaculatory duct (Fig. 1; also see **Chapter 2**). Each of the large, coiled testis is yellowish coloured in wild type (colourless in white eye mutants because in the absence of *white* gene encoded ABC transporters (McKenzie *et al.*, 1999), the testis sheath cells fail to incorporate the pigments. Testis can be arbitrarily differentiated into three main regions. The small proximal region 1 is marked by the presence of mitotically dividing male germinal stem cells, spermatogonia and the meiotic early primary spermatocytes; the region 2 houses cysts of later stages of primary and secondary spermatocytes and early spermatids while the region 3 contains bundles of elongating spermatids and sperms. As the sperms mature, they move basally to seminal vesicles for storage till released through the ejaculatory duct into the female genital tract during mating.

The apical blind end of each testis is the hub region or the stem cell niche (Demarco *et al*, 2014; Siddall and Hime, 2017) which houses a cluster of 10–15 non-dividing somatic hub cells, the germline stem cells (GSC), and somatic cyst stem cells (CySCs). Each GSC divides asymmetrically to produce a GSC and a smaller gonoblast (GB), which undergoes four mitotic divisions with incomplete cytokinesis to generate interconnected 16 spermatogonial cells. The CySCs also divide asymmetrically to produce CySCs and cyst cells. A pair of cyst

cells ensheath a GB to produce a cyst of 16 interconnected primary spermatocytes. The 16 primary spermatocytes in a cyst enter meiotic division to generate 64 haploid round spermatids within the cyst. The 64 round spermatids undergo a complex process of spermiogenesis during which each of the round spermatids elongates, assembles the sperm flagellum to produce bundle of 64 sperms in the cyst (Fig. 3A). Actin-rich individualization complexes are formed around each of the 64 elongating sperm nuclei in a cyst. Following the completion of actin cone formation on each developing sperm, the individualization complex moves away from the nuclei that are located towards the basal or seminal vesicle end of testis. The individualization complex moves along the axonemes towards the apical end of the testis and carries with it the spermatid's cytoplasm as cystic bulge. As the 64 individualization complexes with their cystic bulges reach the tail end of the axonemes, located towards the apical side of testis, they form the Waste bags which are finally degraded, while the sperms become individualized and motile (White-Cooper, 2010; Giansanti *et al.* 2012; Fabian *et al.*, 2012; Demarco *et al.*, 2014; Siddall and Hime, 2017).

Nucleus in the differentiating male germ cells shows remarkable changes in size and chromatin organization. These can be conveniently seen in live testis cells by phase-contrast microscopy. As the small spermatogonia enter the meiotic division cycle, the primary spermatocyte and its nucleus become very large. The large primary spermatocyte nucleus is remarkable for complete decondensation of the heterochromatic Y-chromosome so that its different chromatin regions generate lamp-brush chromosome-like transcriptionally active loops; the Y-chromosome loops are especially distinct in primary spermatocytes of *Drosophila hydei* (Hennig, 1996). The Y-chromosome transcripts are essential for successful completion of spermiogenesis. Following the completion of meiosis, the round spermatid nuclei become small. As the spermatids elongate, the nuclear chromatin also undergoes further remarkable changes to form the narrow and elongated sperm nucleus.

Mitochondria, the membrane bound cytoplasmic organelles that generate the adenosine triphosphate (ATP) to power various cellular activities, show remarkably dynamic movements and rearrangements during transformation of spermatogonia into the highly differentiated mature sperm cells. The most striking mitochondrial transformation takes place during spermiogenesis. At this stage, all the mitochondria fuse to form two long mitochondrial derivatives which wrap around each other to form spherical giant mitochondria called “*Nebenkern*” (German *neben* = next to, and *kern* = nucleus). The nebenkern is of same size as the nucleus and is located close to it during the onion stage of spermatogenesis. Since the nebenkern looks like onion slices, this stage of spermiogenesis is also called ‘onion’ stage. Later, the nebenkerns disassemble as both mitochondrial derivatives unroll and extend parallel to the developing flagellar axoneme during spermatid elongation and fill the entire length of sperm tail. Another transient mitochondrial remodelling occurs in primary spermatocytes as they enter prophase of meiosis 1. At this stage a large mitochondrial cluster is formed adjacent to the nucleus called “*Mitoball*” which colocalizes with Golgi complex, endoplasmic reticulum and the fusome. The Mitoball is a membrane and cytoskeletal-rich structure at the inter-connections between sister cells formed by incomplete cytokinesis in a growing cyst (Li *et al.*, 2023).

The different stages of spermatogenesis in the fly are very similar to those in mammals with comparable developmental regulatory signals that drive the complex process of spermatogenesis involving the complex dynamics of chromatin and mitochondrial organizations required for the development of highly motile sperms. Therefore, spermatogenesis in *Drosophila* has been widely studied to understand the genetic and physiological factors that govern the normal process of spermatogenesis and whose perturbations lead to male infertility (also see Chapter 69).

This chapter describes a phase-contrast microscopy-based study of live testis cells to visualize the remarkable changes in nuclear and mitochondrial organizations that occur during spermatogenesis.

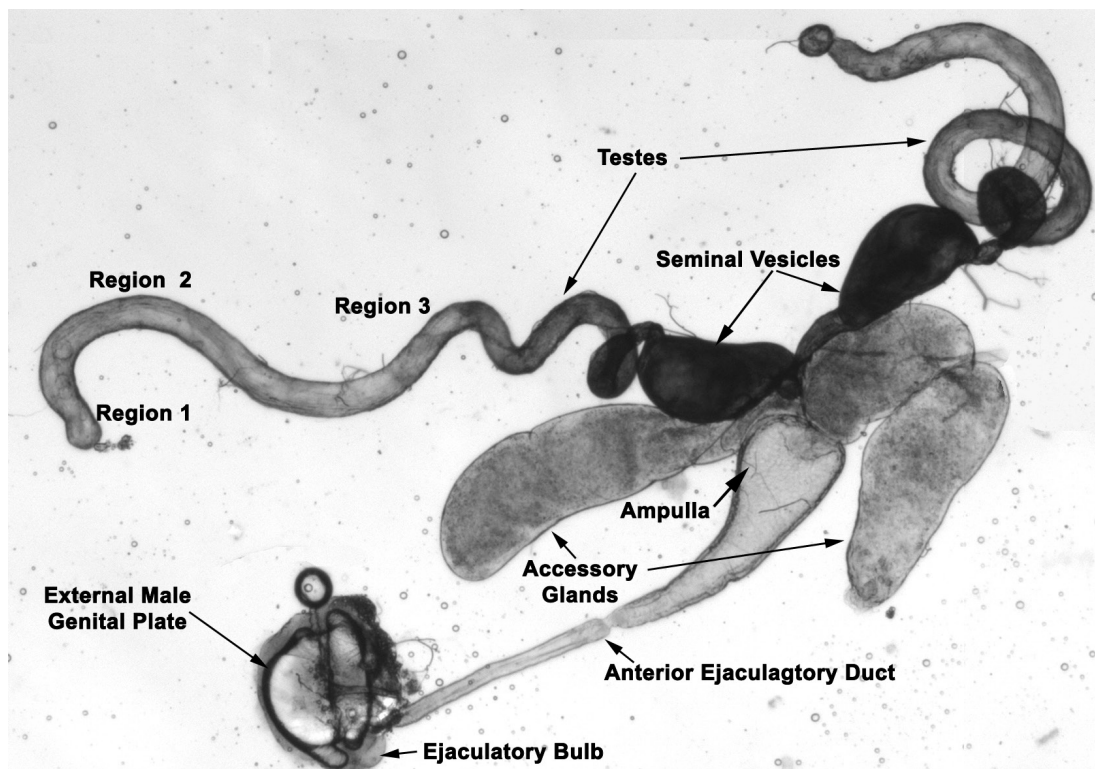


Fig. 1. Male reproductive system of adult *Drosophila melanogaster*. Light micrograph image of five-day old adult male reproductive organs that include a pair of coiled testis connected via the seminal vesicles to the ampulla of the anterior ejaculatory duct which becomes the posterior ejaculatory duct beyond the location of a pair of ejaculatory bulbs. A pair of large accessory glands also opens in the ampulla of the anterior ejaculatory duct. Regions 1-3, marked on testis, indicate the location where a tiny puncture is made in the testis wall to get higher frequencies of cells of desired spermatogenesis stage.

Objective

To study the changes in nuclear and mitochondrial organization during spermatogenesis in *Drosophila*.

Materials Required:

- A. **Fly stocks:** Wild type and other mutant flies, as desired.
- B. **Equipment and other materials:**

- i. Stereo-binocular microscope.
- ii. Phase contrast microscope (optional: fluorescence/confocal microscope).
- iii. Dissecting needles, forceps, fine hairbrush and other fly handling items.
- iv. Slides and Coverslips (22 mm²).
- v. Micropipettes and their tips.

C. Solutions and Reagents

- i. 1X PBS (pH 7.4):

Reagent	Concentration	Amount
NaCl	1.37 M	8.0 gm
KCl	27 mM	0.2 gm
Na ₂ HPO ₄	100 mM	1.44 gm
KH ₂ PO ₄	18 mM	0.24 gm

Serially dissolve the above salts in 800 mL of distilled water (dH₂O). Adjust the pH to 7.4 and make up the volume to 1 L with dH₂O. Autoclave and store the 1X PBS at room temperature (RT) or at 4°C (for long-term storage).

- ii. 4% Paraformaldehyde (PFA): Dissolve 4.0 gm PFA in 75 mL 1X PBS at 60°C with intermittent stirring for 10 min. Slowly add 5N NaOH to the cloudy mixture until a clear solution is formed. Filter the solution with a Whatman filter paper (no. 1) and adjust the pH to 7.0 with HCl or NaOH. Make up the volume to 100 mL with 1X PBS and store in small aliquots at -20°C. Thaw an aliquot at room temperature just before starting the experiment and consume it on the same day of experiment.

Notes:

- a PFA is a polymer with a wide range of monomeric and oligomeric units of formaldehyde, which depolymerises to monomeric formaldehyde form when dissolved in water. Formaldehyde fixes the cellular constituents by crosslinking proteins, especially those with lysine.
- b Formaldehyde is a toxic chemical and, therefore, always wear mask and gloves while handling PFA.
- c It is preferred that small volumes of PFA solution are prepared fresh, as required. A long storage of formaldehyde leads to its polymerisation in paraformaldehyde form which reduces its fixative properties.

Experimental details:

A. Dissection of adult testis (Fig. 2)

1. Lightly anesthetize freshly emerged healthy flies of desired genotypes using diethyl ether or CO₂ (see Chapter 1).

2. Using a fine hairbrush, transfer 2-3 male pharate/adult flies on a clean dry slide kept on the black background plate of a dissecting stereo-binocular microscope.
Note: Testes from pharate or 0-2 days old flies contain high numbers of pre-meiotic and post-meiotic cells but fewer elongated spermatids/sperms which becomes convenient for live study of nuclei and mitochondria in spermatogonia, spermatocytes, and early post-meiotic spermatids. On the other hand, 3-5 days old flies are good for examination of mitochondrial dynamics in elongating spermatids or mature sperm.
3. Remove the wings of flies with fine forceps to prevent the fly from floating in the liquid drop during dissection (removal of wings may not be necessary in the case of pharate stage).
4. Add 200 microliter of 1X PBS on the fly to keep it submerged on the clean glass slide, with its ventral side facing up.
5. Use one pair of forceps to grasp the fly at thorax and another pair of forceps to grasp the hard and black external male genital plate (Fig. 2A, [see Chapter 45](#)).
6. Pull the external genital plate to detach it from abdomen so that the internal male reproductive organs (testes, seminal vesicles, accessory glands and the ejaculatory duct) get pulled out with the external genitalia and separated from the abdominal cuticle (Fig. 2B-D). If the internal reproductive organs do not get pulled out with the external genitalia, use a pair of forceps to tease them out of the abdomen and remove the cuticle pieces, gut etc (Fig. 2E-H).
7. Remove debris and replace the PBS with fresh drop (~50 μ L) of PBS. Examine the dissected out male reproductive system under the stereo-binocular microscope to familiarize with its different components.
8. Using fine forceps or needles, cut away the accessory glands from the coiled and yellowish (in wild type) testes (Fig. 2E-H).
9. Examine testes, without placing a coverslip, under 10X objective of a microscope using phase-contrast optics to get familiar with different regions of testis (Fig. 3A, B).

B. Sample preparation for live imaging of germline cells in testis:

1. Set up the phase-contrast microscope to ready state before preparing a slide with live testis cells.
2. Carefully cut away the seminal vesicle and transfer the testis, with a pair of fine needles to a fresh slide in 50 μ L of 1X PBS.
Note: Seminal vesicles are full of mature sperms. Any damage to their walls releases huge number of actively moving mature sperms which hinder examination of the earlier spermatogenesis stages.
3. Using fine needles, tear open the testis at a specific point (see Fig. 1) to get higher frequency of desired male germline cell types, and soon thereafter, gently lower

a coverslip on the testes without application of any additional pressure. The cells within testis egress from the torn region onto the slide as soon as a coverslip is placed on the tissue.

Note: Tearing at apical region (Region 1 in Fig. 1) will result in a higher frequency of spermatogonia and early primary spermatocytes to egress first while spermatocytes and spermatids will egress with high frequency when tearing at the Region 2 (Fig. 1). On the other hand, opening the testis closer to the highly coiled basal region (Region 3, Fig. 1) releases more of the elongating spermatid sperms bundles and mature sperms.

4. Remove excess liquid present under the coverslip with help a small piece of dry tissue/filter paper placed close to one of the edges of coverslip. This helps in optimal flattening of testis and egression of cells from within the testis.
5. Immediately examine live testis cells using 10X/20X objective of a phase contrast microscope. After selecting the desired field under low power, change to 40X (dry) or 100X (oil immersion) phase-objective for a more magnified and resolved view of cells (Fig. 3C-L).

Notes:

- a. Microscopy should be completed within 30-45 min at 24°C before cells begin to die.
- b. Since the meiotic and post-meiotic cells within a cyst remain interconnected because of incomplete cytokinesis, some cells appear 'multinucleate' because of fusion of cell membranes of the interconnected cells following pressure of the coverslip (Fig. 3M).
- c. For viewing mitochondria more clearly, the Mitotracker Red-CMXRos, (peak excitation at 579 nm and peak emission at 599 nm), a lipophilic cationic fluorescent dye that specifically stains live mitochondria by utilizing their membrane potential, can also be used for fluorescence microscopic examination of live cells. For examination of mitochondria in live testis cells, transfer freshly dissected testes in Mitotracker stain solution (0.1µg/mL, dissolved in DMSO) and after tearing the testis at the desired region, immediately place a coverslip on the tissue in the same solution and examine the mitochondrial fluorescence, using appropriate excitation and emission filters. Higher concentration or extended exposure (>10-15 min) to Mitotracker can result in nonspecific binding with other cell organelles.

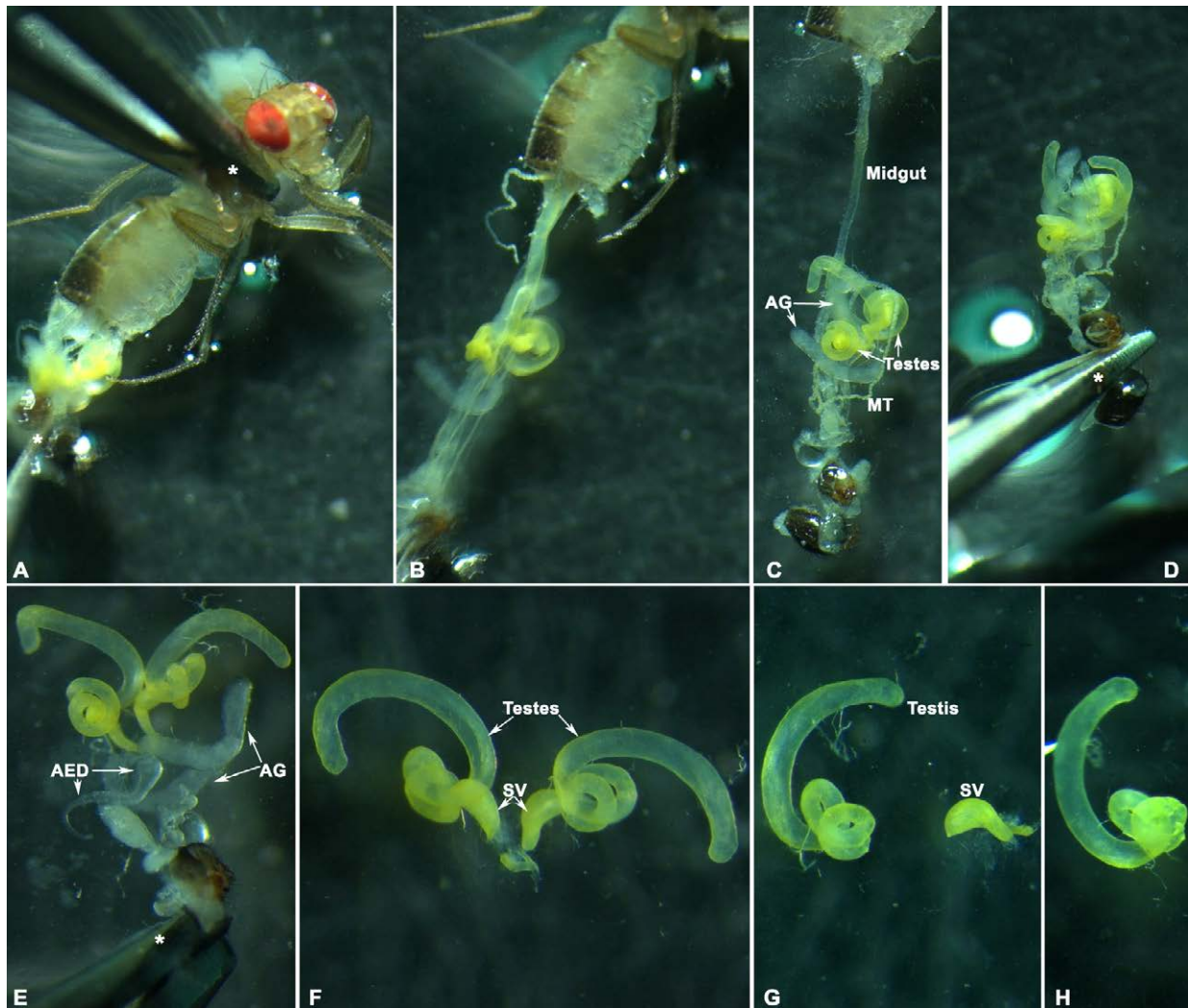


Fig. 2 : Dissecting out testes from young fl . A-D. Steps in pulling out of the reproductive organs from abdomen of male fly using two pairs of forceps (marked with white *) placed at thorax and the external male genital plate, respectively (A); D shows emergence of the reproductive organs from abdomen when the black chitinous abdominal tip (hard external male genital plate) is pulled with forceps (* mark). E-F. Sequential removal of the external male genital plate (E), accessory glands and ejaculatory duct (F), and the seminal vesicle (G) to keep only the testis (H) on slide. AED = anterior ejaculatory duct; AG = accessory glands; MT = Malpighian tubules; SV = seminal vesicles.

Observations:

Examine the complete male reproductive system to familiarize with all the components (Fig. 1).

Examine the freshly mounted live testis under phase-contrast optics using 10X/ and 20X objective to familiarize with locations of the different cell types in the intact testis using the images in Fig. 3B as guide.

Use 40X dry and 100X (oil immersion) phase objectives to identify the different male germ cell types like spermatogonia, primary spermatocytes, round spermatids, and bundles of elongating spermatids/sperms (Fig. 3C-K). Note the changes in nuclear size and chromatin morphology in different cell types.

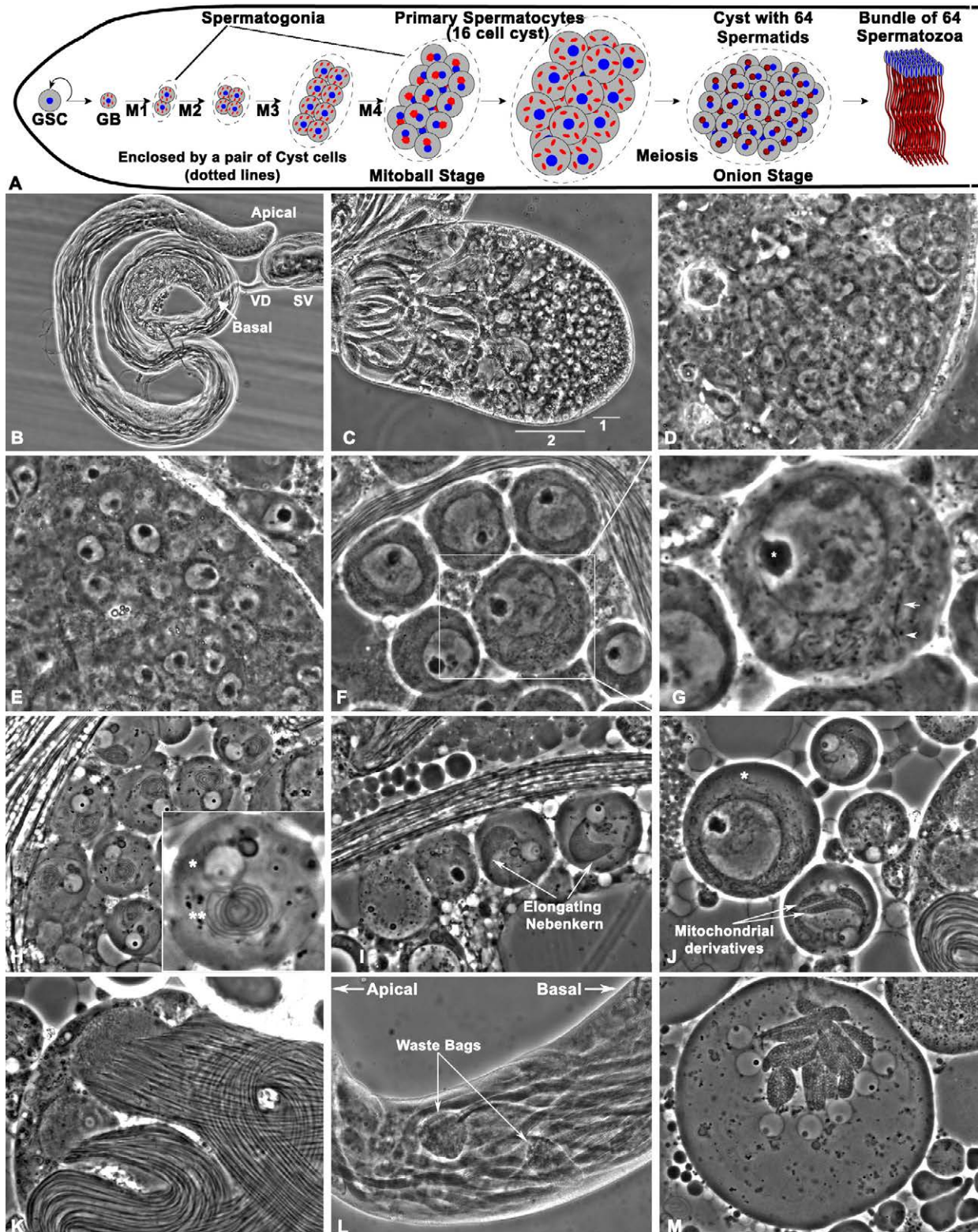


Fig. 3. Phase-contrast microscopy of live testis shows remarkable changes in morphology and organization of cells, nuclei, and mitochondria during spermatogenesis in *Drosophila*. **A.** Schematic representation of the germ cell lineage during spermatogenesis: GSC = germline stem cells; GB = gonioblast; M1-M4 = gonial mitotic divisions; mitochondria in red and nuclei in blue. **B.** Intact testis with the short vas-deferens (VD) at its basal end that leads to the seminal vesicle (SV) (seen under a 5X objective). **C.** Apical region of testis (20X objective): Region 1 houses the germ cell niche and gonial cells while spermatocytes are predominant in the Region

2. **D-F.** Enlarged views (100X oil objective) of apical region that houses niche and gonial cells (**D**), early primary spermatocytes (**E**), mid and late primary spermatocytes (**F**); the boxed late spermatocyte is further enlarged in **G**, where white * marks the very dense nucleolus; the thread-like structures in the large nucleus are transcriptionally active extended regions of the Y-chromosome; white arrow and arrowhead in the cytoplasm point to elongated and rounded mitochondria, respectively. **H-J.** ‘Onion’ (**G**) and later stages of spermatids (100X oil objective) when the nebenkern associated mitochondrial derivatives elongate (**I, J**); inset in **H** is enlarged view of an ‘onion’ stage spermatid (white * and ** indicate the nucleus and nebenkern, respectively); a late primary spermatocyte visible in **J** is marked with white *. **K.** A bundle of 64 maturing sperms in a cyst (100X oil objective). **L.** Waste bags towards the apical side of testis at the tail ends of individualizing sperms (20X objective). **M.** An example of false multi-nucleate appearance of several post-onion stage spermatids (100X oil objective) due to coverslip pressure induced fusion of inter-connected cells in a cyst.

Identify the large number of small rounded or elongated dark and rapidly moving mitochondria in cytoplasm of spermatogonia and spermatocytes (Fig. 3D-G).

Follow the dramatic changes in mitochondrial morphology from early spermatids to onion-stage spermatids as the nebenkern is formed. As the spermatids begin to elongate, the nebenkern also changes its appearance to generate the elongated mitochondria which gradually align with the developing axoneme of spermatid/sperm tail flagellum (Fig. 3H-K).

Locate the ‘waste bags’ in intact testis (Fig. 3L).

Questions for self-study and interpretation:

1. What role is played by the non-dividing hub cells present at the apex of testis?
2. What is the significance of incomplete cytokinesis during gonial and spermatocyte divisions?
3. What is the function of cyst cells that enclose the differentiating germ cells to form a cyst?
4. Why is the heterochromatic Y-chromosome decondensed and transcriptionally active in primary spermatocytes?
5. Why is the number of mitochondria high in spermatocytes?
6. What is the significance of ‘nebenkern’ and why does its organization change as the spermatid begins its elongation phase.
7. What changes occur in the spermatid nucleus during formation of mature sperm?
8. How does the sperm flagellum develop?

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Chapter 108

Collection and identification of *Drosophila* flies

V. Shakunthala (drshakunthalav2@gmail.com)

Department of Zoology, *Drosophila* Stock Centre, University of Mysore, Manasagangotri, Mysore

Recommended Level: UG and PG

Sub-discipline: Animal Diversity/Ecology

Background:

Drosophila is a cosmopolitan species and is an excellent material to study questions related to diversity of species and it may help us to examine and understand several issues in population ecology. Some species are closely associated with human habitations and residences (e.g. fruits and vegetables in kitchen), fruit/vegetable shops and are often called as domestic. *Drosophila* species found in gardens, forests etc., are referred to as semi-wild/wild populations. At global level, more than 1500 species have been described and about 150 species of the *Drosophila* genus have been reported from India (Singh, 2015).

Objectives:

Collection of natural populations of *Drosophila* and taxonomic identification of some common species.

Materials Required:

A. **Flies:** As collected from field

B. **Equipment and other items:**

- i. Stereo-binocular microscope.
- ii. Insect Sweeping Net.
- iii. Ice cream cups.
- iv. Fruits (orange, apple, banana or any seasonal fermenting fruits).
- v. Baker's Yeast granules.
- vi. 250 mL milk bottles and vials with wheat cream agar media seeded with yeast (see [Chapter 1, Appendix I](#)).
- vii. Thread

- viii. Cotton.
- ix. Dissection needles and soft fine brushes.
- x. Glass plates.
- xi. Anaesthetic ether and etherizer.

Experimental Details:

A. Collection of natural populations of *Drosophila*: Collect domestic (associated with human inhabitation like kitchen, fruit/vegetable shops) and/or wild/semi-wild species using the following one or more of the following collection procedures:

- i. Trap flies on fermenting banana pieces kept in a 250 mL milk bottle placed in kitchen, garden or hung on tree branches (**Fig. 1A, A', F and G**).

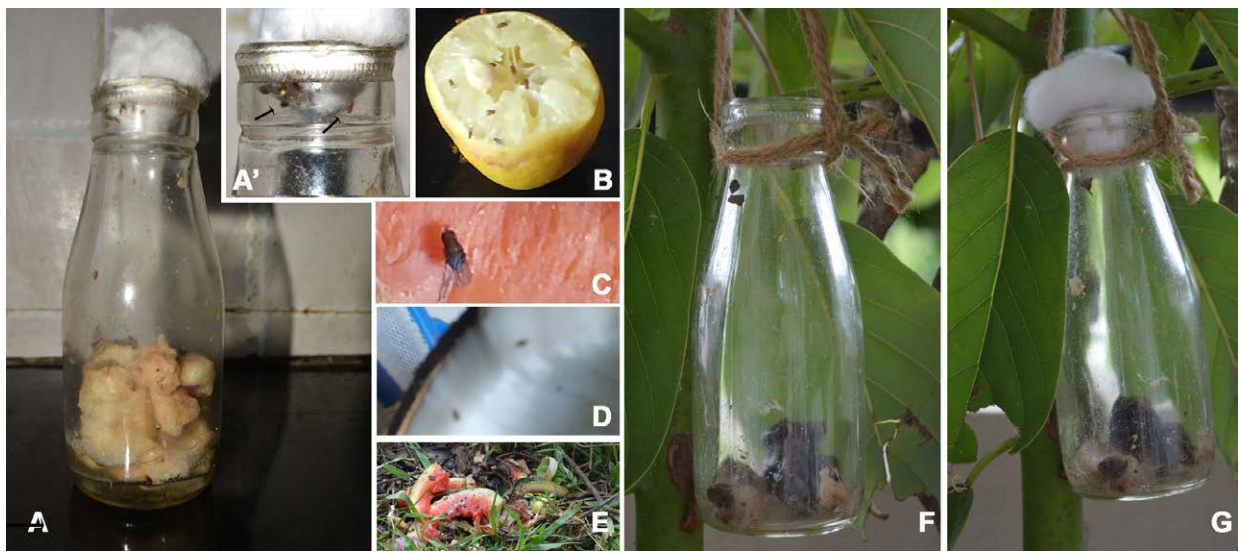


Fig. 1. Collection of flies from natural populations. A. Banana Trap, A'. Flies trapped in the bottle, B. Flies sitting on cut lemon in kitchen. C. Flies on a carrot piece, D. Flies on cut coconut. E. Fruits bait spread on the ground. F. Bait set on a tree for trapping flies. G. Flies trapped in the bait.

- ii. Spread fermenting fruits/vegetables in kitchen or in garden/forests (in cool shaded areas) for one/two days (**Fig. 1B-E**). Trap the flies visiting them by net (**Fig. 2**) sweeping.



Fig. 2. Insect sweeping net

- iii. Transfer the trapped flies to food bottles containing wheat cream-agar medium and bring to the laboratory.

B. Identification of flies

Flies collected and brought to the laboratory would be a mixture of males and females of different species. They need to be taxonomically identified as follows:

- i. As a first step, sequentially identify the taxonomic characters for the Phylum Arthropoda, Class Insecta, Order Diptera and family Drosophilidae, followed by the unique characters for the genus *Drosophila*.
- ii. Identify flies belonging to the family Drosophilidae by the presence of plumose (feathery) arista, bristles on head and thorax, and pattern of wing venation.
- iii. Identify *Drosophila* species by their small size (2-4 mm long) and typical pale yellow to reddish brown to black body colour and red eyes.
- iv. Since males of different species can be more easily identified because of their sex-comb (see [Chapter 44](#)) and their genital plate (see [Chapter 45](#)) than females, set up iso-female cultures from the collected flies. Keep single females, inseminated in nature, in vials with food seeded with yeast. The progeny in each vial will have males and females of one species.
- v. Identify *Drosophila* species of the iso-female progenies using the major features of some of the common *Drosophila* species found in India as given in **Table 1**.

Table 1. Significant morphological features used for identification of some common species of *Drosophila*

<i>Drosophila</i> Species	Abdominal pigmentation	Sex comb on the first pair of legs in males (meta-tarsal segment)	Male genital plate
<i>D.melanogaster</i> Domestic	Only males have dark abdominal pigmentation	One row of sex comb with 10-14 teeth.	One - primary clasper with 15-20 thick bristles
<i>D.ananassae</i> Domestic	Insignificant difference between males and females in pigmentation.	No sex comb	Two clasps. Primary clasper with two sets of teeth and secondary clasper with one long curved and 4 small teeth.
<i>D.rajasekari</i> Wild/semi wild	Males : Posterior segments darkly pigmented. Only males have a Dark patch on the wings.	Two rows, proximal row with 6-7 teeth and distal with 3 teeth.	One Primary clasper roughly triangular and bent anteriorly with 6-7 medium size teeth.

<i>D. nasuta</i> Wild/Semi wild	No abdominal pigmentation in males and females. Males: Head with silvery whitish frons.	No sex comb, Femur of the first pair of legs has a row of characteristic cuniform bristles	Primary clasper with 7-8 short cone-shaped setae
<i>D. bipectinata</i> Wild/Semi wild	Insignificant difference between males and females in the pigmentation	Two obliquely placed sex combs with 4-6 teeth in the proximal and 4-8 teeth in the distal comb.	Primary clasper with small bristles while secondary clasper with only one pointed teeth.
<i>D. malerkotliana</i> Wild/Semi wild	Insignificant difference between males and females in pigmentation.	Sex comb in 2 sets: proximal with 1-4 brown teeth and distal comb with two transverse rows of brown teeth.	Genital arch is elongated and broad in the middle. Primary clasper with two sets of teeth: 2 anterior and 3 posterior. 12 Marginal bristles. Secondary claspers with single long tooth.
<i>D. jambulina</i> Wild/Semi wild	Insignificant difference between males and females in pigmentation except two posterior most tergites being darkly pigmented in male	Sex comb in two sets: proximal with 22-24 dark teeth and distal comb with 12-15 teeth.	Primary claspers with 4-5 teeth and with marginal bristles. Secondary claspers with 3 long black curved teeth.
<i>D. agumbensis</i> Wild/Semi wild	Abdominal tergites of male shiny yellow, posterior most darkly pigmented. In female, tergites with posterior dark bands.	Two sets of sex comb. Proximal comb with 18 teeth and distal comb with 13 teeth.	Primary clasper with laterally located six teeth. Secondary clasper with 3 large curved teeth.

<i>D.nagarholensis</i> Wild/Semi wild	Female has yellowish broad apical band. In males, the posterior most tergites are shiny dark.	Two sets of sex-comb. Proximal set with 28-30 teeth and the distal set with 20 teeth.	Primary clasper with laterally arranged 4-5 pointed teeth. Secondary clasper carries three large bristles.
<i>D.kikkawai</i> Wild/Semi wild	Yellow tergites with dark band in posterior part of each tergite in female and pale band in male.	Two sets of sex comb. Proximal set with 22-26 teeth and the second set with 17-24 teeth.	Primary claspers with 4-6 dorsal teeth and a cluster of 7 ventral bristles. Secondary clasper, fused to anal plate, consists of strong median tooth and a few smaller ones below.

Reference can also be made to publications on keys for identification of *Drosophila* species (Strickberger, 1962; Gupta, 2005). Additional help can also be sought from appropriate fly researchers working on taxonomic aspects in India.

1. Populations collected from domestic areas:

The fly population from domestic areas in India usually include *D. melanogaster* and *D. ananassae*. At higher altitudes species like *D. nigra*, *D. repleta*, *D. immigrans* may also be trapped. Flies can be collected throughout the year. Record the total number of flies of each species collected and the numbers of males and females in each case.

2. Populations collected from forest, garden etc (non-domestic):

The species diversity from these areas is more than in domestic collections. The number of species may vary and depends on the geographic location and season of the year. Some of the common wild/semi-wild species are: *D. malerkotliana*, *D. nasuta*, *D. bipectinata*, *D. rajasekari* etc. Record the total number of flies collected for each species and numbers of males and females in each case.

Questions for self-study and interpretation:

1. What are quantitative and qualitative differences between i) domestic, semi-wild and wild populations; ii) urban v/s rural populations; iii) from different geographic areas, and iv) from different altitudes?

2. Are seasonal fluctuations in population sizes and species diversity expected?
3. Is there any preference noted for different fruits/baits, sugars, odors etc.?
4. How does species diversity relate with habitat diversity?

References and further reading:

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Chapter 109

Comparison of some life history traits in sympatric *Drosophila* species

V. Shakunthala (drshakunthalav2@gmail.com)

Drosophila Stock Centre, Department of Studies in Zoology, Manasagangotri, Mysore-6, Karnataka

Recommended Level: UG and PG

Sub-discipline: Ecology/Evolutionary Biology

Background:

Life history traits are important from the population ecology perspective as they reflect the ‘species’ or population’s reproductive strategies. Life history traits are critical components of fitness and frequently reflect adaptive responses to environmental pressures. Some of the life history traits are age distribution in a population, lifespan, fecundity, viability of eggs, viability of larvae, development time, body size, stress resistance, etc. (Annalise, 2009; Behrman *et al.*, 2015). Many of the natural populations of *Drosophila* are found to be heterogeneous assemblages of different species. Depending on the geographic area of study/season, one can witness coexistence of several species of *Drosophila* in the same habitat. These species may use common resources for feeding, mating, breeding and for their existence. Such a sympatric association of species facilitates inter-species interactions and competitions, and will have an impact on the life history traits and the fitness of species.

Objectives:

To undertake a comparative study of life history traits like female fecundity, egg to adult developmental time and egg to adult viability during intra- and inter- species competitions in sympatric species of *Drosophila*.

Materials Required:

A. Flies: select any two sympatric species of *Drosophila* collected from domestic (e.g., *Drosophila melanogaster* and *D. ananassae*) or wild/forest areas (e.g., *D. nasuta*, *D. malerkotliana* and *D. rajasekari* or other species available in the region). If need be, cultures can also be obtained from other fly labs (e.g., *Drosophila* Stock Ctr at Mysore University). Avoid species which are morphologically indistinguishable. For the present chapter, the two selected sympatric species are labelled as ‘Species A’ and ‘Species B’.

B. Equipment and fly handling items

- i. Stereo-binocular microscope

- ii. Bottles and vials with fly food, seeded with extra yeast paste
- iii. Petri dishes with egg-collection medium (see **Chapter 1**)
- iv. Cotton
- v. Needles and fine hair brushes
- vi. Glass plates
- vii. Anaesthetic ether and etherizer,
- viii. Aspirator

Experimental details:

A. Assay for fecundity of females:

- i. Isolate virgin females (see **Chapter 1**) of Species A and Species B, and after ageing them for 5 days, organise 20 sets of pair mating by placing one male and one female of the given species in each vial containing equal amount of food seeded with yeast. Transfer the pair of flies without etherisation from one vial to fresh food vial with every 24 hr for the next 10 days.
- ii. Record the number of eggs laid in each vial over the period of ten days for the 20 sets for each species.
- iii. Calculate the total number of eggs laid by 20 females of both the species. Compare the fecundity of species with appropriate statistical tests.

B. Egg to adult development time:

- i. Egg collection: Take equal numbers of 5-6 days old flies of both species and separately collect eggs over a 2 hr period (for egg collection method, see **Chapter 32**). Count the total number of eggs laid by females of each species and distribute these eggs into vials with normal food and yeast paste (see steps ii and iii below for numbers of eggs in a single food vial). Number and label the vials for 'Species A' and 'Species B'.
- ii. For intra-specific competition: Place eggs of only the Species A or Species B (pure cultures) in a given food vial at two different densities, namely 100 eggs/vial (A1, A2 and A3; B1, B2 and B3), and 200 eggs/vial (A4, A5 and A6; B4, B5 and B6). Maintain a minimum of three vials at each density for each species. Consequently, there will be two sets (Species A and Species B) of 'Pure cultures' in triplicate at each eggs/vial density.
- iii. For inter-specific competition: Place eggs of two species together in each vial (mixed cultures) at different densities. For each set maintain a minimum of three vials. In one set, keep 50 eggs of each species (total 100 eggs/vial; AB1, AB2 and AB3), and in another set, 100 eggs of each species (total 200 eggs/vial; AB4, AB5 and AB6).
- iv. When the flies start emerging, record the numbers of flies eclosed on each day and calculate the mean developmental time in days for the species under study at different densities in pure and mixed cultures. Present the data graphically and statistically compare the mean development times at different densities under intra- and inter-species competitions.

C. Egg to adult viability:

i. Collect the data for this from the same set of vials that are used above for determining the time of egg to adult development.

ii. Record the total number of flies of species A and species B emerged at different densities of eggs (A1, A2 and A3); (B1, B2 and B3) and (A4, A5 and A6); (B4, B5 and B6). Compare the total number of flies emerging for each species under the two egg density conditions in intra- and inter-species competitions. The total number of eggs in the 3 sets of pure cultures will be 300 and 600 at the two densities. Similarly, in the mixed cultures (inter-specific competition), it will be 150 eggs of species A and 150 of species B in the lower density sets while at the higher density, it will be 300 eggs of species A and 300 eggs of species B.

iii. Calculate the ratio of total eggs to total flies that emerge in each set to determine the egg to adult viability of species under intra- and inter-specific competitions. Statistically compare the egg to adult viability in different sets.

Note: An additional variable in these experiments may be introduced by rearing cultures at different temperatures e.g. 22°C or 25°C or 29°C. Likewise, nutrient variations may also be introduced, e.g., using different sugars or species of yeast in the food medium.

Questions for self-study:

1. Were there any differences in the fecundity, egg to adult development time and egg to adult viability between intra- and inter-specific competition conditions? What can you infer from these results about conditions in nature when these two species co-exist?
2. Is the body size of flies developing under different densities same or different? What are its implications for fitness of populations?
3. What is the relative importance of life history traits on overall fitness of species?

References and further reading:

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Evolutionary Genetics from Prediction to Validation

Genetics is the first biological science which got in the position in which physics has been in for many years. One can justifiably speak about such a thing as theoretical mathematical genetics, and experimental genetics, just as in physics. There are some mathematical geniuses who work out what to an ordinary person seems a fantastic kind of theory. This fantastic kind of theory nevertheless leads to experimentally verifiable prediction, which an experimental physicist then has to test the validity of. Since the times of Wright, Haldane, and Fisher, evolutionary genetics has been in a similar position.

(In: Provine, W. B. 1989. Sewall Wright and Evolutionary Biology. Univ. Chicago Press. P. 277)

Chapter 110

To estimate competitive fitness of *Drosophila* species

V.Shakunthala (drshakunthalav2@gmail.com)

Drosophila Stock Centre, Department of Studies in Zoology, Manasagangotri, Mysore-6, Karnataka

Recommended Level: UG/PG/Research

Sub-discipline: Evolutionary Biology

Background:

Competition is an important dimension of population ecology. Intraspecific competition is among individuals of the same species while interspecific competition is between individuals of different species. These interactions have profound effects on fitness of species. Many species of the genus *Drosophila* coexist in nature and may compete for similar resources for feeding, breeding, and for their existence. Darwin (1859) stated “As the species of the same genus usually have, though by no means invariably, much similarity in habits and constitution, and always in structure, the struggle will be more between them if they come into competition with each other than between species of different genera”. Competitive interactions can have large effects on fitness and, therefore, are likely to drive adaptive divergence within species (Hargreaves *et al.*, 2020). Such effects need systematic analysis. The present experimental protocol is designed to explore intra- and inter-specific competitive interactions under laboratory conditions.

Objective:

To examine competitive fitness of species in terms of overall productivity and population size during intra- and inter- species interactions, and impact of inter-species competition on their fitness parameters.

Materials Required:

A. **Flies:** Cultures of two species of *Drosophila* collected from nature.

B. **Equipment and fly handling items**

- i. Stereo-binocular and bright-field microscopes
- ii. Food bottles
- iii. Cotton

- iv. Needles and brushes
- v. Glass plates
- vi. Anaesthetic ether, etherizer and re-etherizer

Experimental Details:

A. Productivity and population size during Intra-species competition:

Establish cultures from *Drosophila* flies collected from nature (kitchen or garden) and identify the species. Alternatively, cultures of different species can also be obtained from other fly labs that maintain them. Maintain the experimental populations by adapting the Serial Transfer Technique (Ayala, 1966; Ranganath and Krishnamurthy, 1986), which brings overlapping generations together during the experimental period.

For intra-species competition, maintain two 'pure cultures' of any two *Drosophila* species (designated here as species 'A' and species 'B').

- i. Place 5 days old 25 males and 25 females of species 'A' and 'B' in separate bottles with standard yeast seeded food (bottle nos. **A1** and **B1**, respectively).
- ii. After one week, etherise the flies in each bottle (**A1** and **B1**) and record the numbers of males and females that have survived in each case.
- iii. Transfer the surviving flies to yeast seeded fresh food bottles (bottle nos. **A2** and **B2**, respectively). At this stage, bottle nos. **A1** and **B1** will have eggs and larvae but no adult flies.
- iv. Next week, count the numbers of males and females available in bottles **A2** and **B2** and transfer them to fresh food bottle nos. **A3** and **B3**, respectively.
- v. At the same time, check bottle nos. **A1** and **B1** for the progeny flies that would have emerged. Etherize them and record the number of progeny, males and females for each species. Transfer these new flies to bottle nos. **A3** and **B3**, respectively. The bottle nos. **A3** and **B3** thus contains young progeny flies from bottle nos. **A1** and **B1** the flies surviving in bottle nos. **A2** and **B2**, respectively. This is the population size of bottle nos. **A3** and **B3**.
- vi. After one more week, repeat the step v, i.e., count the numbers of males and females surviving in bottle nos. **A3** and **B3**, and transfer them to bottle nos. **A4** and **B4**, respectively. At this stage, the bottle nos. **A3** and **B3** will have eggs, larvae but no adult flies. At the same time, record the numbers of new born males and females from the bottles nos. **A1**, **A2**, **B1** and **B2**. Introduce these progeny flies to bottle nos. **A4** and **B4**, respectively. Thus the bottle nos. **A4** and **B4** contain young flies from respective bottles **1** and **2** as well as flies survived in bottle nos. **A3** and **B3**. These are the population sizes of bottles **A4** and **B4**, respectively.
- vii. After one more week, repeat the process, i.e., count the numbers of males and females survived in the bottles **A4** and **B4** and transfer them to the bottle nos. **A5** and **B5**, respectively. At this stage, the bottle nos. **A4** and **B4** will have eggs and larvae and no adult flies. Also check, bottles nos. **1**, **2** and **3** for each species, for new born flies. Record the number of males and females, and transfer them to bottle nos. **A5** and **B5**, respectively. Thus the bottle nos. **A5** and **B5** contain young flies from bottles **1**, **2** and **3** as well as flies survived in bottle nos. **A4** and **B4** for the respective species. These are the population sizes of bottles **A5** and **B5**.

Note: At this stage of the experiment, discard the bottle nos. **A1** and **B1** which have

completed four weeks and retain the bottle nos. **2, 3, 4** and **5** for each species to continue the experiment.

Summary schedule of transfers:

Date: 1 – Bottle (Bot) no1: Beginning of the experiment.

Date: 8 (after one week): Bot. 1 and Bot 2 for each species;

Date:15 (after two weeks): Bot.1, Bot 2 and Bot 3 for each species.

Date: 22 (after three weeks): Bot 1, Bot 2 Bot 3 and Bot 4 for each species.

Date: 29 (after four weeks): Bot 2, Bot 3, Bot 4 and Bot 5 for each species.

Note: Three to five replicates of the above schedule is advisable. Take care that bottles of different replicates do not get mixed up. Depending upon the facilities available, continue the experiment, following the weekly steps as above for eight or twelve weeks. Discard the bottles which have completed four weeks in the experimental regime, which means that at any one time, each replicate will not have more than four bottles.

B. Productivity and Population size during Inter-species competition:

- i. Select two sympatric species (**A** and **B**) and establish mixed cultures by introducing 5 days old 25 (12 males + 13 females) flies of each species into a single yeast seeded food bottle (bottle no.1).
- ii. Follow the steps ii-vii above for intra-species competition. During each weekly change, record the numbers of males and females of each species.
- iii. Maintain the experiment for 8 to 12 weeks or till a complete elimination of one of the competing species, whichever is earlier.

Results:

For each of the above two sets of experiments, calculate the following:

- i. Productivity of each species **A** and **B**: calculate the mean Productivity per week, with Productivity being the sum of new born flies in each week.
- ii. Population size for each species **A** and **B**: calculate the total population size in the last bottle of respective species at the end of the experiment (8 or 12 weeks) and the mean population size per week for species **A** and **B**.
- iii. In each case, statistically compare the mean values of productivity and population size of species **A** and **B** and present these graphically.

Note: The above data provide weekly oscillations in productivity and population sizes for the two species. Productivity is a comprehensive measure of outcome of many processes like the prevailing adult population density, fecundity, pre-adult crowding, egg to adult viability, speed of development, changing constitution of the food during the experimental period etc. The overall population dynamics, i.e., weekly fluctuations in productivity and population sizes during the experimental period reflect intra-species and inter-species competitions.

Further Studies:

1. The above experiments may be carried out at different temperatures, for example 22° or 25 ° or 29 °C.

2. Experiments may also be conducted using different nutritional conditions viz., varying quantity or quality of sugars, species of yeast etc.

Questions for self-study and interpretation:

1. What factors affect a correlation (positive or negative) between productivity and population size of a given species?
2. Compare the reproductive fitness of species **A** and **B** during intra- and inter-specific competition. If this is different, what may be possible reasons for the difference?

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Chapter 111

Demonstration of reproductive isolating mechanisms (pre-zygotic and post-zygotic) between two closely related and sympatric species of *Drosophila*: *D. bipectinata* and *D. malerkotliana*

B. N. Singh (bnsingh@bhu.ac.in)

Department of Zoology, Institute of Science, Banaras Hindu University, Varanasi, India

Recommended Level: Research

Sub-discipline: Evolutionary Biology

Background:

Dobzhansky (1937) coined the term “Isolating Mechanisms” for the first time for all the factors which alone or in combinations prevent interbreeding of closely related species and thus prevent gene exchange between them since evolution is not possible without reproductive isolation. Speciation (cladogenesis) is defined as origin of new species through splitting of pre-existing ones. During the process of speciation, genetic changes accumulated in the populations lead to origin and development of reproductive isolating mechanisms which are considered important and pre-requisite for the process of speciation. For the maintenance and stability of species, reproductive isolation is essential for preventing gene flow between different species/populations. Reproductive isolating mechanisms are classified into different kinds: premating and postmating/prezygotic and postzygotic (Mayr, 1966; Dobzhansky, 1970; Chatterjee and Singh, 1989; Singh, 2014). *Drosophila* has been extensively used in the study of reproductive isolating mechanisms to show that species may have complete or incomplete isolation. Incomplete isolation may be symmetrical or asymmetrical, which have been used to decipher phylogenetic relationships and the direction of evolution among different species. The experimental process described here is used to demonstrate sexual isolation (pre-zygotic/pre-mating) and hybrid sterility (post-zygotic/post-mating) taking example of two closely related and sympatric (populations living in the same geographical area) species of *Drosophila*, viz., *D. bipectinata* and *D. malerkotliana* which are commonly found in India (Singh and Singh, 2001).

Objectives:

- (i) To test sexual isolation (pre mating/prezygotic) between *D. bipectinata* and *D. malerkotliana* by employing male-choice technique (placing two species females with one species male).
- (ii) To test hybrid sterility (post mating/postzygotic) between *D. bipectinata* and *D. malerkotliana* by making reciprocal crosses as well as backcrosses.

Materials Required:

1. Stocks of *D. bipectinata* and *D. malerkotliana*
2. Materials required for handling, culturing and maintaining flies.
3. Stereo binocular microscope

Experimental Details:

1. *D. bipectinata*, *D. malerkotliana* and their hybrids can be identified with the help of colouration of male abdominal tips and sex combs as described below (Fig. 1):

a. *D. bipectinata*: General body colouration yellow, each abdominal tergite with a dull brown, narrow posterior band. Male prothoracic legs with two obliquely placed sex combs on metatarsal segment, upper comb with about 5-8 teeth, lower one with 6-9 teeth, 1-2 teeth on distal part of first tarsal segment of the same leg.

b. *D. malerkotliana*: General body colouration pale yellow, each abdominal tergite with a black band except in male where terminal tergites completely black. Sex comb in two sets, upper metatarsal comb with two transverse rows of 1 and 3-4 tough bristles; lower tarsal comb with similar rows of 1 and 3 tough bristles.

c. Hybrids: General body colouration pale yellow, each abdominal tergite with a narrow black band, except in male where terminal tergites light black. Male prothoracic legs two two obliquely placed sex combs on metatarsal segment, upper one with about 3-5 teeth, lower one with 5-7 teeth, first tarsal with two transverse rows of 1 and 2-3 teeth.

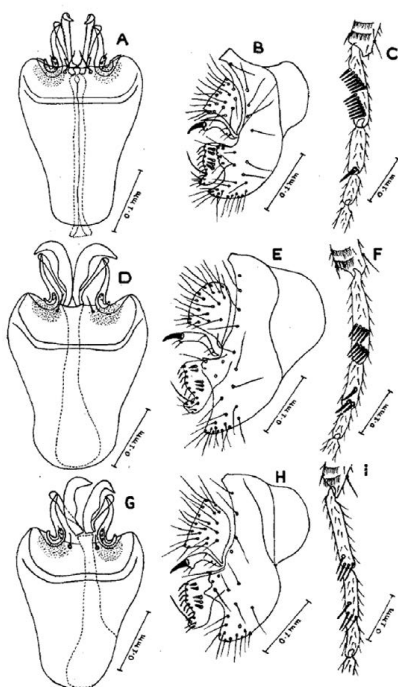


Fig. 1. Distinguishing features of the phallic organs (A, D and G), peripheral organs (B, E and H) and male prothoracic legs (C, F and I) of *D. bipectinata* (A-C), inter-specific hybrids of *D. bipectinata* and *D. malerkotliana* (D-F) and *D. malerkotliana* (G-I) (From J. P. Gupta, 1973, *Experientia* 29: 224-225).

2. Culture both the species separately in food bottles, collect virgin females and males in separate food vials and grow them for a few days prior to setting up crosses.
3. Male-choice technique is used to test sexual isolation between the *D. bipectinata* (bi) and *D. malaerkotliana* (ma). Accordingly, set up following crosses:
 - a. bi females + ma females + bi males
 - b. bi females + ma females + ma males

Females of both species and males of one species are kept in food vials (separate for each cross) for a week, following which the females are separated and placed individually in new food vials. The progeny flies in these vials are observed for the presence of parental species or hybrids. This permits scoring of homogamic and heterogamic matings.

4. To test hybrid sterility, two species are crossed and hybrids are tested for fertility. If no progeny is seen when the hybrid females and males are crossed, hybrid females and males are crossed with the parental species males and females, respectively, to know whether both sexes of the hybrid progeny are sterile or only one sex (female or male) is sterile.

Results/Observations:

1. Male Choice Experiments for measuring Reproductive isolation: On the basis of data obtained with respect to homogamic and heterogamic matings, Isolation Index is calculated to measure the sexual isolation using the following formula:

$$\text{Isolation Index (I. I.)} = \frac{\% \text{ Homogamic matings} - \% \text{ Heterogamic matings}}{\% \text{ Homogamic matings} + \% \text{ Heterogamic matings}}$$

If the **I. I.** is one, there is complete isolation. If **I. I.** is zero, there is no isolation. If heterogamic matings are more frequent than homogamic ones, the **I. I.** is negative. Incomplete isolation shows close phylogenetic relationship between the species.

2. Hybrid sterility test: If hybrids do not produce progeny, they may be sterile. The results of backcross will show whether both sexes are sterile or hybrids of only one sex (male or female) are sterile.

Questions for self-study and interpretation:

Besides the ‘male-choice’ method described here, learn about other approaches like ‘No-choice’, ‘multiple-choice’ and ‘female-choice’ to study sexual isolation (ethological or behavioural isolation). These approaches can be used to examine reproductive isolation between any two related species of *Drosophila*.

1. It is stated that race and species formation without isolation is not possible. Why is isolation so important?
2. Dobzhansky remarked “Nothing in biology makes sense except in the light of evolution”. Why is this stated?

3. What is the important key point in the Biological species concept, which is most widely accepted concept about species?
4. Genus *Drosophila* is rich in species diversity with more than 1500 species. Why have these species been so extensively used in evolutionary studies with particular reference to ethological isolation (pre-mating or pre-zygotic) and hybrid sterility (post-mating or post-zygotic)?
5. What are the different types of sexual isolation mechanisms?
6. Why is asymmetrical mode of mating preference or one-sided sexual isolation considered important and often used to predict the direction of evolution between species/strains?
7. There are two methods to measure the degree of sexual isolation between related species: Isolation Index and Isolation Estimate. How do they differ and how are the results interpreted?

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Chapter 112

Effect of natural selection, artificial selection and random genetic drift on allele frequencies in *Drosophila melanogaster* populations

Bimalendu B. Nath (bbnath@gmail.com)

Department of Zoology, S.P. Pune University, Pune-411007, India

Recommended Level: PG

Sub-discipline: Evolutionary Biology/Population Genetics

Background:

Towards the mid-twentieth century, a historic fusion of Mendelian genetics with Darwinian evolution marked the genesis of unified concept of evolutionary biology, popularly known as the ‘modern synthetic theory of evolution’. The fundamental tenets of the theory are based on the population genetics work of R.A. Fisher and Sewall Wright and seminal contributions of a number of scientists like Th. Dobzhansky, H. J. Muller, J.B.S. Haldane, E. Mayr, J. Huxley, G.L. Stebbins, among others (see Borges, 2005). Integration of genetics and evolutionary biology helped us to understand how populations evolve by natural selection acting on genetic variation. The synthetic theory of evolution has also clarified an old misconception regarding the ‘unit’ of evolutionary change. Now we understand that it is the populations that evolve rather than individuals. Accordingly, micro-evolutionary changes begin at the population level through changes in the frequencies of alleles and the chromosome configuration in any population. *Drosophila* has proved to be a useful model for many discoveries in population and evolutionary genetics. Theodosius Dobzhansky and his co-workers’ work on *Drosophila* provided the first insight into the natural selection and other evolutionary processes that shape genetic variation in a population (Nath, 2000). Laboratory based selection experiments require data from multiple generations comprising interbreeding individuals. *Drosophila* has an advantage in such experimental evolutionary studies by offering rapid generation time and availability of a large repertoire of mutants. Evolution is difficult to comprehend through short-term observation in the natural world around us. However, modeling an evolutionary process through simulated exercise in the laboratory is feasible. The protocol presented here will exemplify this.

The genetic make-up of a population’s gene pool may change over time. Several factors act to increase or decrease the frequency of alleles and drive the evolutionary changes. In view of this, one can conceptually deduce a population which is ‘non-evolving’.

Hardy-Weinberg (HW) principle describes a non-evolving, large, randomly (also called 'panmictic') as well as non-randomly mating population where allele frequencies remain stable over generations. Such stability of allele frequencies in a population is maintained primarily when there are no mutations, selection, migration and immigration (<https://www.nature.com/scitable/knowledge/library/the-hardy-weinberg-principle-13235724/>). Evolutionary forces act on the gene pool affecting allele frequencies by selective actions which can vary from time to time in direction and intensity. Therefore, evolutionary changes may not always be slow and gradual, but can also occur rapidly. When factors like natural selection, gene flow, genetic drift and human mediated artificial selection act on a natural population, the population violates the HW equilibrium. This initiates evolutionary changes. From the exercise presented here, one can infer how natural selection, artificial selection and random genetic drift alter allele frequencies over time in successive generations reflecting diverse mode and tempo of evolutionary changes.

In the broadest sense, 'natural selection' can be perceived in a population where environment determines which genotype/s are the most 'fit' to perpetuate while 'artificial selection' can be recognized where humans play the role to determine chosen genotype/s for propagation. Artificial selection is also known as selective breeding when humans (breeders) actively choose individuals of one genotype to be selected while remaining individuals of other genotype/s are selected against for breeding. The first part of the protocol described here employs both natural as well as artificial selection strategies and experimental design using wildtype and vestigial winged *Drosophila melanogaster* flies. In this exercise, one can get a glimpse of natural selection in action while comparing it with the outcome of artificial selection experiments. The experiments are carried out by performing crosses between normal winged (vg^+/vg^+) and vestigial winged (vg/vg) flies in the presence or absence of external factors to influence the genotype frequencies of vg^+/vg^+ and vg/vg flies in subsequent generations.

The second part of the protocol deals with the genetic drift (GD) that occurs as a result of any sudden fluctuations in the allele frequencies from one generation to the next. GD is common after a population 'bottleneck' caused by sudden environmental catastrophic events and habitat fragmentation (e.g. cyclone, flood, forest fire, earthquake, landslide etc.) leading to random mortality of individuals irrespective of their genotypes (i.e. in absence of natural and artificial selection). Such stochastic events cause random and sudden alternation in the allele frequencies. Thereafter, the residual chance-survivors constitute the 'founder population' and shape the genetic profile of future generations. In this exercise, GD in a population of *D. melanogaster* consisting of wildtype and vestigial wing genotypes is simulated by introducing a chance event leading to random mortality of flies. The effect on the allelic frequencies can be observed in successive generations thereafter. After completing the three different experiments to measure fluctuations in allelic frequencies under NS, AS and GD, the contrasting profile of allele frequencies under these conditions can be appreciated.

Objective:

To develop a basic understanding of natural and artificial selection, and genetic drift through laboratory based experiments using *Drosophila melanogaster* populations.

Materials Required:

A. Flies: Wild type *Drosophila melanogaster* and vestigial *vg/vg* mutant flies

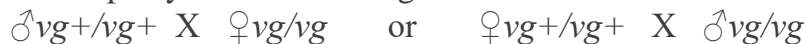
B. Equipment and other supplies:

- i. Stereo-binocular microscope.
- ii. Biological Oxygen Demand (B.O.D.) incubator or a room maintained at $23 \pm 1^\circ\text{C}$.
- iii. Culture bottles containing fly-food (large culture bottles with a volume of about 250 mL to accommodate up to 500-800 progeny flies).
- iv. Etherizer, re-etherizer, fine brush.
- v. Plastic fly-flapper.
- vi. Drawing board (12 inch x 18 inch)

Experimental Details:

In these experiments, wildtype and mutant (vestigial winged, *vg/vg*) flies are to be bred over a few generations in two different set ups, marked, respectively, as NS (natural selection experiment) and AS (artificial selection experiment).

- i. Set up any of the following crosses:



To initiate the cross, take a population of approximately 300 wildtype (*vg+/vg+*) and mutant flies (*vg/vg*) in 1:1 ratio (frequency of *vg+* and *vg* alleles in this population is 0.5 each).

- ii. Self-cross the F_1 *vg+/vg* (normal winged flies) flies using equal numbers of virgin females and males.

iii. Examine F_2 progeny and separate normal winged (*vg+/vg+* and *vg+/vg*) and vestigial winged (*vg/vg*) flies. Count and record the numbers to calculate the frequency of *vg* allele (**Table 1**). Since the population maintained under similar conditions is expected to be at Hardy-Weinberg (HW) equilibrium, the frequency of recessive allele *vg* can be calculated using HW formula (<https://www.nature.com/scitable/definition/hardy-weinberg-equation-299/>).

Table 1. Frequency of *vg* allele in F_2 generation

Date of scoring F_2 flies	Number of normal winged flies (A)	Number of vestigial winged flies (B)	Total number of flies (A + B)	Ratio A : B	Frequency of <i>vg</i> allele $q = \sqrt{[B/(A+B)]}$

iv. Use wild type and vestigial winged male and female flies to establish two sets of subsequent generations as follows: (a) **NS experiment**: breeding without any artificial selection (No Selection or NS set up) such that both normal and vestigial winged flies are reared and perpetuated randomly, and (b) **AS experiment**: breeding through artificial selection (AS) such that only normal winged flies are selected at each generation for parenting the next generation.

v. **NS Experiment**: The cross is set up using a total of 300 (approximate) flies. The ratio of wild type: vestigial winged male and female flies used in the cross should be 1:1 as observed in the F_2 progeny for generating the F_3 for NS experiment. Similar crosses are set up for all the successive generations (F_4 , F_5 and F_6 generations) and the results recorded in **Table 2** under the NS section.

vi. **AS Experiment**: For the AS experiment, discard the F_2 male and female vestigial winged (*vg/vg*) flies. Take only normal winged male and female (virgin) flies in equal numbers (total ~300 flies) to obtain the F_3 generation. Repeat this step with the same number of progeny flies to obtain F_4 , F_5 and F_6 generations. Record results in **Table 2** under AS section.

vii. **GD Experiment**: For the GD experiment, repeat the procedures of NS by setting up cross of wild type and vestigial winged male and female flies to obtain the F_3 generation. Once F_3 progeny flies have been counted and numbers and phenotypes recorded in **Table 2**, mix and spread all the anesthetized F_3 flies on a drawing board. In order to simulate a catastrophic event leading to large scale mortality of flies irrespective of their genotypes, either a) use a fly-flapper and strike randomly twice or thrice to crush many flies and remove the dead flies or b) randomly handpick a large number of flies from the mixed population spread on the board (preferably done by a blindfolded volunteer) and discard the rest. Quickly pick up all the live male and female flies (irrespective of their phenotypes) who survived the simulated catastrophic episode and put them in a fresh culture bottle irrespective of the population size. This is the ‘founder’ population for the F_4 generation. Score F_4 progeny resulting from this founder population and then continue setting up crosses for F_5 and F_6 as done for NS experiments above. Each time, take approximately 300 flies for setting up these crosses.

Table 2. Frequencies of *vg* allele in subsequent generations under NS, AS and GD conditions

Natural Selection (NS) Experiment

Date of scoring flies	Generation	Number of normal winged flies (A)	Number of vestigial winged flies (B)	Total number of flies (A + B)	Frequency of <i>vg</i> allele $q = \frac{B}{A+B}$
	F_2				
	F_3				
	F_4				
	F_5				
	F_6				

Artificial Selection (AS) Experiment

Date of scoring flies	Generation	Number of normal winged flies (A)	Number of vestigial winged flies (B)	Total number of flies (A + B)	Frequency of vg allele $q = \sqrt{[B/(A+B)]}$
	F ₂				
	F ₃				
	F ₄				
	F ₅				
	F ₆				

Genetic Drift (GD) Experiment

Date of scoring flies	Generation	Number of normal winged flies (A)	Number of vestigial winged flies (B)	Total number of flies (A + B)	Frequency of vg allele $q = \sqrt{[B/(A+B)]}$
	F ₂				
	F ₃				
	F ₄				
	F ₅				
	F ₆				

Observations:

1. Plot graphs for the frequencies of vg allele in each generation of NS and AS experiments, using X-axis for indicating generations (F₂, F₃ ... F₆) and Y-axis for calculating vg allele frequency.
2. Separately compare the frequencies of the vg allele at subsequent generations in the NS and AS experiments and the frequencies of the vg allele in all the three experiments (NS, AS and GD).
3. Explain the results.

Note: The protocol presented here can be modified in a number of ways using other mutants (Heil *et al.*, 2012) and can also be extended (Bixler and Schnee, 2005) to estimate if frequencies of vg⁺ and vg alleles show any deviations from HW equilibrium in NS and AS experiments.

Questions for self-study and interpretation:

1. In NS set up, there are no external factors that could be implicated for the selection operating against vg allele. Did you find the vg allele frequency in subsequent generations to remain similar to that in the F₂ generation? Why or why not?

2. In spite of removing vg/vg flies in every generation while carrying out the artificial selection experiments and allowing only normal winged flies to reproduce, did the frequency of vg allele reach a zero value over time? Using the trends seen in your results, how many generations of artificial selection may be required to obtain a population with vg^+ allele only?
3. On the basis of the results obtained in the **NS experiment**, predict the allelic (vg and vg^+), genotypic (vg^+/vg^+ , vg^+/vg and vg/vg) and phenotypic (wildtype and vestigial winged flies) frequencies in the progenies of the following crosses: (i) $vg^+/vg^+ \times vg^+/vg$ (ii) $vg^+/vg \times vg/vg$. What will be the ratios of wild type and vestigial winged flies if the progenies are self-crossed in each case? Attempt the same under the artificial selection experimental design given in the protocol and follow up till 6th generation. Explore and interpret the results.
4. Three different geographically distinct natural populations of *Drosophila melanogaster* have been surveyed by a team of research students and the frequency of *sepia eye* (*se*) allele (autosomal recessive) was monitored in the population over a period of twelve months. The estimation of *se* allele frequencies in the three populations is shown in the table below. Find out whether there exist any differences in the data sets in terms of the trends of *se* allele frequencies over months. If you do not find a difference then justify your inference using the HW principle. If yes, then suggest the most plausible cause/s which can lead to observed trends of *se* allele frequency in the population (assume that the Sepia eyed flies in the three populations exhibited no differences in their fitness compared to wild type flies regarding viability, lifespan, reproductive success and mating propensity in all months and seasons).

Month of sampling	Frequency of <i>se</i> allele		
	Population # 1	Population # 2	Population # 3
January	0.35	0.41	0.38
February	0.28	0.39	0.35
March	0.29	0.32	0.37
April	0.31	0.35	0.09
May	0.34	0.30	0.25
June	0.28	0.32	0.42
July	0.32	0.08	0.62
August	0.31	0.01	0.59
September	0.32	0.00	0.58
October	0.34	0.00	0.63
November	0.33	0.00	0.59
December	0.29	0.00	0.62

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From Observationalism to Experimentation

At the beginning of its existence as a science, biology was forced to take cognizance of the seemingly boundless variety of living things, for no exact study of life phenomena was possible until the apparent chaos of the distinct kinds of organisms had been reduced to a rational system. Systematics and morphology, two predominantly descriptive and observational disciplines, took precedence among biological sciences during the eighteenth and nineteenth centuries. More recently physiology has come to the foreground, accompanied by the introduction of quantitative methods and by a shift from the observationalism of the past to a predominance of experimentation.

(In: Dobzhansky, Th. 1937. *Genetics and the Origin of Species*. Columbia University Press)

Chapter 113

Allozyme variation in natural populations of *Drosophila melanogaster*

Bimalendu B. Nath* (bbnath@gmail.com) and Leena Thorat (leenathorat@gmail.com)

Department of Zoology, S.P. Pune University, Pune-411007, India

* For correspondence

Recommended Level: PG/Research

Sub-discipline: Population Genetics

Background:

Genetic variation for a trait in any natural population is contributed by a wide assortment of genes and alleles (<https://www.nature.com/scitable/ebooks/essentials-of-genetics-8/118523195/>). Extent of genetic variation is often pronounced amongst 'ecotypes', which are natural populations of a species inhabiting diverse geographical zones and adapted to local environmental conditions. In order to obtain a preliminary status of genetic heterogeneity at the population level, 'allozymes' have been used in many population genetic studies in both plants and animals.

In several cases, multiple forms of an enzyme are encoded in the genome. Allozymes are enzymes which are encoded by different alleles at the same locus in individuals from the same as well as different populations. Unlike allozymes, isozymes (also called isoenzymes) are multiple forms of an enzyme that catalyze the same reaction but are encoded by genes at different loci. Allozymes catalyze the same reaction but display different kinetic parameters because of small variations in the amino acid sequence. Consequently, they exhibit variable electrophoretic mobilities when separated by gel electrophoresis. Unlike allozymes, isozymes show restricted tissue/organ expression and specific physiological role. In order to distinguish allozymes and isozymes of a given enzyme, one can carry out isoelectric focusing and compare parameters of enzyme kinetics (e.g. K_m , V_{max}).

Electrophoretic mobility variations in allozymes are studied by population geneticists since it provides a direct reflection of underlying genetic polymorphism at a given gene locus (see [Chapter 66](#)). Nevertheless, it is to be noted that allozyme analysis has a limitation and cannot provide information on nucleotide level variation (e.g. silent mutations). In recent times, allozyme based assessment of genetic variation is mostly used for obtaining only the base-line assessment of ecotype based genetic heterogeneity. The various DNA-based genetic markers are commonly used (**Fig. 1**) to identify nucleotide level intraspecific variation in natural populations (FAO 2007).

In many population genetics and evolutionary studies, allozymes have been associated with adaptation of a species to its local environment. One such group of enzymes is L-lactate dehydrogenase (LDH, EC:1.1.1.27) which reduces pyruvate to L-lactate during glycolysis and oxidizes L-lactate to pyruvate during gluconeogenesis. In adult flies, LDH is present as homotetramer composed of four subunits of ~152 kDa size (Onoufriou and Alahiotis, 1982). *Drosophila melanogaster* adults express only one *Ldh* gene [https://flybase.org/reports/FBgn0001258.html#genomic_location] and thus LDH allozyme assay provides a quick reflection of electrophoretic profile at the *Ldh* loci between populations inhabiting two different ecotypes.

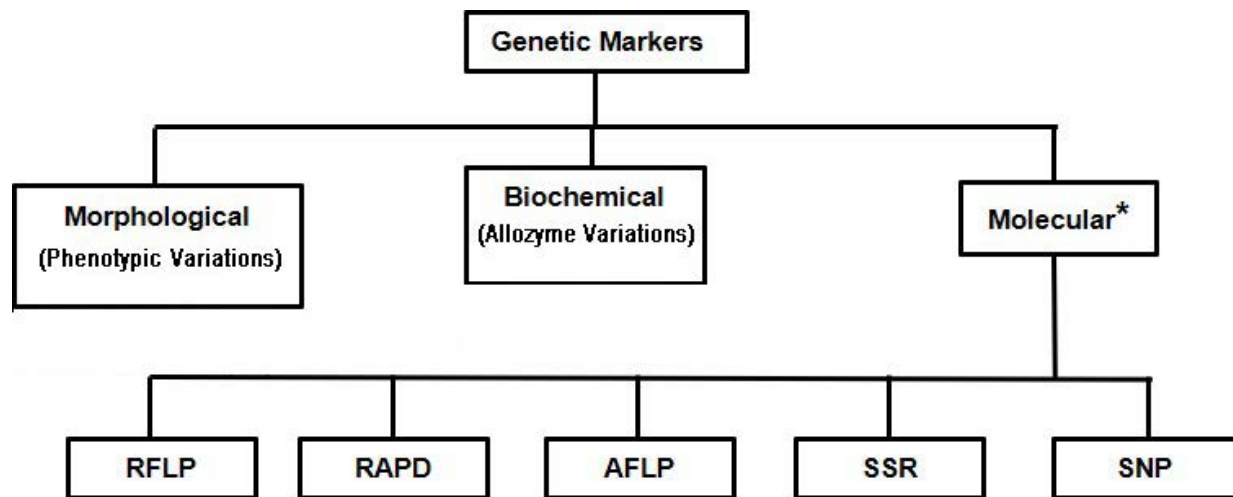


Fig. 1. Markers in population genetics: RFLP = Restriction Fragment Length Polymorphism; RAPD = Random Amplified Polymorphic DNA; AFLP = Amplified Fragment Length Polymorphism; SSR = Simple Sequence Repeat; SNP = Single Nucleotide Polymorphism; *Nonpolymorphic molecular markers: ESTs = Expressed Sequence Tag; STS = Sequence Tagged Sites.

Objective:

To detect Lactate Dehydrogenase (LDH) allozyme variations in same species of *Drosophila* flies from natural populations of two different ecotypes.

Materials Required:

A. Biological: Collect *Drosophila* flies from two geographical areas (preferably with distinctive ecological features) and select flies belonging to one desired species, based on the taxonomical keys (Markow and Grady, 2006). Flies from wild can be trapped by attracting adults to ripened fruit-baited (e.g. banana) collection bottles (see **Chapter 108**). Gravid females are separated and individually transferred to food vials and allowed to produce progeny for successive generations to produce 'Iso-female' lines (David *et al.*, 2005). Select samples of two such iso-female lines established from two different ecotypes for the experiment.

B. Chemicals:

i. Tris-glycine Buffer: pH 8.2 (dissolve 3.0 gm of Tris-Cl and 14.4 gm Glycine in 800 mL distilled water. Check pH of the buffer. It should be ~8.2; if required, adjust the pH using 1N NaOH or HCl. Make up the volume to 1 L with distilled water.

ii. Bromophenol blue (BPB): 0.25% BPB stock (dissolve 0.0125 gm BPB in 5 mL 85% glycerol)

iii. Agarose gel: 1% agarose gel (dissolve 0.5 gm agarose in 100 mL Tris-glycine buffer (sufficient for a small gel of 10 X 10 cm size) by heating up the mixture in a microwave (or on a heater) as described under 'Experimental details'.

iv. Phosphate-buffered saline (PBS): Add Sodium Chloride (1.37 M) 8.0 g, Potassium Chloride (27 mM) 0.2 g, Disodium hydrogen phosphate (100 mM) 1.42 g and Potassium dihydrogen phosphate (20 mM) 0.272 g in 800 ml distilled water, and adjust pH to 7.4 by adding 1N-HCl, as required. Finally add distilled water to make the final volume 1000 mL. Autoclave and store at 40C.

v. LDH activity staining solution (100 mL):

- | | |
|--|---------|
| a) Nitro Blue Tetrazolium (NBT) | 50 mg |
| b) Phenazine metho sulphate (PMS) | 2.5 mg |
| c) Nicotinamide Adenine Dinucleotide (NAD) | 0.18 gm |
| d) D- Lithium lactate | 19.2 gm |

Add the above components serially in 100 mL Tris-glycine buffer in a 150 mL capacity amber colored bottle. Cover the bottle with its cap/stopper and shake thoroughly for 5-7 min to dissolve the components completely.

Principle of LDH activity staining:

At the end of the electrophoresis, activity of LDH is visualized by incubating the gel in the staining solution containing the substrate (Lactate), coenzyme (NAD), mediator of electron transfer (PMS) and a tetrazolium dye (NBT).

- | | | |
|------------------------|-------|----------------------------------|
| 1. Lactate + NAD | ————— | Pyruvate + NADH |
| 2. NADH + PMS | ————— | NAD ⁺ + PMS (reduced) |
| 3. NBT + PMS (reduced) | ————— | NBT (reduced) + PMS |

The product (NADH) of Step 1 reacts with PMS, which acts as the electron carrier (Step 2) and finally, the PMS (reduced) reacts with the last hydrogen acceptor NBT (Step 3) to produce the colored NBT (reduced)

C. Equipment:

- i. Horizontal gel electrophoresis apparatus (gel size 10 X 10 cm),
- ii. Power supply (output range up to 300 V, 100 mA)
- iii. Incubator set at 37°C
- iv. Stereo-binocular microscope

Experimental Details:

1. Take 30 adult flies from different ecotypes for each sample.

2. Anaesthetize the flies, decapitate and cut off their legs and wings while the slide with flies is kept over a cool pack to maintain cold conditions (samples should not get heated during the procedure). Discard head, legs and wings.
3. Transfer the decapitated fly samples into 1.5 mL centrifuge tubes with chilled 100 μ L PBS kept on ice.
4. Homogenize the samples on ice and centrifuge at 10,000 rpm for 15 min at 4°C.
5. Transfer the supernatant to a fresh 1.5 mL centrifuge tube kept on ice. Estimate the protein content of the supernatant and calculate the volume required to load equal amounts of the protein samples from different ecotypes on the gel.
6. Cast the gel by mixing the components (described above) in a 150-200 mL glass beaker or conical flask. Heat the mixture in a microwave (on maximum power of the equipment for 30 sec) or on a heater. Swirl the solution and heat again until it starts boiling. Check for crystals and repeat boiling until the solution is homogeneous. Handle carefully and cool to 50-55°C, swirl and pour into the casting stand. Allow 30-45 min for gel to solidify.
7. Meanwhile, prepare samples to be loaded into the wells. Use 50 μ g equivalent volume for each protein sample. For example, 50 μ L sample (volume equivalent to 50 μ g protein based on protein estimation) + 5 μ L BPB (as tracking dye).
8. Perform electrophoresis using chilled Tris-glycine buffer at 10°C at 100 V with the electrophoresis tank placed inside a refrigerator or in a cold-room. Run the gel till the BPB dye front reaches the end of the gel (~4-5 hr).
9. Remove the gel from its tray/ platform and transfer the gel to a dark box and pour in the staining solution. Incubate the gel at 37°C (in the dark) for 2-3 hr until blue bands appear on the gel.
10. Rinse the gel in water and proceed for imaging and compare the locations of LDH activity bands in the different samples.

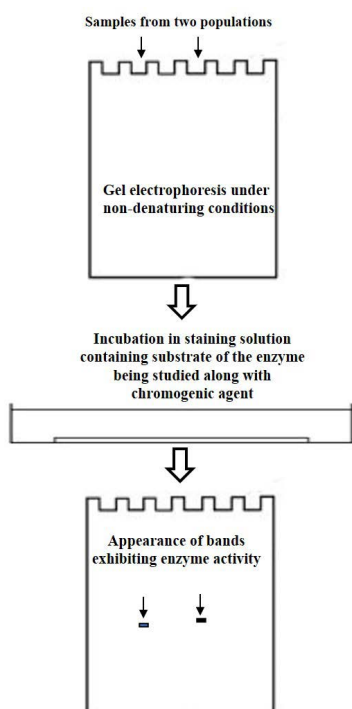


Fig. 2: Schematic of steps in electrophoretic separation and detection of allozyme variants.

Observations:

The bands exhibiting specific enzyme activity but which have moved varying in the gel indicate *LDH* allozyme variants with different electrophoretic mobility (**Fig. 3**). The allozyme variants reveal intra-specific genetic variation between different alleles of the *LDH* gene.

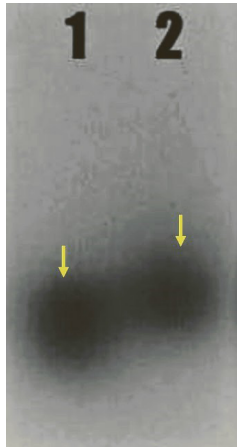


Fig. 3: Showing LDH allozyme variants (bands with different electrophoretic mobility indicated by arrows) in two natural populations (1 and 2) of *Drosophila melanogaster*.

Applications:

Allozyme variations in different enzymes have been a valuable tool for population geneticists to ascertain the genetic profile of a population using Hardy-Weinberg (H-W) principles. An enzyme which is a dimer (quaternary structure) would exhibit an electrophoretic pattern consisting of either 'fast' moving or 'slow' moving bands for the homozygotes and both 'fast' and 'slow' bands for heterozygotes for the enzyme encoding co-dominant genes. Therefore, one can apply Hardy-Weinberg principles to assess the equilibrium status and genetic profile of a given population using allozyme variation.

Questions for self-study and interpretation:

1. How would the observed differences in the electrophoretic mobility of LDH allozyme assay have originated?
2. Why are the following components of the staining solution used in the assay and what chemical roles played by each?
(a) D-Lithium Lactate (b) NBT (c) PMS (d) NAD
3. How can allozyme assays be useful in evolutionary biology?

References & Further reading:

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<https://www.wur.nl/en/show/Allozymes.html> [Webpage: Drvan Treuren (Wageningen University and Research site)]

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Onoufriou, A. and Alahiotis, S. N. (1982) *Drosophila* Lactate Dehydrogenase: Molecular and genetic aspects. *Biochemical Genetics*. 20:1195-1209.

Richardson, B. J., Baverstock, P. R. and Adams, M. (1986) Allozyme electrophoresis: A Handbook for animal systematics and population studies. Academic Press Inc. San Diego.

Thorpe, J. P. and Solé Cava, A. M. (1994) The use of allozyme electrophoresis in invertebrate systematics. *Zoologica Scripta* 23: 3-18.

Chapter 114

To study chromosomal inversion polymorphism in natural populations of *Drosophila*

H.A. Ranganath (haranganath@gmail.com)

Centre for Human Genetics, Bangalore; *Drosophila* Stock Centre, University of Mysore.

Recommended Level: UG/PG

Sub-discipline: Genetics/Cytogenetics/Evolution

Background:

Inversion is one of the types of structural rearrangements of chromosomes. This is due to breaks at two points on a chromosome, followed by re-joining of the broken piece in reverse direction. This results in a chromosome segment with inverted sequence of genes in that region. If the centromere of the concerned chromosome is not included in this rearrangement, it is called 'paracentric inversion'. On the other hand, if the centromere is also part of the chromosomal segment that gets inverted, it is called 'pericentric inversion'. Natural populations of many species of *Drosophila* are known to be polymorphic for inversions, particularly for paracentric inversions. Three types of individuals are seen in a 'Polymorphic population'. They are: i) homozygous for 'normal' or the most common sequence, ii) homozygous for inverted sequence, and iii) heterozygous for the 'normal' and inverted sequence. Of these three types, meiosis in heterozygous individuals has interesting consequences. In such individuals, the inverted segment forms an inversion loop for synapsis with its homolog (**Fig. 1**). Studies in *Drosophila* and Maize (Sturtevant and Beadle, 1936; McClintock, 1941) showed that if a single event of crossing over occurs between the non-sister chromatids within the inversion loop during meiosis, the recombinant chromatids carry duplication or deficiency for several genes, while the two non-sister chromatids that do not participate in the recombination event, produce gametes carrying the 'normal' or the inverted sequence, respectively. Due to the genetic imbalance because of duplication/deficiency, gametes receiving the recombined chromatids do not produce viable zygotes. Protective cytogenetic mechanisms to overcome such harmful effects of inversions include absence of meiotic recombination in *Drosophila* males, and a preferential segregation of such chromatids in polar bodies in females. If any gamete carrying the recombinant chromatid (with duplication or deficiency) does become part of the oocyte, the resulting zygote would be an euploid and, therefore, inviable. Consequently, inversion heterozygous individuals do not produce progeny carrying recombination in the inverted segment (Sturtevant and Beadle, 1936). This led to their use in developing the balanced lethal system (see **Chapter 8**) and balancer chromosomes (see **Chapter 1**).

The giant polytene chromosomes seen in *Drosophila* (see **Chapter 31**) have greatly

helped in understanding of configurations of different types of inversions, such as simple, overlapping, included, tandem, complex etc. An important feature of polytene chromosomes is synapsis of homologous chromosomes or somatic pairing. When the two homologs of a chromosome carry similar sequences without any visible rearrangement, complete synapsis is observed throughout the length of chromosome. (**Fig. 1A**). Depending on the nature and extent of difference in the homology, synapsis is affected and is reflected in different configurations of the synapsed chromosomes. In the case of an inversion heterozygous individual, synapsis is seen to be affected near the inversion breakpoints while the rest of the inverted segment pairs with the un-rearranged homolog by forming a loop, termed as the ‘inversion loop’ (**Figs. 1B and C**). Thus the presence of easily identifiable inversion loop in polytene chromosome spreads provides a convenient mark for heterozygosity for inversion.

Evolutionary geneticists like Theodosius Dobzhansky focused on chromosomal inversion polymorphisms existing in wild populations of *Drosophila* species. Frequencies of several commonly seen inversions in natural populations were found to correlate with geographical and other climatic conditions. Absence of recombinant progeny for genes included in the inverted segment helps maintain integrity of allele combinations in that region which may have adaptive significance. Consequently, inversions can act as potential drivers of divergence and speciation (Dobzhansky, 1970). Much of our understanding of the evolutionary significance of inversion polymorphisms has come from studies in *Drosophila pseudoobscura*, *D. subobscura*, *D. willistoni*, *D. ananassae*, *D. nasuta*, and other species (Singh, 2019).

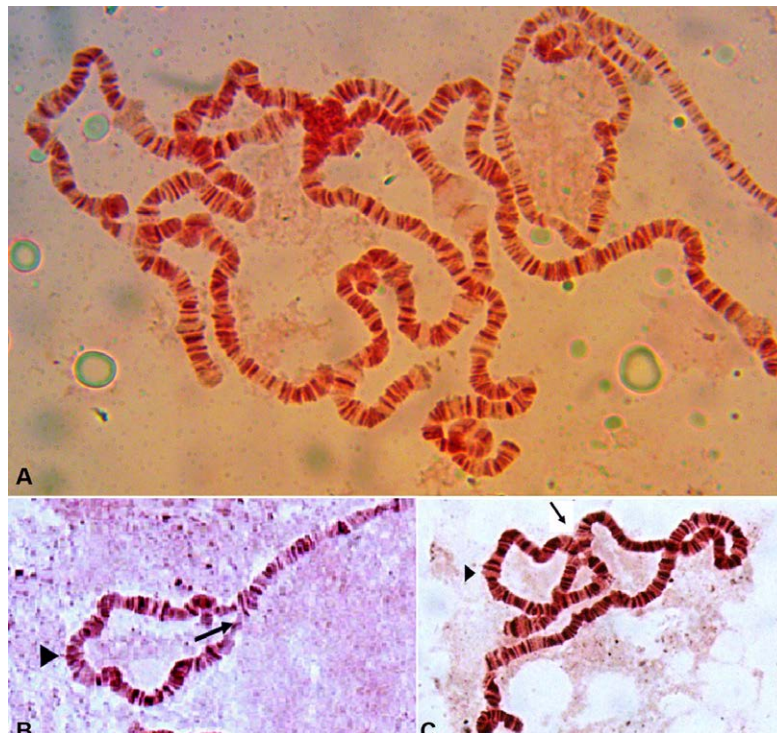


Fig. 1. Polytene chromosomes to show normal pairing of homologs and pairing in inversion heterozygotes. A. Polytene chromosomes from wild type *Drosophila melanogaster* with all homologs paired along their entire lengths (image by Miss Sneha Pai of Ctr for Human Genetics, Bangalore). **B.** Inversion loop in a polytene chromosome of *D. ananassae* heterozygous for an inversion (image by Dr. M.S. Krishna). **C.** Inversion loop in a polytene chromosome of *D. nasuta* heterozygous for an inversion (image by Prof. S. R. Ramesh). Arrow heads in **B** and **C** indicate the normally synapsed homologs in the inversion loop while long arrows point to the asynapsed regions at the inversion breakpoints.

Objective:

To examine inversions in polytene chromosomes of domestic and semi-wild/wild populations of *Drosophila*.

Materials Required:

A. **Flies:** Isofemale cultures of *Drosophila* species collected from nature, preferably of *D. ananassae/D.nasuta* (see **Chapter 108**). Alternatively, larvae from one of the balancer chromosome carrying stocks (see **Chapter 1**) can also be examined to get familiar with chromosome configuration in inversion heterozygous condition.

B. Equipment and fly handling items

- i. Stereo-binocular and bright-field microscopes
- ii. Food bottles
- iii. Cotton
- iv. Needles and fine hair brushes
- v. Glass plates
- vi. Anaesthetic ether and etherizer
- vii. Slides and cover slips
- viii. Nail polish/wax

C. Reagents:

- i. Insect saline
- ii. 1N HCl
- iii. 2% Lacto-Aceto-Orcein stain
- iv. 45% Acetic acid

Experimental Procedure:

1. Dissect and remove salivary glands from well fed third instar larva in insect saline (see **Chapter 31**). Keep the salivary glands from different larvae separate.
2. Fix glands in 1N HCl for 5 min.
3. Stain the glands for 15 min in 2% Lacto-aceto-orcein stain.
4. Wash the stained salivary glands with 45% Acetic acid and squash the salivary glands in 45% Acetic acid (for squashing method, see **Chapter 31, 53**).
5. Blot excess Acetic acid.
6. Seal the edges of the coverslip with was or nail polish.
7. Observe under bright-field microscope.

Observations:

1. Examine polytene chromosome squash preparations from >25 progeny of the established isofemale lines and look for presence of inversion loop in each case. The pairing configuration may vary depending on the nature of inversion (simple, overlapping, included, tandem type). Record the types of inversions as well as the frequency of each (see references for familiarization with various common inversions seen in natural populations).

2. Calculate the frequency of individuals with and without inversion loop. The latter may be due to homozygosity for 'normal' or inverted sequence. Following the banding pattern of chromosomes, identify the individuals as homozygous for the 'normal' and for inverted sequence.

Questions for self-study and interpretation:

1. Can the inversion loops observed in polytene nuclei be also seen in mitotic chromosomes of the individual?
2. What is adaptive and evolutionary significance of inversions?
3. What may be the cause for an inversion to occur in populations in nature?
4. Can inversions be induced in laboratory?

References:

Dobzhansky, Th. (1970) Genetics of the evolutionary process, Columbia University Press, USA. McClintock, B. (1941). The stability of broken ends of chromosomes in *Zea mays*. Genetics. 26:, 234-282.

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Sturtevant, A. H. and Beadle, G. W. (1936) The relations of inversions in the X chromosome of *Drosophila melanogaster* to crossing over and disjunction. Genetics. 21: 554-604.

<https://www.genetics.org/content/21/5/554>

Chapter 115

Quantification of genomic variants and cross-species comparisons from whole genome/chromosome alignments in *Drosophila*

Koushik Ponnanna (koushikponnanna@genetics.uni-mysore.ac.in) and Nallur B. Ramachandra* (nallurbr@gmail.com/ramachandra@genetics.uni-mysore.ac.in)

Department of Studies in Genetics and Genomics, University of Mysore, Manasagangotri, Mysuru, India

*For correspondence

Recommended Level: UG/PG/Research

Sub-discipline: Genetics/Evolutionary Biology/Taxonomy/Genomics/Bioinformatics

Background:

Current advances in sequencing technologies with a paralleled surge in genomic analysis tools have facilitated biologists to explore a large number of sequenced *Drosophila* species. This practical session will serve as a primer for performing pairwise whole-genome/chromosome alignments, visualization of the generated alignments using dot plots, and quantifying genomic variants from the alignments. Whole-genome/chromosomal level alignments refer to the correspondence between the genomes/chromosomes between species. For each segment in the genome A, the sequence alignment will predict its homologous segment in the genome B. This is of interest in *Drosophila* research as it permits analysis of molecular evolution at both large and small scales. Large scale analysis includes the estimation of frequency and location of rearrangements along with duplication events and synteny. At the small scale, the alignments are used to examine the rate of substitution, insertions and deletions (also known as InDels) across entire genomes. Further, these events aid in inferences about species-tree and reconstructions of ancestral genome. Genomic differences within species influence phenotypic variations, and quantification of this allows deciphering the evolutionary past as well as gene functions. Differences in genomes range from single nucleotide to highly complex genomic rearrangements quantifiable from genomic alignments (Dewey, 2019).

The *melanogaster* subgroup comprises of nine species and is placed under the subgenus *Sophophora*. The subgroup has garnered the attention of biologists due to the availability of rich genomic resources. The present exercise provides a stepwise approach from generating chromosome level alignments to genomic variant quantification using examples of genomic sequences of the right arm of chromosome 3 (3R) in three species belonging to the *melanogaster* subgroup. This principle can be extrapolated to whole-genome alignments. The tools used are easy to follow, and do not require an understanding of programming language to obtain and interpret results. The analysis starts with input data preparation, which relies on downloadable chromosome

sequences from the NCBI repository—followed by whole chromosome alignment generation through the Galaxy web interface or Mummer tool. Subsequently, pair-wise alignment files generated are used downstream for creating dot plots that are routinely used for visualization of sequence comparisons and large structural rearrangements. Quantifying genomic variants from the alignment files using the Assemblytics web interface allows the user to generate productive graphical outputs promoting more explicit biological interpretations.

Objectives:

To generate pair-wise chromosome alignments for the given genomic sequences to identify and quantify genomic variants and to identify and visualize fixed paracentric inversion in the 3R chromosomal arm.

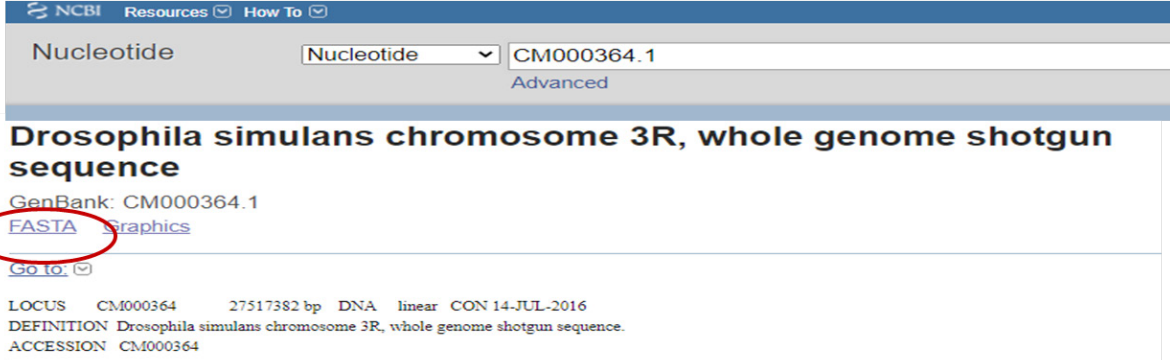
Materials Required:

- A. Laptop or a desktop computer with Windows/Linux operating system.
- B. Internet connection.
- C. Chromosome or scaffold level nucleotide sequences from the NCBI website.

Experimental Details:

Section 1: Downloading the chromosome data and preparing the *FASTA* inputs.

- i. For the example here, 3R chromosomal sequences of three species belonging to the *melanogaster* subgroup are used.
- ii. Access the NCBI website and, in the search drop-down menu, select nucleotide. Search for *D. melanogaster* 3R chromosome (AE014297.3), *D. simulans* 3R chromosome (CM000364.1), and *D. sechellia* 3R chromosome (NC_045952.1) using either names or accessions.
- iii. On the search result page, click on *FASTA* followed by *SEND TO (check, complete record-file- ASTA-create file* to download the data in *FASTA* format (**Fig. 1A-1B**).
- iv. Use a suitable text editor (E.g., *EmEditor*) to open and rename the *FASTA* headers to *>D. melanogaster*, *>D. simulans* and *>D. sechellia* in respective files (**Fig. 1C**). Make sure no additional characters, including spaces are present in the *FASTA* headers as this can lead to input incompatibility issues downstream. Save the files and proceed to section 2.

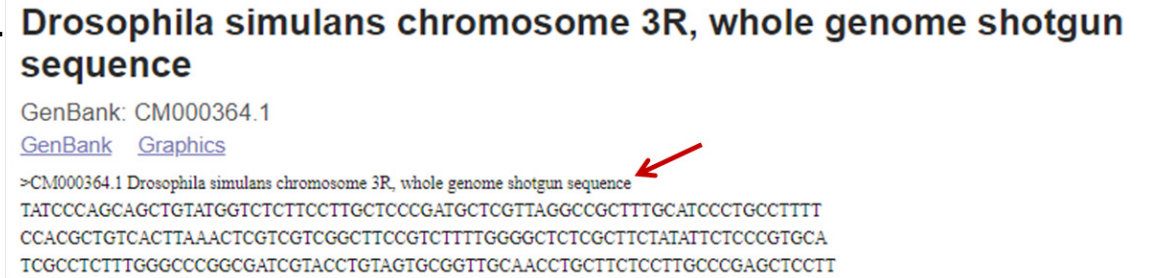
A. 

Nucleotide
Advanced

Drosophila simulans chromosome 3R, whole genome shotgun sequence
GenBank: CM000364.1
[FASTA](#) [Graphics](#)
[Go to:](#)

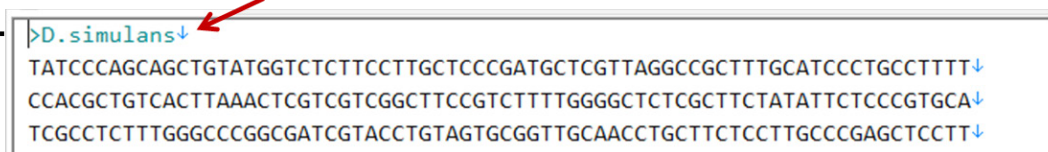
LOCUS CM000364 27517382 bp DNA linear CON 14-JUL-2016
DEFINITION Drosophila simulans chromosome 3R, whole genome shotgun sequence.
ACCESSION CM000364

FASTA Send to: ▾

B. 

Drosophila simulans chromosome 3R, whole genome shotgun sequence
GenBank: CM000364.1
[GenBank](#) [Graphics](#)

>CM000364.1 Drosophila simulans chromosome 3R, whole genome shotgun sequence
TATCCCAGCAGCTGATGGTCTCTTCCCTTGCTCCCGATGCTCGTTAGGCCGCTTTGCATCCCTGCCTTTT
CCACGCTGTCACTTAAACTCGTCGTCGGCTTCCGTCTTTTGGGGCTCTCGCTTCTATATTCTCCCGTGCA
TCGCCTTTGGGCCCGGCGATCGTACCTGTAGTGC GGTTGCAACCTGCTTCTCCTTGCCCGAGCTCCTT

C. 

```
>D.simulans↓
TATCCCAGCAGCTGATGGTCTCTTCCCTTGCTCCCGATGCTCGTTAGGCCGCTTTGCATCCCTGCCTTTT↓
CCACGCTGTCACTTAAACTCGTCGTCGGCTTCCGTCTTTTGGGGCTCTCGCTTCTATATTCTCCCGTGCA↓
TCGCCTTTGGGCCCGGCGATCGTACCTGTAGTGC GGTTGCAACCTGCTTCTCCTTGCCCGAGCTCCTT↓
```

Fig. 1. Snapshots representing step-wise sequence data retrieval: **A)** using accession numbers to retrieve sequences from NCBI database **B)** *FASTA* sequence with respective information in default and **C)** edited *FASTA* header in a text editing tool for downstream compatibility.

Section 2: Generating pair-wise chromosome alignments for the three sequences (*D. melanogaster* versus *D. simulans* 3R chromosomal arms; *D. melanogaster* versus *D. sechellia* 3R chromosomal arms; and *D. sechellia* versus *D. simulans* 3R chromosomal arms).

Strategy 1: To generate the three pair-wise alignments using an open-source, web-based Galaxy platform.

- a. The user must register and request for an account to access the features hosted in *Galaxy* (Blankenberg *et al.*, 2010). Once the *Galaxy* account is set up, proceed with the prepared *FASTA* inputs for generating pair-wise chromosome alignments using *Nucmer* program in *Galaxy*.
- b. Users are advised to familiarize with the *Galaxy* interface and follow the stepwise best practices guide provided below for generating a delta file, which is the primary pair-wise alignment file.
- c. Use the link <https://usegalaxy.org/> to access *Galaxy*. As the datasets (*FASTA* files) are <100 MB, these can be directly uploaded through a local disk in which the files are stored. For uploading larger files of size >100 MB and for a

detailed tutorial, refer the link <https://galaxyproject.org/tutorials/upload/>. Once the upload is finished, the datasets reside in the history panel that is towards the right on the webpage.

d. Access *Nucmer* from the tools panel, found on the left on the web page.

e. Follow the sequence *Tools* → *Genomics Analysis* → *Multiple Alignments* → *Nucmer* to access *Nucmer* interface in *Galaxy* (**Fig.2**).

f. In the *nucmer* interface, in the Reference Sequence section, choose *D. melanogaster_3R_chromosome.fasta* from the dropdown, and *D. simulans_3R_chromosome.fasta* for Query Sequence.

g. Scroll down to notice various parameters that can be adjusted based on the study objective. For this example, change the Anchoring parameter to *--maxmatch*, *Minimum match length (-l)* to 100, *Minimum cluster length (-c)* to 500, and set the *Filter* parameter to *YES* for reporting only the best alignments. Leave all other parameters as default and press execute.

h. Perform a similar analysis to generate the two other pair-wise combinations mentioned earlier. The program allows uploading all three combinations at once with run time being ~30- 45 min.

i. Monitor the job status on the history panel. Successful execution generates two files per combination. An alignment file in *delta* format and a *dot plot* file aiding alignment visualization. These result files are downloadable to a local disk for further analysis.

j. The generated *delta* file is our primary input for variant calling in the next section.

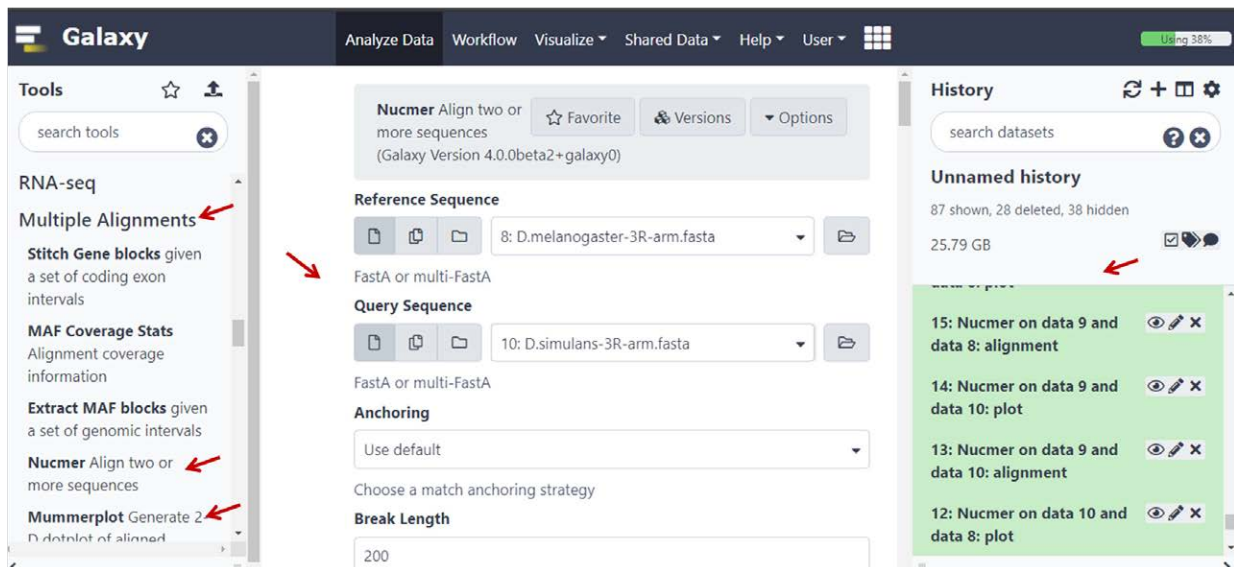


Fig. 2. Snapshot of Galaxy web interface and the tools used in this exercise are indicated by arrows.

Strategy 2: To generate alignment file in *delta* format (recommended for those familiar with the usage of Linux systems).

- a. The prerequisite for running *nucmer* is an installation of MUMer4 (Marcais *et al.*, 2018) hosted on Github (<http://github.com/gmarcais/mummer>). For complete installation, tutorials, and system requirements, access the following link <http://mummer.sourceforge.net/>).
- b. Proceed to align the chromosome sequences using the parameters given below in the box. Perform a similar analysis to generate two more combinations.

```
nucmer --maxmatch -l 100 -c 500 D.melanogaster_3R_chromosome.fasta  
D.simulans_3R_chromosome.fasta --prefix melanogaster_simulans_3
```

Note: Successful execution of the above commands will generate pair-wise alignments in *delta* format similar to the one generated through *Galaxy*. Refer to the *mummer* manual for detailed parameter usage and to plot the results using *mummerplot*.

In case the *Galaxy* server takes more than the expected run time, or if found challenging to run either of the two strategies, the users may make use of our Github repository (https://github.com/koushik1989/KPCR_tutorials) to access pre-computed *delta* files. However, using *Galaxy* or Linux based result generation is recommended, which would allow the users to explore available features and tools. With the generated *delta* files the users may proceed with the following two exercises.

Exercise 1: To identify and quantify genomic variants from pair-wise alignments.

In this section, let us explore the alignments generated between species pairs and quantify genomic variants. *Assemblytics* (Nattestad, 2016) is a web app for detecting and analyzing variants from pair-wise genome alignments. It incorporates a unique anchor filtering approach to increase robustness to repetitive elements and identifies six classes of variants based on their distinct alignment signatures. The classes of variants detected by *Assemblytics* include insertions, deletions, tandem expansions, tandem contractions, repeat expansions, and repeat contractions. Usage of *Assemblytics* is relatively simple as the only input the user supplies is the *delta* file generated in section 2 (from either of the strategies used). Here, the genomic variants in the query sequence are reported relative to the reference sequence used.

- i. Access *Assemblytics* from the following link using any web browser. (<http://assemblytics.com/>).
- ii. Upload the *delta* file in the option provided in the right panel. Provide a suitable description name and retain the unique sequence length parameter at the default value of 10,000 bp (**Fig. 3**).
- iii. The maximum and minimum variant size can be modified as per the user's requirement. In this analysis we will set to a maximum of 10,000 bp to a minimum of 2 bp.

iv. A valid alignment file will prompt the user to proceed further, and successful job completion will generate a summary report, graphs, interactive dot plot, and a downloadable zip file with generated results. Extract the results from the downloaded zip folder and further interpret the results.

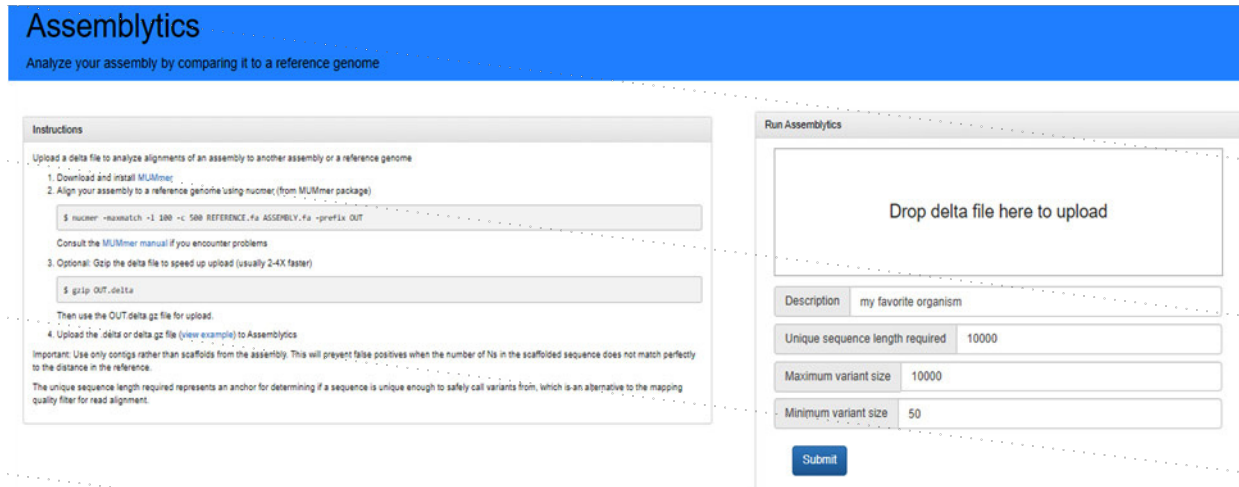


Fig. 3. Snapshot of Assemblytics web interface.

Exercise 2: To identify and visualize fixed paracentric inversion in the 3R chromosomal arm. In this section, we will use a web interface tool *D-GENIES* (Dot plot large Genomes in an Interactive, Efficient and Simple way) (Cabanettes, 2018). *D-GENIES* is a web-interface tool for generating dot plots aiding convenient visualization of sequence alignments. *D-GENIES* uses *minimap* or *mashmap* to align the sequences and render plots with a detailed summary. It supports large genomes, and the user can interact with the dot plot to improve the visualization. The input files are the *FASTA* sequences used in section 2. The aim is to compare *D. simulans* and *D. sechellia* 3R chromosomal arm with *D. melanogaster* 3R chromosomal arm.

- i. Access *D-GENIES* through the link <http://dgenies.toulouse.inra.fr/>.
 - ii. On the top taskbar, choose Run option which will guide the launch panel for uploading *FASTA* sequences (Fig. 4). Run *D-GENIES* twice, keeping *D. melanogaster* as a reference and successively changing the query to *D. simulans* and *D. sechellia*.
 - iii. Provide a name for the job followed by a valid email ID and set the uploading option to local and upload the reference and target *FASTA* files.
 - iv. Users can choose between *minimap* and *mashmap* aligners and submit the job.
 - v. Once the job completes successfully, click on the results to view the interactive dot plot with associated summary information.
 - vi. In the result page, the user can explore various features like opting to view strong precision matches, hide noise in the data and enhance dot/stroke width.
 - vii. Dot plots and summary information exportable in various file formats.
- Note:** Dot plots provide an excellent overview of the genome-genome alignments from the perspective of both genomes. In a dot plot the reference and query sequences are

represented on the two different axes aiding visualisation and a dot is plotted at each co-ordinate where there is a similarity between bases. A reference guide for interpreting the dot plot can be found at the following links.

http://resources.qiagenbioinformatics.com/manuals/clcgenomicsworkbench/650/Examples_interpretations_dot_plots.html

<https://omicstutorials.com/interpreting-dot-plot-bioinformatics-with-an-example/>

<http://mummer.sourceforge.net/manual/AlignmentTypes.pdf>

The screenshot shows the D-GENIES web interface. At the top is a navigation bar with links: D-GENIES, About, Run, Results, Gallery, Documentation, Install, and Contact. The main heading is 'Launch map analysis'. Below this are input fields for 'Name of your job' (containing 'meDzH_20200909071857') and 'E-mail'. There are two tabs: 'New alignment' and 'Plot alignment'. Below the tabs are fields for 'Target fasta' and 'Query fasta', both set to 'Local' with file selection icons. The 'Aligner' section has radio buttons for 'Minimap2' (selected) and 'Mashmap'. At the bottom are 'Submit' and 'Load example' buttons. Footnotes explain that the email field is for result delivery and that FASTA files can be gzipped.

Fig. 4. Snapshot of D-GENIES web interface.

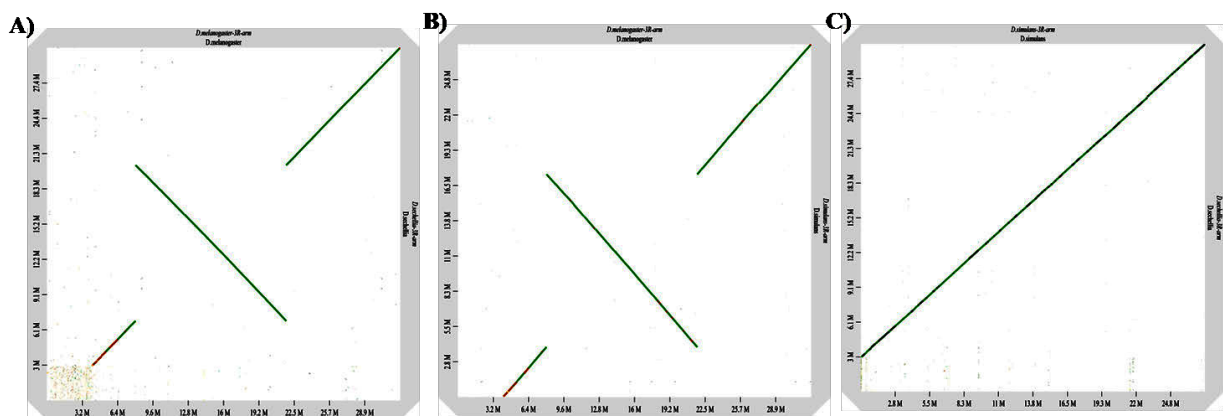


Fig. 5. Dot plots generated using D-GENIES for **A.** *D. melanogaster* versus *D. simulans* 3R chromosomal arms, **B.** *D. melanogaster* versus *D. sechellia* 3R chromosomal arms; and **C.** *D. sechellia* versus *D. simulans* 3R chromosomal arms. The reverse diagonal line seen intersecting in **A** and **B** denotes inversions in query relative to the reference genome.

Results/Observations:

1. Analyze the dot plots generated in *galaxy* or *mummer* programs for all the three comparisons and infer the differences seen in the derived dot plots between species.
2. Tabulate all the six types of genomic variants and summarise the results in comparative plots. User may use the *Assemblytics* outputs and utilize the generated summary files. The summary file contains various statistics pertaining to quantified variants along with bases affected by these variants. This data can be plotted separately using either *Graph Pad prism* software or using *Microsoft Excel programme*.
3. Use the BED file generated in *Assemblytics* to quantify variants common between *D. simulans* and *D. sechellia*. The genomic coordinates can be used for this quantification.
4. Observe the large fixed paracentric inversion seen as a diagonal line in the middle, intersecting the slope in both *D. melanogaster* versus *D. simulans* 3R chromosomal arms, and *D. melanogaster* versus *D. sechellia* 3R chromosomal arms comparisons (**Fig. 5**).
Note: These steps can be used for cross-species genomic sequence comparisons as required.

Questions for self-study and interpretation:

1. *D-GENIES* outputs for *D. melanogaster* versus *D. simulans* 3R chromosomal arms, and *D. melanogaster* versus *D. sechellia* 3R chromosomal arms appear relatively similar. Do *D. sechellia* versus *D. simulans* 3R chromosomal arms reveal similar breakpoints in the alignment? Generate and infer the results.
2. Explore more about dot plots and its usage in comparative genomics. How are various genomic rearrangements like insertions, inversions, deletions, duplications, translocations and low complexity regions represented in a dot plot?
3. Quantify genomic variants in *D. sechellia* considering *D. simulans* as a reference. How different is your result from the comparison made with *D. melanogaster*?
4. The number of the quantified genomic variants (Point 2 in Results/observation) differs in the pair-wise comparisons made (results generated in section 2). Delineate species relatedness in terms of variants and correlate the results obtained. Quantifying shared and unique variants between species will help in answering this question.
5. Perform whole genome alignments between *D. melanogaster* and *D. virilis* which has a divergence history of ~40 MY and between *D. melanogaster* and *D. simulans* which has a divergence history of ~5.4 MY. Visualize the alignments using dot plots and infer the observations made. This exercise helps to understand sequence divergence relative to divergence time.

References and further reading:

1. Blankenberg, D., Kuster, G. V., Coraor, N., Ananda, G., Lazarus, R., Mangan, M., Nekrutenko, A. and Taylor, J. (2010) *Galaxy*: A web-based genome analysis tool for

experimentalist. *Current Protocols Molecular Biology*. 89: 19-10.

2. Cabanettes, F. and Klopp, C. (2018) *D-GENIES*: dot plot large genomes in an interactive, efficient and simple way. *Peer. J.* 6: 958.

3. Dewey, C. N. (2019) Whole-genome alignment in evolutionary genomics. *Springer Protocols*. 121-147.

4. Marçais, G., Delcher, A. L., Phillippy, A. M., Coston, R., Salzberg, S. L., Zimin, A. (2018) *MUMmer4*: a fast and versatile genome alignment system. *PLoS Computational Biology*. 14:1005944.

5. Nattestad, M and Schatz, M. C. (2016) *Assemblytics*: a web analytics tool for the detection of variants from an assembly. *Bioinformatics*. 32: 3021-3023.

Integrating DNA Sequence Divergence & Phenotype

Recent studies have provided a large amount of information about the divergence of DNA between closely related species. The cause of this divergence, however, can only be completely understood if it is related to phenotypic differences. This will require integrating DNA information with protein and morphological studies of variation.

(In: Morton, R.A., Choudhary, M., Cariou, ML. et al. 2004. A reanalysis of protein polymorphism in *Drosophila melanogaster*, *D. simulans*, *D. sechellia* and *D. mauritiana*: Effects of population size and Selection. *Genetica* 120: 101–114. <https://doi.org/10.1023/B:GENE.0000017634.17098.aa>)

Chapter 116

Molecular Phylogenetics of *Drosophila* species

C. Rajanikanth (rajanikanth.cnayak@gmail.com) and N. B. Ramachandra* (nallurbr@gmail.com)

Department of Studies in Genetics and Genomics, University of Mysore, Manasagangotri, Mysuru-560006

*For correspondence

Recommended Level: PG/Research

Sub-discipline: Evolutionary Biology/Bioinformatics

Background:

The species-rich biodiversity needs to be organized and classified systematically for understanding the biology and evolutionary history of species. A classification of organisms was first formalized by Carolus Linnaeus with establishment of a new field called 'Taxonomy', which involves naming, ranking, and hierarchically classifying the organisms. As studies in the field of evolutionary biology progressed, it was apparent that the living organisms should be classified based on their evolutionary relationships. This led to the birth of a new area called 'Systematics.' According to Systematics, the organisms are placed under a common group based on shared characters or similarities. Among the several schools of systematics, 'Phenetics' and 'Cladistics' are the leading schools of classification. Phenetics classifies organisms based on the overall similarity in many characters, whereas Cladistics groups the organisms based on a subset of similarities, the shared characters or 'synapomorphies'. These characters are then used to derive overall similarities or dissimilarities index, which is used as a matrix for clustering the taxa based on similarity. A clustering algorithm such as Unweighted Pair Group Method with Arithmetic mean (UPGMA) is used and represented as a tree (Sokal and Michener, 1958).

A phylogenetic tree is a graphical representation of evolutionary relationships among entities that share a common ancestor. These entities can be species, genes, genomes, or any other Operational Taxonomic Unit (OTU). Any given character can be taken into consideration while grouping the organisms. For example, in *Drosophila*, characters such as the presence of abdominal pigmentation, pattern of wing pigmentation, presence or absence of sex comb or frons, etc. can be considered. The intensity of pigmentation can be lightly pigmented, moderately pigmented, or highly pigmented; accordingly they can be coded as '1', '2', and '3', respectively. Presence and absence of a trait such as sex comb can be coded as 1 and 0, respectively. Below is an example of various such characters and how they are coded while inferring a phylogenetic tree.

Table 1: Species 1-5 representing the character state for individual traits

	Trait 1 (Presence of Sex comb in males)	Trait 2 (Presence of frons)	Trait 3 (Abdominal pigmentation)	Trait 4 (wing pigmentation)	Trait 5 (Presence of thoracic stripes)
Species 1	0	1	1	1	0
Species 2	1	1	1	1	0
Species 3	0	0	1	0	1
Species 4	1	0	2	2	0
Species 5	1	0	2	2	1

These values can be represented in a matrix by calculating the pairwise dissimilarity.

	Species 1	Species 2	Species 3	Species 4	Species 5
Species 1	0				
Species 2	1	0			
Species 3	3	4	0		
Species 4	4	3	5	0	
Species 5	5	4	4	1	0

This index can now be used to cluster each taxon based on the similarity using the clustering algorithm, such as UPGMA. The output is usually a phenogram or dendrogram. A phenogram or dendrogram links OTUs by estimating the overall similarity or dissimilarity.

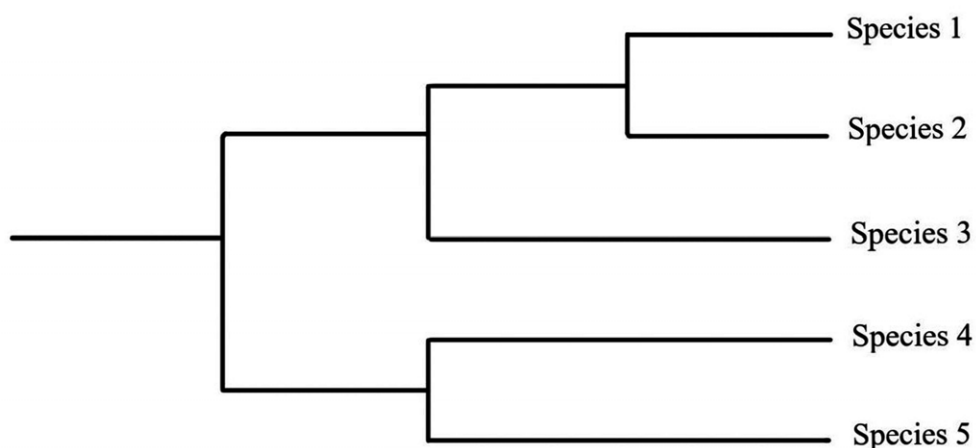


Fig. 1. A phenogram/dendrogram showing relationship between the species 1-5, with reference to the characters analysed

Until about 40 years ago, biologists mostly used only visible features of anatomy and morphology to reconstruct the ancestry of living species. This was based on the

assumption that organisms with similar morphology also have similar genes, and thus they are closely related. With the advent of DNA sequencing technology, one can directly compare the gene sequences and reconstruct phylogenies. Use of DNA sequence in phylogenetic reconstruction is based on a reasonable assumption that species having similar DNA sequences are more closely related. A multiple sequence alignment or a pairwise alignment of a set of homologous sequences involves comparison of nucleotides at each specific site in a set of homologs. A phylogenetic tree can then be constructed based on the similarity or dissimilarity index. Of the many different methods available for phylogenetic inference, main categories (Parsimony, distance, likelihood, and Bayesian methods) are based on their scheme of evaluation. In the parsimony method, possible trees are compared, and each is given a score, which is a reflection of the minimum number of character state changes (nucleotide substitutions). Distance method generates a tree by calculating the estimated evolutionary distance between all pairs of sequences. Maximum likelihood method is similar to parsimony methods in that possible trees are compared and given a score. These scores are based on how likely the given sequences may have evolved in a particular tree given the model of nucleotide substitution. Bayesian approach also works on the basis of maximum likelihood, but attempts to infer the tree topology by determining the posterior probability of an inferred tree. In this exercise, the maximum likelihood method is discussed for inferring phylogenetic relationship between *Drosophila* species using DNA sequences.

Objective:

Introduction to the fundamentals of molecular phylogenetics using DNA sequence information to assess relationships amongst taxa.

Materials required:

A. Homologous sequences: The homolog sequence for the given exercise can be downloaded from the github repository (<https://github.com/RajanikanthC/Molecular-Phylogeny-Timeless-and-Cytb5>).

B. Alignment tools: *MUSCLE* (Edgar, 2004) (<https://www.ebi.ac.uk/Tools/msa/muscle/>) and *Clustal Omega* (Madeira *et al.*, 2019) (<https://www.ebi.ac.uk/Tools/msa/clustalo/>).

C. Phylogenetic tree building software: *iqTree* (<http://iqtree.cibiv.univie.ac.at/>) (Trifinopoulos *et al.*, 2016).

D. Tree visualization software: *Seaview* (Gouy *et al.*, 2010) (<http://doua.prabi.fr/software/seaview>): Download the software and install it in your computer.

Experimental Details:

The process of carrying out sequence-based phylogenetic analysis involves four steps:

1) Selecting gene(s) of interest: In principle, anything from a single gene to a whole genome can be compared. A whole gene or only the coding or intronic region can be used. When comparing a specific gene, sequences from the different species should be of orthologous genes.

2) Identification of homologs: In the present example, nucleotide sequences of a nuclear gene *Timeless* and a mitochondrial gene *Cytochrome-b5 (Cyt-b5)* from 12 *Drosophila* species available in the public domain are considered. Using the *BLAST* program, *Cyt-b5* and *Timeless* orthologs were extracted from *flyDIVaSv1.2*, (Stanley and Kulathinal *et al.*, 2016) a comparative genomic database resources for divergence and selection (<http://www.flydivas.info/>). Use ‘ClustalW with character counts’ option and move to step 3.

3) Alignment of homologous sequences: In phylogenetic inference using DNA sequences, each sequence could be treated as a single character or trait with a different number of states. The advantage of using DNA sequence is that each nucleotide position in the sequence can be treated as a separate character state. One can compare individual homologous positions found in different homologous sequences. This is done by making sequence alignments. Multiple Sequence Alignment (MSA) is the critical step in phylogenetic reconstruction from gene sequences. Each sequence is assigned to a separate row in a matrix, and homologous sequences are lined up in columns, just like the character matrix explained above.

4) Alignment tools: *MUSCLE* and *Clustal Omega* are two widely used alignment tools. Open any of their links, input the ortholog sequences which are to be aligned. In step 1, select the DNA sequence option and paste the downloaded sequence in the slot. This alignment serves as the data matrix for phylogenetic inference. Once the alignment is complete, download the aligned sequences, save in *fasta* file format and use in tree inference.

Multiple Sequence Alignment

Clustal Omega is a new multiple sequence alignment program that uses seeded guide trees and HMM profile-profile techniques to generate alignments between three or more sequences. For the alignment of two sequences please instead use our [pairwise sequence alignment tools](#).

Important note: This tool can align up to 4000 sequences or a maximum file size of 4 MB.

The screenshot displays the Clustal Omega web interface, organized into three distinct steps:

- STEP 1 - Enter your input sequences:** This section includes a dropdown menu for selecting the sequence type (currently set to 'DNA'), a large text area for pasting sequences, and a file upload option with a 'Browse...' button. At the bottom of this step, there are links for 'Use a example sequence', 'Clear sequence', and 'See more example inputs'.
- STEP 2 - Set your parameters:** This section features a dropdown menu for the 'OUTPUT FORMAT', which is currently set to 'ClustalW with character counts'. Below the dropdown, a note states 'The default settings will fulfill the needs of most users.' and a button labeled 'More options...' with a link to view or change settings.
- STEP 3 - Submit your job:** This final step contains a checkbox for 'Be notified by email (Tick this box if you want to be notified by email when the results are available)' and a prominent 'Submit' button.

Fig. 2. GUI of MSA tool *Clustal Omega*

5) Inference of phylogenetic tree: After multiple sequence alignment, a Phylogenetic reconstruction can be carried out using the maximum likelihood approach. An online tree building software *iqTree* (<http://iqtree.cibiv.univie.ac.at/>) can be used to infer the phylogenetic tree.

Step 1: Go to <http://iqtree.cibiv.univie.ac.at/> ; you should be able to see the window displayed in **Fig. 3**.

Step 2: Click on ‘Tree Inference’, Input the alignment file by clicking on ‘Browse...’ and navigate to the folder you saved the alignment file in the previous step.

IQ-TREE web server: fast and accurate phylogenetic trees under maximum likelihood

Server load: 8% Trifinopoulos J, Nguyen LT, von Haeseler A, Minh BQ (2016) *Nucl. Acids Res.* 44 (W1): W232-W235. doi: 10.1093/nar/gkw256

Tree Inference Model Selection Analysis Results

For a quick start, take a look at the [tutorial](#) for the IQ-TREE web server.
 Please visit the [IQ-TREE homepage](#) for more information or if you want to download the main software.
 Data Privacy Statement: All your personal data are strictly confidential and will not be shared with any third parties. Your data will be automatically deleted after 180 days.

Input Data

Alignment file : Browse... Show example >

Use example alignment: Yes ?

Sequence type: Auto-detect DNA Protein Codon ?
 DNA->AA Binary Morphology

Partition file: This field is optional. Browse... Show example >

Fig. 3. GUI of *IQTree* web server showing details of input selection

Step 3: Choose ‘Codon’ as the ‘Sequence Type’.

Step 4: Choose ‘GTR’ as the ‘Substitution model’ as in **Fig. 4**.

Step 5: Choose ‘FreeRate heterogeneity’ by clicking ‘Yes’ [+R].

Step 6: Choose ‘Ultrafast’ or ‘Standard’ option for bootstrap analysis.

Step 7: Specify a name to your job in the slot and click the submit button.

It takes a few minutes to build the phylogenetic tree. The results can be downloaded by selecting the job and clicking on the ‘Download Selected Jobs’.

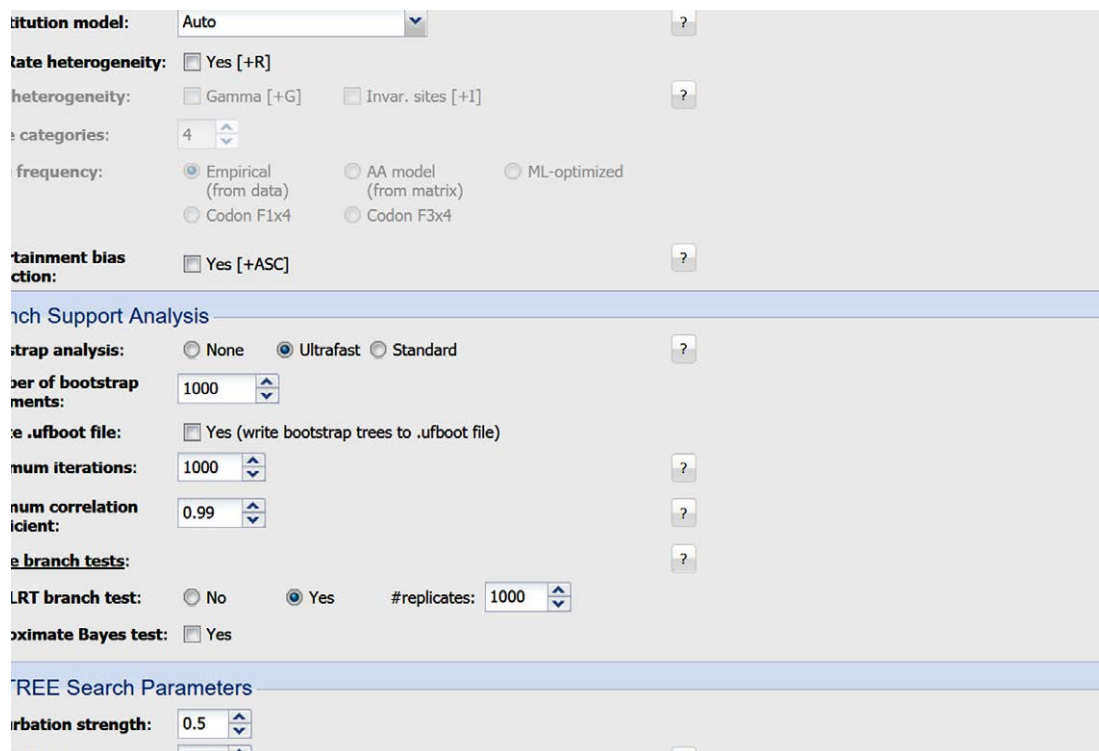


Fig. 4. GUI of *IQTree* webserver showing details of model selection.

Results / Observation:

Visualization of Phylogenetic tree:

The downloaded .zip result file of *IQTree* will have four files in it, extract the .tree file. Open the Seaview application, click on the file option or press Ctrl+O and open the .tree file you extracted in the previous step. You should be able to see the phylogenetic tree similar to the one in Fig. 5.

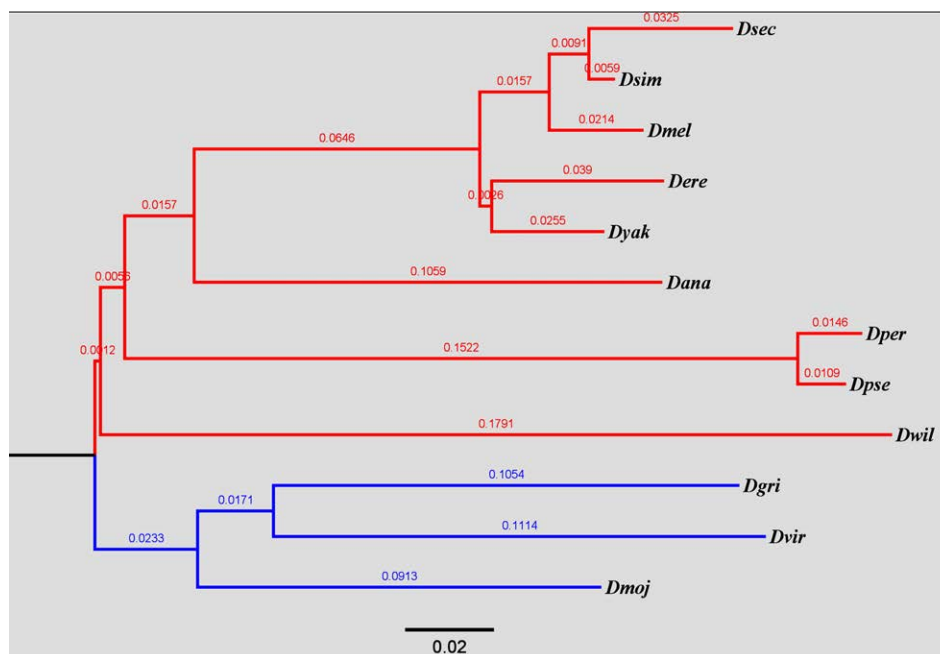


Fig. 5. Maximum likelihood tree of *Timeless* gene constructed using *IQTree*. The branch length is noted along branches.

Note: A phylogenetic tree depicts the branching history of species with common ancestry. The pattern of branching is called topology of the tree. The tips (also called as ‘leaves’ or ‘terminal node’) of a phylogenetic tree represent a species or a sequence. The node at which two branches split, represents the last common ancestor of the daughter lineage. The root of the phylogenetic tree (depicted in black line) represents the last common ancestor of the whole tree. The phylogenetic tree constructed using the *Timeless* gene of *Drosophila* is consistent with the known phylogeny of 12 species. The phylogeny consists of two major lineages, *Sophophora* (indicated in red lines) and *Drosophila* (indicated in blue lines). The first split separated the lineage that gave rise to *Sophophora* and *Drosophila*. It occurred approximately 40 million years ago. The *Sophophora* lineage consists of three subgroups depicted in the upper part (in red) of the phylogenetic tree. The *Melanogaster* group consists of *Drosophila melanogaster*, *D. simulans*, *D. sechelia*, *D. yakuba*, *D. erecta* and *D. ananassae*; the Obscura group consists of *D. pseudoobscura* and *D. persimilis*, while the Willistoni group consists of *D. willistoni*. *Drosophila* lineage consists of three groups, with one example of each being represented in the present analysis. The branch length indicated on each branch represents the number of substitutions per nucleotide site, and the scale indicated below represents 0.03 substitutions.

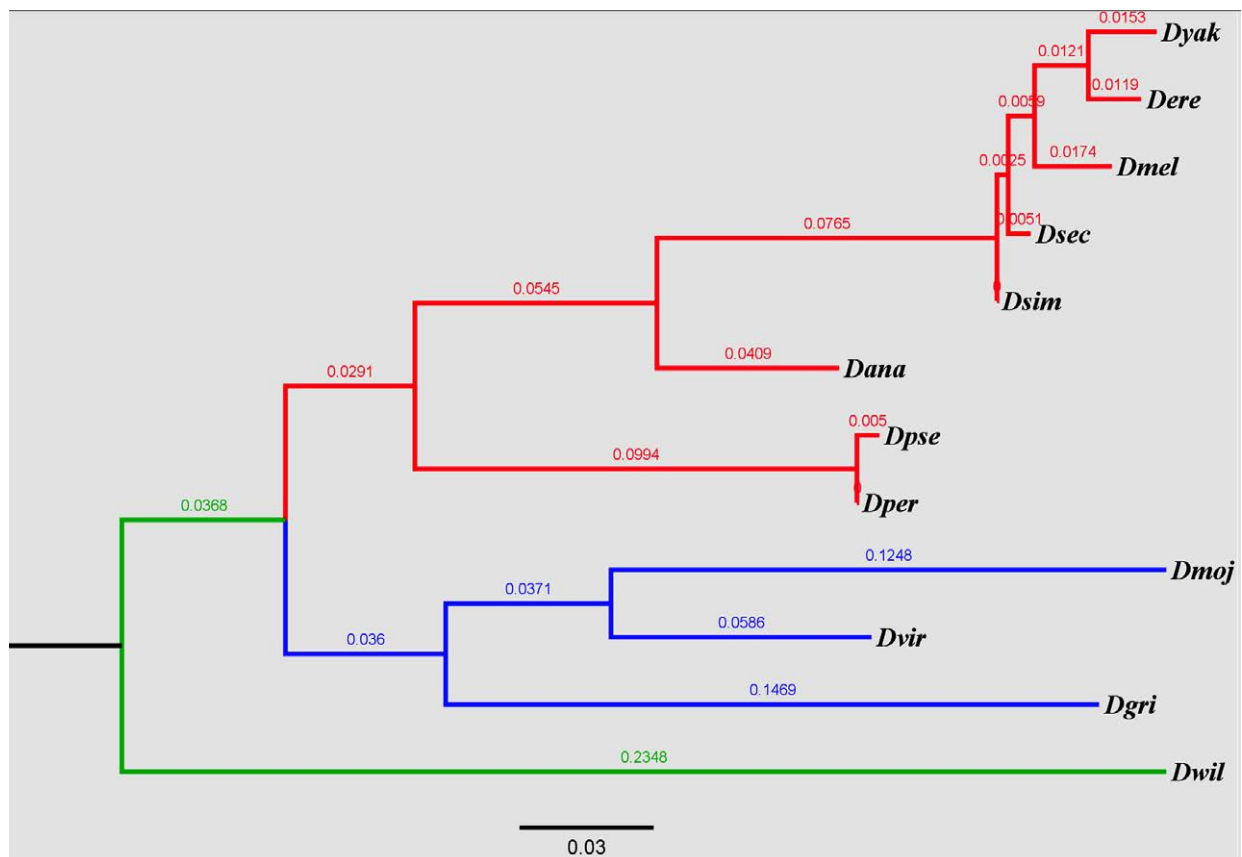


Fig. 6. Maximum likelihood tree of *Cytochrome-b5* gene constructed using *IQ Tree* (Branch length is represented)

The phylogenetic tree constructed using *Cytochrome-b5* gene is not entirely consistent with the known phylogenetic tree of the 12 species. This is because *Cytochrome-b5* is a mitochondrial gene and, therefore, inherited maternally. Moreover, mtDNA represents just one linked and relatively small segment, and therefore, inference is based on a very small amount of DNA.

Questions for self-study and interpretation:

1. What other molecular information can be used in phylogenetic analysis?
2. What other factors make mtDNA linked genes relatively less reliable markers for use in inferring phylogenetic relationship of species?
3. How can recombination and hybridization affect the phylogenetic inference?
4. **Additional exercise:** Download orthologs from *flyDI aS* for a gene of interest and perform the analysis and interpret the results as above. One can also choose an ortholog from diverse taxa (bacteria, fungi, plants, invertebrates, and vertebrates) and construct a phylogenetic tree (<https://github.com/RajanikanthC/Molecular-Phylogeny-Timeless-and-Cytb5/blob/main/CytochromeC.fasta>)

References:

Edgar, R. C. (2004) *MUSCLE*: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32:1792–7.

Gouy, M., Guindon, S. and Gascuel, O. (2010) SeaView version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Molecular Biology Evolution.* 27:221-224.

Madeira, F., Park, Y. M., Lee, J. *et al.*, (2019) The EMBL-EBI search and sequence analysis tools APIs in 2019. *Nucleic Acids Research.* 47(W1):W636-W641. Doi 10.1093/nar/gkz268.

Sokal, M. (1958) A statistical method for evaluating systematic relationships. *Univ. Kansas Science Bulletin.* 38:1409-1438.

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Trifinopoulos, J., Nguyen, L. T., von Haeseler, A. and Minh, B. (2016) *W-IQ-TREE*: a fast online phylogenetic tool for maximum likelihood analysis. *Nucl. Acids Res.* 44 (W1): W232-W235.

Chapter 117

Understanding molecular evolution using *Drosophila* genes

C. Rajanikanth (rajanikanth.cnayak@gmail.com) and N. B. Ramachandra* (nallurbr@gmail.com)

Department of Studies in Genetics and Genomics, University of Mysore, Manasagangotri, Mysuru-560006

*For correspondence

Recommended Level: UG and PG

Sub-discipline: Evolutionary Biology/Bioinformatics

Background:

Proteins, made up of amino acids as encoded by the corresponding genes, are fundamental players of all life processes. Genes undergo random mutations providing the raw material for evolution to act. The fate of a new mutation is determined by the nature of mutation, selection and the random genetic drift. Most large-scale mutations, like large deletions, duplications, insertions, and translocations, are generally deleterious. The single nucleotide mutations such as deletion, insertion, and duplications result in frame-shift, which may result in the formation of a truncated protein. Nucleotide substitutions are types of mutations that do not affect protein formation but may alter its composition. A synonymous substitution does not change the amino acid, and hence, it may not affect the protein's function. However, nonsynonymous substitution changes the amino acid, allowing the natural selection to act on it. If a nonsynonymous mutation has a deleterious effect on the organism that carries it, it is less likely to propagate in a population. However, a nonsynonymous substitution with beneficial effect can spread in the population and reach fixation by natural selection.

DNA Sequence ...CAA...ATG...TGG...

Amino acid sequence Q M W

Synonymous substitution

...CAG...ATG...TGG...

Q M W

(Amino acid sequence not altered)

Non-synonymous substitution

...CAA...ATC...TGG

Q I W

(Altered amino acid sequence)

The ratio of nonsynonymous to synonymous substitution dN/dS , (where dN is rate of nonsynonymous substitution at a non-silent site and dS synonymous substitution at a silent site) is denoted as ω . The value of ω is particularly useful in determining the mode and strength of selection acting on a nucleotide site. The $\omega > 1$ indicates positive ($s > 0$)

selection (adaptive or diversifying), $\omega < 1$ indicates negative (purifying) selection ($s < 0$) and $\omega = 1$ indicates neutral selection ($s = 0$). The value of ω is a very informative feature because it helps to identify the genes under selection. It is important to understand the mode and strength of selection acting on genes to understand organisms' biology better. Let us consider a mutation occurring in a protein-coding gene. If this mutation is synonymous and does not change the amino acid sequence of the gene, it would generally be neutral in nature. Nonsynonymous substitutions, however, are affected by selection since they change the amino acid sequence. The ratio of nonsynonymous to synonymous substitutions can be calculated for a set of orthologous sequences in the phylogenetic framework (by comparing all or some orthologs/species in a phylogenetic tree). With the advent of DNA sequence techniques and the accumulation of sequence data for a wide variety of taxa, understanding evolution at the molecular level has gained momentum.

Selection acts differently on different genes. Not all genes evolve through positive selection. For example, most housekeeping genes are least diverged even among distantly related species and show high sequence conservation. Many genes involved in reproduction and immunity are known to evolve through positive selection across a wide variety of taxa. *Drosophila* reproduction is of particular interest as one of the best models to understand evolution. The availability of genome information for hundreds of different species of *Drosophila* makes it a suitable candidate to study and understand molecular evolution. Species divergence occurs through many different established mechanisms. Sympatric species originate by achieving pre-mating reproductive isolation. *Drosophila* courtship behavior has been studied for decades and has been a great tool to understand reproductive behavior. *Drosophila* males display a series of ritualistic courtship behavior, allowing the female to decide whether to mate (Gwenaelle and Claude, 2014; also see [Chapter 101](#)). Male orients with females to identify the species-specific visual and chemosensory cues. Females also gather species-specific visual, olfactory, gustatory, and auditory cues for making a decision to allow the male to copulate. There will be several species of *Drosophila* living in the natural habitat. Both male and female flies of different species identify conspecific members through cuticular hydrocarbons (CHC) acting as pheromones, which are species-specific and highly divergent.

Olfactory and Gustatory receptors are a class of 6 and 7-transmembrane receptors expressed in the olfactory and gustatory neurons, respectively. *Gr33a* is one of the gustatory receptor genes, which is expressed on the tarsi of foreleg of male flies. Male taps the female abdomen to recognize the female by its corresponding conspecific cuticular hydrocarbon profile. *Gr33a* is primarily involved in this sex-specific CHC identification. Closely related species and incipient species at the early stage of speciation tend to have close similarity in courtship related phenotypes/behaviour. In such cases, a fly must choose the correct mating partner to ensure effective fertilization. Since *Gr33a* is involved in mate recognition, this particular gene is expected to be under strong selection pressure and, therefore, to show the signature of positive selection.

Several different methods can be employed to detect positive selection acting on a gene. Phylogenetic Analysis by Maximum Likelihood (PAML) (Yang, 2007) is one of the widely used tools in the detection of positive selection. PAML is robust, but requires considerable statistical expertise to perform and interpret the results. Mixed Effects Model of Evolution

(MEME) (Murrell *et al.*, 2012) is an adaptive evolution analysis package that employs a mixed-effects maximum likelihood approach to test the hypothesis that individual sites have been subject to episodic diversifying or positive selection. It is simple and easy to use with its user friendly interface. MEME aims to detect sites evolving under positive selection under a proportion of branches of the alignment. This chapter illustrates the use of this approach to identify the nature of selection acting on a gene taking *Gr33a* as an example.

Objectives:

To infer the mode and strength of natural selection acting on a protein through bioinformatic comparison in different *Drosophila* species.

Materials required:

A. Orthologs: In most cases, the gene of interest from a group of species should be PCR amplified, and Sanger sequenced to infer natural selection. If the genome sequences are available, one can perform ortholog search using BLAST tool kit. Gene of interest from one species can be used as query to perform *tBLASTn* (Gertz *et al.*, 2006) against the available genomes. The best BLAST hit will be used to predict the gene and CDS of the gene. The predicted CDS from all the species is used to build the alignment. For the current experiment, the ortholog sequences for the gene of interest (*Gr33a* taken as an example here) were extracted using *tBLASTn* against genomes of 12 *Drosophila* species and provided in .fasta format in the github repository (https://github.com/RajanikanthC/Gr33a_Selection_Analysis). Both aligned and unaligned data sets are provided in the repository. Students can use an alignment tool to align it themselves or use the aligned ortholog set to perform the analysis.

B. Alignment tool: In case students want to try building an alignment using the ortholog set, they can use MAAFT (Kato *et al.*, 2002), MUSCLE (Edgar, 2004), CLUSTAL OMEGA (Madeira *et al.*, 2019), or PRANK (Loytynoja and Goldman, 2005) alignment tools, which are freely available online. (It is important to remove the stop codons in the alignment)

C. Selection analysis tool: MEME package which is available in Data monkey Adaptive Evolution Server HYPHY (Pond *et al.*, 2005) (<https://www.datamonkey.org/meme>)

Experimental Details:

The orthologous sequences of *Gr33a* gene are given in .fasta format. FASTA is one of the widely used text file formats in bioinformatics. It is used for representing nucleotides or aminoacids sequences wherein base pairs or amino acids are represented using a single letter code. A sequence in FASTA format begins with a single-line description (commonly called a header) followed by lines of sequence data.

A. Building Multiple Sequence Alignment (MSA)

MSA can be built using tools such as MUSCLE (<https://www.ebi.ac.uk/Tools/msa/muscle/>), MAAFT (<https://www.ebi.ac.uk/Tools/msa/mafft/>), CLUSTAL ω (<https://www.ebi.ac.uk/Tools/msa/clustalo/>), or PRANK (<https://www.ebi.ac.uk/goldman-srv/webprank/>) by copying and pasting the ortholog sequences in the given slot in any of the above web servers. Select .fasta as the output format. Copy and paste into a file or downloaded and save as .fasta file.

1. Selection analysis

Step 1: Go to <https://datamonkey.org/>

Click on ‘Selection’ in the window displayed as below (**Fig. 1**) since we aim to study selection acting on the gene of interest.

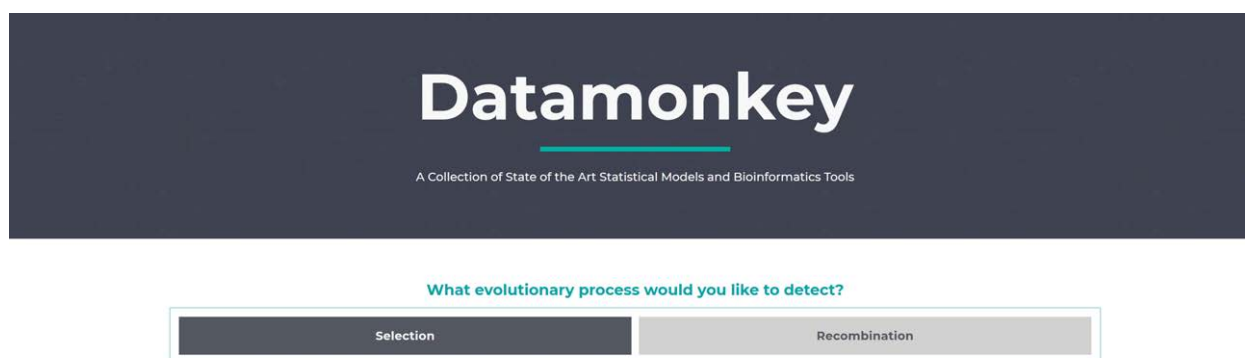


Fig. 1. GUI window of Data monkey web server showing options for selecting the evolutionary.

Step 2: Click on ‘Sites’ (**Fig. 2**) to examine selection acting on individual sites in the aligned ortholog sequences,

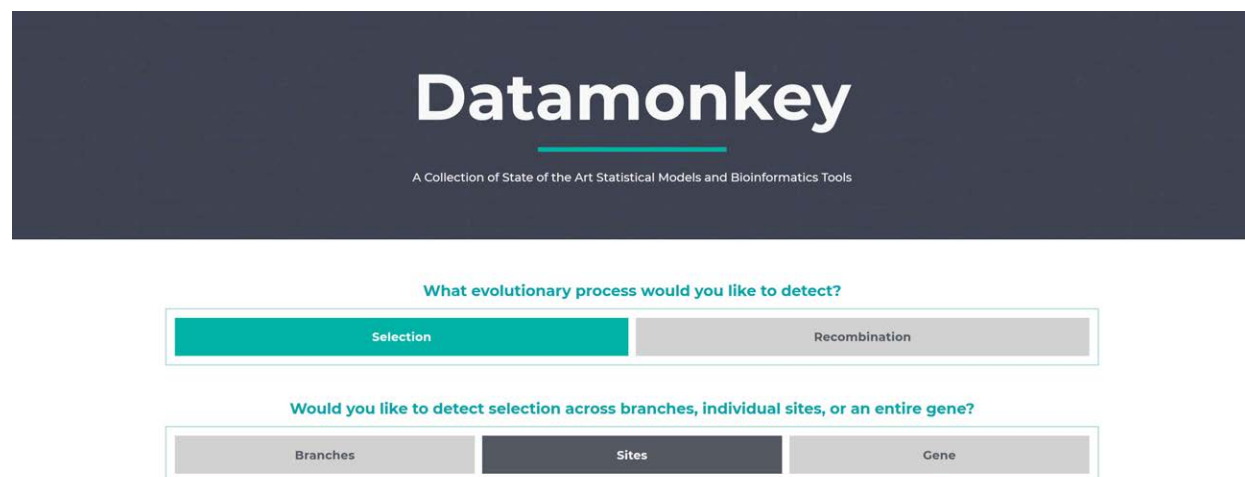


Fig. 2. GUI window of Data monkey web server showing the options for selection acting at different levels

Step 3: Select ‘Episodic’ (**Fig. 3**) to check whether the gene of interest is under positive or episodic diversifying selection.

What evolutionary process would you like to detect?

Selection Recombination

Would you like to detect selection across branches, individual sites, or an entire gene?

Branches Sites Gene

Do you want to detect episodic or pervasive selection?

Episodic Pervasive

Fig. 3. GUI window of Data monkey web server showing options for analysing the mode of selection.

Step 4: Next click on MEME (Fig. 4)

Do you want to detect episodic or pervasive selection?

Episodic Pervasive

Datamonkey recommends that you use...

MEME

Fig. 4. GUI window of Data monkey web server suggesting MEME for further analysis of selection.

Step 5: Browse and navigate on the MEME window to the folder where the alignment file is stored. Select the desired file. (Fig. 5).

- i. Select 'Universal Code' in the Genetic Code option
- ii. Provide your email address if you would like to be intimated when the analysis is over

MEME Detect Individual Sites Subject to Episodic Diversifying Selection

Browse... No file selected.

Genetic Code? Universal code

NOTIFY WHEN COMPLETED

Email Address

Run Analysis ▶

Fig. 5. GUI window of Data monkey web server for selecting the alignment file to be uploaded for analysis.

Results:

The analysis output opens as the window displayed in Fig. 6. MEME has found evidence of positive selection at 14 different sites (codons) on *Gr33a* gene with a p-value threshold of 0.05. The p-value threshold can be adjusted according to the desired stringency of analysis. Generally, the p-value threshold of 0.05 or less is significant enough to infer a

positive selection at a given site. The output consists of a summary of the analysis (Fig. 6), a Table with calculated values for each site (Table 1), an alignment-wide plot (Fig. 7) for Likelihood Ratio Test (LRT) statistic and other values, a phylogenetic tree topology for *Gr33a* gene (Fig. 8), and a model-fit summary.



Fig. 6. GUI window of Data monkey web server showing summary of the performed analysis.

Table 1. Details of individual sites and the values calculated by MEME

	Site	Partition	α	β^-	p^-	β^+	p^+	LRT	p-value	# branches under selection	Total branch length	MEME LogL	FEL LogL
summary	87	1	0.00	0.00	0.95	545.48	0.05	20.29	0.00	1.00	28.62	-26.09	-13.87
table	460	1	0.23	0.00	0.95	68.44	0.05	9.27	0.00	1.00	3.88	-22.95	-16.78
plot	464	1	0.00	0.00	0.94	14.19	0.06	9.57	0.00	1.00	0.87	-16.18	-11.77
tree	465	1	0.19	0.00	0.94	61.08	0.06	11.78	0.00	1.00	3.71	-24.71	-17.64
fits	495	1	0.29	0.00	0.94	47.40	0.06	10.14	0.00	1.00	2.91	-22.85	-15.85
	507	1	0.08	0.08	0.94	56.15	0.06	10.00	0.00	1.00	3.75	-21.98	-17.12
	496	1	0.10	0.00	0.92	46.99	0.08	6.10	0.02	1.00	4.14	-20.29	-17.20
	505	1	0.75	0.00	0.95	96.36	0.05	6.42	0.02	1.00	5.71	-23.09	-17.73
	420	1	0.00	0.00	0.95	58.42	0.05	5.16	0.03	1.00	3.30	-13.78	-11.32
	462	1	0.39	0.00	0.94	13.50	0.06	5.63	0.03	1.00	0.98	-21.40	-16.64
	508	1	0.09	0.08	0.94	73.54	0.06	5.29	0.03	1.00	4.72	-22.13	-16.72
	151	1	0.00	0.00	0.81	3.87	0.19	4.67	0.04	1.00	0.77	-15.56	-14.54
	461	1	1.20	0.00	0.94	62.05	0.06	4.87	0.04	1.00	4.31	-28.12	-22.39
	502	1	0.88	0.10	0.94	69.19	0.06	4.74	0.04	1.00	4.97	-29.79	-25.48
	494	1	0.34	0.00	0.94	57.57	0.06	3.96	0.06	1.00	3.61	-17.71	-15.09
	156	1	0.00	0.00	0.57	3.78	0.43	3.90	0.07	1.00	1.74	-25.33	-24.91

A. How to interpret the Table?

- i. The first column in the output Table 1 refers to a particular site, which is usually listed in order. However, this can be re-ordered as desired.
- ii. α (column 3) is the synonymous rate (e.g., it is 0.08 for the site 507)
- iii. β^- (column 4) is nonsynonymous rate constrained to be $\leq \omega$

- iv. $\beta+$ (column 6) is not constrained to be $\leq \omega$
- v. For purifying or neutral selection at the site 507, $\alpha = 0.08$, $\beta^- = 0.08$ ($\omega = 1$, i.e. neutral selection) at a weight of 0.94
- vi. For positive selection at the site 507, $\alpha = 0.08$, $\beta^+ = 56.15$ ($\omega = 701.8$, which is greater than 1, positive selection) and weight of 0.06
- vii. Accordingly, the site **507** can be described as ‘**to be under purifying/neutral selection ($\omega = 1$) in 94% of the times and under diversifying/positive selection ($\omega = 708.1$) in 6% of the times**’.

The plot in the **Fig.7**, based on data in **Table 1**, depicts the probability of individual sites being under positive selection (Y-axis) across the entire alignment (X-axis). As observed in the **Table 1**, the sites 87, 460, 464 and eleven other sites have the p-value threshold of ≤ 0.05 .

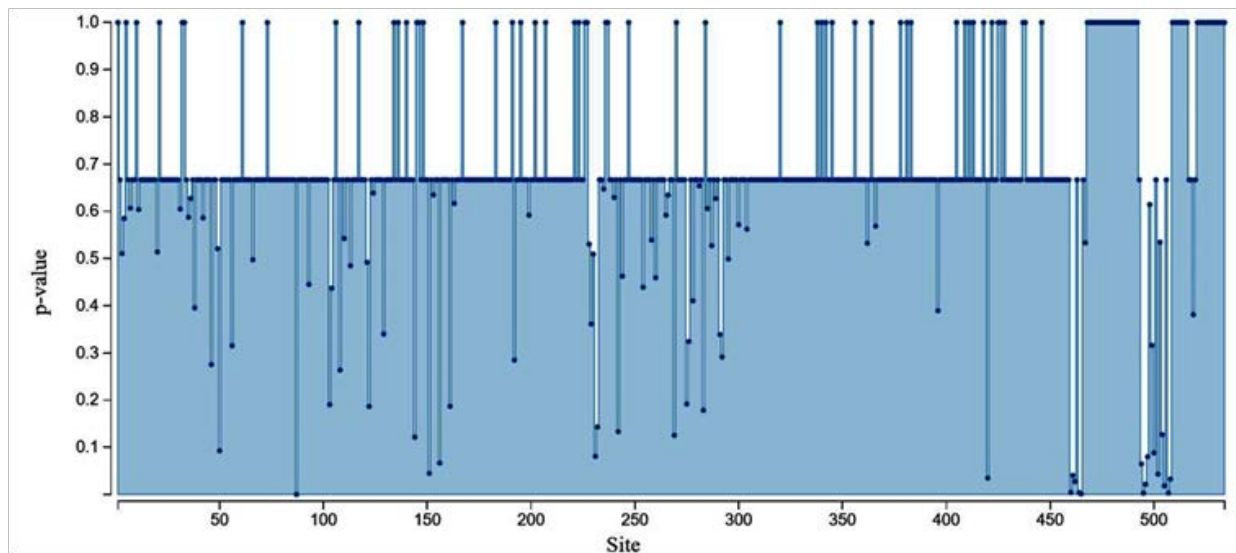


Fig. 7. GUI window of Data monkey web server with the plot of p-value distribution across the gene

The MEME analysis also generates a phylogenetic tree (**Fig. 8**) which is built, in the present case, using the *Gr33a* gene sequences in the 12 species. It is a gene tree and represents species relationship. The tree is constructed by calculating the number of substitutions per site across the alignment.

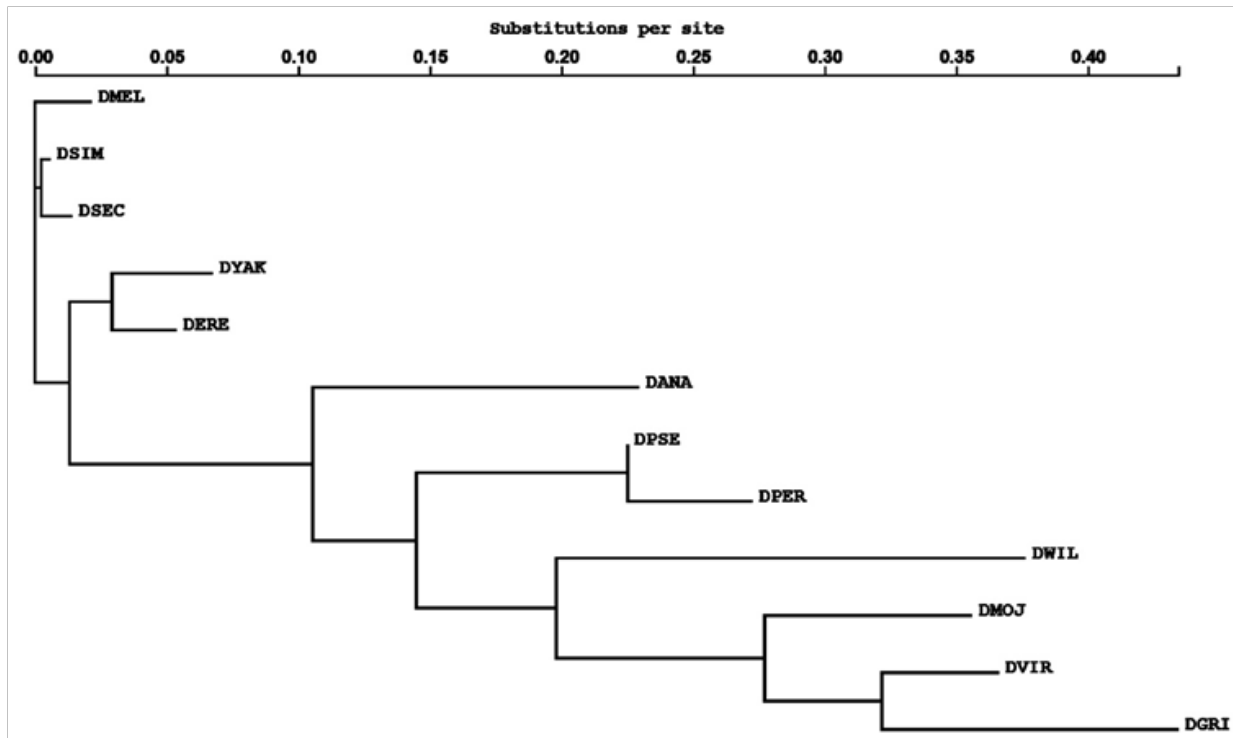


Fig. 8. Phylogenetic tree inferred by MEME, X-axis represents the number of substitutions per site. (Species abbreviations: DMEL= *D. melanogaster*, DSIM= *D. simulans*, DSEC= *D. sechellia*, DYAK= *D. yakuba*, DERE= *D. erecta*, DANA= *D. ananassae*, DPSE= *D. pseudoobscura*, DPER= *D. persimilis*, DWIL= *D. willistoni*, DMOJ= *D. mojavensis*, DVIR= *D. virilis*, DGRI= *D. grimshawi*)

B. What does this mean in the context of molecular evolution?

The above analysis of the *Gr33a* gene, which is involved in mate recognition, reveals this gene to be, as expected, under strong selection. Mutations in such genes have a distinct beneficial or detrimental phenotypic effect. A mutation that makes the mate recognition stringent, confers the advantage of mating with appropriate female and, therefore, would be enhancing the probability of more offspring. A mutation that makes mate selection less stringent, on the other hand, may make a fly recognize a wrong female. This may result in less or unsuccessful mating. Therefore, such mutations would be under negative selection and not established.

Questions for self-study and interpretation:

1. What would be the theoretical spontaneous mutation rate per replication cycle in *Drosophila* genome?
2. What other class of genes do you think would evolve through positive selection considering the biology of *Drosophila*?
3. Issues for further exploration:
 - i. The signature of positive selection can also be inferred by specifying a particular branch of the phylogenetic tree using other packages available in HYPY (<https://www.hyphy.org>)

- ii. Orthologs of any *Drosophila* gene can be downloaded from fly DIV as (<http://www.flydivas.info/>) and selection analysis can be performed.
- iii. The nucleotide sequence of *Gr33a* can be reverse translated and used to predict membrane topology since this is a trans-membrane protein (<http://www.cbs.dtu.dk/services/TMHMM/> and <http://phobius.sbc.su.se/>)
- iv. It is also possible to map the positively selected site onto different domains of the receptor and see if a particular domain is under positive selection

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Determinants of Molecular Evolution

Determinants of molecular evolution include mutation rates and patterns, effective population sizes, rates of recombination and biased gene conversion, and the fitness effects of mutations. Strict constancy of all these factors is implausible. However, the timescale of parameter fluctuations determines their relevance to molecular evolution; variability in evolutionary forces cause heterogeneous substitution patterns if parameters changes occur on a similar timescale as molecular evolution.

(In: Akashi, H. *et al.*, 2006. Molecular Evolution in the *Drosophila melanogaster* species subgroup: frequent parameter fluctuations on the timescale of molecular divergence, *Genetics*. 172: 1711-1726. <https://doi.org/10.1534/genetics.105.049676>)

Chapter 118

Detection of microbes in *Drosophila* through Sanger sequencing

Sujata Mohanty* (sujata.mohanty@jiit.ac.in) and Kopal Singhal (kopalsinghal7@gmail.com)

Drosophila Genetics Lab, Department of Biotechnology, Jaypee Institute of Information Technology, Noida 201309, India

* For Correspondence

Recommended Level: PG/Research

Sub-discipline: Molecular Biology/Genomics/Bioinformatics

Background:

Microbes and their communities dominate our earth and occupy a wide range of ecological niches. Large number of microbial species and their complicated ecosystem has made their research very challenging. Most organisms, including Dipteran insects, harbour a number of microbial species in their gut, reproductive system and other organs. The microbiome present in a living being is crucial for growth and immunity of the host. These symbiotic microbes regularly interact with the host body for their growth and support several host metabolic processes. The gut microflora are dependent on many parameters like age, gender, species, host's diet and geographical background of the host (Broderick and Lemaitre, 2012; Singhal *et al.*, 2017). Roles of bacteria like *Bacillus sp.*, *Spiroplasma*, *Acetobacter sp.*, *Lactobacillus sp.* etc in affecting different developmental processes in *Drosophila* host have been studied (Storelli *et al.*, 2011). In order to understand the effect of microbiome, it is necessary to identify the bacterial species present in the host. The availability of DNA sequencing technology permits more convenient identification of different bacterial species compared to the earlier methods based on culturing of microbes. Sanger sequencing based on dideoxy chain termination is a standard method for species identification using marker genes (Sanger *et al.*, 1977). The high conservation of 16S rRNA sequence (~1500 bp) has been widely used as a marker for identification and phylogenetic analysis of bacterial species. Because of its functional constancy as part of the 30S ribosomal subunit, it provides precise assessment of phylogenetic relatedness of organisms since the DNA region includes conserved and variable sequence regions, which evolve at very different rates. The conserved regions permit use of universal primers for amplification of the 16S rRNA sequence by polymerase chain reaction (PCR), while the enclosed variable regions allow species identification and measurement of phylogenetic relationships (Srinivasan *et al.*, 2015). In addition, there are several other conserved molecular marker genes like *rpoB*, *gyrB*, *dnaK*, *amoA*, *amoB*, *recA* etc, whose sequences have also been used for studying bacterial

taxonomy (Liu *et al.*, 2012). The present chapter provides a protocol for identification of bacteria present in different fly strains/species for further comparative studies to understand host-symbiont association.

Objective:

To understand gene markers and their role in bacterial species identification using PCR, Sanger sequencing and bioinformatic analysis.

Materials Required:

A. Flies: Healthy *Drosophila* flies (wild type from lab, field and/or mutants, as desired).

B. Equipment:

- i. Hot water bath
- ii. Microcentrifuge
- iii. Agarose gel electrophoresis unit
- iv. UV trans-illuminator
- v. Micropipettes (10, 100 and 1000 μ L)
- vi. Thermocycler

C. Reagents and Solutions:

- i. Nuclease free water
- ii. Cell lysis buffer (1 M Sodium chloride (NaCl); 10% w/v Sodium Dodecyl Sulfate (SDS); 1 M Ethylene Diamine Tetra Acetic acid (EDTA)(pH 8.0);
- iii. 1 M TRIS hydrochloride (Tris-HCl) (pH 8.0)
- iv. RNaseA
- v. 8 M Potassium acetate
- vi. Iso-propanol (2- propanol)
- vii. Agarose (molecular grade)
- viii. TAE buffer (1 X)
Stock solution (50 X, Volume 1 L): dissolve 242 gm of Tris Base in 500 mL distilled water; add 57.1 mL of Glacial Acetic Acid and 100 mL of 0.5 M EDTA solution; make the final volume to 1 L with distilled water.
- ix. Gel loading dye solution (6X), containing 0.25% Bromphenol Blue, 0.25% Xylene Cyanol, 30% Glycerol.
- x. Ethidium Bromide (EB) staining solution (0.01 gm of (EB) in 1 L of MilliQ H₂O)
- xi. PCR mix (PCR buffer with MgCl₂, dNTPs (200 μ M), Taq polymerase, nuclease free water)
- xii. Primers (0.1 μ M) – for sequence of primers, see **Table 1**.
- xiii. Exonuclease I and Shrimp Alkaline Phosphatase solution

Table 1: Sequences of Forward and Reverse universal primers for amplification of 16S rRNA sequences from any bacterial species

Name*	Primer sequences (Forward and Reverse)	Annealing temperature	Amplicon size (in basepairs)
Universal 16S rRNA bacterial primer pair 1	27F (5'-AGAGTTTGATCCTGGCTCAG-3') 1492R (5'-GGTTACCTTGTTACGACTT-3')	55°C	1500
Universal 16S rRNA bacterial primer pair 2	Forward-5'CCAGCAGCCGCGG TAATACG-3', corresponding to nucleotides 518 to 537 of the <i>E. coli</i> 16S rRNA gene, Reverse -5'-ATC G G (C /T) TACC T TG TTACGACTTC3', corresponding to nucleotides 1513 to 1491 of <i>E. coli</i> 16S rRNA gene	58°C	996

* The primer pair 1 (27F and 1492R) is considered to be the best primer pair for amplification of nearly full-length 16S rRNA sequences. However, to obtain a taxonomic resolution of bacteria at species and strain level, other primer pair (e.g, the primer pair 2) is used.

Experimental Details:

1. Extract genomic DNA (gDNA) from a single fly or larva of desired genotype (for single fly gDNA isolation, see [Chapter 61, 62, 63](#)). Replicates of gDNA should be collected from different individuals.
2. Check quality of the extracted gDNA by running it on agarose gel (see [Chapters 60, 61](#)).
3. Amplify the microbial 16S rDNA sequences in the extracted gDNA by PCR amplification with the universal primers listed in [Table 1](#).
4. For PCR, include the following steps: a) initial denaturation for 2 to 10 min at 94°C, b) denaturation (45-60 sec at 94°C), c) annealing (45 sec at the determined optimal annealing temperature), d) extension (60 sec at 72°C), e) Repetition of steps a-d for 30 cycles, f) final extension for 5 min at 72°C and g) holding the PCR amplification products at 4°C for 10 min ([Fig. 1](#)).

Note: Since PCR is a very sensitive process, concentration of each component in the PCR mix, including the amount of gDNA, the annealing temperature etc. need to be standardized to obtain optimal PCR product (Joshi and Deshpande, 2010).

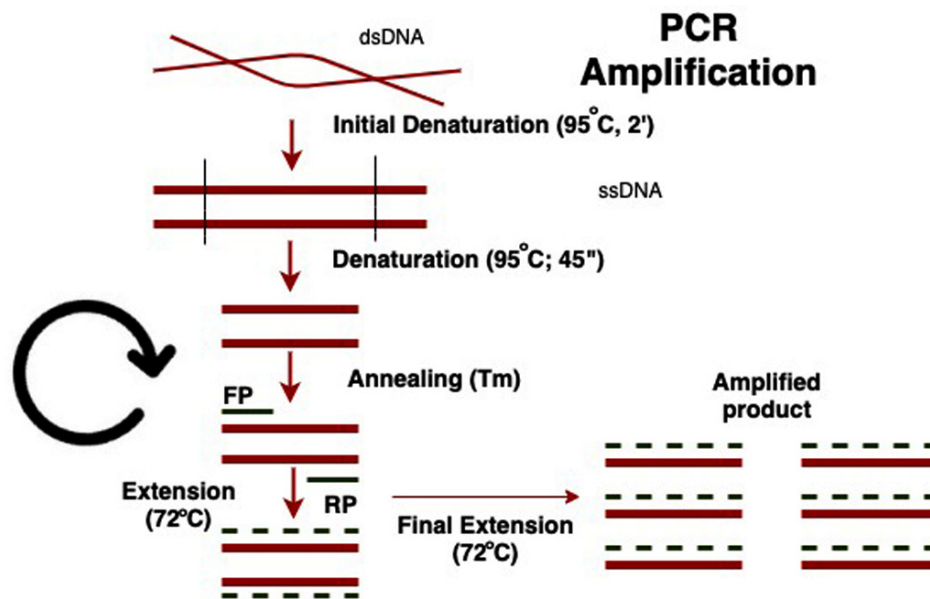


Fig. 1. Schematic of steps in PCR amplification

5. Visualise the amplified PCR product on 1% agarose gel and purify the PCR products using two enzymes Exonuclease I and Shrimp Alkaline Phosphatase to remove DNA or primer impurities (for details of these steps refer to Mardis and McCombie (2016).

6. Nucleotide sequences of the purified PCR fragments using Sanger sequencing technique can be obtained by outsourcing or through the sequencing facility if available in-house. Sequencing should be done using both forward and reverse primers for accuracy.

7. Align both forward and reverse sequences by choosing ‘contig formation’ using an appropriate software (e.g. BioEdit free software); editing of nucleotide sequences can be done by comparing the forward and reverse aligned sequences. Finally, save the consensus sequences in appropriate file.

8. Search homology for the PCR amplified 16S rRNA sequences at the NCBI database using the Blastn tool to identify the bacterial populations present in the given DNA isolated from the fly sample.

Note: If some microbial sequences show <98.7% sequence identity with those in the NCBI database, these can be considered as novel bacterial species.

Results/Observations:

1. Observe the *Drosophila* gDNA and PCR products after electrophoresis in agarose gel to check that all the DNA bands are sharp, without any trailing smear, which indicates random fragmentation of gDNA during extraction or nonspecific amplification during PCR amplification.

2. Small sized DNA bands (~30-50 bps) PCR amplified DNA lanes in the agarose gel represent formation of primer dimers rather than amplified products.

Note: While performing homology search with Blastn, the E-value should be close to zero

since this value represents the expectation of finding that sequence by random chance. Search for a short sequence (<100 bps) is likely to generate a lot more hits with high E-value (low significance), while search with a long sequence (> 500 bps) would give fewer hits with lower E-value (greater significance). The lower the E-value, or the closer it is to zero, stronger is the matching.

Percent identity reflects the percentage of similar nucleotide sequences present among the query and the Blastn-searched homolog target sequences at the NCBI database.

3. List the bacterial species identified in the examined *Drosophila* gDNA samples. Compare results among different species/populations of *Drosophila* to know more about host species-specific nature of microbes.

Questions for self-study and interpretation:

1. Are there variations in microbial diversity on the basis of host's gender, geographical location or different host species or mutant and wild type stocks maintained in laboratory?
2. Why should the microbiome vary between different populations of the same species? What factors may affect the microbiome of an individual?
3. What should be the minimum percentage of matching in Blastn result to identify a bacterial strain/ species?

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Experiments with *Drosophila* for Biology Courses



(Credit: CalTech Archives)

*Few laboratory creatures have had such a spectacularly successful and productive history as *Drosophila*, the friendly fruit fly. It first entered laboratories around 1901, revealed its talent for experimental genetics in 1910, and with some ups and downs is still going strong.*

(In: Koehler, R. (1993) [J. History of Biology. 26: 281-310](#))

Morgan's Reversal of Ideas

In the first decade of the twentieth century Morgan strongly opposed three of the most prominent ideas of the day: Darwinian natural selection, the chromosome theory of heredity, and the newly-rediscovered Mendelian genetics. Moreover, and quite remarkably, he went on to change his mind on all three theories and developed a vibrant research program that ultimately helped pave the way for the unification of genetics and Darwinian evolution in what has been called “the evolutionary synthesis” of the 1930s. Morgan’s is a rather unusual case in which a scientist makes an about-face on not one, but three different theories in a relatively short period of time (between 1910 and 1915).

(In: Garland, E. A. 2013. How Many Times Can You Be Wrong and Still Be Right? T. H. Morgan, Evolution, Chromosomes and the Origins of Modern Genetics. Science & Education. https://nosyevolucion.files.wordpress.com/2015/09/allen_morgan-and-hos.pdf)

S. P. Ray-Chaudhuri – Founder of *Drosophila* Research In India

It is an interesting story how Ray-Chaudhuri's *Drosophila* laboratory in Calcutta was established. When JBS Haldane, adjudicator of Ray-Chaudhuri's doctoral dissertation (*under H. J. Muller at Edinburgh*), visited him in 1954-55 in Calcutta University, he noticed Ray-Chaudhuri doing only chromosomal studies and he was not making use of his expertise in *Drosophila* genetics because of lack of an air-conditioned laboratory. Haldane had a high opinion of Ray-Chaudhuri and wanted his research talents to be fully utilised. Haldane had decided by then to settle in Calcutta as a naturalised Indian citizen. He wrote to Pandit Nehru, the then Prime Minister of India, from England before leaving for Calcutta about his need of a *Drosophila* laboratory for genetics research. Nehru phoned the then Vice-Chancellor of Calcutta University Sir JC Ghosh and requested him if he could help in the matter. Haldane had suggested that SP Ray-Chaudhuri of Zoology Department of Calcutta University be consulted for details of the laboratory. The Vice-Chancellor called Ray-Chaudhuri to his office and asked him that *Drosophila* laboratory for Haldane be made ready at the earliest in the Zoology Department. He was told that the required fund would be made available immediately. The airconditioned lab was promptly made and Ray-Chaudhuri arranged some *Drosophila* stock from Muller in the USA. Haldane shifted to Calcutta and he brought with him a few *Drosophila* mutants which he gave to Ray-Chaudhuri. When Ray-Chaudhuri was handing over to Haldane the key of the *Drosophila* laboratory, Haldane smiled and said that the laboratory was asked not for his research but for Ray-Chaudhuri to work on *Drosophila* genetics. This was how the first *Drosophila* laboratory of the country was established. (In: Sharma, T. (1994) Sachi Prasad Ray-Chaudhuri. [INSA Biographical Memoirs. 22: 103-113](#)).

For details of Fly Community and Resources in India, visit <https://sites.google.com/view/indian-fly-reseachers/home>

Experiments with *Drosophila* for Biology Courses

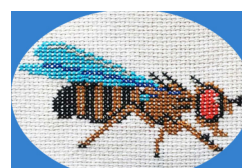
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