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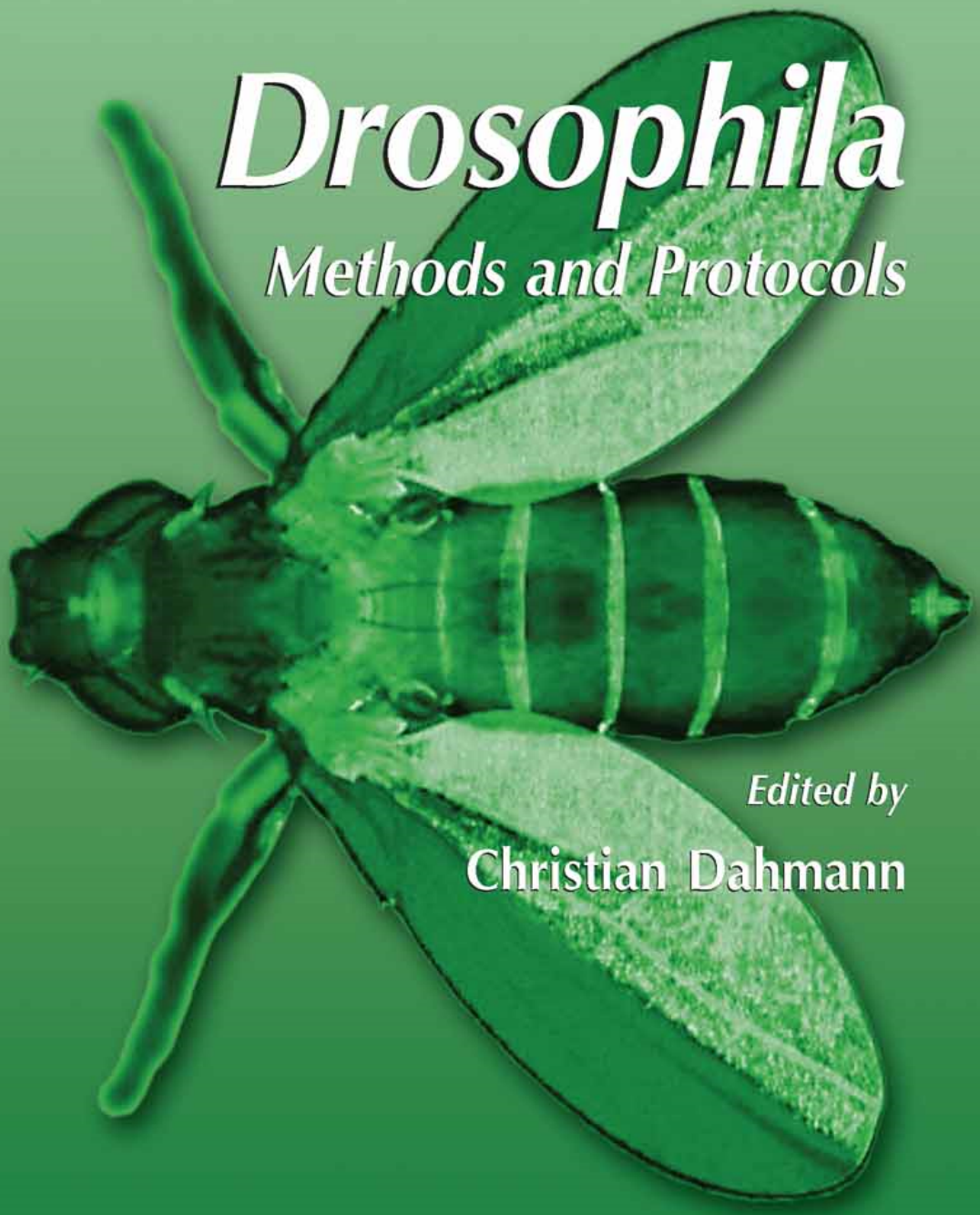
Methods and Protocols



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Methods and Protocols

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Cover illustration: An artistic presentation of a *Drosophila* fly expressing green fluorescent protein under control of the *engrailed* gene in all cells of the posterior compartments.

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Preface

Drosophila melanogaster has been the model system of choice for many investigators over the past hundred years. Due to its long-standing tradition as a model organism, many techniques used in *Drosophila* have been established and continue to be developed to answer a variety of scientific questions. The recent invention of techniques allowing the knock-down of genes by RNA interference and gene replacement by homologous recombination are two important demonstrations of the ongoing efforts to broaden the technical repertoire at hand for researchers working with *Drosophila*. This volume is a collection of protocols covering both standard techniques as well as novel methods. An introductory chapter highlights the importance of *Drosophila* as a model system for the development of Biology in the 20th century and review chapters provide concise and up-to-date overviews on selected experimental systems.

This book makes no attempt to be comprehensive. A number of frequently used standard techniques were selected and are described in-depth to allow novices to get started with *Drosophila*. In addition, detailed protocols of recently developed methods that we felt will become of broad use in the *Drosophila* community within the next years are included.

I am grateful to Konrad Basler, Suzanne Eaton, Elisabeth Knust, Pavel Tomancak, and Andreas Wodarz for alerting me to novel techniques and for their advice on selecting the protocols for this book. I would also like to thank all the authors for their expert contribution to this Volume.

Christian Dahmann

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1

Drosophila melanogaster and the Development of Biology in the 20th Century

Alfonso Martinez Arias

> "When I see *Drosophila* under moderate magnification of a binocular microscope I marvel at the clearest form of the head with giant red eyes, the antennae, and elaborate mouth parts; at the arch of the sturdy thorax bearing a pair of beautifully iridescent, transparent wings and three pairs of legs……"
>
> C. Stern (1954) "Two or three bristles" *Am. Sci.* **42,** 213–247.

Summary

The fruit fly *Drosophila* has played a central role in the development of *biology* during the 20th century. First chosen as a convenient organism to test evolutionary theories soon became the central element in an elaborate, fruitful, and insightful research program dealing with the nature and function of the gene. Through the activities of TH Morgan and his students, *Drosophila* did more than any other organism to lay down the foundations of *genetics* as a discipline and a tool for *biology*. In the last third of the century, a judicious blend of classical genetics and molecular biology focused on some mutants affecting the pattern of the *Drosophila* larva and the adult, and unlocked the molecular mechanisms of development. Surprisingly, many of the genes identified in this exercise turned to be conserved across organisms. This observation provided a vista of universality at a fundamental level of biological activity. At the dawn of the 21st century, *Drosophila* continues to be center stage in the development of *biology* and to open new ways of seeing cells and to understand the construction and the functioning of organisms.

Key Words: Development; *Drosophila;* fruit fly; genes; genetics; history.

1. Introduction

Biology is about experiments rather than theories, about observation and description rather than prediction. For this reason, it is often difficult to separate what one knows from how one knows it, the observation from the method.

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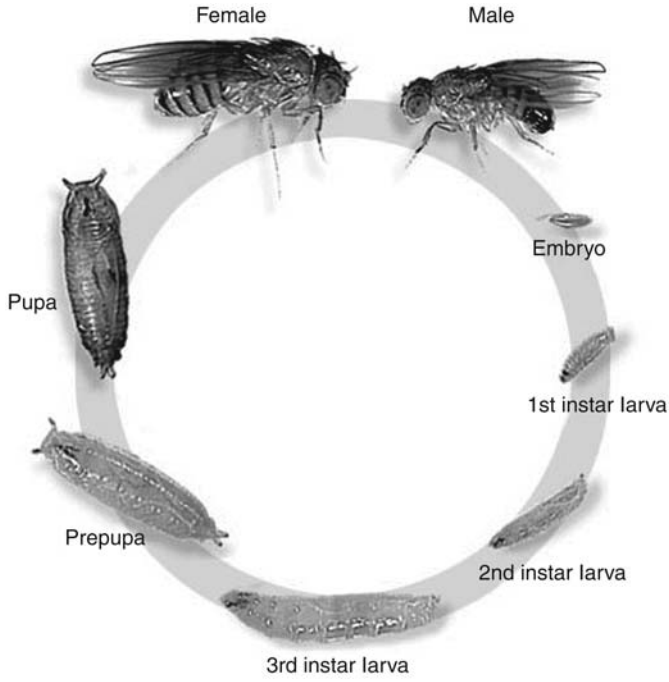

Fig. 1. Male and female *Drosophila* and their life cycle (courtesy of Christian Klämbt in FlyMove: http://flymove.uni-muenster.de/Homepage.html).

Once we notice the presence of wings in an organism we can check whether they are present in others; however, even if we see them in many, this does not allow us to say that they will be present in all: all birds have wings but mammals do not. The same is true at whichever level of organization one looks at. Successful predictions are rare in *biology*. It is perhaps, for this reason, that the notion of *model organism* has been crucial in the development of *biology* as a *science* in the 20th century. A model organism is one which allows us to analyze a particular problem in the hope that the answer it gives us will be general and perhaps universal. Thus, peas and plants in general, were essential to develop the key notions of *heredity*, finches for *evolution*, and bacteria for unravelling the molecular nature of the gene, the genetic code, and the fabric of metabolism. With few exceptions model organisms come and go and are limited to specific fields and moments but one has had a constant presence in the 20th century and has made significant contributions to multiple areas of *biology*: the fruit fly *Drosophila melanogaster*.

Drosophila **(Fig. 1)** was introduced as an experimental animal at the beginning of the 20th century, probably around 1901 in the context of evolutionary biology but soon became a workhorse of biological research ***(1,2)***. Its main

attributes were then the same as they are now: rapid generation time, ease and robustness of culturing, and low maintenance cost. Over the years it has left its mark in a wide range of questions from the nature and organization of the hereditary material to the effect of space trips on embryogenesis. However, much of this mark is not only by way of the concepts it has generated but also in terms of ways to approach problems that have been exported to other organisms. This volume is a compilation of methods used with *Drosophila* in the last few years to tackle a number of problems of cell biology and developmental genetics. The methods are described by active practitioners and therefore, have the flavor that only a cook can give to a meal. It is a very up-to-date compilation and adds significantly to others of a more general nature ***(3–5)***.

In this introductory chapter I have been asked to comment on *Drosophila* "as a model system." Instead of providing an annotated foreword to the technical chapters that follow, I have decided to illustrate the development of *Drosophila* as the sophisticated experimental organism that it is today and how this development has resulted from its rising to the challenge of specific biological problems. My account will be couched in a historical framework but this is no abridged history of the contributions of *Drosophila* to biological knowledge, a subject that would take more space than is available here and that probably would take a different approach.

2. TH Morgan, his Students, and the Foundations of Genetics

Around 1908, in room 613 of Schmerhorn Hall at Columbia University in New York, Thomas H. Morgan, an embryologist of some renown at the time, begins to grow *Drosophila* in large quantities with an interest in exploring the existence of what today we would call "macromutations." At the time, classical animal and plant breeding are turning into the new *science* of *genetics* and for people with an interest in the relationship between genes and phenotypes, mutations provide an intriguing, albeit mysterious, link. Sometime in 1910, Morgan came across a fly with white eyes, which was going to sidetrack him from his interests for 20 yr. This was the first allele of *white*, and its linkage to the sex chromosome ***(6)*** triggered a revolution in our understanding of heredity and led to the establishment of *genetics* as a subject with defined concepts and experimental methods.

Much has been made of how TH Morgan and his students Sturtevant, Bridges, and Muller laid down the foundations of modern *genetics* and I shall not dwell on this here (but *see* **refs.** ***1,2,7–9***). When Morgan begins to grow *Drosophila* in 1908, genes are hereditary particles in the abstract mendelian sense, a concept Morgan was suspicious of ***(1,6)***, without a subcellular location, fabric, or clear connection with their products, the phenotypes. By 1928 when Morgan moves to the California Institute of Technology to establish the biology division, there is an impressive edifice on which much of modern *biology* will be built.

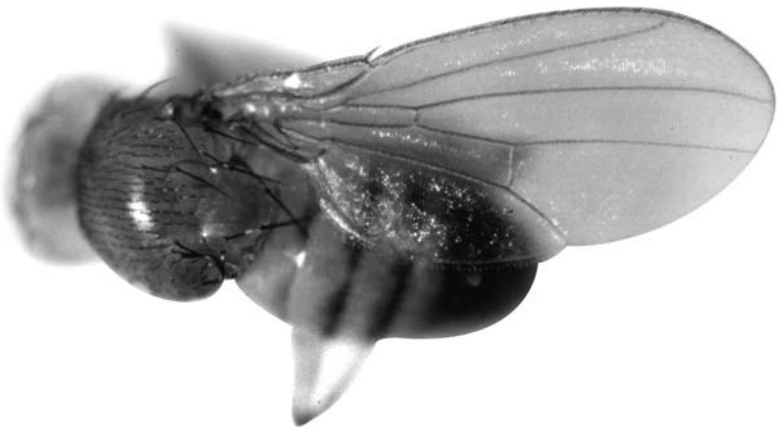

Fig. 2. Adult fly homozygous for the *wingless*[1] mutation, which leads to the loss of wing tissue and its transformation into notal tissue. Often the phenotype is not fully penetrant and only shows on one side, which by comparison with the other side, highlights its features (courtesy of Elizabeth Wilder).

In a period of about 20 yr, Morgan and his students showed the universality of Mendel's factors, that they could be arranged in linear order and that this order can be used to create genetic maps, that these factors exist in different forms, alleles, which can mutate in forward and reverse manner, and that their functioning depends on their position within the chromosome. In addition they demonstrated that the genes lie on the chromosomes and elucidated the existence of chromosomal aberrations (inversions, duplications, and deletions) from genetic data before observing them directly, i.e., they could use genetics as a predictive algebra for chromosomal structure. Altogether these findings created a foundation and a language, which enabled *biology* to progress for much of the 20th century. Finally, one of Morgan's students, H. Muller demonstrated that mutations could be induced by X-rays. In doing this he opened the door not only for the understanding of the chemical nature of the gene, but also, and perhaps most importantly, for the generation of alleles and mutants that have been an essential element of progress. At the same time with a system of balanced lethals, or what we call today "balancer" chromosomes, Muller introduced the ability to keep stable stocks, a system which is the envy of other organisms and which has enabled a huge amount of genetics in *Drosophila*.

In addition to concepts, a most important legacy of this period is mutants **(Fig. 2)**, carefully kept in stocks, some of which have survived almost 100 yr. They were used to probe into the nature of the gene and thus, mutations like *achaete* and *scute*, *bithorax*, or *Notch* served as battle grounds to define notions

like allele, dominance or recessiveness, cross over, *cis–trans* tests, and the logic of complex complementation or of gain and loss of function. However many of these genes were hiding other revelations for which the ground work of these years would prove invaluable. Inadvertently, *Drosophila* was doing the first homework of any model organism: good genetics and mutants.

3. Difficult Problems as Tractable Questions

TH Morgan was not a geneticist at heart and never stopped being an embryologist/developmental biologist. Two anecdotes betray this fact. Intriguingly, he only published one paper in the journal Genetics and this was on the subject of the obituary of C. Bridges (referred in **ref. *10***). In addition, and perhaps most significantly, after he moved to Caltech in 1928, he left Genetics to C. Bridges, A. Sturtevant, and his new recruits and returned to the embryological investigations with marine organisms that had occupied him in his pre-Columbia days ***(6,11)***. It some times appears as if, for Morgan, *genetics* was a necessary distraction, a "deviation" as S. Brenner has put it, to develop tools to tackle, with more hope of success, the issues that preoccupied him: the secrets of animal development and evolution. At several times in his career he tried to bridge the two fields ***(12,13)*** but never made much progress; although he did feel that *genetics* one day would provide the answers he was seeking ***(13)***. Notwithstanding this impasse, progress was being made in linking genes and embryos, although it took time to appreciate this.

Part of the difficulty to bridge the two subjects was that embryology is about embryos, and the Morgan school, engaged as it was in the abstractions that genes were at the time, never paid too much attention to what was going on inside fertilized eggs. In the late 1920s and 1930s, the embryogenesis of *Drosophila* began to be described ***(2)***. D. Poulson ***(14)*** did the most thorough work summarized in a classic article, which served as an obligatory reference until the publication of the treaty of J. A. Campos Ortega and V. Hartenstein ***(15)***, the standard modern reference, which describes the process with modern histological techniques **(Fig. 3)**.

Poulson did not simply describe the histology and development of the embryo, but also tread into the relationship between the genome and development. At the time it was felt that there was a correlation between the amount of chromosomal material and the development of the animal. This, no doubt, was derived from the classical experiments on dispermic fertilization of sea urchin eggs by T. Boveri, who showed a correlation between the number and identity of chromosomes present in an embryo and its degree of development ***(16)***. A quantitative correlation between chromosomes and development was reinforced by observations on the development of flies with large intrachromosomal deletions until the issue was systematically analyzed by Poulson in a study of the

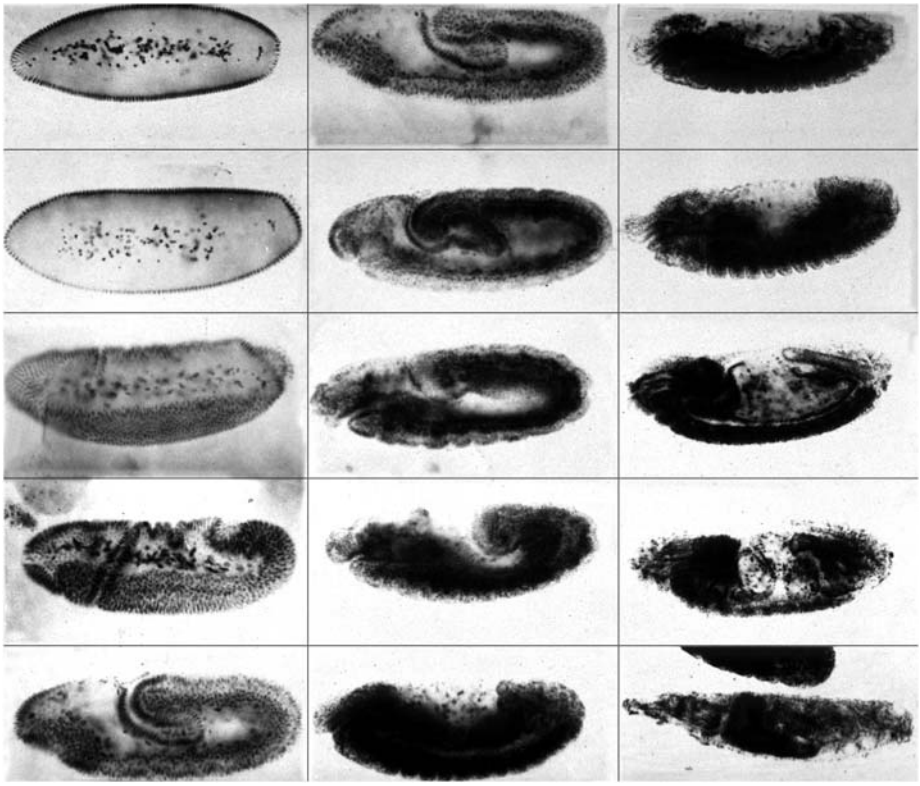

Fig. 3. Whole mounts of *Drosophila* embryos stained with Fuchsin (which highlights the nuclei) and which reveal the development and morphogenesis of different tissues. Blastoderm is at the top left and the hatching larva at the bottom right. The density in the stain is associated with an increase and density of cells. These preparations were very useful in the analysis of internal organs in the 1980s.

Notch region. Focusing on the phenotype of embryos mutant for *Notch*, which had precise defects in the development of the epidermis, the nervous system, and the mesoderm, he noted that the defects of embryos with variable deletions that included the *Notch* locus were the same as those bearing mutations in *Notch*, as long as the deletion included *Notch* ***(17)***, i.e., he established the first correlation between a gene and a specific developmental defect.

A few years later, in Switzerland, E. Hadorn coming from a tradition of experimental embryology began to use imaginal disks to ask similar and related questions. In the course of this work he developed techniques to generate mosaics between cells of different genotypes, which allowed him to probe into issues of cell differentiation and cell determination that were going to play an important role later on. Two important contributions of his work are the notion of stability

of certain differentiated states ***(18)*** and his treaty "developmental genetics and lethal factors" ***(19)***, which represents an important account and discussion of the significance of lethal mutations in a large number of organisms.

These studies were exceptions, and for the most part, work with *Drosophila* still focused on visible mutations in the adult, for example the effects on bristles of mutations in *achaete* and *scute*, many that affected the pattern of the wing and to a fewer extent, the legs. These mutations were used to probe the nature and behavior of the gene, but the connection between these mutations and the developmental events they affected took time to come to the fore. A particularly interesting and significant case in point is the development of the work of E. Lewis on the *bithorax* mutants, a collection of mutations initially uncovered by C. Bridges and which appeared to transform one segment of the fly into another. E. Lewis ***(20)*** began to study *bithorax* mutants to explore abstract notions of the fine structure of genes, but he slowly realized that they reflect homeotic transformations ***(21)*** and contain information about the relationship between genes and development ***(22,23)***. His work moved slowly from gene structure to gene function and pioneered a logic and an approach that were to prove very important for the developments of the 1980s ***(24)***.

After the 1930s, *Drosophila* moves out of the limelight, which becomes occupied by phages and *Escherichia coli*. The objective was to unravel the molecular nature of the gene and *Drosophila* was not the organism of choice for this task. However, *Drosophila* researchers continued to probe into other problems in the way they knew best: using mutations and the cook book of *genetics*. In doing this, it became steadily clear that *genetics* harnesses an enormous potential to turn difficult problems into a set of tractable and answerable questions, and much of the progress that led to the watershed of the end of the century relied on the steady accumulation of information and reagents that took place during the 1940s and the 1950s. It is along these lines that the work of E. Lewis tranforms itself from abstract genetics to the relationship between genes and development, and that of E. Hadorn from experimental embryology to developmental genetics and to cite another important example a few years later S. Benzer decides to use the fly to tackle problems of neurobiology and behavior with excellent dividends ***(25)***.

4. Mosaics

The significance of the work of Lewis and Hadorn would take some time to be noticed. There were several reasons for this and one of them is that in the context of developmental biology mutations can be dangerous weapons. It is good thing to have a mutation that disrupts a process and generates an abnormality but it is a different one to know the cause of the phenotype, as it is often not clear whether the phenotype is a direct or an indirect consequence of the mutation

(19). It is important to know if the effect that one observes is specific or not, whether it is instructive or responsive. Some of these issues were resolved by the introduction of mosaic analysis for the study of gene function, an approach that has now become widespread using the guidelines derived from *Drosophila*.

Development is not just about generating different tissues or structures but about organizing cells in space. It was C. Stern who first noticed that one could use genetics as an analytical tool to probe this problem and find the relationship between gene function and pattern ***(26)***. In his initial studies he used mutants in the *achaete/scute* complex, which affect the pattern of bristles in the adult. Seizing an observation by A. Sturtevant that it was possible to generate individual mosaic for particular mutations ***(27)*** he generated flies in which some of the epidermis was wild-type and some mutant for specific alleles of *achaete* and *scute*. The technique was a genetic enhancement of the loss of the X chromosome in females, which allowed the generation of gynandromorphs in which some of the tissue is male (XO) and some female (XX). If the fly contained a mutation in one of the X chromosomes, its phenotype reveals itself in the male tissue. Taking advantage that *achaete* and *scute* are on the X chromosome he analyzed their requirement in the generation of the pattern of bristles. He observed that bristles were affected only when the producing cells were mutant for *achaete* or *scute*, i.e., the mutations behaved cell autonomously ***(26)***. He extended these studies with mosaics obtained by mitotic recombination and concluded that these genes were involved in the read out or response to some underlying prepattern that he struggled to find for most of his scientific career ***(28)***. Stern's analysis of the *achaete/scute* complex has withstood well the passage of time and his conclusions served as an inspiration for further work on the nature and function of these genes ***(5,29,30)***. E. Lewis used the mosaic technique to analyze the mutations of the Bithorax complex (BX-C) and show that these genes, also, act in a cell autonomous manner ***(31)***. This work was the beginning of an important link between genes and cells.

The use of mosaics to analyze gene function was taken to a modern level of possibilities with the work of A. Garcia Bellido and his students. Garcia Bellido had worked with Hadorn and was aware of the possibilities of mosaics to analyze issues of determination and differentiation in imaginal disks. In the late 1960s, he went to Caltech to work with E. Lewis and to learn some of the genetic techniques that were being developed to analyze developmental events. His interactions with Sturtevant and Lewis led him to use genetic mosaics for the study of cell lineages and in collaboration with J. Merriam extended Stern's technique of mitotic recombination to generate large numbers of temporally controlled mosaics ***(32)***. This allowed him to trace precise lineages of different tissues and to ask very defined questions about gene requirements in time and space, observations that would lead him to the discovery of compartments ***(33)***

and to the connection between gene activity in the cellular realm with a particular emphasis on *bithorax* and the adult lineages ***(34)***.

The visit of A. Garcia Bellido to E. Lewis blent traditions and approaches in a manner that increased the conceptual and technical repertoire of *Drosophila* as a model organism. At about the same time, a parallel blending exercise was taking place at Yale when another student of Hadorn, W. Gehring, met the last student of Poulson, E. Wieschaus. While Garcia Bellido, Merriam, and Lewis were interested in adult lineages, gene function, and determination, Wieschaus and Gehring began to explore related issues in the embryo and its relationship to the imaginal disks ***(35)***. This fusion of Europe and the US was going to have important consequences and allow *Drosophila* to tackle as an important model system the problem of development.

Mosaic analysis was not just used to tackle simple developmental problems but began to have an important role in neurobiology where the pioneer work of Hotta and Benzer ***(36)*** attempted to map behaviors to particular loci and organs. Mosaic analysis is an indispensable tool of modern fly genetics and was taken to a level of ease and interest with the FLP system ***(37,38)*** and its extension to internal organs with the availability of histological markers ***(39,40)***. It is easy to underestimate the significance of these developments as mosaic analysis is, today, a tool of choice for the analysis of any gene within a developmental context with a significant impact in the analysis of vertebrate development.

5. The Molecular Nature of Genes

Morgan never gave up wondering about the links between *genetics* and *embryology* and in his Nobel address, considering how genes might regulate development remarked that "it is conceivable that different batteries of genes come into action one after another, as the embryo passes through its stages of development" ***(13)***. To test and probe into this remarkable statement required understanding the molecular nature and biology of the gene and the ability to identify, read, and interpret those batteries of genes. Unfortunately *Drosophila* was not the organism of choice to answer these questions. Solving these problems required the development of molecular genetics and molecular biology in bacteria and phage, which provided a new set of tools and concepts that made classical genetics more powerful.

Once the link between genes, DNA, and the genetic code became clear, and the technology to obtain and characterize DNA was at hand, one could turn to issues of how genes look like and what they encode. Answers to the genetic control of development or behavior were at hand. Waiting on the wings were *bithorax* and *achaete–scute*, *Antennapedia*, and *Notch*, and also genes involved in behavior like *Shaker* or circadian rythms like *period*. What did they encode? Enzymes? Structural proteins? A new kind of entity dedicated to development

and behavior? Anecdotes of those musings could fill many pages. If genetics and experimental embryology began to give us an inkling of the relationship between genes and cells, a second fusion exercise between molecular biology and developmental genetics broke the code of development and began to place names to the cogs and wheels of behavior.

6. Fusion Biology

The molecular era of *Drosophila* genetics begins with D. Hogness proposal in 1971 to use large overlapping pieces of DNA to obtain maps of chromosomes at a molecular resolution (referred in **ref. *41***). For the next few years this work leads to the development of techniques and materials, which allow the assembly and mapping of large chromosomal regions. Throughout the late 1970s, a collaboration between Hogness and Lewis initiates an assault on the BX-C through a fusion of molecular biology and classical genetics, spearheaded by W. Bender and P. Spierer ***(42)***. The BX-C is what the studies of E. Lewis had turned the collection of *bithorax* mutants into and which he interpreted as a gene complex regulating the differentiation of the posterior half of the fly ***(23)***. The objective of the collaboration was to obtain a molecular map of the region and uncover the functions of the resident genes. Shortly afterward two other foci of activity target a group of genes, the Antennapedia complex, identified by T. Kaufman and then hypothetically related to the BX-C. MP Scott in Bloomington and R. Garber with W. Gehring in Basel (Switzerland) undertook the exploration of the corresponding region of DNA. It might seem strange that hour long seminars on restriction maps and locations of inversions, insertions, and deletions filled rooms with excitement, but this was the way it was in the 1980s when everybody was reading much in those maps where often transcripts were not easy to spot. This work revealed an intriguing landscape of large transcription units with small exons and mutations peppering noncoding regions **(Fig. 4)** ***(42–45)***. The correlation with function required a visualization of the spatial distribution of the transcripts and it was M. Akam in the Hogness group who began to work out the methods to do this in *Drosophila* embryos and imaginal disks. The patterns of transcription that emerged were roughly in agreement with the requirements laid down by the genetics, i.e., genes were expressed in defined patterns that overlapped the regions in which they were required, but they also revealed surprising temporal dynamicity and intriguing tissue and cell type specificity **(Fig. 5)** ***(46,47)***. The mutants were the landmarks that colored the otherwise arid landscape of restriction maps of large DNA stretches and a picture of these complexes began to emerge. Much is rightly made of these specific studies, but it is important to mention how parallel studies were undertaken to analyze the molecular nature of genes shown to influence the development and activity of the nervous system, in particular *Notch* and *period*.

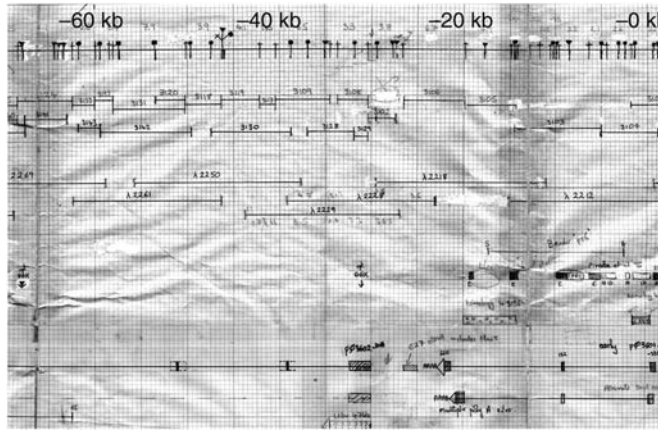

Fig. 4. Molecular map of the *Ultrabithorax* region of the BX-C as used by Michael Akam (modified from W. Bender) in his studies of the spatial expression of the *Ultrabithorax* gene (courtesy of Michael Akam).

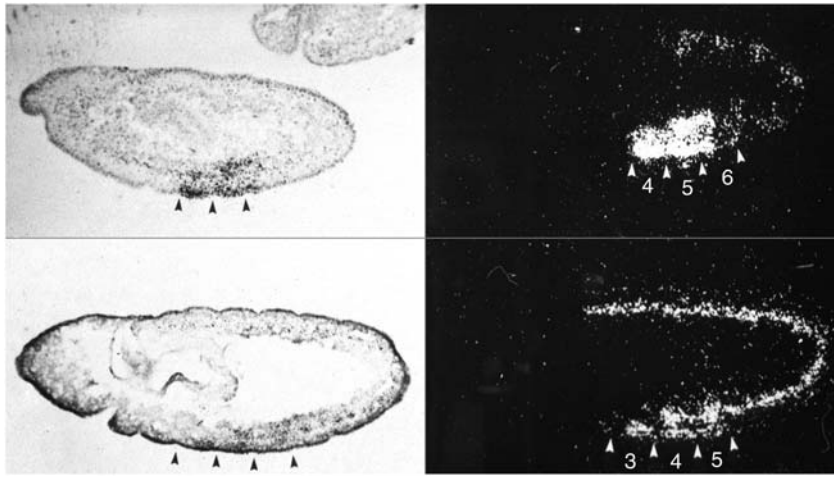

Fig. 5. Differential activity of the two promoters of the *Antennapedia* gene from the Antennapedia complex, as shown by radioactive (^{3}H) *in situ* hybridization to sections of stage 9 embryos. The P1 promoter is predominantly expressed in the ectoderm of parasegments (PS) 4 and 5 and the mesoderm, of PS5, which span the first and second thoracic segments, whereas the P2 promoter is expressed in the whole of the central nervous system and in the ectoderm of parasegments 3, 4, and 5. The frames on the left represent bright field images and those on the left dark field (Alfonso Martinez Arias).

The ability to manipulate and put genes back into organisms had been an important element in the success of *E. coli* and *Saccharomyces cerevisiae* to understand the molecular underpinnings of gene structure and function. This was achieved in *Drosophila* by an ingenious domestication of wild transposable elements, P elements, which were discovered in studies of wild-type *Drosophila* populations in the 1970s, as mutagenic elements. A. Spradling and G. Rubin generated a series of vectors and associated technology that allowed, albeit without control of location, the transformation into the genome of engineered pieces of *Drosophila* DNA ***(48)***. *Drosophila* was the first higher eukaryote to be transformed and this served as an inspiration for attempts in other organisms. The technique has continued to evolve and recent developments allow easy targeted insertion into the genome.

The molecular analysis of *Drosophila* genes was initiated by a fusion between classical genetics and molecular biology and the results proved the benefits of such exercise with high dividends, which would have an impact on the way similar problems would be tackled in other organisms.

7. Mutants, Stripes, and Boxes

At the beginning of the 1980s, methods had been developed to target any gene, to find out its sequence, analyze its regulatory logic, and observe its pattern of expression. It was low throughput and low resolution but it was good enough to begin to uncover the logic of development. The BX and ANT complexes had led the way and their revelations raised many questions: What did the gene products do? How was the tight spatial expression of these genes regulated? How did the patterns emerge? What had made these complexes such objects of interests was their *genetics*, and it was *genetics* again, which was going to lead to the answers to many of these questions with most unexpected results.

Mutants are the bread and butter of *Drosophila* biology and they had been collected in a haphazard manner through the years, sometimes as curiosities, others as ways of asking questions about chromosomal mechanics or gene structure. However, with the exception of the *period* gene and a few exceptions including a focus in the absence of disks ***(49)*** or sterility ***(50)***, screens for mutants affecting a particular function or structure had not been performed in a systematic way and most certainly not with embryonic development as an object. It was the realization of the meaning of the phenotypes of mutants like *Notch*, *Kruppel*, or *bicaudal* and particularly the observation by E. Lewis in his 1978 paper that the cuticle secreted by the embryo could be used as a readout of genetic processes ***(23)*** what changed things (**Fig. 6**). The potential of these observations was brought to bear by the systematic screens for lethals conducted by Nüsslein-Volhard and Wieschaus in collaboration with Jürgens at EMBL in Heidelberg ***(51–54)***. The mutant collection that resulted from this

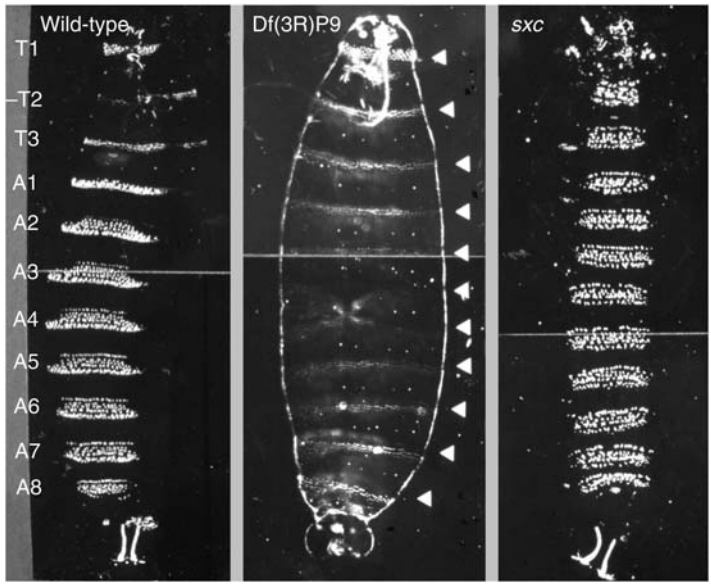

Fig. 6. Cuticles of embryos showing that the patterns of denticles are a readout of gene activity. Wild-type showing the thoracic (T) and abdominal (A) segments. *Df (3R) P9*, which removes the complete BX-C and results in all segments posterior to T2 developing like T2. *Sxc* mutant embryos in which all segments develop like A8 (courtesy of Phil Ingham).

effort uncovered a treasure trove, which provided the raw material for the molecular analysis of the corresponding genes. The emphasis turned from the adult to the embryo and what had been invisible until then revealed an intriguing choreography of stripes and spots. Disturbances in the pattern of the cuticle turned out to provide information about signaling, transcription, the cell cycle, the cytoskeleton, and cell adhesion. As a result of these findings, by the end of the 1980s the genetic regulatory network that establishes the coordinates and patterns of the early *Drosophila* embryo had been outlined ***(55)*** and with this work many now current notions about gene regulation in space and time were laid out. The notion of prepattern put forwarded by Stern ***(26,28)*** acquired a molecular ground in the observation that a gene expression pattern acts as a scaffold for a more complex one.

The cloning experiments of J. Gurdon in the 1960s had indicated that development was about controlling the expression of the genome in space and time ***(56)*** and *Drosophila* provided sound evidence for this. Furthermore, from very early on it became clear that development was about transcription and many of the mutants with pattern defects were mapped to genes encoding transcription factors. Also, there were some surprises and one of them was that these transcription

factors could be clustered into classes based on the structure of their DNA-binding domains. One of these domains, or boxes as they often were called, shared by members of the BX and ANT complexes ***(57,58)***, was termed the homeobox and seeded a large group of proteins with variations in the structure of this domain. The homeobox of the BX and ANT complexes turned out to be conserved in metazoa ***(59)*** and this structural conservation was accompanied with a functional one that provided the first glimpses of universality in the genetic make up of the developmental tool kit. The significance of these findings, with its roots in the genetic studies of E. Lewis, represent another highlight in the history of biology and vindicate *Drosophila* as a significant model system. It is possible that the homeobox would have been found by genome research methods, but it is only the work in *Drosophila* that provided the context within which to interpret what otherwise would have been a conserved protein-coding domain.

Soon, as the genes identified in the large Heidelberg screens began to be analyzed at the molecular level, a picture emerged of development being driven by transcriptional networks modulated by signaling molecules. The prescient comment of Morgan in his Nobel lecture became a reality: there were batteries of genes operating in spatio-temporal sequences. The molecular analysis of the existing mutants not only yielded a large number of new transcription factors but also new signaling pathways. Thus, to the then familiar EGF/FGF-Ras-MAPK were added other with the colorful names of Wnt and Hedgehog. The development of enhancer traps ***(60)*** and of techniques to establish spatio-temporal control of gene expression ***(61)***, which followed related techniques that had proven very successful in *E. coli* and yeast, added to an arsenal that developed as fast as it was making new findings about the structure and function of genes. As a result of this effort *Drosophila* was seen in a new light ***(61)***.

8. From More Genes to Genomes

The study of the genes that are required to make a fly embryo revealed that very few are dedicated to this task. It quickly became clear that genes are just tools that configure circuits and that most of those involved in early development are redeployed later in the imaginal disks ***(5,62)***. Slowly a picture emerged of how the very large number of cell types and tissue patterns required to make a fly resulted from combinatorials and redeployment of a few genes. The same picture of course is true of other organisms and, like the homeoboxes, the "fly genes" pop up in mice and humans as they do in jellyfish. The lesson was clear: what defines an organism is not its genes but their networks. Furthermore, the genes appeared to be conserved.

These conclusions were drawn from the work of many laboratories and individuals but the contribution of the laboratory of G. Rubin in Berkeley, California from the mid-1980s until 2000 is particularly significant. Using the

compound eye as an experimental system with a blend of classical and molecular genetics, mosaic analysis, and modern cell biology, several generations of postdocs and graduate students pursued the mechanisms required to specify and pattern the cells that make up the ommatidia of the fly. The result is a large harvest of genes, interactions, and mechanisms in the best Morgan tradition and one that has contributed very significantly to the modern development of *Drosophila* as a model system (*see* as two examples **refs.** ***63*** and ***64)***. With hindsight it is surprising that a structure so specialized has revealed so much that is universal; but the power might not lie in the structure, the bristle can be deemed to have done as much, but it was the method, above all, the genetics and the sustained and concentrated energy.

In the late 1990s, sequencing of genomes had become an important priority of the biological community. The main thrust was to decipher the information hidden in the human genome but, as ever, work on model organisms was paving the way for this enterprise. *Caenorhabditis elegans* was the first organism to display its genome ***(65)***, and *Drosophila*, with a more complex genome, its long tradition and high standing in the field of *genetics* was coming along at the pace and with the returns of other genome projects. It is at this time that C. Venter proposed a radical new sequencing approach to the human genome. In contrast to the ordered and progressive sequencing in vogue at the time he proposed shotgun cloning, large-scale sequencing, and computer programming power to assemble the genome. To test the method he looked for a suitable organism, and *Drosophila*, with a complex genome and a scaffold in place against which to test the sequence was an appealing prospect. And so, within a short period of time, the shotgun approach pioneered by Celera was shown to function ***(66,67)***. The publication in 2000 of the sequence of the euchromatic genome of *Drosophila* ***(67,68)*** and its analysis was a landmark in genome research and opened a bright new era of possibilities and information with which to take the information accumulated over the years.

9. Of Flies and Men

In 1794, William Blake dedicates one of his "Songs of innocence and of experience" to a fly and ends by musing: "am I not a fly like thee or art not thou a man like me?" Two hundred years later there might be reason to believe that there was something in this statement, for it is in the similarities in genetic make up and programming logic between flies and humans that *Drosophila* reveals itself as a consumate model organism ***(69–71)***. Fortunate as this might be, it is also true. Did we think that lungs would be made in such a similar fashion to the trachea of the fly ***(72)***? Did we think that the development of the fly nervous system was going to have the pervasive influence that it has on the development and function of the vertebrate nervous system ***(73,74)***? Did we think

that stem cells were going to find a model system in the fly ***(75)*** or that we could use the fly to study the cellular basis of cancer ***(76–78)***? Perhaps most significantly, over 70% of the proteins involved in the disease in humans exist in the fly and this means that the point can be made that *Drosophila* may act as a model system for human disease ***(69,70,79)***, learning and behavior ***(80)***, and even alcoholism and addiction ***(81)***.

The ability to shuttle at the genetic level between *Drosophila* and other organisms, and humans in particular, is founded on the conservation of the core genome. Whenever a gene is highlighted by its association with a disease, it is not surprising that the first port of enquiry is the multiple databases associated with the biology of *Drosophila* (*see* Chapter 3). If there is a similar gene (and chances are high that this will be the case), it is then easy to find useful information about its possible function, biochemical properties, and interactions and thus gain an entry for further studies. However, the main reason why this exercise is useful is not simply because of the conservation, which would have been highlighted by the genome projects and would have just left (as it does in many cases) a structural puzzle difficult to solve. The reason why the exercise is useful is because the deep and rigorous genetic analysis performed in *Drosophila* tells about function and molecular relationships of the gene product in a manner that, with the possible exception of *C. elegans*, is unparalleled. The true Rosetta stone of modern biology is not the homeobox ***(82)***, but the whole genome, and the Champolions are the many workers that gene-by-gene have laid a foundation on which much of modern *biology* is, like it or not, built on.

A short reflection on *C. elegans* might be in place here for it is clear that the worm has played a significant role in the modern era of *genetics*, particularly *developmental genetics*, side-by-side *Drosophila*, and it would not be fair to forget about this. However, for a number of reasons associated with its mode of development and possibly its evolutionary position, the fly has turned out to be a better model system for the vertebrates. This might be because the worm, as the highly specialized system that it is, does not have proliferation-dependent construction of organs and structures and has a very peculiar organization of its nervous system, which is not easy to relate to the operation of vertebrate systems. Be that as it may, despite these strategic issues, which play in favor of the fly when thinking about many aspects of human biology, it would not be right to forget the essential role that *C. elegans* has played in identifying many of the components of the molecular toolkit of an organism and establishing their universality.

10. *Drosophila* in the Postgenomic Era

There is little doubt that *Drosophila* has been an exceptionally useful model organism throughout the 20th century. Its most long-lasting legacy will perhaps be the practical demonstration that *genetics* is the language of *biology*. Much as

mathematics is the language of *physics*, *genetics* allows us to transform abstract problems into concrete experimental questions, which can then be answered through the application of established rules and operations. The formulation of mutant screens tailored to specific problems is, arguably, the best example of the power of this language ***(51–54,63,64,83)***. As a result of this endeavor, today *Drosophila* is not just a model organism but a reference for other organisms and most notably for humans. None of this will change as we move into the 21st century, but, is there more to come? What about the postgenomic/systems era? Will *Drosophila* be diluted in a sea of model organisms? Will it become a virtual reference more than an actual model for a *new biology*? How will *Drosophila* fare with the questions that will emerge in the near future?

We are in a time of transition. Where there were defined big questions that could be answered from many different points of view, today we have many deceptively small questions, all and each, probably interesting. Where we have had fusions of *genetics* and *experimental embryology* ***(34)***, and *genetics* and *molecular biology* ***(41)***, today we have *genetics* and *cell biology* converging to reveal intriguing aspects of the structure and function of the cell and to raise a wealth of questions, which we had not thought of before about protein function in cellular contexts ***(84)***. The ability to visualize molecules in space and time in living cells is one of the important drivers of this new perspective of cells and organisms, and *Drosophila* is playing an important role in the development of these techniques. But if something is changing more rapidly than anything else is the way we observe. Where before there were EMS, X-rays, and flies, today there are (in addition) *Drosophila* tissue culture cells and dsRNA, which allow rapid whole genome screens targeted to particular functions to be analyzed in cellular context (e.g., **refs.** ***85–87***). Instead of looking at one gene at a time, the microarray technology allows us to monitor the activity of the whole genome under defined conditions and it is the activity of the whole genome that becomes the phenotype we look at ***(88,89)***. All these developments are being used to delve into fundamental and general principles of the role, which ensembles of genes and cells play in the organization and function of *Drosophila* and by extension other organisms. Most remarkably, these observations are also having a big impact in *evolutionary biology* where through the extrapolation of these findings to other organisms, it has been possible to bring developmental biology into the fold of classical evolutionary theory and create the new discipline of "EvoDevo" deeply rooted in what we have learnt about *Drosophila* **(Fig. 7)** ***(90,91)***.

These developments will pave the way to the future, but the most interesting aspect of the future is that it is unknown, unpredictable and that although its seeds lie in the present we only see this with hindsight. In this regard, it is perhaps pertinent to ask about questions that remain to be answered and problems that we have not seen or that perhaps, in the deluge of genes of the last few

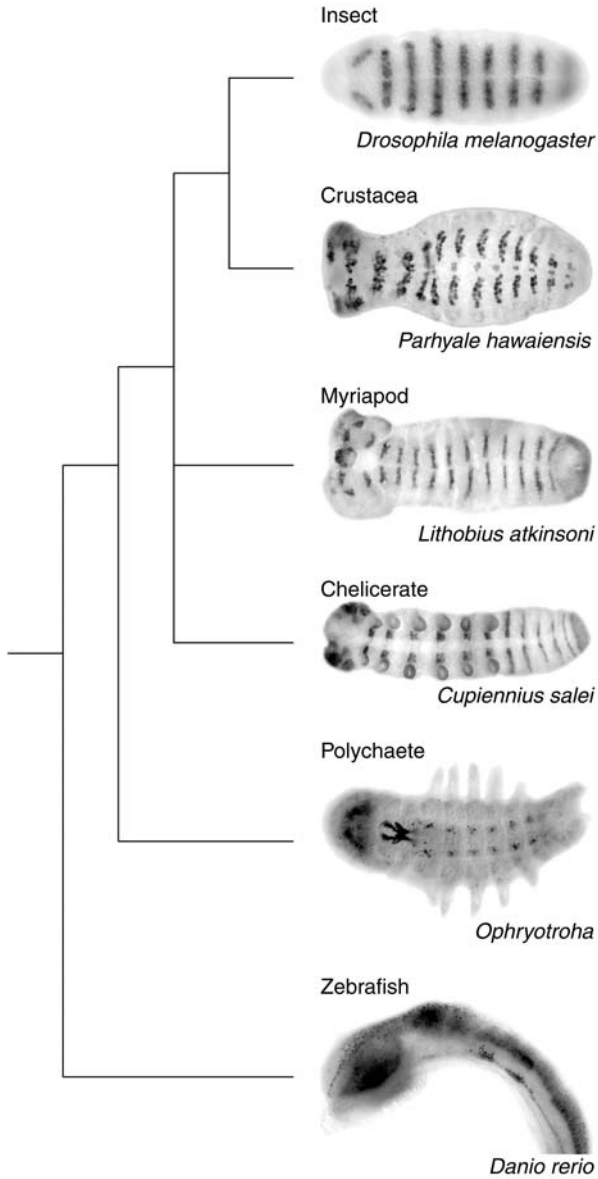

Fig. 7. Expression of *Pax3/7* family in embryos of various species revealed with Mabs DP311/DP312. In *Drosophila* this antibody recognizes the paired and gooseberry genes and, as in other arthropods, delineates a subset of neuroectodermal cells in each segment. In the annelid shown, the staining is mostly neural and in zebrafish it highlights a subset of cells in the neural tube and the neural crest. Notice how the pattern can be easily related from one organism to another indicating that not only the sequence of the gene and probably its function have been conserved but also the regulatory network associated with the regulation of its expression (courtesy of Nipam Patel and Greg Davis).

years, lie dormant or we have forgotten. Are there important biological questions left in need of an answer? Can we enunciate them and test them in our favorite model organism? Will *Drosophila* succumb to the temptation of becoming a genomic cottage industry at the service of an ever-increasing number of publications and databases? Or will it rise to new challenges and once more show its potential to find general solutions? What are or can be those questions? Of course, the most interesting one probably escape our attention but at the moment there are some themes emerging, which might play a role in the future. One of them, as hinted at previously, is the new *cell biology* driven by live imaging and the links it poses between structure and function. Most important though, biology is becoming quantitative because we can measure variables unthinkable a few years ago. In this exercise we realize that, at the molecular level, the processes we observe are subject to fluctuations that sometimes are used and sometimes are dampened to generate cell fates or pattern them in space. Appreciating this is leading us to analyze the mechanisms that regulate the transitions from stochastic molecular events to smooth and deterministic cellular processes **(Fig. 8)** ***(92–95)***. As a result of the precision in the analysis of some events, like the patterning of the blastoderm, *Drosophila* emerges as an important reference for modeling and *synthetic biology*, which aims at reproducing from first principles the circuits that produce stable patterns in space and time ***(96)***.

An important task in the immediate future is to unravel how the components uncovered in the 20th century are put together to make up a functional organism. It is almost certain that this will yield significant insights into biological processes and that we shall discover new laws in *biology*. In this enterprise, *Drosophila* is likely to emerge again as a useful model organism, as at the beginning of the 21st century it is still true that "When with foresight and luck Morgan selected this species for studies in heredity and together with Sturtevant, Bridges, and Muller derived from it the evidence for the existence, arrangement, and complex transmission of genes in the chromosomes, the significance of the results was not owing to *Drosophila* as a unique organism, but as a representative of all organisms" ***(27)***.

Acknowledgments

First of all I want to thank C. Dahmann for the opportunity to organize some ongoing thoughts into this preface, and very special thanks to M. Akam, M. Bate, A Friday, A. Garcia Bellido, P. Lawrence, J. Modolell, MP Scott, and the late JA Campos Ortega for over the years and often unknowingly, furnishing comments, information, and views on the history of an interesting subject. Also many thanks to M. Akam, P. Ingham, C. Klämbt, N. Patel, J. Reinitz, and E. Wilder for the images that allow me to illustrate some of the points I try to make, an image is always worth a thousand words. The text has a slight developmental genetic slant and I apologize for this. It is what I work on and it is the

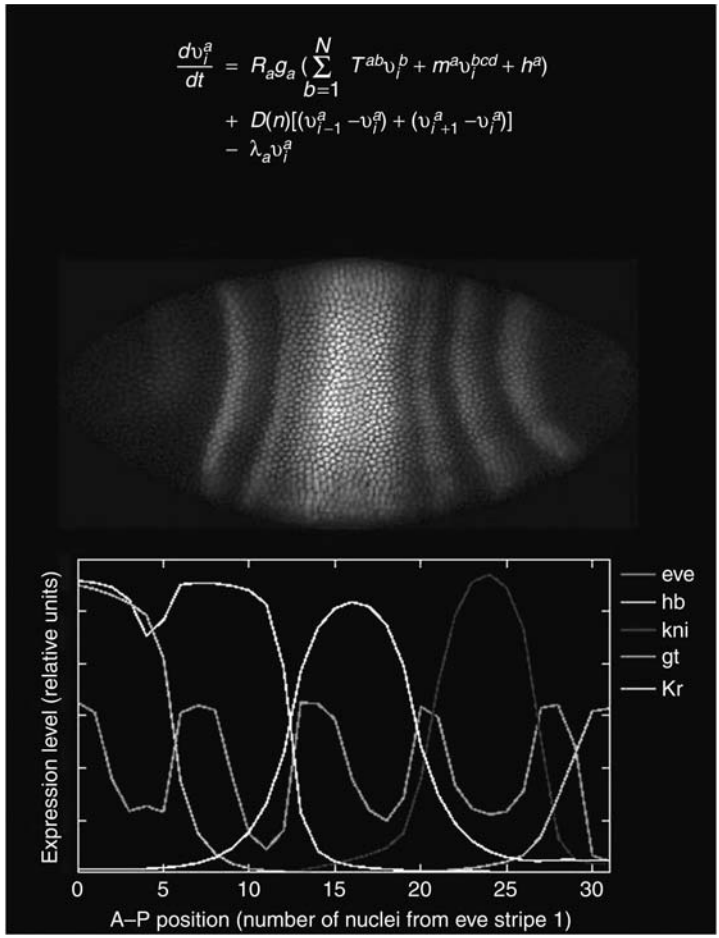

Fig. 8. A quantitative analysis of gene expression during the deployment of *gap* gene expression allows the development of models and simulations that can reveal the wiring and functioning of gene networks (courtesy of John Reinitz and *see* http://flyex.ams.sunysb.edu/).

perspective from which I look at the field (I do not think it is a bad one). I have nonetheless tried to point out here and there how *Drosophila* has and does contribute to the other big questions of *biology* like the development and function of the nervous system or evolutionary biology. If at times I have oversimplified or omitted significant contributions, it was not intentional. As I indicate at the outset, this is no history of *Drosophila* but a glimpse at the track record of *Drosophila* as model organism extraordinaire. The real history would require more space and, in this day and age, perhaps a different format. My work is funded by The Wellcome Trust and the BBSRC.

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2

Getting Started

An Overview on Raising and Handling Drosophila

Hugo Stocker and Peter Gallant

Summary

Drosophila melanogaster has long been a prime model organism for developmental biologists. During their work, they have established a large collection of techniques and reagents. This in turn has made fruit flies an attractive system for many other biomedical researchers who have otherwise no background in fly biology. This review intends to help *Drosophila* neophytes in setting up a fly lab. It briefly introduces the biological properties of fruit flies, describes the minimal equipment required for working with flies, and offers some basic advice for maintaining fly lines and setting up and analyzing experiments.

Key Words: Balancer; *Drosophila melanogaster*; genetics; model organism; nomenclature; stock keeping.

1. Introduction

Drosophila melanogaster has served as a genetic model system for a century. It has populated research laboratories all over the planet because of its many advantages: it is modest regarding dietary and spatial requirements, allows easy observation and manipulation at most developmental stages, produces large numbers of offspring, and is robust against plagues and pathogens. Above all, the plethora of sophisticated genetic tools developed by an ever increasing number of "Drosophilists" over many years makes *Drosophila* the model system of choice to study biological phenomena as diverse as pattern formation, behavior, aging, and evolution.

A big advantage of *D. melanogaster* is its rapid development. Under standard laboratory conditions (25°C, *see* **Subheading 2.2.**) the whole life cycle does not take longer than some 10 d. Embryogenesis occurs within the egg that is deposited into the food, and after slightly less than 24 h, the first instar larva

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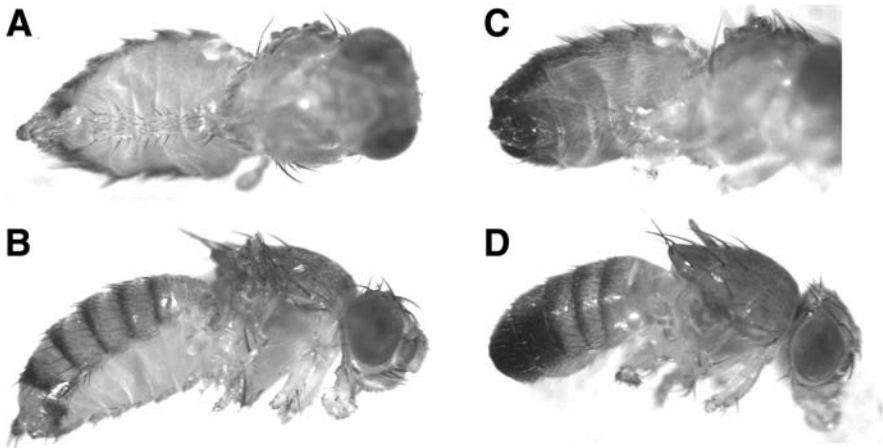

Fig. 1. Bottom **(A,C)** and side **(B,D)** views of a female (A,B) and a male (C,D) abdomen. Males can be recognized by the chitinous structure at the ventral side of their abdomen (the clasper, used during copulation), by their continuous pigmentation at the posterior end, and by the round shape of the abdomen. Wings and legs have been removed for better visibility, and therefore the *sex combs*, found exclusively on the male forelegs, are not shown.

hatches. Immediately after hatching, the larva takes up its main task: feeding! The growth period lasts 4 d and includes two molts. During this time, the larva increases approx 200-fold in weight. This astonishing mass accumulation is aided by the endoreplication of larval tissues, i.e., those tissues that will be destroyed during metamorphosis and will not contribute to the adult fly. In contrast, the so-called imaginal discs consist of diploid cells and during metamorphosis will be transformed into the adult body structures. Toward the end of the third larval instar (about 5 d after egg deposition), the larva stops feeding and leaves the food (wandering stage) in search of a dry place suited for pupariation. Metamorphosis takes place in the pupal case during the following 4 d, and the imagines eclose 9–10 d after egg deposition. The emerging adult flies are some 3 mm in length with females being slightly larger than males. The distinctive features of the two genders are illustrated in **Fig. 1**. Females weigh about 1.4 mg, whereas males are only about 0.8 mg (much of this weight difference is accounted for by the ovaries in the female abdomen). The dry weight is about one-third of the wet weight. Evidently, both environmental conditions (food quality, temperature) and genetic makeup impact on body size and weight.

The females are already receptive less than 12 h after eclosion, and they start to lay eggs soon after mating. Therefore, two weeks usually suffice for each generation in a crossing scheme. Egg production reaches up to 100 eggs per day and female (with a fecundity peak between day 4 and day 15 after eclosion).

Thus, a single pair of flies can give rise to a substantial number of offspring. However, this is an inadmissible simplification, as each stock keeper knows how poorly some fly stocks (usually the most important ones) perform.

2. Handling Flies

2.1. Fly Pushing

Although fruit flies are not very demanding, each laboratory intending to do fly work should be equipped with certain basic tools. It is possible to start out with minimal equipment, and many of the tools can be self-made with a bit of imagination. Furthermore, personal preferences result in fly laboratories that hardly resemble each other. Nevertheless, some tools are quite essential and will be described in the following sections. A typical collection of such tools is shown in **Fig. 2**. Please contact a local fly laboratory (can be found at the FlyBase web site) or the Bloomington stock center web page for the addresses of local suppliers.

Even though some "fly pushers" recognize the sex of flying flies with bare eyes, the use of dissecting microscopes is essential. As you will spend many hours observing flies under the stereomicroscope, you should refrain from buying the cheapest one. Good optical quality and a magnification range from 6× (for handling live adult flies and larvae) to 40× (for dissections) are desirable. Transmitted light is not required. Use heat filters or—preferably—either fiber-optic transmission from a distant light source or light-emitting diodes (LEDS) to avoid overheating of the flies. A ringlight is appropriate for inspection of flies as it reduces unwanted optical reflections. For dissections, flexible optical fibers—ideally mounted directly on the microscope—are recommended. As green fluorescent protein (GFP) is widely used as a marker, a stereomicroscope suitable for fluorescence analysis is often required. In order to examine dissected animals or individual tissues, you will also need a (fluorescence) compound microscope with higher magnification objectives and phase contrast optics.

Obviously, you need to anesthetize the flies before inspection. Although the use of ether has a long-standing tradition, modern fly labs are relying on carbon dioxide as anesthetic. Industrial grade CO_2 in tanks of 40–50 L can be purchased from gas suppliers. The tanks should be secured by solid racks. An automated switch between tanks makes your life easier, as CO_2 tanks tend to run out of gas at the very moment you are chasing the long-sought-after fly. If your laboratory intends to do a large volume of fly work, permanent piping of CO_2 at the individual benches in combination with a large remote CO_2 source (e.g., two batteries of 12 CO_2 tanks each placed in the basement) is an attractive (but expensive) option. Pre-existing air lines can also be adapted to provide the workspaces with CO_2 (contact a professional plumber and check for safety regulations!). The CO_2 source needs to be fitted with a pressure reduction valve.

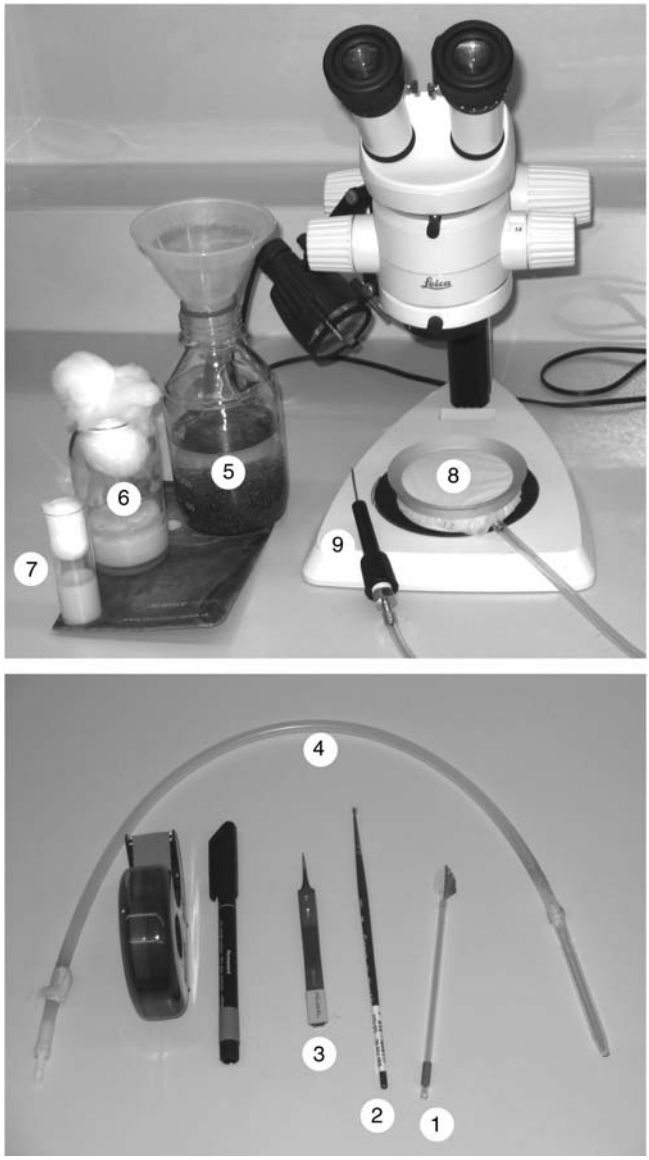

Fig. 2. The figure illustrates the essential tools of a fly pusher: feather (1), brush (2), forceps (3), and spit-tube (4) for moving flies, stereomicroscope for looking at flies. the final destination of most flies—a morgue (5), standing on a mouse pad are a culture bottle (6), and a vial (7) (plus the tools to label them). The fly pad (8) and the "CO_2 needle" (9) (containing a valve that is opened by bending the needle) are located under the microscope.

Also keep in mind that the expanding CO_2 cools the environment—without heating, the valves and pipes may freeze.

At each workstation, an additional valve should allow to regulate the supply pressure of CO_2. From this valve, a pipeline consisting of plastic tubing of about 5 mm inner diameter and bifurcating by means of a Y-junction supplies two devices: one of the two branches leads to a special plate (fly pad), the other one ends in a robust syringe needle connected to a spring valve. The needle can be inserted into vials and bottles (*see* **Subheading 2.2.**) between the stopper and the rim of the culture vessel, and CO_2 is infused by bending and thereby opening the valve. The fly pad consists of a porous plate (e.g., made of polyethylene) surrounded by a metal or plastic rim. The CO_2 passes through the porous plate and forms a sea of gas in the shallow vessel. Thus, flies lying on the pad will be anesthetized by the lack of oxygen and can be readily inspected and handled. Flies can survive several minutes in this unconscious state, which leaves plenty of time for extensive analysis. However, exposure to CO_2 for more than 20 min will result in lethality, and even before that, the flies' fertility begins to suffer. A further unwanted consequence of prolonged exposure to such a CO_2 stream can be dehydration. This problem can be minimized if the CO_2 is passed through a flask of water before arriving at the fly pad.

The use of ether may still be required in certain situations. If you intend to take pictures of the animals or to measure their weights, you need to immobilize the flies for several minutes. This can be achieved either by freezing or by treatment with ether. Leaving flies in an ether atmosphere for about 30 s renders them unconscious, whereas a minute suffices to kill them. Be aware of the rapid dehydration that will change the wet weight significantly within minutes. Therefore, the flies should always be treated identically if you want to compare their weights (e.g., 1 min in ether atmosphere). If you want to avoid ether, you can also measure the flies' dry weight by placing them into an Eppendorf tube in a 95°C heat block. Once the flies have stopped moving, open the lid and continue the incubation for 10–15 min, then put the Eppendorf tube at room temperature to equilibrate with ambient humidity. After such a treatment, flies can be stored for several days without any change in weight.

Although tiny and seemingly delicate, flies are not particularly fragile. They can be moved around with fine paintbrushes or bird feathers. Another convenient tool to transport individual flies and to add them to culture vials already containing other flies is the spit-tube. It consists of a piece of plastic tubing (~70 cm long and 5–7 mm in diameter) with a mouthpiece at one end and a small glass (Pasteur) pipet with a wide opening at the other end. The spit-tube allows you to pipet up and down individual flies—just make sure that you place a filter (e.g., a little ball of cotton) between the glass pipet and the plastic tubing, lest you swallow your favorite fly.

In the course of your genetic experiments, a large number of flies will be produced that are of no use (any more). Dump these flies into the "morgue"—a medium-sized glass vessel filled with 70% ethanol, fitted with a funnel. Once the morgue is full, the dead flies should be discarded according to your local biosafety regulations (e.g., autoclaved).

A few other items will support your daily work: forceps (typically watchmaker's forceps, size 5, essential for dissection), a hand-held counter (either mechanical or digital, as also used by tissue culture experimentalists), and a little piece of carpet or a computer mouse pad (to dampen the hits when you bang vials or bottles against the bench). Furthermore, you need some fly traps to catch escapees. Either hang up sticky flypapers or place a reasonable number of unused fly food bottles with a funnel on top all over the fly room (or, even better, do both).

2.2. Vials and Hardware for Raising Flies

Flies need a cozy home and good food. Space is usually not limiting—although maintaining thousands of different lines does require large cultivation rooms. For small cultures (up to about 200 progeny flies), fly pushers make use of different kinds of vials. Standard volumes are 30–45 mL (25 mm in diameter, 70–100 mm in height), and the vials can be made of plastic or glass. Whereas plastic vials are typically for single use only, glass vials can be reused a number of times (after autoclaving, washing, and intense rinsing). The use of disposable plastic vials may be more expensive, and some fly pushers do not like their electrostatic features (flies tend to stick to the walls when you want to push them into the vial). Furthermore, there are anecdotal reports that the fly food detaches more quickly from plastic walls on drying (although the reason for this phenomenon is unknown). Nevertheless, plastic vials may be preferable if there is no efficient cleaning facility available. Larger cultures (up to 1000 progeny flies) are set up in bottles (volumes of about 200–250 mL) that are also made of glass or plastic. Special conditions apply for very large cultures (*see* Chapter 23).

The vials and bottles can be closed by various kinds of stoppers, the most common ones being paper or foam plugs and cotton. Using nonabsorbent cotton is the only reliable way to keep mites out of the vial (*see* **Subheading 2.5.**). However, many fly pushers are irritated by cotton fibers in the air. Plugging the vials in the fume hood may offer some relief. Paper and foam plugs can be washed and reused several times. However, it is crucial that the stoppers are autoclaved after every use, and this harsh procedure certainly does not contribute to an extension of their half-lives.

The vials can be placed into cardboard boxes, and the bottles are usually transported and stored on trays. Make sure that both the boxes and the trays are regularly cleaned to prevent the accumulation of microorganisms or mites (it is

recommended to incubate the trays and boxes between uses at 60°C for several hours).

To ensure the reproducibility of the experiments, the fly cultures have to be maintained at standard conditions. A frequently used temperature is 25°C, and the relative humidity should be around 70%. There are two ways to meet these criteria: either you use stand alone incubators, or preferentially, you have access to a climate-controlled room. Incubators have several disadvantages: there is a tremendous exchange of air (and a rapid drop in temperature) every time you open the door. Furthermore, incubators capable of controlling temperature and humidity are expensive and noisy. However, incubators are very useful if an experiment requires switching to an unusual temperature or, for example, a repeated incubation at 37°C (e.g., to induce expression from a transgene under heatshock promoter control). Especially the latter is a painful experience without a programmable incubator. For single heatshock treatments, the vials can be placed in a water bath.

Climatized fly culturing rooms are very convenient for both controlled experiments and stock keeping. The temperature should be kept within a narrow range (±0.5°C), and the circulating air needs to be humidified (70% relative humidity is ideal). It is crucial that both overheating and freezing of the climate room cannot occur under any circumstances. Both temperature and humidity should be constantly monitored, and an alarm needs to be triggered whenever the temperature falls outside an acceptable range (e.g., 22–27°C for the 25°C room). The inside of the chamber (including the shelves) should be designed such that it provides maximal accessibility for cleaning and minimal opportunities for hiding (of unwanted guests, *see* **Subheading 2.5.**). Automated doors are desirable as fly pushers often approach the climate room with both hands filled with fly boxes. Finally, the lighting in the room should be controlled to achieve a 12 h light/12 h dark cycle. Obviously, the transition times between dark and light need not coincide with the outside day/night cycle. Instead, they can be adjusted to the experimenter's needs, as adult flies tend to eclose around dawn.

2.3. Feeding Flies

The well being of your flies depends on the food even more than on the environment. Our limited survey among fly labs on most continents revealed that there are probably not two laboratories that produce exactly the same fly food. This may cause problems when growth-related aspects are under investigation. Therefore, instead of relying on published findings, you should always carry out the controls under the same nutritional and environmental conditions.

Most fly food recipes are based on similar ingredients: water, agar, sugar, corn meal, yeast, and fungicides. The main difference is the source of carbohydrates.

Whereas laboratories in the United States tend to use molasses (a byproduct of the processing of sugarcane or sugar beet), fly pushers in Europe and Asia seem to prefer glucose or dextrose. In principle, fly food can be prepared in a simple cooking pot. However, to prepare large quantities, you will need a stirrer kettle (volume up to 100 L) and a peristaltic pump.

We prepare our fly food as follows (the volume depends on the demand; the following indications are for 1 L of water): while the water is warming up, 100 g of live yeast is added and dissolved. Glucose (75 g), agar (8 g), and corn meal (55 g) are mixed and added to the boiling water under constant stirring. Wheat flour (10 g) is dissolved in 100 mL cold water and added to the boiling mixture. After at least 30 min of boiling, the heating is reduced and the mixture is allowed to cool down slowly. The fungicide (either 15 mL of a 1:1 mixture of Nipagin (methyl paraben, 33 g/L ethanol) and Nipasol (propyl paraben, 66 g/L ethanol) or 5 mL of 8.5% phosphoric acid plus 5 mL of 85% propionic acid is added at a temperature of approx 60°C, and the mixture is stirred for another 15 min before dispensing into vials (roughly 12 mL per vial) and bottles (roughly 40 mL per bottle). The vials and bottles are placed in open plastic boxes on a table and allowed to cool and dry. Constant subtle ventilation accelerates this process (and keeps hungry flies away). As soon as the fly medium is dry enough (after about 5 h), a drop of autoclaved yeast paste is added on top. When kept in closed plastic boxes, the fly food can be stored for several days (always check for invaders before use!).

2.4. Culturing Flies

The vials are now ready for use. For most crosses, 5 virgins and 2–5 males per vial will give you a reasonable number of progeny. At least 20 virgins and 5–15 males are needed to populate a bottle. Carefully check whether the unconscious flies stick to the food (especially when the yeast drop is still wet). Laying the vials on the side until the flies have recovered helps to avoid early losses.

As soon as a culture is set up, the vial must be labeled. Use a waterproof marker to write date and the genotypes of the females and males directly onto the vial. For stocks, the use of labels (e.g., sticky tapes) is convenient.

After 2–3 d, the flies should be transferred to a new vial. There is no need to anesthetize the flies again—simply shake the flies down, open the old and the new vials, press them together, and shake the flies into the new vial. With a bit of exercise, you will manage to transfer your flies quantitatively. Repeat as needed—and then dump the flies into the morgue.

Rule number one of stock keeping is diligence—to avoid contamination or mixing up of fly stocks. Stocks are usually maintained in vials at 18°C (which slows down development to a generation time of about 20 d). A dedicated constant temperature room is strongly recommended. Again, there are several

schedules for stock collections. Many labs prefer to simply flip stocks into new vials in order to save time. However, we recommend inspecting your flies at least twice a year for their phenotype (to recognize contamination of a stock and allow the rescue of the correct genotype) and for mite infestations. The inspection under the dissecting microscope also has the advantage of an accurate population control. Either way, the cultures should be changed over to a new vial after 1 wk, and a second time after another week. Thus, you will have three copies of each stock. Keep an old vial until larvae are visible in the new ones. Under optimal conditions (vials not overcrowded), you can wait up to 5 wk before starting the same procedure again.

There will always be some stocks that are difficult to maintain. Keep a special tray for the sick stocks, usually at 25°C because many stocks perform better at this temperature. However, some stocks do prefer lower temperature, especially those that carry genetic elements to achieve Gal4-mediated overexpression.

Finally, good practice of stock keeping involves a database harboring all information about the stocks, including any special requirements for stock keeping.

2.5. Plagues

Cleanliness is key to healthy fly cultures. Always keep an eye on the places that could convert into sites of infection: the working spaces in the fly room, the cultivation rooms, and the fly food kitchen. Make sure that all lab members keep their working areas clean. Especially the fly pads should be cleaned with ethanol after work. It is crucial that old vials are not given the chance to get spoilt, as rotten cultures are the main source of nasties. Not only should you appeal to the discipline of your colleagues, but you should also appoint a person to regularly inspect the fly room and the cultivation rooms.

Contaminations can also be favored by insufficient precautions taken in the fly food kitchen. Double doors to avoid flies being attracted by the smell of the food are helpful. Also pay attention to the quality of the ingredients of the fly food (especially live yeast is a potential carrier of infectious agents).

Incoming stocks should be treated with special caution. Keep them under quarantine in an isolated place for two generations (e.g., a dedicated incubator far away from your fly room, or even your office may do), and only transfer them to your fly room on careful inspection.

The main causes for sleepless nights of fly pushers are molds and mites. Molds appear rapidly in the absence of fungicide. Whereas healthy fly stocks can usually cope with mold infections, weak stocks are heavily endangered by the fungi. Make sure that fungicide is always added to the fly food in proper quantities. It is also suggested that two fungicides (e.g., Nipagin/Nipasol and propionic/phosphoric acid) are used in an alternating manner to prevent resistance

formation. For example, add propionic/phosphoric acid on a particular weekday and Nipagin/Nipasol on all the others. Furthermore, the relative humidity in the climate room should not exceed 70% and, importantly, all the reused items (vials, stoppers) must be autoclaved after every use. These few and simple rules usually suffice to fight the molds successfully.

Mites can be more renitent. There are two types of mites, those that feed on fly food and those that feed on flies. Food mites are much more common but, fortunately, far less dangerous. They tend to appear out of nowhere and spread rapidly. Probably, they are imported into the laboratory by the raw ingredients of the fly medium (corn meal, flour). If you notice mites, the affected cultures should be quarantined or, if possible, autoclaved. Quarantined cultures should be transferred daily—a procedure that is, however, no option for weak stocks. If the mites persist, manual removal of adult mites and their eggs from fly eggs or pupae may help. Finally, placing dechorionated eggs (by means of "bleaching," i.e., treatment with sodium hypochlorite) into fresh vials is a promising but tedious strategy to get rid of mites. You may want to choose chemical warfare instead: filter papers soaked in Tedion (Tetradifon) are effective weapons against some mite species.

3. Experimental Use of Flies

3.1. Genetic Makeup of Flies

Drosophila is, above all, a genetic model organism, and working with flies requires a minimal knowledge of their genetic makeup. The fly's genome is distributed onto 8 chromosomes: 2 sex chromosomes (two X chromosomes in females, also called 1st chromosomes; one X and one Y chromosome in males) and 2 sets of autosomes in both sexes (simply called 2nd, 3rd, and 4th chromosomes). These chromosomes differ substantially in their sizes: 21.9, 42.5, 51.3, and 1.2 Mb of euchromatin are located on the X, 2nd, 3rd, and 4th chromosome, respectively. The Y chromosome consists entirely of heterochromatin and carries just a few genes that are only required for male fertility, but not for viability. To indicate a specific position within a chromosome, different coordinate systems are used: molecular nucleotide sequence, genetic map, and cytological location. The first is based on the completed 120 Mbp sequence of the *Drosophila* euchromatin. The genetic map is derived from experimentally determined recombination frequencies between genes; the left tip of each chromosome is arbitrarily set to map position 0, and a map distance of 1 corresponds to a 1% recombination rate—however, notice that the one-to-one relationship between map distance and recombination frequency holds only for closely spaced loci (and, of course, that the maximum frequency of meiotic recombination between any two loci is 50%). The cytological map is based on the appearance of the massively polyploid (and polytene) chromosomes found in larval

salivary glands; the alternating darker bands and lighter interbands that can be discerned under a light microscope each have been assigned an identifier of the type "xay," where "x" is the band number, "a" the lettered subdivision (ranging from "A" through "F"), and "y" another number subdividing the lettered subdivision. Each major chromosome arm is divided into 20 such bands (X: 1–20; left arm of the 2nd chromosome: 21–40; right arm of the 2nd chromosome: 41–60; left arm of the 3rd chromosome: 61–80; right arm of the 3rd chromosome: 81–100; 4th chromosome: 101–102). As an example, the *white* gene is localized close to the tip of the X chromosome at cytological region 3B6, map position 1.5, and it starts at nucleotide position 2,646,755.

3.2. Nomenclature

Genes are often named for the first mutant phenotype observed (frequently the phenotype of a weak or hypomorphic mutant allele). If this phenotype is dominant to wild-type, the gene name begins with an uppercase letter, else with a lowercase letter. For example, mutation of the *white* gene has no phenotypical consequences as long as a wild-type copy of the gene is present, but when both copies of the *white* gene are mutant the fly has white eyes. Each gene also carries a unique symbol (or abbreviation), and superscripts or brackets are used to distinguish between different alleles; for example, w^{1118} or *w[1118]* refer to the allele "1118" of the *white* gene. A "+" designates the wild-type allele (e.g., w^{+}) and an asterisk a mutant allele whose identity is not known (e.g., w^{*}).

Some frequently encountered names of mutations (and, consequently, also of genes) are lethals, steriles, minutes, enhancers, suppressors, and transposon insertions. Lethal mutations in unknown genes are designated *l(x)n*, for a recessive lethal mutation located on chromosome "x" (1, 2, 3, or 4), where "n" either corresponds to a code for the gene or to the cytological location of the mutation; for example *l(1)1Aa* corresponds to a lethal mutation mapping to cytological band 1A on the X chromosome. Mutations resulting in male or female sterility are abbreviated *ms(x)n* or *fs(x)n* if they act recessively, *Ms(x)n* and *Fs(x)n* if they act dominantly; for example, *fs(1)3* would be a recessive female-sterile mutation located on the X chromosome and having the name "3". The *Minute* mutations are characterized by a dominant growth defect manifested (amongst others) as a delay in development and a reduction in bristle size. Most *Minute* mutations disrupt a gene coding for ribosomal proteins—example: *M(3)66D* is a mutation of the *RpL14* gene, which is located on chromosome 3 at cytological position 66D. Enhancer or suppressor mutations were initially isolated based on their ability to modify the mutant phenotype of a different mutation "m" and named accordingly as *e(m)n* or *su(m)n—E(m)n* and *Su(m)n* if their effect on the mutation *"m"* is dominant. For example, the mutation *Su(Pc)35CD* is located at the cytological bands 35C/35D and dominantly

suppresses mutations in the *Pc* (*Polycomb*) gene (which itself has dominant mutant phenotypes). A special class of modifier mutations has an influence on "position effect variegation", a phenomenon linked to the control of transcription and chromatin structure. Such mutations are called *E(var)* or *Su(var),* for example, *Su(var)3–9.* Finally, tens of thousands of mutant fly lines have been created using transposable elements, mainly P-elements (*see* Chapter 6). Insertions of such transposons are labeled as *P{c}n,* where "c" describes the "payload" of the P-element (i.e., the transgene carried by the P-element) and "n" a code or (if applicable) the gene into which this P-element has inserted; an example would be $P\{GawB\}h^{1J3}$, which expresses both *white* and the yeast transcription factor Gal4 as indicated by the term "GawB" and has inserted into the *h* (*hairy*) gene and now constitutes allele "1J3" of *h*. At this point, we should also mention the very large class of genes named "CGz". This name is not derived from any observed mutant phenotype but based on a gene prediction—CG is an acronym for "computed gene," and "z" stands for a 4- to 5-digit identifier.

In addition to mutations affecting a single locus, several types of large-scale chromosomal abnormalities are commonly encountered. *Deficiencies* are denoted as Df(x)n (where x specifies the chromosome arm, i.e., 1, 2L, 2R, 3L, 3R, 4), and they are characterized by the deletion of large regions of the chromosome, often containing dozens of genes. *Duplications* are denoted as Dp(x1;x2)n, whereby "x1" denotes the chromosome from which a segment is duplicated onto chromosome "x2," and "n" denotes a code or "designator". A combination of duplications and deletions is encountered in *transpositions* and *translocations*, denoted Tp(x1;x2)n and T(x1;x2)n, respectively. *Inversion* chromosomes, In(x1)n, contains segments that are inverted in their arrangement as compared with a wild-type chromosome. Importantly, such a configuration suppresses meiotic recombination.

3.3. Balancers

This attribute is exploited in so-called *balancer chromosomes*. Balancers are among the most important genetic tools in *Drosophila* (and the envy of non-Drosophilists). They contain multiple inversions to suppress meiotic recombination with an un-rearranged chromosome. In addition, balancers carry dominant mutations with an easily visible phenotype and recessive lethal or recessive sterile mutations. Thus, suppose you are crossing a fly of the genotype "$hippo^{42–47}$ $yorkie^{B5}$/SM5, *Cy*" to a wild-type fly. As "SM5, *Cy*" is a balancer (of the 2nd chromosome) marked with the dominant wing mutation *Curly* (*Cy*), you know that half of the offspring of this cross will be "$hippo^{42–47}$ $yorkie^{B5}$/+" and the other half will be "SM5, *Cy*/+". These latter flies will be easily recognized as they have bent-up (curly) wings, so all the flies with normal wings are heterozygous both for *hippo* and *yorkie*—even though you cannot recognize the

presence of these mutations themselves by visual inspection. Importantly, you also know that you will never encounter *hippo* or *yorkie* alone. Now suppose you are crossing this *hippo*$^{42–47}$ *yorkie*^{B5}/SM5, *Cy* fly with a partner of the same genotype. A *priori*, you might expect to obtain three types of offspring: *hippo*$^{42–47}$ *yorkie*^{B5}/*hippo*$^{42–47}$ *yorkie*^{B5} (homozygous mutant), SM5, *Cy*/SM5, *Cy* (homozygous for the balancer), and *hippo*$^{42–47}$ *yorkie*^{B5}/SM5, *Cy*. However, life without *hippo* (or without *yorkie*) is impossible for flies, and the SM5 balancer is also not homozygous viable, hence you only get the third genotype, which is identical to the genotype of the parents—you have just established a *balanced stock*. This means that you can transfer the offspring from the above cross into a new vial, let them have offspring of their own, and repeat this procedure for many generations more—you will always only have one type of flies in your vials, so you can maintain your fly line without having to molecularly genotype them.

Given their usefulness, balancers have been developed for each major chromosome: the FM6/7 series for the X chromosome (where F stands for the *f*irst chromosome and M for the *m*ultiple inversions), CyO and SM5/6 for the 2nd chromosome (S for *s*econd), TM2/3/6 for the 3rd (T for *t*hird). There is no need for a balancer chromosome for the 4th chromosome as it does not undergo meiotic recombination—and there is also no meiotic recombination in males (so theoretically balancers are only needed in female flies). Amongst the dominant markers found on these balancers—as well as on other marked chromosomes—are mutations affecting adult eye shape (*Bar*/*B*, on the X; *Glazed*/*Gla*, on the 2nd), wing shape (*Curly*/*Cy*, 2nd; *Serrate*/*Ser*, 3rd), bristle shape (*Stubble*/*Sb*, 3rd), and bristle number (*Sternopleural*/*Sp* and *Scutoid*/*Sco*, both on the 2nd; *Humeral*/*Hu*, 3rd). To mark earlier stages of development one uses *Tubby*/*Tb* (carried on the TM6B chromosome; *Tb* makes larvae short and fat, but it is only suitable for older larvae and pupae) or transgenes expressing *Drosophila yellow*/*y* (this requires the use of a y^- background), a fluorescent protein (typically GFP), or bacterial lacZ. Such transgene insertions exist for several different balancers.

Despite all the enthusiasm about balancers, we should add some words of caution. Depending on the chromosomal location and on the particular balancer, considerable meiotic recombination on the "balanced" chromosome may still be possible. Moreover, the recombination rates on the other chromosomes are increased by the presence of a balancer (e.g., a fly carrying the 2nd chromosome balancer CyO will have increased recombination between the two homologous 3rd chromosomes). Also, flies carrying balancers are not as fit and do not produce as many offsprings as wild-type flies. This is particularly obvious when balancers for two different chromosomes are used at the same time—and it is virtually impossible to work with flies that are simultaneously balanced on the 1st, 2nd, and 3rd chromosomes. Furthermore, some visible markers cannot

be combined, either because they interact genetically or because they affect the same trait. For example, *singed/sn* and *Sb* both destroy bristle architecture and the mutant phenotypes cannot be scored simultaneously. Along the same line, a balancer chromosome (i.e., one of the mutations carried on this balancer) can also modify the phenotype one is trying to study (e.g., the rough eyes that are caused by overexpression of your favorite gene), and hence it is advisable to analyze such phenotypes in flies lacking any balancer chromosomes.

After all this talk of mutations and mutant chromosomes, we also need to mention wild-type lines that are commonly used for comparison purposes, typically Oregon R (OreR) and Canton S (CS). In addition, many researchers use "w^{1118}" and "$y^{*}\ w^{*}$" lines as reference lines, as many transgenes (marked by the expression of *white* or *yellow*) have been generated in these backgrounds.

Finally, if we want to put together all the genetic elements mentioned above into a coherent genotype, we need to observe a few rules of syntax. These can be illustrated with the genotype "*y w*; *Kr*[If-1]/CyO, *Cy*; *D*/TM3, *Ser*." First, only genes with mutant alleles are mentioned (and none of the 14,000 other genes). Second, the mutant alleles are listed according to their cytological position without intervening comma, whereby different chromosomes are separated by semicolons. Third, two homologous chromosomes are only listed if they differ, and then they are separated by a forward slash "/". Fourth, a "named" chromosome (e.g., a balancer such as "TM3") is followed by a comma and a list of specific mutations on this chromosome. You will also notice in the example shown above that the different chromosomes are not explicitly numbered, but if you know that "*y*" and "*w*" are located on the first chromosome, that CyO is a 2nd chromosome balancer, and that TM3 is a 3rd chromosome balancer, you will figure out which chromosomes are described here. Occasionally, however, the situation is less clear; for example, without any further information you cannot know whether the P-element in "w; P{w+}xxx" flies is inserted on the 2nd, 3rd, or even the 4th chromosome.

3.4. Crossing Flies

Only rarely will you obtain flies of exactly the right genotype from an outside source. Instead, you will usually need to cross different mutant flies together in order to generate the desired flies. This will confront you with one of the most common tasks in fly husbandry: virgin collection. As you want to force the females to mate with the partners you have chosen for them (rather than with their brothers or fathers from the stock), they have to be virgins before you introduce them to their selected mates. Female flies start mating only a few hours after eclosion, therefore you can safely identify virgins by collecting freshly eclosed flies. Such flies can be recognized by the light color of their cuticle (as it tans only later) and by a greenish spot that can be easily seen

through the white abdomen—the meconium (waste products the fly will get rid off with the first defecation). Alternatively, you can empty a vial or bottle of all adult flies and then wait for 8–10 h (at 18°C); all females that have eclosed in the meantime will be virgins. It is a good idea to keep virgin females in a separate vial for a few days. Virgins will lay a small number of eggs, but if any of these hatches into a larva you know that (at least) one of the flies had already lost its virginity. Whenever possible, you should also include markers in your crosses such that illegitimate offspring (e.g., originating from nonvirgin mothers) can be recognized.

Many crossing schemes involve more than one generation. In such cases it is important to start with enough flies (e.g., by setting up the first cross with many flies in bottles rather than in small vials). Otherwise, you risk collecting fewer and fewer flies with each passing generation (and end up with none) because the "correct" flies typically make up only a small fraction of all the offspring. Also, you should make sure that all the used mutations are mutually compatible. It could be that one marker cannot be recognized in the presence of another one (*see* **Subheading 3.3.**), or that flies carrying a combination of two particular mutations are not viable. Often it is not possible to predict such problems and test-crosses might be required.

3.5. Basic Phenotypic Analysis

Several chapters in this volume describe the generation of mutations, starting either with a mutant phenotype (*forward genetics*) or with a gene of interest (*reverse genetics*). Below we provide some suggestions for a general and basic characterization of such mutants. As there is always a risk of unrelated background mutations, in particular if the mutation of interest was generated using chemical mutagens, it is essential to carry out such an analysis in a heteroallelic situation, i.e., in an animal carrying mutant allele 1 over mutant allele 2 (or over a deficiency uncovering the mutant gene). If only one allele is available, one should try to rescue the mutant phenotype with a transgene carrying the wild-type version of the gene or a cDNA.

Arguably, the most distinctive aspect of a mutation is its effect on viability. If mutant adult flies are viable, they can be compared with control flies with respect to their external morphology (e.g., size and shape of their wings, eyes, legs, and bristles), weight, and fertility. Also, the duration of development from egg to adult should be determined, because a number of mutations significantly delay larval development (by up to several days). A method for weighing flies has been described in **Subheading 2.1.** To determine fertility, set up several parallel single fly crosses between a mutant fly and a wild-type tester mate and count the number of offspring. A reduction in fertility can be caused by different defects that can be investigated specifically, for example, behavioral or morphological abnormalities

that prevent the adults from efficiently mating, developmental abnormalities that disrupt gametogenesis within the parent, maternal effects that interfere with the development of the offspring (zygote). Note that for any of the analyses mentioned here it is important to raise the flies under controlled conditions (temperature, humidity, day/night cycle). Furthermore, variations in the number of flies developing in a culture vial can strongly influence several parameters—overcrowding delays the duration of development and results in small flies.

If a mutation causes partial or complete lethality, it will be important to establish the lethal phase—or phases, as lethality is often not confined to a single moment during development. To detect a possible embryonic lethality, a large number of flies with the appropriate genotype (e.g., 20 females *allele 1*/+ and 20 males *allele 2*/+) are placed in an empty culture vial (or a plastic yoghurt beaker) and placed on a Petri dish with apple (or grape) agar, topped with yeast paste. Let the flies lay eggs onto the agar for a few hours, then remove the adults and place the covered Petri dish at 25°C for at least 24 h. During this time, wild-type and heterozygous zygotes will complete embryogenesis and hatch as larvae, leaving empty egg shells behind. If the examined mutation results in embryonic lethality, at least 25% of eggs containing only partly developed, unhatched embryos will remain behind; in a wild-type control cross, a few eggs also suffer this fate, but unless the "wild-type" stock is in extremely bad shape this fraction is below 10%. In case of embryonic lethality, it will be interesting to examine the cuticles of such dead mutant embryos (*see* Chapter 11). Cuticular structures are secreted by the developing embryo and they reflect its segmentation pattern; thus, mutations in numerous patterning genes (e.g., *wingless* [*wg*], *decapentaplegic* [*dpp*], *hedgehog* [*hh*]) result in characteristic cuticle defects, and any mutation with similar phenotype is likely to affect a gene that functions in the corresponding pathway.

Many lethal mutations allow survival to larval or pupal stages, though. Death during metamorphosis can be easily determined by scoring the fraction of empty pupal cases (normally >>95% for a control cross) at a sufficiently late time-point when all normal flies have eclosed (e.g., at 20 d after egg deposition at 25°C). To characterize larval lethality in more detail, a similar cross as described above for embryonic lethality determination can be set up. However, in this case the nonmutant chromosomes should carry a fluorescent marker. At >24 h after egg deposition the nonfluorescent first instar larvae are collected—these must be of the genotype *mutant 1*/*mutant 2*; they are then transferred at controlled densities to normal food vials. At regular intervals, the food (including the larvae) is extracted from these vials and submerged in glycerol; this floats the living larvae to the surface so they can be counted. Larval stages can be determined by examining the mouthhooks or the anterior spiracles (for a detailed

description, *see* **ref. *1***). However, in many instances such a detailed analysis is not required and researchers are happy to state that their mutation causes death during larval development.

3.6. Stock Centers

Drosophila biologists have a long-standing tradition of sharing their animals freely. Many of these lines have been deposited at one of the official stock centers (and that is where you always should look first before contacting individual researchers): Bloomington, Indiana (http://flystocks.bio.indiana.edu/); Szeged, Hungary (http://expbio.bio.u-szeged.hu/); Kyoto, Japan (http://www.dgrc.kit.ac.jp/); and Ehime, Japan (http://kyotofly.kit.jp). Additional large collections of P-element insertions and deficiencies are accessible at Baylor College of Medicine, Texas (http://flypush.imgen.bcm.tmc.edu/pscreen/); at Harvard Medical School, Massachussetts (http://drosophila.med.harvard.edu/); and University of Cambridge, UK (http://www.drosdel.org.uk/). A commercial collection of P-element insertions is found at http://genexel.com/eng/htm/genisys.htm. Of interest is also the Drosophila Genomics Resource Center (DGRC; http://dgrc.cgb.indiana.edu/), which distributes cDNA clones, cell lines, and microarrays. The conditions of use of these facilities are described under the different home pages.

3.7. Sending Flies

If you want to ship flies yourself, you can do so quite easily—flies are sturdy and usually survive the hardships of international travel quite well. However, especially during the year-end's holiday season such a travel can take quite a long time, and any shipment in the midst of winter (or summer) risks exposing the freight to extreme temperatures. To maximize the chances of survival under these conditions, for each line send two vials that contain flies at different stages of development. Importantly, make sure that one vial contains embryonic and larval stages and do not only send adult flies, as extreme temperature can deprive them of their fertility quite easily. The lids on the vials should be secured with adhesive tape, without blocking air access. The vials can be sent with regular mail (in our experience in 95% of the cases this works well for the trip from the US to Europe) or with an express carrier (if this carrier accepts the transport of life animals—check beforehand). If your parcel crosses borders you should include a customs declaration stating that it contains *D. melanogaster*, which are to be used for research purposes only, are nonhazardous and of no commercial value. Import into the US additionally requires an "import permit" from the USDA—detailed information about which is provided at the Bloomington home page (*see* **Subheading 3.6.**).

3.8. Further Reading

By necessity, this text can only provide a brief introduction to the use of *D. melanogaster* as a laboratory animal. We refer you to the references listed below for extensive (and highly readable) information about fly pushing ***(2)***, about the development of flies from eggs to adults and back ***(3–5)***, and about everything else you possibly ever wanted to find out about these critters ***(1,6,7)***.

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3

FlyBase

A Database for the Drosophila *Research Community*

Rachel Drysdale and the FlyBase Consortium

Summary

FlyBase (http://flybase.org) is the primary database of integrated genetic and genomic data about the Drosophilidae, of which *Drosophila melanogaster* is the most extensively studied species. Information in FlyBase originates from a variety of sources ranging from large-scale genome projects to the primary research literature. Data-types include sequence-level gene models, molecular classification of gene product functions, mutant phenotypes, mutant lesions and chromosome aberrations, gene expression patterns, transgene insertions, and anatomical images. Query tools allow interrogation of FlyBase through DNA or protein sequence, by gene or mutant name, or through terms from the several ontologies used to capture functional, phenotypic, and anatomical data. Links between FlyBase and external databases provide extensive opportunity for extending exploration into other model organism databases and resources of biological and molecular information. This review will introduce the FlyBase web server and query tools.

Key Words: FlyBase; genome analysis; BLAST; gene function; mutant phenotype; comparative genomics; biological ontologies; database query; *Drosophila.*

1. Introduction

FlyBase (http://www.flybase.org) houses information about the structure and function of the *Drosophila* genome. More than 55,000 gene records from over 500 species of Drosophilid accommodate data regarding gene function and expression patterns, and phenotypes and genetic interactions owing to mutant alleles of those genes. Allele, chromosome aberration, and transgene insertion records are linked to strains available for ordering from *Drosophila* stock centers, and where possible, to their location in the genome sequence.

The determination of the euchromatic genome sequence of *Drosophila melanogaster*, and initial gene annotation effort was followed by a systematic

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genome-wide annotation review by FlyBase ***(1–4)***. The ongoing incorporation of updated data is based on research publications curated by FlyBase, and on information submitted to FlyBase by members of the research community. At time of writing the *D. melanogaster* genome annotation has reached Release 5.1, which now includes several megabases of centric heterochromatin. In addition, the *D. pseudoobscura* genome has been the subject of particular analysis ***(5)*** and is currently at annotation Release 2.0. During 2006, the genome assemblies and gene predictions for an additional 10 species of *Drosophila*, generated in a systematic comparative analysis effort by the Assembly, Alignment, and Annotation groups (http://rana.lbl.gov/drosophila/), became public (*see* **ref. *6*** for review).

FlyBase curators and annotators curate data from the primary research literature, genome sequencing projects and online resources such as GenBank, large-scale gene disruption projects (e.g., the GDP, *see* http://flypush.imgen.bcm.tmc.edu/pscreen/), and protein databases (e.g., Uniprot, *see* http://www.ebi.ac.uk/uniprot/). Additionally, they work with curators of other databases such as the Gene Ontology (GO) Consortium (*see* **ref. *7***, and http://www.geneontology.org/) to apply consistent standards of functional annotation, for example, with the GO vocabularies, across databases. The FlyBase curators ensure that each object (gene, transcript, transgene construct, and so on) is uniquely named and identified in the FlyBase data structure, and disciplined "controlled vocabularies" are applied to the relevant item, with each piece of gathered information attributed to the publication in which it appeared. The controlled vocabularies of anatomical and developmental stage terms used to describe mutant phenotypes and expression patterns, and the GO, are part of the Open Biomedical Ontologies project (*see* **ref. *8***, and http://obo.sourceforge. net/) used by the majority of model organism databases. FlyBase also uses the Sequence Ontology ***(9)*** to describe aspects of the genome annotation. The application of these vocabularies throughout FlyBase facilitates the indexing of the data and permits comprehensive retrieval of related items in searches of FlyBase data. Prior knowledge of the contents of the vocabularies is not required for use, as queries are framed using pull-down menus or term selection routines where the users choose between terms displayed in hierarchies (*see* **Subheading 3** and **6.**).

In this review an introduction to the FlyBase web server and query tools will be presented. Although it is not possible to provide a comprehensive guide to all features and uses of FlyBase here, the aim is to provide sufficient background to encourage further exploration.

2. The Home Page

The FlyBase home page (http://flybase.org) is shown in **Fig. 1**. The header panel with dark menu bar persists on all the reports on the FlyBase web site,

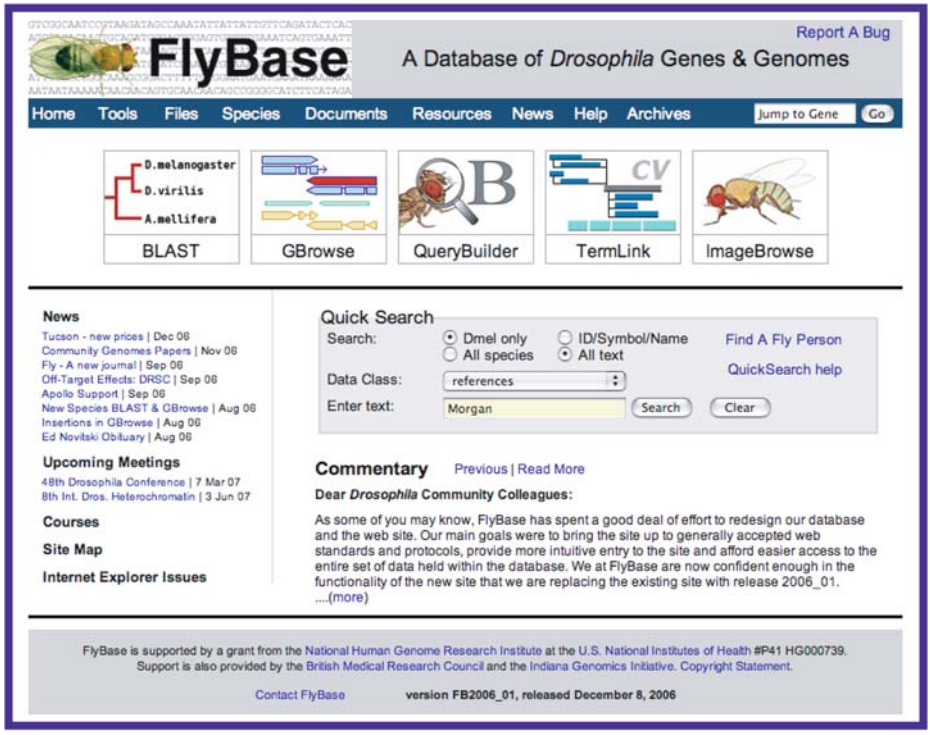

Fig. 1. The FlyBase home page. The FlyBase home page (http://www.flybase.org): notice the horizontal menu bar (Home, Tools, Files…), the News section (Meetings, Courses…), Search tools (BLAST, GBrowse, QueryBuilder, TermLink, and ImageBrowse), Quick Search panel, and "Jump to Gene" feature (top right corner). The "Contact FlyBase" hyperlink (bottom of page) appears on each FlyBase page, for convenient reporting of problems or sending of queries, though is omitted from the following figures.

and provides pull-down menu access to **Tools** (all query tools including "Find a person"), **Files** (i.e., data files for downloading), **Documents** (such as the FlyBase reference manual and the nomenclature guide), and **News** (including meeting listings). The **Resources** pull-down menu provides access to Network and Material resources external to FlyBase, along with links to several stock centers. The **Species** pull-down menu provides information about Drosophilid phylogeny and synteny relationships, and a list of species abbreviations used within FlyBase. Finally, the **Help** pull-down menu provides access to an assortment of supporting documentation. Recent news items, upcoming meetings, and courses, along with a site map, are highlighted in the News panel, on the left hand side of the home page.

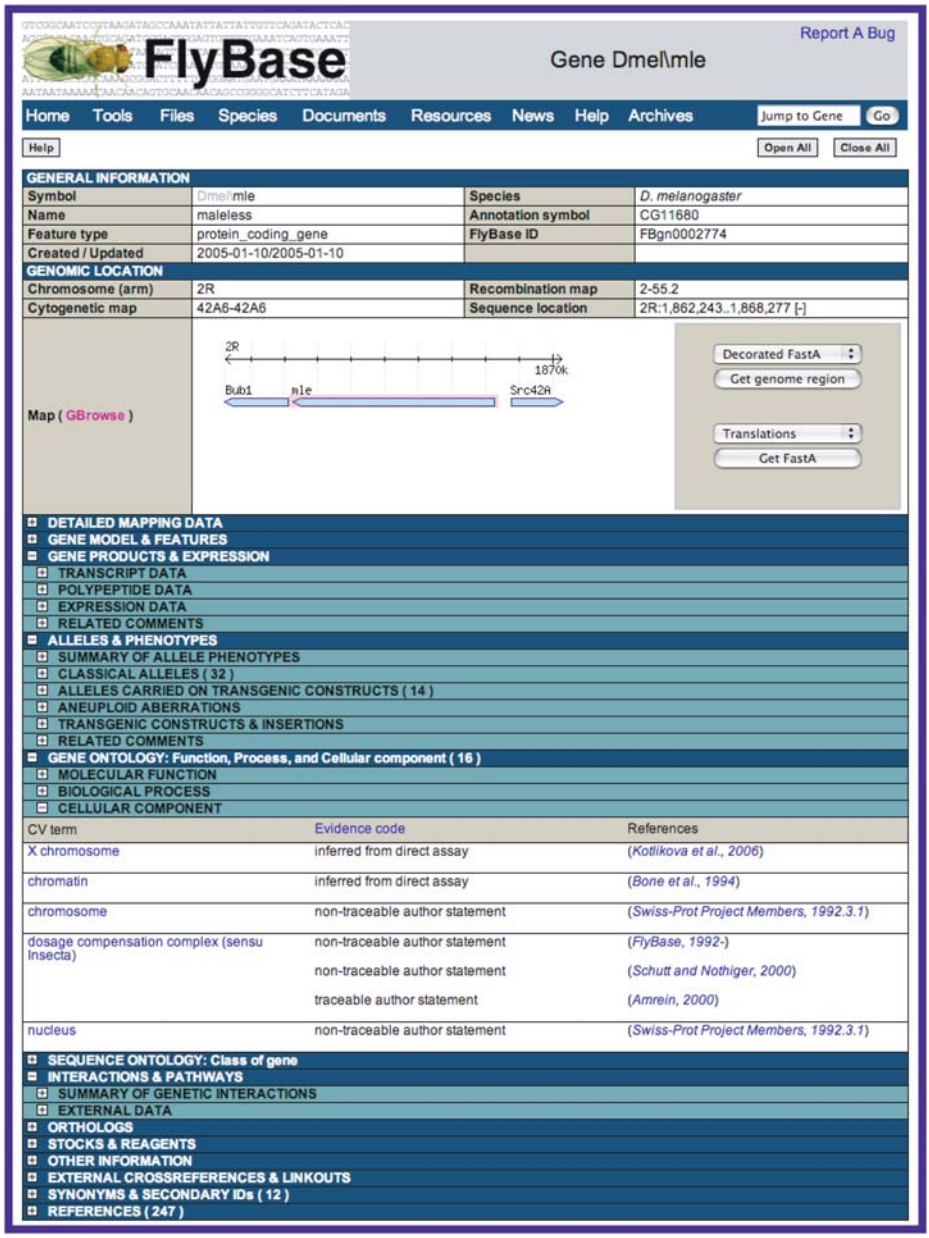

Fig. 2. The gene report for *mle* (FBgn0002774). The entry level gene report includes the horizontal menu bar for direct navigation to other parts of FlyBase, Documentation and Help, a "General Information" panel that identifies the gene that is the subject of the report, a "Genomic Location" panel with a sketch of the gene in the context of the chromosome and a panel of expandable/collapsible subreports, listed with blue background

Moving down the home page, the next panel presents direct access to tools widely used for interrogating the FlyBase data. **BLAST**, **GBrowse**, and **QueryBuilder** will be described in further detail later. TermLink allows navigation through the anatomical terms used to categorize phenotypic and expression pattern data, and **ImageBrowse** allows access to data based on diagrams of morphology that are annotated with those anatomical terms (*see* **ref. *10*** for description of TermLink and ImageBrowse).

Those interested in moving directly to information about a specific gene can enter its symbol in the **Jump to Gene** feature on the home page (top right corner). A central **Quick Search** panel allows querying for a particular report, in combination with data-type, for example, by author name in the "References" data section.

3. The Gene Report

The *mle* gene (*maleless*; FBgn0002774), will be used as an example to demonstrate features of the data stored within the FlyBase gene reports.

Figure 2 shows the gene report page for *mle*. The top section, General Information, identifies the gene by symbol, name, and FlyBase unique identifier (FBgn0002774). The central section, Genomic Location, provides a summary of the recombinational, cytogenetic, and sequence coordinate map data for the gene, and for those genes mapped to the genome, provides a thumbnail sketch of its genomic organization. The right-hand panel of the Genomic Location section provides several options for downloading nucleotide or protein sequence. The left hand panel of the Genomic Location section includes a link to GBrowse, described in **Subheading 4**.

The lower portion of the gene page illustrates a design feature used throughout FlyBase reports: collapsible subreports, which can be opened or closed according to the interests of the user, by clicking the +/– toggle boxes. For example, **Fig. 2** shows the first level of unpacking for the Gene Products and Expression, Alleles and Phenotypes, and Interactions and Pathways subreports. The Gene Ontology subreport has been expanded out to display the specific "Biological process" and "Molecular function" report headers and the complete "Cellular component" report for the *mle* gene product. Each report header panel is labeled according to the type of data that would become visible

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Fig. 2. *(Continued)* and identifiable by the clickable "+/–" box at their left hand limit. The titles of these clickable subreports, and their subcategories (shown for "Gene Products and Expression," "Alleles and Phenotypes," "Interactions and Pathways," and "Gene Ontology") give an overview of the data-types that can be explored in the gene report. The Gene Ontology subreport is unpacked to the maximal extent for the "Cellular Component" division of the Gene Ontology, revealing the data and sources.

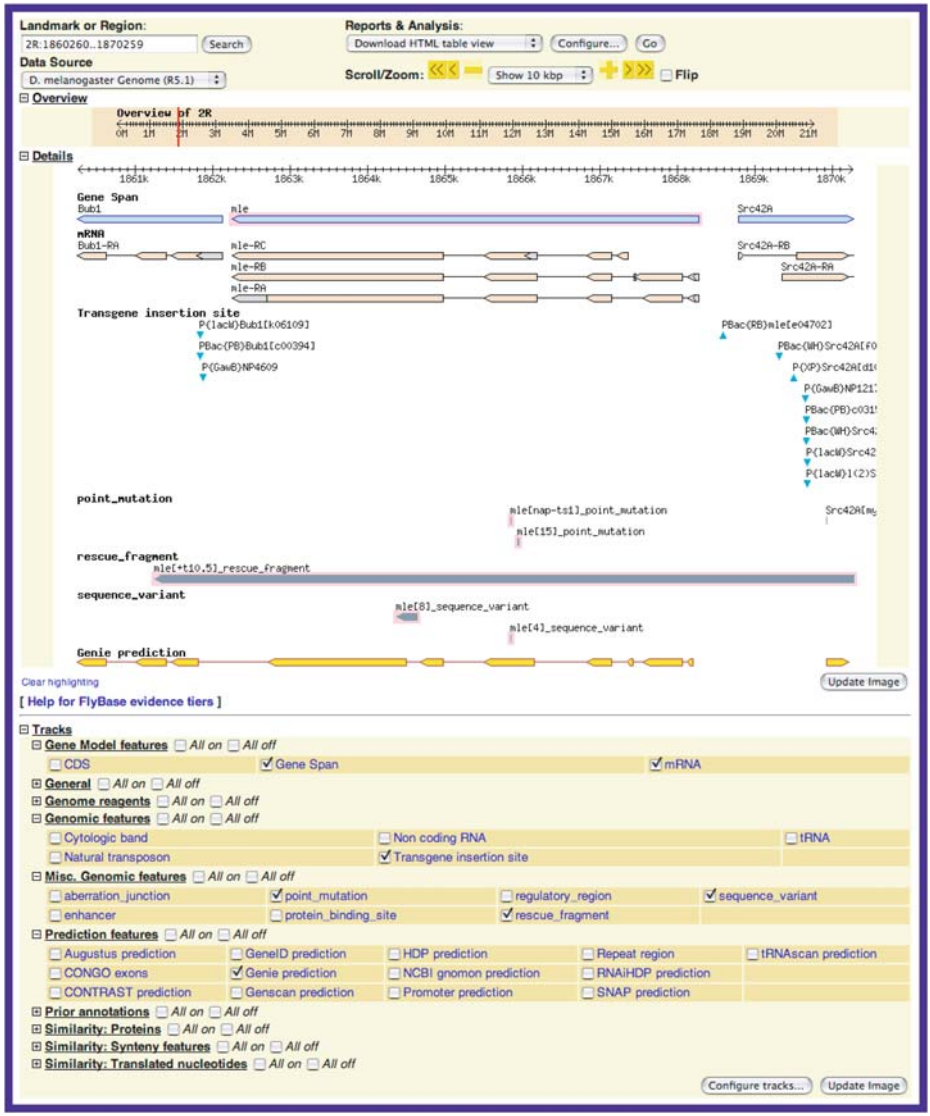


Fig. 3. GBrowse view of *mle* gene region. In this view a 10 kb region including the *mle* transcription unit is shown. Three annotated transcripts (mle-RA, B, and C) are diagrammed beneath the extent of the gene (highlighted blue bar). Flanking genes *Bub1* and *Src42A* are evident on either side of *mle*. A rescue fragment, $mle^{+10.5}$, and four genome-mapped mutant alleles, mle^{8}, $mle^{nap\text{-}ts1}$, mle^{15}, and mle^{4}, are shown above a track of Genie predictions. Many transgene insertions can be seen to either side of, although not within, the *mle* transcription unit. The top section of the window provides tools to change the region of genome being viewed, whereas the bottom "Tracks" section

in that section, were it to be further expanded. Thus "Classical alleles (32)," under Alleles and Phenotypes indicates access to a table of the 32 mutagen-induced or spontaneous alleles of *mle* that FlyBase has cataloged, from which individual allele reports could be accessed. In this view the Detailed Mapping Data, Gene Model & Features, Sequence Ontology, Orthologs, External Crossreferences & Linkouts, Synonyms & Secondary IDs, and References subreports are left unexpanded.

The External Crossreferences & Linkouts subreport includes access to entries in databases external to FlyBase relevant to the gene that is the subject of the report. At time of writing the Linkouts for *mle* include *Drosophila melanogaster* exon database (DEBD), general repository for interaction datasets (FlyGRID), NCBIO gene expression omnibus (GEO), FlyMine, protein analysis through evolutionary relationships (PANTHER), *Drosophila* RNAi screening center (DRCS), integrating genome and high throughput data (FLIGHT), Heidelberg RNAi, Berkeley *Drosophila* genome project (BDGP) *in situ* gene expression database, and *Drosophila* protein interaction map (Dros PIMRider).

Each controlled vocabulary data statement within a report, for example, "X chromosome" in the "Cellular component" subreport in **Fig. 2**, is hyperlinked to a "Term Report" providing information about that term and terms related to it, often including definitions, and for anatomical terms, an anatomical drawing (*see* **ref.** ***10*** for review). Each data statement within a report is displayed alongside the hyperlinked reference from which that statement was recorded by the curators, reflecting the FlyBase policy of attributing all incoming data to its primary source. The hyperlinked reference links to a full Reference Report, which states whether the reference is a primary research publication, a review or book chapter, a note to FlyBase (archived and kept as a "personal communication to FlyBase"), or a sequence database record—to name commonly used sources of information. Each Reference Report is linked, where possible, to the text of the publication itself. Thus researchers can use information found in FlyBase as a spring-board to publications with information specific to their interests, where more detailed information than FlyBase provides is required.

4. The Genome View—GBrowse

The Genomic Location section of the gene report (*see* **Fig. 2**) has a hyperlink (GBrowse, left panel) that links directly to the GBrowse ***(11)*** view of the gene in the context of the genomic region to which it has been localized. The GBrowse view of *mle* is shown in **Fig. 3.** This particular view is set to show a 10 kb

Fig. 3. *(Continued)* allows selection of classes of feature to display on the portion of the genome in view. Data in GBrowse comes from a variety of sources including the whole genome annotation project ***(4)*** and in-depth curation of the primary research literature.

window, the *mle* gene span and its three mRNAs, transgene insertions, a rescue fragment ($mle^{+t10.5}$), four mapped mutant alleles (mle^{8}, $mle^{nap\text{-}ts1}$, mle^{15} and mle^{4}), and Genie predictions. The relative orientation of the insertions is indicated by the orientation of the triangles representing their insertion site. The transcripts, insertions, and alleles are hyperlinked to their respective reports; the $mle^{nap\text{-}ts1}$ allele report is shown in **Fig. 4** (*see* **ref. *12*** for description of the system used to store phenotypic data in FlyBase).

The choice of which features to display in GBrowse is set using the clickable Tracks configuration panel shown in the lower section of **Fig. 3**. The region of the chromosome viewed can be adjusted using the Scroll/Zoom facility. The entire view can be left–right reversed using the Flip function. The Reports & Analysis feature of GBrowse (center top of **Fig. 3**) provides the option of translating the image into a tabular format. The alternatives available for tabular download are Hyper Text Markup Language (HTML) table view (as shown in **Fig. 3**), general feature format (GFF) Format, or fastall (FASTA) format.

GBrowse provides web access to the genome view. Apollo ***(13)*** is a standalone interactive genome annotation viewing and editing tool, enabling viewing down to the DNA sequence level. Apollo can be downloaded from http://www. fruitfly.org/annot/apollo and is available for a range of operating systems.

5. Searching by Sequence—BLAST Queries

FlyBase basic local alignment search tool (BLAST) provides sequence-based query retrieval within FlyBase. The BLAST query page, shown in **Fig. 5**, is divided into three areas. The top portion allows selection of the BLAST parameters, and provides a key to features available to BLAST used in the "Select species to search against" panel below. In **Fig. 5** the query is the *mle* protein product B amino acid sequence and the BLAST program is tblastn. The lower portion of the query page allows selection of the genome(s) to be searched: the 12 Drosophilid species (*pseudoobscura, yakuba, simulans, virilis, ananassae, erecta, willistoni, grimshawi, mojavensis, persimilis,* and *sechellia*), *Anopheles gambiae*, *Aedes aegypti*, *Bombyx mori*, *Apis mellifera*, and *Tribolium castaneum*. The bottom panel of the BLAST query page, not shown in **Fig. 5**, allows selection of Advanced BLAST options with alternatives (Alignment view, Tabular, and Extensible Markup Language [XML]) for displaying the BLAST output. The BLAST output presents each BLAST hit in combination with a "BLAST *hit* on Genome Map" link to GBrowse (not shown). The bottom panel of **Fig. 5** shows the consequent GBrowse view of the result for the *D. sechellia* genome, with selected predictions shown as evidence panels. At time of writing *D. melanogaster* and *D. pseudoobscura* have consensus annotation sets: for other species the GBrowse view consists of gene predictions generated by the Assembly, Alignment, and

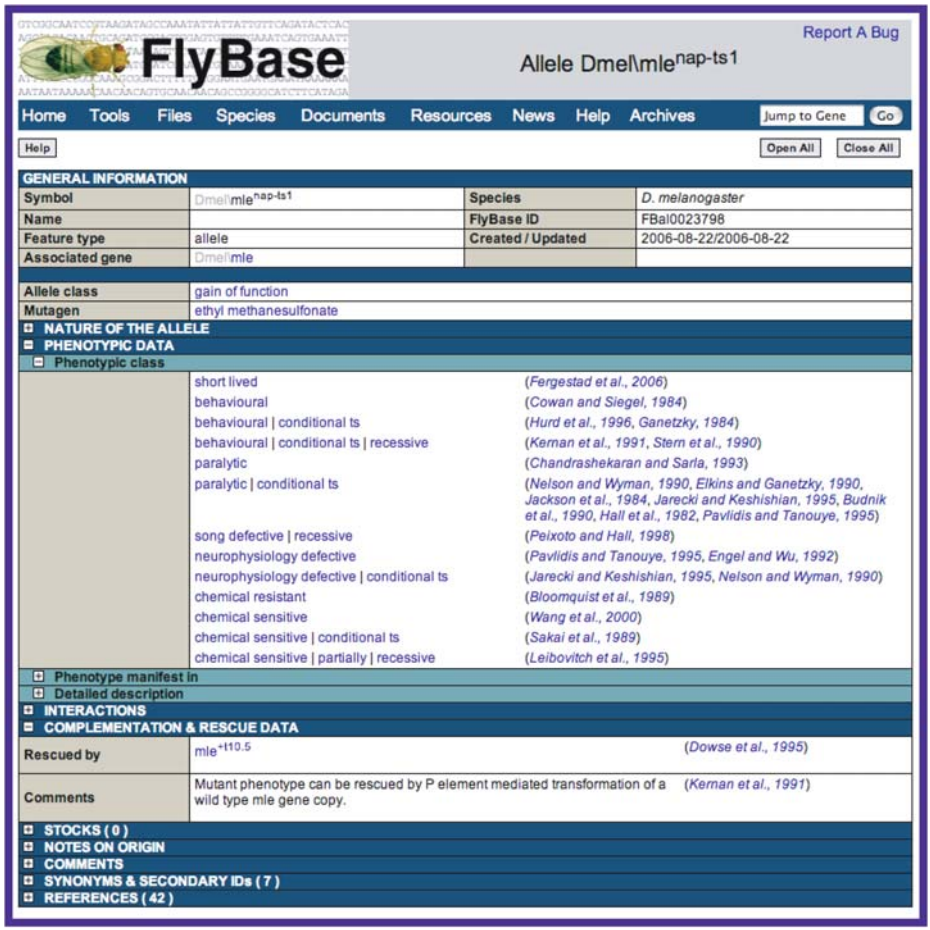
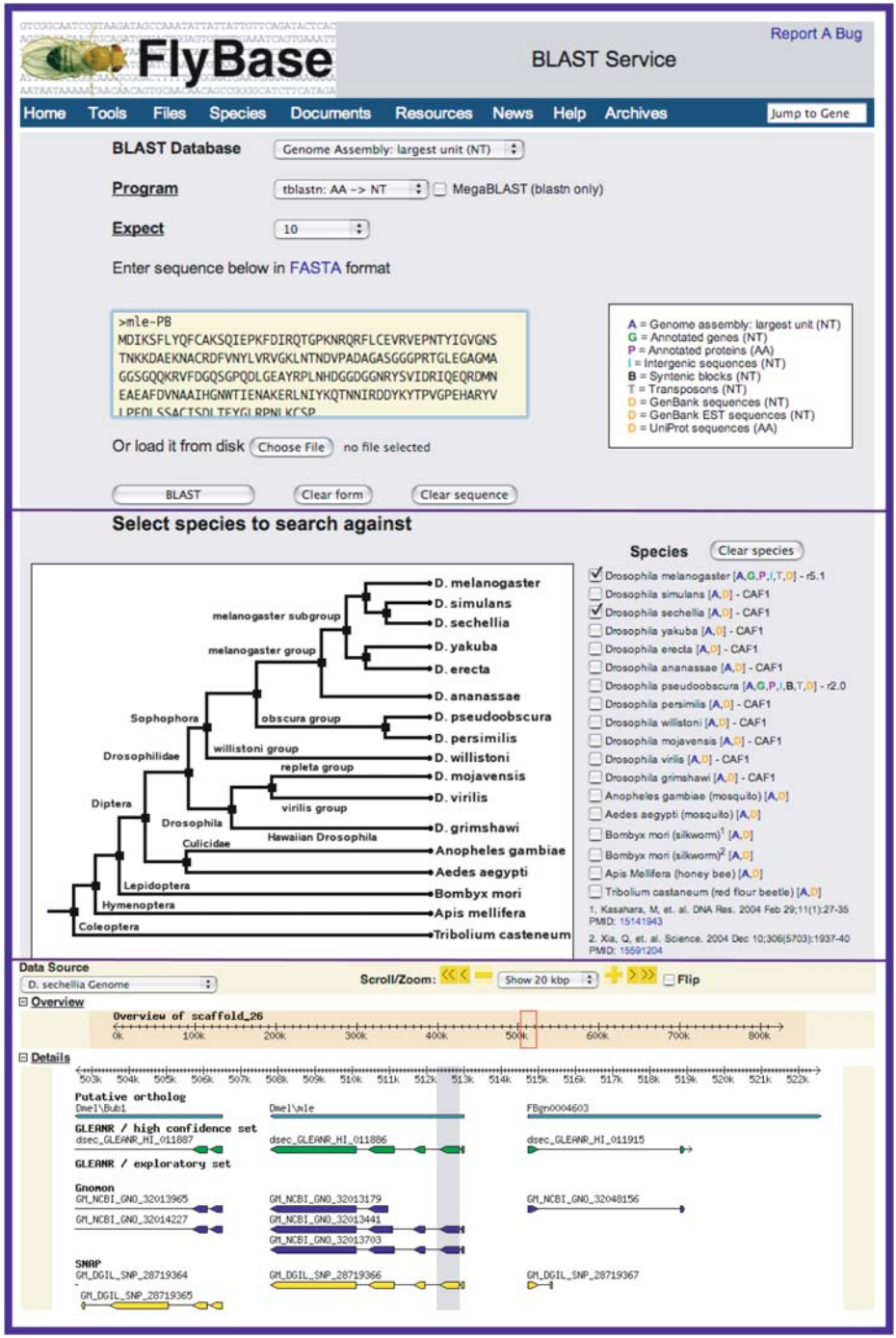


Fig. 4. Allele report for *mle*$^{nap\text{-}ts1}$. The top panel of the Allele Report includes information identifying the allele that is the subject of the report, in this case *mle*$^{nap\text{-}ts1}$ (identifier number FBal0023798). The allele reports use expandable/collapsible subreports to control the view of the information. In this view the "Phenotypic class" subreport is opened, and the "Complementation and Rescue" panel indicates that *mle*$^{+10.5}$ can rescue the *mle*$^{nap\text{-}ts1}$allele. A review of the systems FlyBase uses for describing mutant phenotypes and genetic interactions is given in **ref. *12***.

Annotation projects (http://rana.lbl.gov/ drosophila/), named numerically for the algorithm or prediction group (e.g., GM_NCBI_GNO_32013179), in the context of *D. melanogaster* "Putative ortholog," in this case *Dmel\mle*. The extent of the BLAST hit is shown by a shaded grey vertical bar. The Data Source pull-down menu (top left in lower GBrowse panel of **Fig. 5**) allows the view to traverse from one Drosophilid species to any of the other eleven.


FlyBase
BLAST Service
Report A Bug
Home Tools Files Species Documents Resources News Help Archives
Jump to Gene
BLAST Database
Genome Assembly: largest unit (NT)
Program
tblastn: AA -> NT
MegaBLAST (blastn only)
Expect
10
Enter sequence below in FASTA format
>mle-PB
MDIKSFLYQFCAKSQIEPKFDIRQTGPKNRQRFLCEVRVEPNTYIGVGNS
TNKKDAEKNACRDFVNYLVRVGKLNTNDVPADAGASGGGPRTGLEGAGMA
GGSGQQKRVFDGQSGPQDLGEAYRPLNHDGGDGGNRYSVIDRIQEQRDMN
EAEAFDVNAAIHGNWTIENAKERLNIYKQTNNIRDDYKYTPVGPEHARYV
Or load it from disk
Choose File
no file selected
BLAST
Clear form
Clear sequence
A = Genome assembly: largest unit (NT)
G = Annotated genes (NT)
P = Annotated proteins (AA)
I = Intergenic sequences (NT)
B = Syntenic blocks (NT)
T = Transposons (NT)
D = GenBank sequences (NT)
D = GenBank EST sequences (NT)
D = UniProt sequences (AA)
Select species to search against
D. melanogaster
D. simulans
D. sechellia
D. yakuba
D. erecta
D. ananassae
D. pseudoobscura
D. persimilis
D. willistoni
D. mojavensis
D. virilis
D. grimshawi
Anopheles gambiae
Aedes aegypti
Bombyx mori
Apis mellifera
Tribolium casteneum
melanogaster subgroup
melanogaster group
obscura group
willistoni group
repleta group
virilis group
Hawaiian Drosophila
Sophophora
Drosophilidae
Drosophila
Diptera
Culicidae
Lepidoptera
Hymenoptera
Coleoptera
Species
Clear species
Drosophila melanogaster [A,G,P,I,T,D] - r5.1
Drosophila simulans [A,D] - CAF1
Drosophila sechellia [A,D] - CAF1
Drosophila yakuba [A,D] - CAF1
Drosophila erecta [A,D] - CAF1
Drosophila ananassae [A,D] - CAF1
Drosophila pseudoobscura [A,G,P,I,B,T,D] - r2.0
Drosophila persimilis [A,D] - CAF1
Drosophila willistoni [A,D] - CAF1
Drosophila mojavensis [A,D] - CAF1
Drosophila virilis [A,D] - CAF1
Drosophila grimshawi [A,D] - CAF1
Anopheles gambiae (mosquito) [A,D]
Aedes aegypti (mosquito) [A,D]
Bombyx mori (silkworm)1 [A,D]
Bombyx mori (silkworm)2 [A,D]
Apis Mellifera (honey bee) [A,D]
Tribolium castaneum (red flour beetle) [A,D]
1. Kasahara, M, et. al. DNA Res. 2004 Feb 29;11(1):27-35 PMID: 15141943
2. Xia, Q, et. al. Science. 2004 Dec 10;306(5703):1937-40 PMID: 15591204
Data Source
D. sechellia Genome
Scroll/Zoom:
Show 20 kbp
Flip
Overview
Overview of scaffold_26
Details
Putative ortholog
Dmel\Bub1
Dmel\mle
FBgn0004603
GLEANR / high confidence set
dsec_GLEANR_HI_011887
dsec_GLEANR_HI_011886
dsec_GLEANR_HI_011915
GLEANR / exploratory set
Gnomon
GN_NCBI_GNO_32013965
GN_NCBI_GNO_32013179
GN_NCBI_GNO_32048156
GN_NCBI_GNO_32014227
GN_NCBI_GNO_32013441
GN_NCBI_GNO_32013703
SNAP
GN_DGIL_SNP_28719364
GN_DGIL_SNP_28719366
GN_DGIL_SNP_28719367
GN_DGIL_SNP_28719365

6. Advanced Queries—QueryBuilder

QueryBuilder is a tool designed to allow searching through the entire FlyBase data collection. It allows users to perform complex searches that are not possible using the QuickSearch function on the home page. The strength of QueryBuilder is that queries crossing datasets can be constructed. Queries are built up in a stepwise fashion; specified datasets (e.g., genes, insertions, transcripts, and so on) are interrogated sequentially for values selected from the range of values possible for the dataset specified in each step. The QueryBuilder output provides sets of records that match the query, but are also cross-referenced to related records in FlyBase, to allow traversing interrelated datasets. **Figure 6** shows the QueryBuilder entry page (top panel) and the interface for adding or modifying a query segment (central panel). In the Search DataSet column the dataset to be queried is selected from a pull-down menu, then the DataBase Field to be searched is selected from a scrolling menu, and the Text to find is specified. The choices in the DataBase Field and Text to find columns correspond to the values selected in the DataSet and DataBase Field, thus constraining the field to be searched to the relevant dataset. This prevents nonsensical queries such as looking for "volume" (a property of References) in a query acting on the Genes dataset. In the example shown in **Fig. 6** the Genes dataset is being queried for the intersection of occurrences of the "GO: Molecular Function" term "transcription regulator activity" (or terms below this term in the GO hierarchy) with alleles, which show an "OBO: Phenotypic class" exact value of "circadian rhythm defective." The controlled vocabularies used in FlyBase are extensive, so an interactive "term picker" (not shown) guides the user through choosing a valid term whenever a controlled vocabulary DataBase Field is selected. The bottom panel of **Fig. 6** shows the results page obtained by running this query. Five genes are annotated with "transcription regulator activity" or more specific instances of this term, and have at least one allele annotated with "circadian rhythm defective." The Records matching

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Fig. 5. *(Opposite page)* BLAST search of *D. sechellia* genome with an *mle* protein sequence BLAST query page. In this example the *D. sechellia* genome is queried for sequence corresponding to the *mle* protein product B amino acid sequence. The top panel shows the selection of tblastn and the query sequence. The middle panel shows the selection of the genomes to query against. The bottom panel shows the result displayed on the *D. sechellia* genome in the context of a subset of the available gene predictions. At time of writing additional predictions were available for Contrast, N_Scan, Exonerate, the Oxford pipeline, GeneMapper, and Geneld. Further details of these prediction projects are available from the Assembly, Alignment, and Annotation project—http://rana.lbl.gov/drosophila/.

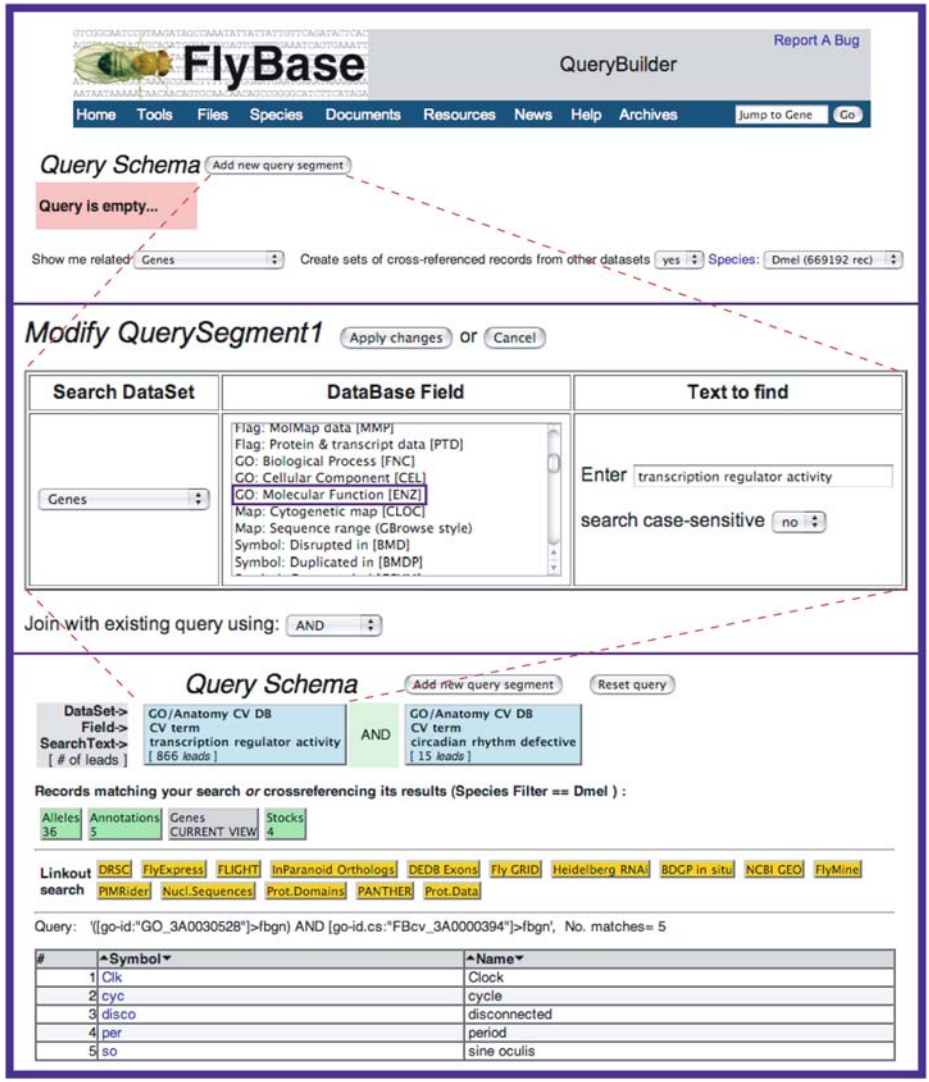

Fig. 6. QueryBuilder. QueryBuilder enables the user to construct multistep queries that traverse distinct datasets within FlyBase. In this query the database is queried for genes that are annotated with the GO term "transcription regulator activity" and which have alleles that show a "circadian rhythm defective" mutant phenotype. The top panel shows an empty query. A query segment is added by using the "Add new query segment" dialog, shown in the middle panel. Once a DataSet to be searched has been selected (left column) and the DataBase field to query has been chosen (middle column) a term-picker chooser allows navigation through the relevant options for that field (not shown) taking into account the hierarchical structure of the controlled vocabulary where this exists. The term-picker drops the term to search for into the third column,

links allow traversing the different data-types in FlyBase that relate to the query. For example, changing the view to Alleles (36) would show the 36 alleles annotated with "circadian rhythm defective" that map to genes annotated with "transcription regulator activity." To further modify the query, additional segments would be added through the "Add new query segment" facility, to modify the first segment in a Boolean "and," "or," or "but not" relationship, specified with the Join with existing query box.

Query Builder was designed to enable users to query FlyBase in ways that are not dependent on knowing the name of a searched item, nor the structure of the data relationships within FlyBase. In addition to the in-page guidance for use of QueryBuilder a comprehensive QueryBuilder Tutorial (downloadable from the QueryBuilder page "full tutorial" link (not shown)) provides several worked examples and further explanation of the menu items and controlled vocabulary/dataset relationships.

7. FlyBase and the Research Community

7.1. Help for Users

Online documentation can be found in the Help section, accessible from any FlyBase page. A comprehensive explanation of FlyBase data-types, the Reference Manual, is kept in the Documents section. Questions about FlyBase data content or query tools can be emailed to FlyBase at flybase-help@morgan.harvard.edu. Such help requests are seen by the majority of the project members and are answered promptly. Additionally, each FlyBase page includes a "Contact FlyBase" link (as shown in **Fig. 1**) to a help request form, which serves the same purpose as a direct email message.

7.2. Contributions and Corrections

Contributions of data, or corrections to data presented in FlyBase, should be emailed to flybase-help@morgan.harvard.edu. Where data submitted is novel, i.e.,

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Fig. 6. *(Continued)* and clicking on the "Apply changes" button in the "Modify Query Segment" window adds that query segment to the Query Schema (bottom panel). This process is reiterated for each step of the query. At each stage the user has the option of asking the query to match the term exactly, or to include all hits that use terms further down the hierarchy of the term in question. In this example "transcription regulator activity" and its descendents were specified for query segment 1, with an exact match to "circadian rhythm defective" requested for query segment 2. Running the query matched five genes—shown in the bottom panel. The "Records matching" links allow traversing the different data-types in FlyBase that relate to the query. The yellow "Linkout Search" provides a table of links to external database entries relevant to the hits displayed in the table below, in this case the five genes retrieved by the query.

not included in any otherwise published research article, a FlyBase curator will work with the contributor to settle on a text that will be archived at FlyBase as a "personal communication." Each personal communication is given publication details (author, date, title) and treated, for the purposes of citation within FlyBase, as a regular research publication.

7.3. The FlyBase Project

FlyBase is a collaboration between three groups, based in the Biological Laboratories, Harvard University, Cambridge, Massachusetts, USA (Principal Investigator W. Gelbart), the Department of Biology, Indiana University, Bloomington, Indiana, USA (co-PIs T. Kaufman and K. Matthews), and the Department of Genetics, University of Cambridge, Cambridge, UK (co-PIs M. Ashburner and R. Drysdale). Members of the project come from both *Drosophila* research and computer science backgrounds; for a current full list *see* http://flybase.bio.indiana.edu/docs/lk/refman/refman-I.html#I.5.

8. FlyBase in the Future

The FlyBase project began in 1992 in order to continue the tradition of the "red book" ***(14)***, which itself was built on a series of publications beginning in 1925 ***(15–17)***. These compendia cataloged mutants, with description and references in a way that was useful for *Drosophila* researchers. The first public release of FlyBase, in 1993, was made using a gopher server; the world wide web was not yet in widespread use. During the time that FlyBase has been in operation the entire genome sequence of first *D. melanogaster* and then 11 other drosophilid species has been determined. The changing requirements for FlyBase have necessitated an overhaul of its database structure and reports, with the new service based on a PostGres chado genome database (**[*18*]**; *see* http://www.gmod.org/schema) released to users in 2006. FlyBase is no longer an isolated database of interest only to fruit fly geneticists, but part of a vast network of internet resources used by biologists, bioinformaticians, and educators across a wide range of interests and specialities. Nonetheless, serving the needs of Drosophilists of all kinds remains our primary goal, even as the fly adds whole-genome comparative genomics to the list of subjects for which it is a valuable model organism.

Acknowledgments

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4

The Use of P-Element Transposons to Generate Transgenic Flies

André Bachmann and Elisabeth Knust

Summary

The development of a technique to stably integrate exogenous DNA into the germline of *Drosophila melanogaster* marked a milestone in the ability to study gene function in the fly.

On the molecular level germline transformation mainly relies on a particular transposable element, the *D. melanogaster* P-element. Based on certain features of the P-element, vectors have been designed for diverse applications like gene disruption, chromosome engineering, gene tagging, and inducible gene expression/repression. Despite the fact that an increasing number of other transposons have been utilized for germline transformation of *Drosophila* most transformation vectors are still P-element based.

Technically, microinjection serves as the method of choice to physically introduce transgenes into preblastoderm *Drosophila* embryos. Besides an appropriate technical equipment including suitable microcapillaries in conjunction with a micromanipulator, a microinjector, and a microscope, proper handling of the *Drosophila* embryos before and after microinjection is the key step to the generation of transgenic flies. Pioneer work in *Drosophila* also served as a general guideline for the transformation of other insect species including those with medical and agricultural importance.

Key Words: *Drosophila melanogaster*; germline transformation; microinjection; P-element; transformation vector; transgenic fly; transposable element.

1. Introduction

The discovery of P-elements in *Drosophila melanogaster* is tightly connected to a syndrome called hybrid dysgenesis. When females of so-called M strains were mated to males of a P stock the offspring displayed a number of defects including sterility, mutation, chromosome breakage, and male recombination. The molecular basis for hybrid dysgenesis turned out to be the presence of a particular type of mobile genetic elements, P-elements, in P stocks but not in M strains. Depending on their capability to transpose, P-elements are classified as autonomous or

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nonautonomous. Autonomous P-elements encode a functional transposase that enables them to move by a cut-and-paste mechanism inside a genome, whereas nonautonomous ones lost this ability owing to internal deletions. The functional P-element is 2907 bp in length with 31 bp inverted terminal repeats and 11 bp inverted subterminal repeats as the major *cis*-acting target sequences for excision by the transposase. Reintegration of an intact P-element at a random chromosomal location generates a flanking direct 8 bp duplication at the target site ***(1)***.

In order to serve as a tool for germline transformation, P-elements have been integrated into plasmid vectors and undergone further modifications ***(2,3)***. To inhibit their autonomous movement the transposase gene has been transferred to another vector named "helper-plasmid", which provides the transposase *in trans*, but is itself unable to integrate into the genome ***(4,5)***. In exchange for the transposase gene a multiple cloning site and a visible marker gene to identify transformants are inserted inside the inverted terminal repeats ***(6)***. The multiple cloning site facilitates the insertion of desired DNA sequences into the transformation vector. In most cases the visible marker gene is a *white* mini-gene, which in a *white* mutant background allows detection of an integration event ***(7,8)***. Besides *white* other marker genes like *rosy* ***(6)***, *vermilion* ***(9)***, or *yellow* ***(10)*** have been applied, which for their detection require injection of the transformation vector into embryos of corresponding mutant recipient strains. However, *white* has turned out to be the most convenient and frequently used marker gene. Nowadays a number of non-P-element transformation vectors use fluorescent proteins like GFP as a visible marker to uncover transformants because of its higher sensitivity ***(11–13)***. The transposase gene on the "helper-plasmid" has been modified, too. Normally the mobility of wild-type autonomous P-elements is restricted to the germline. This is owing to gene regulation at the level of RNA-splicing, which involves the 2-3 intron of the transposase gene. Artificial removal of this particular intron resulted in a transposase, termed Δ2-3, that functions in germline and somatic cells ***(5)***.

Besides the general modifications of a P-element-based transformation vector aforementioned, adaptation of the vector to different tasks requires special alterations. Numerous P-element vectors have been created that are suited for gene disruption, gene-, protein- and enhancer-trapping, transgene misexpression, promotor studies, genome manipulation, gene targeting, and RNA interference ***(14)***. A large collection of diverse transformation vectors is available from the *Drosophila* Genomics Resource Center at http://dgrc.cgb.indiana.edu/.

Germline transformation requires the stable integration of the DNA of interest into the germ cells of the recipient embryo. This is accomplished by the physical delivery of the desired DNA at the posterior pole of syncytial blastoderm *Drosophila* embryos where the precursors of the germ cells form. On cellularization the DNA will then, in theory, be incorporated into the so-called pole

cells and integrated into their genome. Deposition of the DNA of interest is fullfilled by penetrating the preblastoderm embryos with a suitable injection capillary and application of an injection mix including transformation vector and "helper-plasmid" ***(15)***. Coinjection of the transformation vector with purified P-element transposase has also been described, but appears to be less convenient ***(16)***. Thirdly, injection of only the transformation vector into embryos with a stably integrated source of transposase is possible, too ***(17)***.

Microinjection requires a couple of technical devices like a micropipet puller to produce the injection capillaries, a micromanipulator, a microinjector, and a microscope. Whereas the microscope is needed to optically control the microinjection procedure, the micromanipulator helps to gently align the injection capillary with the embryos. Finally, the microinjector is used to control the delivery of a reproducible amount of injection mixture to the embryos.

Apart from technical prerequisites correct treatment of the *Drosophila* embryos is critical for successful germline transformation. This includes collection of properly staged embryos, careful handling before and after microinjection, and selection and characterization of the transformants.

It should be noted that besides the P-element a couple of other transposons like *Minos* ***(18)***, *hobo* ***(19)***, *mariner* ***(20)***, *Hermes* ***(21)***, and *piggyBac* ***(22)*** have been introduced as tools for germline transformation in *D. melanogaster*. The germline transformation technique described in **Subheading 3**. should be applicable to these transposons, too. However, with the exception of the *piggyBac* transposon their usage for germline transformation in *D. melanogaster* is limited owing to the lack of a broad range of transformation vectors for the diverse applications listed above. On the other hand and in contrast to the P-element some of them display a broad host range allowing their usage as vectors for germline transformation in a wide variety of non-drosophilid insect species ***(23)***.

2. Materials

Depending on the equipment of your institute you might use other devices (micropipet puller, micromanipulator, and so on) than described here to generate transgenic flies. Ask coworkers familiar with a particular device for appropriate handling. We will refer mainly to the tools that we use and can recommend from our experience with P-element-mediated germline transformation. Therefore, some details on the set up and performance of germline transformation might not be useful for everyone, but could nevertheless serve as a general guideline.

2.1. Embryo Collection/Handling

1. A w^{1118} stock (Bloomington stock 5905) as the source of embryos for injection when using the *white* mini-gene or a fluorescent marker for the selection of transformants.

2. As egg laying cages we use self-made cylindric acrylic glass vials (1000 mL) that fit into standard Petri dishes (94×16 mm^2) filled with apple juice agar and are covered on their top side with a commercially available fly screen.
3. For apple juice plates take 40 g of agar, 340 mL of filtered apple juice (100%), 17 g of table sugar or sucrose and scale up to 1000 mL with sterile water. Boil until the agar and the sugar have dissolved completely and cool down to 60°C. Add Nipagin, a preservative and fungicide, to a final concentration of 0.3%, mix and immediately pour it into Petri dishes (94×16 mm^2). Avoid bubbles and make sure that the surface of the apple juice plates is smooth. After they have hardened and cooled down plates can be stored at 4°C for approx 2 wk.
4. Nipagin/Methylparaben: prepare a 10% stock solution in ethanol and store it at 4°C.
5. Yeast paste: dissolve Baker's yeast in sterile water until it has a paste-like consistency. Fill it into a plastic syringe to facilitate the dosage and store it at 4°C.
6. 10% Acetic acid.
7. To prepare steel mesh baskets cut a plastic tube (15 mm diameter) into pieces of 10 mm height, heat the plastic briefly on one side under a Bunsen burner and press it with the melted side onto a steel mesh whose mesh size will retain embryos (≤0.1 mm). Let it cool down and cut out the basket exactly from the steel mesh.
8. 7% Bleach/sodium hypochlorite.
9. A fine paintbrush.
10. Microscope slides ($76 \times 26 \times 1$ mm^3).
11. A blunt straight teasing needle.
12. For glue-coated cover slides incubate 10 pieces of adhesive tape (5×1 cm^2) in 10 mL of heptane for several days at room temperature. The heptane will dissolve the glue from the tape. Centrifuge the liquid part to precipitate indissoluble components and store the supernatant/liquid glue in a glass bottle at 4°C (*see* **Note 1**). Distribute 1–2 drops of the liquid glue on one half of a cover slide (20×20 mm^2), place it on a planar surface and let the heptane evaporate. The glue will remain on the cover slide. Test adhesiveness with your fingertip (the cover slip should stick to it tightly). Do not prepare more glue-coated cover slides than you will need for 1 d of injection.
13. A stereo dissecting microscope, for example, a Stemi 2000 from Zeiss, Göttingen, Germany.
14. A small spatula.
15. Medium-sized vials of fresh fly-food (~15 mL).

2.2. DNA Mix Preparation

1. A commercially available midi plasmid purification kit (e.g., NucleoBond PC100 from Macherey-Nagel, Düren, Germany).
2. 10X injection buffer: 1 m*M* sodium phosphate buffer, pH 6.8, 50 m*M* KCl. Filter-sterilize and store aliquots at −20°C.
3. Phenol red: dissolve in sterile water at 20 mg/mL, filter-sterilize, and store aliquots at −20°C.

2.3. Injection Needle Preparation

1. Micropipet puller: we obtained good results with the horizontal Flaming/Brown Micropipet Puller P-97 from Sutter Instrument, Novato, CA. We also successfully used the PUL-1 micropipet puller from World Precision Instruments, Berlin, Germany.
2. Microcapillaries: the choice of microcapillaries depends on your micropipet puller. We take borosilicate glass capillaries (100 mm length, 1.2 mm outside diameter, 0.69 mm inside diameter), for example, GB120F-10 from Science Services, Hofheim, Germany.
3. Tip grinder/beveler: We use a tip grinder from Saur, Reutlingen, Germany.
4. Petri dishes (94×16 mm^2) with a bar of plasticine for storage of the injection needles.

2.4. Microinjection

1. Microscope: almost any microscope equipped with ×10 eyepieces, a ×10 objective, and a microscope stage that allows a fine two-dimensional micrometer movement will do, so choice may depend on availability and personal preferences. We use a BH-2 upright microscope from Olympus, Tokyo, Japan.
2. Micromanipulator: a manual micromanipulator, which allows fine adjustment in all three axes (x, y, and z), is sufficient. We use a MM33 micromanipulator from Märzhäuser, Wetzlar, Germany.
3. Ball-joint/pipet holder: ball joints attach pipet holders to micromanipulators and enable that the pipet holder angles can be set freely and easily. We use ball-joint B-8 and pipet holder H-7 from Narishige, Tokyo, Japan.
4. Microinjector: a microinjector is a syringe driver for pneumatic or hydraulic control of injection needles. We use a manual microinjector: a 1-mL glass syringe (Fortuna Optima, Poulten & Graf, Wertheim, Germany) is inserted into a self-made syringe driver. A motor-driven microinjector is not necessary for the generation of transgenic flies in *D. melanogaster*. Microinjectors can also be purchased from Narishige, Tokyo, Japan, from Sutter Instrument, or from World Precision Instruments, Berlin, Germany.
5. A self-made heavy metal plate with rubber bumpers to mount the microscope and the micromanipulator and minimize vibrations.
6. Silicone tube (3 mm outside diameter, 0.5 mm inside diameter) (e.g., Rotilabo Silicone tube from Roth, Karlsruhe, Germany).
7. 3S Voltalef oil (VWR International, Darmstadt, Germany, cat. no. 24626.185).
8. 10S Voltalef oil (VWR International, Darmstadt, Germany, cat. no. 24627.188).
9. In order to coat cover slides immerse them briefly in Repel-Silane, let them air-dry for a few minutes, rinse them with water, and let them air-dry again.
10. Petri dishes (35×10 mm^2) for incubation of the injected embryos.
11. Petri dishes (145×20 mm^2) for preparation of a moist chamber.

3. Methods

3.1. Flies

1. If the construct of interest contains a mini-*white* reporter gene or a fluorescent marker a *white* mutant strain (e.g., w^{1118}) is suited best for this protocol (*see* **Note 2**).

Begin to amplify the *white* mutant strain a couple of weeks before the injections are scheduled.

2. Three days before injections start transfer an appropriate number of *white* mutant flies into egg-laying cages to allow them to get used to the new environment (with our egg-laying cages we take up to 800 flies per cage; we do not especially balance the number of females and males). Cover the cages with apple juice plates and add a drop of yeast paste to the plates to make sure that the flies are well nourished **(Fig. 1A)**. Until the day of injection, incubate flies at 25°C in a quiet place and change the plates at least once a day (*see* **Note 3**).

3.2. Injection Mix Preparation

1. Make midi preparations of your construct of interest and of the "helper–plasmid," which serves as the transposase source by using a commercially available midi plasmid purification kit and quantify DNA by OD_{260} measurement (*see* **Note 4**).
2. Mix approx 4 µg of your plasmid of interest with approx 1 µg of the helper plasmid, add 1 µL of 10X injection buffer, 1 µL of 2% phenol red and scale up to 10 µL with sterile water (*see* **Note 5**).
3. Spin the injection mix for 15 min at maximum speed (~16,000*g*) in a desk centrifuge to precipitate junk. Carefully take 1 µL of the supernatant to fill the injection needle. The rest of the injection mix can be stored at −20°C and should be centrifuged again before each usage.

3.3. Preparation of the Injection Needle

1. Preparation of the injection needle requires glass capillaries and a micropipet puller. In order to produce appropriate needles the settings of the puller have to be adjusted according to the capillaries and the puller used. Several parameters influence the shape of the needle and have to be tested individually. In general, length and tapering of the needle depends on the temperature of the glass and the velocity of the pulling process. Needles that are too long will bend and break and those that are too short will damage the embryos and reduce the survival rate (*see* **Note 6**). Injection needles can be stored in a Petri dish on a bar of plasticine before and after they are sharpened **(Fig. 1B)**.
2. To sharpen the injection needle to an appropriate inner diameter (~5 µm) a tip grinder is used. The injection needle is inserted into the tip grinder under an angle of 30° and sharpened for about 5 min on a wet rotating grinding stone (*see* **Note 7**).

3.4. Filling of the Injection Needle

1. Insert the sharpened injection needle into the pipet holder of the micromanipulator. We use a manual micromanipulator with a ball joint, which attaches the pipet holder to the headstage of the micromanipulator. The ball joint enables the adjustment of the angle height and distance of the pipet holder. A rather simple microscope is sufficient for microinjection. To ensure a vibration-free surface it is recommended to place the micromanipulator and the microscope on a heavy metal plate with rubber bumpers **(Fig. 1C)** (*see* **Note 8**).

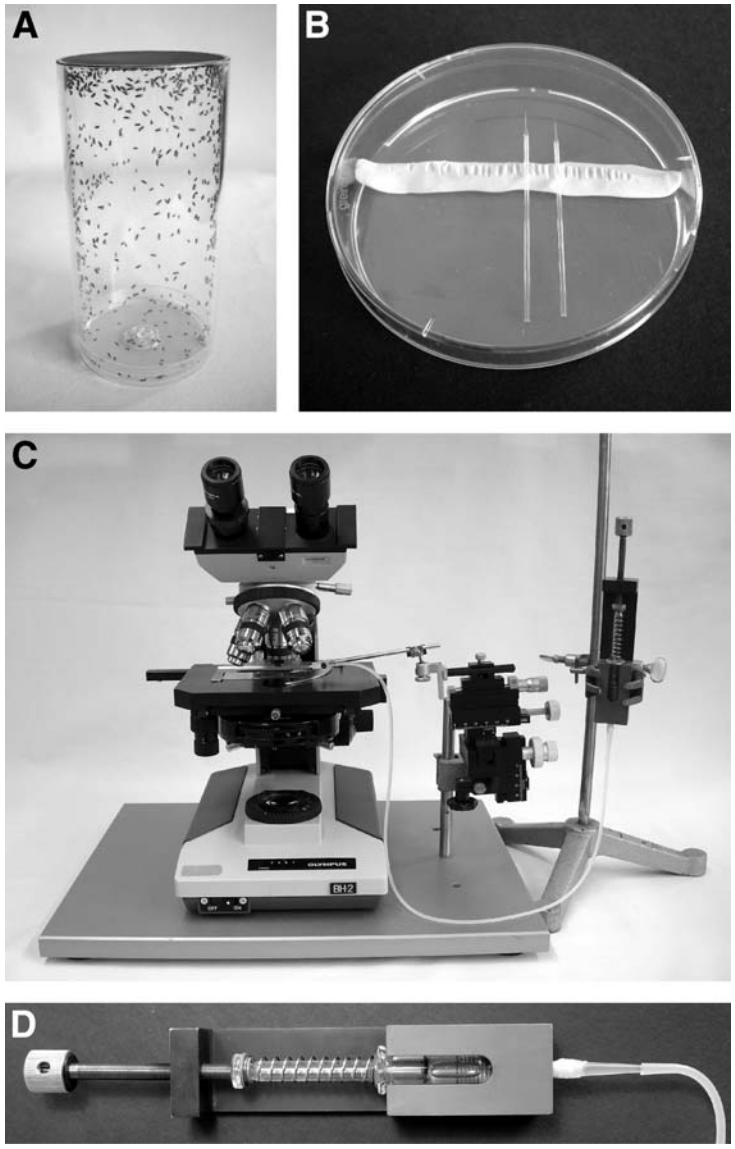

Fig. 1. Tools required for P-element-mediated germline transformation I. **(A)** A self-made 1000 mL acrylic glass egg-laying cage covered on the top side with a fly screen and mounted onto an apple juice agar plate with yeast paste. **(B)** Injection needles stored on a bar of plasticine in a Petri dish to protect them from dust. **(C)** The manual microinjection apparatus used by the authors. Microscope and micromanipulator are mounted on a heavy metal plate with rubber bumpers to reduce vibrations. **(D)** Higher magnification of the self-made manual microinjector. An oil-filled 1-mL glass syringe is inserted into the self-made syringe driver and connected through a silicone tube to an injection capillary (not shown).

2. Fill the 1-mL glass syringe of the microinjector, which is a manual syringe driver for hydraulic control of injection needles **(Fig. 1D)**, and the attached silicone tube completely with 3S Voltalef oil. Connect the injection needle through the silicone tube to the prepared microinjector.
3. Fill the injection needle with 3S Voltalef oil by gently turning the driver of the injector until oil droplets appear on the tip of the needle (*see* **Note 9**).
4. Put 1 µL of the centrifuged injection mixture on a cover slide (20×20 mm^2) that has been coated with Repel-Silane. This treatment will facilitate the uptake of the injection mixture into the injection needle (the injection mixture forms a rounded drop). Fix the cover slide on a microscope slide by placing it in a small drop of 10S Voltalef oil and gently pressing both slides together and place the microscope/cover slide sandwich on the microscope stage.
5. Under optical control bring the oil-filled injection needle and the injection mixture into the field of vision, lower the injection needle into the injection mix, and gently turn the driver of the injector so that the injection mixture is sucked constantly and slowly into the tip of the needle.
6. Once a sufficient amount of injection mixture has been taken up by the needle (uptake of half of the injection mixture is normally sufficient for 1 d of injection) turn the driver of the injector so that an equilibrium is formed between influx and efflux. Place a drop of 3S Voltalef oil next to the injection mixture so that both fluids contact each other and move the filled needle into the 3S Voltalef oil. Adjust the pressure in the injector so that neither injection mixture leaves the needle nor oil enters it (*see* **Note 10**).
7. If flies are kept under a normal day/night cycle they usually start laying eggs from the early afternoon on until late in the evening. Plan your microinjections accordingly, i.e., do the setup of the system described in **Subheadings 3.2–3.4.** preferentially in the morning and schedule microinjections for the afternoon.

3.5. Embryo Collection

1. On the day of injection change the apple juice plates every hour to empty females from old embryos and synchronize the egg laying (*see* **Note 11**). For injections harvest embryos from a 30 min egg-laying period with a fine paintbrush, transfer them into a small steel mesh basket **(Fig. 2A)** and wash them thoroughly with water (*see* **Note 12**).
2. Place the steel mesh basket with the cleaned embryos into a Petri dish with 7% sodium hypochlorite solution for 2 min to chemically remove the chorion (*see* **Note 13**). Wash dechorionated embryos thoroughly with water and place the steel mesh basket in a Petri dish with water. Dechorionated embryos will appear glossy and float on the surface.
3. Pick up the dechorionated embryos with a brush, remove excessive water by briefly touching a paper towel, and transfer them to a rectangular piece of apple juice plate (20×20 mm^2) that has been placed on a microscope slide. This will protect embryos from drying out.
4. Under a stereo dissecting microscope orient the embryos with a blunt straight teasing needle in a straight row (do this near the edge of the apple juice plate and leave

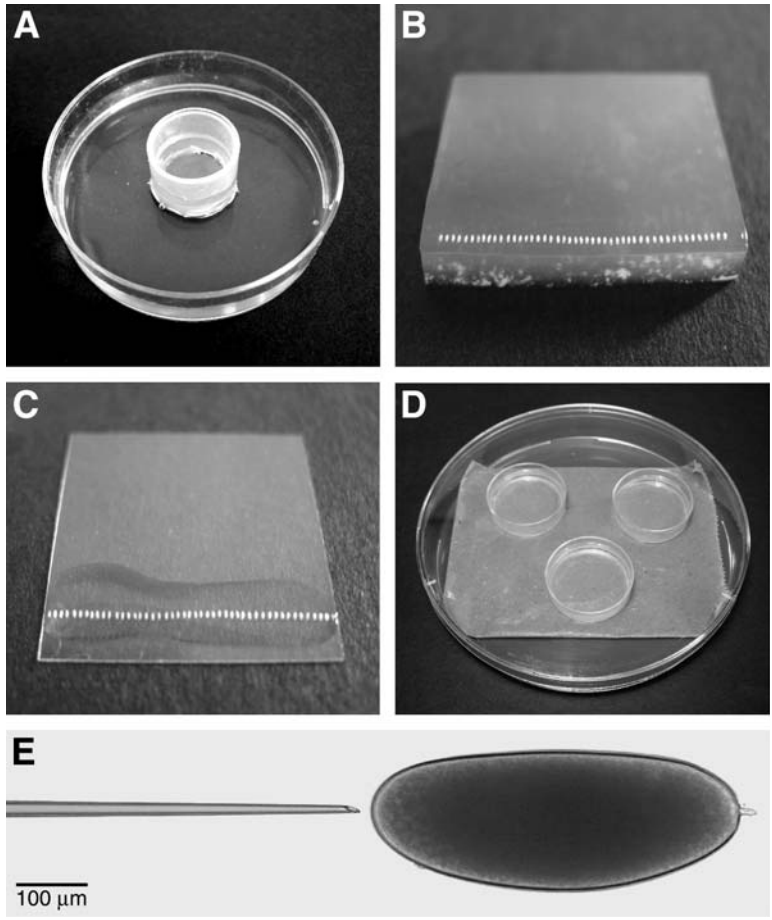

Fig. 2. Tools required for P-element-mediated germline transformation II. **(A)** A self-made steel mesh basket for dechorionating and washing of *Drosophila* embryos placed in a Petri dish (35 × 10 mm^2). **(B)** Alignment of dechorionated and oriented embryos on a rectangular piece of apple juice plate. **(C)** Embryos on a glue-coated cover slide under 10S Voltalef oil after transfer from a rectangular piece of apple juice plate and drying. **(D)** A water-moist chamber for incubation of injected embryos. **(E)** A preblastoderm embryo and a suitable injection needle shortly before injection into the posterior pole. Notice the relative size of the beveled tip of the injection needle in comparison with the embryo.

some space between them to prevent oxygen depletion) so that their anterior poles marked by the small outgrowth of the micropyle all point to the edge of the apple juice plate **(Fig. 2B)** (*see* **Note 14**).

5. By gently pressing a glue-coated cover slide (20 × 20 mm^2) onto the lined-up embryos transfer them to the cover slide. Their posterior ends should point to the nearby edge of the cover slide.

6. Before injecting them embryos have to be dried correctly. The time necessary for drying depends on the surrounding temperature and air humidity and has to be tested individually (*see* **Note 15**). To determine the correct drying grade gently touch an embryo with a blunt straight teasing needle and move the needle carefully over its surface. A furrow should form that has to disappear once the needle has been retracted. If you have not yet reached the correct drying grade you will only observe a small indentation but no furrow at the point of contact. In the case of an overdried embryo the furrow will not disappear but remain after needle retraction. Overlay properly dried embryos immediately with a drop of 10S Voltalef oil. This prevents them from drying out any further **(Fig. 2C)**.

3.6. Microinjection

1. Fix the cover slide with the embryos on a microscope slide by placing it in a small drop of 10S Voltalef oil and gently pressing both slides together.
2. Exchange the microscope/cover slide sandwich with the 3S Voltalef oil, where the injection needle has been stored in so far, for the microscope/cover slide sandwich with the dried embryos submerged in 10S Voltalef oil. Orient it so that the posterior ends of the embryos point into the direction of the injection needle.
3. Bring the first embryo in the row and the injection needle into the field of vision and into the same focal plane **(Fig. 2E)**. The tip of the injection needle should be in the center of the field of vision. During one injection session there should be no further need to move the injection needle beside minor corrections in the *z*-axis. Instead embryos are shifted by gently moving the microscope stage.
4. Touch the tip of the injection needle with the center of the posterior back of the first embryo. A small indentation forms. By retracting the back of the embryo from the needle tip the indentation should vanish proving correct desiccation of the embryo.
5. Penetrate the embryo with the tip of the injection needle and avoid entering it to more than 1/4 of its overall length (*see* **Note 16**). Retract the embryo until the needle is barely inside of it. Only minimal leakage of cytoplasm is tolerable. If the pressure from the microinjector is balanced correctly the injection mixture will leak automatically and slowly out of the injection needle. If this is not the case gently adjust the pressure by turning the driver of the microinjector in small steps. The influx of the injection mixture is visualized by the phenol red dye. A sufficient amount of injection mixture is injected once you faintly see the red dye. Overcome the tendency to inject too much, because this will not help but harm the embryo.
6. Retract the embryo from the injection needle, move over to the next one by moving the microscope stage and repeat the injection procedure.
7. You may encounter embryos that are too old for injection and therefore, are no longer suited for germline transformation. They can be killed by penetrating them completely (*see* **Note 17**).
8. Embryo preparation and microinjection should take no longer than 25 min to be ready before the next 30 min egg-laying period is finished.

Table 1
Transformation Results From Microinjection of a P-Element-Based Transformation Vector

Injected embryos	Hatched larvae	Eclosed flies	Fertile flies	Transgenic flies
1013	514	217	175	23

Exemplary transformation frequency of a P-element transformation vector for enhancer detection, $pCAB_{70}$, with an overall vector-insert size of 22 kb ***(29)***. From 175 fertile G_0 flies 23 independent transgenic lines could be established, which corresponds to a transformation rate of 13.1%. Related to the number of injected embryos this equals an overall transformation frequency of 2.3%. In the described experiment three transgenic lines could be mapped to the 1st, six to the 2nd, and fourteen to the 3rd chromosome. Four of them were derived from double-insertion events that could be separated during the mapping procedure.

3.7. Postinjection Care

1. After injection place the cover slide with the injected embryos in a small Petri dish (35×10 mm^2) and overlay it with 3S Voltalef oil. This on the one hand allows gas exchange as embryos need oxygen for development and on the other hand prevents their desiccation.
2. Put the small Petri dish with the embryos into a water-moisted chamber (e.g., Petri dish, 145×20 mm^2; **Fig. 2D**) and incubate them at 18°C.
3. On the day after tomorrow those larvae that survived the injection procedure will hatch (*see* **Note 18**). They will either crawl on the cover slide, the walls of the Petri dish, or float in the oil. Under optical control take a blunt straight teasing needle, carefully reach under the larvae and lift them with the needle. Larvae tend to adhere to it. Do not use tweezers! You could squeeze the larvae to death! Transfer the larvae to a medium-sized vial with fresh food (~15 mL), which in advance has been prepared as follows: stir the food with a small spatula, add some sterile water to make it more fluid (this will facilitate the larvae to invade the food), and add a small drop of yeast paste. Always check the needle under a stereo dissecting microscope for successfull transfer of the larvae into the vial. Collect approx 50 larvae into one vial and incubate them at 18°C. Larvae derived from the injection of different constructs should of course be transferred to separate vials. After about 14 d flies will eclose. Shortly before hatching, lay the vial on the side to avoid newly eclosed flies from falling into the food (*see* **Note 19**).

3.8. Establishment and Mapping of Transgenic Lines

1. Collect hatched flies (G_0) and cross males and virgins individually with three *w⁻; Gla/CyO* flies of the opposite sex (*see* **Note 20**). Incubate the crossings at 25°C.
2. Screen each single crossing for offsprings with pigmented eyes (F_1) (*see* **Note 21**). The eye color of these transgenic flies can vary from bright yellow to dark red depending on the insertion site of the transgene (for simplicity we will generally refer to transgenic flies as "red-eyed") **(Table 1)**. From one vial you may also encounter

Table 2
A Simple Table to Determine the Chromosomal Insertion of a Transgene

w⁻;Gla/CyO virgins x	F_2:*w⁺; CyO* m	f	F_2:*w⁺, Gla* m	f	F_2:*w⁻; Gla/CyO* m	f	F_2:*w⁺; Gla/CyO* m	f	F_2:*w⁻, CyO* m	f	F_2:*w⁻, Gla* m	f	Insertion on chromosome
F_1 male:	–	+	–	+	+	–	–	+	+	–	+		1st
w⁺; *Gla*	+	+	+	+	+	+	–	–	–	–	–	–	2nd
or *CyO*	+	+	+	+	+	+	+	+	+	+	+	+	3rd

According to the table, write down the occuring phenotypes of the F_2 generation and directly determine the chromosomal insertion site of your transgene. Note that the table is only valid for a distinction between a 1st and a 3rd chromosomal insertion when transgenic F_1 males are used.

F_1-flies with different eye colors. This is indicative of multiple insertions (*see* **Note 22**). In this case choose those with brighter eye colors (putative single insertions) for further crosses. "Red-eyed" *CyO* males should be preferred for further mapping the localization of the transgene, although in general "red-eyed" *Gla* or *CyO* flies of both sexes can be used (*see* **Note 23**). If all transgenic flies from one vial display the same eye color it is likely that they are derived from one single transgene-insertion event. In this case cross two transgenic F_1-flies individually for mapping the chromosomal insertion site. Otherwise take two flies of each individual eye color (*see* **Note 24**).

3. Cross each transgenic F_1-fly individually with three *w⁻; Gla/CyO* flies of the opposite sex and incubate the crossings at 25°C.
4. Collect virgins and males from the offspring of each individual crossing (F_2), determine their genotypes, and identify the chromosomal insertion site of the transgene with the help of **Table 2** (*see* **Note 25**).
5. In case of a second chromosome insertion cross "red-eyed" *CyO* males with "red-eyed" *CyO* virgins. If 25% of their descendants (F_3) have straight wings this means that the transgene insertion is homozygous viable. Establish a stock by crossing straight-winged flies (*see* **Note 26**). Otherwise keep the homozygous lethal stock balanced over *CyO*.

   If the crossing scheme suggests a third chromosomal insertion, cross "red-eyed" *CyO* males with virgins from a third chromosomal balancer stock (e.g., *w⁻; TM3/TM6B*). Interbreed transgenic *TM3* males with "red-eyed" *TM3* virgins. If 25% of their descendants (F_3) lack the dominant *TM3* marker gene *Stubble* (short, thick bristles) the transgene insertion is homozygous viable. Establish a stock by crossing non-*Stubble* flies. If all the offsprings carry the *TM3* balancer chromosome keep the transgene balanced over *TM3*. Depending on your personal preferences establishment of a third chromosomal insertion can of course also be done using the *TM6B* balancer chromosome.

   For a first chromosome insertion mate "red-eyed" *CyO* virgins with *FM7/Y* males. If "red-eyed" males show up in the offsprings (F_3) this demonstrates that the transgene is homozygous viable. Mate them with "red-eyed" *FM7* virgins from the

same crossing and from their descendants interbreed "red-eyed" males with "red-eyed" non-*FM7* virgins to establish a stock. If no "red-eyed" males appear in the F_3 generation "red-eyed" *FM7* virgins have to be crossed against *FM7/Y* males again to balance the homozygous lethal transgene (*see* **Note 27**).

6. It is desirable to obtain at least two independent transgenic lines with P-elements located on the 1st, the 2nd, and the 3rd chromosomes. Insertions on different chromosomes facilitate bringing your transgene into certain genetic backgrounds without the need to recombine it. Additionally, the activity of the same transgene can vary considerably depending on its integration site. If your transgene will be used for misexpression experiments (e.g., UAS-effector lines) ***(24)***, it is always advantageous to have strong- and weak-expressing strains. In many instances there seems to be a correlation between the activity of the transgene and the marker gene, for example, the *white* mini-gene. In this case the intensity of the eye color can be used to roughly estimate the probable expression level of the transgene.

4. Notes

1. Depending on the kind of adhesive tape you are using the amount of heptane may have to be adjusted to obtain a suitable liquid glue. Ingredients of some adhesive tapes are toxic to the embryos. We obtained best results when using Packaging Tape (tesapack) or Extra Power Perfect Tape from tesa, Hamburg, Germany.
2. As an alternative to the w^{1118} stock a *y w* stock can be used for injection. The *y* phenotype provides an extra security to ensure that pigmented flies in the F_1 generation are not contaminants. Besides the w^{1118} or a *y w* stock it is also possible to use flies with a stable source of transposase for P-element-mediated germline transformation (Δ2-3[99B], Bloomington stock 3629). Its potential advantage is that the presence of the transposase is ensured in any germ cell that takes up the plasmid vector ***(17)***. On the other hand, in following crosses one has to get rid of the Δ2-3(99B) chromosome to avoid further transposition events.
3. The size of the egg-laying cages has to be coordinated with the number of flies. Overcrowding stresses the flies and reduces the egg-laying rate. The flies should also not be too old and between 4 and 8 d of age.
4. Different "helper-plasmids" can be used and appear to be equally suited, for example, the "wings-clipped" pπ25.7wc construct ***(4)*** or the pUChsπΔ2-3 plasmid ***(5)***. Check the purity of your construct of interest and of the "helper-plasmid" by measuring the OD_{260}:OD_{280} ratio. Contaminated DNA may harm injected embryos and reduce the survival rate.
5. The transformation efficiency of a construct also depends on its size. Large constructs (>12 kb) show a significantly lower integration rate ***(25)***. This might be partially compensated by scaling up their concentration in the injection mixture. However, too highly concentrated injection mixtures are more viscous and may clog the injection needle.
6. Reproducible production of suitable injection needles is critical for successful performance of germline transformation and requires a high-quality micropipet puller. We obtained best results with the Flaming/Brown-Type Micropipet Puller P-97 (Sutter Instrument) using the following settings: 1. cycle: heat = 460, pull = 0, vel = 30,

del = 50; 2. cycle: heat = 460, pull = 100, vel = 60, del = 5. However, these settings depend on the current heating filament and are only valid when used in conjunction with appropriate glass capillaries (in our case GB120F-10 from Science Services, Hofheim, Germany). These injection needles are sealed at their tip and have to be opened and beveled by grinding.

7. Instead of sharpening the injection needle on a tip grinder it can also be adapted to a suitable inner diameter by breaking it. This can be done under a stereo dissecting microscope with a tweezer or a sharp scalpel. However, in our hand usage of a tip grinder gave more reproducible results and injection needles of constant quality.
8. It is recommended but not absolutely required to do the microinjections in an air-conditioned room at about 18°C. This gives you more time flexibility as embryonic development is slowed down and the time for the injections is prolonged. Furthermore, it has been suggested that the survival rate of embryos injected at 18°C is higher owing to reduced leakage of cytoplasm.
9. Make sure that there are no air bubbles in the injection needle and the connected silicone tube. This often leads to occlusion of the needle. Use the Voltalef 3S and Voltalef 10S oil sparingly, because it is very expensive.
10. Whenever you are not injecting store the injection needle in 3S Voltalef oil to prevent the injection mixture from drying. Long air-exposure will clog the needle and normally requires preparation and filling of a new one.
11. Warm the apple juice plates to room temperature before usage. Flies do not like to lay eggs onto cold plates. Egg laying can further be stimulated by putting a drop of 10% acetic acid on the apple juice plates and distributing it evenly. However, avoid moistening the plates too much because otherwise flies will stick to the surface. To save apple juice plates they can be reused: remove all remaining eggs with water, briefly dry the apple juice plate, and put fresh yeast paste onto it. At 1 d of injection two apple juice plates can be used in rotation for one egg-laying cage.
12. Embryos must be injected before pole cell formation (stage 2), which at 22°C starts approx 80 min after egg laying ***(26)***.
13. Do not incubate the embryos for more than 2 min in sodium hypochlorite because this will harm them. Alternatively, the chorion can also be removed manually by hand peeling: collect and wash the embryos as described, dry them briefly on a paper towel and transfer them to a microscope slide that is partially covered with a piece of double-sided adhesive tape. Under a stereo dissecting microscope carefully move the adherent embryos with a blunt straight teasing needle. The chorion will break open and stick to the adhesive tape whereas the dechorionated embryos will adhere to the needle. Transfer them with the needle to a rectangular piece of apple juice plate and proceed with orienting them.

    It is also possible to inject *Drosophila* embryos without removing the chorion. Although the general procedure of germline transformation is the same, this requires preparation of an injection needle with different properties ***(25)***.
14. Dim the light source of your stereo dissecting microscope. Full light may produce too much heat and quicken the drying of the embryos. Sort out embryos that are too old for injection (beyond stage 2). Suitable embryos appear cloudy. The number of embryos to line up for injection mainly depends on your experience. As a beginner

start with about 20–40. If you are more experienced you may manage to inject up to 100 embryos (arranged in two rows) during one round of injection.

15. Proper drying of the embryos is a critical step. Insufficient dessication results in leakage of cytoplasm from the embryo when it is penetrated by the injection needle. Excessive drying leads to embryonic death, too. Doing injections in a humidity- and temperature-controlled room helps estimating the correct drying point.
16. Entering the embryo too deeply may disturb morphogen gradients and lead to developmental defects and death.
17. Killing over-aged embryo with the injection needle is risky. Embryonic material can attach to the needle and finally occlude it. Alternatively, number lined-up embryos and note down those that are over-aged. Kill them after finishing the whole injection procedure with a blunt straight teasing needle.
18. If you are familiar with germline transformation expect 30–70% of the injected embryos to hatch.
19. The G_0 flies will be white-eyed, because the insertion of the transgene is restricted to the germ cells. Do not expect all hatched larvae to survive until adulthood. The survival rate will be around 50%. Moreover up to 50% of the G_0 adults can be sterile owing to disruption of pole cell formation or damage of other posterior structures.
20. Crossing the G_0 flies directly to a balanced w^--stock will save you one generation during the mapping process. In general any well-reproducing 2nd or 3rd chromosomes balancer stock can be used. We use a *w^-; Gla/CyO* balancer stock, because it can be kept at all standard temperatures (18–25°C) and reproduces quite well. Usage of three *w^-; Gla/CyO* flies for the G_0-crossing turned out to be a good compromise between the effort of virgin collection and successful reproduction of the G_0 flies. Note that the *Cy* phenotype (curled wings) is temperature-sensitive and at 18°C may only barely be visible. After approx 7 d transfer each G_0 cross once to a new vial to increase the number of offspring and minimize the loss of transformants.
21. The transformation efficiency is measured by the ratio between fertile G_0 flies and established transgenic stocks and in our hands ranges between 10 and 25%. Based on the number of injected embryos this corresponds to an estimated transformation efficiency between 1 and 3% **(Table 1)**. Remember that besides your personal experience and technical equipment the size of your transgene considerably influences the transformation rate.
22. Owing to a certain degree of dosis compensation, transgenic males have a slightly darker eye color than the corresponding females (even autosomal insertions). Do not confuse this with a separate insertion of the transgene.
23. Whenever possible avoid using the *Gla* chromosome, as this is not a balancer. It is just marked with the *Gla* mutation (smaller eyes with a glass-like texture) as a dominant, homozygous lethal marker.
24. From every separate transgene-insertion event cross two F_1-flies individually to *w^-; Gla/CyO* balancer flies. This reduces the probability to lose a whole transgenic line if one transformant dies.
25. **Table 2** helps you to map the transgene insertion to a particular chromosome. However, the scheme is only valid without limitation when "red-eyed" males are

used. Otherwise discrimination between an insertion on the 1st or on the 3rd chromosome is not possible.

26. In comparison with the heterozygous situation, homozygosity of the transgene leads to a darker eye color as now two copies of the *white* mini-gene are present. Therefore, homozygotes may also be selected just on the basis of their eye color.
27. In general transgene insertion can occur randomly on any of the four chromosomes with the probability being relative to the length of the corresponding chromosome. However, transgene insertions on the 4th chromosome are very rare as it is very small and mainly heterochromatic. P-elements appear to favor euchromatic regions on a chromosome for integration ***(27)*** and have a tendency to integrate at the 5′-end of genes ***(28)***.

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5

The GAL4 System

A Versatile System for the Expression of Genes

David A. Elliott and Andrea H. Brand

Summary

Over the past decade the adoption and refinement of the GAL4 system by the *Drosophila* field has resulted in a wide array of tools with which the researcher can drive transgene expression in a precise spatiotemporal pattern. The GAL4 system relies on two components: (1) GAL4, a transcriptional activator from yeast, which is expressed in a tissue-specific manner and (2) a transgene under the control of the upstream activation sequence that is bound by GAL4 (UAS_G). The two components are brought together in a simple genetic cross. In the progeny of the cross, the transgene is only transcribed in those cells or tissues expressing the GAL4 protein. Recent modifications of the GAL4 system have improved the control of both the initiation and the spatial restriction of transgene expression. Here we describe the GAL4 system highlighting the properties that make it a powerful tool for the analysis of gene function in *Drosophila* and higher organisms.

Key Words: Conditional gene expression; *Drosophila*; transcriptional activation; transgenesis; UAS; GAL4.

1. Introduction

Ectopic expression has proved an excellent technique for analyzing gene function in *Drosophila* and other model organisms. By altering the gene expression profile of a given cell, one can: (1) induce a cell fate change ***(1)***; (2) induce altered cell fates in neighboring cells ***(2)***; and (3) alter the cell's physiology ***(3)***. Therefore, ectopic expression of a gene can test whether it is sufficient for cell identity and whether its mode of action is autonomous or nonautonomous. Furthermore, ectopic expression is useful in the determination of signaling pathways and the response of a given cell or tissue to these pathways ***(4,5)***. As it has been estimated that up to 60% of *Drosophila* genes will have no loss-of-function phenotype ***(6)***, ectopic gene expression will be important in obtaining functional data on a large

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number of genes. In addition, with the advent of RNA interference (RNAi) technology it is now possible to use ectopic expression to perform gene knockdown experiments to determine if a gene is necessary for a given process ***(7–9)***.

The GAL4 system is a genetic tool allowing the ectopic expression of any given sequence, be it protein coding or a noncoding RNA (e.g., RNAi) ***(10)***. A key strength of the GAL4 system is its remarkable flexibility, with modifications and improvements of the original system giving the *Drosophila* geneticist a large selection of tools with which to drive expression in a restricted spatiotemporal pattern. This flexibility has facilitated the development of many ingenious procedures including labeling mutant cells within chimeric tissue, performing tissue-specific gain-of-function or loss-of-function screens, analyzing the developmental and/or functional role of defined cell populations, inhibition of neuronal function, and genetic rescue experiments as well as functional characterization of a given gene.

The GAL4 system was built on the characterization of transcriptional regulation in yeast. GAL4 is an archetypal eukaryotic transcription factor isolated as an activator of the genes responsible for galactose metabolism in *Saccharomyces cerevisiae* ***(11)***. Analysis of the GAL4 protein revealed that it binds DNA as a dimer through a Zn(2)-Cys(6) zinc finger and has two transactivation domains ***(12,13)***. Furthermore, the activity of GAL4 is repressed by a physical interaction with the GAL80 protein, which is alleviated when galactose is the only carbon source ***(14,15)***. The target sequence of GAL4 was defined as a 17-mer, four copies of which are found in the upstream activation sequence (UAS_G, hereafter UAS) of the galactose metabolism genes, *GAL10* and *GAL1* ***(16–18)***. The high level of conservation in the eukaryotic transcriptional machinery means that GAL4 can activate transcription in other species, as distantly related as humans and plants ***(18–20)***. Crucially, GAL4 is able to regulate transcription from the UAS in *Drosophila* ***(21)*** enabling the GAL4 system to be developed.

2. Key Features of the GAL4 System

The GAL4 system is a bipartite system in which one transgenic line, the driver, expresses GAL4 in a known temporal or spatial pattern and a second transgenic line, the responder, contains a UAS-dependent transgene (**Fig. 1A**) ***(10)***. When using the GAL4 system the most important requirement is an appropriate expression pattern of GAL4. From the outset the GAL4 system was designed to generate a range of driver lines in which a diverse array of regulatory elements controlled GAL4 expression ***(10)***. By adapting the enhancer-detection technique ***(22,23)*** a vector, pGawB ***(10)***, was constructed in which GAL4 is under the control of a weak P-transposase promoter. GAL4 expression depends on the regulatory elements surrounding the integration site of the vector. Thus, the spatial and temporal control of GAL4 expression is based on endogenous

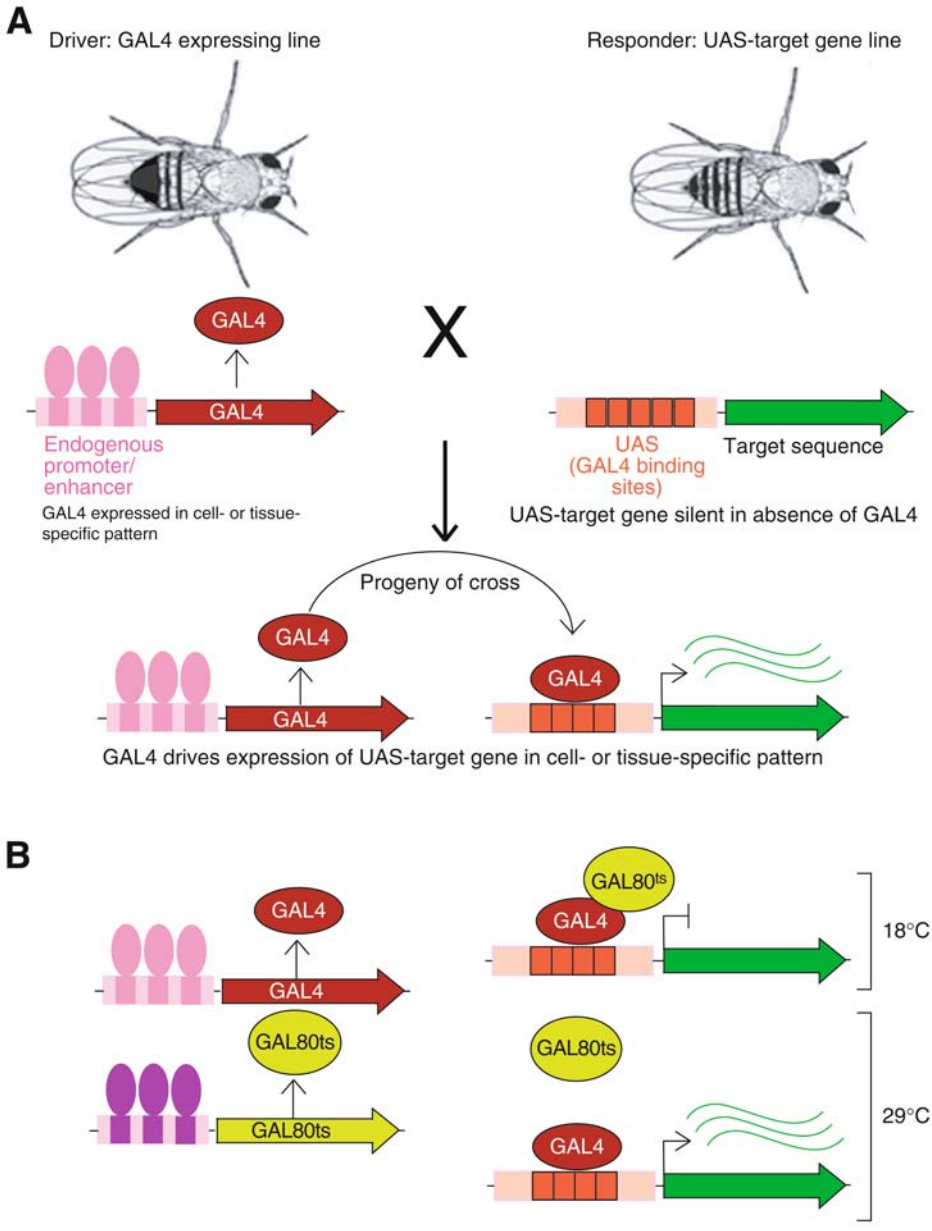

Fig. 1. *(Continued)*

enhancers. A large number of lines can be generated and screened for GAL4-expression pattern to obtain drivers in the tissue of interest. Almost 7000 GAL4 drivers are available and documented online at the GAL4 Enhancer Trap Data Base (*see* **ref. *24***).

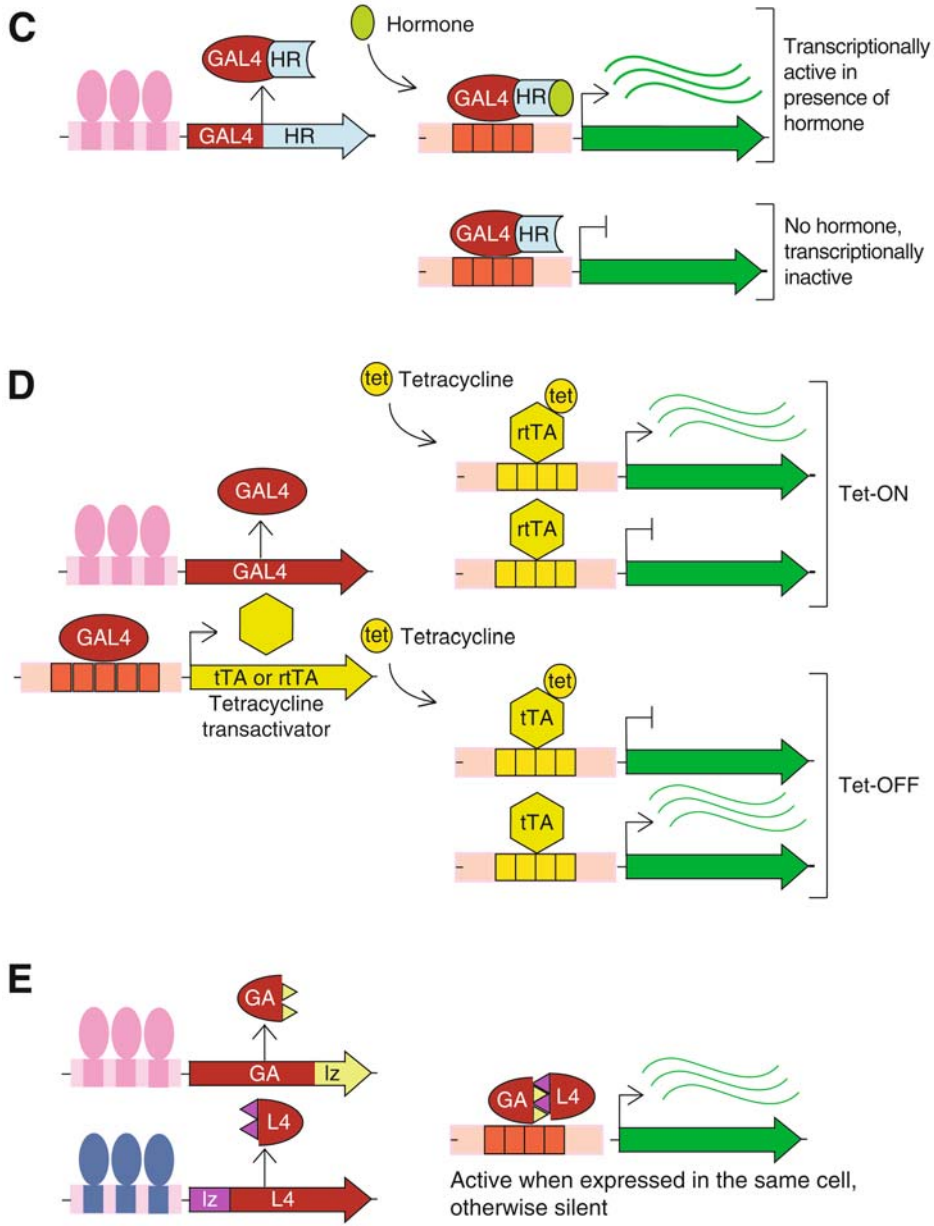

Fig. 1. *(Continued)*

Separation of the GAL4 driver and the UAS responder offers several key advantages. First, a single UAS-dependent transgene can be analyzed in multiple tissues and/or time-points through the use of different drivers. Second, as the UAS construct is effectively silent in the absence of GAL4, transgenics encoding

toxic proteins such as ricin, or apoptotic proteins such as reaper or head involution defective (hid), can be generated ***(25,26)***. Furthermore, rescue experiments can be performed by recapitulating endogenous gene expression with the appropriate drivers in a mutant background (e.g., **ref.** ***27***).

Temporal control, without cell or tissue specificity, of GAL4 activity can be achieved by driving GAL4 expression from a heat-shock promoter ***(28)***. Although the heat-shock procedure does offer good temporal control of the onset of expression a disadvantage is ubiquitous transgene expression. An important advance of the GAL4 system has been the development of several methods offering improved temporal control of the GAL4 system.

3. Temporal Control of the GAL4 System

3.1. Antagonizing GAL4 With the GAL80 Repressor

One approach to regulate temporal expression of a UAS-responder is to make use of the yeast protein GAL80, which binds the transactivation domain of GAL4 and prevents GAL4 from activating transcription in yeast ***(14,15)***. GAL80 can also repress GAL4 in *Drosophila* ***(29–31)*****,** and when expressed ubiquitously under the control of the *tubulin 1*α promoter, represses GAL4 activity in all tissues (**Fig. 1B**) ***(29)***. This feature paved the way for Lee and Luo to develop the mosaic analysis with a repressible cell marker (MARCM) technique to generate marked mutant clones (*see* **refs.** ***29*** and ***30***).

Recently, a series of balancer chromosomes bearing the *tub-GAL80* transgene have been generated ***(32)***. Vef et al. present a strategy in which *tub-GAL80* containing balancer chromosomes can be used in mutagenesis screens to rapidly identify homozygous mutants based on expression of green fluorescent protein (GFP). Briefly, lines containing a chromosome bearing a GAL4 driver and *UAS-GFP*

Fig. 1. *(Opposite page)* Schematic representation of the GAL4-based systems for transgene expression. The original scheme for the GAL4 system is shown in **(A)** GAL4 activity can be antagonized by GAL80 and forms the basis of the TARGET method of transgene regulation **(B)**. Use of a temperature sensitive GAL80 ($GAL80^{ts}$) allows the greater temporal control over the onset of expression. Panel **C** illustrates the hormone induction of transgene expression using a GAL4-hormone receptor (HR) fusion, hormone receptors used include estrogen and progesterone receptor. The Tet-ON and Tet-Off systems have been combined with GAL4 **(D)** affording inducible control of transgene expression by the feeding or withdrawal of tetracycline. The Split-GAL4 system is shown in panel **E**, the GAL4 DNA-binding domain is fused to a heterodimerizing leucine zipper (lz) motif as is the activation domain. The different fusion proteins are expressed in overlapping expression patterns using endogenous promoters/enhancers. When the fusion proteins are expressed in the same cell the leucine zippers direct heterodimerization, resulting in the formation of a functional activator. Either the GAD or a heterologous activation domain (e.g., VP16) can be used. B–E show only the progeny of the cross.

responder (i.e., GFP expressing) are mutagenized, and subsequently, balanced over the *tub-GAL80* balancer. Homozygous mutants are easily distinguished by GFP expression (from the GAL4/*UAS-GFP* on the mutagenized chromosome), which is lacking in heterozygotes because of GAL80 repression of GAL4 activity.

Perhaps the most promising and flexible development of temporal control of the GAL4 system is the development of the temporal and regional gene expression targeting (TARGET) technique ***(33)***. In the TARGET technique a temperature-sensitive version of the GAL80 protein ($GAL80^{ts}$) ***(34)*** is expressed ubiquitously under the control of the *tubulin 1α* promoter. GAL80 repression of GAL4 is alleviated by a simple temperature shift, giving the researcher exact control of the onset of expression. Crucially, the TARGET system is fully compatible with the vast array of GAL4 lines already established. The strength of the TARGET system is illustrated by the dissection of *rutabaga*, a type I adenylyl cyclase, function in short-term memory in *Drosophila* ***(33)***. Using TARGET to activate UAS-*rutabaga* expression in the mushroom bodies of adult flies rescued the memory defect of *rutabaga* mutants. Thus, the olfactory memory phenotype of *rutabaga* mutants is not owing to abnormal mushroom body development but abnormal neural physiology in adult neurons lacking the *rutabaga*-encoded type I adenylyl cyclase.

3.2. Inducible GAL4

Another approach to regulate temporal expression are hormone inducible variants of GAL4 ***(35–37)***. Two GAL4-hormone receptor chimeras are available: GAL4-estrogen receptor ***(35)*** and a second called GeneSwitch, which is a fusion of GAL4-progesterone receptor, and the activation domain of p65 ***(36,37)***. Transcriptional activation in both systems requires the addition of the appropriate ligand as both are transcriptionally silent until bound by the ligand (**Fig. 1C**). The onset of expression is controlled by feeding the fly or larva the ligand at an appropriate time, thereby circumventing possible detrimental effects of early transgene expression. Expression levels in the GeneSwitch system are ligand dose-dependant ***(36–38)***. Furthermore, the system is reversible by removal of the fly or larva from feed containing the ligand. However, reversal is restricted by the slow off-rate kinetics of GeneSwitch ***(36)*** and the perdurance of the transgene product.

The major limitation of this approach is that drug treatment requires feeding, so the embryonic and pupal stages of the lifecycle are not amenable to study. In addition, new driver lines must be generated expressing either GeneSwitch or GAL4-estrogen receptor. Nevertheless, elegant experiments can be performed using these drug inducible GAL4 variants. For example, in a recent demonstration, signaling from the heterotrimeric G(o) protein is required for associative learning ***(39)***; GeneSwitch, expressed from a mushroom body-specific promoter,

was used to drive expression of an inhibitor of G(o) signaling, *pertussis toxin* (PTX) (UAS-PTX), in the mushroom body. PTX expression was induced by feeding adult flies the GeneSwitch ligand, RU486, ensuring that the phenotypes were not the result of developmental defects. In this way, inhibition of G(o) signaling in the mushroom body was shown to impair olfactory associative learning and memory. In another study ***(40)***, GeneSwitch was used to express dFOXO, the downstream transcription factor of the insulin/insulin-like growth factor signaling pathway, in the adult fat body. Adult fat body-specific activation of the transcriptional response to insulin signaling resulted in an increased life span.

3.3. FLP-Out GAL4

One approach to refining temporal regulation of the GAL4 system is to combine it with the FLP-out technique ***(41–44)***. For example, a terminator cassette flanked by FLP recognition target (FRT) sites can be placed between the UAS promoter and the gene to be expressed, rendering the transgene silent ***(42)***. To activate the transgene requires the expression of the FLP recombinase to remove the terminator cassette. Use of a heat shock inducible *FLP recombinase (hs-FLP)* affords temporal control to the onset of transgene expression. A similar result can be achieved by placing a FRT-flanked terminator cassette in front of the GAL4-coding sequences ***(41,43)***. In this case, *GAL4* expression is reliant on the removal of the terminator cassette by FLP recombinase, which can be conveniently supplied using *hs-FLP* to control the timing of expression. In addition to the commonly used *hs-FLP* it is possible to use other characterized promoters to drive FLP expression in a spatiotemporal fashion (e.g., the eye-specific driver *ey-FLP* ***[45]***).

Recent work combined the FLP-out technique ***(44)*** with GeneSwitch to investigate the role of the decapentaplegic (DPP) morphogen gradient in controlling growth in the wing imaginal disc ***(46)***. A FRT-flanked terminator cassette was placed between the actin promoter and the GeneSwitch-coding sequence. The cassette was removed by induction of *hs-FLP* during larval stages, resulting in a mosaic animal in which GeneSwitch expression was clonally restricted. The GeneSwitch ligand RU486 was fed to transgenic larvae promoting the activation of the UAS transgene. This methodology was used to manipulate levels of DPP signaling within the clones through the expression of a constitutively active variant of thick veins (the DPP receptor). In imaginal wing discs cellular proliferation was stimulated both within the clones (i.e., autonomous) and in cells adjacent to the clone boundary (i.e., nonautonomous) indicating that cells sense differences in DPP-signaling activity. Therefore, the uniform growth of the entire wing disc in response to DPP signaling, and not just of those cells close to the DPP source, can be explained by the noncell autonomous stimulation of cell proliferation resulting from cells reacting to different levels of DPP signaling in adjacent cells ***(46)***.

3.4. Tetracycline-Transactivator System

Transcriptional activity of the tetracycline-transactivator (tTA), a fusion between the *Escherichia coli* tetracycline repressor (tetR or the reverse tetR for Tet-On) and the strong transcriptional activation domain of the herpes simplex virus VP16, is regulated by tetracycline or its derivatives (e.g., doxycycline). The convenience of inducible expression has led to the widespread use of tTAs in transgenic mouse models and mammalian tissue culture ***(47,48)***. Two versions of the tetracycline systems exist: Tet-On, in which the addition of the drug results in an active reverse-tetR (rtTA) causing transgene activation from the tet operator and Tet-Off, in which addition of the drug inactivates tTA, and in turn, expression from the tet operator is switched off. The introduction of the tetracycline inducible systems, in particular the Tet-On method, to *Drosophila* were initially hindered by a few technical problems, such as the level of transgene induction and adverse effects of high doses of tetracycline ***(49–52)***. A modified version of rtTA, rtTAs-M2-altTA, has largely rectified these problems ***(53,54)***. However, this system is not directly compatible with existing UAS transgenes, such as the existing collection of enhancer promoter (EP) lines (*see* **Subheading 5.**).

To take advantage of the number of established tissue-specific GAL4 lines, both the Tet-On and Tet-Off expression systems have been linked to the GAL4-UAS system (*see* **Fig. 1D**) ***(52,53)***. To do this the tetracycline transactivators were placed under the control of the UAS. Therefore, expression of rTA and rtTAs-M2-altTA can be regulated by crossing the *UAS-rTA* (or *UAS-rtTAs-M2-altTA*) transgenics to a given GAL4 driver ***(52,53)***. This protocol has been used to investigate the role of the calcium/calmodulin protein kinase II (CaMK II) in regulating mating behavior ***(55)***. Expression of a constitutively active CaMKII (T287D CaMKII) in adult neurons was achieved by combining a GAL4 driver, in which GAL4 is expressed in the mushroom body and antennal lobes, regulating *UAS-rTA*, and inhibition of rTA by growth on doxycycline until adulthood. Indeed, this experiment relies on silencing by doxycycline, as expression of T287D CaMKII during development is lethal ***(55)***. Removal of doxycycline permits T287D CaMKII expression. In behavioral assays female flies expressing T287D CaMKII displayed enhanced courtship plasticity during training implying a role for CaMKII in behavioral plasticity.

4. GAL4-Based Methodologies for Improved Spatial Restriction of Gene Expression

4.1. Dual Binary System

A second binary system, which functions in an analogous manner to the GAL4 system, is based on the bacterial lexA protein ***(56,57)***. In a recent report, Lee and Lai have combined the GAL4 and lexA systems to facilitate

more precise cell labeling by MARCM ***(58)***. The lexA DNA-binding domain is fused either to the GAL4 activation domain (lexA::GAD) or the VP16 activation domain (lexA::VP16) resulting in GAL80-sensitive and -insensitive proteins, respectively. All cells can be marked with lexA::VP16 whereas the GAL80 sensitive lexA::GAD can be kept silent until the generation of mutant clones using the MARCM protocol. Using this approach to study the cell lineage in the optic lobe, Lai and Lee ***(58)*** identified a novel neuroglioblast cell type. As use of the lexA system becomes more widespread, modifications of the system are sure to follow. Many of these modifications are likely to be derived from those already in use with the GAL4 system.

4.2. Split GAL4

A new method, "Split GAL4," takes advantage of the modular nature of the GAL4 transcription factor ***(59)***. In this technique the separate DNA binding domain (DBD) and activation domain (AD) of GAL4 are fused to a heterodimerizing leucine zipper motif and each fusion protein is expressed separately. Only when they are present in the same cell can the leucine zippers direct heterodimerization, resulting in the formation of a functional activator (**Fig. 1E**). The fusion proteins, ZIP::GAL4DBD and GAL4AD::ZIP (or a second activation domain VP16AD::ZIP), are transcriptionally inactive when expressed panneuronally using the *elaV* promoter. The inactive constructs are referred to as "hemi-drivers." Transactivation requires the reconstitution of GAL4 by the presence of both ZIP::GAL4DBD and GAL4AD::ZIP, which will only occur in the domains of overlapping expression of the hemi-drivers. Coexpression, through the *elaV* promoter, of the DBD and AD fusion proteins results in nervous system-wide expression of a *UAS-GFP* reporter.

The authors demonstrate that the Split GAL4 system can target gene expression to a restricted cell population at the intersection of the expression patterns of the hemi-drivers ***(59)***. For example, to identify cholinergic neurons within the *Crustacean Cardioactive Peptide* (*CCAP*) neuronal population the GAL4AD::ZIP was expressed in CCAP neurons whereas the *choline acetyltransferase* promoter drove ZIP::GAL4DBD in all cholinergic neurons. The split GAL4 system was able to label a subset of cholinergic neurons within the larger CCAP neuronal population.

5. Genome Wide Screening With GAL4

The GAL4 system has also been used in large-scale gain-of-function genetic screens. A modular misexpression screen has been designed combining P-element mutagenesis and the GAL4 system ***(60)***. The P-elements used contain a GAL4-regulated promoter, consisting of GAL4 DNA-binding sites upstream of a basal promoter, arranged to drive transcription of flanking

genomic sequences after integration ***(60–62)***. Transgenic lines generated by integration of these P-elements are referred to as EP lines and, when GAL4 is present, these P-elements will direct expression of the gene, which lies next to the integration site. Many thousands of EP lines are now available to the research community, from both public and private consortia ***(61,63,64)***. By crossing a driver, which is expressed in the tissue of interest to these libraries one can screen for gain-of-function phenotypes. Such screens have been used to identify genes involved in many processes, including eye morphology ***(65)***, vein formation in the wing ***(66)***, muscle pattern formation ***(64)***, blood cell activation ***(67)***, and axon guidance and synaptogenesis ***(68)***. Furthermore, screens can be performed for modifiers of signaling pathways. For example, Leptin and colleagues screened for modifiers of the fibroblast growth factor (FGF)-signaling cascade by looking for rescue or enhancement of the rough eye phenotype resulting from coexpression of the FGF receptor Breathless and downstream-of-FGF receptor, identifying the small GTPase sar-1 and the cell surface receptor robo-2 as components of the FGF pathway ***(5)***.

Mutations that cause early lethality hinder analysis of the mutated gene's function during later development and adult life. Duffy and colleagues ***(69)*** have developed a GAL4-based technique, termed directed mosaics, which permits the study of recessive lethal mutations by generating mosaic flies in which only select cells or tissues are homozygous for the mutation whereas the rest of the animal is wild-type. This method uses recombination between FRT sites that have been engineering into all five major chromosomal arms in *Drosophila.* FLP is supplied in a tissue-specific manner using characterized GAL4 drivers and *UAS-FLP.* FLP catalyzes mitotic recombination between chromosome arms carrying a particular mutation, resulting in wild-type and homozygous mutant daughter cells. In this manner approx 95% of the genome can be tested in mosaics ***(69)***. With certain modifications, the directed mosaics method can be used to generate an entire tissue made solely of mutant cells ***(70)***. An FRT-marked chromosome is engineered to carry a tissue-specific transgene expressing a dominant cell death gene (e.g., *hid* or *reaper*) ensuring that all daughter cells in the tissue of interest inheriting this chromosome will undergo apoptosis. As a result, the tissue of interest will be entirely made up of cells in which recombination between FRT sites has resulted in homozygous inheritance of the lethal mutation of interest ***(70)*** but not hid or reaper. This has been achieved in the eye using the *ey-GAL4* driver, *UAS-FLP*(EGUF), and a glass multimer reporter (GMR)-hid transgene as a tissue-specific apoptotic factor, and is referred to as the EGUF/hid method ***(70)***. Mutant screens, or examination of existing mutants, can now be performed scoring any aspect of eye biology (e.g., morphology, neurophysiology, and light response) in an otherwise wild-type animal ***(70–72)***.

6. Repressing Genes Using GAL4

Although most studies use GAL4 solely as an activator GAL4 can also be used to switch off a gene of interest. GAL4 can be turned into a repressor by inserting the GAL4-DBD domain into the suppressor of hairy wing isolator domain, which has enhancer-blocking activity. This activity is presumably mediated by, as yet undefined, protein interactions ***(73)***. One can use this chimeric protein in loss-of-function studies in an analogous manner to the gain-of-function studies using the EP lines. In this case the chimera binds to the UAS element that is integrated in the genome, and the suppressor of hairy wing isolator causes a localized inactivation of transcription. The technique offers the promise of performing conditional loss of function screens that switch off gene expression only in the tissue or process of interest.

7. GAL4 Methods are Universally Applicable to Higher Eukaryotes

Early reports demonstrated that GAL4 can activate transcription from the UAS in a range of species including human tissue culture cells and plants ***(18,20)***. The success of the GAL4 system in *Drosophila* has encouraged researchers working on other model organisms, including *Arabidopsis* ***(74,75)***, zebrafish ***(76)***, *Xenopus* ***(77)***, and mouse ***(78,79)***, to adopt the GAL4 system. In mice the GAL4 system was used to analyze both the sonic hedgehog (shh) and BMP-signaling pathways in neural tube development ***(78,79)***. GAL4 was expressed in the roof plate of the neural tube by placing the GAL4 sequence under the control of *Wnt-1* regulatory sequences ***(79)***. Using the Wnt1-GAL4 line to drive expression of *shh* revealed that prolonged exposure to shh results in an increased proliferative rate of neural precursors. Furthermore, at later stages of development differentiation was blocked, demonstrating proliferation and differentiation are separately regulated by shh signaling in the developing spinal cord. Given the success of the GAL4 system, in its various guises in *Drosophila*, it is likely that GAL4-based technologies will continue to be adapted to work in other species.

8. What Does the Future Hold for GAL4?

The GAL4 system is now well-established in the armory of the *Drosophila* geneticist. Modification of the original GAL4 system has led to a more precise level of control of transgene expression in both space and time. To increase the resolution of the GAL4 system it is likely that researchers will combine the GAL4-based reagents listed to manipulate transgene expression in the tissue or cells of interest. For example, the Split GAL4 system can be combined with $GAL80^{ts}$ and MARCM to drive expression in a subset of cells within a given organ. One can ablate given cells within a tissue by driving expression of the proapoptotic protein reaper in clones within an already restricted domain and

examine the biological outcome. In the context of neuronal networks, a similar experiment using tetanus toxin to block activity in selected neurons may reveal precisely which neurons govern a particular behavior.

Other systems of promise are the hormone inducible systems, which use a transactivation domain requiring the presence of a hormone to be active. Obviously, the time delay in switching the transgene off might be a complicating factor for some experiments as a transcriptionally active protein remains after the withdrawal of the drug. This difficulty may be overcome by the introduction of a ligand-sensitive destabilizing domain, which in the absence of ligand results in rapid protein degradation ***(80)***. This approach has been shown to function in a range of mammalian cell lines ***(80)***. However, although such a feature would inactivate GAL4 time would still be required for the degradation of the existing transgenic products. An alternative approach based on fusion to a temperature sensitive intein domain has been demonstrated to function in GAL80 in *Drosophila* and GAL4 in yeast ***(81)***. At permissive temperatures the intein domain is spliced out and the resultant protein is active, whereas at nonpermissive temperatures the intein domain remains resulting in a nonfunctional protein. Incorporation of an intein domain into GAL4 would allow GAL4 activity to be regulated merely by moving the flies to different temperatures, which offers considerable advantages over the presence of a second protein or reliance on the administration of ligands to regulate expression. In particular, the embryonic and pupal life stages are amenable to temperature shift experiments but less easily to drug treatments.

The success of the GAL4 system in addressing basic biological questions has led to the adoption of this technique in more applied situations. In particular, the development of GAL4-based transgenesis in pest insects will help in the identification of new insecticide compounds ***(82)***. More importantly, for diseases where insects are the vectors it is possible that the GAL4 system or one of its variants may provide a mechanism to prevent the transmission of disease or control the population of the insect vector. Finally, *Drosophila* is now used as a screening organism by the pharmaceutical industry ***(83–85)***. Given the flexibility and robust nature of the GAL4 system it is likely that the GAL4 system will be a key methodology for future *Drosophila*-based drug discovery programs.

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6

P-Element Mutagenesis

Thomas Hummel and Christian Klämbt

Summary

Mobile elements were first used as a mutagenesis tool that introduces a molecular tag in the genes of interest. This facilitated subsequent molecular cloning and eventually promoted molecular analysis of a large number of fly genes. Soon after, P-elements were modified to detect genes not only based on a mutant phenotype but rather through revealing RNA or protein expression patterns (enhancer trap, gene trap). Owing to the typically imprecise mobilization of the P-elements these enhancer trap or gene trap insertions also provided means to generate (excision) mutants. Whereas the excision mutants are valuable deletions they are induced in a random fashion and the exact breakpoints have to be determined following molecular analysis. More recently, the introduction of recombination targets (flipase recombination targets) into P-elements has provided the ability to generate precise chromosomal deletions between preselected sites. Here we will summarize the current genetic approaches to generate different type of insertional and deletion mutations using specifically designed P-elements.

Key Words: *Drosophila*; genetic screens; mutation; recombination; P-element.

1. Introduction

Chromosomal DNA is far from being a static organization of DNA, but during evolution a number of mobile elements emerged—also called transposons—which provide the basis for many types of chromosomal rearrangements. First noted in maize by B. McClintock, transposable elements are found in almost all species.

One very common class of (autonomous) transposons is characterized by inverted repeat sequences at their ends. These transposons encode an enzyme, the transposase, which specifically catalyzes the mobility of the host transposon. Transposition occurs through an excision and reintegration or through a replicative mechanism. *Drosophila* harbors a prominent member of this family of transposable elements, the P-element. Owing to pioneering work from Spradling and Rubin the P-element has gained fundamental importance for *Drosophila* genetics as a mutagen as well as a tool to generate transgenic animals.

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1.1. The P-Element

In the 1960s, population geneticists crossed different wild-type strains to study the variation of fitness of single chromosomes. Surprisingly, the offspring of these crosses (*wild-type* × *wild-type*) often showed increased mutagenesis rates—a phenomenon introduced as hybrid dysgenesis in 1977. Wild-type fly strains could be grouped as either being a P- or and M-cytotype. When male P-cytotype males are crossed to M-cytotype females the F_1 offspring were often sterile. In the few fertile males recombination was observed—which is never found in wild-type male flies—and the F_2 offspring were characterized by a high mutagenesis rate. This hybrid dysgenesis effect is confined to the germline. When P-cytotype females are crossed to M-males only very mild dysgenesis effects are found and hybrid dysgenesis is never found in pure P- or M-cytotype crosses.

Today we can explain these initially quite puzzling results. P-cytotype flies carry a transposable element called P-element. The normal P-element is 2907 bp in length and carries a 31 bp inverted repeat structure at its ends **(Fig. 1)**. The P-element harbors a single gene that is spread over 4 exons (0, 1, 2, 3) and encodes a transposase specific for the P-element ***(1)***. The P-element promoter activates constitutive transcription and generation of a functional transposase is regulated through differential splicing. The intron between exon 2 and 3 is only removed in the germline and the resulting mRNA then directs expression of the functional transposase of 87 kDa in size. In the soma, splicing of the last intron does not occur and the resulting mRNA directs expression of a 66 kDa large transposase fragment. Thus, in vivo transposition activity is regulated through differential splicing and occurs only in the germline. The last intron has been removed in the so-called Δ2-3 constructs, which are then constitutively generating an active transposase.

The transposase activity has been studied extensively and was shown to act as an endonuclease with a 17 nucleotide 3′ overhang that recognizes the terminal sequences to conduct a GTP dependent cut and paste reaction ***(2,3)***. Efficient transposition requires only about 150 bp of the P-element termini including the 31 bp inverted repeat, the transposase binding sites, and another 11 bp inverted repeat of regulatory sequences. The 31 bp inverted repeat terminal sequences are bound by *Drosophila* proteins such as inverted repeat binding protein (IRBP), the DNA binding subunit of the DNA-dependent protein kinase that may have helicase function. Both P-element ends are functionally distinct and both are required for transposition. The insertion of a P-element results in an 8 bp duplication of the target sequence. The excision of a P-element leads to the generation of two complementing 17 nucleotide 3′ extension sequences. Following repair, the excision of the P-element most often leaves a 32 bp sequence (and the 8 bp

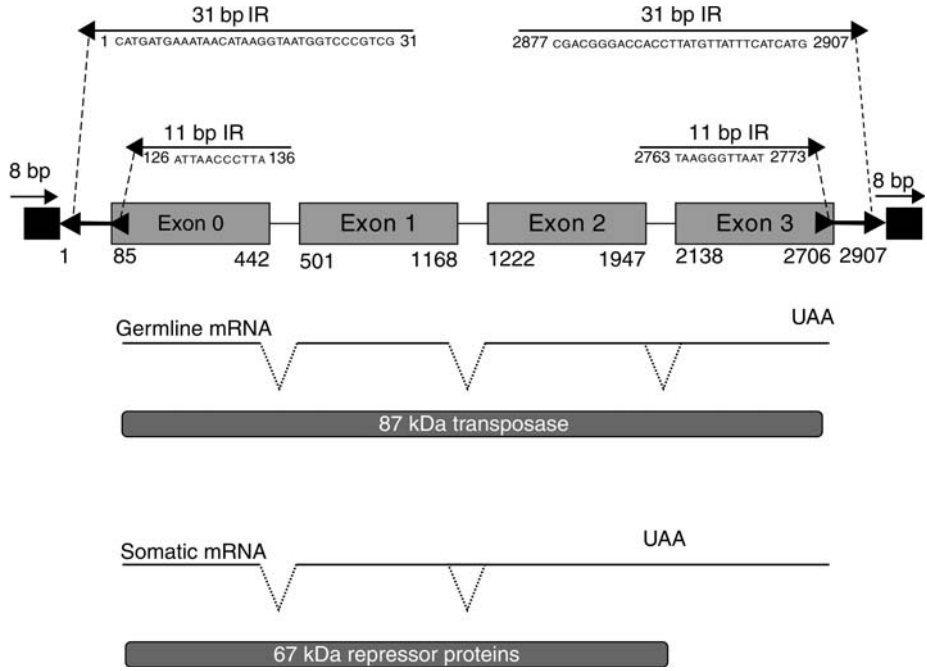

Fig. 1. Structure of the 2.9 kb P-element. Insertion of a P-element results in the generation of a 8 bp large duplication of genomic DNA. The position of the different P-element exons is indicated. Splicing of intron number 3 between exons 2 and 3 occurs only in the germline. The resulting protein products are indicated. For further details *see* text.

target site duplication from the initial integration event ***(4,5)***. To generate a precise P-element excision it is thus required to remove both 17 nucleotide extensions and one of the 8 bp duplications before repair of the DNA strand brake.

The insertion of the P-element does not require a specific target sequence but is clearly biased toward the 5′ regions of genes. The insertion position is mostly found within a 100 bp distance of the transcriptional start site within a region with a 14 bp long palindromic pattern of hydrogen bonds ***(6)***. The integration preferences appear to be owing to the transposon type as, for example, piggyBac or Minos transposons do not show a specificity for promoter regions ***(7,8)***.

P-element based mutagenesis strategies offer a number of experimental advantages. P-elements show a relative high frequency of mobilization, which can be controlled by expression of the active transposase. Owing to efforts of the Gene Disruption consortium (laboratories of Bellen, Hoskins, and Spradling) P-element or piggyBac insertions in more than 50% of all *Drosophila* genes have been isolated ***(9)***. Even if the P-element insertion does

not result in a disruption of gene function, loss-of-function alleles can be easily generated by imprecise excision of the P-element integration. In the following, we first describe the generation and isolation of new P-element insertions. Subsequently, we discuss the generation of chromosomal deletions starting from previously defined P-element insertions.

2. Materials

P-element insertion into genes of interest can be obtained through several public stock centers:

- Bloomington Stock Center http://flystocks.bio.indiana.edu/
- Stock Center Kyoto http://www.dgrc.kit.ac.jp/en/about/history/index.html
- Stock center Szeged http://expbio.bio.u-szeged.hu/fly/
- P-screen database Huston http://flypush.imgen.bcm.tmc.edu/pscreen/
- DrosDel collection http://www.drosdel.org.uk/
- Exelixis stock collection http://drosophila.med.harvard.edu
- GenExel http://genexel.com/eng/htm/genisys.htm (commercial supplier of P-element insertion lines)

3. Methods

3.1. P-Element Insertional Mutagenesis

In most cases a transposon insertion into a gene of interest has already been described and can be obtained from a public stock collection center (*see* **2. Materials**) or individual laboratories (http://flybase.bio.indiana.edu/people/). If no insertion line is available or the available insertion is not of the desired transposon type one can follow a number of experimental strategies to generate new transposon insertions or exchange existing ones (*see* **Note 1**). Next we present some information on:

1. The most common transposable elements used in the field.
2. P-element mobilization and random new integration on different chromosomes (random insertion).
3. P-element mobilization and new integration on the same chromosome (local hopping).
4. P-element mobilization and distinct new integration on different chromosome (replacement).

3.1.1. Different Transposable Elements Used

Several different transposons types can be used in a mutagenesis experiment. Beside the choice of the transposon backbone (P-element, piggyBac, Minos, or Hobo) it is important to consider the dominant selectable marker. The most commonly used P-element of piggyBac-based constructs are shown in **Fig. 2**. In most cases the *white* gene is used as a dominant marker, which makes identification of

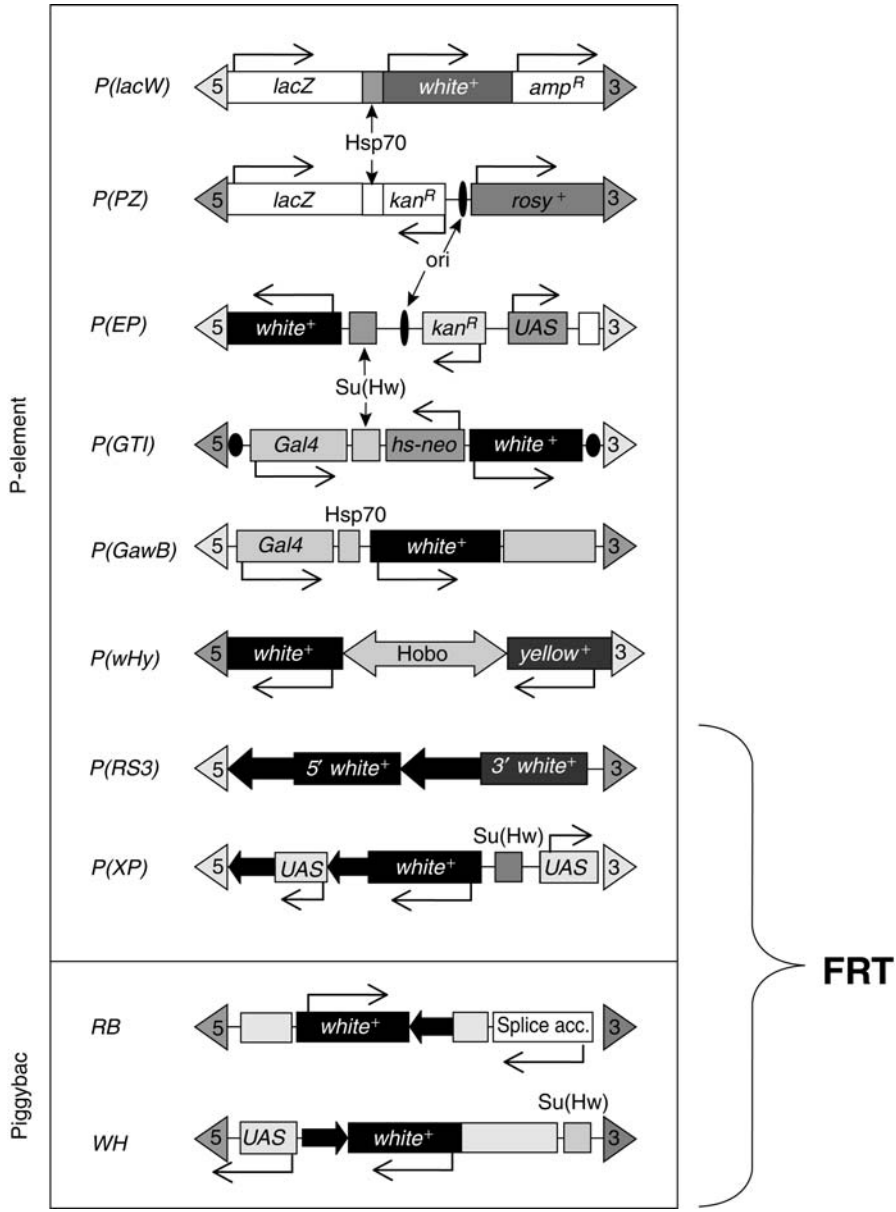

Fig. 2. List of different transposon types. The table summarizes the most frequently used transposons. Individual sequence motives are explained in the Figure text. Data according to **refs. *9*,*11*,*12***.

characterization of insertion events easy. The expression of the *white* gene results in a cell autonomous induction of a red eye phenotype that is highly dosage sensitive, and thereby facilitates separating different insertion events. It should be noted that the mutagenic efficiency of the different P-element based constructs also differs ***(9)***.

3.1.2. Isolation of New P-Element Insertions

Disruption of gene functions through insertional mutagenesis has been tremendously useful in analyzing *Drosophila* gene functions, and transposon-induced alleles provide several advantages: (1) sequences adjacent to P-elements can be easily cloned using inverse polymerase chain reaction (IPCR) and thus, the position of the element can be determined within few days. (2) P-elements can be excised in the presence of a stable transposase ***(10)***. Precise excision that can be correlated to reversion of the phenotype uniquely confirms the given insertional mutation as the direct cause of the phenotype. (3) Finally, imprecise excision allows the generation of new alleles that can become valuable in phenotypic analysis (*see* **Subheading 3.2**, P-induced Deficiences, following).

Figure 3 presents a crossing scheme for the generation of new autosomal P-element transposon insertions. In the lab, the exact genetic background of course depends on the nature of the genetic selection marker (e.g., *white*; *yellow*, *rosy*, green fluorescent protein [*GFP*]). To induce insertions on the heterosomes a jumpstarter strain is used that carries multiple insertions on a balancer chromosome. Following the crossing scheme with P(*white*) elements **(Fig. 3)** about 1/150 flies carries a new insertion. The use of P(*rosy*) results in somewhat higher jumping frequencies, with 1/100 flies carrying a new insertion. This is most likely owing to the higher sensitivity of the nonautonomous *rosy* marker.

Several transposase sources are available from the Bloomington stock center: no. 3629 w; wg^{Sp-1}/CyO; ry^{506} Sb^1 P(ry Δ2-3)/TM6B insertion at 99B ***(10)***, no. 4368 y^1 w^1; Ki^1 p^p P[ry Δ2-3] 99B ***(10)***, and no. 8200 y^1 w^{1118}; Pbac[w Δ2-3]; +/+ ***(11,12)***.

3.1.3. Local Hopping

As the genome projects approach saturation it will not be necessary to establish a standard transposon mutagenesis. Instead, if no insertion into your favorite gene is available, you can generate your own P-element insertion by locally mobilizing a nearby P-element insertion utilizing a phenomenon called "local hopping" ***(13,14)***.

A P-element insertion line is crossed to a stable source of transposase (*see* above), the offspring are then crossed to appropriate balancer flies. Mobilization of the P-element occurs in the germline of both males and females but is reported to be somewhat more effective in females ***(14)***.

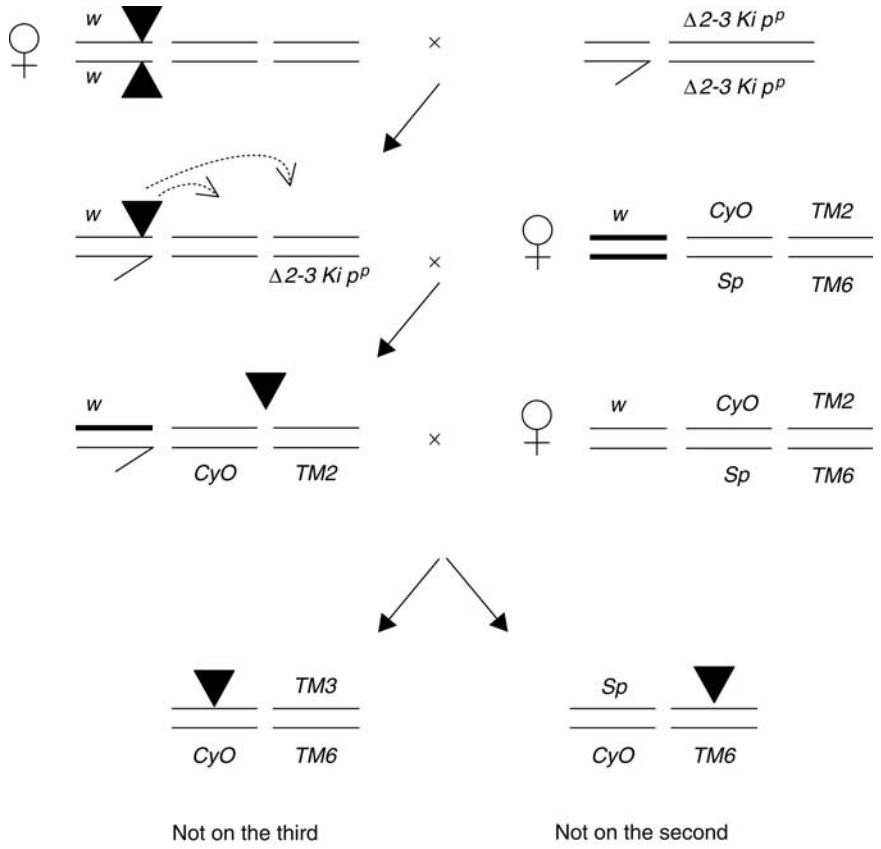

Fig. 3. Crossing scheme for the isolation of new autosomal P-element insertions. The determination of the chromosomes carrying the new P-element insertion occurs through exclusion of marker combinations. In the F_1 generation, the X-chromosomes from the mother are marked by a thick line to clarify the separation of the jump-started.

Transposition takes place with a high tendency to integrations sites with 0–150 kb ***(13–15)***. To select the new integration sites it can be advantageous to start with a P[*white*] element. Here changes in the intensity of the eye color can be used as valid indication for a new transposon integration. In addition, one has to keep in mind that local hopping will not only generate new insertions but the starting insertion can be left behind as well, as deletions are induced around the starting P-element (*see* **Subheading 3.2**).

3.1.4. P-Element Replacement

As can be seen from **Fig. 2** many different P-elements have been generated. They allow expression of downstream genes (EP-elements), or function as

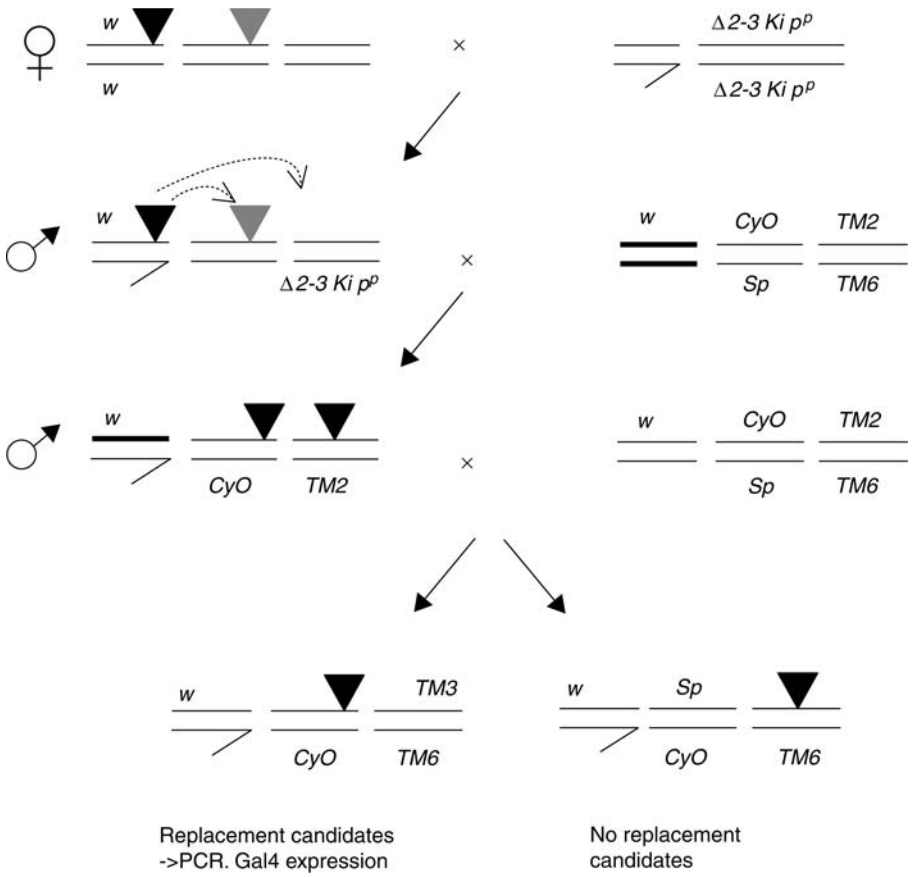

Fig. 4. Crossing scheme for the replacement of P-element insertions. In the F_1 generation, the X-chromosomes from the mother are marked by a thick line. Replacement candidates must be confirmed by PCR.

enhancer detector strains driving the expression of a reporter gene such as Gal4 in a specific temporal and spatial pattern. It would thus be desirable to convert one P-element insertion into another one. Early observations suggested that during P-element mobilization a cut-and-paste process that leaves a double-strand gap. The repair of such gaps resulted in the replacement of sequences from a homologous template by a process similar to gene conversion ***(16)***. Auld and colleagues have adopted this finding and established a method for targeted replacements of different P-elements **(Fig. 4)** ***(17)***. The efficiency of gene conversion/targeted transposition is variable and depends on the location of the different elements. It is usually in the 1% range, which requires the set up of 300 single pair crosses according to the scheme shown in **Fig. 4**.

3.2. P-Induced Deficiencies

The crosses mentioned above describe the generation of new transposon insertions. Existing insertion events can then be used to induce chromosomal deletions, which provide valuable tools during the functional analysis of any gene function. In the following, we review the different approaches that can be used to obtain deletions around a given P-element insertion.

1. Deleting the flanking genomic DNA through imprecise excision of single P-elements.
2. Deletion of genomic DNA between two P-elements (*cis*/*trans*) using *P*-transposase (hybrid element insertion [HEI] strategy).
3. Deletion of genomic DNA between two P-elements carrying flipase recombination target sites using flipase recombinase (Ex/DrosDel methods).
4. Deletion of genomic DNA between two hybrid transposons *P[wHy]* using *hobo* transposase.

3.2.1. Excision Mutagenesis

Besides its potential to generate insertional gene disruptions, P-elements are extremely useful for generating deletions of flanking genes. When a P-element is excised from a chromosome, a double-stranded break is created. If the ends of such double-stranded break are degraded before repair, a deletion of the genetic material will occur. This event is known as imprecise excision and occurs with a frequency of approx 1%. The size of the chromosomal deletions can range from a few base pairs to several kbp. Deletions can be found starting from the 5′ end of the P-element or its 3′ end. They can also include both ends and remove the entire P-element including its flanking sequences. Additionally, internal deletions may be induced. There is no way to predict the extent of one specific deletion orientation and in some cases a very strong bias for one deletion direction has been noted. The underlying mechanism for this specificity is unknown. In case the *white* gene is used, the selection of revertants with an altered eye color may not only reveal local transpositions but may also indicate directional deletions. If the excision event leaves the *white* gene intact but deletes flanking genomic sequences with regulatory influence on *white* expression a change of eye color can be the consequence.

In a standard cross, independent reversion events are selected on the basis of the loss of the dominant marker carried by the P-element **(Fig. 5)**. Excision mutagenesis works in both males and females. In general, 200–300 independent excision events should be established **(Fig. 5)**. For this, 350 single pair crosses of P[*white*]/Δ2-3 × TM3/TM6 are set up. One *white* reversion per vial is selected and is used to establish an excision mutant stock. Subsequent alterations in the genomic sequence are determined by PCR technology or Southern blotting.

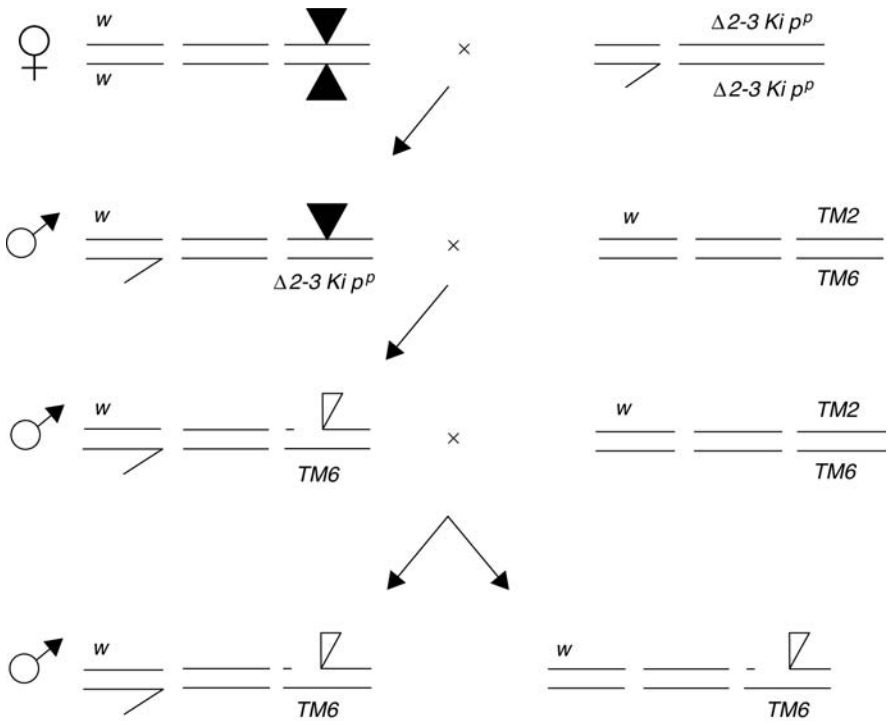

Fig. 5. Crossing scheme for the isolation of excision mutants. The excision mutant is indicated by an half P-element symbol.

3.2.2. HEI Strategy

Simultaneous mobilization of multiple P-elements can lead to chromosomal aberrations owing to mistakes in the transposition process. Here the insertion of a transient hybrid P-element consisting of paired 5′ and 3′ ends from different P-elements inserted at a new chromosomal site causes a deletion or duplication of the material between the elements **(Fig. 6)**. This process has been called HEI ***(18,19)***. HEI preferentially but not exclusively occurs between P-elements that are located *in trans* on sister chromatids. The approach enriches for local deletions because hybrid elements tend to insert in or near one of the original *P* insertions. The determination of the chromosomal breakpoints is facilitated by the fact that deletions generated by *P* transposase in the presence of *trans*-heterozygous *P* are often flanked by one of the initial P-elements providing a convenient molecular tag.

The HEI event can involve two *P* insertions regardless of their orientation on the chromosome. In addition, the HEI strategy can be employed using a large

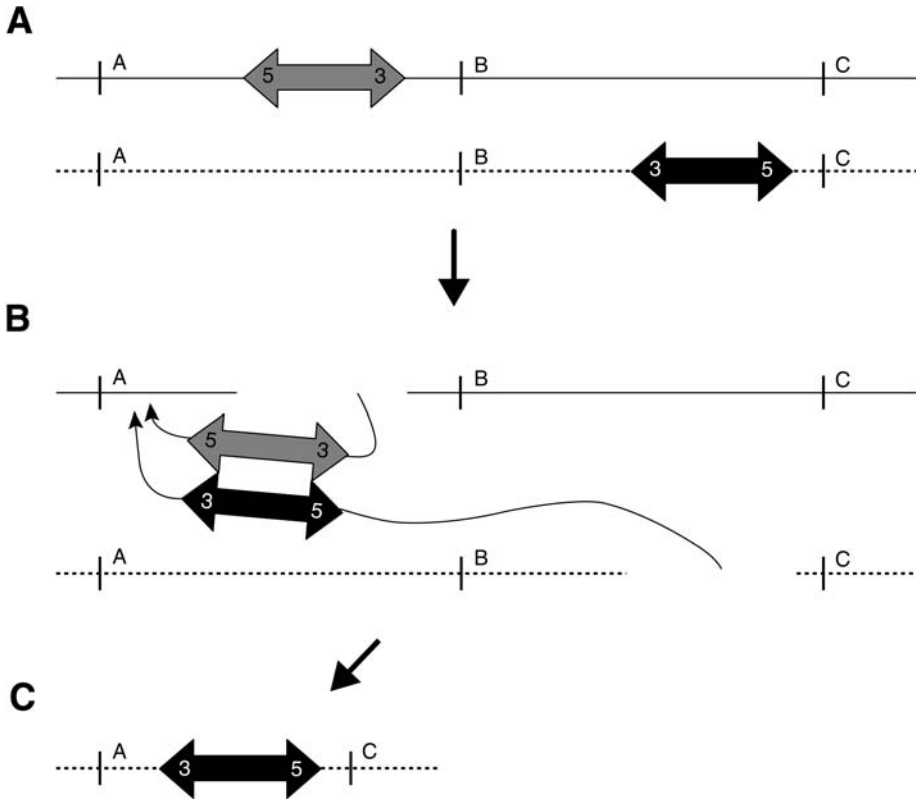

Fig. 6. **(A)** Creating genomic deletions using HEIs. **(B)** Two P-elements are located *in trans* on sister chromatids. **(C)** A hybrid element transposes locally and results in a deletion of the intervening chromosomal region.

variety of types of P-elements (successful combinations of P[PZ], P[lacW], P[SUPor-P], P[EP], and P[GT1] insertions have been reported).

Following the crossing scheme outlined in **Fig. 7**, meiotic recombination of markers (*m1, m2,* and *m3*) *in cis* with each *P* insertion is induced in males by the presence of constitutive transposase (for a list of convenient marker stocks *see* **ref. *12***). Following the combination of two P-elements *in trans* with a transposase source complementation tests for the visible markers (*m1, m2, and m3*) have to be conducted to determine the breakpoints of the deletion. The approach requires the screening of 20,000–30,000 progeny. On an average 25% of the recombinant premeiotic clones were associated with a deletion extending from one *P* insertion site to the other, a unique *P*-to-*P* deletion clone was detected in 1 of every 3000 progeny. However, the exact efficiency depends on the nature of the involved P-elements.

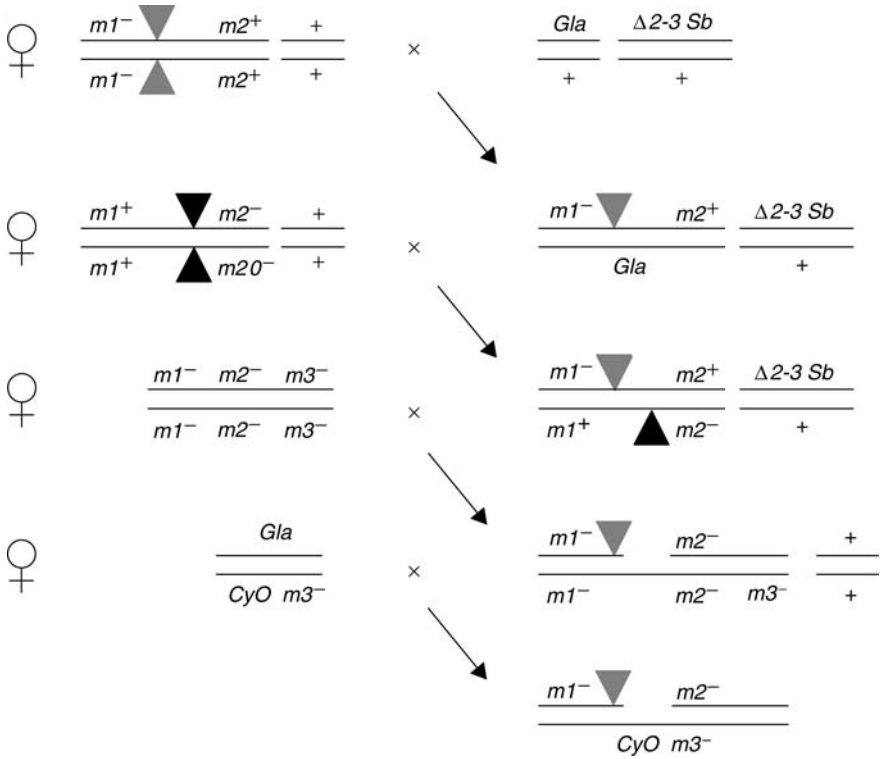

Fig. 7. Crossing scheme for the isolation of genomic deletions using HEIs. The different P-elements are indicated by color-coding. For details *see* text.

3.2.3. FRT/FLP-Induced Deletion

The HEI strategy makes it difficult to create a deletion of a specific size or in a precisely defined region. To overcome this problem more effective site-specific recombination events have to be used to engineer chromosome deletions. In contrast to the HEI strategy is the FRT/FLP-induced deletion generation system, where chromosome rearrangements can be generated precisely between preselected sites. The FLP site-specific recombinase acts on chromosomal target sites located within specially constructed *P-e*lements to provide an easy screen for the recovery of rearrangements with breakpoints that can be chosen in advance ***(20,21)***. Deficiencies can be recovered by recombination between two elements that lie in the same orientation on the same chromosome or on homologues chromosomes. In the presence of FLP recombinase, FRT-bearing transposon insertions can be used to efficiently isolate deletions with precisely defined end points. The recombination frequency between the *FRT* elements decreases with their distance.

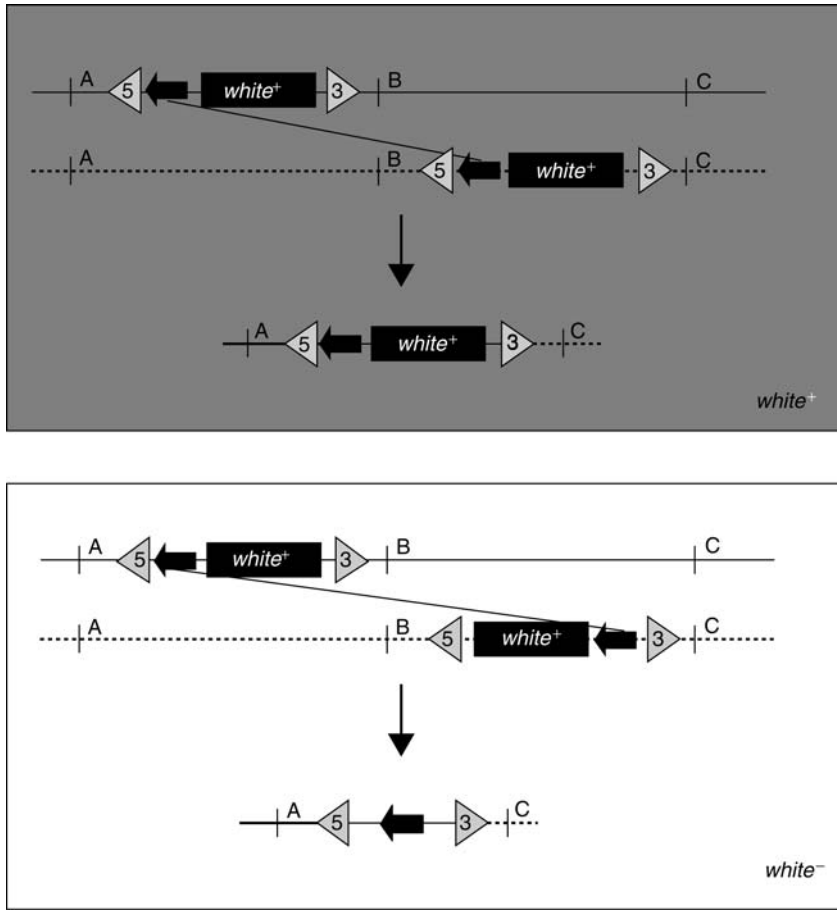

Fig. 8. Principles underlying FRT/FLP-induced deletion. Two different recombination schemes are shown. In the upper panel both P-elements are inserted in the same orientation. Recombination results in a deletion of genomic material but leaves the *white* gene intact. In the lower panel the two P-elements are arranged in the opposite orientation. Flp-induced recombination results in a deletion of chromosomal DNA and in a loss of the *white* marker.

A large collection of different P-element and *piggyBac* insertion lines containing FRT sites was recently established by Exelixis Inc. and the DrosoDel consortium ***(11,12,22)*** (*see* **Fig. 2**). These transposon insertions contain FRT sites either 5′ or 3′ of the *white*$^+$ mini gene. In the presence of FLP recombinase, efficient *trans*-recombination between FRT elements results in a genomic deletion with a single residual element tagging the deletion site **(Fig. 8)**. Depending on the pair of starting P-element insertions, their genomic orientation

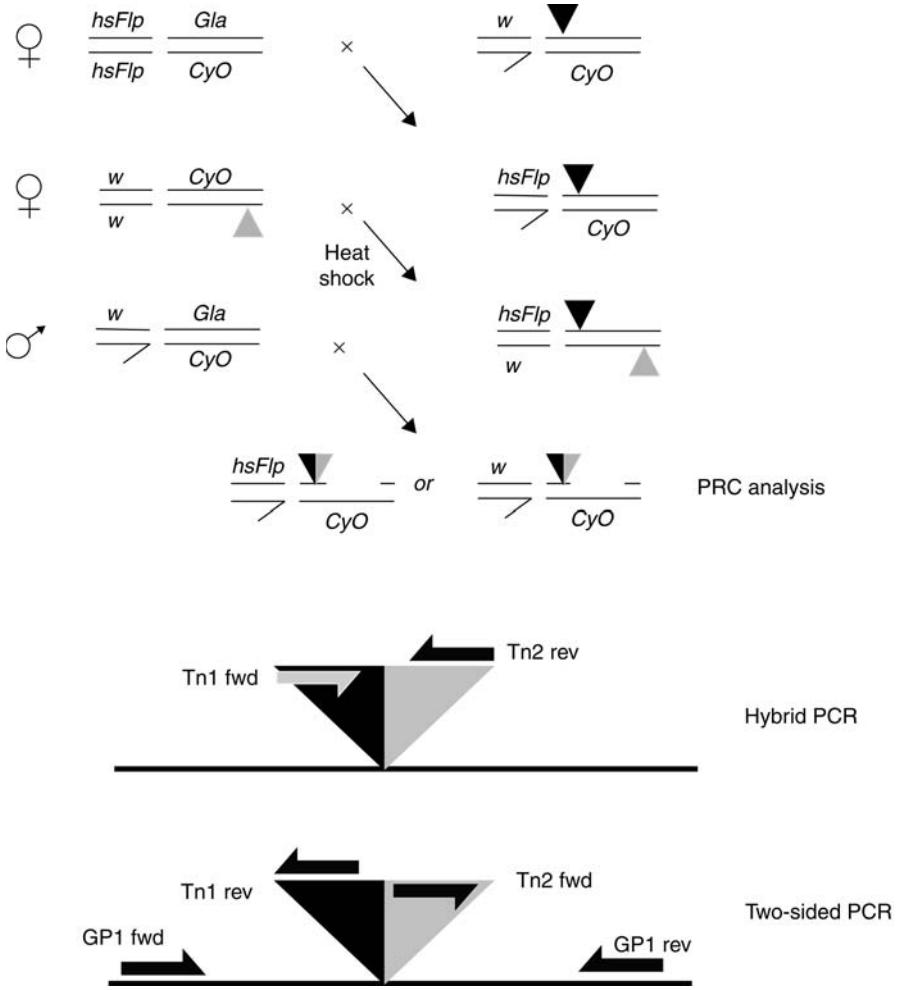

Fig. 9. Crossing scheme for the isolation of FRT/FLP-induced genomic deletions. The different P-elements are indicated by color-coding. For details *see* text.

and the relative position of the FRT element with respect to the *white*$^+$ mini gene, some deletions can be easily detected in the progeny by the loss of the *white*$^+$ marker. **Figure 9** shows a four-generation crossing scheme resulting in the generation of a defined deletion mutant. The candidates identified should be screened for the presence of the residual P-elements by PCR. Both, the resulting hybrid element (e.g., *XP:WH*) should be detected using element-specific primers as well as the position of the residual element that ends in the genome should be checked, because chromosomal rearrangements can occur during the FRT/FLP-induced deletion generation. In general, PCR screening of five

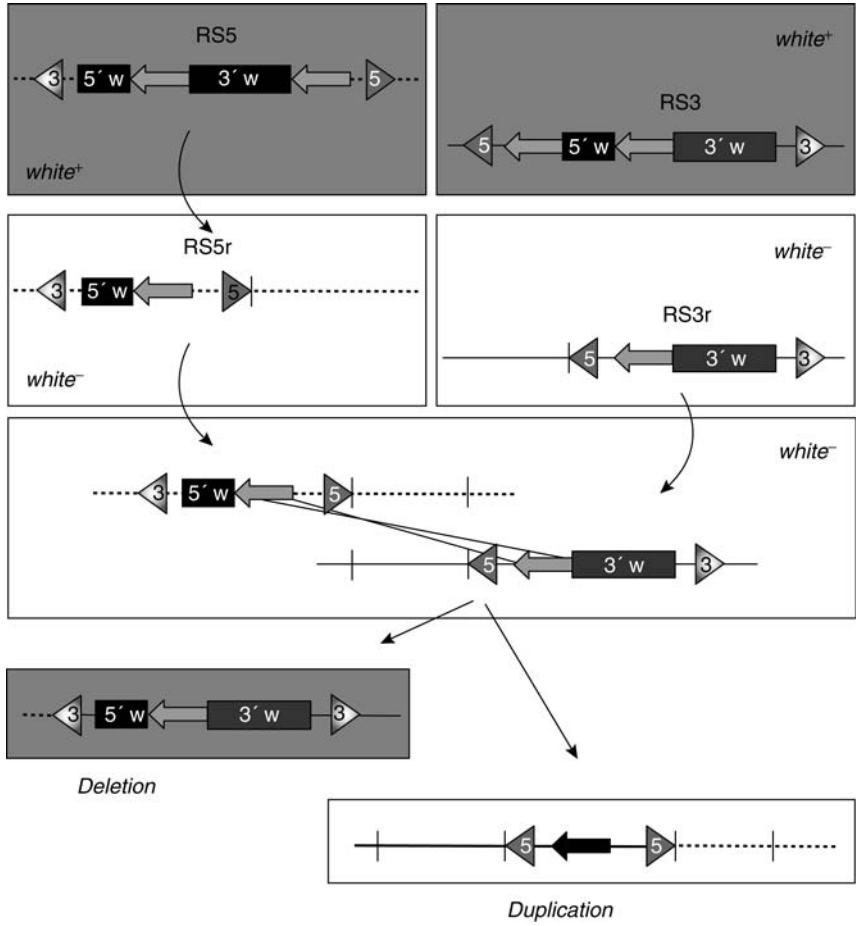

Fig. 10. Principles underlying the generation of genomic deletions with RS elements. Color shading indicates presence or absence of a functional *white* gene. In the series of crosses, recombination *in cis* removes either the 3′ or the 5′ half of the *white* gene, rendering the *white* marker gene unfunctional. If two such deleted P-elements are brought together, Flp-induced recombination *in trans* results in a deletion of chromosomal DNA and restores a functional *white* marker.

individual *white*$^{-}$ progeny from a *white*$^{-}$ deletion generation cross, or PCR screening of 50 progeny from *white*$^{+}$ deletion generation cross, resulted in four confirmed deletions.

The DrosDel consortium followed a similar FRT/FLP-induced deletion generation approach ***(22)***. However, as a major difference *white* mutant flies are crossed and the offspring is screened for the presence of *white*$^{+}$ flies that carry a deletion between two P-element insertion sites. **Figure 10** depicts the rational

of the approach. Two distinct P-elements, P[RS3] and P[RS5] **(Fig. 2)**, were generated such that each would contain a *white* mini gene construct but would differ by the position of the FRT sites. The activity of FLP recombinase on each of these elements alone produces a *cis* chromosomal excision within each element, resulting in truncated P-elements each containing a nonfunctional part of the white gene and a single FRT site, P[RS3r] or P[RS5r] **(Fig. 10)**. When both truncated P-elements are combined in one fly, a second FLP-mediated recombination event between a pair of the remnant P[RS3r] and P[RS5r] elements will result in a reconstituted functional *white* gene. If the two elements are *in trans* and in the correct orientation with respect to each other, the FLP-mediated recombination will generate a deletion of the chromosome material between the sites **(Fig. 10)**.

Figure 11 describes the generation of an autosomal deletion taking the example of the 2nd chromosome. Males carrying the P[RS3] or P[RS5] elements were mated with females carrying a FLP recombinase transgene. In the F_1 generation, progeny males carrying both the element and the FLP recombinase were mated to females carrying the second element. After 2 d, parents and progeny were subjected to a 1-h heat shock by placing the bottles into a 37°C water bath. Parents were removed after 3 d of total egg-laying time and the bottles were subjected to daily 1-h heat shocks for four further days. In the F_2 generation, virgin females were collected and crossed to males containing marked balancer chromosomes. Five individual *white*$^-$ males progeny were then crossed pair wise to appropriate balancer virgins, to generate the stocks in an isogenic background, and to subsequently confirm the internal deletion by PCR.

In the F_3 generation, two internally deleted P[RSr] elements were combined. In the F_4 generation, males with two P[RSr]-elements *in trans* were collected and crossed to balancer carrying virgin females. After 2 d, parents and progeny were subjected to a 1-h heat shock in a 37°C water bath. Parents were removed after 3 d of total egg-laying time and the bottles were subjected to daily 1 h heat shocks for four further days. *white*$^+$ progeny were selected that should carry the desired deletion between the flanking P[RSr] elements. In the F_5 generation, about 50 *white*$^+$ males were selected and crossed to balancer carrying virgin females. The presence of the reconstituted P[RS] element must be confirmed by PCR analysis.

3.2.4. P[wHy] Strategy

The recently described P[wHy] strategy allows to generate a set of nested deletions in a particular region ***(23,24)***. The method is based on a hybrid transposable element, P[wHy], which carries a *hobo* "deleter" element flanked by two genetic markers, *yellow*$^+$ and *white*$^+$, inside a *P*-element **(Fig. 12)**. First,

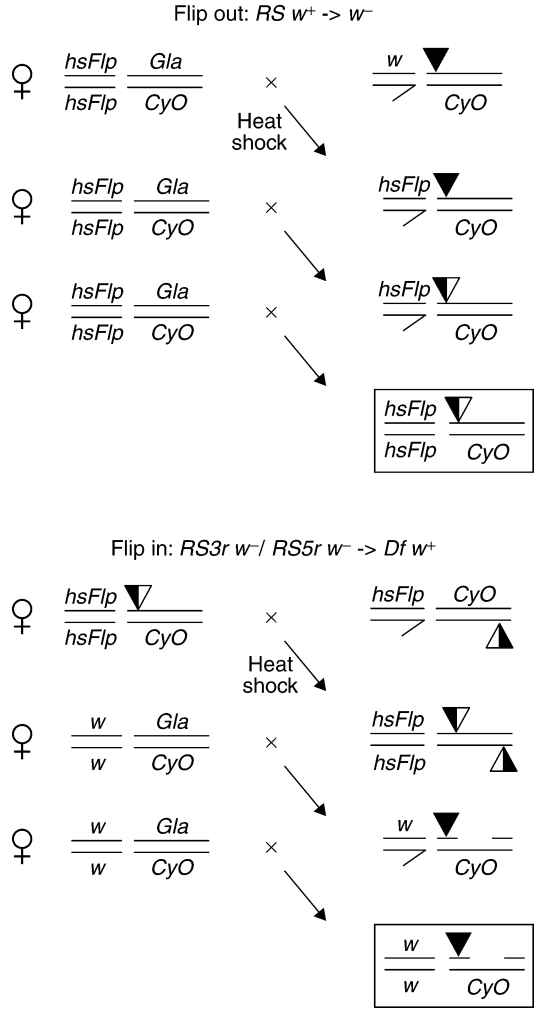

Fig. 11. Crossing scheme for the generation of genomic deletions with RS elements. Top box depicts the generation of the starter P-element (deletion *in cis*). Bottom box depicts the generation of the deletion that is detected by the restoration of a functional *white* gene.

replicative local *hobo* hopping results in the integration of a second *hobo* element close by. Then, following recombination between the two *hobo* elements, all genes that are located between both *hobo* insertions are removed, provided that the hobo elements are inserted in the same orientation. The concomitant removal of one of the two genetic markers of the hybrid transposable element, P(wHy), *yellow*$^+$ or *white*$^+$, allows to determine the orientation of the

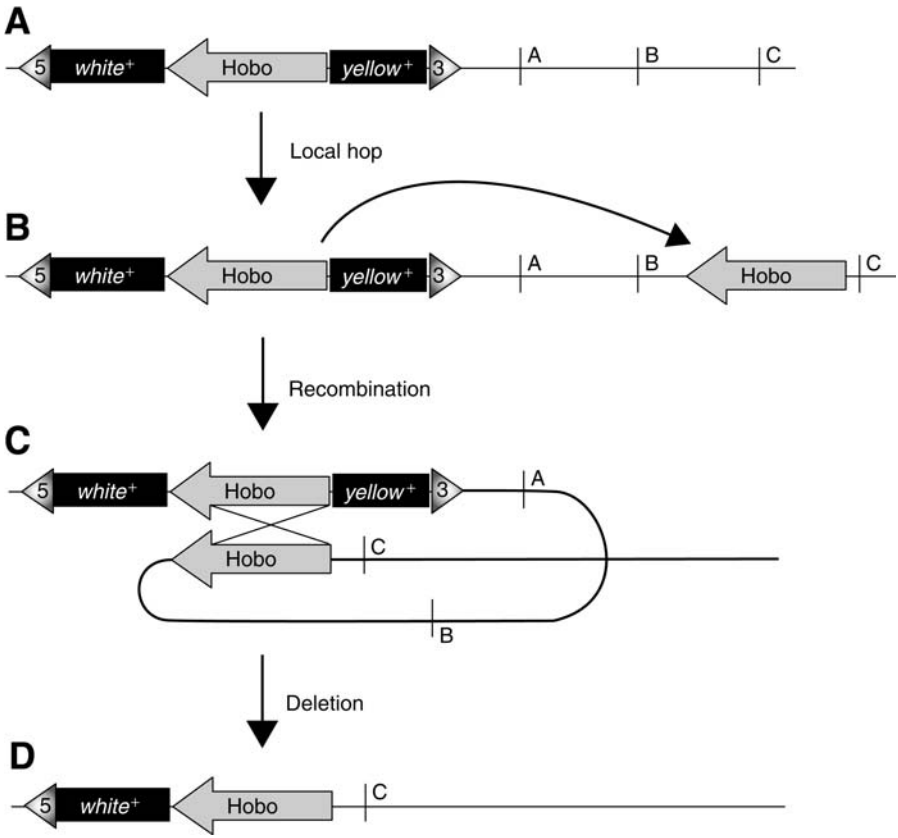

Fig. 12. Principles underlying the generation of genomic deletions following the P[wHy] *strategy.* Following a local transposition of a hybrid element to random sites, recombination results in the deletion of the intervening genomic material. A truncated hybrid element is left behind.

deletion event by genetic means. As one copy of *hobo* and a partial *P*-element remains, the deletion can be molecularly defined by inverse PCR technology. This method has been useful for generating nested deletions within 60 kb of the original insertion site. The deletion end points are staggered every 1–3 kb and deletions up to 400 kb have been reported. Deletions must be recovered and maintained in a *hobo*-free genetic background.

3.3. Additional Approaches

P-elements can be used in several other ways to generate mutant alleles. In the most direct approach a number of P-element transgenes have to be generated that eventually allow gene targeting through homologous recombination. A

more detailed account of this strategy is presented by Maggert et al. in this book. In addition, EP-elements and related transposon constructs **(Fig. 2)** have been successfully used to obtain *gain-of-function* phenotypes. Provided that overexpression of a given gene results in an easily detectable and penetrant phenotype, an ethane methyl sulfonate mutagenesis (*see* Chapter 6) can be used to generate mutants that revert this dominant phenotype. In most cases, these mutants are either hypo- or amorphic alleles of the overexpressed gene. Thus, loss-of-function alleles can be easily generated.

Finally, the upstream activating sequence (UAS) cannot only be used to cause an overexpression phenotype. The use of specific Gal4 derivatives also allows a sequence specific transcriptional inactivation of the chromosomal region close to the P-element insertion. Such silencing occurs through binding of a chimeric Gal4 protein that contains the Gal4 DNA-binding domain and the repression domain of the isolator suppressor of hairy wing to UAS sequences. Binding results in a chromatin remodeling and a conditional repression of genes located downstream of a UAS sequence ***(25)***.

4. Notes

1. Some of the techniques described in this chapter require the knowledge of the exact transposon integration site. A number of methods have been described to efficiently obtain information on flanking sequences. In our hands inverse PCR technology as described by the Berkelay *Drosophila* genome project is most reliable (http://www.fruitfly.org/about/methods/ inverse.pcr.html). Alternative methods such as plasmid rescue or universal fast waking may also be applied ***(26,27)***.

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7

EMS Screens

From Mutagenesis to Screening and Mapping

Christian Bökel

Summary

The success of *Drosophila* as a genetic model organism is based on the efficient generation, recovery, and identification of new mutations. Various agents have been used to induce *de novo* DNA lesions. However, the use of mutagenic alkylating agents, especially ethyl methanesulfonate (EMS), has become a standard approach for mutagenesis that has been succesfully used in the classic forward genetic screens that have defined the field of developmental genetics, as well as in many alternative screening schemes that have since been developed. In this chapter, a basic EMS mutagenesis protocol is introduced, and examples for the fly crossing schemes used in several different types of screen are presented. In addition, some new genome sequence-based approaches are discussed that have alleviated the notoriously difficult molecular mapping of EMS induced point mutations. Together these protocols should allow researchers as yet unfamiliar with *Drosophila* genetics to take advantage of all the benefits of this mutagenesis method, which include its wide and largely unbiased coverage of the genome, the high mutation frequency, and the variety of null, hypomorphic, conditional (e.g., temperature sensitive), or domain specific mutations that can be caused by EMS treatment.

Key Words: *Drosophila*; EMS; forward genetics; genetic screens; mutagenesis; mutation mapping.

1. Introduction

Ethyl methanesulfonate (EMS) mutagenesis has become a standard method for the *de novo* induction of DNA lesions in *Drosophila* ***(1)***. This approach is complemented by X-ray mutagenesis and methods based on mobile genetic elements, which have been presented in the preceding chapter, and to a lesser extent by other mutagens, which are discussed in detail in **ref.** ***2***. EMS genotoxicity is mediated by the formation of O^6-methylguanine adducts that can mispair with thymine during replication. Thus, the bulk of all EMS-induced mutations (75–100%) was

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found to consist of GC→AT transitions ***(3–5)*** that may form missense or nonsense codons or destroy splice sites. Other DNA lesions such as transversions, frameshifts, and deletions can also be recovered, albeit at lower frequencies and especially if females are forced to store sperm for several days before laying ***(2,6)***. Aside from a preference for G residues preceded by a purine there is no intrinsic bias for mutation sites ***(3)***, and in principle the entire genome can be mutagenized.

At average EMS concentrations (25–50 m*M*), new mutations are induced at random sites on average once every 150–300 kb ***(3,5)***. One new mutation per 150 kb corresponds to about 50 DNA lesions inside actual open reading frames (ORFs) per chromosome, and it can be calculated that about 12% of these cause gene inactivation ***(3,5)***. As roughly only one in three genes can be mutated to lethality ***(7)***, this mutation frequency corresponds well with the observed average of approximately two lethal mutations per chromosome observed under these conditions ***(5)***. Clearly, the several hundred other newly induced mutations represent a change in genetic background, and "genetic cleaning" (i.e., replacement of the irrelevant regions of the mutagenized chromosome by recombination), or at least the use of independent alleles *in trans* instead of homozygous mutagenized chromosomes is highly advisable.

Flies respond to mutagenesis by triggering DNA repair mechanisms. As this can still occur in the embryo after the first cleavage division rather than during spermatogenesis, a high percentage of F_1 flies are actually genetic mosaics. In screens where the phenotype is already scored in the F_1 generation, the germline of a candidate fly may therefore not necessarily transmit the responsible mutant allele. However, the resulting fraction of "false positive" nonrecoverable mutants is more than made up by the high mutagenesis rate achievable by EMS treatment. Mosaicism is generally not a problem for screens in which phenotypes are scored later ***(2,8,9)***.

For practical reasons, screens are in most cases aimed at individual chromosomes or chromosome arms. Mutations generated in EMS screens were notoriously difficult to map in comparison with transposon insertions or X-ray induced deletions. However, the time and effort required to pin down the molecular nature of a given EMS mutation has been drastically reduced since the release of the genome sequence and molecular mapping strategies based thereon. Whereas a detailed treatment of such mapping approaches would exceed the scope of this chapter, a short overview will be given.

2. Materials

2.1. Isogenization of Starter Fly Stock

1. Flies: 10 males of starter genotype to be mutagenized, 40 dominantly marked balancer virgins.
2. Fly equipment: 31 small fly food vials (26 mm opening, e.g., Greiner 217101).

2.2. Basic EMS Mutagenesis Method

1. Flies: 1000 males of starter stock. Preferably these males are homozygous, have been isogenized, and carry visible or molecular markers for subsequent mapping (*see* Bloomington *Drosophila* Stock Center website [*see* Appendix]). Virgins: 1500 for G_0 cross; 30,000 for F_1 cross.
2. Fly equipment: 30 empty plastic fly vials with 50 mm opening (big vials, e.g., Greiner 960177), 10 extra 50 mm foam stoppers (e.g., Greiner 332070), 60 big vials with food, 10,000 small (26 mm diameter) vials with food per subsequent generation (F_1,F_2) as appropriate. Plastic tray for 10 large bottles, second tray as cover, thick filter paper (e.g., Whatman GB004) fitting into the tray.
3. 2.6 mL of 25 m*M* EMS (e.g., Sigma M-2880).
4. 100 mL of 1% sucrose solution in H_2O.
5. 10 L of inactivation solution (10% sodium thiosulfate in H_2O) (*see* **Note 1**).
6. Kimwipes (e.g., Kimtech Science 7102).
7. Double-sided sticky tape (e.g., Tesa 5338).
8. Nitrile gloves (e.g., Ansell Touch'N'Tuff).
9. Disposable 30-mL syringes (e.g., Terumo) with wide gauge needles.
10. Disposable 1-mL syringe or 1-mL pipet with filter tips.
11. Wide neck chemical waste disposal barrels (e.g., Roth Sekuroka, please also consult the responsible safety officer).
12. Thick filter paper (e.g., Whatman type GB004 in sheets) or Benchkote (Whatman).
13. Four 50 ml screw cap tubes (e.g., BD Bioscience Falcon 352070).
14. Parafilm (Alcan).

2.3. Measuring Mutation Efficiency

1. Flies: 2 × 30 mutagenized males (*see* **Subheading 3.2.**), 2 × 20 nonmutagenized control males, ca. 200 C(1)Dx, y w f virgins.
2. Fly equipment: 4 yeasted big (50 mm) diameter food vials.

2.4. F_3 Lethal/Phenotype Screens

As listed for **Subheading 2.2.** including F_2 vials, 37°C water baths for heat shock.

2.5. Autosomal Allele Screens/Deficiency Saturation

As listed for **Subheading 2.2.**, F_2 vials only for recovery of candidates.

2.6. X-Chromosomal Allele Screens

As listed for **Subheading 2.2.**, upscaling may be required (not all F_1 males usable, F_1 loss owing to compound X-chromosome). F_2 vials only for recovery of candidates.

2.7. F_1 Clonal Screens Using Flp/FRT-Mediated Mitotic Recombination

As listed for **Subheading 2.2.**, upscaling may be required (detection relies on successful clone induction, failure to recover some candidates owing to mosaicism), 37°C water baths for heat shock. F_1 vials only for retest of candidates, F_2 and F_3 virgins and vials only for recovery.

2.8. Suppression/Modulation Screens

As listed for **Subheading 2.2.**, upscaling may be required (mosaicism). F_1 virgins and vials only for recovery.

3. Methods

The basic mutagenesis method outlined as follows can—using different starting strains and crossing schemes—be applied for several different types of screen. For selected strains, Bloomington Stock numbers (BL-xxx) are given (*see* Appendix). Fly numbers and material has been estimated for screening around 10,000 genomes after starting with 1000 homozygous males (e.g., for F_2 allele screens or F_3 lethal screens, *see* **Note 2**). Using a standard concentration of 25 m*M* EMS (inducing on average between one and two lethal mutations/chromosome) this will normally provide sufficient saturation (*see* **Note 3**). For smaller scale pilot screens, mutagenesis schemes that start with heterozygous males, or if higher saturation is required, these numbers can be scaled appropriately. Although the mutagenesis procedure itself will remain unchanged, alternative screening schemes will include additional or specific procedures (e.g., heat shock treatments for clone induction in F_1 screens) that will be discussed in the sections dealing with the crossing schemes for the respective screens. In all examples, mutagenized chromosomes are indicated by an asterisk.

If larger numbers of genomes need to be screened it is advisable to mutagenize males in several smaller batches, for example, once weekly. Such a "rolling" screen design will allow the labor intensive steps of the protocol to stagger, such as virgin collection, outcrossing of F_1 males, and scoring of phenotypes in time.

3.1. Isogenization of the Starter Fly Stock

Before beginning any mutagenesis experiments the starter chromosome (i.e., the genetic background where mutations will be induced) should be isogenized. This ensures that there are no pre-existing DNA polymorphisms within the starter fly stock that may then appear in individual candidates and make the subsequent mapping and recovery of any newly induced mutations more difficult. Thanks to balancer chromosomes, which carry sets of nested inversions and can therefore be used to suppress recombination, isogenization in flies is straightforward and can be achieved using the example in **Fig. 1** for the second chromosome. The genetic markers *b*, *pr*, *cn*, and *bw* are recessive cuticle and

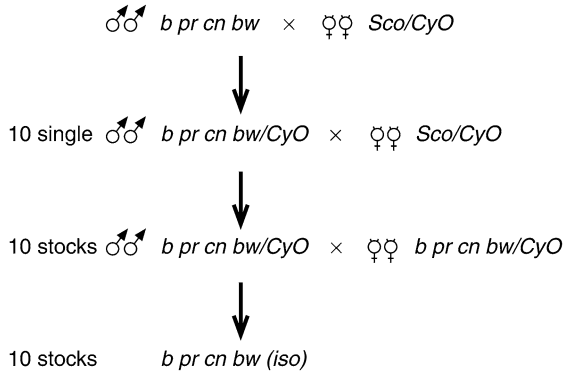

Fig. 1. Isogenization of a second chromosome starter fly stock.

eye color mutations and *Sco* is a dominant visible bristle marker (*see* also **Subheading 3.3.**). The 10 homozygous isogenized fly lines should be compared with respect to viability and fertility, and the most suitable fast growing and healthy fly line should be expanded as the starter fly stock for mutagenesis.

True isogenization is obviously not possible when the starter stock is homozygous lethal (e.g., in some modifier screens). In these cases the starter chromosome should still be derived from a single male so that all recovered candidates share a common background haplotype. Information on useful markers can be found on the Bloomington *Drosophila* Stock Center and Flybase websites (*see* Appendix) and in the "Red Book" of Lindsley and Zimm ***(10)***. It is important to carefully decide, which markers or features (e.g., Flp recombinase target [FRT] sites) the starter chromosome should contain, and even spend some time constructing such stocks if they are not available, as this can save lots of work during later steps.

3.2. Basic EMS Mutagenesis Method

Collect sufficient numbers of virgins and maintain in yeasted food vials at 18°C. Collect the males to be mutagenized on the first day after eclosion, age in food vials for 3 d (*see* **Note 4**).

1. Cut 10 big Ceaprene (foam) stoppers horizontally. Wrap each half into two Kimwipes.
2. Fix Kimwipes with double-sided sticky tape.
3. Push the wrapped stopper halves to the bottom of 20 empty big vials so they are held at the bottom by pressure and the double stick tape.
4. Add 10 mL of tap water each into ten of these vials and let the wrapped stoppers soak up the water. Remove any droplets from wall.
5. In the evening of day 1, add 100 males into each of these ten vials and put them on top of wet filter paper inside a plastic tray. Cover with another tray and incubate overnight at 25°C. This starves the males but prevents dehydration.

6. On the morning of day 2, prepare the chemical hood for the EMS work. Ensure proper safety procedures are in place (*see* **Note 1**). These include that a dedicated mutagenesis hood is coated with benchcoat or some other material able to soak up any accidental spills, that sufficient chemical waste disposal barrels with inactivation solution (10% sodium thiosulfate) are present to inactivate, and discard all EMS-contaminated material such as syringes, fly vials, Falcon tubes, gloves, and benchcoat. In addition, set aside some Kimwipes and inactivation solution to handle accidental spills and decontaminate equipment. Put on a labcoat, goggles, and double gloves before collecting the EMS from the safety cabinet. Make sure that the hood is properly closed when handling EMS as the liquid readily evaporates. Always use fresh EMS, as opened batches may partially lose their activity within a few weeks.
7. Under a chemical hood, prepare three 50-mL Falcon screw cap tubes (e.g., BD Bioscience Falcon 352070) each containing 30 mL of 1% sucrose solution in H_2O, and one containing 10 mL of 1% sucrose solution in H_2O. For a final EMS concentration of 25 m*M* add 0.26 mL EMS per 10 mL of sucrose solution (3×0.78 mL and 1×0.26 mL) using a disposable 1-mL syringe or a pipet with aerosol proof tips. Seal screw caps with parafilm, then shake or vortex first tube under chemical hood until EMS is dispersed into tiny droplets that do not anymore sink. On vigorous shaking EMS is miscible in H_2O (*see* **Note 5**).
8. Suck up EMS solution into 30-mL syringe with wide needle. Inject 10 mL EMS solution through the Kimwipe layer into the wrapped foam stoppers of the first three tubes **(Fig. 2)**. Avoid puddles, but ensure that the Kimwipe is soaked from below. Discard Falcon tube in inactivating solution. Continue until all vials are treated, always briefly resuspending the EMS before moving on to next Falcon tube. Remove any droplets from the vial wall using a Kimwipe.
9. Under the hood, flip the males from the ten starvation vials into the EMS/sucrose vials. Incubate them under the hood in a plastic tray coated with wet filter paper and covered with another tray to prevent dehydration.
10. In the evening of day 2 (for 6–10 h EMS exposures) or on the morning of day 3 (for overnight exposure, *see* **Note 2**), transfer males into empty plastic vials where they can clean off and defecate much of the remaining EMS. Let the G_0 males recover for 30 min (*see* **Note 6**). Carefully discard the mutagenesis vials into the waste barrels under the hood making sure that they are filled with nactivating solution by pushing them below the surface using a plastic pipet.
11. During the recovery period, distribute virgins into vials with fly food and extra yeast for the outcross of the G_0 generation (40–50 virgins/vial). Also prepare four vials with *C(1)DX, y w f* virgins to assay mutagenesis efficiency (*see* **Subheading 3.3.**).
12. Anesthetize males on a dedicated EMS mutagenesis CO_2 plate covered in a discardable Whatman paper or by using ether. Divide the surviving males from each recovery vial in 3 groups and add to the vials containing the G_0 virgins. Set aside some males for the mutagenesis test. Do not use mouth-operated fly pooter (*see* **Note 1**). Carefully discard the recovery vials into the EMS waste. Incubate crosses in 25°C incubator.
13. After 4 d remove all males using a dedicated EMS fly pad to prevent the appearance of clusters of identical mutants derived from mutagenized germline stem cells. Transfer the females into freshly yeasted food vials.

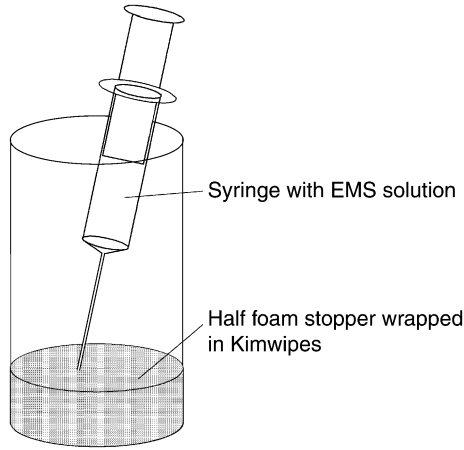

Fig. 2. Injection of EMS solution.

14. After 10 d start collecting F_1 males. Begin distributing virgins for F_1 cross into small (26 mm diameter) yeasted food vials. Place two to three virgins in each vial, depending on virgin availability. Make sure to use F_1 males from all collection days to avoid bias.
15. Cross single males of F_1 generation to F_1 virgins. Keep crosses in a 25°C incubator for around 10 d.
16. Continue by establishing F_2 stocks (for F_3 screens), or by screening the F_2 generation in the case of allele screens/deficiency saturation.

3.3. Measuring Mutation Efficiency

EMS treatment will induce mutations on all chromosomes of the mutagenized sperm including the X-chromosome. This can be used to estimate already in F_1 the average number of lethal mutations induced per chromosome by using Compound (1) females (often also called "Attached X" females) **(Fig. 3)**. In such stocks (e.g., *C[1]DX, y w f*/Y/*w*) females carry two physically linked X chromosomes as well as a Y chromosome. X*/Y_m, where "*" denotes the presence of newly induced mutations and the C(1) female (*C[1]DX, y w f*/Y_f). The other two resulting genotypes (Y_f/Y_m and *C[1]Dx, y w f*/X*) are nonviable. Any newly induced lethal mutations on the mutagenized X chromosomes will therefore kill the F_1 males (genotype X*/Y_f).

By crossing nonmutagenized males to *C(1)DX*, *y w f* females, one can first establish the expected value for the male to female ratio of the cross (variable, about 0.9). Counting the y^+ w^+ f^+ F_1 males and *y w f* females (yellow cuticle, white eyes, kinked, or split bristles) in the test cross with the mutagenized males and dividing by the expected ratio will then yield the fraction *f* of viable

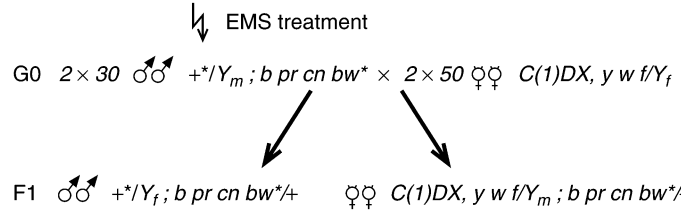

Fig. 3. "Attached-X test" for measuring mutagenesis efficiency.

X chromosomes that did not receive a lethal hit. Under the assumption of a Poisson distribution of lethal hits, the likelihood p(0) for not receiving a lethal mutation (i.e., *f*) is a function of the average number λ of lethal mutations per X chromosome,

$$p(0) = f = e^{-\lambda}, \text{ which can therefore be calculated as } \lambda = -\ln(f)$$

As all four autosomal chromosome arms and the X chromosome each contain roughly 1000 genes that can be mutated to lethality ***(7)***, the value of λ thus derived is a convenient proxy for lethal mutations on the other arms. However, when calculating saturation levels (*see* **Note 3**) one has to keep in mind that owing to F_1 mosaicism a significant fraction of mutations detected by this assay may not be recoverable (*see* **Note 7**).

3.4. F_3 Lethal/Phenotype Screens

F_3 screens with subsequent phenotypic analysis have built the basis for *Drosophila* developmental genetics. The scheme in **Fig. 4** is an example for a simple second chromosome F_3 screen. The same crossing scheme can also be used to generate entire libraries of mutagenized flies, for example, for TILLING (***5,11***; *see* also the Seattle TILLING Project homepage http://tilling.fhcrc.org:9366/fly).

The isogenized (*see* **Subheading 3.1.**) *b cn bw* starter chromosome (Bloomington *Drosophila* stock center number BL-1577) in this example is chosen so that homozygous mutagenized lines can be easily identified at F_3 owing to their straight winged (*Cy*+), black cuticle (*b*), and white eyed (*cn bw*) phenotype. This makes screening the homozygous flies for a given phenotype convenient as they are easily recognized under the stereo microscope. For a corresponding third chromosome example, *see* **Subheading 3.5.**

Establishing thousands of F_2 stocks by individual collection of virgins and males is extremely labor intensive. This can be avoided by introducing a balancer chromosome during the G_0 cross, and a synthetic temperature sensitive chromosome (BL-7757), which carries a transgene expressing the apoptosis inducer *Hid* from a heat shock promoter ***(12)*** in the outcross of the F_1 males.

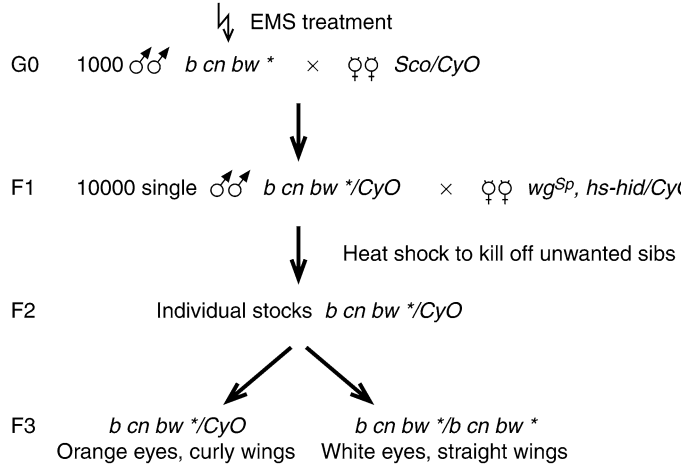

Fig. 4. Crossing scheme for a second chromosome F_3 screen.

This allows the elimination of all unwanted genotypes from the F_2 larvae (wg^{SP-1}, hs-hid/*CyO* and wg^{SP-1}, hs-hid/*b cn bw*) through heat shock driven systemic induction of apoptosis, thus automatically generating stable F_2 stocks. In practise, after sufficient larvae have appeared in the F_1 cross the adult flies are removed and the vials heat shocked by submersion into a water bath for 2 h at 37°C on two subsequent days. It is essential to locally establish heat shock conditions with the actual water baths to be used that reliably kill all transgene carrying larvae without harming the desired genotype.

3.5. Autosomal Allele Screens/Deficiency Saturation

Another common application of EMS screens is to generate additional alleles of existing mutations (allele screens), or to generate mutations in a given gene by mutagenizing all genes, which are uncovered by a deficiency known to include the gene of interest and which are mutable to lethality (deficiency saturation). Here, complementation (i.e., the appearance of flies carrying the mutagenized chromosome over the deficiency or existing allele) can be scored already in the F_2 generation, avoiding labor-intensive establishment and test crossing of stable mutagenized stocks. It is crucial to choose easily visible markers on both the starter and tester chromosomes so that the phenotypic scoring can be done without removing the flies from their vials. In addition, for recovery it is important that the F_2 flies carrying either the starter or deficiency chromosome over the balancer can be separated. For this purpose, on the second chromosome the *b cn bw* marker combination is useful. As the commonly used balancer chromosomes CyO, SM5, and SM6 all are marked with *cn* as

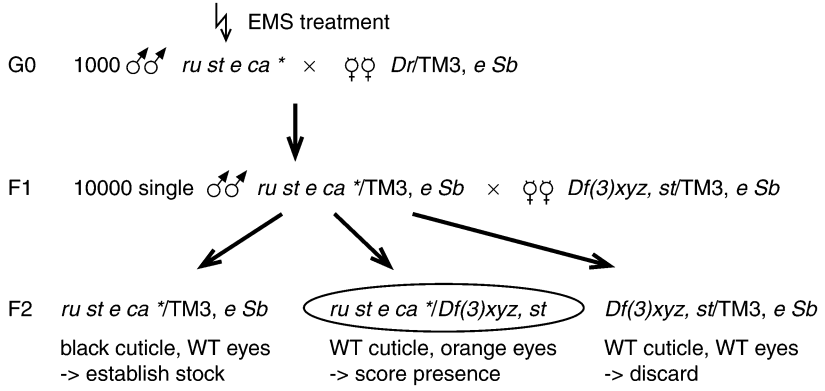

Fig. 5. Crossing scheme for a third chromosomal deficiency saturation.

well as the dominant marker *Cy*, the *b cn bw* starter chromosome can be recovered through its *cn* eye color phenotype. On the third chromosome markers, such as *st* (bright red eyes) and *e* (black cuticle), can be easily scored with the flies still inside the vial.

Thus, in the example in **Fig. 5** vials containing bright red eyed, brown colored flies (whose genotype *ru st e ca**/Df[3]XYZ, *st* shows that the mutagenized chromosome complements the deficiency) are discarded. The remaining vials are carefully rechecked under the dissecting microscope. If indeed no further bright-eyed brown *ru st e ca**/Df(3)XYZ, *st* flies are recovered, a stock can be established from their normal-eyed, black (st^+ *e*) siblings (*ru st e ca**/TM3, *e Sb*) that can then be retested by crossing again to the deficiency fly line.

3.6. X-Chromosomal Allele Screens

The isolation of additional alleles of lethal mutations on the X-chromosomes requires additional genetic tricks, because the males that would normally be used for a complementation cross are by definition dead. One way around that problem is to cross the mutagenized G_0 males to a stock that carries an autosomal duplication of the candidate X chromosomal region. This will allow the survival of males carrying the newly induced lethal alleles. During screening in the F_2 generation the presence of females carrying the mutagenized chromosome over the existing mutation in the absence of the duplication is scored: absence of these females indicates the presence of a newly induced, noncomplementing lethal allele.

In the example presented in **Fig. 6**, new alleles of *mys*, the gene encoding the major fly β-Integrin subunit are generated. The dp^{OVL} mutation and the SM5 balancer are both lethal over CyO, therefore in F_1 only the curly winged males

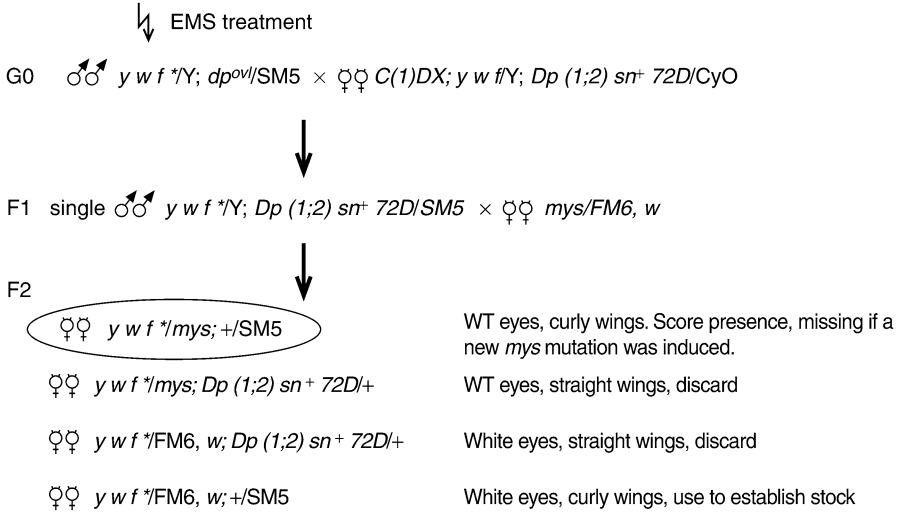

Fig. 6. Crossing scheme for an X-chromosomal allele screen.

will be crossed out as they carry the duplication over a dominant marker, and can therefore carry new *mys* alleles. These males are then crossed to females carrying the *mys* mutation over a balancer marked with *w* and *B*. Thus, in the F_2 generation, females carrying *mys* over the mutagenized chromosome in absence of the duplication can be recognized by their normal eyes (color and shape) and their curly wings again without opening the vial. Vials containing such females are discarded. Newly induced *mys* mutants can be recovered from the other vials through their balanced, duplication free sibs.

3.7. F_1 Clonal Screens Using Flp/FRT-Mediated Mitotic Recombination

From the screening protocol, clones can conveniently be induced using the Flp/FRT system, where overexpressed yeast Flipase (Flp) induces mitotic recombination between chromosomes bearing transgenic FRT sites (*see* also Chapter 10). However, great care must be taken that mutant clones are indeed reliably spotted, and that some way of monitoring the efficiency of clone induction is present. One possibility is to use *w* mutant flies and induce clones with an unmarked FRT starter chromosome over an FRT "tester" chromosome carrying a w^+ transgene, which in the presence of clones will generate a mottled eye color and can be selected against during recovery of the mutagenized chromosome. Alternatively, as shown in **Fig. 7** for chromosome arm 2R, recessive genetic markers can be used to spot clones and distinguish the mutagenized and tester chromosomes.

In this example, the mutagenized chromosome is marked using *shavenoid* (sha). Homozygous clones induced by heat shock expression of Flp-recombinase can

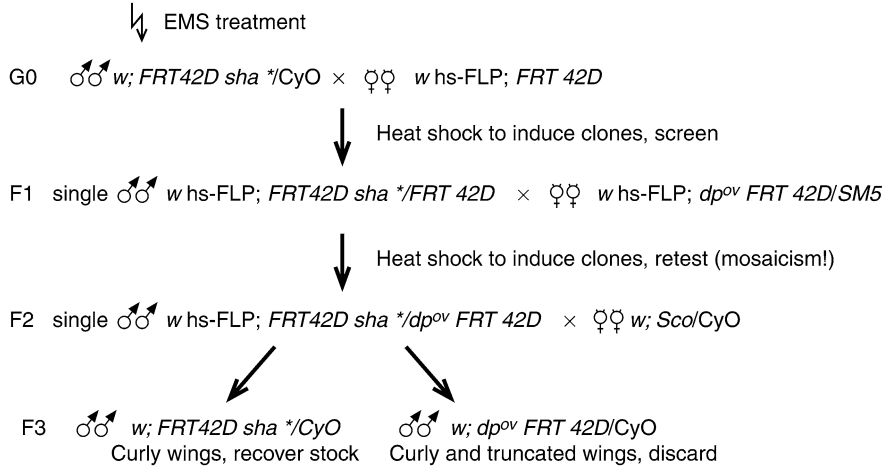

Fig. 7. Crossing scheme for a wing clone screen on chromosome arm 2R.

therefore be recognized by the absence of wing hairs. F_1 males that exhibit the desired phenotype in the clones are immediately retested using flies carrying an FRT chromosome marked with the hypomorphic recessive visible mutation dp^{OV}. This verifies that the F_1 flies (which are largely genetically mosaic, *see* **Note 7**) actually transmit the mutation of interest through their germline. As the CyO chromosome carries an amorphic *dp* allele (dp^{lvl}), the dp^{OV} *FRT* tester chromosome can in the F_3 flies be identified by its truncated wing and deformed thorax phenotype and discarded together with their *Sco* sibs.

A mutation may also be recovered from F_1 females, but with more difficulty because of possible recombination.

3.8. Suppression/Modulation Screens

In this type of screen mutagenesis is used to modulate the phenotype caused by a sensitized genetic background. Sensitization can, for example, be achieved by overactivation of a specific signaling pathway. Mutations affecting the same pathway (either decreasing or further increasing the activation) may then, respectively, either suppress or enhance the assayed phenotype. This approach has been extremely successful to dissect signaling pathways and apoptosis regulation in the fly, especially using the eye as a model tissue (for review, *see* **ref. *13***).

In the postgenomic era it is also possible to use this approach for a kind of "reverse genetics." Here one makes use of so-called EP or EY elements ***(14,15)***. These are P-derived synthetic transposable elements carrying upstream activating sequence (UAS) sites, which allow Gal4-dependent overexpression of the downstream endogenous gene (*see* also Chapter 5). Obviously, such an EP or EY insertion has to be present upstream the target gene of interest, and a Gal4

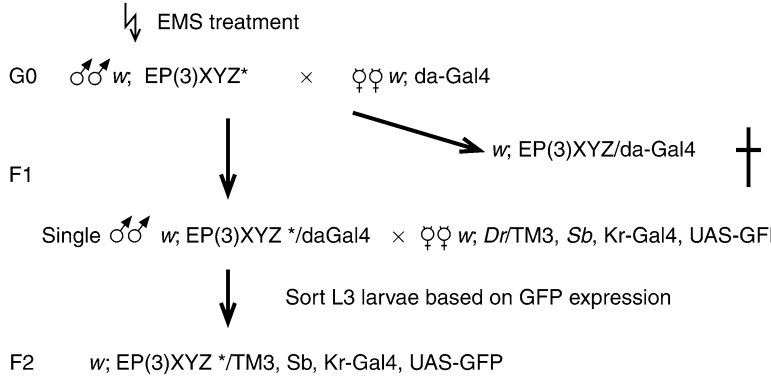

Fig. 8. Crossing scheme for a third chromosomal suppresssion of overexpression screen.

driver needs to be found that causes a scoreable overexpression phenotype. Inactivation of the endogenous gene should then suppress this overexpression effect. Most conveniently the overexpression phenotype is death: in this case all surviving flies of the cross are candidate mutants.

In the scheme in **Fig. 8** the ideal situation has been realized that both the Gal4 driver and the EP element are viable, and are both situated on the same chromosome. Thus, when males with the EY-carrying chromosome are mutagenized and then crossed to Gal4-expressing females, all surviving flies are either (1) candidate mutants, or (2) potentially interesting second site suppressors (that may affect functionally related genes), or (3) escapers. In case the overexpression is not tightly lethal, the latter class will probably be in the majority, and must be eliminated during retesting: true suppressor mutations will give a high frequency of survivors in the retest, whereas nonsuppressing escapers will again have only few surviving progeny.

The use of marked balancers expressing green fluorescent protein (GFP) under UAS control makes recovery of the mutagenized chromosome extremely easy. In this example, one would simply collect third instar F_2 larvae expressing GFP in the mottled larval Kr-Gal4 pattern, but discard both completely green siblings (which are expressing GFP under da-Gal4 control) and those not at all expressing GFP (the unbalanced sibs). GFP expression levels during recovery and retesting can also be used to exclude all candidates where the mutant chromosome affects the activity of the Gal4 system.

If either starting stock is homozygous lethal, balancer chromosomes will have to be removed, either genetically (e.g., using hs-hid, *see* **Subheading 3.4.**) or by sorting during the screening phase. Having balancer larvae around may even help the survival of the mutagenized escapers, which may be lost in a vial full of dead or dying siblings, especially if the overexpression is embryonic lethal. If the candidates are the only surviving larvae, it is advisable to remove

them from the vials containing the corpses, and transfer them to a more healthy environment. In addition, the balancer carrying siblings can be used to estimate the number of mutagenized genomes screened, by counting these progeny in a few sample vials, and taking into account the Mendelian ratio between balanced and overexpressing siblings. Newly induced suppressing mutations should then be identified by sequencing of the overexpressed candidate gene. The last part is then to clean the mutagenized chromosome. This again includes removing irrelevant parts of the mutagenized chromosome by recombination, and finally the mobilization and jumping out of the starting EP or EY element.

3.9. Mapping of EMS-Induced Point Mutations

Identification of the molecular lesions caused by the EMS treatment has been notoriously difficult. However, this has changed owing to the release of the genome sequence, molecular mapping strategies that complement the traditional genetic approaches, and by the massive drop in sequencing costs over the last few years.

The first step of mapping is already the retest: regardless of the specific type of screen, it is essential to ensure that the phenotype of interest is indeed linked to the recovered chromosome, and that this is also transmitted by the germline of the recovered fly. The next step is to ensure that there really is one and only one specific mutation on a recovered mutagenized chromosome that causes the scored phenotype. This is easy when multiple alleles are recovered from the screen: noncomplementation between independent alleles makes it unlikely that a given phenotype is synthetic, i.e., caused by a combination of mutations induced on the same chromosome (*see* **Note 3**). On the other hand, sometimes single alleles are of sufficient interest that one may want to continue working with these, even though mapping and further analysis are generally much more difficult.

Mutations that were isolated by F_3 lethal or allele screens and which therefore have a scoreable homozygous or transheterozygous phenotype, it is often worthwhile to screen for deficiencies that give the same phenotype when put *in trans* to the mutagenized chromosome. The Bloomington deficiency kit (available from the Bloomington Drosophila Stock Center at Indiana University, http://flystocks.bio.indiana.edu) consists of a collection of mostly overlapping large deficiencies that will allow mapping of the mutation to an interval of a few hundred kilobases to megabases within only a few dozen crosses. For finer mapping one can then use smaller deficiencies in the region of interest, many of which have been generated by Flp-FRT-mediated recombination ***(16,17)*** and therefore have molecularly characterized breakpoints. Alternatively, the genome information can be used to narrow down breakpoints of other deficiencies in the region. One quick way of doing this is through single embryo PCR ***(18)***, where amplicons uncovered by the deficiency fail to be amplified in the one quarter of embryos in the stock that is homozygous for the deficiency to be mapped.

However, many mutagenized chromosomes carry more than one lethal hit, and may therefore be lethal over several different deficiencies. In such cases meiotic recombination is required to separate the different lethal mutations, if the responsible region cannot be identified on the basis of independent alleles, or through a characteristic phenotype (e.g., in the cuticle of the transheterozygous embryos).

Meiotic recombination is also the method of choice when it is not known whether the mutation does itself have a homozygous phenotype, for example, when mapping suppressors of overexpression, or other modifiers of a sensitized background. It may also be the quicker approach in comparison with deficiency mapping when the original mutation was identified in clones. Meiotic mapping can either involve the classic recessive visible markers, which are available in many useful combinations, or rely on molecular markers such as PCR fragment length polymorphisms (PLPs), restriction fragment length polymorphisms (RFLPs), or single nucleotide polymorphisms (SNPs), or indeed, a combination of these ***(19–21)***. The initial mapping is most conveniently done using visible markers or PLPs (as these are most easily detected), especially when using isogenized starter and marker strains with previously known polymorphisms.

Whichever markers are used, the underlying principle remains the same: as outlined in **Fig. 9**, after recombination with a polymorphic marker chromosome the resulting recombinant chromosomes are tested whether they still cause the phenotype of interest, and which parts of these chromosomes are derived from the mutagenized starter chromosome. For FRT screens, there is an elegant variant to this basic approach ***(19)***. Here for mapping, clones are induced using many different chromosomes generated by recombination between the unmarked, mutagenized FRT starter chromosome and a tester chromosome that carries a distal w^+ marked P-element but no FRT site. Thus, in one step chromosomes can be identified in which a recombination event occurred between the FRT and the distal P-element (only those can give clones recognizable by their mottled orange eye color) and then be tested as to whether they still carry the mutation of interest (in which case the originally scored phenotype should reappear). The same paper also lists a set of useful starter/polymorphic marker chromosome pairs together with information on polymorphisms able to distinguish them.

Using such molecular approaches the mutation can quickly be narrowed down to several hundred kilobases. Further refinement can then be achieved by identifying additional SNPs between the mutagenized starter chromosome and the marker chromosome used for recombination. This is most easily done by sequencing of PCR products in the region of interest from starter/marker heterozygous flies: for sufficiently unrelated strains, most conveniently sized amplicons (500 bp–1 kb), especially those located in introns, should contain at least one or two SNPs (Sylke Winkler, personal communication, and ***[5]***). New SNPs in the region of interest can then be used to reanalyze those recombinant

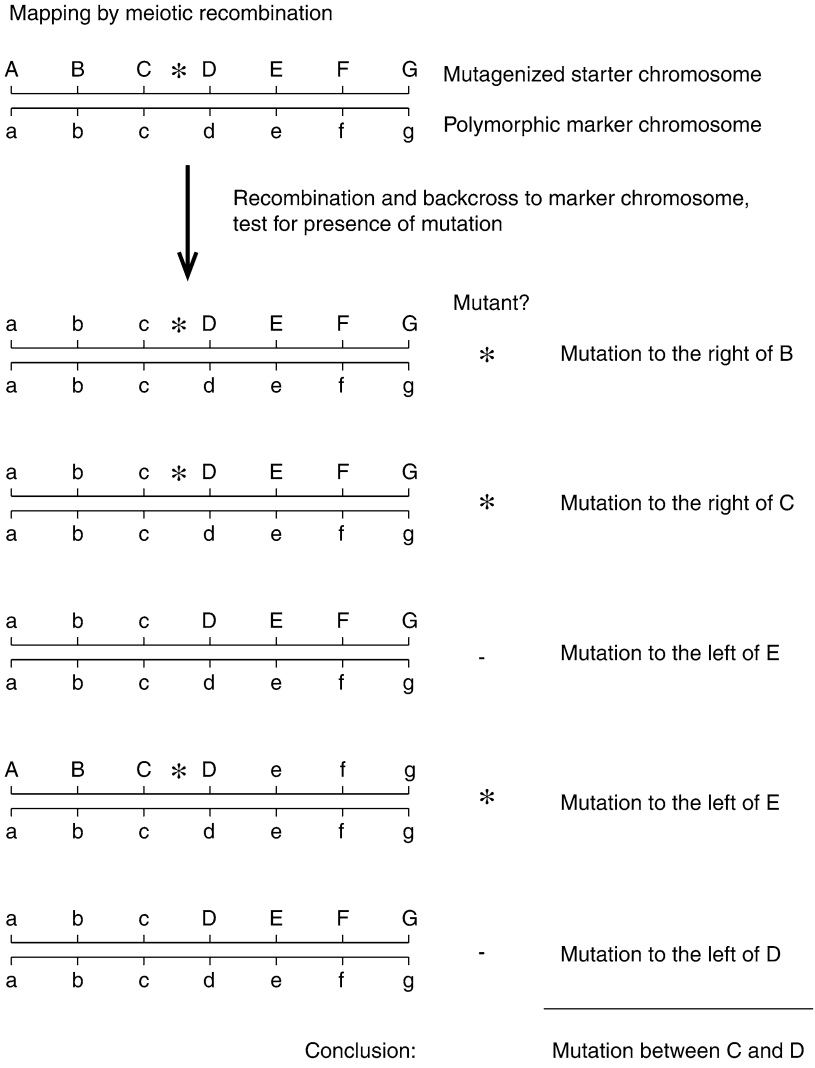

Fig. 9. Meiotic mapping of newly induced mutations.

chromosomes from the previous round of testing, which showed crossover events between the two closest PLPs or other markers flanking the mutation.

Generally, the easiest and in the meantime also cheapest way to detect SNPs or newly induced polymorphisms/mutations (which are literally also SNPs) is straight dye sequencing of PCR products from heterozygous flies, looking for characteristic double peaks in sequencing traces. Calling and flagging of the double peaks caused by each SNP can even be automated in sequence analysis

packages such as PolyPhred *(22)*. A useful protocol for fly DNA preparation and PCR can be found in **ref. *5***.

Once the candidate region has been sufficiently narrowed down, sequencing of candidate ORFs is often the fastest way forward. To save one generation, our experience with TILLING libraries *(5)* has shown that sequencing can effectively be done using PCR products from heterozygous, balanced flies without backcrossing to the isogenic starter stock. As aforementioned, polymorphisms also exist between the starter chromosome and the balancer used to recover the mutation. However, when several candidate lines are analyzed for each amplicon this presents no problem. Any such pre-existing polymorphisms will appear in all samples, whereas the specific, newly induced polymorphisms that include the sought after mutation will stand out.

4. Notes

1. On top of its acute toxicity EMS is a dangerous mutagenic, carcinogenic, and teratogenic substance also for humans. Unlike radioactive spills, EMS contamination is not conveniently detectable by monitoring devices. Any work involving EMS must therefore only proceed when appropriate measures to avoid contamination with EMS and to provide decontamination in the case of spills are in place. Beware that EMS readily evaporates, and that its half-life in water is more than 2 d at 25°C.

   The key elements are working under a closed chemical hood, wearing protective clothing and gloves, and the diligent decontamination of all equipment that has or might have directly or indirectly come into contact with EMS. The present protocol uses a 10% weight to volume aequous solution of sodium thiosulfate to inactivate any EMS, which significantly reduces the half-life of EMS in aequous solution *(2)*. However, some local authorites may mandate different procedures (e.g., the use of NaOH and thioglycolic acid). It is therefore advisable to consult the responsible safety officer before beginning with the EMS work.

   For the entire time of a mutagenesis project, one chemical hood should be set aside for the actual EMS work. This hood should be coated with one layer of Whatman paper or Benchcoat to soak up inadvertent spills. One or two (depending on the expected number of fly vials used for mutagenesis and recovery) chemical waste barrels half filled with inactivation solution should be placed under the hood. Each of these barrels should also contain a disposable plastic pipet with which contaminated vials can be pushed below the surface after disposal. In addition, one open beaker with inactivation solution should also be present under the hood, into which Kimwipes can be dipped before removing small spots of EMS contamination.

   It is advisable to wear double gloves, as the sharp edges of the plastic fly vials pose a risk of tearing the gloves when handled. The highest risk of contamination occurs at two steps: first, when the EMS solution is prepared and distributed, and second when the G_0 males are transferred into the recovery and food vials. For skilled fly handlers, it is possible to avoid the anesthesia step entirely, instead distributing the males through a funnel. In case the G_0 flies have to be handled on the

pad, it is essential to not use a mouth operated fly pooter. This still applies when removing the F_1 males 4 d later.

It is generally advisable to use only disposable equipment. If this is not possible, any equipment that was handled under the mutagenesis hood (e.g., trays, pipets, falcon racks, or fly pads) must be submerged overnight in inactivation solution before removal.

2. The initial number of mutagenized males will be reduced after every treatment step. Although most males should survive the starvation, EMS toxicity will kill up to 10% of flies. Owing to the partial sterility of the G_0 males, the number of F_1 males recovered may be significantly reduced in comparison with nonmutagenized control crosses. Finally, a significant fraction of these F_1 males will be sterile, especially if higher doses of EMS (e.g., 50 m*M*) are used.

   In a recent 25 m*M* overnight mutagenesis, we screened 12,000 F_2 lines starting with 1000 G_0 males, whereas at 50 m*M* EMS, 10,000 starting G_0 males only yielded 1915 stable F_2 lines ***(5)***. Increasing EMS levels therefore incurs significant tradeoff costs in terms of F_1 sterility that have to be set against the benefit of an increased number of mutations per chromosome. Similarly, longer exposures to EMS also give higher mutagenesis rates at lower fertility ***(2,6)***. We have found that 6 h and 12–14 h overnight exposure to EMS both give acceptable results.
3. Based on the measurement of the mutation frequency (*see* **Subheading 3.3.**) one can also estimate the saturation of a screen ***(5)***. Here, this term is used to indicate the likelihood of obtaining at least one inactivating hit in a given gene of interest. As there are approx 1000 genes per chromosome arm that are mutable to lethality, the number of lethal hits per arm (λ) is also equal to the average number N of hits per gene per 1000 genomes screened. Under the assumption of a Poisson distribution of hits the saturation *S* of a screen of n genomes is therefore calculated as

$$S = p(N \geq 1) = 1 - e^{-(n/1000 \times \lambda)}.$$

   Thus, for a standard mutagenesis rate of on average 1 lethal hit per arm, a collection of 1000 mutagenized genomes will with 63% likelihood contain at least one inactivating hit in a gene of interest. Increasing the number of genomes screened to 3000 brings this figure to 95%, and going to 10,000 genomes virtually ensures that the gene of interest has been hit (>99.9%). Similar estimates can also be made on the basis of the number of polymorphisms detected by molecular means after mutagenesis ***(3,5)***.

   However, one may wish to further increase the number of genomes screened to also ensure that genes are covered that present small targets for an EMS mutagenesis (so-called "cold spots"), for example, because of their small size or a low frequency of codons that can be mutated to a stop. Finally, in F_1 screens the number of genomes screened should be increased to compensate for candidate mutants lost owing to mosaicism.
4. Yeasted food vials are standard fly food vials supplemented by a small drop of a thick, toothpaste-like suspension of live yeast in water. Although preaging virgins on yeast will increase their fertility, males are not believed to consume live yeast after hatching, and should be kept on regular food.

5. A commonly used alternative way of dispersing EMS in the sucrose solution is by repeated aspiration and ejection from a syringe. Whereas this undoubtedly is effective, unnecessary generation of EMS-containing aerosols should be avoided.
6. It has been reported (Stefan Luschnig, personal communication) that, letting the mutagenized males recover for 24 h in food vials before mating significantly improves their fertility, thus lowering the required numbers of G_0 males and virgins.
7. Unlike ionizing radiation alkylating agents such as EMS generally affect only one strand of the mutagenized chromosome. If this lesion is not repaired before the first embryonic cleavage (i.e., during spermatogenesis) but later, the resulting F_1 fly will be genetically mosaic. Whereas the presence of the mutation in a part of the somatic cells may be sufficient to identify the candidate mutant, there is no guarantee that the germline of the F_1 fly will share that genotype, in fact it may even be mosaic by itself. The likelihood that candidates fail to retest positively depends on the size of the target tissue where the phenotype is scored, and its distance form the germline in the embryonic fate map. Often this fraction will be around 10–30%, but may for some screens reach 50%.

Acknowledgments

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8

RNAi Screening in Cultured *Drosophila* Cells

Sandra Steinbrink and Michael Boutros

Summary

RNA interference (RNAi) has become a widely used tool to analyze biological functions in vivo and in vitro. With the availability of an increasing number of *Drosophila* cell lines, a variety of different processes can be studied ranging from cell cycle control defects to signaling pathway activities and changes in cell morphology. Owing to the ease of RNAi in *Drosophila* cells, this experimental system has become a preferred method to screen for novel cellular factors, before their in depth analysis. We here describe the experimental procedures for RNAi experiments in cultured *Drosophila* cells, starting from the design of long double-stranded RNAs, their synthesis by in vitro transcription and application for cell-based RNAi experiments from low to high-throughput formats. Finally, we show how phenotype analysis can be performed using cell-based assays for luminescence or flow cytometric analysis as examples.

Key Words: *Drosophila* cells; high-throughput screening; phenotypic readouts; RNAi; double-stranded RNA; cell-based assays.

1. Introduction

RNA interference (RNAi) uses an endogenous cellular mechanism for the degradation of target mRNAs and is triggered through the introduction of homologous double-stranded (ds) RNAs into cells. In animals, RNAi was discovered by Fire and Mello in 1998 when they observed that injection of dsRNAs in *Caenorhabditis elegans* is several 10-fold more efficient in silencing homologous mRNA sequences than single-stranded antisense RNAs ***(1)***. Later, analogous experiments performed in *Drosophila* demonstrated a similar efficiency of target mRNA silencing after injection of dsRNA into embryos ***(2)*** or simply after addition of dsRNA to the culture medium of cell lines ***(3)***.

Since then, RNAi in *Drosophila* has been used as a tool to dissect a variety of cellular processes using microscopy, flow cytometry, or chemiluminescence as cell-based assays ***(4–6)***. Such experiments have been performed both

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using genome-wide RNAi libraries that have become available for *Drosophila*, or limited sets focusing on particular functional groups or homologous genes ***(7–10)***.

Drosophila cell-based assays are widely used because the genome is well-annotated and many cellular pathways are highly conserved. Similar to genetic approaches, RNAi experiments are helped by the fact that *Drosophila* lacks a redundancy often found in mammalian genomes. For example, depleting Dishevelled (Dsh) by RNAi is sufficient to fully recapitulate a Wingless (Wg) loss-of-function phenotype, whereas it is necessary to deplete all three homologs (Dvl1-3) in human cells to observe a Wnt phenotype. More than 20 genome-wide (or genome-scale) screens in *Drosophila* cells have been published since 2003, and an important challenge remains to integrate and compare different data sets.

Although RNAi in cultured cells have become a powerful tool both in small- and large-scale experiments, it is also important to be aware of potential pitfalls and problems. RNAi in model organisms is very efficient because of "natural" pooling of many 21 nt sequences. Long dsRNAs are intracellularly cleaved into 21–22 nt siRNAs by Dicer and direct the degradation of target mRNAs through the RNA-induced silencing complex. Although dsRNAs should be designed in order to match to one specific gene, off-target effects can occur if siRNAs have sequence homology to genes that are not supposed to be targeted. Furthermore, the knockdown of target transcripts might differ depending on the efficiency of siRNAs derived from long ds RNAs.

In this chapter, we provide protocols for RNAi experiments in cultured *Drosophila* cells. We describe the primer design for dsRNA templates, the generation of polymerase chain reaction (PCR) products suitable for in vitro transcription, the in vitro transcription procedure, and the purification step. Finally, we outline how to perform RNAi cell culture experiments both in low- and high-throughput formats.

2. Materials

2.1. Cell Culture

1. Schneider's *Drosophila* Medium with L-Glutamine (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) Gold Category "EU" (PAA), 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen) (*see* **Note 1**).
2. Phosphate-buffered saline (PBS), pH 7.4 (Invitrogen).
3. Solution of trypsin (0.25%) and 1 m*M* ethylenediamine tetraacetic acid (EDTA) (Invitrogen).
4. Cell scraper (Biochrom, Berlin, Germany or similar).
5. Trypan Blue (Invitrogen).

2.2. Polymerase Chain Reaction

1. 10X PCR buffer ***(11)***: 500 m*M* KCl, 100 m*M* Tris-HCl (pH 8.3) and 15 m*M* $MgCl_2$. Aliquot and store at −20°C. Alternatively, use commercially available PCR buffers.
2. Primers (if required, with fused tags or T7-promotor sequences).
3. dNTPs, 10 m*M* stocks (Sigma-Aldrich, St. Louis, MO).
4. cDNA or genomic DNA as template.
5. Taq DNA polymerase: Taq Polymerase, no. 201203 (Qiagen, Hilden, Germany), GeneChoice Taq, no. 62-6086-01 (PGC Scientific, San Diego, CA), TaqPlus, no. 600210 (Stratagen, La Jolla, CA), or Herculase, no. NC9690330 (Stratagene).

2.3. In Vitro Transcription

1. In vitro transcription kit: MEGAscript T7 kit, no. AM1333 (Ambion, Austin, TX), T7 RiboMAX, no. TB316 (Promega, Madison, WI).
2. DNaseI (Fermentas, Burlington, Canada).

2.4. Purification of dsRNA

1. RNA purification kit: RNeasy Kit, no. 74104 (Qiagen), NucAway Spin Columns, AM10070 (Ambion).
2. For 96-well purification: multiscreen PCR plates (Millipore, Billerica, MA, no. MANU03050).
3. Ethanol, absolute.
4. 5 *M* NH_4OAH (Ambion).
5. 1 *M* Tris-HCl (pH 7.0) (Ambion).
6. Aluminum-seals (Corning, Corning, NY, no. 6569).

2.5. RNA Quality Control

1. 2X RNA gel-loading buffer ***(11)***: 95% (v/v) deionized formamide, 0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol FF Roth, Karlsruhe, Germany, 5 m*M* EDTA, pH 8.0, 0.025% (w/v) sodium dodecyl sulfate.
2. TAE-buffer: 40 m*M* Tris Base, 1 m*M* EDTA, 20 m*M* glacial acetic acid. Adjust pH to 8.0.
3. Agarose gel: 1% agarose (Invitrogen) in TAE-buffer, 1:20,000 ethidium bromide (1% stock solution, Roth).

2.6. RNAi Experiments

2.6.1. RNAi by Bathing

1. Serum-free medium: Schneider's *Drosophila* Medium with L-Glutamine (Invitrogen) supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen).
2. Nuclease-free H_2O (Acros Organics, Geel, Belgium).

2.6.2. Liposomal Transfection

1. Liposomal transfection reagents: effectene (Qiagen), cellfectin (Invitrogen).

2.7. Flow Cytometry

1. Ice cold 70% ethanol ((20°C).
2. PBS for washing steps.
3. Staining solution: PBS containing 50 µg/mL pancreatic RNaseA (Sigma) and 50 µg/ mL propidium iodide (Calbiochem, San Diego, CA) (*see* **Note 2**).
4. 24-Multichannelwand (V&P Scientific, San Diego, CA).
5. A 96-well plate with a U-shaped bottom (Greiner, Frickenhausen, Germany).

3. Methods

3.1. Design of dsRNAs

1. DNA-templates for the generation of dsRNA are generated by PCR using cDNA (e.g., expressed sequence tags), first-strand reverse transcription-cDNA, or genomic DNA as a template. Most of the dsRNAs should correspond to exonic sequence, but dsRNA sequences including two or more exons interspersed by small introns also work efficiently. DsRNAs targeting the 3′ and the 5′ untranslated regions of a transcript are preferred target sites when an RNAi phenotype should be rescued by introduction of the wild-type gene that lacks the untranslated regions. Such rescue experiments can be used to demonstrate that an observed RNAi phenotype is specific and not caused by potential off-target effects.
2. We generally aim to generate dsRNA of approx 500 bp in length, although dsRNA probes ranging from 150–3000 bp in length have been efficient for silencing. Silencing with smaller products often yields less efficient knockdowns.
3. One should avoid target region that are homologous to other genes. If possible, the dsRNA should not contain any 19-mer homology to any gene other than the gene of interest, which might otherwise lead to off-target effect. Also, regions which are of low-complexity, for example, caused by glutamine-repeats should be avoided ***(12,13)***.
4. It is recommended to search the various sequence sources (Publications, National Center for Biotechnology Information and Berkeley Drosophila Genome Project genomic and expressed sequence tags) to confirm the primary sequence of a given gene.
5. For the automated design of long dsRNA sequences (RNAi probes) and the corresponding primers for the amplification from genomic DNA or cDNA by PCR, we recommend the E-RNAi website ***(14)***. The E-RNAi webservice automates all tasks required for the design of long dsRNAs, including (1) the identification of the targeted transcript, (2) in silico dicing of the template sequence into all possible siRNAs, (3) calculation of RNAi efficiency of each in silico-diced siRNA, (4) identification of sequences (siRNAs) that potentially target additional genes, and (5) design of optimized PCR primers to amplify dsRNA templates directly from genomic DNA or cDNA using a built-in implementation of the primer3 software ***(15)***. The webservice is accessible via http://e-rnai.dkfz.de/.
6. Another option is to retrieve sequences from previously published RNAi libraries that target different gene regions and have been used in published screens. Several databases have collected target regions and associated phenotypes. Both GenomeRNAi ***(16)*** and FLIGHT ***(17)*** databases contain a large collection of dsRNA target regions and associated phenotypes.

3.2. Generation of In Vitro Transcription Templates (Amplicons) by PCR

3.2.1. One-Step Generation of Amplicons

To generate PCR products, which can be used for production of dsRNA by in vitro transcription, T7-promoter sites must be fused to the gene-specific primers in order for a T7 RNA polymerase-based in vitro transcription reaction to generate dsRNA. When using the "E-RNAi" webservice the T7-promoter sites (or alternatively SP6) can directly be added to the primer sequences.

1. Perform a standard 100-µL PCR reaction using the gene-specific primer pair flanked by the T7 promoter sequences to amplify the region of interest. Use 20 pmol primer, 200 ng DNA as template, 40 µ*M* of each dNTP, and 2.5 U Taq DNA polymerase. We have successfully used Taq DNA Polymerase GeneChoice Taq, TaqPlus, and Herculase to amplify PCR products in sufficient quantity for high-yield in vitro transcription reactions.
2. Run the PCR reaction in a thermal cycler for 5 min at 94°C, followed by 30 cycles of denaturation at 94°C for 45 s, annealing for 30 s at 57°C, and elongation at 72°C for 45 s. End the program with an elongation step for 10 min at 72°C. The annealing temperature and the elongation time can vary according to the template length and the Taq polymerase used.
3. Check the PCR product on a 1% agarose gel containing ethidium bromide to ensure that a single product of the expected size has been obtained.

3.2.2. Two-Step Generation of Amplicons

When generating dsRNAs in a high-throughput format for genome-wide libraries or subsets, a two-step procedure has been used which has the advantage of normalizing the amount of PCR product for subsequent in vitro transcription reactions and preventing potential cross-contamination through the use of varying adaptor tag sequences. To prevent well to well cross-contamination, a two-step PCR can be performed by creating a unique adaptor "neighborhood" for each amplicon by varying tag-adaptors from well to well ***(4,18)***. In the first step, approx 500-bp long fragments are amplified from genomic DNA with the use of gene-specific primer pairs that have the tag-adaptors sequences fused. Thereby a forward primer with a unique tag can be combined with reverse primers carrying one of nine different tags. A second PCR with tag-specific primers allows for efficient reamplification of the amplicon set. This PCR product can then be used for production of dsRNA by in vitro transcription. Therefore, T7-promoter sites are merged with tag-specific primers that will enable the T7 RNA polymerase to bind to the PCR product and generate RNA. For amplification scheme *see* also **Fig. 1**.

1. For a 50 µL reaction, use 100 ng of genomic DNA, 1X PCR buffer, 40 µ*M* of each dNTP, 1 U Taq polymerase (Qiagen), and 20 pmol of each gene-specific primer merged to the tag-adaptor sequences (other Taq polymerases have also been successfully used).

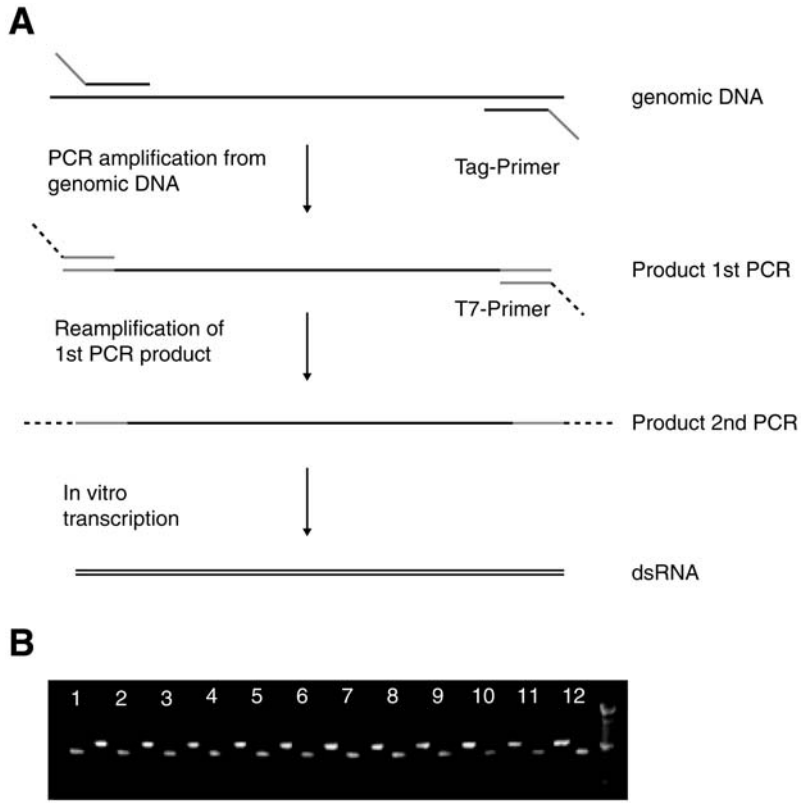


Fig. 1. High-throughput generation of dsRNA libraries by two-step PCR and in vitro transcription. **(A)** Two-step PCRs are performed for normalizing product quantities and to avoid cross-contaminations between wells by using a unique tag environment for each well. In the first PCR, a gene specific product is synthesized from genomic DNA with the help of tag-primers. The primers have one of 10 different tag sequences fused, which allows to use primers specifically amplifying these tag-sequences in the second round of PCR. These in turn are linked to T7-sequences to enable an in vitro transcription with T7-RNA polymerase. **(B)** RNA from an in vitro transcription. In the example shown here, two rows of a 96-well plate are loaded with a multichannel pipet in every other well. One row contains RNAs of smaller size, so that a cross-contamination would become visible as a change in the pattern.

2. Use a thermal cycler and incubate the plates for 5 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealing for 30 s at 55°C, and an elongation step at 72°C for 90 s. During the last 20 cycles the elongation time is extended by 5 s for each cycle.
3. For the second amplification step, a 100 µL PCR reaction is setup with 1 µL product of the first PCR as the template, 50 pmol of each T7-tag-primer, 100 µ*M* of each dNTP, 1X PCR-buffer, and 2 U Taq polymerase.

4. Run the PCR in a thermal cycler for 5 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealing for 30 s at 60°C, and elongation at 72°C for 90 s. Prolong the elongation time in the last 20 cycles by 5 s in each cycle. Conclude the program with 15 min at 72°C and let the reaction cool down to 4°C.
5. Check the PCR product on a 1% agarose gel containing ethidium bromide to ensure that a single product of the expected size has been obtained.

3.3. In Vitro Transcription of dsRNA

1. We have successfully used different high-yield in vitro transcription kits to generate dsRNAs, such as the Ambion MEGAscript T7 kit, T7 RiboMAX, and homemade kits. Follow the manufacturer's protocol to perform the T7 (or SP6) in vitro transcription reaction. It is not necessary to purify the PCR-product before in vitro transcription. 0.5–1 μg PCR product is used as a template.
2. Incubate the reaction in a heat block or thermocycler at 37°C for 4–16 h. Transcription and annealing occur simultaneously and no additional step is required to anneal the two complementary RNA-strands. To generate high amount of dsRNA, it might be necessary to scale the reaction up.
3. Following incubation, degrade the DNA template by adding 1 U DNaseI to the reaction mixture, and incubate for 30 min at room temperature.

3.4. Purification of dsRNAs

3.4.1. Column Purification (Suitable for Low-Throughput)

1. Purify dsRNAs using Qiagen's RNeasy or Ambion's NucAway Spin Columns. When using Qiagen's RNeasy columns, follow the RNA cleanup protocol. Up to 100 μg of RNA longer than 200 bp can be bound to the column, purified, and eluted afterward. We usually elute twice to maximize recovery.
2. For larger synthesis reactions (>100 μg), divide the sample and purify in two or more columns in order to not overload a single column. For synthesis 96-well plates, we have successfully used multiscreen PCR plates to purify the dsRNAs.
3. Measure the optical density (OD) of a 1:50 dilution at 260 nm (OD_{260}). Multiply the OD_{260} by the dilution factor and an extinction coefficient of 45 (dsRNA concentration = $OD_{260} \times$ dilution factor $\times$ 45). The standard yield of 20 μL reaction is 80–200 μg.

3.4.2. Ethanol Precipitation in 96-Well Plates (Suitable for High-Throughput)

For large-scale purification of RNA in vitro transcriptions, precipitation with ethanol is a suitable method.

1. Add 0.1 volumes of 5 *M* NH_4OAH and 2.5 volumes of absolute ethanol to the transcription reaction.
2. Seal the 96-well plates with aluminum-seals, mix by vortexing, and incubate at –20°C for at least 12 h.
3. Centrifuge the plates at 4°C and 3000*g* for 40–50 min.

4. Remove ethanol by turning the plate upside down and carefully drying the surface with a clean paper towel. Dry plates in the laminar flow of a sterile hood for 3 h until pellets become transparent.
5. Dissolve pellets in 100 µL nuclease-free 1 *M* Tris-HCl (pH 7.0) and vortex for 10 min.

3.5. Determination of dsRNA Quality and Concentration

The purified dsRNA has to be checked for quality and correct size.

1. Mix 5–10 µL of a 1:20 dilution of the in vitro transcription reaction with 2X RNA gel-loading buffer and heat to 65°C for 5 min to reduce secondary structures in the RNA. Load on a 1% ethidium bromide agarose gel (TAE-buffer) to check for correct size of the transcript (*see* **Note 3**).
2. Cover plates with aluminium-seals and freeze them at –20°C or –80°C. Store dsRNAs in original in vitro transcription plates or aliquot them ready to use in assay plates.

3.6. RNAi Experiments in Drosophila Cells—General Considerations

Experiments using RNAi as a technique to silence the expression of genes are dependent on a number of experimental parameters. One of the most important factors to consider is the cell line to be used in such experiments. Depending on the biological questions, different cell types are available. *Drosophila* cell lines differ significantly in their growth behavior, their phenotypes, and also their ability to take up dsRNAs. Available *Drosophila* cell lines and their handling are excellently reviewed in Chapter 25. Most cell lines derived from embryonic tissues have the ability to autonomously take up long dsRNAs, whereas cells derived from imaginal discs such as cl-8 require transfection protocols. Both "bathing" and "transfection" protocols can be done in small scale as well as in high-throughput experiments (**Table 1–2**).

3.6.1. Small-Scale RNAi (6-Well) by Bathing

Drosophila cells should be passaged using a strict splitting regime to maintain a constant growth rate and reproducible experimental results. Cells can be kept in disposable plastic cell culture flasks with standard screw caps and are maintained in a cell culture incubator at 25°C without CO_2. Loosely adherent cells can be detached from the plastic by carefully shaking the flask or pipeting the media over the cell layer. More adherent cell types can be detached with a plastic scraper. Cells are split when almost approaching confluency. Depending on the cell line and its growth rate, a certain amount of cells (approximately a third to a fifth of the flask) is transferred to a new flask containing fresh culture medium.

1. Cell cultures that are confluent on the day of transfection or show an abnormal shape should be avoided. Both confluent and "stressed" cells show a lower abilility to take up dsRNA and a lower transfection efficiency.
2. In 6-well tissue culture plates 15 µg dsRNA/well are added before cell seeding.

Table 1
Summary of RNAi Protocol in 6–384-Well Plates

	6-Well	96-Well	384-Well
Serum-free medium containing cells (mL)	1	0.04	0.02
dsRNA (μg)	15	1	0.25
Serum-containing medium (mL)	1	0.05	0.03

Table 2
T7-Primers Used to Generate dsRNA by PCR and In Vitro Transcription for Experiments[a]

Targeted gene	Forward/ reverse	Tag	Primer-sequence
Cyclin E	For	T7	*taatacgactcactatagg*tgcgtcttctgcaccttatg
Cyclin E	Rev	T7	*taatacgactcactatagg*atgacctttggacgacagc
Th/DIAP1	For	T7	*taatacgactcactatagg*ccggcatgtacttcacacac
Th/DIAP1	Rev	T7	*taatacgactcactatagg*gaatcggcactgacttagcc
Anillin	For	T7	*taatacgactcactatagg*cgttgaattccaaaccgaat
Anillin	Rev	T7	*taatacgactcactatagg*aaaatggcaaagccgtattg
EGFP	For	T7	*taatacgactcactatagg*accctcgtgaccaccctgacctac
EGFP	Rev	T7	*taatacgactcactatagg*ggaccatgtgatcgcgcttctcgt
FLuc	For	T7	*taatacgactcactatagg*ggggaagaacgccaaaaac
FLuc	Rev	T7	*taatacgactcactatagg*gctctggcacaaaatcg

For, forward; Rev, reverse.
T7 promoter sequences are given in italics.
[a]Shown in Fig. 2.

3. Cells are scraped from the cell culture flask, and a sample is stained for viability with Trypan Blue and counted with a Neubauer Hemocytometer (Brand, Wertheim, Germany).
4. Cells are pelleted at 300*g* for 5 min and subsequently resuspended in serum-free medium at 1–5 × 10^6 cells/mL. Cell should not be kept for an extended period of time (>1 h) in serum-free medium.
5. 1 mL of the cell suspension is plated into each well together with the dsRNA. Alternatively, cells can be seeded first and dsRNAs added in a second step.
6. The cells are incubated with the dsRNA at room temperature for 30–45 min (bathing procedure).
7. 2 mL cell culture medium, containing 10% FBS, is added to each well.
8. Depending on the assay, the cells are incubated for 2–6 d and subsequently processed depending on the cellular assay (*see* **Note 4**).

3.6.2. Small-Scale RNAi (6-Well) Using Liposomal Transfection

1. For transfection of dsRNA or DNA in cells we recommend using liposomal transfection reagents (effectene, cellfectin) according to the manufacturer's protocol.
2. If a DNA reporter system has to be introduced into the cells, cells can be batch-transfected with the reporter plasmids before "bathing" in dsRNA. Alternatively, the reporter can be transfected together with the dsRNA or subsequently to RNAi. This is dependent on the assay and has to be optimized individually.

3.6.3. Medium-Throughput RNAi (96-Well Plate) by Bathing

1. Long dsRNAs are diluted in nuclease-free water to a final concentration of 100 µg/mL. 1 µg RNA (in a volume of <10 µL) are transferred to each well of 96-well tissue culture plate.
2. Cells are scraped of the cell culture flask and a sample is stained for viability with Trypan Blue and counted with a Neubauer Hemocytometer.
3. Cells are pelleted at 300*g* for 5 min and afterward resuspended in serum-free medium at 1–5 × 10^6 cells/mL.
4. 40 µL of the cell suspension are transferred to each well and incubated for 30–45 min at room temperature (bathing procedure).
5. 50 µL cell culture medium containing 10% FBS are added to each well.
6. Plates can be sealed with aluminium-seals to prevent evaporation.
7. Depending on the assay, incubate the 96-well plate for 4–6 d at 25°C before the analysis of protocols.

3.6.4. High-Throughput RNAi (384-Well Plates) by Bathing

For high-throughput experiment we usually aliquot dsRNAs at working concentrations in assay plates to reduce experimental handling. Assay plates are sealed and stored at –80°C. Each well should contain 5 µL of a 0.05 µg/µL dsRNA solution for a final amount of approx 0.25 µg dsRNA per well (*see* **Note 5**).

1. Spin plates at 500*g* for 1 min and clean the surface and the bottom with 70% ethanol before removing the aluminium-seals.
2. Cells are removed from the cells culture flask, a sample is stained for viability with Trypan Blue and counted with a Neubauer Hemocytometer.
3. Cells are pelleted at 300*g* for 5 min and afterward resupended in serum-free medium at 1–5 × 10^6 cells/mL.
4. 20 µL of the cell suspension are plated into each well of a 384-well assay plate, containing dsRNA using a liquid dispensing device, such as a MultiDrop (Thermo Fisher Scientific, Waltham, MA).
5. Incubate at room temperature for 30–45 min (bathing procedure).
6. 30 µL of serum-containing cell culture medium is added to each well.
7. Plates are sealed with aluminium-seals to prevent evaporation.
8. Depending on the assay, plates are incubated 3–5 d at 25°C and analyzed by platereader (Mithras LB940, Berthold Technologies, Bad Wildbad, Germany) or microscopy.

3.7. Phenotypic Readouts

3.7.1. Homogenous Luminescence Assays

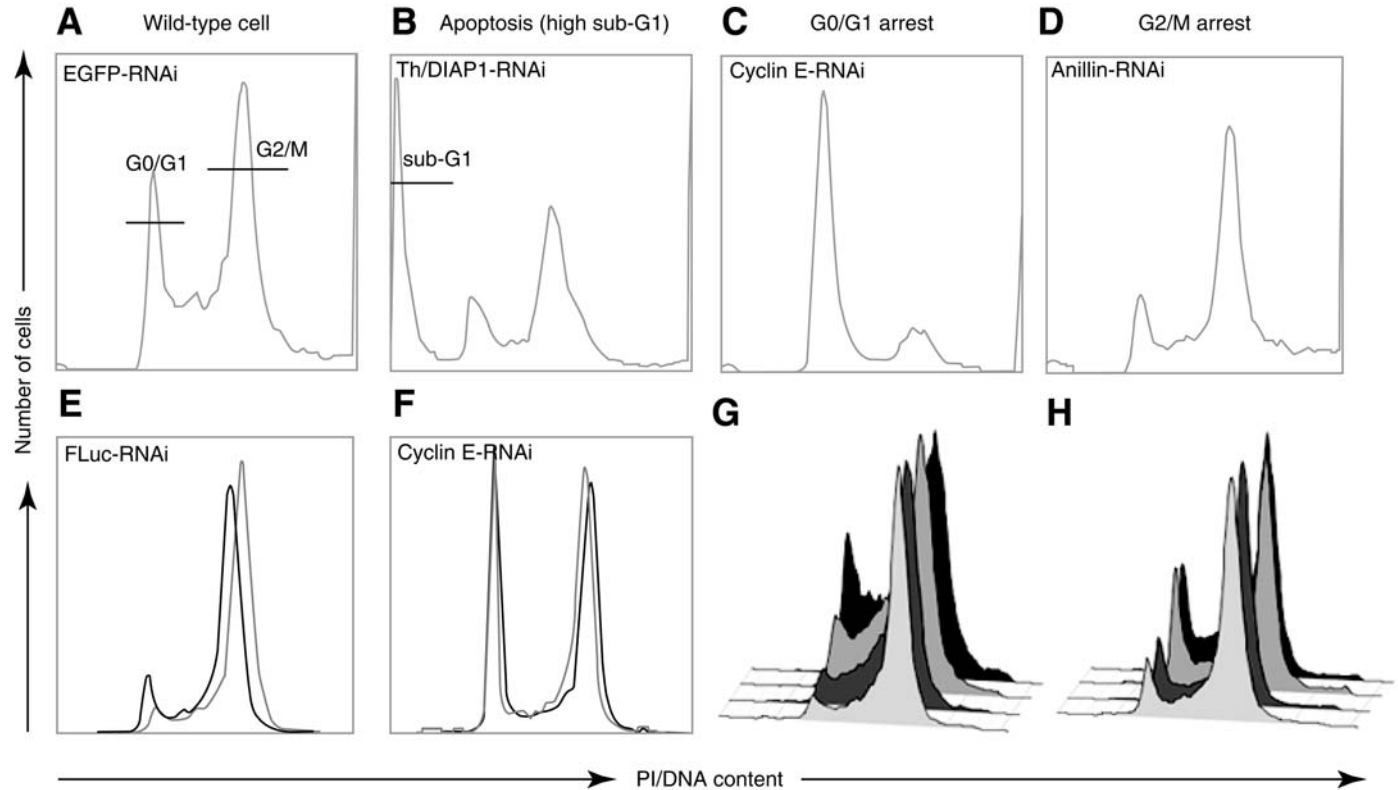
Homogenous assays measure phenotypes across the whole population of cell in a single well. These are usually end point assays, which allow for high-throughtput screening approaches. Typical examples are cell growth, proliferation, and survival assays with a broad phenotypic readout to analyze the effect of dsRNAs alone or in modifier assays. Homogenous assays are also often used for the analysis of signaling pathways using reporter gene assays. For the analysis of cell growth and viability phenotypes, commercially available reagents, such as the CellTiterGlo (Promega) can be adapted to a high-throughput format. Luminescence or fluorescence can be measured by automated multilabel plate reader (such as Mithras LB940, Berthold Technologies, Bad Wildbad, Germany). Homogenous assays are suitable for screening in 96-well as well as the 384-well formats.

1. RNAi treatments are performed as described in **Subheading 3.6.4.** with 0.25 µg dsRNA per well in white 384-well LIA plates (Greiner).
2. The assay plates are incubated for 3–5 d, depending on the cell line.
3. On the day of the readout, the assay plates are spun for 5 min at 300*g* and the supernatant is carefully removed with a 24-multichannelwand.
4. The cells are lysed and treated according to manufacturer's instruction. For CellTiterGlo viability measurements, cells are incubated for 15 min with the reagents before luminescence readouts (Mithras LB940 or similar, Berthold Technologies).
5. Data can be analyzed using the software package "cellHTS," which has been developed for the analysis of high-throughput cell-based assays (http://www.dkfz.de/signaling/cellHTS) ***(19)***. This software package is based on "Bioconductor/R" a free platform for statistical data analysis (http://www.bioconductor.org/).

3.7.2. Flow Cytometric Analysis

High content readouts such as flow cytometric analysis of cell populations can be used to determine a potential cell cycle arrest point, induction of apoptosis or signaling by use of specific antibodies. To analyze depletion of genes involved in cell cycle regulation and cell growth, potential cell cycle phenotypes can be analyzed by flow cytometry after fixation and DNA staining. Flow cytometric analysis can also be used to determine phenotypes such as an altered cell size and morphology **(Fig. 2)**. A more detailed description of flow cytometric analysis of *Drosophila* cells can also be found in Chapter 24.

1. Conduct RNAi-treatment with *Drosophila* cells as described under **Subheading 3.6.3.** with 1 µg dsRNA per well in 96-well flat-bottom cell culture plates (*see* also **Table 2**).
2. Incubate the plates for 4–6 d, depending on the cell line.


A
Wild-type cell
EGFP-RNAi
G0/G1
G2/M
B
Apoptosis (high sub-G1)
Th/DIAP1-RNAi
sub-G1
C
G0/G1 arrest
Cyclin E-RNAi
D
G2/M arrest
Anillin-RNAi
Number of cells
E
FLuc-RNAi
F
Cyclin E-RNAi
G
H
PI/DNA content

3. Cells are harvested by taking from the cell culture supernatant, washing the cells with PBS and incubating them with Trypsin/EDTA solution at room temperature for 10–20 min. Supernatants and PBS from washing steps are collected in a separate 96-well plate with a U-shaped bottom (*see* **Note 6**).
4. After detachment of the cells (check for completeness with microscope) the collected cell culture supernatant is used for resuspension of the trypsinized cells in order to inhibit trypsin activity by the FBS in the medium. The cells are collected in U-shaped cell culture plates, and are then forwarded to the fixation, staining, and flow cytometry procedure (*see* **Note 7**).
5. Cells are pelleted by centrifugation at 300*g* for 5 min.
6. Carefully remove the supernatant with a 24-multichannelwand.
7. Cells are fixed with ice cold 70% ethanol and incubated overnight at –20°C. Cells can be stored for several weeks at this point.
8. Fixed cells are pelleted in a cooled centrifuge at 400*g* for 10 min.
9. To remove remaining ethanol, cells are washed once with PBS.
10. Cells are resuspended in propidium iodide staining solution and incubated at 37°C in the dark for 2 h. Plates are stored afterward at 4°C in the dark until flow cytometry analysis.
11. The DNA content of the fixed cells is analyzed on a flow cytometer suitable for 96-well plates (BD FACSArray Bioanalyzer System, BD Biosciences, San Jose, CA).
12. Use the BD FACSArray System software (BD Biosciences) or FlowJo analysis software (http://www.flowjo.com) to analyze and quantify the cell cycle stages **(Fig. 2)**.

Fig. 2. *(Opposite page)* FACS analysis of cell cycle phenotypes. Cells were incubated for 1 h in dsRNA with and without serum starvation. After 4 d, cells were fixed in ethanol, subjected to RNaseA treatment and propidium iodide DNA-staining and analyzed using a BD FACSArray flow cytometer and FlowJo analysis software (http://www. flowjo.com). T7-primers used to generate dsRNA by PCR and in vitro transcription are shown in **Table 2**. **(A–D)** Cell cycle of SL2 cells shows different phenotypes on knockdown of the indicated cell cycle transcripts. Viability defects with a high amount of cells in sub-G1-phase are shown in (B), cell cycle arrests in G0/G1 (C) and G2/M-phase (D) can be detected. **(E,F)** Serum starvation during the RNAi bathing procedure has only little effect on RNAi-phenotypes in *Drosophila* Kc_{167} cells. The changes in the cell cycle of cells treated with (gray line) and without serum starvation (dark line) are similar in the control (dsRNA targeting *firefly luciferase*, FLuc) (E) as well as after knockdown of the cell cycle regulator Cyclin E (F). **(G,H)** Influence of serum starvation and cell seeding density on the cell cycle of *Drosophila* Kc_{167} cells. The cell cycle of cell lines is sensitive to changes in cell density and use of serum starvation during the RNAi bathing procedure. Cells were seeded in different densities (from back to front: 50,000, 40,000, 30,000, and 20,000 cells per well, respectively) and treated with enhanced green fluorescent protein-targeting dsRNA for 4 d with (G) and without serum starvation during the RNAi bathing procedure (H). On serum starvation the cell cycle is more diffuse and shows an increased S phase content.

4. Notes

1. Testing serum batches before use in *Drosophila* cell culture experiments has been shown to be crucial. Some *Drosophila* cell lines appear to be much more sensitive to changes in sera than many mammalian cell lines. We recommend to carefully test new batches of sera using growth curves and monitoring cell morphology to exclude possible detrimental effects. There also have been reports that batches of Schneider medium (Invitrogen) lead to loss of RNAi-competency.
2. We boil the RNase A before use, to deplete DNase activity caused by contaminations. A stock solution of 10 mg/mL in 10 m*M* sodium acetate (pH 5.2) is prepared and heated to 100°C for 15 min to inactivate the DNase. Adjust the pH to 7.4 using 1 *M* Tris/HCl (pH 7.4), aliquot, and store at (–20°C. The propidium iodide is prepared as a solution of 1 mg/mL in double-distilled water, sterile filtered and stored at 4°C.
3. During ethanol purification of in vitro transcriptions, nucleotides are coprecipitated with the dsRNA and spectrophotometric quantifications are not exact. An additional sample of defined amount (dsRNA, purified with column-precipitation and quantified by photometry, e.g., 100 ng) should therefore be loaded on the gel to estimate the concentration of the ethanol-precipitated dsRNA samples.
4. The incubation time of the RNA treatment can vary depending on the biological question of the assay, the cell line used, and the readout system. Because of differences in growth rates of the cells and turnover times of the protein-equipment, the RNAi effect of different genes becomes visible at different time-points. Therefore, the end point of the assay has to be carefully determined and one should keep in mind it is not possible to end the assay at a time which is perfect for all of the genes.
5. We recommend using an automated dispensing system for seeding the cells and distributing media in 384-well plates.
6. Use multichannel pipets or automated pipeting robots for preparing the cells for flow cytometry.
7. If cells are clumpy raise the EDTA content of the trypsin/EDTA-solution and of the washing buffers to 5 m*M*. This helps separating the cells for flow cytometric analysis.

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9

Methods for Homologous Recombination in *Drosophila*

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Summary

We present detailed protocols for two methods of gene targeting in *Drosophila*. The first, ends-out targeting, is identical in concept to gene replacement techniques used routinely in mammalian and yeast cells. In *Drosophila*, the targeted gene is replaced by the marker gene *white*$^+$ (although options exist to generate unmarked targeted alleles). This approach is simple in both the molecular cloning and the genetic manipulations. Ends-out will likely serve most investigators' purposes to generate simple gene deletions or reporter gene "knock-ins."

The second method, ends-in targeting, targets a wild-type gene with an engineered mutated copy and generates a duplication structure at the target locus. This duplication can subsequently be reduced to one copy, removing the wild-type gene and leaving only the introduced mutation. Although more complicated in the cloning and genetic manipulations (*see* **Note 1**), this approach has the benefit that the mutations may be introduced with no other remnant of the targeting procedure. This "surgical" approach will appeal to investigators who desire minimal perturbation to the genome, such as single nucleotide mutation.

Although both approaches appear to be approximately equally efficient (*see* **Note 2**), each method has separate strengths and drawbacks. The choice of which approach is best depends on the researcher's goal.

Key Words: Ends-in; ends-out; gene targeting; homologous recombination; mutation; replacement.

1. Introduction

Genetic manipulations have long been a recognized strength for research utilizing *Drosophila*, despite the late arrival of techniques for targeted gene disruption or replacement. We present two protocols for generating linear DNA molecules in vivo, capable of stimulating homologous recombination and incorporating desired sequence into specific locations in the genome. Targeting allows an investigator great flexibility by allowing the design of specific alleles, including amorphic or

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antimorphic alleles. A growing number of cases of successful targeting by many groups demonstrates the utility of this approach ***(1–32)***.

The chief requirement for homologous recombination as we describe is the generation of in vivo linear DNA molecules. The linear molecule is generated from an integrated transgenic donor construct using the enzymatic activity of *FLP* recombinase (to excise a circular molecule of DNA from the construct) and *I-Sce*I homing meganuclease (to convert the liberated circle to a linear molecule). Two arrangements of the linear molecule relative to the target genomic sequence are possible—ends-out and ends-in (**Figs. 1** and **2**)—generating distinctly different products after recombination and repair (*see* **Note 3**) ***(33,34)***.

The efficiency of pairing and recombination in *Drosophila* may be similar to the high rate of targeting in fungal cells, but because *Drosophila* does not allow in vitro introduction to cultured germ cells, and positive/negative selection is not used, more complicated genetic manipulation and screening are required for *Drosophila*. In the following protocols, we attempt to make the considerations in construct design clear, and the genetic crosses straightforward. For those who wish to understand the gritty details, we provide ample references, and for those who merely wish to follow the instructions and get their mutant, we provide a step-by-step protocol.

There are three steps of targeting that require some attention—construct design (*see* **Subheadings 3.1.1.** and **3.2.1.**), targeting (*see* **Subheadings 3.1.2.** and **3.2.2.**), and either marker removal (*see* **Subheading 3.1.3.**, for ends-out) or reduction (*see* **Subheading 3.2.3.**, for ends-in). Marker removal is optional and likely unnecessary for most purposes. We assume that a laboratory that attempts these procedures is sufficiently equipped for molecular biology (DNA cloning, the polymerase chain reaction (PCR), Southern blotting, electrophoresis, sequencing, and so on) and *Drosophila* husbandry (incubators, fly food, microscopes, experience with simple genetic crosses, P-element-mediated germline transformation, and so on) to complete the procedures.

2. Materials

2.1. Ends-Out Targeting

1. *FLP* and *I-Sce*I on chromosome *2* (y^1 w^*; $P\{ry^{+t7.2}$ *70FLP*$\}11$ $P\{v^{+t1.8}$ *70I-Sce*I$\}$2B *noc*^{Sco}/*CyO*, S^2) or on chromosome *3* (y^1 w^*; $P\{ry^{+t7.2}$ *70FLP*$\}23$ $P\{v^{+t1.8}$ *70I-Sce*I$\}$4A/*TM6*).
2. *FLP* on chromosome *2* (w^{1118}; $P\{ry^{+t7.2}$ *70FLP*$\}10$; Sb^1/*TM6*, *Ubx*).
3. One or more of: pP[w25.2], pP[w30], pP[w35] ends-out targeting vectors.
4. Optional: *Cre* recombinase on the *X* chromosome (y^1 w^{67c23} $P\{y^{+mDint2}$ *Crey*$\}$1b; *noc*^{Sco}/*CyO* or y^1 w^{67c23} $P\{y^{+mDint2}$ *Crey*$\}$1b; D^*/*TM3*, Sb^1) or on a chromosome *2* balancer (y^1 w^{67c23}; *noc*^{Sco}/*CyO*, $P\{w^{+mC}$ *Crew*$\}$DH1).
5. Optional: an in vivo source of Δ2,3 transposase (e.g., the *TMS* balancer, *TMS*, $P\{ry^{+t7.2}$ Δ*2-3*$\}$99B, *see* **Note 4**).

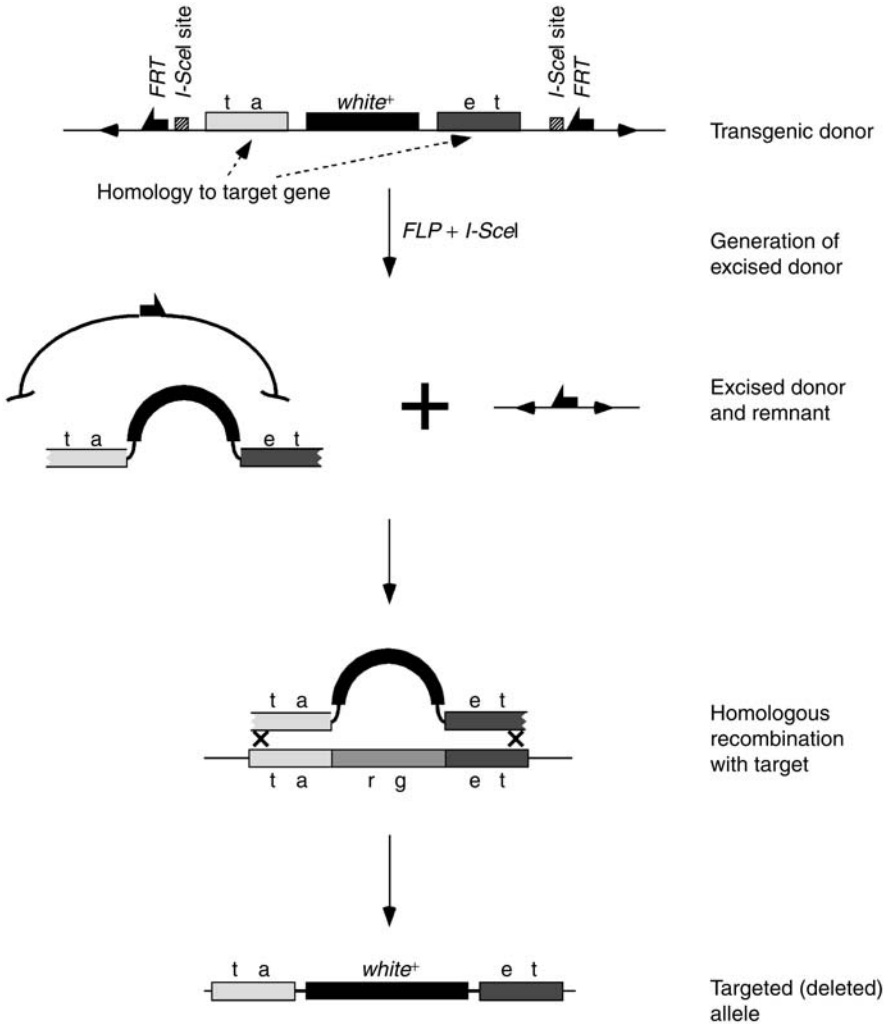

Fig. 1. Diagrammatic representation of ends-out targeting. *Transgenic donor* shows a clone using P[w35] as a basis for targeting. Hatched boxes are *I-Sce*I recognition sites, half-arrows are *FRT*s, arrowheads are *P*-element ends. Black box is the *white*$^+$ marker, used for both transformation and targeting. Gray bars, labeled with "ta" and "et," show cloned homology to target sequence. *FLP* and *I-Sce*I induction is responsible for the *generation of the excised donor*, leaving the *remnant* at the site of original transformant integration. Homology matches between the *excised donor* and *target* sequence cause *homologous recombination with the target*, replacing endogenous "rg" sequence with the *white*$^+$ gene and generating the *targeted (deletion) allele*.

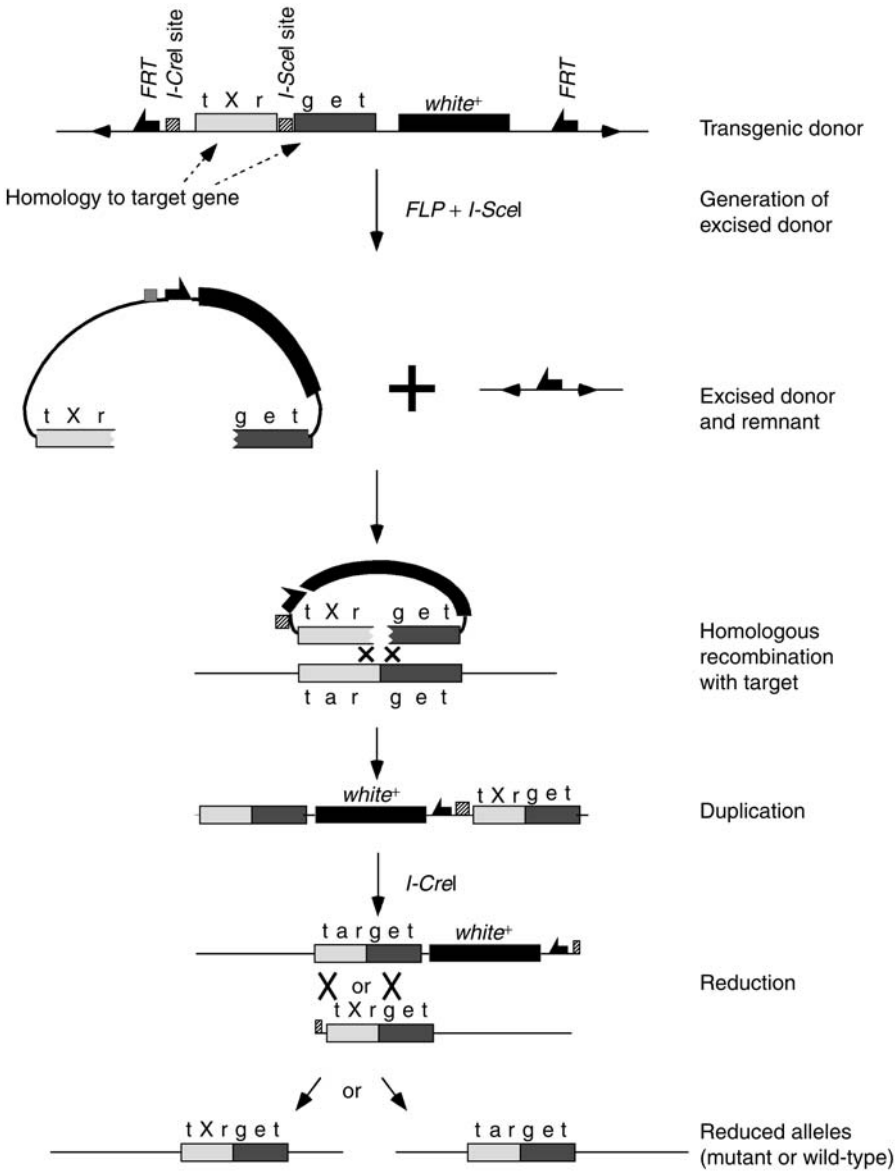

Fig. 2. Diagrammatic representation of ends-in targeting. In the *transgenic donor*, hatched boxes are *I-Sce*I and *I-Cre*I recognition sites, half-arrows are *FRT*s, arrowheads are *P*-element ends. Black box is the *white*$^+$ marker, used for both transformation and targeting. Gray bars, labeled with "tXr" and "get," show cloned homology to *target* sequence, where the "X" represents an introduced mutation. *FLP* and *I-Sce*I induction is responsible for the *generation of the excised donor*, leaving the *remnant* at the site of original transformant integration. Homology matches between the *excised donor* and

6. Optional: a balancer or double-balancer stock to establish a stock of your targeted allele (e.g., w^1; noc^{Sco}/*CyO*; D^*/*TM3*, Sb^1).

2.2. Ends-In Targeting

1. *FLP* and *I-Sce*I on chromosome *2* (y^1 w^*; $P\{ry^{+t7.2}$ *70FLP*$\}11$ $P\{v^{+t1.8}$ *70I-Sce*I$\}2B$ noc^{Sco}/*CyO*, S^2) or on chromosome *3* (y^1 w^*; $P\{ry^{+t7.2}$ *70FLP*$\}23$ $P\{v^{+t1.8}$ *70I-Sce*I$\}4A$/*TM6*).
2. *FLP* on chromosome *2* (w^{1118}; $P\{ry^{+t7.2}$ *70FLP*$\}10$; Sb^1/*TM6*, *Ubx*).
3. *I-Cre*I on the *X* chromosome ($P\{v^{+t1.8}$ *hs-I-Cre*I.R$\}2A$, v^1; ry^{506}) or on chromosome *3* (w^{1118}; $P\{v^{+t1.8}$ *hs-I-Cre*I.R$\}1A$ Sb^1/*TM6*, *Ubx*).
4. The pP[TV2] (*see* ref. ***33***) ends-in targeting vector.
5. Optional: an in vivo source of Δ2,3 transposase (e.g., the *TMS* balancer, *TMS*, $P\{ry^{+t7.2}$ Δ*2-3*$\}99B$, *see* **Note 4**).
6. Optional: a balancer or double-balancer stock to establish a stock of your targeted allele (e.g., w^1; noc^{Sco}/*CyO*; D^*/*TM3*, Sb^1).

All fly stocks are available at the Bloomington Drosophila Stock Center, Indiana (http://flystocks.bio.indiana.edu/), and DNA vectors are available from the Drosophila Genomics Resource Center (Indiana University, Bloomington, Indiana.) (http://dgrc.cgb.indiana.edu/).

3. Methods

3.1. Ends-Out Targeting

Ends-out targeting is so-named because the paired arrangement of donor and target DNA places the cut ends of the donor at the left and right sides of the recombining structure **(Fig. 1)** ***(34)***. Through heat-shock-induced expression of *FLP* recombinase and *I-Sce*I homing endonuclease, you will generate a linear excised donor DNA molecule whose ends are homologous to your target, but whose middle is not. Homologous recombination at both ends will replace the genomic target with the desired donor sequence. For screening, either a sequence alteration of your design (using P[w30], *see* **Subheading 3.1.1.**) or the $white^+$ marker (using P[w25.2] or P[w35]) may be used. The chief advantages of ends-out targeting are that cloning and genetic manipulations are simple. Ends-out is the best approach if your application requires the simple removal of a sequence (for instance, to create a deletion allele of a gene under investigation) or insertion of a sequence to create a disruption allele.

Fig. 2. *(Continued) target* sequence cause *homologous recombination with the target*, generating a *duplication* of the wild-type and mutated sequence separated by the $white^+$ marker gene. Subsequent *I-Cre*I induction generates a double-stranded break and induces recombination between the two copies of the gene. *Reduction* by recombination generates *reduced alleles (mutant or wild-type)*, depending on the location of the recombination relative to the mutation.

The final product of ends-out usually leaves some exogenous DNA at the targeted locus, often a *white*$^+$ marker gene. However, using the *Cre-loxP* site-specific recombination system ***(35)***, the *white*$^+$ gene may subsequently be removed, leaving only a single 34 bp *loxP* site behind. If the targeted gene is expected to have an easily scored phenotype (e.g., reversion of a mutant phenotype to wild-type, or generation of an allele with expected morphological phenotype), you may choose to target without a marker gene.

3.1.1. Construct Design for Deletion

1. Select the appropriate ends-out targeting vector **(Fig. 3)**.
   a. P[w35]: this vector has two cloning sites for cloning of the sequences that flank the region that you have targeted for insertion or removal **(Fig. 1)**. DNA between these flanking sequences in the target will be replaced with the *white*$^+$ gene during the targeting procedure. *white*$^+$ serves as both a transformation marker and a marker for monitoring the targeting procedure. P[w35] is the simplest vector for targeting.
   b. P[w25.2]: this vector, like P[w35], is designed to generate disruption or deletion alleles, but with a few additions. Two six-frame stop codons are included to assure minimal "read-through" from the *white*$^+$ gene, as well as two *loxP* sites flanking the *white*$^+$ marker. These *loxP* sites may be subsequently used to remove the *white*$^+$ gene (and its regulatory elements) and generate "unmarked" alleles. P[w25.2] may be useful if the presence of *white*$^+$ would interfere with subsequent analyses. Examples may include cases where other alleles of *white* are used as reporters, or when the presence of a marker gene is undesirable.
   c. P[w30]: this vector is designed with a *white*$^+$ transformation marker, but no targeting marker. *white*$^+$ is not flanked by FLP recombination target (FRTs) and will not become part of the excised donor during targeting—targeting with P[w30] does not allow secondary screening with *FLP* (*see* **Subheading 3.1.2.**, **steps 9** and **10**). Note that only one cloning site is present in P[w30]; the sequence cloned into this site will replace the endogenous sequence, so P[w30] may be used to introduce site-directed mutations or specific allele structures created by the investigator. The targeting reaction will not be marked with a convenient marker, other than a phenotype introduced by the targeting itself. Examples of application of P[w30] include the generation of an allele with an obvious phenotype (e.g., a GFP fusion gene or a known morphological phenotype).
2. Clone sequences of homology into the appropriate vector, using high fidelity PCR or cloning from a DNA library, to minimize polymorphism between donor and target sequence (*see* **Note 5**). We recommend using as much homology as is practical to increase targeting efficiency. We prefer to use a minimum of 3 kb of homology on each side when using P[w35] or P[w25.2], or 6 kb of total homology when using P[w30] *(34)*.
3. Generate transformed lines carrying this donor construct.
4. Map the insertion site using meiotic segregation.

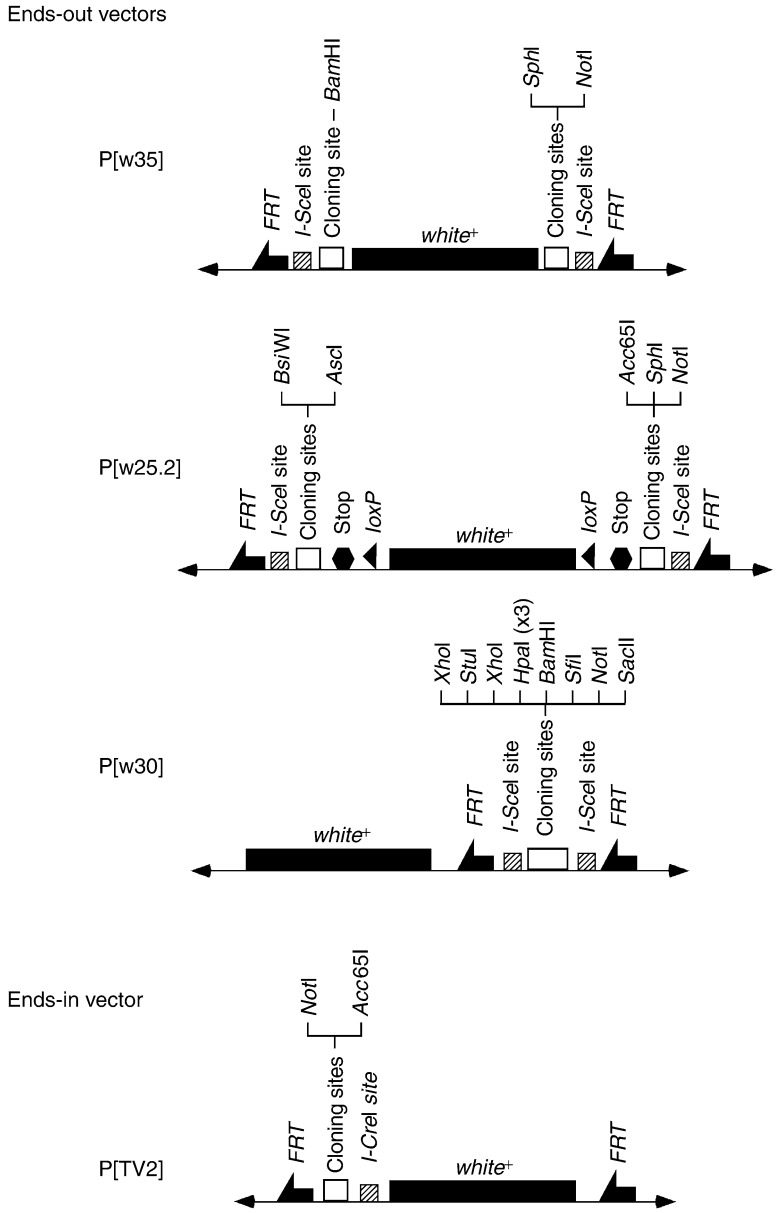

Fig. 3. Vectors used for ends-out and ends-in targeting. Arrowheads are *P*-element ends, half-arrows are *FRT* sequences, hatched boxes are *I-Sce*I and *I-Cre*I recognition sites, open boxes are sites for cloning DNA homologous to targeted sequence, hexagons are six-frame translation stop codons, triangles are *loxP* sites, and black boxes are *white*$^+$ marker gene.

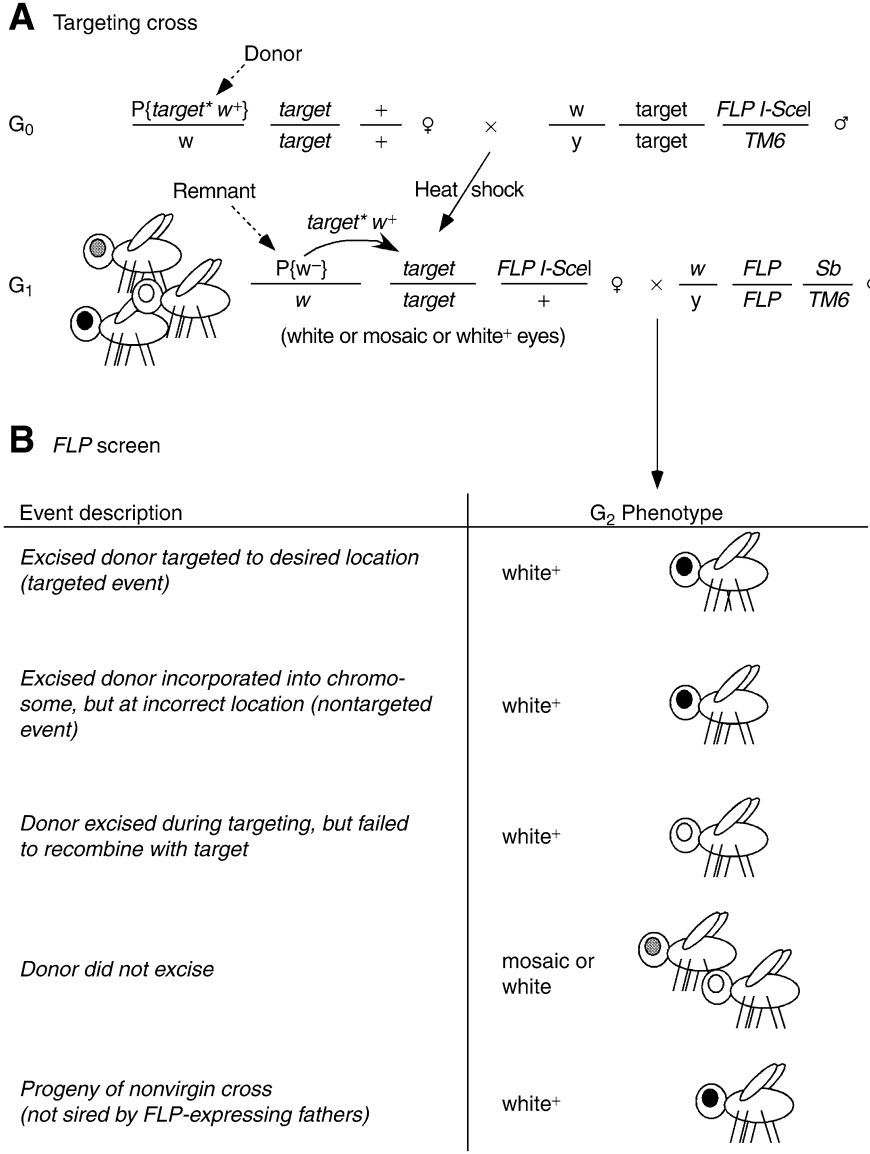

Fig. 4. Genetic crosses for targeting. **(A)** Ends-out and ends-in targeting (*see* **Subheading 3.1.2.**). G_0: transgenic flies, carrying the donor construct (P{*target* w*$^+$}) are crossed to flies expressing *FLP* and *I-Sce*I and heat-shocked (*see* **Subheading 3.1.2.**, **steps 1–7**). G_1: female progeny will have red, mosaic, or white eyes, and are collected and crossed to males expressing *FLP* (*see* **Subheading 3.1.2.**, **steps 8** and **9**). Red-eyed progeny (G_2) are collected and analyzed for proper targeting (*see* **Subheading 3.1.2.**, **steps 10–12**). **(B)** Offspring of the *FLP*-screened potential targeted flies (*see*

5. Confirm that the structure of the donor is unaltered using PCR and Southern-based techniques.

3.1.2. Targeting

The first cross (**Figs. 4** and **5**) is the same, for both ends-out or ends-in targeting. During this cross, portions of the donor are liberated from the chromosome to form the excised donor (*see* **Note 3**), which will recombine with the endogenous target gene. In the second generation, a constitutively expressed *FLP* gene is used as a secondary screen to exclude and discard flies that are white$^+$ because the excised donor was not generated, greatly reducing the amount of genetic and molecular analysis ***(36)***.

1. G_0 generation: cross flies containing your donor transgene to flies carrying *FLP* and *I-Sce*I transgenes **(Fig. 4)**—establish about 20 vials, each with five females and three males. Label this set of vials "A."
   a. The choice of which *FLP*- and *I-Sce*I-expressing lines to use will depend on your genetic scheme. It is best to use one different from your target chromosome, as you don't want to target the gene on the *FLP*- and *I-Sce*I-containing chromosome.
   b. $y^1\ w^*$; *P{ry*$^{+t7.2}$ *70FLP}*11 *P{v*$^{+t1.8}$ *70I-Sce*I}2B *noc*^{Sco}/*CyO*, S^2.
   c. $y^1\ w^*$; *P{ry*$^{+t7.2}$ *70FLP}*23 *P{v*$^{+t1.8}$ *70I-Sce*I}4A/*TM6.*
2. After 2 or 3 d, when you see first and second instar larvae crawling in the food, transfer the adults to new vials, labeling the new set of vials "B."
3. The following day, push the cotton plugs in the "A" vials to just above the food, preventing the larvae from crawling up the walls of the vial.
4. Heat-shock "A" vials at 38°C for 1 h in a circulating water bath, immersed with the water level above the level of the cotton plug.
5. Raise the cotton plugs and return the flies in the incubator.
6. Continue transferring and heat-shocking, labeling successive transfers "C," "D," and so on. Transfer flies 6–10 times **(Fig. 5)**.
7. After heat-shock, allow the flies to grow under standard culture conditions.

Fig. 4. *(Continued)* **Subheading 3.1.2., step 10**) will be of five types. The first type are targeted events, generating the expected replacement (for ends-out) or duplication (for ends-in) structures. These will be *white*$^+$ and may be Stubble (*see* **Note 11**). The second type are nontargeted events, where the excised donor is incorporated at random in the genome, not at the desired location. These will also be white$^+$ and may be Stubble, and must be discriminated from targeted events using molecular or genetic methods. The third type, typically representing the vast majority of offspring, are cases where the donor was excised, but did not target, and was lost. These flies will have white eyes and may be Stubble. The fourth type are cases where the donor was not excised from the chromosome. These flies will possess white or mosaic eyes and may be Stubble. The fifth type are products of nonvirgin matings. Your crossing scheme may vary, depending on the chromosomes that contain your donor and target genes.

Example timeline for targeting

Day	Activity
1	Set “A” vials with G0 parents
2	
3	Transfer flies from “A” vials to “B” vials
4	Heat shock “A” vials
5	Transfer flies from “B” vials to “C” vials
6	Heat shock “B” vials
7	Transfer flies from “C” vials to “D” vials
8	Heat shock “C” vials
9	Transfer flies from “D” vials to “E” vials
10	Heat shock “D” vials
11	Transfer flies from “E” vials to “F” vials; begin collecting G1 females from “A” vials, cross to *FLP* males
12	Heat shock “E” vials
13	Discard flies from “F” vials; begin collecting females from “B” vials, cross to *FLP* males
14	Heat shock “F” vials
15	Begin collecting females from “C” vials, cross to *FLP* males
16	
17	Begin collecting females from “D” vials, cross to *FLP* males
18	
19	Begin collecting females from “E” vials, cross to *FLP* males
20	
21	Begin collecting females from “F” vials, cross to *FLP* males
22	
23	
24	
25	Begin screening G2 offspring from “A” females for white^{+} eyes
26	
27	Begin screening offspring from “B” females
28	
29	Begin screening offspring from “C” females
30	
31	Begin screening offspring from “D” females
32	
33	Begin screening offspring from “E” females
34	
35	Begin screening offspring from “F” females

Fig. 5. Typical timeline for targeting (ends-out or ends-in) crosses. Transfer of parents (*see* **Subheading 3.1.2.**, **steps 2** and **6**) should occur when you see first and second instar larvae in the food. Heat-shock **(steps 3–5)** should occur when third instar larvae are visible. Actual time may differ based on your culture conditions.

8. Collect female flies (*see* **Notes 6** and **7**) as they eclose—remember that “B” vials will begin to eclose 2 or 3 d after the “A” set, and so on.
9. G_1 generation: cross *all* females, two at a time (*see* **Note 8**), to *FLP*-expressing males (*see* **Fig. 4B** and **Note 9**). Set up about 1000 vials.
   a. w^{1118}; $P\{ry^{+t7.2}\ 70FLP\}10$; $Sb^{1}/TM6$, *Ubx*.

10. G_2 generation: screen for nonwhite-eyed progeny (*see* **Note 10**). These flies will be targeted events, nontargeted events, or the progeny of nonvirgin mothers (*see* **Note 11**).
11. Cross to appropriate flies (e.g., w^1; noc^{Sco}/*CyO*; D^*/*TM3*, Sb^1) to establish a stock.
12. Confirm successful targeting using molecular analyses (*see* **Note 12**). PCR-based techniques are sufficient for quick screening, but Southern blot-based analyses are ultimately necessary, as many alternate structures may be produced during targeting ***(36)***.

3.1.3. Marker Removal

If you have used P[w25.2] for targeting and do not wish to have your engineered allele marked with *white*$^+$, it can be removed using the *loxP* sites flanking the *white*$^+$ marker gene ***(37)***. Targeted events can be crossed to *Cre* recombinase-expressing flies according to the following cross **(Fig. 6A)**.

1. G_0 generation: cross *Cre*-expressing females (listed below) to targeted males (*see* **Note 13**)—establish 5–10 vials, each with five females and three males.
   a. The choice of which *Cre* recombinase-expressing line to use will depend on your genetic scheme. Choose a stock to balance your targeted allele because after this cross, it will be unmarked.
   b. y^1 w^{67c23} $P\{y^{+mDint2}$ *Crey*$\}$1b; noc^{Sco}/*CyO*.
   c. y^1 w^{67c23} $P\{y^{+mDint2}$ *Crey*$\}$1b; D^*/*TM3*, Sb^1.
   d. y^1 w^{67c23}; noc^{Sco}/*CyO*, $P\{w^{+mC}$ *Crew*$\}$DH1.
2. Grow the flies in standard culture conditions. Heat-shock is not necessary because the *Cre* recombinase exhibits considerable maternal expression ***(35)***.
3. G_1 generation: select male (or, if your targeted gene is *X*-linked, female) progeny that possess *Cre* recombinase and the targeted allele.
4. Cross to appropriate flies (e.g., w^1; noc^{Sco}/*CyO*; D^*/*TM3*, Sb^1) to balance your targeted allele.
5. G_2 generation: select white-eyed progeny. These should possess the targeted allele now devoid of the *white*$^+$ marker. Collect individuals and cross them to appropriate flies (e.g., w^1; noc^{Sco}/*CyO*; D^*/*TM3*, Sb^1) to establish stocks (G_3 generation).
6. Confirm marker removal using molecular analyses (e.g., PCR or Southern blot-based analyses).

3.2. Ends-In Targeting

This approach uses a two-step method to introduce a mutant gene copy in tandem to the endogenous (wild-type) allele, and subsequently, to remove a portion from each of the two copies to leave a single-copy mutated allele in the chromosome **(Fig. 2)** ***(33,36,38)***. The benefit of this approach is that alleles carrying only the desired mutation, and no other alterations, can be recovered. Some have used this approach to make single nucleotide mutations within a gene ***(39)***. Ends-in targeting typically adds one additional genetic step compared with ends-out targeting, and construct design is somewhat more involved (though not always ***[36]***). Nevertheless, for some needs, it is the best solution.

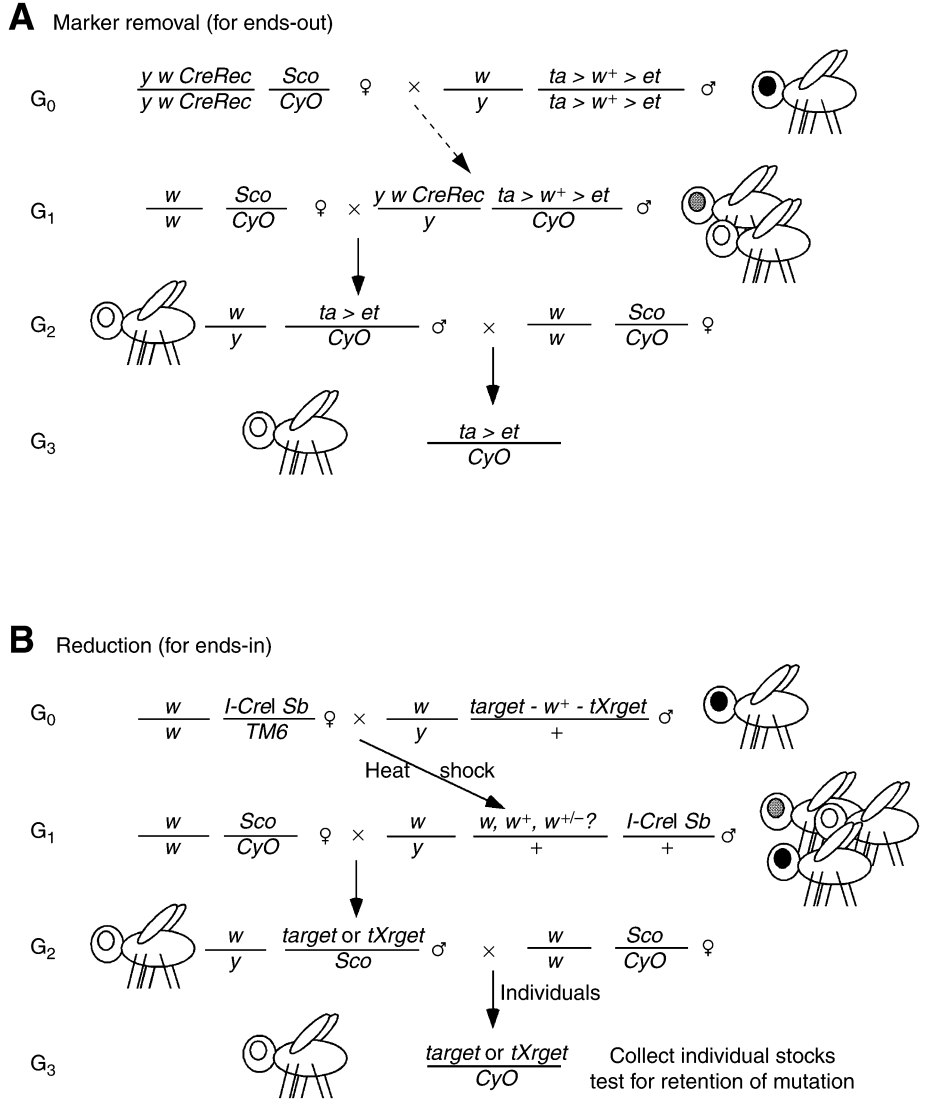

Fig. 6. Genetic crosses for marker removal and reduction. **(A)** Marker (*white*$^+$) removal when using P[w25.2] as an ends-out targeting vector (*see* **Subheading 3.1.3.**). G_0: *white*$^+$ targeted males (containing the target gene disrupted by *loxP*-flanked *white*$^+$, "ta > w^+ > et") are crossed to *Cre* recombinase-expressing females (*see* **Subheading 3.1.3.**, **step 1**). G_1: progeny will have mosaic or white eyes, and are collected and crossed to balancer-containing flies (*see* **Subheading 3.1.3.**, **steps 3** and **4**). G_2: white-eyed flies have lost the *loxP*-flanked *white*$^+$ gene, retaining only a *loxP* site (>) and are crossed to establish a stock (G_3, *see* **Subheading 3.1.3.**, **step 5**). **(B)** Reduction of anends-in targeted allele to a mutant allele (*see* **Subheading 3.2.3.**). G_0: *white*$^+$ targeted

3.2.1. Construct Design

1. Clone sequences of homology into a convenient plasmid (e.g., pBluescript [Strategene, La Jolla, CA], pGEM [Promega, Madison,WI]) for easy manipulation, using high fidelity PCR or cloning from a DNA library, to minimize polymorphism between donor and target sequence (*see* **Note 5**). We typically use DNA sequence with 5–6 kb of total homology or more.
2. Introduce an *I-Sce*I recognition site in the middle of the sequence (*see* **Note 14**).
3. Introduce your mutation—a small deletion or insertion, a point mutation, and so on. This mutation should preferably be at least approx 1 kb from the *I-Sce*I recognition site, although shorter distances may also produce successful alterations (*see* **Notes 1, 15**, and **16**).
4. Clone the constructed allele into P[TV2] **(Fig. 3)**. It possesses the other features necessary for ends-in targeting.
   a. A *white*$^+$ transformation and targeting marker gene.
   b. Two *FRT* sequences.
   c. An *I-Cre*I recognition site for Reduction (*see* **Subheading 3.2.3.**).
5. Generate transformed lines carrying this donor construct.
6. Map the insertion site using meiotic segregation.
7. Confirm that the structure of the donor is unaltered using PCR and Southern-based techniques.

3.2.2. Targeting

The procedure to induce targeting, and subsequently, screen with *FLP*-expressing males, is identical to that of ends-out targeting (*see* **Subheading 3.1.2.**). However, the final product differs in structure **(Fig. 2)**.

3.2.3. Reduction

With ends-in targeting, the theoretically expected product is a duplication at the targeted locus **(Fig. 2)**: the wild-type gene followed by the donor (mutated) gene with the *white*$^+$ marker in between. However, because recombinants with unexpected structures do arise ***(38)***, the structure should be confirmed by molecular methods, including Southern blotting. The duplication is reduced by

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Fig. 6. *(Continued)* males (containing the duplication of wild-type gene and mutant gene separated by *white*$^+$, "target - w^+ - tXrget") are crossed to *I-Cre*I-expressing females and heat-shocked during development (*see* **Subheading 3.2.3.**, **steps 1–7**). G_1: progeny will have red, mosaic, or white eyes, and are collected and crossed to balancer-containing flies (*see* **Subheading 3.2.3.**, **steps 8** and **9**). G_2: individual white-eyed flies are again crossed to balancer-containing flies to establish many independent lines (*see* **Subheading 3.2.3.**, **step 10**). G_3: each line, once established, is tested for reduction to wild-type or to mutant allele **(step 11)**. Genetic markers are listed in **Subheading 2.**

a recombination event that removes one copy of the gene and the marker ***(33)***. The result is the replacement of the wild-type copy with a specifically designed mutation, and no other alterations to the genome.

1. G_0 generation: cross *I-Cre*I females to targeted (duplication) males **(Fig. 6B)**—establish five vials, each with five females and three males.
   a. The choice of which *I-Cre*I-expressing line to use will depend on your genetic scheme.
   b. $P\{v^{+t1.8}$ *hs-I-Cre*I.R$\}$2A, v^1; ry^{506}.
   c. w^{1118}; $P\{v^{+t1.8}$ *hs-I-Cre*I.R$\}$1A Sb^1/*TM6*, *Ubx*.
2. After 2 or 3 d, when you see first and second instar larvae crawling in the food, transfer the adults to new vials, labeling the new set of vials "B."
3. The following day, push the cotton plugs in the "A" vials to just above the food, preventing the larvae from crawling up the walls of the vial.
4. Heat-shock "A" vials at 36°C (*see* **Note 17**) for 1 h in a circulating water bath, immersed with the water level above the level of the cotton plug.
5. Raise the cotton plugs and return the flies in the incubator.
6. Continue transferring and heat-shocking, labeling successive transfers "C," "D," and so on. Transfer flies approximately four times.
7. After heat-shock, allow the flies to grow under standard culture conditions.
8. Collect males as they eclose—remember that "B" vials will begin to eclose 2 or 3 d after the "A" set, and so on.
9. G_1 generation: cross males, two or three at a time, to appropriate flies (e.g., w^1; noc^{Sco}/*CyO*; D^*/*TM3*, Sb^1) to introduce marker mutations so that you may monitor potential reduced targeted alleles.
10. G_2 generation: select an *individual* white-eyed male (or, if your targeted gene is *X*-linked, individual females) from each vial, which will assure that each collected event is independent, and cross to appropriate flies (e.g., w^1; noc^{Sco}/*CyO*; D^*/*TM3*, Sb^1) to establish stocks (G_3 generation). Establish at least 20 (or more) individual lines to test for reductions to the mutant allele.
11. Confirm successful reduction using molecular analyses (e.g., PCR and Southern blot-based analyses).

4. Notes

1. The orientation of elements in the donor, the resulting duplication, and final reduction allele of ends-in can be very confusing, and a poorly designed donor construct may decrease efficiency or prevent targeting altogether. We recommend diagramming the structure of each step. The orientation of the *white*$^+$ gene relative to the donor allele sequence, the location of the *I-Sce*I recognition site, and the location of the introduced mutation are all critical factors **(Fig. 2)**. A few minutes of drawing can save months of work leading to a dead end.
2. It is difficult to compare frequencies because variance in targeting efficiency is high, between different laboratories, target genes, and donor transformant lines (*see* **Note 4**). We and others have seen frequencies of targeting as high as one event

per three females, and as low as one event per 1000 females, depending on the gene being targeted and the donor transformant line ***(32)***.

3. For ease and consistency, we define the following nomenclature: *donor*—a *P*-element-based transgene, carrying sequence homologous to the gene to be targeted, as well as necessary homing endonuclease sites (i.e., *I-Cre*I and *I-Sce*I), *FRT* sites, and marker genes used during targeting. *Excised donor*—The linear DNA, from the *donor*, that will recombine with the endogenous gene; *FLP* excises the DNA from the *donor* site and *I-Sce*I creates the double-stranded break(s) to expose the ends that stimulate recombination. *Remnant*—The exogenous DNA left behind by the *donor* during the targeting reaction. In most cases (except ends-out targeting with P[w30]), it consists of a single *FRT* flanked by *P*-element ends. *Targeted event*—desired movement of a portion of the *donor* to the target endogenous location, based on homology. *Nontargeted event*—although initially appearing as a *targeted event*, this is an undesirable movement of the *Donor*, perhaps to a random location in the genome. *Marker removal*—For ends-out targeting using P[w25.2], the removal of the *white*$^+$ marker gene, using Cre recombinase, to generate an unmarked but targeted allele. *Duplication*—the product of a *targeted event* during ends-in targeting; the juxtaposition of the *donor* (mutated) copy of the gene of interest and the endogenous copy (*see* **Fig. 2**). *Reduction*—For ends-in targeting, the *I-Cre*I-induced recombination between the two copies of the targeted gene, resulting in a single copy (wild-type or mutant, *see* **Fig. 2**). The crosses that we show (**Figs. 4** and **6**) illustrate a target gene on chromosome *2*. The details of your genetic scheme may differ and will be based on the chromosome on which your target gene is found.
4. Chromosomal position effects—proximity of a transformed transgene to regulatory elements, heterochromatic chromosome features, and so on—may affect the ability of a transgene to act as a donor for targeting. Although we have not methodically tested different insertion sites for their efficacy in targeting, we routinely notice that some insertions fail to lead to targeting events, whereas others do so at high frequency. Hence, use of Δ2,3 Transposase to mobilize a targeting transgene to other locations within the genome (or use of multiple independent transformation events as targeting donors) is recommended.
5. In genomic regions where there are nearby genes, some parts of the donor may include coding regions of neighboring genes. To prevent inadvertently introducing mutations to these genes, we recommend the use of high-fidelity polymerases during PCR to clone the gene from flies. Some problematic genes may require PCR amplification and cloning, or simply direct subcloning, from a bacterial artificial chromosome or bacteriophage P1 genomic clone. Although no controlled comparison has been made to our knowledge, DNA polymorphism appears to have little effect on targeting efficiency in *Drosophila* ***(10,39)***.
6. Females have a higher rate of targeting than do males, and so we recommend using only females. Males may be used and crossed to *FLP*-expressing females, but we find that it is not worth the extra effort owing to an appreciably lower frequency of targeting. Females do not need to be virgin, although use of virgins will make subsequent crosses to identify targeting events easier. Nonvirgins may have mated with

white$^+$ male siblings, but progeny from nonvirgin matings will be obvious in the next generation (*see* **Note 7**). We feel that it is better to cross all females, whether virgin or not, and screen through the progeny later.

7. Expect to see all sorts of patterns of your marker gene: white eyes, red eyes, and eyes with patches of both. We have not seen a strong correlation between expression of *white*$^+$ in the eyes (variegation caused by *FLP*-mediated *white*$^+$ removal in the soma) and successful targeting (where *FLP*-mediated excision is in the germline). *Be safe*: cross every female, regardless of eye phenotype, to *FLP*-expressing males.
8. Most researchers have reported frequencies of targeting to be relatively low, approx 1/100 vials of progeny from the cross of heat-shocked females to *FLP*-expressing males. For this reason, we routinely culture the progeny of two (or three) females together: the vials are healthier and we rarely encounter multiple independent targeting events from two females in the same vial.
9. The *FLP*-expressing stock appears to be an enhancer-trap line and does not require heat-shock to express efficiently. Other labs use eye-specific expression of *FLP* with similar effect ***(40)***.
10. The *white*$^+$ marker used during targeting may come under the influence of chromosomal position effects, and thus properly targeted alleles may have red, orange, or yellow eyes. Any nonwhite eyed fly should be treated as a potential targeting event.
11. As the *FLP*-expressing stock is marked with *Sb*, it is possible to discriminate some of the white$^+$ flies that represent targeting events from those that are products of nonvirgin mothers by the presence of the Stubble phenotype. However, only 50% of the progeny of *FLP*-expressing fathers will be Stubble (their siblings will be Ultrabithorax, which could also come from nonvirgin mothers). Although white$^+$ *Sb* flies can only be from transgene movement, and indicate a targeted (or nontargeted) allele, excluding *white*$^+$ Stubble$^+$ flies may result in discarding 50% of your targeting events. Although some germ cells may show targeting, many more will not. Typically, we see fewer than five *white*$^+$ flies from a vial, the remaining white$^-$ siblings are derived from germ cells where targeting did not occur.
12. After recovery of white$^+$ individuals, and before more tedious molecular analyses, it is often worthwhile to repeat the *FLP* screen (*see* **Subheading 3.1.2.**, **steps 9** and **10**, **Fig. 4A** generation G_1, **Fig. 4B**) to confirm that the recovered *white*$^+$ gene is not flanked by *FRT*s.
13. Cre recombinase expression is controlled by an *Hsp70-Mos1* promoter fusion, and is expressed efficiently without heat-shock ***(35)***. In our experience, nearly 100% of the flies with a *loxP*-flanked *white*$^+$ gene and expressing *Cre* recombinase give offspring with white eyes.
14. The *I-Sce*I recognition sequence is 5′-TAGGGATAACAGGGTAAT-3′. Note that the consensus is nonpalindromic, and has a T_m of approx 50°C. Although orientation of the *I-Sce*I site does not affect targeting (in most cases the *I-Sce*I sequence will be removed before or during targeting by cellular exonucleases), PCR can be used to determine orientation. We use versions of this sequence that can be cloned into blunt-ended or cohesive restriction sites. Remember that oligonucleotides must be 5′ phosphorylated (chemically during synthesis, or afterwards using polynucleotide kinase) in order to be efficient substrates for ligation.

15. For mutagenesis, we often use annealed oligonucleotides that introduce translational stop codons in all three reading frames. This sequence may be designed with cohesive ends to suit your cloning scheme. In addition to stop codons in all three frames, we recommend making the oligonucleotides of correct length to introduce a frame shift. Easy molecular analysis can be accomplished by simultaneous introduction of a diagnostic restriction site.
16. As the "homologous recombination" during Reduction (*see* **Subheading 3.2.3.**) may involve single strand annealing and mismatch repair, multiple mutations on a donor may not all appear in the final mutated allele. Although this may be a problem for some, it has been used to great advantage by one laboratory to generate a suite of alleles with different combinations of lesions that otherwise would have taken significant time and effort ***(39)***. Additionally, another laboratory has used the "unexpected" structures generated during ends-in targeting as additional alleles ***(40)***.
17. *I-Cre*I endonuclease recognizes a sequence in the *Drosophila rDNA* arrays on the *X* and *Y* chromosomes. High levels of expression can cause *rDNA* deletion or translocation between the *X* and *Y* chromosomes ***(41)***. Even higher levels of expression can cause death. You will note that offspring with the *I-Cre*I transgene are underrepresented in the second generation. A reduced heat-shock temperature will facilitate survival if this is a problem.

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10

Recombinases and Their Use in Gene Activation, Gene Inactivation, and Transgenesis

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Summary

The site-specific recombinase FLP is used in *Drosophila* to precisely manipulate the genome, in particular, to eliminate gene function by mitotic recombination and to activate transgenes in discrete populations of cells. These approaches are already part of the standard tool kit for studying gene function. The number of applications for the FLP recombinase has increased over the years and further members of the large family of site-specific recombinases are being added to the arsenal of fly geneticists, most recently, the ϕC31 integrase. This chapter will introduce these recombinases and describe how such instruments are utilized to accurately manipulate the *Drosophila* genome.

Key Words: *Drosophila melanogaster*; Cre/loxP; FLP/*FRT*; ϕC31 integrase; mitotic recombination; mosaics; recombinases; transgenesis.

1. Introduction

The impact of *Drosophila melanogaster* on the understanding of biological processes has been strongly influenced by the ever increasing ability to manipulate its genome in a precise manner. An important step in this process was the ability to introduce transgenes into the genome through P-element-mediated germline transformation (*see* Chapter 4) ***(1,2)***. A further tier of manipulation was achieved with the introduction of the Gal4-UAS system, which opened up a myriad of possibilities to control the expression of transgenes (*see* Chapter 5) ***(3,4)***. The genetic tools for *Drosophila* were further enhanced by the yeast FLP/*FRT* (FLP recognition target) system that allowed for site-specific recombination in the genome ***(5)***. This tool has experienced many modifications and refinements since then, allowing one now to perform various manipulations in the genome with high precision. These manipulations include the controlled loss of gene function by mitotic recombination, the activation of transgenes,

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and more recently, even the insertion of transgenes into preinstalled landing sites. Such site-specific integration has also been accomplished with the bacterial Cre/*loxP* system and with a recombination system derived from the phage ϕC31. It is likely that the already adopted recombinases, as well as additional ones from the large arsenal of site-specific recombinases, will in the future play an increasingly important role in the genetic analysis of *Drosophila* and other model organisms. As follows we provide an overview about various techniques established in *Drosophila* that rely on the use of such site-specific recombinases, starting with a more general introduction to this class of enzymes.

1.1. Classes of Recombinases

Site-specific recombinases recognize short DNA sequences (typically between 30 and 40 bp) and mediate the recombination between these elements resulting in excision, integration, inversion, or exchange of DNA fragments ***(6)***. They are generally classified into two families or subgroups based on distinct biochemical properties ***(7)***. The members of the integrase family, sometimes referred to as the "λ integrase family," cleave one strand of each of the two DNA molecules involved, then exchange this strand, and subsequently cleave the second DNA strand. The members of this family use a conserved tyrosine residue to establish a transient covalent bond between the recombinase and the target DNA (and are therefore also termed "tyrosine recombinases"). Well-known members include the Cre recombinase from the phage P1 and the FLP recombinase (FLP) from *Saccharomyces cerevisiae* **(Box 1)**.

The members of the second group, the invertase/resolvase family, cleave all four DNA strands and then exchange them. The catalytic residue used by this family to initiate the DNA cleavage is a serine ("serine recombinases"). Members of this family include the Tn3 resolvase and the ϕC31 integrase **(Box 2)**.

The Cre/*loxP* recombinase system is the prevailing tool used for manipulating mammalian genomes ***(8,9)***, although it has found only limited application in *Drosophila*. In mammalian cells the Cre/*loxP* system has been demonstrated to be more effective than the FLP/*FRT* system ***(10)***. As typical experiments in mammalian cells require very efficient recombination, leading to the desired alteration in all cells under investigation (e.g., conditional excision), the Cre/*loxP* system is the tool of choice. However, the generation of mosaics, which represents the most frequent application of site-specific recombination in *Drosophila*, requires low recombination efficiency and therefore, FLP/*FRT* has been more suitable.

There are only a few reports about the use of the Cre/*loxP* system in *Drosophila* so far. It was first introduced in a strategy aimed at targeting two genes to the same site in the genome (**ref. *11***). Later, transgenic lines allowing for the expression of Cre by the Gal4-UAS system were generated ***(12)***. However,

Box 1

FLP and Cre recombinases

Both belong to the integrase family (also termed "λ integrase" family) of recombinases and do not need any accessory factors to mediate recombination. The FLP recombinase (FLP) is responsible for maintaining the copy number of the yeast 2 μm plasmid ***(43)***. It recognizes *FRT* sites and mediates recombination between them ***(44)***. A genuine *FRT* site consists of 48 bp, containing an 8-bp spacer (or core) region, two flanking almost perfect 13-bp inverted repeats, and an additional upstream 13-bp direct repeat, plus an isolated basepair **(Table 1)**. This direct repeat and the single basepair are dispensable for recombination, making a functional *FRT* site 34-bp long. Shorter *FRTs* of only 28 bp are still functional with respect to excision, but are, like the 34-bp FRTs, practically not substrates for integration ***(9,45)***.

Recombination is executed by FLP monomers binding to the 13-bp repeats and takes place in the asymmetric spacer region, including strand cleavage, exchange, and ligation ***(46)***. Because of the asymmetry of the spacer region, the strand exchange can only occur if the two *FRT* sites are orientated in the same direction. Therefore, a recombination event results in distinct outcomes, depending on the relative orientation of the *FRT* sites with respect to each other **(Fig. 1)**.

The FLP/*FRT* system was first introduced into the fly field in 1989 ***(5)*** and since then it has become the dominant tool for genetic in vivo manipulations in *Drosophila*.

The Cre (causes recombination) recombinase of the bacteriophage P1 recombines *loxP* [locus of crossover (×) in P1] target sites ***(47)*** and shares the common integration mechanism with FLP. The overall structure of the *loxP* site is similar to the *FRT* site; however, different at the nucleotide level, containing two 13-bp inverted repeats flanking an 8-bp spacer region **(Table 1)**. Here again, the asymmetry of the spacer region imparts directionality on the recombination reaction and hence on the outcome **(Fig. 1)**.

A considerable limitation of both the FLP/*FRT* and the Cre/*loxP* system exists when used for integration and inversion. In both cases two identical target sites (homotypic sites) are present in close proximity after the recombination event, which then can serve again as substrates for a further recombination event, i.e., for excision or reinversion **(Fig. 1)**. In other words, FLP and Cre are "bidirectional" recombinases. Because excision reactions are kinetically favored over integrations, the integrated DNA is highly unstable ***(32)***. Therefore, strategies have been devised to make integrations and inversions almost irreversible, mainly by specifically altering the spacer region of the *FRT* and *loxP* target sites. (For thorough overviews on these topics, mainly dedicated to applications in mammalian systems, *see* **refs. *9,48***; **ref. *49*** provides a very informative account on genetic screens in *Drosophila*, involving various applications of the FLP/*FRT* system.)

Table 1
Target Site Sequences of Cre, FLP, and ϕC31 Recombinases

	Recognition sequence				
Site	Direct repeat	1 bp	Inverted repeat 1	Spacer	Inverted repeat 2
FRT	5′GAAGTTCCTATTC	C	GAAGTTCCTATTC	TCTAGAAA	GTATAGGAACTTC 3′
	3′ CTTCAAGGATAAG	G	CTTCAAGGATAAG	AGATCTTT	CATATCCTTGAAG 5′
loxP			5′ATAACTTCGTATA	ATGTATGC	TATACGAAGTTAT 3′
			3′ TATTGAAGCATAT	TACATACG	ATATGCTTCAATA 5′
attP	5′ CCCCAACTGGGGTAACCTT T GAGTTCTCTCAGTTGGGGG 3′				
	3′GGGGTTGACCCCATTGGAA A CTCAAGAGAGTCAACCCCC 5′				
attB	5′GTGCCAGGGCGTGCCCT T GGGCTCCCCGGGCGCG 3′				
	3′CACGGTCCCGCACGGGA A CCCGAGGGGCCCGCGC 5′				

The *FRT* sequence is a full-length target site consisting of 48 bp; the *loxP* site consists of 34 bp. For FLP-mediated excision, the direct repeat and the following single bp are dispensible (***[9]***; for further details *see* **Box 1**). The recombination between two *FRT* or two *loxP* sites, respectively, occurs in the spacer region. The heterotypic *attP* (39 bp) and *attB* (34 bp) sequences depicted represent the minimal extent necessary to still achive 100% integration efficiency compared with longer attachment sites ***(52)***. Both sequences share an identical 3-bp region (bold) that is flanked by imperfect inverted repeats. The recombination between *attP* and *attB* occurs in this highlighted 3-bp region.

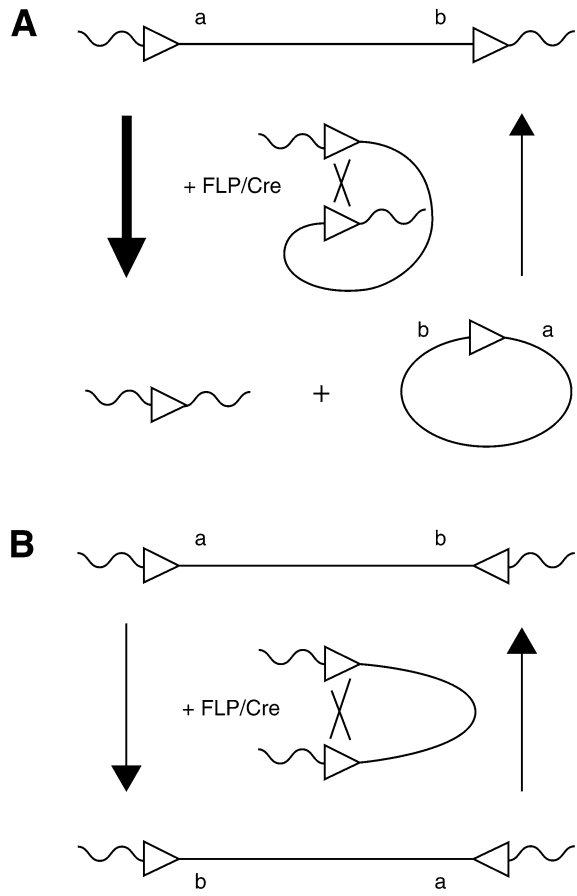

Fig. 1. Reactions catalyzed by Cre or FLP. Depending on the relative orientation of the *loxP* (or *FRT*) sites with respect to each other, the Cre- (or FLP-) mediated recombination event leads to different outcomes. **(A)** Cre or FLP will cause the excision of the sequence between two homotypic sites (*loxPs* or *FRTs*) oriented in the same direction. **(B)** If the sites are oriented in an opposite direction relative to each other, the outcome will be an inversion of the intervening sequence. (The inversion event can in principle occur in a repeated fashion as long as the recombinase is present.)

activation through this system resulted in high toxicity in proliferating cells, which was caused by the high levels of Cre recombinase that presumably acted on pseudo-*loxP* sites present in the genome and caused chromosomal aberrations. By fusing the Cre recombinase to the ligand-binding domain of the human estrogen receptor, the activity of this modified Cre recombinase could be regulated hormonally, and toxicity effects were avoided by administering appropriate estrogen concentrations ***(12)***.

Box 2

ϕC31 Integrase

The integrase of the *Streptomyces* phage ϕC31 normally mediates the integration of the phage genome (41.5 kb) ***(50)*** into the bacterial chromosome through heterotypic recombination sites, termed *attB* and *attP* site. Like FLP and Cre, the ϕC31 integrase does not need any accessory factors to mediate this integration ***(51)***. The minimal size for a fully functional *attB* site is 34 bp and for the *attP* site, 39 bp (*see* **Table 1**) ***(52)***. The two sequences, though largely different, share a 3-bp central region, where the crossover occurs, and this central region is flanked by imperfect repeats.

Recombination between the two attachment sites creates the hybrid sites *attL* and *attR* that are no longer substrates for the ϕC31 integrase ***(37,53)***. This distinguishes it from Cre and FLP, and thus the ϕC31 integrase, as a unidirectional recombinase, is an efficient tool to insert transgenes into a genome. This has been demonstrated in a number of organisms in recent years, including *Drosophila* ***(38,51,52,54,55)***.

In most transgenesis approaches the *attB* site, which in its natural context is in the bacterial host genome, has been moved into the incoming plasmid, and the *attP* site into the genome instead, where it serves as a docking site. This arrangement appeared to be more effective in earlier studies ***(54,56)***.

In principle, when combined with the FLP/*FRT* system, the Cre/*loxP* system offers an additional level of flexibility to perform more sophisticated clonal analysis. However, as there are only a few reports so far on the use of this system in *Drosophila*, we will concentrate below on the FLP/*FRT* system and some new strategies developed for site-specific transgenesis that use the ϕC31 integrase system.

1.2. Genetic Mosaics

Genetic mosaics serve as an important tool for characterizing gene function in flies. Mosaic animals contain cells or groups of cells (often clones) with a distinct genotype that differs from most other cells in the animal. The creation and analysis of such animals has allowed one to address many key questions in the field of developmental biology. Applications include the tracing of cell lineages for the establishment of fate and specification maps, the analysis of the function of essential genes in various body parts and at various developmental stages, and the determination of the autonomy of gene function.

Over the decades many techniques have been developed to create genetic mosaics in *Drosophila* (a recent excellent review is **ref. *13***; for an extensive coverage *see* **ref. *14***). These include surgical manipulations (transplantation), chromosome losses, mitotic recombination induced by irradiation, and in a different

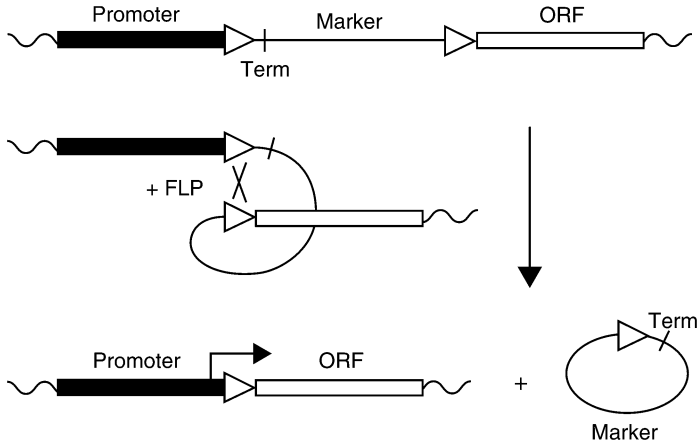

Fig. 2. The FLPout technique for gene activation (clones will be "negatively" marked). In the "ground state" the promoter will not transcribe the gene of interest, as they are separated by a FLPout cassette, which contains a transcriptional termination signal (Term). The termination signal can also be present after the marker gene; in such a case the marker can consist of a simple ORF that is driven by the promoter upstream of the FLPout cassette, and transcriptional termination would occur at the stop downstream of this ORF, still within the cassette. The cassette is flanked by *FRT* sites (open triangles) that are oriented in the same direction. The induction of FLP expression leads to a recombination between the FRT sites, resulting in the elimination of the cassette and allowing the promoter to direct transcription of the coding sequence. The cassette itself is excised and is lost in subsequent cell divisions.

way, the use of the Gal4-UAS system to activate expression of a particular gene or RNA interference (RNAi)-construct in subsets of cells. However, the currently dominant technique to create mosaics in *Drosophila* makes use of the site-specific FLP. The recent application of the ϕC31 integrase offers yet another tool to create mosaics in the future; however, to date, this enzyme has been mainly used to direct transgenes to distinct genomic sites.

2. Recombinases and Their Use for Gene Activation

2.1. FLPout Technique

This technique is mainly used to generate constitutive expression of a transgene in marked cells or clones of cells ***(15)***. The expression of the transgene is switched on by the excision of a so-called "FLPout" cassette, which separates the transgene from its promoter and contains a transcriptional termination site. The removal of the FLPout cassette leads to the fusion of the promoter to the coding sequence of the transgene and consequently to its expression **(Fig. 2)**.

In its simplest form, the promoter is an ubiquitously active one, such as that of the *actin5c* (Act5C) or *tubulin1*α (tub1α) genes. The FLPout cassette is made up of a marker gene (e.g., *yellow*, CD2) and a transcriptional termination site (polyA signal), and is flanked on both sides by an *FRT*. These *FRT*s are orientated in the same direction (direct repeats) and therefore FLP-mediated recombination between these two sites will lead to the excision of the intervening sequence. Finally, the FLPout cassette is followed by the coding region of the transgene that will be activated. The FLPout cassette, and hence the transcriptional stop site that prevents the expression of the transgene by the promoter, is removed by induction of the FLP recombinase, and this event can be controlled through the timing and the levels of FLP expression. The release of the cassette leaves a single *FRT* behind and fuses the promoter to the coding sequence of the transgene, thereby resulting in its heritable expression.

In most applications FLP is expressed under the heat-shock-inducible promoter of the *hsp70* gene ***(5,15)*** and thus, the short application of a heat shock leads to ubiquitous expression of the recombinase, resulting in the excision of the cassette (a "FLPout" event). The timing and the strength of the heat shock allows one to influence "clone formation". Whereas early clone induction generally results in larger clones, the strength of the heat shock influences the number of cells undergoing a FLPout event. The released cassette, carrying a marker gene, is lost in subsequent cell divisions, allowing "negative" identification of the cells or clones of cells that underwent an excision event.

Since its original implementation, the FLPout system has been further modified and combined with other tools, most notably the Gal4-UAS system. In the most commonly used combination of these two methods, which is often referred to as "FLPout-Gal4 system" ***(16–18)***, the transgene driven by the ubiquitous promoter after the FLPout event encodes the Gal4 transcription factor **(Fig. 3)**. Clones expressing Gal4 will in turn activate any UAS-transgene present. Therefore, these Gal4-expressing clones can be positively marked, for example, by the presence of an UAS-GFP or UAS-*lacZ* construct.

2.2. UAS-FLP

A variation of the system described earlier is the combination of "Gal4 enhancer trap" insertions together with UAS-*flp* and FLPout constructs. The main advantage of this "directed mosaic" system is that recombination events can be induced in spatially restricted areas ***(19)***. Here, the expression of Gal4 occurs in a subset of cells or in a given tissue, depending on the genomic regulatory elements acting on the Gal4 transgene insertion. The Gal4 expression drives the expression of the UAS-*flp* transgene. The FLP recombinase in turn will act on the *FRT* sites of the FLPout construct, leading to the excision of the

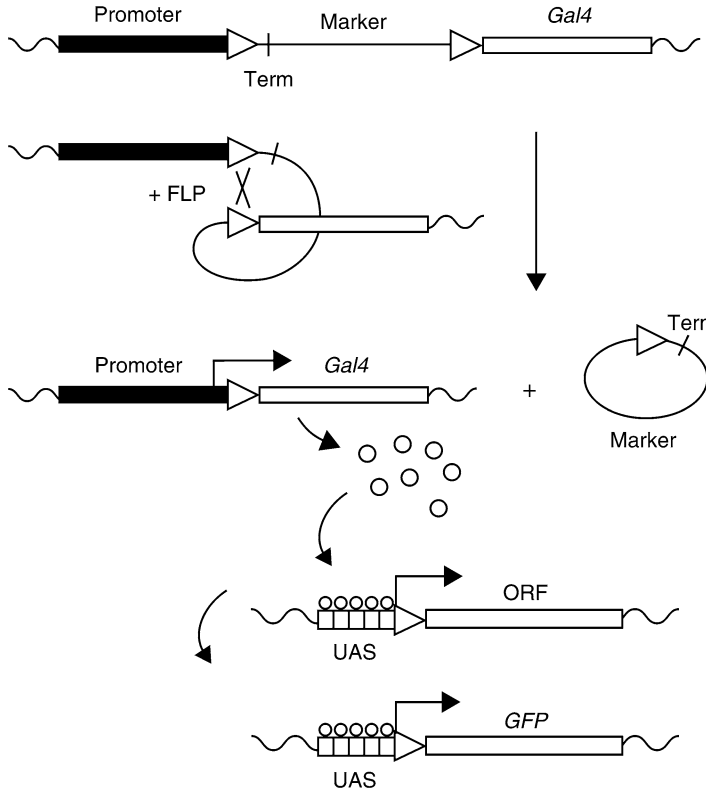

Fig. 3. The FLPout-Gal4 technique for gene activation (clones will be "positively" marked). The activation of FLP leads to the release of the cassette and thus to the expression of the *Gal4* transgene. Gal4 (open circles) will in turn activate UAS-regulated transgenes, including UAS-driven marker genes. This has the advantage that the generated clones will be positively marked. With this system multiple UAS-controlled transgenes can be activated in the FLPout clones (UAS-binding sites are indicated as small squares in front of the *FRT* symbol).

marker cassette and activation of, for example, another marker gene such as *lacZ* ***(20)***. If the FLPout construct contains a constitutive promoter, the cells undergoing the FLPout event will irreversibly continue to express the marker gene, even if these cells cease to express Gal4. Such a setup allows us to trace the fate of these cells, and their descendents. However, a prerequisite for this is that the Gal4 transgene insertion used expresses enough Gal4 to generate FLP levels that are sufficiently high to catalyze the FLPout reaction, and this is not always the case.

3. Recombinases and Their Use for Gene Inactivation

3.1. Mitotic Recombination

Tools for generating mosaics are especially useful to study recessive mutations that cause embryonic lethality when present in the entire organism. Such mutations *per se* would prevent analysis of their effects at later developmental stages. However, homozygous clones of such mutations can be created in an otherwise heterozygous animal by means of the "mitotic recombination" technique. This allows the characterization of gene function in various tissues at later developmental stages. Although mitotic recombination was induced by irradiation in the past, today this is generally done with the FLP/*FRT* system. We will concentrate on this latter method to generate mitotic clones.

In *Drosophila*, recombination is not only restricted to meiosis but can also occur spontaneously, albeit rarely, between the arms of homologous chromosomes during mitosis ***(21,22)***. If such an event occurs at a position proximal to a heterozygous mutation it can lead to the generation of two daughter cells that genetically differ with respect to each other and with respect to the heterozygous parent cell. One daughter cell is homozygous for the mutation (and the entire chromosome arm distal to the site of recombination), and the other is homozygous for the "wild-type" arm. The frequency of such naturally occurring mitotic recombination can be massively increased by providing *FRT* recombination sequences in conjunction with FLP, thereby also predetermining the site of mitotic recombination ***(23)***.

FRT sites were introduced into each of the major chromosome arms of *Drosophila* through P-elements, and for further use those lines were selected, wherein the P-elements had inserted into proximal locations (i.e., close to the centromere ***[24,25]***). The *FRT* insertion stocks were equipped with a transgene encoding FLP that is driven by a heat shock promoter (hsp70). In this setup, mitotic recombination between homologous *FRT* sites can be induced by applying an appropriate heat shock, causing expression of FLP, which in turn mediates recombination between the *FRT* sites **(Fig. 4A)**.

In a heterozygous setup, recombination between nonsister chromatids can create one daughter cell, which is homozygous for the mutation(s) distal to the *FRT*. All cells generated from this cell in subsequent cell divisions will be genetically identical, giving rise to a mutant clone, which can be analyzed phenotypically. The other daughter cell will give rise to a so-called "twin-spot" clone, which is genetically wild-type. Both these clones normally coexist in their heterozygous environment, the twin-spot often serving as a control for the mutant sibling clone. To visually follow the clones, the wild-type *FRT*-chromosome arm is usually equipped with a marker gene (e.g., *ubi*-GFP, GFP driven by an *ubiquitin* promoter). The twin-spot clone expresses two copies of

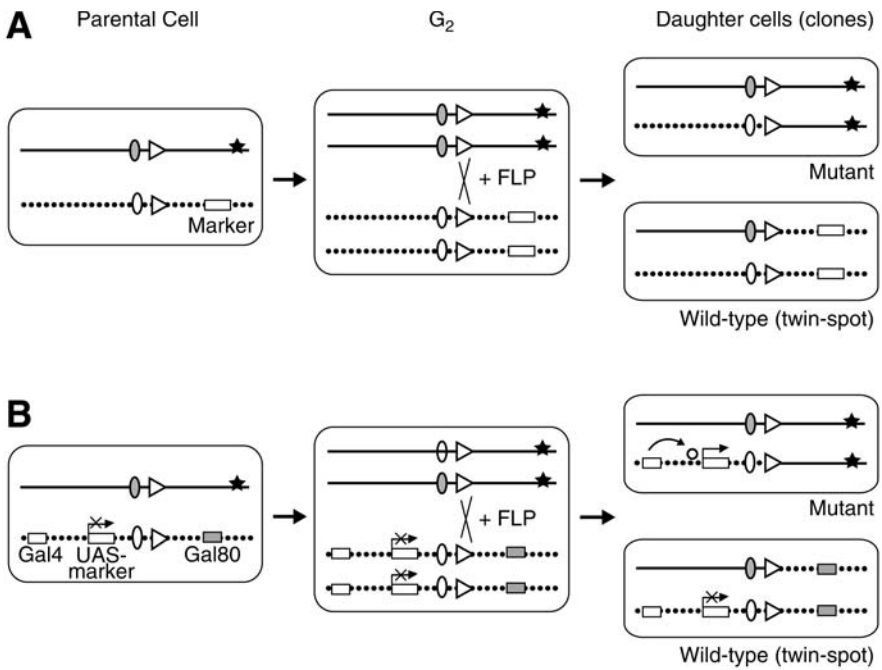

Fig. 4. Mitotic recombination with the FLP/*FRT* system. **(A)** One chromosome arm harbors a mutant allele (asterisk), the homologous arm a marker gene (e.g., *ubi*-GFP). Activation of the FLP recombinase can lead to recombination between two FRT sites present on homologous chromosome arms in the G_2 phase of the cell cycle (sister chromatids are drawn for the G_2 phase). If the sister chromatids segregate appropriately, two genetically different daughter cells are generated, of which one is homozygous for the mutation and lacking the marker gene, the other is wild-type (giving rise to the "twin-spot") and is expressing two copies of the marker gene. (The sister chromatids can also segregate such that the daughter cells are heterozygous for both the mutation and the marker gene, thus being of the same genetic constitution as the original parent cell. This possibility is not indicated in this scheme. Centromers are represented as small ovals.) **(B)** Mitotic recombination with the MARCM system. The characteristic feature of this technique is that the homozygous mutant cell (clone) will be labeled by expression of a marker gene. This is achieved by the use of the Gal80 repressor transgene, which represses Gal4 activity. In the absence of Gal80, as occurs in the mutant daughter cell, Gal4 can drive the expression of a UAS-marker transgene (e.g., *GFP*). For convenience the Gal4 gene, which is driven by a constitutive promoter, and the UAS-marker gene were drawn on the same chromosome; however, they can also be located on different chromosomes. A second useful application of the MARCM system is the expression of an experimental UAS-transgene in a marked cell clone that is accompanied by a wild-type sister clone for comparison. The FLPout systems described earlier do not generate such twin-spots (*see* **Figs. 2** and **3**).

the marker, the mutant clone none, and all other cells one copy. If the marker used allows discrimination between one vs two copies (by means of expression levels), all three genotypes can conveniently be distinguished by immunofluorescence microscopy.

The major advantages of the FLP/*FRT* mitotic recombination system are: (1) the recombination rates are much higher than those achieved by irradiation, (2) the site of FLP-mediated recombination is precisely determined by the location of the *FRTs*, and (3) the system does not cause significant cell death or developmental delay. Disadvantages of this system include: (1) the requirement to first recombine a mutation onto the appropriate *FRT* chromosome and (2) the fact that mutations on the fourth chromosome and proximal to the *FRT* sites on the other chromosomes cannot be analyzed (recombining a mutation and an FRT site is not easily possible on the fourth chromosome, because no meiotic recombination occurs on this chromosome).

3.2. Germline Clones: Dominant Female-Sterile Technique

Another use of the FLP/*FRT* system is to generate mutant clones in the female germline. The effect of a mutated gene on early development is often not seen in a zygotically homozygous mutant animal, because it is masked by the maternal contribution of wild-type gene product. However, if a homozygous mutant situation is established in the germline early on, no or an insignificant level of wild-type product is present in the oocyte and the resulting embryo, allowing the examination of so-called "maternal-effect phenotypes."

By using the dominant female-sterile (DFS) mutation ovo^{D1} in combination with the FLP/*FRT* system it is possible to create and select for the earlier described situations very efficiently ***(24)***. Female germ cells expressing the ovo^{D1} mutation die at an early stage and therefore do not give rise to any eggs. However, if mitotic recombination is induced early on in the female germline, some germ cells in the ovaries arise that lack the ovo^{D1} mutation, giving rise to clones permissive for egg production. If mutations are present on the *FRT* chromosome arm *in trans* to the ovo^{D1} mutation, their maternal effect phenotype can be examined in the resulting embryos. *FRT* lines carrying ovo^{D1} transgenes on all major autosome arms were generated ***(26,27)*** and therefore, mutations on all these chromosome arms and the X chromosome (where the *ovo* gene resides) can be analyzed with this technique.

3.3. Mosaic Analysis With a Repressible Cell Marker

The creation of mutant clones is generally coupled to the loss of a marker in these clones, as described under **Subheading 3.1.** This is usually not problematic in tissues such as mosaic imaginal discs, where the mutant clones reside in an essentially flat cell layer, and can therefore be relatively easily identified, as they appear as patches of

"nonmarked" cells. However, in more complex tissues, like for instance the nervous system, it can be hard to clearly identify unlabeled mutant cells or clones of cells among their marked sibling, and impossible to carefully assess cellular morphology.

To overcome this problem a strategy was developed that marks the mutant daughter cell and its descendants following a recombination event in a positive manner ***(28)***. This approach, called "mosaic analysis with a repressible cell marker" (MARCM), relies on the combined use of a Gal4 activator and a Gal80 repressor transgene, arranged in a manner such that the FLP-mediated mitotic recombination event causes the genetic loss of the Gal80 repressor and the concomitant derepression of Gal4 activity **(Fig. 4B)**. Such cells are homozygous for a potential mutation that was situated *in trans* to the Gal80 repressor, but they are at the same time positively marked by the expression of a UAS-marker transgene (e.g., *UAS-GFP* or *UAS-lacZ*). All other cells express the Gal80 repressor, which inhibits the activity of Gal4, and are therefore unmarked. Importantly, this system offers the possibility to drive, in addition to the marker, other UAS-transgenes in the mutant clone.

3.4. Creating Molecularly Defined Deletions

Deletions (deficiencies) are important tools in the genetic analysis of any organism. They are especially valuable for mapping recessive mutations to intervals on a chromosome. The failure of a specific deletion to complement a recessive mutation can be taken as an indication that the affected gene lies within the interval defined by the deletion. Overlapping deletions can be used to further narrow down the location of a mutant gene.

However, because many available deficiencies are not precisely characterized with respect to their "breakpoints," strategies were developed to create molecularly defined deletions ***(29)***. The approach uses two nearby located transposons *in trans*, each of them carrying one *FRT* site, which is orientated in the same direction as the other **(Fig. 5)**. The Flp-mediated mitotic recombination (*in trans*) between the *FRT*s causes the excision (and duplication) of the sequence lying between these transposons (the mechanism is essentially a controlled translocation). If the position of the two transposons is exactly known, the extent of the deficiency created is molecularly defined. Based on this strategy two libraries of such deletions have been created and are freely available ***(30,31)***. However, not all combinations of compatible *FRT* insertions have been used for these libraries, especially for pairs of *FRT*s that are located in close proximity to each other. These novel combinations can be exploited for the generation of small deletions that allow the fine-mapping of a mutant gene.

4. Recombinases and Their Use for Transgenesis

One fruitful strategy to characterize the function of genes is to manipulate them in vitro and then to examine the effect of these modifications in vivo. This

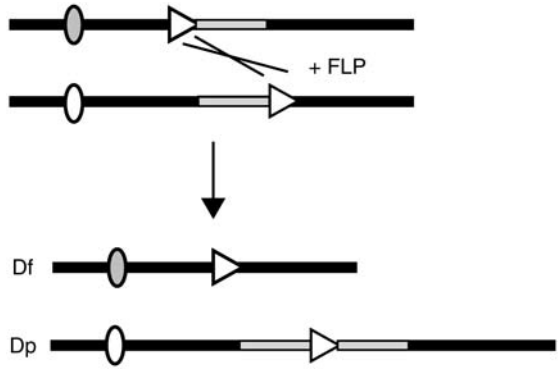

Fig. 5. Creating defined deletions with the FLP/*FRT* system. The scheme represents the basic principle to create molecularly defined deletions. Two precisely mapped *FRT*-containing transposons are brought *in trans* to each other. Induction of FLP expression results in a recombination event between the two *FRTs,* on which the indicated region (light gray) is either lost (Df) or duplicated (Dp). (The *FRT*s are indicated as triangles, but the transposons, carrying these *FRT*s, are not indicated.)

requires the stable insertion of the constructs into the genome and the prevailing method for this over the last 25 yr has been germline transformation with P-elements ***(1,2)***. As P-elements integrate in a near-random fashion, this method has found many other applications that specifically make use of this "randomness" (e.g., insertional mutagenesis). However, with respect to transgene analysis, this randomness is mainly regarded as a disadvantage.

Different integration sites do not permit a precise comparison between various transgene constructs, as the transgenes might be under varying influences from the neighboring chromosomal environment, so-called "position effects." A rough assessment can be achieved by examining several independent integrations. However, for precise structure/function analyses (e.g., comparison of differently mutated constructs), transgenes should only be compared quantitatively if they are situated at an identical location. If this is a predetermined integration site, it offers the additional advantage of eliminating the efforts associated with mapping and testing insertion sites. Further, predetermined target sites eliminate unwanted disruption of genes as can occur with "random" integration techniques. With this in mind strategies have been developed to achieve integration at predefined sites, based on the following recombinases: Cre, FLP, and ϕC31 integrase.

4.1. Transgene Coplacement and Cassette-Exchange Approaches

An early attempt used a combination of the Cre/*loxP* and the FLP/*FRT* system ***(11)***. In this approach, termed "transgene coplacement", two transgenes are introduced within the same P-element vector into a random position in the

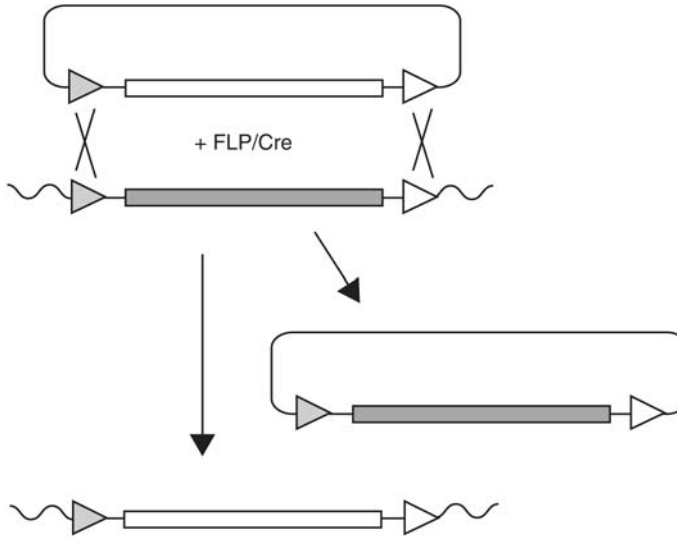

Fig. 6. Basic mechanism of RMCE. There are numerous RMCE strategies. The one depicted here was used by Oberstein et al. ***(33)*** and made use of heterotypic *loxP* sites. The general intention to use heterotypic *loxP* (or *FRT*) sites is that sites of different types do not recombine with each other—neither in the donor construct nor in the landing site. Thus, recombinations only occur between sites of the same kind (homotypic sites, indicated as blank or light gray-colored triangles), causing the exchange of the donor cassette and the cassette present in the landing site. Each of the cassettes contains a different marker gene (*white* and *yellow*), allowing for convenient scoring of exchange events in the F_1 generation.

genome. Each of the transgenes is flanked by either *FRT* or *loxP* sites, thus allowing the excision of one or the other of the transgenes by inducing either FLP or Cre recombinase expression. This method generates two lines, each with one of the transgenes at the same genomic locus. The obvious limitation of this approach is that only two transgenes can be compared with each other.

More recently, the Cre/*loxP* system was used for a so-called "recombinase-mediated cassette exchange" (RMCE; reviewed in **ref. *32***). In this system a marker gene cassette, which is flanked by heterotypic (incompatible) *loxP* sites, is first introduced through P-element transformation and subsequently this "landing site" is used to integrate transgenes through Cre-mediated cassette exchange **(Fig. 6)** ***(33)***. The transgenic integration frequency of this strategy is slightly less than the one obtained with P-elements; however, this system allows analysis and comparison of any number of transgenes at the very same genomic position.

Another study presented a FLP-mediated RMCE approach for site-specific targeting ***(34)***. To increase the efficiency of the cassette exchange the authors included, apart from heterotypic *FRTs*, a homing sequence from the *linotte*

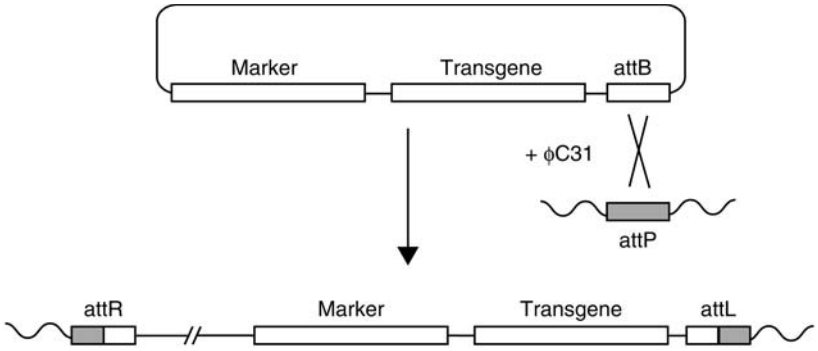

Fig. 7. Basic mechanism of a ϕC31-mediated plasmid integration. The ϕC31 integrase mediates recombination between the *attP* site (here present in the genome) and the *attB* site (here present on a vector plasmid). In *Drosophila*, an *attP* landing site is installed in the genome by transposon-mediated germline transformation and its position is molecularly characterized. The transformation vector carries a transgene, an *attB* site, and a marker gene (e.g., *white*). The marker gene will allow the scoring of transformants in the F_1 generation, just as in a conventional transgenesis procedure. The difference here is that the ϕC31 integrase on its own can only mediate the integration reaction, but not excision. Furthermore, integration occurs at the provided docking site with a high degree of specificity, thus allowing for site-specific integration.

locus, which has been reported to efficiently mediate the homing of P-element vectors to the *linotte* locus. The *linotte* sequence was inserted both into the cassette of the landing site and of the donor construct. However, the influence of these *linotte* sequences on targeting frequency was not assessed. This approach resulted, on average, in targeted integrations at a frequency of 23%.

Although this FLP-RMCE strategy is more efficient than the Cre-RMCE version, the latter may be favored when FLP/*FRT* is used for additional manipulations in the same fly line. An important advantage of any RMCE approach is that sequences outside the cassette will not be integrated (e.g., vector backbone); however, the same result can also be achieved with the ϕC31 integrase system (*see* **Subheading 4.2.**).

4.2. Transgenesis With the ϕC31 Integrase System

The integrase of the *Streptomyces* phage ϕC31 normally causes the integration of the phage genome into the bacterial chromosome by mediating a sequence-directed recombination between two short attachment sites, the bacterial attachment site (*attB*) and the phage attachment site (*attP*) ***(35–37)***. It has been shown that this integrase system causes recombination through these sites in a number of other organisms and it has recently been applied to *Drosophila* (*see* **Fig. 7**) ***(38)***. An attractive feature of the ϕC31 system is that the integrase

solely mediates integration ***(37)***, which distinguishes it from the Cre/*loxP* or FLP/*FRT* system, where the recombinase can catalyze both the integration and the excision reaction.

In its first implementation ***(38)***, a P-element was used to install an *attP* site in the genome. Embryos harboring an *attP* landing site were then coinjected with an *attB*-containing plasmid and in vitro transcribed ϕC31 integrase mRNA.

Recently, this integrase system has also been applied in an RMCE strategy in *Drosophila* ***(39)***. This approach used cassettes that were flanked by either *attP* or *attB* sites. Cassette exchange events were detected by the loss of the target site marker and the appearance of the donor cassette marker. The use of the ϕC31 system for RMCE has the advantage that FLP/*FRT* (or Cre/*loxP*) can be used for other genetic manipulations.

In another recent application ***(40)*** BAC (bacterial artificial chromosome) vectors with *attB* sites were developed; the ϕC31 integrase was shown to have the capability to insert very large DNA constructs (of up to 130 kb) into various *attP* lines, that they have generated, with frequencies that are generally not achievable with P-elements (about 10% for 15–20 kb and about 2–4% for plasmids >50 kb). Thus, this new approach will be relevant, for example, when studying the entire complexes of genes, and it will facilitate genetic engineering in *Drosophila* (for an overview on the tools involved and related subjects, *see* **ref. *41***).

Further improvements to efficiency and convenience of the ϕC31 integrase system were presented in another study ***(42)***. A large collection of lines with precisely mapped *attP* sites on the four major chromosomes was created and many of these landing sites were tested for accessibility and expression behavior. These target sites were additionally designed to facilitate various subsequent in vivo modifications, either with the Cre/*loxP* or the ϕC31 system itself. These modifications also allow for the elimination of the vector backbone after the integration event, an advantage usually attributed to RMCE approaches. A very useful advancement of the ϕC31 integrase system is the generation of transgenic lines expressing ϕC31 integrase in a germ cell-specific manner ***(42)***. These lines render the production and handling of integrase mRNA superfluous. The combination of such an "endogenous" ϕC31 integrase source with selected *attP* sites yielded integration rates up to almost 70%.

Given the combination of the site specificity and the high efficiency, the ϕC31 will undoubtedly become the transgenesis method of choice, superseding the P-element system for most transgenesis experiments.

5. Concluding Remarks and Future Directions

In the above review we have tried to highlight the immense contribution that the application of site-specific recombinases has had, and will have, on *Drosophila* research. As biological questions become increasingly complex, we need to

develop more and more sophisticated tools, and site-specific recombinases are an integral part of this tool kit. Such experiments may, for instance, require controlling the activation or the inactivation of several genes, and this in both a temporally and spatially controlled fashion. By way of a specific example: cell–cell communication could be more carefully investigated by inducing new clones in pre-existing clones, both clones being genetically distinct. However, this induction of clones within clones, and other experimental setups, will require the development of new ways to independently control the activity of various recombinases. Such efforts have been described, so far mainly with respect to the Gal4-UAS system, where several different inducible techniques were recently developed (reviewed in **ref. *4***).

The ϕC31 system will strongly influence the field of transgenesis, not only because of its efficiency, but also because this system allows one to get considerable control over position effects, which influence expression patterns and levels of transgenes. Thus, this system will permit precise in vivo structure/function analyses, the integration of large DNA constructs, and it will practically lead to a reduction of the number of transgenic lines that have to be generated to analyze a specific transgene, as already characterized landing sites can be targeted. Because of its integration efficiency the ϕC31 system will also be a promising tool to handle large DNA sets, for instance to create systematic UAS-ORF (open reading frame) libraries for mis- or overexpression screens. It can further be expected that this system will shortly also be used to enable site-specific integration in commonly used *Drosophila* cell lines, offering further improvements for cell-based assays. Clearly, therefore, site-specific integrase systems will strongly influence research with *Drosophila* in the coming years.

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11

Cuticle Preparation of *Drosophila* Embryos and Larvae

Cyrille Alexandre

Summary

The *Drosophila* embryonic ventral epidermis has served as a unique tissue for the genetic analysis of patterning. Two types of epidermal cells are easily distinguished: those that secrete short, thick hair-like structures called denticles and cells that only secrete smooth cuticle. Denticle-secreting cells form segmentally repeated belts. Within each belt, six types of denticles can be recognized according to size, shape, and orientation (types 1–6). They are arranged in a stereotypical manner within each denticle belt. This pattern results from the spatially organized activation of several signaling pathways during embryogenesis. Cuticle patterns therefore provide a sensitive readout of signaling activity and other patterning mechanisms. Here, I describe methods of preparation and analysis of cuticles from 1st instar larvae as well as from 3rd instar larvae. In addition, a protocol to simultaneously analyze cuticles and β-galactosidase activity of embryos expressing *lacZ* reporter genes is presented.

Key Words: β-galactosidase staining; cuticle preparation; denticles; *Drosophila*; polarity; signaling pathways.

1. Introduction

In *Drosophila* the epidermis is a one-cell-thick layer of epithelial cells covered by a cuticle that protects the insect from the environment ***(1)***. At the end of embryonic development, the ventral epidermis becomes decorated by segmentally repeated belts of denticles, short hair-like protrusions secreted by individual cells ***(1,2)***. The abdominal region has eight denticle belts. In each belt, there is a precise orientation and arrangement of the denticle hairs. This orientation is under the control of a least four signaling pathways, those activated by Hedgehog, Wingless, epidermal growth factor (EGF), and Notch ***(3,4)***, and therefore, the precise analysis of the ventral cuticle of 1st instar larvae is an important diagnostic tool for defective signaling. In this chapter, I describe a detailed procedure for preparing the cuticle of embryos reaching the end of embryogenesis and for the analysis of

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the cuticle pattern at high magnification. A modification of this protocol to prepare cuticles from 3rd instar larvae (*see* **Note 1**) is also provided. Finally, I detail a method for the simultaneous analysis of cuticle morphology and β-galactosidase activity in embryos expressing a *lacZ* reporter gene (*see* **Note 2**). The latter method allows, for example, to relate the expression of specific genes, visualized by a *lacZ* reporter, to the location of specific denticle types in embryos.

2. Materials

1. Egg-laying cages (home made) with apple juice agar plates (for description, *see* Chapter 4).
2. Sodium hypochlorite solution (8% [bleach] [Fisher Scientific, UK]).
3. Very fine hypodermic needles (27 g × 1/2 TW) (Sherwood Medical, UK).
4. Hoyer's-based medium: add 30 g of gum arabic (Sigma-Aldrich, UK) to 50 mL of distilled water. Stir overnight until completely dissolved, then gradually add 200 g of chloral hydrate (anhydrous) (Sigma-Aldrich). Add 20 g of glycerol. Centrifuge for 2 h at 25,000*g*. The medium is stable for several years at room temperature. If the medium becomes too viscous over the years, it can be diluted with water.
5. Lactic acid. The addition of lactic acid to Hoyer's-based medium increases the contrast in preparation and reduces clearing time. The 1:1 (v/v) mixture of Hoyer's-based medium and lactic acid is referred to here as Hoyer's-based mountant. This solution will digest all internal tissues but will leave the cuticle intact.

3. Methods

I will describe a protocol that requires the removal of the vitelline membrane. Other procedures, like the quick preparation of undechorionated eggs, has been described elsewhere ***(5)***, and therefore, will not be mentioned here. Removal of the vitelline membrane before embedding is essential for high-quality cuticle preparations.

3.1. Cuticle Preparation

1. Put flies into egg-laying cages with apple juice agar plates and incubate at 25°C in the dark. Change collection plate when 50–100 eggs have been laid.
2. For wild-type or viable larvae, allow embryos to age for 24 h at 25°C. Recover larvae before they hatch because crawling larvae will have yeast in the digestive track and this can spoil the preparations. For preparations of larvae carrying a lethal genetic combination, collect the unhatched embryos from a 24–36 h old plate. Wild-type crawling larvae can be rinsed off the plate with phosphate-buffered saline (PBS) and discarded.
3. Perform the subsequent steps under a stereo microscope. Add a drop of distilled water (5 µL) to a Petri dish. Place a few (~10) nondechorionated embryos in this drop using fine forceps (Dumont). Remove the water using a P-20 pipet (Gilson) and replace it with a drop of 8% sodium hypochlorite solution. Leave for about

2 min until the chorion is seen to dissolve. Replace the bleach with a drop of water using the P-20 pipet. Do not worry about overdechorionating, the vitelline membrane is still present and protects the embryo. Rinse one more time with another drop of distilled water.

4. Roll the embryo to the edge of the drop with the side of a very fine hypodermic needle. Poke the embryo with the needle around the head region. The vitelline membrane will remain at the water surface, whereas the larvae will sink into the water. Repeat with the other embryos, or proceed to the next step.
5. Remove the vitelline membrane(s) from the water drop with the help of the needle. Replace the drop of water with a drop of 1:1 lactic acid:Hoyer's-based medium (Hoyer's-based mountant).
6. Transfer the Hoyer's drop containing the embryo, using the P-20 pipet, onto a glass microscope slide. Orient the larvae so that the denticle belts are upward. This step will be difficult if the embryos are mutant for the *yellow* (*y*) gene as these embryos become translucent within the mounting medium. Try to use *yellow*$^+$ flies if possible. Around 7 µL of Hoyer's-based mountant should be applied if a 18×18-mm^2 cover slip is used in the next step. The exact volume depends on the viscosity of the mountant.
7. Place one edge of a cover slip next to the drop using forceps and slowly apply the cover slip onto the drop. If this is performed under the stereo microscope, the orientation of the embryo can be modified in the process. If the right amount of Hoyer's-based mountant is used, it will spread by capillary action under the entire cover slip resulting in a flat preparation. If too much Hoyer's-based mountant was applied, use forceps to press gently on the cover slip to flatten the larvae. Avoid lateral movement as this will ruin the morphology of the cuticles. Monitoring the whole procedure under a stereo microscope (using a Leica MZ16 [Leica, Germany] or equivalent) usually ensures even flattening of the embryo.
8. Incubate the slide at approx 60–65°C overnight in an oven to allow digestion of internal tissues and clearing of the larvae. Optional: seal the cover slip with nail varnish for extra-long storage.

3.2. Analysis of Cuticle Preparation

After a few hours in the oven the preparation can already be evaluated under a compound microscope equipped with phase contrast optics (however, for best results incubate overnight in the oven). We use A Zeiss Axiophot (Grom Zeiss, Germany). The Ph1 setting of the condenser should be used with ×10 Plan-Neofluar Zeiss objectives. A darkfield image of the whole larvae can be obtained by setting the condenser on phase 3 and using the ×10 Plan-Neofluar Zeiss Ph1 objective **(Fig. 1A)**. Use the Ph2 setting for ×20 and ×40 Plan-Neofluar Ph2 objectives for phase images **(Fig. 1B**,**C)**. At ×20, one can see the whole larvae. At ×40, some details of the denticle belts can be observed **(Fig. 1B**,**C)**; however, a ×100 Plan-Neofluar oil objective should be used for detailed observation of denticle shape and orientation. For this objective, set the condenser on Ph3. This magnification gives very good details on denticle diversity

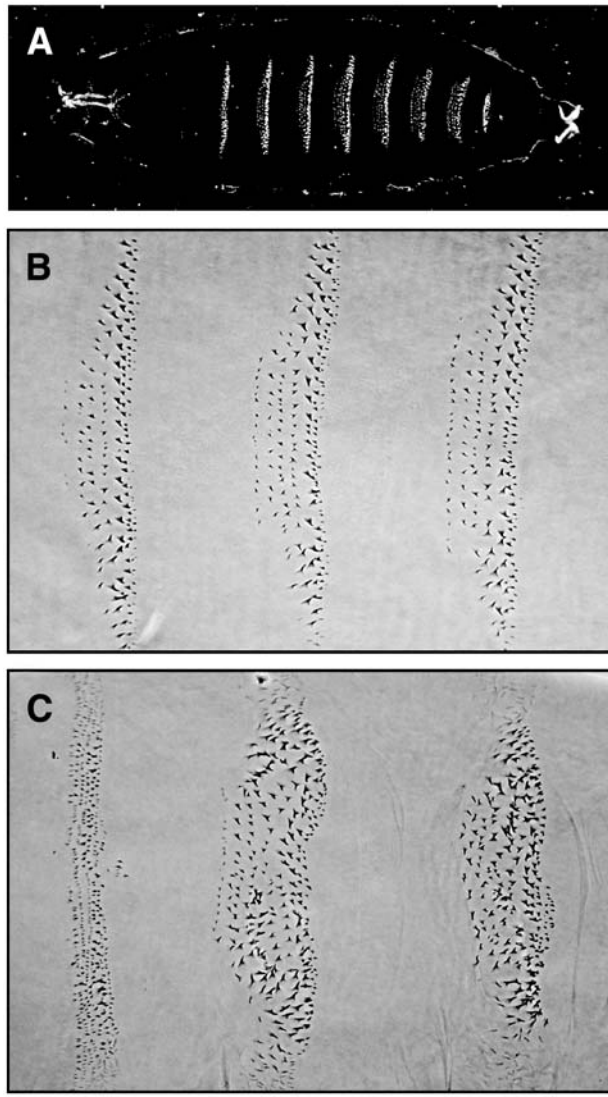

Fig.1. **(A)** Darkfield image of a wild-type embryo with the ×10 objective and phase 3 condenser. Note the eight abdominal denticle belts. **(B)** Wild-type abdominal segments (A2–A4) using the ×40 objective and phase 2 condenser. **(C)** *Wingless* mutant (*wg* ^{CX4}) ***(6)*** embryo overexpressing an activated form of Armadillo under the control of the *paired*-Gal4 driver, which is expressed in every other segment in the embryo. The presence of activated Armadillo changes the fate of the epidermal cells from denticle to smooth cell type (naked cuticle). In this and subsequent figures, anterior is to the left.

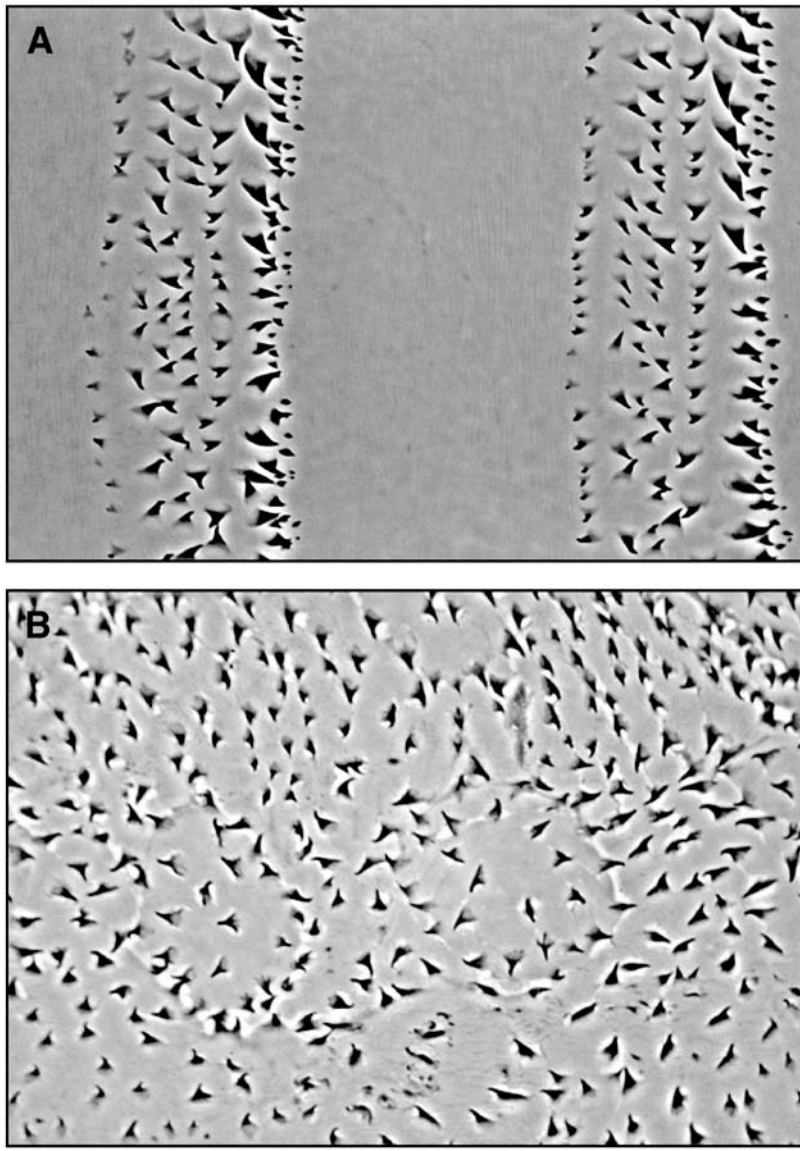

Fig. 2. **(A)** Detailed region of a wild-type embryo using the ×100 oil objective and phase 3 condenser. Note the stereotypical arrangement of denticles in both belts (A2 and A3). Rows 1 and 4 point anteriorly whereas rows 2, 3, 4, and 6 point posteriorly. **(B)** *Hedgehog* mutant (hh^{AC}) ***(7)*** with the same optics as (A). In this segment polarity mutant, the early disappearance of Wingless expression leads to the loss of the smooth cuticle specified by Wingless signaling. The image corresponds to approximately three segments.

(Fig. 2A,B). To increase the contrast with the different magnifications, the opening of the field diaphragm should be such that only the field of view is illuminated. We Capture Images on a CCD Leica camera and process them using Adobe Photoshop.

4. Notes

1. Protocol to simultaneously analyze cuticles and β-galactosidase activity of embryos expressing *lacZ* reporter genes: although the protocol described earlier gives cuticle preparations that allow high-resolution analysis at ×40 and ×100, it does not allow reporter gene expression to be assessed. This can be important in mutant situations when the position of specific denticle types must be related to the expression of specific genes. I have devised a method to reveal β-galactosidase expression in preparations that also display good denticle morphology. The first steps of the protocol (from **steps 1** to **3**) are as described in **Subheading 3.1.**
   a. After the addition of bleach, dechorionated embryos are selected and mounted on a sticky surface for injection. The procedure to achieve this is similar to that used for generating transgenic flies (for description, *see* Chapter 4). The aim here is to prepare embryos for injection with the posterior side facing the needle.
   b. Prepare 8% of glutaraldehyde (diluted in PBS). Load the injection needle with this solution of fixative. The internal diameter of the tip of the needle should be three or four times larger than needles used for transgenesis. A sharp needle that will easily penetrate the cuticle is essential.
   c. Inject the fixative solution into the middle of the larvae. The vitelline membrane will burst whereas the larva elongates and becomes fixed. Remove the needle as quickly as possible (it tends to stick to the internal fixed tissues).
   d. After injection, collect the elongated larvae with forceps and transfer them into a Petri dish containing PBS Triton (0.1%). Make them sink to the bottom of the Petri dish by removing as much of the oil as possible. With a piece of razor blade cut the larval head off, as well as the posterior side, in order to help the diffusion of the Lac-Z buffer into the larvae.
   e. Transfer the cut larvae into the Lac-Z buffer containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) substrate. Prepare an 8% (w/v) stock solution of X-Gal in dimethylformamide. Add 0.08 volume of the X-Gal stock solution to prewarmed (37°C) LacZ buffer (3 m*M* $K_3Fe[CN]_6$, 3 m*M* $K_4Fe[CN]_6$, 0.9 m*M* $MgCl_2$, and 0.1% [v/v] Tween-20 in PBS). The X-Gal stock solution can be stored at −20°C. Let it develop until the appropriate staining is visible.
   f. Transfer the stained larvae to a Petri dish containing PBS Triton (0.1%). While holding the larva with the forceps in one hand, cut it longitudinally with the razor blade in order to obtain two halves: the dorsal "half pipe" and the ventral "half pipe." Clean the inside of the larva with fine forceps or a tungsten needle, i.e., remove the fixed gut and central nervous system (CNS) while holding the half pipe with forceps.
   g. Mount the stained epidermis with Hoyer's-based mountant as described earlier (**Fig. 3A**).

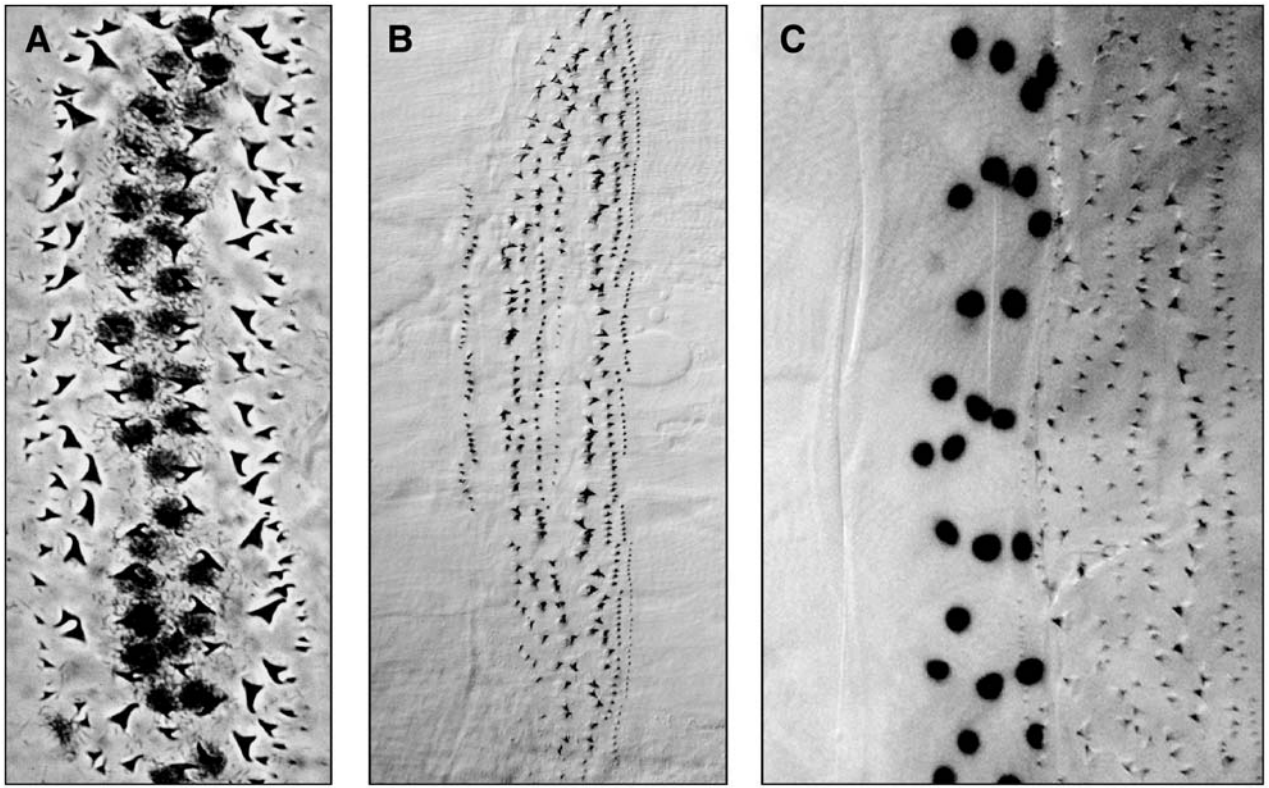

Fig. 3. **(A)** Denticle pattern of a larva overexpressing under the control of *engrailed*-Gal4, a truncated form of the transcription factor Ci, which represses the expression of *hh*. This larva also carries a *rhomboid*-nuclear *lacZ*-enhancer trap gene. The early loss of *hh* changes the denticle pattern. There is now a mirror-image 5, 4, 4, 5 pattern flanked by row1 anteriorly and row 6 posteriorly. The Lac-Z staining of this 1st instar larva before mounting helps to visualize the upregulation of *rhomboid* expression in rows 3 and 4 (*see* **ref. *3*** for more details). **(B)** Denticle belt (A3) from a 3rd instar larva using the glutaraldehyde fixation method. **(C)** Visualization of engrailed-expressing cells in 3rd instar larvae fixed as in (B) and stained for Lac-Z. The genotype is *engrailed*-Gal4, UAS-nuclear Lac-Z.

2. Protocol for preparing cuticle from 3rd instar larvae: the protocols described so far are for 1st instar larvae. However, it is also possible to examine the denticle pattern at later larval stages (2nd and 3rd instar). The protocol described next can be used for wild-type 3rd instar larvae as well as 3rd instar larvae that express lacZ or GPP under the control of a specific promoter. The main goal in this protocol is to obtain a flat epidermis. Because of their size, 3rd instar larvae cannot be mounted easily under a cover slip. The way around this problem is to mount only the epidermal tissue. In order to get an elongated preparation, live 3rd instar larvae are fixed in heptane saturated with glutaraldehyde and are subsequently dissected.
   a. To prepare the heptane, mix 166 µL of 50% glutaraldehyde with 166 µL of PBS and 333 µL of heptane in an Eppendorf tube (Trefflab, Switzerland). Vortex the Eppendorf tube several times for 30 s and then leave the tube for 1 min on the bench. Transfer the upper phase (heptane) to a new tube. Incubate the tube at 56°C for 15 min to warm up the heptane.
   b. Then, open the tube and quickly put the 3rd instar larvae straight into the heptane with the help of the forceps and place the tube back at 56°C for 5 min. During this time, the heat will elongate the larvae and the larvae will become fixed.
   c. Transfer the saturated heptane to a new Eppendorf tube (saturated heptane can be used for additional fixations) and replace it with PBS.
   d. Transfer the fixed larvae to a Petri dish containing PBS. Use blue pipet tips cut with scissors to enlarge their diameter.
   e. As described in **Note 1**, cut the head region and the posterior part of the larvae.
   f. Cut the larvae longitudinally with the razor blade and remove all internal tissues.
   g. Transfer the ventral dissected epidermis into the Lac-Z buffer containing the X-Gal. Let it develop until appropriate staining is visible. Stop the color reaction by transferring the stained epidermis to a dish containing PBS and 0.1% (v/v) Tween-20.
   h. Mount epidermis in Hoyer's-based mountant. To avoid folds when mounting the epidermis, mount only two to three segments at a time by cutting the appropriate epidermal region of interest with a razor blade (*see* **Fig. 3B**,**C**).

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Immunolabeling of Embryos

H.-Arno J. Müller

Summary

The molecular mechanisms controlling *Drosophila* embryogenesis are among the best-studied examples in animal development. Whereas the formation of developmental pattern in embryos was intensely examined in the past three decades, the cell biological basis of morphogenesis is now entering the center stage of the research on fly embryos. A fundamentally important procedure has always been to determine the subcellular localization of proteins in embryos by immunolabeling. The challenge of the commonly used whole mount-staining procedures is to balance a good structural preservation during fixation and allow at the same time the penetration of the antibodies through the tissue. Different procedures have been developed that allow the preservation of distinct compartments of the cell and thus, optimize for the specific subcellular localization of proteins. This chapter provides a general immunolabeling protocol with variations suitable for a broad panel of antigens.

Key Words: Antibodies; *Drosophila*; embryos; immunolabeling; microscopy; whole mount.

1. Introduction

One of the most widely used techniques to study the function of proteins during embryogenesis is to determine their localization in fixed samples using specific antibodies. The specificity of the antibodies, the abundance of the epitopes of the proteins under investigation, and the general conservation of the cell and tissue structure are the most important factors to obtain reliable staining results. The choice of the immunolabeling protocol and the interpretation of immunolocalizations will therefore always need to take all of these factors into account.

In nature, *Drosophila* embryos develop in the open air and hence need to be protected from dehydration. This protection consists of the eggshell, the chorion, and the vitelline envelope, which render the embryo inaccessible to aqueous solutions, including fixatives. In particular the outer, waxy layer of the vitelline

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envelope needs to be permeated by the fixatives ***(1)***. The removal of the vitelline envelope often causes problems with the preservation of antigens and structural conservation. Although manual removal of the vitelline envelope reveals the best results regarding fine structure, low permeability of the tissue may be a problem. Physical removal of the vitelline envelope by methanol treatment is suitable for large amounts of embryos and results in decent staining results because of good tissue permeability; however, the structural preservation is severely affected by methanol treatment. Some of these technical hitches have been addressed by the traditional phase partition fixation by Zalokar and Erk ***(2)***, where embryos are fixed at the interphase of a heptane/aldehyde aqueous solution. The Zalokar fixation also provides a good structural preservation for electron microscopy **(Fig. 1A–C)**.

Immunolabeling procedures must consider the accessibility of antigens within the cell. Antibodies have to penetrate into the tissue; a process strongly improved by using detergents. However, different cellular proteins might require diverse fixation procedures, because of distinct requirements to maintain certain subcellular structures and to maintain the availability of epitopes. These considerations make it almost impossible to advise a single immunolabeling procedure that will simultaneously fulfill all requirements for different antibodies and antigens; therefore, many improvements specific for particular antigens or cellular structures have been developed throughout the years.

Fixations that allow for whole mount immunolabeling of fly embryos are generally not sufficient to provide good preservation of the fine structure of the cell **(Fig. 1D–L)**. Cellular structures are often destroyed or extracted by detergents or organic solvents used during fixation and labeling. In particular, extraction of cytosolic proteins and membranes have to be considered when interpreting results of whole mount-stained embryos. On the other hand, protocols that maintain the fine structure of the cells—for example, in transmission electron microscopy—will usually not allow for whole mount immunolabeling of embryos. It is therefore pivotal to keep in mind that the localization of antigens by whole mount immunolabeling might not reflect the precise subcellular localization, simply because the particular cellular compartment or its normal cellular position has been destroyed or altered ***(3)***. The problem is most evident in the case of membrane proteins; for example, to get access in a whole mount preparation to a luminal epitope within the endoplasmic reticulum (ER), the ER membrane itself needs to be at least partially destroyed, because antibodies will need to permeate the ER membrane. In such cases costaining with established subcellular markers and/or electron microscopy is essential to provide some evidence for specificity. Although whole-mount procedures might therefore often not provide the exact localization of proteins in most cases, these procedures are still valid when immunolabeling in wild-type is directly compared with mutant embryos. The problem of the

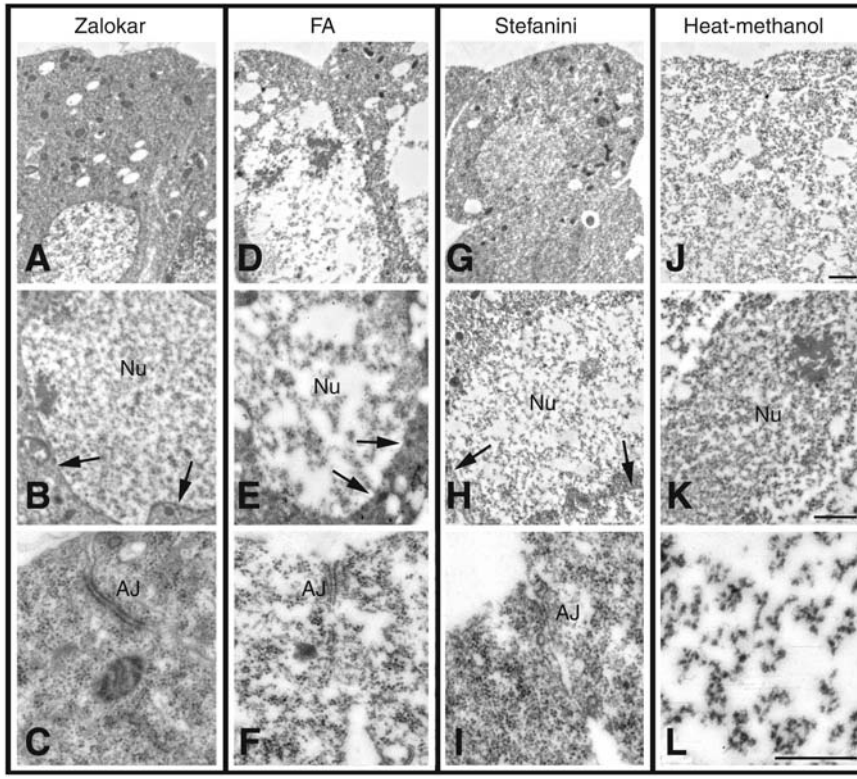

Fig. 1. Preservation of fine structure by different fixations for whole mount immunolabeling of embryos. Embryos (postgastrulation stages, extended germ band) were fixed by standard methods for transmission electron microscopy (Zalokar; **A–C**) or by methods described in this chapter: formaldehyde fixation (FA; **D–F**), modified Stefanini fixation (Stefanini; **G**,**H**), or heat-methanol fixation **(J–L)**. After primary fixation all samples were treated the same way and then processed for transmission electron microscopy, as described elsewhere ***(9)*** (A,D,G,J) overviews show general structural preservation and evidence of extraction in FA and heat-fixed samples, whereas Stefanini's fixation provides much less extraction. Heat-methanol fixed samples (J) did not exhibit much recognizable structure. (B,E,H,K) structure of nucleoplasm (Nu) and nuclear membranes (arrowheads). FA-fixed samples (E) show evidence of extraction of chromatin and nuclear membrane. Stefanini-fixed embryos (H) show more evenly distributed chromatin as well as heat-methanol treated embryos (K). In heat-fixed embryos, nuclear membranes were not observed. (C,F,**I**,L) Adherens junctions (AJ) between epidermal cells are well preserved in FA and Stefanini-fixed samples, but in heat-fixed samples ZA structure cannot be resolved. Bars represent 1 μm in (J,K) and 0.5 μm in (L).

exact subcellular localization is less important, because the fixation artifacts that are being produced will usually be identical in both cases—except when extraction is a problem for localizing a specific antigen.

The present chapter will provide protocols for immunolabeling of embryos that take into consideration some of the problems described. Additional procedures have been developed for the staining of particular cellular components and will not be described here. For example, staining of microtubules or the actin cytoskeleton requires specific fixation procedures and are described elsewhere ***(4–6)***. In this chapter, I will provide a protocol for immunolabeling of embryos, which can be broadly used with a particular emphasis on different fixation procedures with respect to tissue preservation and permeability.

2. Materials

2.1. Harvesting and Dechorionization of Embryos

1. Egg collection: flies are placed in egg collection cages as advised by Wieschaus and Nüsslein-Volhard ***(7)***. Briefly, 100-mL plastic beakers (Kendall) (TRI-Pour® polypropylene) are prepared to contain tiny holes. Each beaker will hold a 60 mm Petri dish attached with rubber band.
2. Apple juice plates: preparation of apple juice plates is described elsewhere ***(8)***. The medium contains 17.5% agar, 12.5 g/L sucrose, 25% apple juice, and 0.2% Nipagin M. Apple juice plates are covered with a little smear of yeast (from dry yeast or bakers yeast). Halocarbon oil 27 (Sigma-Aldrich, UK) is used for making the chorion transparent for microscopic inspection.
3. Dechorionization: a basket that fits regular depression slides can be prepared of a stainless steel wire mesh; alternative devices have been successfully used, like Nitex® mesh used as a sieve with a cut-off 15-mL Falcon tube (Fisher, UK). For 3% sodium hypochlorite, commercial bleach can be used.

2.2. Fixatives

1. Formaldehyde fixative: 4% formaldehyde (p.a. grade, 37% stock solution, methanol-free) in phosphate-buffered saline (PBS) or 0.1 *M* sodium phosphate buffer pH 7.4.
2. Modified Stefanini's fixative ***(9,10)***. 4% formaldehyde (p.a. grade, 37% stock solution, methanol-free), 15 mL of a saturated aqueous solution of picric acid, 75 m*M* PIPES pH 7.4.
3. Heat/methanol fixation ***(11)***. 10X Triton-X-Salt solution (TSS): 3 mL Triton-X-100, 40 g NaCl on 1 L distilled H_2O.
4. Heptane and Methanol should be p.a. grade only.

2.3. Immunolabeling Procedures

1. Incubations are performed in 1.5-mL test tubes, if volumes between 350 µL and 500 µL are used. If smaller volumes are required—for example, to save primary antibody—0.5 mL test tubes are used to incubate embryos in 150–300 µL.

Alternatively, embryos can be stained in 24-well COSTAR® plates. All surfaces that come into contact with fixed embryos, in particular glass and plastic pipets need to be preabsorbed with excess protein solution (e.g., blocking solution) to avoid sticking of embryos.

2. Blocking solution: 10% serum (normal goat, horse, or donkey) in 1X PBS (130 m*M* NaCl, 7 m*M* $NaHPO_4$, 3 m*M* NaH_2PO_4, pH 7,4) with 0.1% Tween-20. Instead of Tween-20, Triton-X-100 can be used, which is a stronger detergent and thus promotes permeability of the tissue by extraction of membranes.
3. PBS with Tween-20 (PBT): 1X PBS with 0.05% Tween-20.
4. Antibody solutions: in blocking solution (*see* **Subheading 2.3.**, **step 2.**). A collection of excellent monoclonal antibodies for various *Drosophila* antigens is available through the Developmental Studies Hybridoma Bank (DSHB) in Iowa City (http://www.uiowa.edu/~dshbwww/).
5. Tubes/plates are incubated on a nutating shaker (VWR, UK) or Rocker Plate (Heidolph, Germany).

2.4. Antibody Detection

1. Fluorescence-conjugated antibodies: continue with **Subheading 2.5.**
2. Fluorescent DNA staining: 4′6′ diamidino-2-phenylindol (DAPI) can be used at a concentration of 1 mg/mL. Alternatively, other DNA stains can be used, for example, YOYO®-1 iodide (Invitrogen, UK); note that many fluorescent nucleic acid dyes (except DAPI or Hoechst 33342 [Invitrogen]) will also stain RNA and so embryos must be pretreated with RNAse (at 0.2 mg/mL for 1 h at room temperature) **(Fig. 2)**.
3. Alkaline phosphatase (AP) detection; AP buffer: 100 m*M* Tris-Hcl pH 9.5, 50 m*M* MgCl2, 100 m*M* NaCl, 0.1% Tween-20. AP buffer should be stored without Tween-20 at 4°C. 1 mL AP buffer + 4.5 µL nitroblue tetrazolium chloride ([NBT]; stock solution at a concentration of 18.75 mg/mL) + 3,5 µL 5-bromo-4-chloro-3-indolyl-phosphate ([BCIP], toluidine; stock solution at a concentration of 9.4 mg/mL).
4. Horseradish-Peroxidase (HRP) detection: for detection commercially available enhancement systems can be used following the manufacturer's protocols. DAB-solution: 0.5 mg/mL 3,3′ diaminobenzidine (DAB); DAB staining solution: per 1 mL DAB solution add 2 µL H_2O_2 (from 3% stock-solution).

2.5. Mounting of Specimen

1. Fluorescently labeled specimen: Mowiol/DABCO: 1.5% Mowiol 4-88 (Polysciences Europe, Germany), 33% glycerol, few crystals (1–2 per mL) of 1,4-diazabicyclo (2.2.2) octane (DABCO). To prepare Mowiol: dissolve 1.5% (w/v) Mowiol in PBS (pH 7.4) and stir overnight. Spin down nondissolved Mowiol particles by centrifugation and add 33% (v/v) glycerol and stir overnight. Mowiol without DABCO can be stored at –20°C for months. DABCO is added to Mowiol solution, gently mixed, and centrifuged. This working solution can be kept for a few days at 4°C.
2. AP/HRP color precipitates: ethanol series: 30%, 50%, 70%, 90%, absolute, 100% ethanol or acetone (maintained on molecular sieve). Araldite/Durcupan (Sigma).

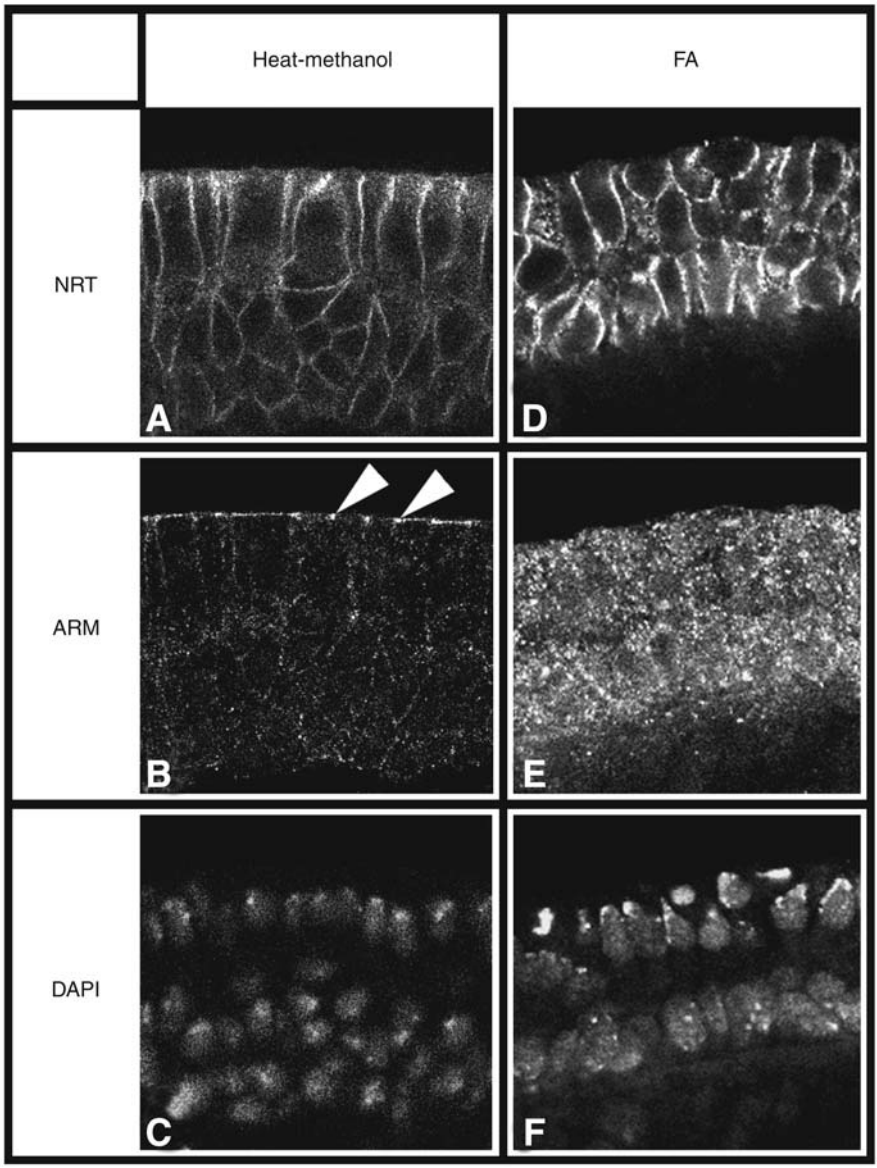

Fig. 2. Comparison of fluorescent immunolabeling of heat-fixed and formaldehyde-fixed embryos. Embryos at postgastrulation stages (extended germband) were fixed by heat-methanol **(A–C)** or by FA **(D–F)** and labeled with anti-Neurotactin (NRT), anti-Armadillo (ARM) antibodies and DAPI for DNA. Secondary antibodies were Cy2®- and Cy3®-conjugated antibodies. (A,D) Plasma membranes are labeled with NRT; note that labeling of NRT is very similar regarding the two fixation methods, although plasma membranes were not preserved by heat-fixation on the fine-structural level

3. Methods

3.1. Embryo Collection and Dechorionization

1. Collect 3–5 d old female and male flies to set up at a ratio of 1:3 (male to female) in egg collection cage to which a yeasted apple juice plate has been attached (place a small amount of yeast on the center of the plate and spread with a spatula [Fisher Scientific]).
2. Change apple juice plates every 8 h (25°C) or 18 h (18°C). Flies will require 2–3 d to adjust to the cage until egg production is best.
3. For staged embryo collections change plate after 30 min and incubate at a constant temperature to the desired developmental stage. Alternatively, a 0–3 h collection of embryos can be obtained and embryos will then be staged after treatment with halocarbon oil under a dissection microscope (Zeiss, Germany) equipped with transmitted light (for staging *see* **ref.** ***7*** and ***12***). On covering embryos with a drop of halocarbon oil, the chorion will become transparent and the embryo will be visible using a stereomicroscope with transmitted light.
4. For dechorionization, embryos are collected in a wire mesh basket that fits to a depression slide. Gently rinse embryos off the apple juice plate with tap water using a cut shorthair paintbrush. Rinse in tap water to remove yeast and remove excess water by blotting dry on a paper towel. Transfer embryos within the mesh into 3% sodium-hypochlorite solution. A 3 min treatment at room temperature removes the chorion; dechorionated embryos will float on the surface of the solution. Microscopic observation of the efficiency of the bleaching procedure is advised, because different batches and age of sodium-hypochlorite as well as the genotype of the embryos might affect the timing. Overbleaching might produce abnormal morphology.
5. If embryos were hand-selected under halocarbon-oil, staged embryos will be collected using watchmaker forceps (Fisher Scientific) and transferred to a wire mesh basket in water. Make sure to remove remaining oil by blotting the basket on paper towels or briefly rinse and blot using bleach. Continue with **Step 4** of **Subheading 4.3.1.**
6. After dechorionization, rinse embryos with distilled water in the wire mesh basket and keep in depression slide on distilled water. Note that dechorionated embryos are very sensitive to dehydration, which will cause severe artifacts. Prolonged treatment with sodium hypochlorite will increase the frequency of such problems.

3.2. Formaldehyde-Based Fixation Procedures

1. Preparation of fixatives: as fixative, buffered formaldehyde solution or modified Stefanini's fixative can be used; the latter exhibiting a better preservation of both

Fig. 2. *(Continued)* (*see* **Fig. 1**). (B,E) ARM protein is present at plasma membranes, in the cytoplasm and—in response to wingless signaling—in the nucleus. Heat-fixation results in loss of most of the cytoplasmic ARM (compare B with C) and to a pronounced staining of ARM associated with adherens junctions (arrowheads in B). (C,F) Nuclear staining is more pronounced in formaldehyde-fixed samples compared with heat-fixed embryos.

structure and antigenicity ***(9,13)***. To improve permeabilization through an increased extraction of cytosolic proteins and membrane lipids, 0.05% Tween-20 can be added to the formaldehyde fixative. Do not add Triton-X-100 to fixing solutions as it will interfere with phase separation. Fixatives can be generally stored at 4°C for at least a week.

2. Add 4 mL of fixative and 4 mL of heptane to a glass scintillation vial (Fisher Scientific). Mix the solution by vigorously shaking for 30 s to allow the formaldehyde to partition into the heptane phase.
3. Transfer the embryos to the vial using the wire basket or a paintbrush. Embryos will float between the two phases. Remove the wire basket after transfer of the embryos.
4. Incubate embryos for 25 min at room temperature on a nutating mixer device.
5. After fixation remove the lower, formaldehyde layer of the fixative and add 4 mL of methanol (*see* **Note 1**). Close the vial quickly and immediately shake the solution for 15 s. Embryos will partition into the lower methanol phase and can be collected using a glass Pasteur pipet (Fisher Scientific). Embryos do not stick to the glass while in methanol.
6. Embryos from multiple fixations can be pooled in one test tube and stored in methanol at −20°C for months. The success of immunolabeling of stored embryos depends on the antigen under examination. For several antigens, immunostaining of embryos was successful after storage in methanol over several years.

3.3. Heat-Methanol Fixation

This fixation procedure essentially represents a methanol fixation. The advantage of this method is that the preservation of epitopes is generally very good, because methanol provides a mild fixation. The disadvantage is that the preservation of the structure is very poor **(Fig. 1J–L)**. In particular, cytosolic antigens will be extracted during the procedure.

1. Fill 5 mL of 1X TSS into a scintillation vial and close lid loosely. Place into boiling water bath.
2. Prepare embryos (as described in **Subheading 3.2.**) and dump dechorionated embryos on wire mesh basket into the hot TSS.
3. Immediately add ice-cold TSS to completely fill the vial and remove wire mesh basket. Place vial on ice and let it sit for at least 5 min. If several fixations are to be carried out it might be convenient to store different samples on ice at this stage.
4. Carefully remove TSS and add 4 mL of heptane. Then add 4 mL methanol (p.a. grade is essential), quickly close cap, and vigorously shake the solution immediately. Fixed embryos will sink to the lower methanol phase.
5. Collect embryos with Pasteur pipet, rinse 2 times with methanol, and store at −20°C.

3.4. Staining Procedures

Immunolabeling procedures start by rehydration of fixed embryos. Following rehydration, unspecific binding sites are blocked by incubation with excess protein. The best results are obtained by using serum protein, preferably

from the same species as the secondary antibody was obtained. Antibody incubations are being performed at 4°C overnight or 1.5 h at room temperature (25°C) in blocking buffer.

1. Rehydration: after fixation, rinse embryos twice with methanol and ensure that traces of heptane have been removed, as it will interfere with the rehydration. Discard the methanol and add PBT. Let embryos settle and rinse three times for 5 min with PBT. It is also possible to rehydrate in a methanol or ethanol series, but we did not see any major differences in the staining results.
2. Blocking: remove PBT and add PBT containing 10% serum (e.g., goat, donkey). Incubate for at least 1 h at room temperature to overnight at 4°C.
3. Primary antibody incubation: dilute primary antibody to desired concentration in blocking buffer. Incubate for 1.5 h at room temperature or overnight at 4°C. When using a new batch of antibodies, test runs of different dilutions are often critical to obtain specific labeling. In the case of purified antibodies a concentration between 1 and 5 µg/mL immunoglobulinG is likely to show specific staining. Some antibodies might require a preclearing step. Dilute primary antibody in PBT at 1:5 up to 1:20 and incubate with fixed and rehydrated embryos overnight at 4°C. Spin down embryos for 5 min in a cooled microcentrifuge and store supernatant at 4°C with a preservative (e.g., 0.02% sodium azide).
4. Wash step: remove primary antibody (in some cases this solution can be retained and used for another staining). Rinse twice with PBT by letting embryos sediment and resuspend in PBT. Wash three more times for 15 min at room temperature using a nutating mixer device.
5. Secondary antibody incubation: prepare secondary antibody mix in blocking buffer. Dilute antibodies following the supplier's protocol. For double- or triple-immunolabeling, multiple secondary antibodies can be applied at the same time. Although we do not find it necessary to preclear secondary antibodies, unspecific binding of secondary antibodies might be a potential problem of specificity (for preclearing secondary antibodies, incubate antibodies at a 1:10 dilution in PBT with fixed embryos at a ratio of 1:10 [v/v] overnight at 4°C). Incubate secondary antibody overnight at 4°C on a nutating mixer device.
6. Wash step: the wash step is identical to **step 4** of **Subheading 3.4.**

3.5. Antibody Detection

1. Immunofluorescence: in the case of fluorescently conjugated secondary antibodies, embryos are mounted as advised under **Subheading 3.6.** and examined under an epi-fluorescence or confocal laser-scanning microscope (Leica, Germany). Moreover, a number of fluorescent substrates for enzyme-conjugated antibodies have been developed and can be applied according to the manufacturers' protocols. One method that works well in fly embryos is signal amplification of HRP-conjugated antibodies with fluorescently labeled tyramide conjugates ***(14)***.
2. Color detection: various methods have been described to detect enzyme-conjugated secondary antibodies in embryos. HRP (*see* **Note 2**) and AP remain the most commonly used enzyme conjugates. The traditional detection of HRP is the colorless substrate

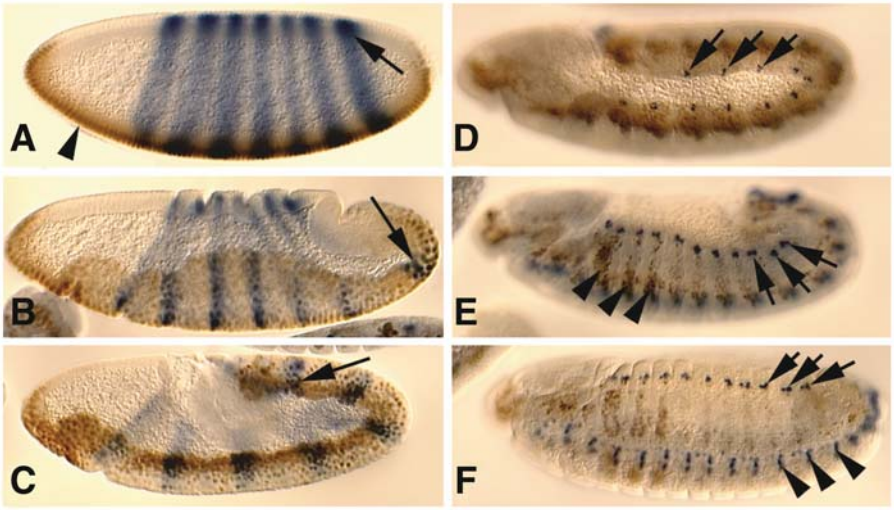

Fig. 3. Double-immunolabeling followed by color detection. Embryos at different developmental stages were fixed with modified Stefanini's fixation and stained for Twist (Twi) (brown, HRP-DAB precipitate) and Even Skipped (Eve) (blue, AP-NBT/BCIP precipitate). **A**: stage 5—blastoderm; **B**: stage 7—gastrula; **C**: stage 8—germ band extension; **D**: stage 11—extended germ band; **E**: stage 13—germ band retraction; **F**: stage 15—retracted germ band. Eve is first expressed in seven stripes up until after germ band extension (the posterior of these stripes is labeled with an arrow (in A–C). After germ band extension Eve is expressed in 13 segmental clusters of dorsal mesoderm cells (arrow in D–F marks the posterior three clusters) as well as in the developing nervous system (arrowheads in F). Twi is expressed in all mesoderm precursor cells from early on; initial expression is seen in the ventral cells of the blastoderm (arrowhead in A). During morphogenesis, the mesoderm cells are being internalized (B,C) and later redistributed in the interior of the embryo; note segmental accumulation of mesoderm cells in late stage embryos (arrowheads in E).

DAB, which will turn into a brown precipitate by the HRP activity in the presence of H_2O_2. This signal can be enhanced using metal ions, for example, 0.5% NiCl solution. For staining, rinse embryos in PBT once and replace with DAB solution. Stop reaction by adding PBT, 0.02% sodium azide, or excessive rinsing with PBT. For AP staining, embryos have to be adjusted to AP-buffer to amend appropriate pH and salt conditions for AP. For color detection, NBT and BCIP are dissolved in AP-buffer. Stop color reaction at desired point by adding excess PBT. For each staining procedure transfer embryos to a depression slide and monitor staining under a stereomicroscope. AP and HRP staining reaction can be performed sequentially to obtain a double immunolabeling **(Fig. 3)**. Although both ways are possible, it is advised to stain HRP first followed by AP detection because the DAB precipitate tends to be more stable.

3.6. Mounting of Specimens

1. Fluorescently labeled specimen: after rinsing in PBT the stained embryos are being transferred to a drop of Mowiol/DABCO onto a glass slide using a cutoff yellow pipet tip. Mix the embryos carefully with the mounting medium and place a cover slip onto the sample. Let the sample settle for an hour before microscopy. Over time the Mowiol/DABCO will solidify and the samples can be kept for several months at 4°C.
2. Light microscopy: after color reaction rinse with PBT and dehydrate with a graded ethanol series (*see* **Subheading 2.5., step 2.**) followed by 2 incubations with 100% acetone (each incubation 5 min). For infiltration with araldite incubate with acetone/Araldite (50:50) for 3 h up to overnight at 4°C. Although some of the NBT/BCIP precipitates will be extracted during infiltration, the amount is usually minimal. If this poses a problem increase AP staining reaction time and reduce the incubation in acetone/Araldite solution. After infiltration place embryos onto a slide and orient the embryos using an eyelash mounted on a holder, like a glass pipet or syringe needle (Becton Dickinson, UK). Let the acetone evaporate for 3 h at room temperature and incubate at 60°C overnight. Let slide cool down and add few droplets of pure araldite solution, just enough to completely cover the specimens after placing a cover slip onto the slide. Carefully place a cover slip onto the slide and incubate 24 h at 60°C.

4. Notes

1. The methanol treatment affects both the antigenicity and the localization of proteins. To circumvent these problems, the vitelline envelope can also be removed manually after fixation. Remove embryos from fixative using a wire mesh basket and rinse with PBS several times. Blot semidry on paper towels and place into a depression slide with PBS. Attach a piece of double-sided tape (Scotch/3M, UK) in the middle of the lid of a 60 mm Petri dish. Transfer embryos using a paintbrush onto the tape and cover with PBS. Rapidly add the PBS to make sure that the embryos do not dry out. Remove vitelline envelopes using a 27-gauge syringe needle under a dissecting scope; if that turns out to be difficult try to slightly over-fix the embryos, as only well-fixed embryos will readily devitellinise.
2. For signal enhancement of HRP-conjugated antibodies, the avidin: biotinylated enzyme complex staining kit Vecta-stain® by Vector Labs, UK has been very successfully applied to *Drosophila* embryos.

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13

Imaging Cellular and Molecular Dynamics in Live Embryos Using Fluorescent Proteins

Matthieu Cavey and Thomas Lecuit

Summary

With the live imaging of embryos, the dynamics of developmental processes, such as tissue remodeling, cell morphogenesis, and protein dynamics can be observed and quantified. This has greatly improved the mechanistic understanding of biological processes. Here we describe how embryos can be prepared for imaging mainly, but not only, fluorescent proteins and probes. This chapter is a users' guide that addresses the following aspects of fluorescent embryo imaging: (1) How to handle and prepare embryos for live microscopy. (2) What microscopic setups are available for embryo imaging and what should they be used for. (3) How to practically use fluorescent imaging setups depending on the experimental context: large-scale imaging of multiple embryos, high-resolution four-dimensional imaging of single embryos, studies of protein dynamics, and so on. (4) Finally, we focus on pitfalls and how to overcome a variety of possible problems encountered during live imaging.

Key Words: *Drosophila*; dynamics; embryo; FLIP; FRAP; GFP; live imaging; time-lapse confocal microscopy.

1. Introduction

Imaging the dynamics of biological processes during development has considerably changed the way we describe and understand gene function. New hypotheses sometimes emerge from unbiased observations and efforts to describe with great precision the phenomenology of a process. It is possible to image the dynamics of a developmental tissue and the changes in tissue shape during gastrulation in relation to cell morphogenesis. Protein dynamics can also be scored and quantified to extract relevant parameters such as diffusion coefficients, exchange rates in a given cell compartment, and so on. Imaging embryos thus

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makes it far easier to understand in a quantitative manner development at the molecular and cellular level.

The live imaging of *Drosophila* embryos has been greatly facilitated in recent years by the development of new microscopic setups that increase the speed of acquisition and the sensitivity of detectors of fluorescent signals. Several companies have developed packages that automate the four-dimensional (4D) acquisition of biological specimens while scanning different positions on a slide. It is thus possible to access a large amount of biological information through the description and quantification of even subtle phenotypes in new ways.

Meanwhile, the development of numerous strains that express green fluorescent protein (GFP) fusion proteins under various promoters (such as with the GAL4 system ***(1)*** or endogenous promoters in GFP protein trap screens ***[2,3]***) has extended our ability to image cell organization in a developing tissue.

The *Drosophila* embryo, as we shall see, is particularly amenable to fluorescent imaging. The specimen can be immobilized and is readily accessible at the surface. Furthermore, in early embryos, the possibility to inject various compounds before or during cellularization renders the early embryo as convenient as cell culture for certain experiments.

2. Materials

2.1. Embryo Preparation and Mounting

1. Heptane/glue mix (e.g., from Sigma): prepared in advance (*see* **Note 1**, store at room temperature, for months to years, carefully seal the cap with parafilm): Heptane; Tesa 4124—Premium grade UPVC packaging tape (Beiersdorf AG Hamburg, www.tesatape.com).
2. Embryo collection: plastic laying cages, apple juice plates and yeast paste (stored at 4°C up to 1 mo, bring to room temperature before use), and brushes.
3. Embryo dechorionation: basket, bleach (commercial), Milli-Q or distilled water in squirt bottles.
4. Embryo positioning and mounting: dissecting microscope, needles with handle, 22×40 type 1 cover slips, Halocarbon oil 200 or 700 (Halocarbon Products Corp., PO Box 661, River Edge NJ 07661, www.halocarbon.com) or Voltalef oil 3S or 10S (MERCK-Prolabo) (*see* http://vwr.com/).

2.2. Imaging Equipment

1. Dissecting stereomicroscope with diascopic illumination (transmitted light) to align and handle the embryos on agar (*see* **Note 10**).
2. Stage: universal mounting frame (*see* **Note 2**).
3. Inverted microscope.
4. Spinning disk confocal system (Perkin-Elmer).
5. Laser scanning microscopy (LSM) confocal system.
6. Objectives: ×20, ×40, ×63, ×100 water or oil immersion (*see* **Notes 3** and **4**).

3. Methods

3.1. Embryo Preparation

Careful handling of embryos as described in the following sections allows them to survive up to hatching. In order to visualize GFP in embryonic tissues, the chorion—the outermost protective layer of the embryo—is removed by chemical treatment using bleach. The vitelline membrane—inner envelope—which is left intact by this treatment is transparent enough to allow the GFP signal to go through without much loss. Embryos are extensively washed with water after being dechorionated, and are glued to a cover slip for observation. The glue is dissolved from packaging tape in heptane. A couple of drops of the heptane/glue mix are transferred with a glass Pasteur pipet on a cover slip, the heptane evaporates and the glue remains stuck to the cover slip. Start by preparing a large batch of heptane/glue mix that can be stored at room temperature for years if properly sealed. Once the embryos glued to the cover slip, the main challenge then is to prevent them from dehydrating while allowing gas exchanges to occur. This is achieved by covering the embryos with viscous oil.

3.1.1. Embryo Collection

The whole procedure up to observation under the microscope routinely takes about 20 min. Collect embryos young enough so that they will reach the right developmental stage once the preparation phase is done.

1. Place well-fed females in a plastic cage pierced with small holes on the top to allow breathing, and an apple juice plate at the bottom with a small dollop of yeast paste on it. Allow females to lay eggs on the agar for the required length of time depending on the embryonic stage of interest and at the desired temperature (*see* **Note 5**).
2. Add distilled water to the plate and resuspend the embryos and yeast paste with a brush. Dump the resuspended embryos in a basket and wash away the yeast paste using a squirt bottle with distilled water **(Fig. 1A)**.

3.1.2. Dechorionation and Wash

1. Place the basket with embryos in a Petri dish cover, add bleach to the basket, and shake gently to mix the embryos with the bleach. Dechorionation takes about 30 s when using 100% bleach (*see* **Note 6**) **(Fig. 1B)**.
2. Place the basket on a paper towel to soak-up the bleach. Wash the embryos using squirt bottles with distilled water to fill the basket and absorb the water on a paper towel (*see* **Note 7**). Make sure this step is repeated several times (5–10 times) (*see* **Note 8**) **(Fig. 1C)**.

3.1.3. Positioning the Embryos

1. Cut a piece of agar with a razor blade from the apple juice plate and put it on a glass slide. Transfer the dechorionated embryos from the basket to the agar using a slightly wet brush (*see* **Note 9**) **(Fig. 1D)**.

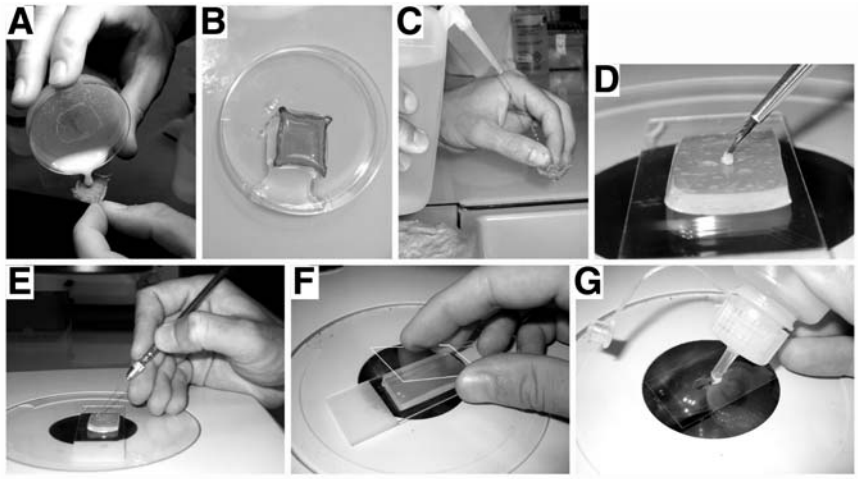

Fig. 1. Details of the different steps of embryo preparation for imaging. **(A)** Eggs laid on an agar Petri dish are washed with water and transferred to a mesh basket (*see* **Subheading 3.1.1.**, **step 2**). **(B)** Addition of bleach to the mesh causes dechorionation of the embryos (*see* **Subheading 3.1.2.**, **step 1**). **(C)** The embryos are then extensively rinsed with distilled water using a squirt bottle (*see* **Subheading 3.1.2.**, **step 2**). **(D)** Using a paint brush, the embryos are then collected from the mesh where they clump, and transferred to a strip of agar placed on a glass slide (*see* **Subheading 3.1.3.**, **step 1**). **(E)** Embryos can be carefully handled with the tip of a needle to align them for instance, and select embryos of the appropriate stage (*see* **Subheading 3.1.3.**, **step 2**). **(F)** Embryos are then lightly and carefully sandwiched between the agar strip on which they are and a cover slip covered with a thin layer of glue (*see* **Notes 1** and **12**). This allows efficient transfer of the embryos onto the cover slip (*see* **Subheading 3.1.3.**, **step 3**). **(G)** Finally, halocarbon oil is added on top of the embryos to prevent further dehydration and allow proper imaging (*see* **Subheading 3.1.3.**, **step 4**). The embryos are then ready for imaging on a microscope. The cover slip is directly clipped on the stage of the microscope.

2. Using the needle under the dissecting microscope, put aside the embryos that are slightly younger than the desired developmental stage (*see* **Note 10**), and orient/position them so that the region to be observed faces up (*see* **Note 11**) **(Fig. 1E)**.
3. Add a small drop of heptane/glue mix prepared in advance onto a 22×40 cover slip (*see* **Note 12**) with a glass Pasteur pipet and spread it over the surface required for all the embryos to fit (*see* **Note 13**). Wait for the heptane to completely evaporate. Transfer the embryos by pressing gently the cover slip upside down on the agar (*see* **Notes 14** and **15**) **(Fig. 1F)**.
4. Cover the embryos with Halocarbon oil 200 or Voltalef 10S (*see* **Note 16**), add a sufficient amount to cover all the embryos but avoid the oil from spreading extensively on the side **(Fig. 1G)**.
5. Fix the cover slip on a Universal Mounting Frame of an inverted microscope, embryos facing up, making sure the cover slip is tightly held to avoid defocalization

problems. Using an inverted microscope is much more convenient and also better for the viability of the embryos. However, if only an upright microscope is available, you may image from the top, placing two small cover slips on top of each other on the sides of the embryos, and adding another one on top to sandwich the embryos. However, breathing and viability are severely reduced in these conditions, especially if long imaging is done, so we do not recommend this.

3.2. Live Imaging

A number of techniques can be used for imaging fluorophores, depending on the purpose of the observation and the amount of details searched. We first describe the main characteristics of four imaging systems: structured illumination using epifluorescence (e.g., Apotome, Zeiss), the spinning disk confocal (e.g., Perkin-Elmer), the laser scanning microscope (LSM) single photon confocal system, and the 2-photon microscope (e.g., Zeiss, Leica). A quick outline to obtain your first images and learn how to optimize them is given. We then describe three protocols/applications with these different systems for live imaging.

3.2.1. Imaging Systems

Structured illumination using epifluorescence is a relatively inexpensive system to obtain good optical sections in an embryo. The main advantage is that it can be mounted on a microscope set up with a motorized stage that captures images on several positions overtime, in conjunction with phase-contrast imaging for instance. We recommend this system to screen a large number of embryos (such as the progeny of genetic crosses, embryos following injection of RNA interference (RNAi) probes, and so on ***[4]***) before performing high-resolution imaging. High-resolution imaging of the surface of the embryo is best done with a confocal setup. A spinning disk confocal system is recommended for fast and high-resolution imaging over a wide field (*see* for instance **ref. *5***). The LSM system is best suited for studying the dynamics of subcellular processes using photobleaching techniques, although it is also very good for wide field imaging when the speed of acquisition is not critical. Finally, for imaging deeper regions of an embryo, one needs to use a 2-photon system.

3.2.1.1. Epifluorescence/Structured Illumination

A large number of embryos (up to 100) can be imaged automatically during a single experiment with this system. If a certain project requires that you obtain time-lapse recordings over several thousand embryos in total, you can use this system (Zeiss offers a remarkable setup, with the Cell Observer containing the Apotome system, the AxioVision software, and the Mark&Find module, *see* http://www.zeiss.com). However, as a consequence, the spatial and temporal resolutions will be lower as you acquire more embryo positions in a cycle.

3.2.1.2. Spinning Disk Confocal (Perkin-Elmer)

The main advantage of a spinning disk confocal is that it allows simultaneously high-resolution and wide-field imaging. Increasing the field of view with a constant resolution does not affect the time of acquisition unlike with a LSM. Moreover, photobleaching during acquisition is much lower than with a standard LSM.

In this system, the laser beam is shaped to produce a field illumination and travels through a spinning disk containing thousands of small holes (pinholes). As the disk rotates, the whole field of view is excited. The signal emitted travels through the pinholes on the spinning disk and is detected by a charged coupled device (CCD) camera.

One can adjust mainly the exposure time on the CCD camera. The pinhole dimension is fixed (equivalent to about 2 airy units when using fluorophores emitting at wavelengths around that of GFP) and cannot be adjusted. The pinhole dimension determines the z resolution (thickness of the slice from where the photons are detected), depending on the wavelength of the fluorophore. No zoom function is available with this system, thus the spatial resolution can only be increased by increasing the magnification of the objectives so that each fixed pixel on the CCD camera corresponds to a smaller structure in the focal plane. The amount of signal collected and thus the quality of images can be adjusted by changing the laser power (excitation), exposure time of the camera (detection), or even the binning of the CCD camera (several pixels can be grouped to collect photons, thus increasing the sensitivity, although this reduces the resolution accordingly). The speed of acquisitions depends on the exposure time and the number of z planes selected.

Before you start imaging, you should know what resolution is required for observing your phenomenon of interest. Choose the objective magnification and the binning on the CCD camera appropriately.

Getting started with a spinning disk confocal:

1. Choose an embryo at the right stage, using epifluorescence to focus in the region of interest.
2. Acquire images in «live» mode. Start with laser power reasonably low and increase exposure time until signal appears on the image. You should adjust the exposure time and laser power to find a good trade-off between bleaching (to minimize), signal-to-noise ratio (to optimize), and the desired temporal resolution.
3. The appearance of the image can be modified by adjusting the contrast and brightness levels.

3.2.1.3. LSM Confocal System

One of the advantages of the LSM over the spinning disk confocal is that it allows one to define regions of interest (ROIs), which are essential for studying

protein dynamics using photobleaching techniques described in **Subheading 3.2.2.3.** (fluorescence recovery after photobleaching [FRAP], fluorescence loss-induced by photobleaching [FLIP], and photoactivation). However, fast imaging at a high resolution in a large region is not possible or not as good as with a spinning disk confocal.

In the LSM system, the laser beam is controlled by a scanner (system of movable mirrors), and thus can be oriented at will, allowing to excite the fluorophore in the desired region of the embryo. As the laser scans the sample, the signal emitted goes back through the adjustable pinhole and reaches photomultiplier (PMT) detectors. These detectors amplify the signal when converting photons to electrons.

There are several ways to modify the amount of signal recovered with a LSM confocal system. Some parameters affect the excitation per se and thus can lead to photobleaching of the specimen. For instance, one can adjust the level of excitation of the fluorophore by changing the transmission of the laser to the sample through Acousto-Optical Tuneable Filters (AOTF). Second, the scan time can be set at will. The scan time corresponds to the time spent by the laser on each pixel, analogous to the exposure time for CCD cameras. Increasing scan time per pixel (dwell time) results in more excitation and hence more emission. The image may also be averaged by multiple scanning of each pixel. Other parameters concern the detection and thus, do not affect bleaching. For instance, the pinhole size is adjustable. Opening the pinhole results in more emitted photons reaching the detectors. Also, the detector gain, which controls the photon-to-electron amplification process can tune the signal detected on the PMT and thus, increase the signal-to-noise ratio up to the limit produced by electronic noise.

Acquisition softwares include three additional functions that help to digitally improve image quality. The «amplified detector gain» function exacerbates the detector gain. The «offset» function enables changing image contrast. The «average» function computes the average signal intensity in each pixel over several scans. This last function is very efficient for removing noise but increases acquisition duration and photobleaching.

The scan speed corresponds to the time required to acquire a whole image. It depends on the dwell time per pixel, the image dimension (number of pixels), and mode of scanning. The scanning process can be performed bidirectionally (scanning left to right and then right to left on the next pixel line) instead of unidirectionally to increase the scan speed.

A digital zoom function allows increasing spatial resolution, although we strongly recommend relying mostly on high magnification objectives to increase resolution and limit digital zoom to ×5 maximum.

Getting started with a LSM confocal:

1. Choose an embryo at the right stage, using epifluorescence to focus in the region of interest.
2. Start with laser power at its minimum value (25% for argon, not adjustable for other lasers), and AOTF at 2–4% maximum (for GFP).
3. Set the pinhole to 1 airy units.
4. Raise the detector gain, keeping the amplified gain at zero and the offset at its maximum value.
5. Set the image size to 512 × 512 pixels.
6. Scan speed: default value, about 1 second per image.
7. Acquire first image, increasing the detector gain until electronic noise appears on the image.
   To adjust the image:
8. Change the speed of acquisition (dwell time) depending on the desired temporal resolution.
9. Raise the amplified detector gain, trying to keep it reasonably low as well as the detector gain to avoid collecting too much background.
10. Use the average function to remove noise.
    To increase scan speed:
11. Use the bidirectional scan mode.
12. Change image size to 512 × 300 or 250 pixels.
13. Decrease scan time.

3.2.1.4. Two-Photon Microscopy

Traditional confocal systems can produce good quality images with fluorescent signals emitted from up to 50 µm depth in the embryo. For deeper objects such as 100–200 µm, one should use a 2-photon microscope, which allows detecting fluorescent signals much deeper in the sample. This was used for instance to image the dynamics of germ cell migration in *Drosophila* ***(6)***. Imaging with this type of system is performed as described in **Subheading 3.2.2.2.** (high-resolution approach) with slight modifications regarding the equipment. The laser has to emit at wavelengths greater than 1000 nm to be able to excite fluorophores such as dsRED. As a consequence, the objectives should be corrected for infrared to increase the transmission of the laser beam.

One major problem encountered with this setup is that the excitation wavelength for eGFP is very close to the absorption range of water. This results in heating of the embryos because of absorption of the energy by water molecules. We recommend using lower wavelengths (e.g., 870–910 nm), which is a good compromise to reduce water absorption and efficient 2-photon excitation of eGFP.

3.2.2. Protocols For Time-Lapse Imaging

Note: irrespective of the equipment used to acquire images of fluorescently labeled specimens, it is essential to make sure the intensity histogram

of the acquired image is not skewed. In some conditions (e.g., too much excitation), the image may be overexposed or saturated, i.e., a large number of pixels are at the maximum brightness (white), which will be evident in the histogram. Conversely, the image may be underexposed, i.e., a large number of pixels may be at the minimum intensity (black). In both cases, the image produced lacks important information about the process observed and the data are skewed. This may bias the interpretation of the data and should thus be avoided in all situations. As a result, when setting up the conditions to obtain an image (*see* **Subheadings 3.2.1.2.** and **3.2.1.3.**), check the shape of the intensity histogram to make sure the image is perfectly exposed and carries unbiased information.

3.2.2.1. Large-Scale Imaging of Multiple Embryos

A large-scale, low-resolution approach is a good starting point for observing new phenomena which are poorly characterized or, for example, screening for specific phenotypes In this setup, several embryos are imaged automatically using the multiple stage position function of the acquisition software. Images are acquired at one or only a few z planes for each embryo, and with fairly long time intervals between acquisitions to allow a maximum of embryos to be imaged during one experiment. The total observation time is fairly long (many cycles) so that different stages of the phenomenon can be recorded in a single time-lapse movie. The main advantage of this system is that one can acquire many (up to 50) positions, each containing two embryos, in time-lapse, thus making it possible to quantify phenotypes with large quantities of embryos. Note that with this approach the spatial and temporal resolutions are limited by the number of embryos observed per cycle of acquisition.

1. Use moderate magnification objectives (×20–40), with water immersion. (*see* **Note 4**). Select the embryos to be observed using the «multiple stage position» function on the acquisition software/menu.
2. For each embryo, select one or only a few z planes to be imaged. The number of z planes and number of embryos are inversely related: the more embryos, the less time available to acquire images over several z planes (and vice versa).
3. Select long time intervals (30 s–1 min) between each time-point. Verify that this interval is long enough so that every embryo and every z plane can be imaged during one cycle. Some softwares now calculate the minimum time interval required for a cycle to be completed correctly. If this function is not available, measure manually how long one cycle takes under various conditions (different exposure times, numbers of embryos, numbers of z planes) and adjust the cycle intervals accordingly.
4. The total number of cycles/length of experiment can be fairly long and depends mainly on the biological process of interest. Determine empirically at what embryonic stage the experiment should be started and ended.

3.2.2.2. High-Resolution Surface Imaging With a Spinning Disk or LSM Confocal

When more detailed observations are required, one needs to focus on a single embryo at a time, using higher magnification objectives and imaging the embryo over a wide z range. The images of each z plane are then projected digitally on a single plane to obtain a global picture of the phenomenon over the whole z range (*see* **Subheading 3.3.2.** and **Fig. 2**). Short time intervals are used in this approach to maximize the amount of information recorded but fairly long movies can still be produced. This approach is more time-consuming but can reveal details that are simply not observable with the large-scale, low-resolution approach described earlier.

1. Use high magnification objectives (×63, ×100), focusing on a single embryo at a time. Obviously, lower magnification objectives may also be used if the field of observation needs to be large.
2. Select a wide range of z planes to be observed, spanning all of the fluorescent signal range. The optimal z distance between each z plane depends on the pinhole size and the wavelength of the fluorophore. 0.5–1 μm z increments are usually optimal.
3. Use short time intervals (5–10 s) between each cycle. However, as for the large-scale method, the cycle interval needs to be long enough for every selected z plane to be imaged. Verify manually that it is the case if the minimum time interval function is not included in your software.
4. Adjust the total number of cycles/length of experiment according to how long the biological process needs to be recorded.

3.2.2.3. Studying the Dynamics of Cellular Processes

Time-lapse imaging of fluorescently labeled proteins as described throughout this chapter is restricted to observing fairly slow processes at cellular or tissue scales. However, to get insight into processes occurring at the subcellular level and over much shorter time intervals such as the dynamics of protein complexes assembly and turnover, one has to turn to photobleaching techniques *(7)*. Photobleaching consists in eliminating the fluorescence from a specific ROI by exposing it to high laser power for a short period of time. Fluorescent molecules in the ROI are permanently damaged by the amount of energy they receive and are not able to re-emit photons anymore—the fluorescent signal is «bleached». Nonfluorescent molecules in the ROI are not damaged. FRAP and FLIP techniques are based on eliminating fluorescent signal from one specific compartment of a cell or tissue and observing how the signal recovers in this region (FRAP) or how is the signal affected in adjacent compartments (FLIP). These behaviours directly reflect the dynamic exchange of molecules between the two compartments. For example, when a fluorescent molecule is constantly and rapidly

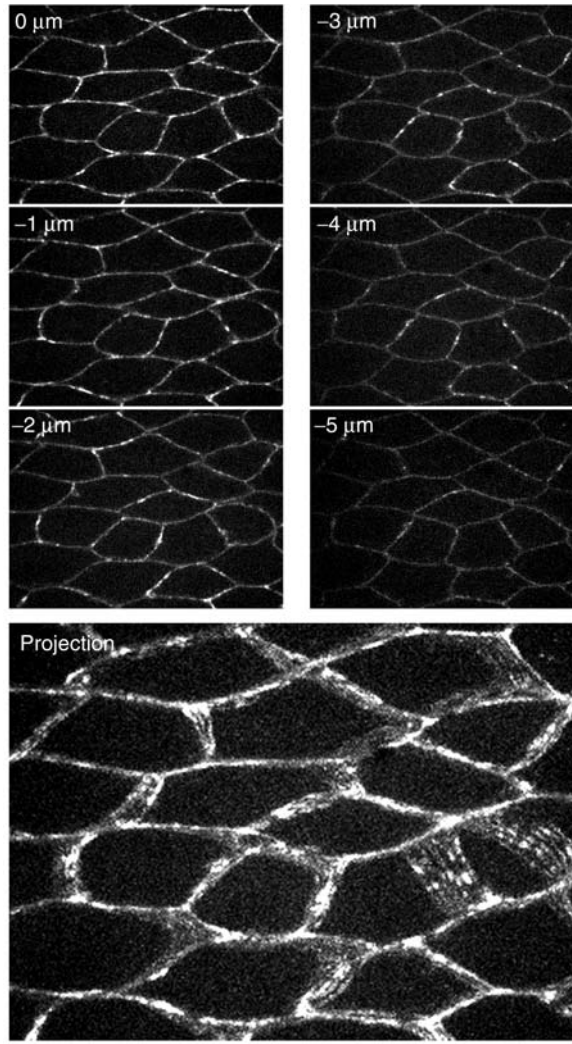

Fig. 2. Projecting a z-stack on a single plane. Images are taken from a 4D acquisition (*x*, *y*, *z,* and time) of DE-Cadherin-GFP in gastrulating embryos obtained with a spinning disk high-resolution setup. A single time-point is shown here, but the projection can be performed automatically for every single time-point. The 6 planes (z depth indicated in microns) are projected onto a single plane shown on the bottom image. The signal is heterogeneously distributed at the cell membrane within a single z plane but appears more uniform on the projection. *Note:* the cell on the right hand of the image is tilted relative to the *z*-axis, resulting in a 3D-like representation on the projection.

cycling between different subcellular regions, fluorescent signal will quickly recover in the photobleached ROI in a FRAP experiment. In a FLIP experiment where the ROI is repetitively being photobleached, the cycling of such a molecule between this fluorescence-free region and adjacent fluorescent-full regions will result in a progressive loss of signal from the adjacent regions.

Performing photobleaching experiments is possible with traditional LSM confocal systems. Here are some guidelines for performing FRAP and FLIP experiments with fly embryos. For more details about the theory, data fitting, and interpretation, the reader is referred to ***(8)***.

3.2.2.3.1. FRAP

1. Use a high-resolution setup (*see* **Subheading 3.2.2.2.**), with fast scan speed. Open the pinhole, reduce image size to 512×300 pixels, scanning continuously (no time interval), in bidirectional mode and over one or a few z planes only.
2. Set laser power to 100% (*see* **Note 17**) and AOTF to 1–2% to avoid photobleaching in the whole image where FRAP is performed. Adjust detector gain, amplified detector gain, offset, and scan average to obtain a reasonably good image. Remember, image quality and speed of acquisition are inversely related. Speed is more critical for this type of experiment, at least initially when the dynamics of the process of interest are completely unknown. If your protein of interest turns out not to diffuse much, slower scan speeds and better spatial resolutions can be used instead.
3. Set the cycle parameters so that two images are acquired before bleach. These will serve as the initial/prebleach fluorescence intensity value in the region. The total length of the experiment should be determined empirically: signal recovery eventually reaches a plateau.
4. The number of bleach iterations (number of scans at full power in the ROI) should also be determined empirically. The rule of thumb is that less than 80% of the initial signal should be bleached. Bleaching more than 80% of the initial signal results in a non-Gaussian distribution of fluorescent molecules after bleaching, and the recovery curves are not interpretable ***(8)***.
5. The ROI size should be fixed and constant for all experiments.
6. Tracking softwares (*see* **Subheading 3.3.2.**) should be used to precisely monitor signal intensity in the ROI after photobleaching.
7. The mobile fraction and diffusion coefficient parameters can be extracted by fitting the data to the Axelrod equation ***(8)***.

3.2.2.3.2. FLIP

1. Use the same setup as for FRAP (scan speed and so on).
2. Use the "Repeat bleach" function to bleach in the ROI between each acquisition scan.
3. Bleach with very few iterations each time. You want the bleach to be progressive and intervals between two scans as short as possible to collect the maximum amount of information on signal behaviour in adjacent regions.
4. The total length of the experiment should also be determined empirically, fluorescence loss in adjacent regions eventually reaches a plateau.

3.2.2.3.3. Photoactivation of Fluorescent Molecules. To probe protein dynamics it is sometimes very informative to do "pulse chase" analysis using photo-activated GFP fusion proteins (PA-GFP) ***(9,10)***. Photo-activable (PA)-GFP is very weakly fluorescent when excited at 488 nm. However, after photo-activation with ultraviolet (UV)-light, (e.g., with a UV laser at 410 nm on a confocal microscope or around 800 nm with a 2-photon microscope), PA-GFP emission after excitation at 488 nm is increased nearly 100-fold. This allows one to control spatially and temporally the fluorescence of a tagged protein in a given ROI and to follow the diffusion of a protein from this ROI. For further technical details on photo-activation of PA-GFP *see* **ref. *11*** and for its adaptation in *Drosophila* embryos *see* **ref. *12***.

Other fluorescent proteins have been discovered since then following the same principle (reviewed in **ref. *13***). Kaede is a photo-convertible protein, shifting from green to red emission after UV-light-induced cleavage of the protein ***(14)***. However Kaede tends to multimerize so this is not very useful for fusion proteins. Nonetheless, cell tracking or lineage analysis can be performed with photo-conversion of a cytoplasmic or membrane Kaede, which is more convenient than the photoactivation of injected caged fluorescent dextran ***(15)***. Other photo-convertible monomeric fluorescent proteins have been reported since then (reviewed in **ref. *13***).

3.3. Pitfalls and Data Manipulations

3.3.1. Photobleaching and Defocalization During Acquisition

Fluorescence signals can change considerably during an experiment for several reasons. Changes in the expression levels and/or localization patterns of a fusion protein can lead to a considerable weakening or strengthening of the signal in matters of minutes to tens of minutes. For example, within about 20 min, E-Cadherin::GFP signal increases and concentrates apically in ectodermal cells during gastrulation (personal observations). These types of variations can be of biological significance, but it is important to control that they are not artifactual, especially when signal appears to decrease overtime. Defocalization of the sample and photobleaching owing to repetitive excitation are the major sources of signal deterioration in live imaging.

When using a LSM confocal setup, one can easily control for photobleaching by briefly scanning adjacent parts to the region of interest or the whole embryo after acquiring a time-lapse movie. Severe photobleaching will become obvious.

When using a spinning disk setup where the whole specimen is illuminated by the laser, or when controlling for defocalization using both LSM or spinning disk setups, one has to rely on another method. The glue used to stick the embryos to the cover slip is autofluorescent and appears as small dots when

focusing above the embryo (i.e., closer to the cover slip surface). This can serve as a useful marker for controlling if photobleaching or defocalization occurs during an experiment. Use the same settings as for a regular experiment (image acquisition settings, cycle parameters, and length of experiment) to acquire images spanning the z range where the glue dots are visible (including z planes above and below the glue). If defocalization or photobleaching occur in the course of the experiment, the glue dots will progressively disappear from the initial plane of focus. When only defocalization is responsible for this loss, the dots should become visible in upper or lower planes. If photobleaching is involved, the dots will simply fade away.

To solve defocalization problems, make sure the cover slip is tightly fixed to the mounting frame (*see* also **Notes 4**, **11**, **12**, and **14**) and acquire images over a wide z range to make image projections as described in **Subheading 3.3.2.**

Photobleaching is usually hard to completely avoid. However, try to minimize it by lowering laser power and increasing exposure time/detector gain so that less than 10% of the signal is lost during an experiment. Photobleaching becomes negligible when the signal emitted is strong enough. The brightness of the signal depends on the levels of expression of the transgene used. When several insertion lines are available for a given transgene, it is usually worth testing a couple of them under the same conditions to determine which one(s) is (are) best suited for live imaging (*see* **Note 18**).

3.3.2. Drift in the z-Axis and Within the Plane of Observation

The main problem encountered when imaging live developing embryos is the drift of objects (e.g., cells) in the *x*/*y*-axes as well as in the *z*-axis caused by cell/tissue movements. This is particularly relevant to high-resolution imaging at high magnification where objects movements can be very rapid relative to the field of observation.

Drift in the *z*-axis causes the objects of interest to become out of focus, like defocalization because of mounting problems. This is dealt with by scanning over a wide range of z planes and projecting images from each z plane over a single plane once acquisition is finished. The resulting image is a 2D representation (*x* and *y*) of a 3D object (*x*, *y,* and *z*). This type of data manipulation is best done with Metamorph (Molecular Devices Corporation; www.moleculardevices.com) but can also be done with operating softwares of LSM confocal systems (*see* **Fig. 2**).

Drift in the *x*/*y*-axes causes the objects of interest to eventually leave the field of observation. This cannot be corrected for during acquisition with a spinning disk setup, but the spinning disk microscope allows the imaging of a larger field of view without increasing the time of image acquisition. With the LSM systems this problem can be overcome by mechanically moving the microscope

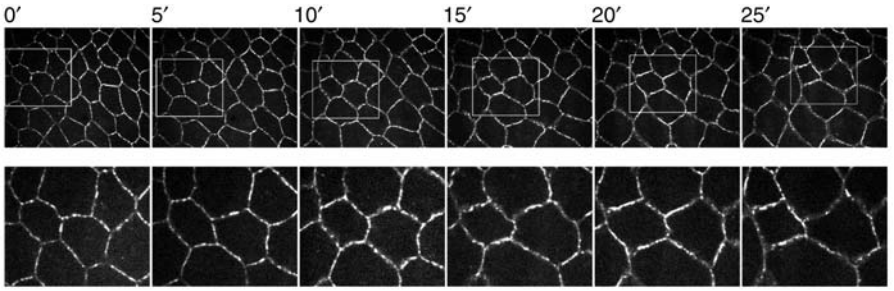

Fig 3. Correcting for x/y drift. Time-lapse sequence of DE-Cadherin-GFP in gastrulating embryos obtained with a spinning disk set up at high resolution. The first row shows the full image at the indicated time intervals, with the sub-region (white rectangle) centered on two cells drifting to the right. The resulting sequence cut from the full images is shown on second row.

stage during acquisition. The operating software includes a fine-moving function (arrows) to move the stage very slowly. Once the main direction of drift during an acquisition is known, adjust the stage position to cancel out the effects of drift. Repeat this regularly as the movie is being recorded. When possible, try to move the stage between two scans instead of during one scan to avoid distorting the image as scanning is under way.

Postacquisition object tracking softwares are very useful with both setups for reducing the effects of drift in a movie. Such functions can be set up in the form of macros in the Metamorph package. At each time-point of a movie, a manually centered subregion of the image is cut. The cut images are assembled into a new time-lapse movie. Determine the time range where a drifting object is visible in the field of observation. Define the region dimensions according to the object shape and size and how these characteristics vary overtime. Use the "track ROI" function of the program to center the region on the object of interest at each time-point (*see* **Fig. 3**). This function is also very useful for quantifying signal intensity in a specific region and observing how this evolves with time.

4. Notes

1. Heptane/glue preparation.
   a. Working in a fume hood, fill a glass scintillation vial with a maximum of packaging tape (wrapped in balls) and add 10 mL of Heptane. The tape brand is critical, as most glues used by tape manufacturers tend to be toxic to the embryos. In our hands, the Tesa 4124 packaging tape and 3M double-sided tape (12 mm × 6.3 m) are not toxic. However, a lethality test should be performed even when using these brands to make sure the glue does not interfere with embryonic development. Prepare embryos as described in **Subheading 3.1.** and let them

age 24 h at 25°C on the cover slip. Measure the survival rate by counting how many embryos hatch and give rise to viable larvae. The survival rate is also dependent on the quality of the oil.

b. Shake vigorously overnight.
c. Transfer the dark brown heptane/glue mix to a clean glass ultracentrifuge tube and centrifuge at 12,000 g for 30 min. The heptane/glue mix should turn yellow/light brown after centrifugation (when using dark brown packaging tape) and should be translucent. When the glue is not completely dissolved, the supernatant color is yellow but it appears cloudy after centrifugation. In that case, add more heptane (a few ml) and repeat centrifugation to completely dissolve the glue.
d. Transfer the supernatant to a new scintillation vial and seal with parafilm. Heptane is highly volatile, if the vial is not sealed properly with parafilm, heptane will evaporate and the glue mix will become more concentrated and darker. Add more heptane to lower the concentration.

2. Some experiments, such as the use of temperature sensitive mutants require temperature to be precisely controlled during time-lapse acquisition. Two systems are available for this purpose: heatable universal mounting frames and incubation hoods.

The advantages of heatable mounting frames is that they are very versatile as they can be adapted to a wide range of microscopes, and they can heat up to the desired temperature in a short time. However, the cover slip is heated from the sides and the diffusion of heat is slowed down in glass. Thus, the temperature of embryos should be precisely monitored with a thermometer equipped with a probe that is dipped in the oil to determine how long the embryos should be warmed up before acquisition. Heatable mounting frames are quite sensitive to room temperature variations and work best when the room temperature is fairly close to the desired temperature. Some heatable stages are quite thick and if the cover slip is fixed on top of the stage, the distance between the objective and the embryos becomes too long for focusing properly. Instead, one has to fix the cover slip from below the stage. For this purpose, use vaseline to glue the cover slip to the stage. Do not use double-sided tape as it does not stick properly when heated and causes serious defocalization problems.

The advantages of incubation hoods is that the temperature is much more stable than with heatable mounting frames and humidity can be controlled. As a result, drifts in the focalization point owing to small fluctuations in the temperature are absent, which is better when performing time-lapse recordings on a confocal microscope for instance. Moreover, the temperature can be either increased or decreased as some hoods can be directly linked to air conditioning equipment. However, the time required to bring the hood to the desired temperature is longer than for heatable stages, therefore experiments should be planned ahead accordingly.

Dehydration problems might be encountered with both systems when performing long acquisitions at high temperature because the oil spreads on the cover slip with time and this is enhanced at elevated temperatures. In that case, use more

viscous oil such as Halocarbon 700 to slow down the spreading process and try to increase the humidity level in the room/hood.

3. The apochromat objectives provide a better correction of chromatic and spherical aberrations and are best for high-resolution fluorescence imaging. Large numerical aperture (between 1.2 and 1.4) is obviously the best. For 2-photon imaging, use infrared corrected objectives that allow a better transmission in the long wave length (beyond 1000 nm).
4. There are several advantages of using water immersion compared with oil immersion objectives. In principle, water with 20% glycerol very closely matches the refractive index of halocarbon oil. However, in practice, it makes no detectable difference and water immersion or oil immersion have little differences in terms of imaging quality. The numerical aperture of the objective is more important. Practically however, here are the main relevant issues. First, the working distance is far greater with water immersion objectives, allowing imaging through the whole embryo with a 2-photon microscope for instance. Second, with water immersion, the suction between the objective and the cover slip is greatly reduced compared with oil. As a consequence, one can change from one stage position (i.e., one embryo) to another very quickly without having the water lagging behind as oil does. In addition, fast acquisition of z-stacks, such as with a piezo-electric device (e.g., Physik Instrumente [PI], GmbH & Co. KG, Karlsruhe/Palmbach, Germany), is limited by the viscous drag of the oil that pulls the cover slip together with the objective as it moves downward, thus preventing z-stacking. With water immersion, this problem is overcome and very fast z-stack (<100 ms per image) acquisition can be obtained. However, there are some problems with water immersion objectives: their numerical aperture is at present not more than 1.2, which is very good but may be insufficient in some cases where the signal intensity is weak and optimal resolution required. Second, water tends to evaporate when the temperature is near 30°C, and when the air is dry. This can be overcome with new immersion liquids that have the same refractive index as water but with the same viscosity as oil, with the disadvantage of oil viscosity.
5. Females start to lay many eggs when about 10-day old. Keep 5–10 d old females on regular fly food for 2 d before transferring them to the cage. Well-fed, healthy females ready to lay have a big abdomen, filled up by the ovaries. Put one male per 5–10 females in the cage. When using virgins, cross them with the appropriate males in tubes for two days before transferring them to the cage.

   Keep the laying cages between 18°C and 25°C. The ideal temperature is 25°C as females lay more eggs and the embryos develop faster. However, some temperature-sensitive strains require higher or lower temperatures. For example, some GAL4 drivers work best at 18°C.
6. Fifty percent bleach can be used instead of 100% bleach if the embryos seem to suffer from bleach treatment. When using 50% bleach, dechorionation takes about a minute and seems more efficient if embryos are rinsed shortly with water after 30 s and the bleach solution is then replaced by a new aliquot for another 30 s.
7. When properly dechorionated, embryos rinsed in distilled water tend to stick to each other and form clumps at the surface of the water. However, this is not observed when

embryos are rinsed with regular tap water or when they are not fully dechorionated. Using distilled water is thus a good way to make sure dechorionation is efficient.

8. Extensive wash is required to completely remove any traces of bleach, which is highly toxic to the embryos. Cleavage cycles and cellularization seem particularly sensitive to bleach treatment. When embryos are not washed well enough, cellularization occurs in an asynchronous way, with waves of cells cellularizing from one end of the embryo to the other. We strongly recommend verifying that embryos are properly washed by making a control movie of cellularizing embryos under differential interference contrast (DIC). Use 1–2 h-old embryos for this purpose, following the protocol described in **Subheading 3.1.** As a rule of thumb, rinse the embryos about 10 times, blotting each time the basket containing the embryos on a thick and clean paper towel to drain the water.
9. Do not leave the dechorionated embryos in the basket for more than 5–10 min, as they quickly dehydrate and eventually will die. Dehydrated embryos are usually soft and very fragile, they tend to blow up easily when manipulating them with a needle. However, when the embryos directly touch the agar, dehydration is considerably slowed down. Dechorionated embryos can be kept on agar for up to 1 h if required.
10. Embryonic stages can be best recognized when embryos are under Halocarbon oil with transmitted light on a microscope or even with a dissecting scope with sufficient magnification. We encourage training first using the Campos-Ortega and Hartenstein manual as a reference ***(16)***. With a little practice it is easy to distinguish embryos that are in cycle 13 or 14, namely entering cellularization. When such expertise is acquired you can also recognize this stage without oil in embryos that are placed on a piece of agar under the dissecting scope with diascopic illumination.
11. We find it useful to orient the embryos in a specific way (e.g., anterior left, posterior right, dorsal up, ventral down) to facilitate subsequent observations especially when using high magnification objectives. If large numbers of embryos are to be observed on a single cover slip, align them in several parallel lines but keep the lines shorter than the cover slip (about 5 mm at each end of the lines, *see* also **Note 14**).

    Group the selected embryos close to each other so that they will occupy a fairly small space once transferred to a cover slip. This is important for several reasons. When the drop of halocarbon oil that covers the embryos is too wide, it tends to spread easily and will eventually reach the edges of the cover slip. The oil might leak on the objective and damage it, and embryos will dehydrate during the experiment. In addition, minimizing the distance between embryos is a good way to gain time when using a multiple stage position setup (described in **Subheading 3.2.2.1.**). Up to two embryos can fit in the field of view of a ×20 objective.
12. Use 22 × 40 type 1 cover slips. Do not use longer cover slips (e.g., 22 × 50 and so on) as they aggravate suction and defocalization problems described in **Note 4**. Most objectives are corrected for type1 cover slips. In some cases, an iris allows one to adapt the objective to different cover slip thicknesses.
13. A very small drop of heptane/glue is usually sufficient, spread it well and quickly after adding it to the cover slip. Do not add too much as it will take time

to evaporate the heptane and will leave too much glue on the cover slip. Too much glue can affect imaging.

14. Try to stick the embryo at the center of the cover slip, especially when the line of embryos is fairly long. Avoid observing embryos too close to the edges of the cover slip as the objective might touch parts of the universal mounting frame. This leads to defocalization during acquisition.
15. At this point, embryos can be microinjected with drugs, RNAi, fluorescent markers, and so on before covering them with halocarbon oil. Do not forget to dehydrate the embryos at this stage before injection!
16. Oil prevents dehydration of the embryos while allowing gas—in particular oxygen—exchanges (breathing). The more viscous, the more effective it will be against dehydration but will also limit breathing. Halocarbon oil 200 or 700 is suitable for short-term imaging (a few hours). However, when embryos are to be kept for longer periods of time (e.g., up to hatching), more fluid oils such as Halocarbon oil 95, 56, or 27, or Voltalef oil 3S or 10S are more suitable. As these oils are less effective against dehydration, the embryos should be kept in a humid place as much as possible.
17. Bleaching fluorophores emitting at wavelengths close to GFP (490–550 nm) is easily performed with the laser used to image the specimen (i.e., Argon laser, 488 nm). This is in fact why photobleaching during simple acquisition occurs even when exciting the fluorophores at low laser power. However, when using fluorophores emitting at longer wavelengths (e.g., Rhodamine, >550 nm) the laser used for imaging (emitting at 546 nm) is not powerful enough and bleaching is not efficient. One requires more powerful lasers emitting at shorter wavelengths such as an Argon or UV lasers.
18. Some GFP fusion proteins compete with the endogenous protein for localization, like for sqh-GFP for instance (*sqh*: *spaghetti squash*; myosin regulatory light chain, which is localized at the cortex). Therefore, in the presence of the endogenous protein, the GFP fusion is diffusely localized throughout the cell and the GFP signal in specific areas of interest is very weak ***(17)***. Reducing the gene dosage for the endogenous protein using deficiencies or protein-null mutants can considerably improve signal brightness. However, this works best with GFP fusions that are able to fulfil the endogenous proteins' function. Thus, one should control that reducing endogenous gene dosage does not cause any phenotypes in the process of interest.

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14

Analysis of Neuromuscular Junctions

Histology and In Vivo Imaging

Andreas Schmid and Stephan J. Sigrist

Summary

The formation of new synapses within neuronal circuits is considered a primary mechanism of long-term synaptic plasticity to allow an increase in synaptic strength. Thus, understanding mechanisms of synapse formation in detail is pivotal for understanding circuit development, as well as learning and memory processes. Unlike the fairly static vertebrate neuromuscular junctions (NMJs), arthropod NMJs are dynamic, and in terms of structure and function similar to central excitatory synapses of the vertebrate brain. The *Drosophila* NMJ, unlike most other synaptic models, allows combining genetics with physiological, ultrastructural, and as described here, histological analyses.

Following "the life history" of identified synapses over time in the intact organism by monitoring their molecular dynamics and functional features is important for a full understanding of synapse formation and plasticity. Thus, there has been a long-standing motivation to follow cellular and synaptic events in vivo. However, to date few preparations have been studied, and often only with great difficulty. New perspectives in this field are opened up by the continuous development of powerful, genetically encoded fluorescent probes for in vivo imaging, most prominently green fluorescent protein (GFP). The *Drosophila* system allows the easy expression of relevant GFP-fusions (e.g., with synaptic proteins) from genomic transgenes to ensure physiological expression levels, and to test the functionality of GFP fusions by genetic rescue assays. Here, we provide protocols for immunolabeling of fixed NMJs in *Drosophila* embryos and larvae. Moreover, molecular in vivo imaging of *Drosophila* NMJs within developing larvae, a recent methodological addition of the NMJ model, is described. Finally, we present simple procedures how to extract quantitative information concerning synapse size and number from NMJ images.

Key Words: Active zone; confocal microscopy; *Drosophila melanogaster*; GFP; immunolabeling; in vivo imaging; Nc82; neuromuscular junction; PSD; quantification; synapse.

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1. Introduction

When analyzing synapses with immunohistochemistry, one should bear in mind that potential defects are often rather subtle, involving only quantitative changes in morphological size, number of synapses, and distribution or amounts of synaptic proteins. The late embryonic and larval neuromuscular junctions (NMJs) of *Drosophila*, established between identified motor neurons and muscles ***(1–4)***, show a comparatively simple architecture, thereby favoring quantitative histological analyses to reliably and efficiently describe such changes. NMJ terminals consist of strings of boutons. Each larval bouton entails several individual synaptic sites **(Fig. 1)**. On their presynaptic site, active zones, the places where vesicle fusions take place, are found. The monoclonal antibody Nc82 (recognizing Bruchpilot; ***[5,6]***) labels individual active zones as discrete spots (ϕ~300nm; *see* **Fig. 1**, right panel, red), offering "digital information" about synapse number and density. Opposite active zones, ionotropic glutamate receptors (subunits GluRIIA to GluRIIE; ***[7–11]***) cluster in postsynaptic densities (PSDs, ϕ~500 nm; *see* **Fig. 1**, right panel, green). Although many morphological features are already manifested at embryonic NMJs, larval NMJs show extensive elongation with a dramatic increase in the number of synaptic sites. Thus, whereas embryonic NMJs also allow the characterization of mutations leading to embryonic lethality, studying the addition of synapses is restricted to the larval NMJ. Recently, synapse addition was imaged at developing larval NMJs in vivo ***(12)***.

2. Materials

2.1. Immunolabeling of Embryonic and Larval NMJs (for Subheadings 3.1. and 3.2.)

1. Ca^{2+}-free hemolymph-like saline (HL-3): 70 m*M* NaCl, 5 m*M* KCl, 20 m*M* $MgCl_2$, 10 m*M* $NaHCO_3$, 5 m*M* trehalose, 115 m*M* sucrose, 5 m*M* HEPES, pH adjusted to 7.2 ***(13)***.
2. Phosphate buffered saline (PBS): 8 g NaCl, 1.15 g $Na_2HPO_4{\cdot}2H_2O$, 0.2 g KCl, 0.2 g KH_2PO_4, H_2O ad 1 L.
3. Phosphate buffered saline with Triton TX100 (PBT): PBS with 0.05% Triton TX100 (Sigma, St. Louis, MO).
4. 4% Paraformaldehyde (PFA): 12 g PFA are dissolved in 24 mL H_2O for 15 min at maximum 60°C. 30 mL 10X PBS and H_2O ad 300 mL are added and the solution is filtered.
5. Methanol, –20°C cold.
6. Goat serum ("NGS," Sigma no. S-2007).
7. 50% Bleach.
8. 3 *M* KOH.
9. Vectashield Mounting Medium for fluorescencence (Vector, Burlingame, CA).

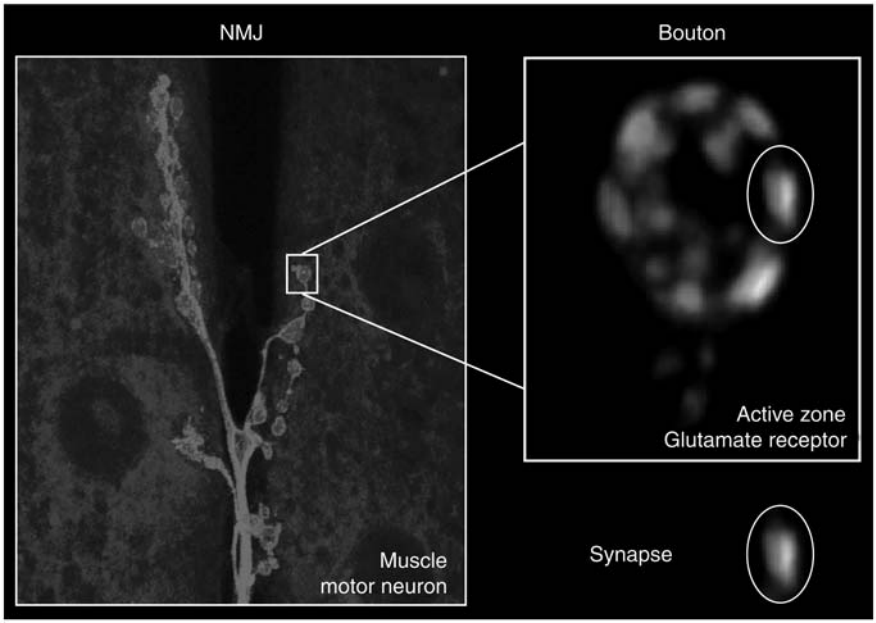

Fig.1. Morphological structure of the *Drosophila* larval NMJ. Third instar ventral-longitudinal muscles 6 and 7 (orange) innervated by motoneurons (green) branching into numerous boutons (left panel). One bouton (type Ib) consists of 10–20 synapses that are characterized by postsynaptic glutamate receptors opposing the presynaptic active zone (right panel).

10. Sylgard (Dow Corning, Midland, MI).
11. Antibodies: several useful monoclonal antibodies are available through the Developmental Studies Hybridoma Bank (DSHB) at the University of Iowa (www.uiowa.edu/~dshbwww) **(Table 1)**.
12. Fine forceps, size 4 or 5 (Fine Science Tools).
13. Dissection spring scissors (Fine Science Tools).
14. Micro sieve.
15. Fine insect pins, 0.1×10 mm^2 (e.g., Thorns, Göttingen, Germany).
16. Rubber dissection pads (Vetter, Wiesloch, Germany) (laboratory supply).
17. Tungsten wire, 0.075 mm^2 (World Precision Instruments, Sarasota, FL).
18. 9V battery, cables (Conrad, Hirschau, Germany), crocodile clips (Conrad), carbon cathode (custom made).
19. Scotch tape (Tesa, Hamburg, Germany).
20. Silicone tubes (Roth, Karlsruhe, Germany).
21. Glass tube, ϕ1.5 mm^2.
22. Vertically rotating disk (custom made).
23. Petri dishes.

Table 1
Antibodies Found Particularly Useful for Histological Description of NMJ Synapses

Protein	Type of antibody	Dilution	Stains at NMJ	Reference/ source
GluRIIA	Mouse monoclonal	1:100	PSD	MAb 8B4D2, DSHB
GluRIIB	Rabbit polyclonal	1:2000	PSD	***8***
GluRIIC	Rabbit polyclonal	1:500	PSD	***9***
GluRIID	Rabbit polyclonal	1:500	PSD	***9***
PAK	Rabbit polyclonal	1:2000	PSD, weak muscle cytoplasm	***14***
Nc82	Mouse monoclonal	1:100	Active zone	***6***, DSHB
HRP	Goat, Cy5 conjugated	1:250	Presynaptic plasma membrane	Dianova
Fasciclin II	Mouse monoclonal	1:10	Pre- and postsynaptic membrane, SSR subsynaptic reticulum	MAb 1D4, DSHB
Dlg	Mouse monoclonal	1:500	Pre- and postsynaptic membrane, SSR	MAb 4F3, DSHB
GFP	Mouse monoclonal	1:200	–	Molecular Probes (Eugene, Oregon)
	Rabbit polyclonal	1:500	–	Molecular Probes
	Goat polyclonal	1:2000	–	Rockland

For an excellent overview of antibodies labeling the NMJ ***(15)*** *see* also **ref. *16***.
HRP, horse raddish peroxidase; SSR, subsynaptic reticulum.

24. Artist's brush (Merck, Darmstadt, Germany).
25. Object slides and cover slips, 24×50 mm^2 and 24×24 mm^2.
26. Nail polish.

2.2. In Vivo Imaging (for Subheading 3.3.)

1. Suprane (Desflurane, Baxter, Deerfield).
2. Voltalef H 10S oil (Atofina, Puteaux, France).
3. Fly cultivation medium.
4. Inverted confocal microscope, for example, DM IRE2 AOBS (Leica Biosystems, Heidelberg, Germany).
5. Imaging chamber (*see* **Fig. 2**).
   a. Petri dish, ϕ60 mm^2.
   b. Round cover slips, ϕ50 mm^2, 0.12 mm^2.

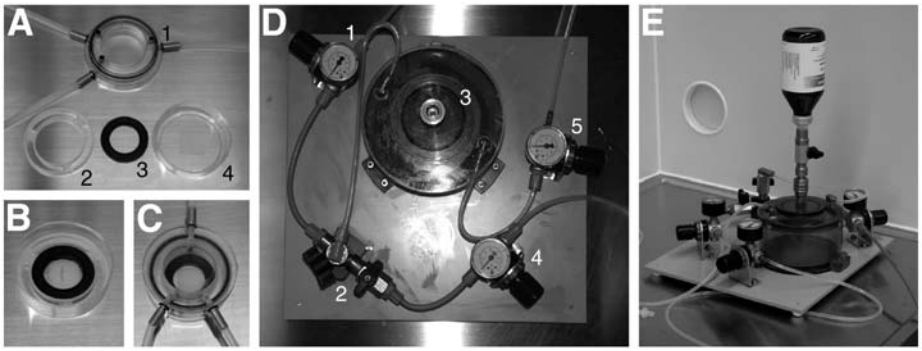

Fig. 2. Components of the device for in vivo imaging of *Drosophila* larvae. **(A)** Components of the imaging chamber: cover plate with three hose connections (1), plastic guide ring (2), iron ring (3), petri dish with cover slip bottom and plastic disk with slit (4). **(B)** Assembled imaging chamber without the cover plate. **(C)** Completely assembled chamber. **(D)** Anesthetization device: compressed air supply (1), two-way valve (2), vaporization chamber (3), air supply (4), anesthetic supply (5). **(E)** Anesthetization device with Suprane supply through an outlet adapter.

   c. Plastic disc (custom made), 0.2 mm^2.
   d. Plastic guide ring (custom made).
   e. Iron ring (custom made).
   f. Airproof cover plate with three hose connections.
6. Anesthetization device (custom made) **(Fig. 2)**.
7. Compressed air supplying device (custom made).
8. Outlet adapter valve (Riegler, Bad Urach, Germany).
9. Silicone tubes (Roth).
10. Plasticine (Uhu, Bühl, Germany).
11. Parafilm (American Can Company, Greenwich, Connecticut).
12. Binocular microscope (Zeiss, Jena, Germany) with a scaled ocular.

2.3. Microscopic Analysis and Quantitative Analysis of NMJ Morphology

1. Confocal microscope (e.g., Leica SP2,3,5, Wetzlar, Germany).
2. Epifluorescence (Axioskop 2 MOT, Jena, Germany) microscope with a scaled ocular.
3. ImageJ (NIH, Bethesda, Maryland; Li rsb.info.nih.gov/ij).
4. Microsoft Excel (Microsoft Corporation, Redmond, Washington).

3. Methods

3.1. Immunolabeling of Embryonic NMJs

1. Electrolytic preparation of ultrathin sharp tungsten needles (*see* **Note 1**).
   a. Attach a piece of tungsten wire to the positive pole of a 9 V battery using a crocodile clip.

   b. Immerse a carbon cathode in 3 *M* KOH and attach it to the negative pole.
   c. To produce a sharp tip, gently move the tungsten wire up and down in the electrolyte.
2. Detach embryos (stage 17, 20–22 h after egg laying) from apple agar plates (custom made) using H_2O and an artist's brush.
3. Transfer the embryos into a micro sieve, which is placed within a small Petri dish using a truncated pipet tip and wash the embryos with H_2O.
4. Removal of chorion: cover the embryos with bleach until the chorion is removed (about 2 min). Rinse thoroughly with H_2O. Then spray the embryos from the sieve into a Petri dish.
5. Carefully press an embryo, to stick to the bottom of the Petri dish, out of its vitellin membrane using fine forceps.
6. Transfer the embryo to the dissection sylgard plate by pipeting (10 µL tip).
7. Fix the embryo at the anterior and posterior end with two fine clips (made from tungsten wire, fixed with tape besides the sylgard plate), dorsal side of the embryo facing up (*see* **Note 2**).
8. Cover the embryo with Ca^{2+}-free HL3 (kept on ice) and stretch the embryo taut.
9. Open the embryo dorsally along the midline using two ultrathin sharp tungsten needles. With the first needle penetrate the embryo at the posterior end, then lift up and span the epidermis. With the second needle zig-zag rip open the epidermis from the posterior to the anterior end.
10. Suck off all internal organs using a blunt glass electrode connected to a silicone tube.
11. Pin down the epidermis laterally with a tungsten needle on each side.
12. Remove HL3 and apply 4% PFA (kept on ice) for 10 min (for alternative fixation media *see* **Note 3**). Replace the fixative with PBT.
13. Remove the lateral tungsten needles and detach the posterior and anterior clips.
14. Prepare a humid staining chamber consisting of a large Petri dish with a staining platform in the center (on top a 24×24 mm^2 cover slip). Fix tungsten wire clips around the platform with sticky tape and bend them in a way that allows fixation of the embryos on the cover slip.
15. Place a water-soaked tissue into the chamber and add PBT (300 µL) onto the staining platform.
16. Pipet the fillet into the staining chamber and fix the embryo with a clip.
17. For further fillet preparations repeat **steps 4–16**. Note the position of each embryo.
18. For immunostaining: remove PBT and add PBT containing 5% natural goat serum (NGS) and the respective volume of primary antibodies (*see* **Note 4**).
19. Incubate overnight at 4°C.
20. Wash the fillets twice briefly and three times for 20 min with PBT.
21. Cover the fillets with PBT containing 5% NGS and the respective secondary antibodies.
22. Incubate at room temperature for 2 h.
23. Wash the fillets twice shortly and three times for 20 min with PBT.
24. Prepare an object slide. Put a small piece (5 mm^2) of sticky tape on both ends of the object slide and distribute drops of 1 µL mounting medium for each embryo.
25. Detach the embryos from the clips on the staining platform and pipet (<1 µL volume) them one by one into the prepared mounting medium drops.

26. Arrange the embryos with the dorsal side facing up, and to avoid them drifting later pull them to the edge of the drops, respectively.
27. Uniformly spread about 40 µL mounting medium in between the drops containing the embryos.
28. Slowly put a 24×50 mm^2 cover slip on top of the samples.
29. Seal the cover slip with nail polish.

3.2. Immunolabeling of Larval NMJs

Usually, mid third instar stage larvae are selected shortly before onset of wandering stage for histological analysis. These are the largest larvae, which are still found within the food slurry but have not yet started crawling up the walls of the culture vial.

1. Transfer the cultivation medium containing the larvae into a micro sieve.
2. Wash the larvae under the water tap and spray them from the sieve into a Petri dish.
3. Carefully transfer a larva to the dissection rubber pad with a pair of fine forceps.
4. With the dorsal side of the larva facing up, fix the larva first at the posterior, then at the anterior end with two fine insect pins.
5. Cover the larva with Ca^{2+}-free HL3 (kept on ice) and stretch the larva taut.
6. Make a small hole at the dorsal midline near the posterior end using dissection scissors.
7. Use the dissection scissors to open the larva dorsally along the midline from the hole at the posterior to the anterior end.
8. Carefully remove internal organs with fine forceps.
9. Stretch and pin down the epidermis laterally with two pins on each side.
10. Remove HL3 and apply 4% PFA (kept on ice) for 10 min (for alternative fixation media *see* **Notes**).
11. Replace the fixative with PBT.
12. To enable parallel immunostainings of several genotypes mark the fillet by cutting off either the posterior tracheal ends, the whole posterior end, or the head segment.
13. Remove all pins.
14. Prepare a cup containing PBT (1–10 fillets → 500 µL cup with 500 µL PBT, 10–20 fillets → 1.5 mL cup with 1 mL PBT).
15. Take hold of either the anterior or posterior end of the fillet and transfer it into the provided cup.
16. For further fillet preparations repeat **steps 4–15**. Note the mark of each genotype.
17. To block unspecific antibody binding incubate the fillets for 30 min in PBT containing 5% NGS. Rotate the cup on a vertically rotating disk (this applies for all following staining and washing steps).
18. For the immunostaining refresh the PBT/NGS mixture and add the respective volume of primary antibodies.
19. Incubate overnight at 4°C.
20. Wash the fillets twice briefly and three times for 20 min with PBT.
21. Cover the fillets with PBT containing 5% NGS and the respective secondary antibodies.

22. Incubate at room temperature for 2 h.
23. Wash the fillets twice briefly and three times for 20 min with PBT.
24. Remove all PBT and incubate the fillets at least 30 min at 4°C in Vectashield mounting medium.
25. Spread about 50 µL of mounting medium on an object slide.
26. Transfer the fillets onto the object slide and arrange them with the dorsal side facing up.
27. Remove the mouth hooks from all fillets.
28. Slowly put a 24 × 50 mm^2 cover slip on top of the samples.
29. Seal the cover slip with nail polish.

3.3. Long-Term In Vivo Imaging of NMJs in Intact Larvae

The following protocol describes the in vivo imaging of synapse populations at the NMJ of intact alive larvae, expressing transgenes encoding fusions of suitable fluorescent labels (green fluorescent protein [GFP], monomeric red fluorescent protein, and so on) to relevant synaptic proteins (e.g., glutamate receptors, *see* **ref. *12***); rich sources for suitable GFP lines are also produced by exon trap screening (***(17)*** (http://flytrap.med.yale.edu). Series of images of identified NMJs are taken at intervals of few hours to follow the development and formation of individual synapses over time. We describe imaging on an inverted confocal microscope using high numerical aperture oil objectives (NA >1.3), which offer sufficient resolution to properly resolve individual synaptic sites of 500 nm in size or smaller. In **Fig. 3**, an example of a NMJ expressing GFP-labeled glutamate receptors within postsynaptic sites is shown with small and large magnification (taken from **ref. *12***).

3.3.1. Imaging Procedure

1. Assemble in vivo imaging chamber.
   a. Take the lower part of a Petri dish and excise the base plate leaving a circular border of about 2–3 mm^2 width.
   b. Use strong glue to tightly attach a round cover slip (ϕ: 50 mm^2, thickness 0.12 mm^2) on the remaining border. Watch out for air bubbles to achieve an airproof seal.
   c. Cover the side of the cover slip facing inwards with a thin coating of Voltalef oil (*see* **Note 5**).
   d. Place a thin plastic disk onto the oil layer. The disk contains a slit in its center, which must match the size of the larva. Moreover, the disk includes two nonstop notches from the slit to the disk border.
2. Prepare the anesthetization device.
   a. Close all valves of the anesthetization device.
   b. Install the outlet adapter valve on top of the Suprane bottle.
   c. Connect the outlet adapter headlong on top of the vaporization chamber of the anesthetization device.
   d. Fill Suprane into the vaporization chamber until the ground is fully covered with the anesthetic.

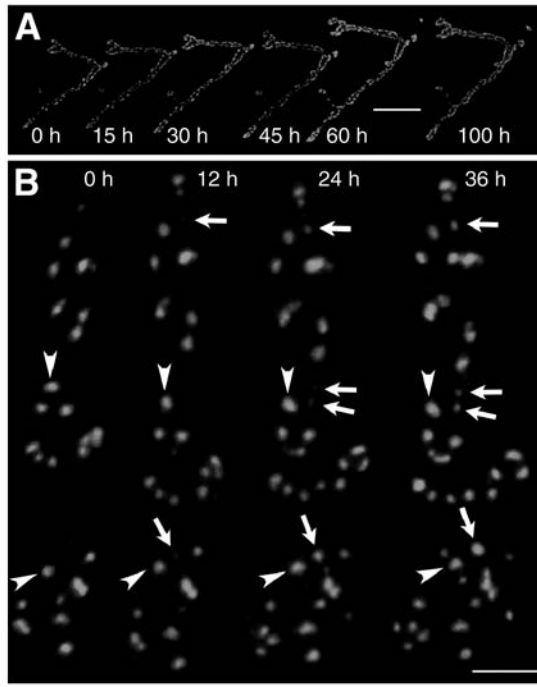

Fig. 3. In vivo imaging of synapse formation using GFP-labeled glutamate receptors. **(A)** Whole image frame following an identified NMJ 27 over 100 h at 16°C. Note increase in NMJ size. **(B)** Higher magnification: new synapses form *de novo* (arrows). Mature synapses (arrow heads) remain stable. Bars: in A, 10 µm, in B, 4 µm. Taken from **ref. *12***.

3. Transfer the cultivation medium containing the larvae into a micro sieve.
4. Wash the larvae under the water tap and spray them from the sieve into a Petri dish.
5. Select a larva of chosen size/stage (e.g., early third instar leaving an observation interval of about 24 h at 25°C until wandering stage), dab it dry, and place it into an airproof anaesthetization chamber.
6. Put the larva with the dorsal side facing up into the slit within the imaging chamber.
7. Place a 24×24 mm^2 cover slip on top of the larva. Carefully arrange the position of the larva by moving the cover slip back and forth with an artist's brush. For optimal optical access turn the larva about 30° aside.
8. Insert a plastic guide ring into the chamber. To fix the larval position, place an iron ring on top of the 24×24 mm^2 cover slip in the middle of the guide ring (*see* **Note 6**).
9. Measure and note the overall length of the larva. In our hand, early third instar larvae with a length of 3.00–3.50 mm^2 are well suitable.
10. Close the chamber with the cover plate, which contains an implemented rubber ring to guarantee a hermetical seal.
11. Attach silicone tubes to the three hose connections of the cover plate.

12. Install the imaging chamber on the inverted confocal microscope and fix it with plasticine.
13. Connect two of the three tubes with the anesthetization device (*see* **Fig. 2**). The first tube provides compartment air. The second is connected with the vaporization chamber and provides the anesthetic. Put the third tube into a small container filled with water.
14. For anesthetization apply a short air pressure surge into the vaporization chamber. Open the valve controlling anesthetic flow into the imaging chamber for 10 to 12 seconds. If no air bubbles ascend in the water container the imaging chamber is leaky.
15. Imaging
    a. Use transmitted halogen light to control for a complete stop of movements within the larva (particularly heart beat and muscle movements). This is absolutely necessary for acquisition of highly resolved images.
    b. Identify the abdominal muscles 26 and 27 ***(3)***, which are beneath the cuticle and thus, well accessible (from the outside). Segments A2 to A6 can be used likewise.
    c. Scan z-stacks of NMJ 27, and if desired as well of NMJ 26. However, NMJ 26 is often too large to be fully covered with a single frame when using a ×63 or ×100 objective. Note the segments being imaged. Go for saturated signals but avoid overexposure.
    d. Take care not to exceed an overall anesthetization time of 30 min (less is preferable).
    e. Use high numerical oil objectives with numerical apertures of 1.3 or higher (for high resolution), choose a voxel size for about twofold oversampling (i.e., when efficient resolution is 200 nm, use a voxel size of 100 nm).
16. Detach the imaging chamber from the anesthetization device and the confocal microscope.
17. Carefully open the chamber, remove the cover plate and tip over the iron and the guide ring. Remove the cover slip and place the larva into a Petri dish containing mashed fly cultivation medium (take care that the posterior endings of the trachea are uncovered). Seal the Petri dish with parafilm.
18. Return the larva back to the cultivation conditions for the desired imaging interval.
19. Before the second imaging: check the vitality of the larva. Wash the larva in a drop of tap water and dab it dry.
20. Repeat **steps 6–14**. From our experience, an increase of larval length of at least 10% (12 h interval) or 20% (24 h interval, both at 25°C) should be noted to approve later image quantification (*see* **Note 7**).
21. Reidentify and reimage the formerly recorded NMJs.
22. Based on the cultivation temperature and the initial animal size, the in vivo imaging procedure can be repeated several times over days.

3.4. Quantitative Analysis of NMJ Morphology: NMJ Size, Total Synaptic Area, and Synapse Density

The following procedure is based on immunostainings for horse raddish peroxidase (HRP) (labels presynaptic plasma membrane, representing NMJ

size), Nc82 (labels discrete active zones; ***[5,6]***), and the glutamate receptor subunit GluRIID ***(9)***, staining the PSDs (*see* **Notes 8–11**).

Image analysis and quantification procedures are performed with ImageJ, freely available at: http://rsb.info.nih.gov/ij.

1. Scan a confocal z-stack of a selected NMJ of a fixed abdominal segment (preferentially NMJ 4 or 6/7, segment A2 or 3). Go for saturated signals in all three channels but avoid overexposure. Recommended resolution: 1024 × 1024 pixels, 8 bit.
2. In ImageJ: set measurements before you start (*Analyze/Set Measurements*). Activate the following fields: *Area*, *Limit to threshold*.
3. Set the color picker to 0, 0, 0.
4. Open the z-stacks (*Import/Image sequence*).
5. Generate maximum projections of all three z-stacks (*Image/Stacks/Z Project/Max intensity*).
6. For better contrast change the lookup table (LUT) to Fire (*Image/Lookup Tables/Fire*). Remove nonsynaptic image matters, predominantly trachea, using the *Freehand selections* (*Edit/Fill*).
7. Mark part of the image background (*Freehand selections*). Measure (*Analyze/Measure*) and subtract the mean background gray value (*Process/Math/Subtract*), respectively.
8. Apply Gaussian blur filtering (radius 2 pixels) to each channel (*Process/Filters/Gaussian Blur*).
9. To normalize the intensity, measure the maximum gray value (*Analyze/Measure*) for each channel and set it to 255 by multiplication of the whole image (*Process/Math/Multiply*).
10. Set the threshold to a fixed gray value. From our experience, a threshold of 50 is well applicable (*Image/Adjust/Threshold/Set, Lower Threshold Level 50, Upper Threshold Level 255*).
11. Measure the total number of the remaining pixels (*Analyze/Measure*). Convert it to area. Area (μm^2) = Pixel number × (voxel size [nm])^2 × 10^{-6}. The voxel size depends on the specific microscope settings.
12. The overall HRP area represents the NMJ size, the Nc82 area represents the total presynaptic area, and the GluRIID area represents the total postsynaptic area. Calculate the synapse density by relating either the Nc82 or the GluRIID area to the HRP area.
13. To normalize the obtained values to the animal size, the muscle dimensions are required. To determine the muscle length and width use epifluorescence microscopy and a scaled ocular. For NMJ 6/7 measure the common area of muscles 6 and 7.
14. Relate all obtained values (except for the synapse density) either to the respective muscle area or alternatively, to the segment length.
15. If applicable, the total synapse number additionally can be determined either by manually counting (preferentially using the Nc82 projections) or by manual segmentation of individual synapses (*Freehand line selections*, *line width: 2 pixels*, *Edit/Draw*).

4. Notes

1. Regularly clean and resharpen the tungsten needles.
2. Periodically change the dissection spot on the sylgard plate for better steadiness of the tungsten needles.

3. Fixation protocols used can strongly influence the results of your immunolabeling. For example, the αGluRIIA monoclonal antibody (8B4D2, DSHB) does not produce any PSD label with PFA fixation but works with either Bouins fixative (*see* **ref. *18***) or cold methanol (freshly from –20°C) for 5 min (our unpublished observation).
4. When characterizing antibodies, use genetic controls (protein null alleles) where possible, as well as preimmune serum controls. Use rabbit sera at least in a dilution of 1:500.
5. Only a thin layer of Voltalef oil is needed. Too much oil might inhibit proper anesthetization.
6. Do not tilt the imaging chamber when a larva is fixed inside, as movement of the iron ring might squeeze the animal.
7. Exclude larvae that did not grow adequately from analysis (for a third instar larva at 25°C at least 10% or 20% length increase after 12 h or 24 h, respectively).
8. To determine the NMJ size always compare only NMJs from the same abdominal segment as systematic differences between segments exist.
9. Try to immunolabel larvae of similar size. For this, measure length of larvae and NMJs with a scaled ocular.
10. Exclude PSDs that cannot be reassigned without doubt at the second time-point from further analysis.
11. Only consider images of high quality, and compare only images of fully identical imaging settings.

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15

Immunolabeling of Imaginal Discs

Thomas Klein

Summary

Drosophila melanogaster imaginal discs are a widely used model system to study signal transduction, developmental, and cell biological processes. An important tool to study these processes is immunolabeling. Fluorochrome-conjugated antibody staining allows the researcher to determine the expression pattern and subcellular localization of several proteins in one imaginal disc. In this chapter, I will describe how to dissect, fix, and immunolabel imaginal discs. The protocol is streamlined so that the whole procedure can be performed in 1 d, including image acquisition.

Key Words: Antibody staining; *Drosophila*; imaginal discs; immunofluorescence; preparation of imaginal discs; larva.

1. Introduction

Most of the adult body wall of the *Drosophila* adult is assembled from parts that are generated by imaginal discs. The "factory" in which these parts are formed is the larva, whereas the assembly takes place in the pupa. Imaginal discs are autonomously developing units that differentiate into specific parts of the adult body, such as wings, legs, and eyes. Imaginal discs are monolayered epithelia that are defined during embryogenesis and proliferate during larval life. Because imaginal discs do not participate in larval life, they can be manipulated without consequence on the survival of the larva. This property makes them ideal systems for genetic and physical manipulations. Because of their relatively easy accessibility, simplicity, and manipulability, imaginal discs have become important systems to study pattern formation in a cellular context, as well as signal transduction and several cell biological processes, such as cell polarity and endocytosis.

Each type of imaginal discs can be recognized by its location and characteristic shape. Examples of imaginal discs and their location in a larva at a late larval instar stage are depicted in **Fig. 1**.

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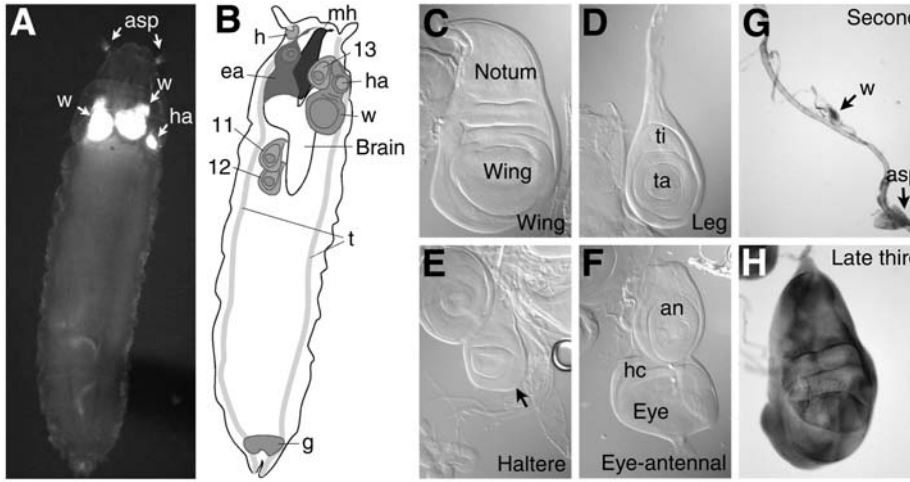

Fig. 1. Location of the imaginal discs in the *Drosophila* larva. **(A)** Wing and haltere imaginal discs in a living mature third instar larva. The imaginal discs are revealed by their fluorescence under a fluorescent dissecting microscope caused by *ap*Gal4-mediated activation of an UAS-green fluorescent protein (GFP) construct. w, wing disc; ha, haltere disc; asp, anterior spiracle. Like most other imaginal discs, the wing and haltere discs are located in the anterior half of the larva. The other imaginal discs are not visible. Most of the imaginal discs come in pairs, which are located symmetrically at the same position on each side of the longitudinal body axis. **(B)** Schematic representation of the localization of the various imaginal discs in the mature third instar larva. For the sake of simplicity only one of each pair of imaginal discs is shown. ea, eye-antennal disc; g, genital disc; ha, haltere disc; l1, l2, l3, first, second, and third thoracic leg discs; mh, mouth hook; t, trachea; w, wing disc, **(C–F)** Live images of imaginal discs prepared from a larva at a late stage of the third larval instar stage. **(C)** Wing disc. This gives rise to the wing and half of the notum. **(D)** Leg disc. ta, tarsal region; ti, tibia. **(E)** Haltere disc. **(F)** Eye-antennal disc. This disc generates the eye, head capsule (hc), and the antenna (an). **(G,H)** A comparison of second and mature third instar wing imaginal discs to reveal the dramatic difference in size. The size difference highlights the massive cell proliferation that takes place between both stages. Shown in the Figures is the expression pattern of the Ser protein, revealed by DAB antibody staining. w, wing disc; asp, anterior spiracle.

After their definition during embryogenesis, each imaginal disc invaginates into the inside of the embryo, but remains attached to the epidermis through a small stalk. Through this invagination the imaginal discs form flattened, sac-like structures with two distinct sides: one side forms the imaginal disc epithelium proper, whereas the other develops into the peripodial membrane. The peripodial membrane is made of very thin flat epithelial cells. In contrast, imaginal disc cells have a columnar shape, with the apical side facing the lumen that forms between the peripodial membrane and the imaginal disc epithelium

during the second larval instar. During all three larval stages of *Drosophila*, the cells of the imaginal discs proliferate extensively and each imaginal disc includes thousands of cells at the end of the larval period. As a consequence of the large-scale proliferation, the imaginal disc epithelium becomes folded in a manner characteristic to each type of imaginal disc (e.g., *see* **Fig. 1C–F**).

At 25°C the larval life of *Drosophila* lasts for about four days: the first two larval instar stages last about 24 h and the third larval stage is twice as long (48 h). At the end of the third larval stage, the larva stops feeding and wanders along the walls of the vial to find an appropriate place for pupariation. The wandering stage last about 12 h but is often longer and ends with the formation of a white prepupa (around 120 h after egg laying [AEL]). For a more detailed description of the larval and pupal stages of *Drosophila*, *see* ***(1–3)***.

Here I will describe the procedure of antibody staining of imaginal discs using flurochrome-conjugated secondary antibodies. This technique allows the comparison of the expression and localization of several antigens in one imaginal disc using primary antibodies raised in different species. The protocol is described for imaginal discs at the end of the third larval instar stage (wandering stage). Imaginal discs of this stage are easy to prepare and are often used for analysis. Imaginal discs of younger ages, for example, second instar imaginal discs, are more difficult to prepare and demand experience in preparation (*see* **Fig. 1G**,**H** for comparison of size of imaginal discs at different stages). However, the protocol is also suitable for imaginal discs at early stages.

2. Materials

2.1. Equipment

1. Plastic multiwell plate with 24-wells (24-well plate, e.g., from Falcon, Falcon tubes, Becton Dickinson, USA).
2. Two very thin forceps (Dumont Biology No. 5, Outils Dumont SA, Switzerland) (*see* **Note 1**).
3. Three-well spot plate (e.g., from Hampton research, California). Other small preparation vessels are also fine.
4. Baskets made from plastic reaction tubes (e.g., 1.5-mL test tubes). To generate a basket, cut the test tube in half and dispose of the bottom half. Seal the bottom of the remaining part of the tube with a fine nylon grid. We seal the tube by carefully melting the plastic at the open bottom of the tube using the heated blade of a knife. The test tube is then pressed onto the nylon grid with the melted side down. After cooling, the grid seals the opening of the tube and the basket is ready.
5. Dissecting microscope: best with ×20 oculars for the highest possible magnification.
6. Rocking plate: vertical or horizontal movement.
7. Fluorescent microscope with appropriate filters.
8. Optional: Cold room at 4°C or refrigerator large enough to fit a rocking plate for overnight incubation.

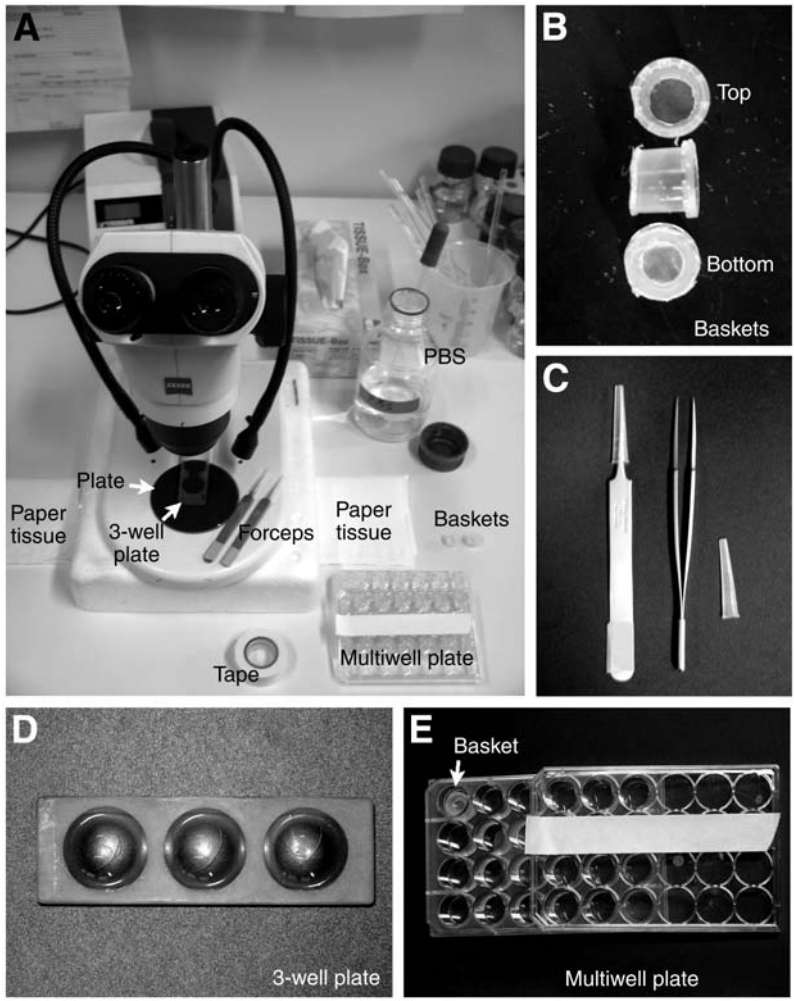

Fig. 2. Equipment required for the preparation of imaginal discs. **(A)** A preparation place with all the equipment at one glimpse assembled around the stereo-microscope. Turn the microscope platform to the black side for better contrast. **(B)** Baskets for staining made of test tubes. The tubes are cut in half and the bottom of the upper half is sealed with a plastic mesh. **(C)** A pair of fine forceps is essential for dissecting larvae. **(D)** Three-well spot plate. **(E)** 24-well plastic plate with a basket immersed in PBS in one-well to collect the dissected larvae.

9. Pasteur pipets.
10. Fine tungsten needle.
11. Microscope slides (ca. 76×26 mm^2) and cover slips (e.g., 24×24 mm^2).

The equipment required is depicted in **Figs. 2** and **3**.

Fig. 3. Rocking platform and other equipment required for antibody staining of imaginal discs. *See* text for further information.

2.2. Solutions and Reagents

1. Phosphate-buffered saline (PBS): 130 m*M* NaCl, 7 m*M* Na_2HPO_4, 3 m*M* NaH_2PO_4, adjust to pH 7.4 with HCl. Store at 4°C.
2. Fixative: paraformaldehyde (4%) in PBS: dissolve paraformaldehyde in 80 mL distilled water on a hot plate with a stirrer, add three drops of 1 *M* NaOH to clear the solution, cool and add 10 mL of 10X PBS stock solution, adjust to pH 7.2 with NaOH and fill up to 100 mL. Aliquot and store at –20°C. (*See* **Note 2** for an alternative fixative.)
3. PBT: 0.3% Triton X-100 dissolved in phosphate buffered saline (PBS).
4. Blocking solution: PBT + 5% fetal calf serum or normal goat serum (*see* **Note 3**).
5. Primary antibody/-ies and fluorochrome- or horseradish peroxidase (HRP)-conjugated secondary antibodies/-sera (*see* **Note 4**).
6. Hoechst 33258 (Sigma-Aldrich, Germany) staining solution: 5 µg/ mL in PBT. Prepare stock solution at 5 mg/mL, dissolve in H_2O, store at –20°C.
7. Mounting medium: Vectashield H-1000 from Vector Laboratories, California, USA. (*See* **Note 5** for an alternative mounting medium).
8. Diaminobenzidine (DAB) stainings:
   a. DAB-stock solution: 2 mg/mL in PBS.
   b. $NiCl_2$ (8%).
   c. H_2O_2 (3%).
9. To prepare 400 µL DAB staining solution, mix 300 µL PBS, 100 µL DAB-stock solution, 2 µL $NiCl_2$, and 2 µL H_2O_2.
10. 30, 50, 70, and 100%, respectively glycerol: dilute in PBS.

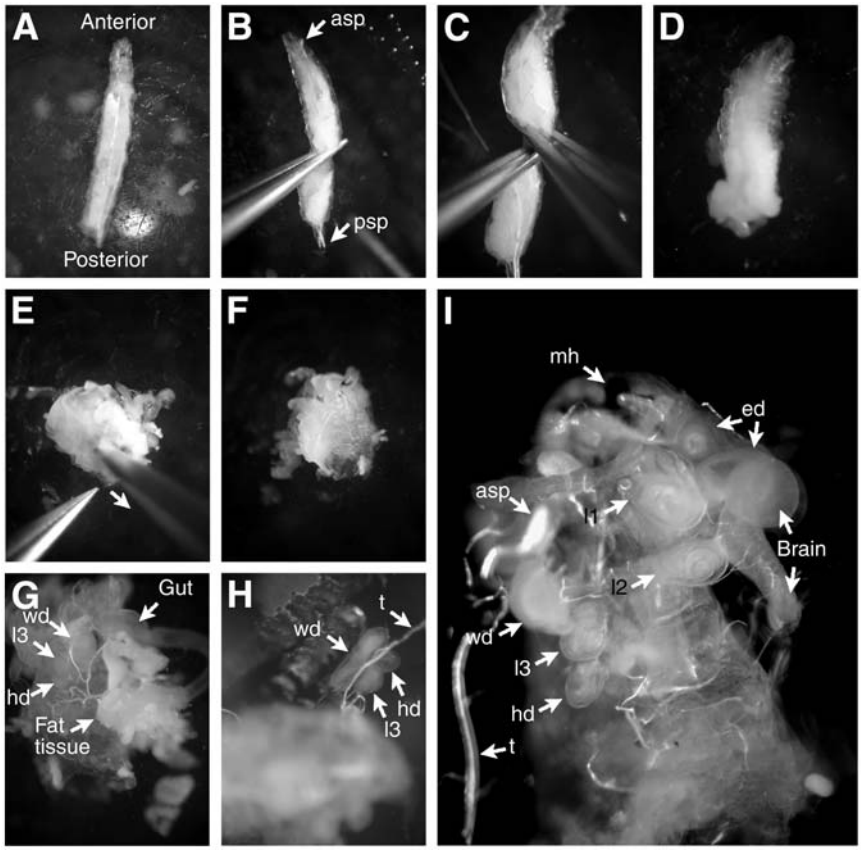

Fig. 4. Important steps during the preparation of imaginal discs. **(A)** A mature third instar larva in a PBS filled preparation well. The anterior of the larva is to the top. **(B)** Grab the larva with one of the forceps (left hand). asp, anterior spiracle; psp, posterior spiracle. **(C)** Grab the larva with the second forceps close to the place of the first one and then tear the posterior half away. Take the posterior half out of the well and discard it onto the paper towels placed beside the microscope (*see* **Fig. 2A**). **(D)** The anterior half of the larva. Anterior is to the top. This half should now be inverted to expose the imaginal discs to the medium. **(E)**. Invert the larva by pushing the tip of the forceps held in the right hand from the anterior side into the larva and pulling the edges over the sides of the tip of the forceps. **(F)** An inverted anterior half of a larva with all tissue still attached. It is shown in higher magnification in **(G)**. The imaginal discs are covered in part by the larval tissue, such as fat and gut. These tissues have to be removed. The wing discs (wd), third thoracic leg discs (l3), and haltere discs (hd) are already visible as transparent entities. **(H)** The wing disc, the third thoracic leg discs, and the haltere discs are attached to the trachea (t) close to the anterior spiracles. **(I)** An inverted anterior half of a larva freed from larval tissue. All imaginal discs are recognizable. This half is ready for the staining procedure and should be transferred to the basket in a PBS-filled well

3. Methods

The principle underlying immunolabeling/antibody staining is to expose the imaginal discs to a primary antibody or antiserum that is directed to the epitope of choice and then detect this antibody with a fluorochrome-conjugated secondary antibody, or more frequently, antiserum raised in another species that detects the heavy chain of the primary antibody or serum in a species-specific manner. Before exposing them to antibodies the imaginal discs are incubated with fixative to preserve the structure and to facilitate handling. Several primary antibodies can be used for multiple staining of imaginal discs, if these antibodies are raised in different species and can be detected by independent, noncross-reacting secondary sera (*see* **Note 6**). Our protocol is based on the standard protocol used for staining of embryos (e.g., *see* **ref. *4***).

3.1. Dissection of Imaginal Discs

A critical step in the staining procedure is the preparation of the imaginal discs. The aim of the preparation is to expose the imaginal discs to the staining solutions, but prevent the loss of these tiny entities during the staining procedure. This problem is solved by inverting the larvae and removing the unnecessary tissue such as fat and gut, but leaving the imaginal discs attached to the remaining body wall or other larval structures. Preparation of the imaginal discs is carried out under a dissecting microscope (*see* **Note 7**). The critical steps of preparation are shown in **Fig. 4**.

1. Fill two of the wells of a three-well spot plate with cold (4°C) PBS (*see* **Note 8**). Keep the three-well spot plate on ice during dissection.
2. Collect wandering stage larvae from the wall of the food vials with a paintbrush and transfer to one of the wells filled with cold PBS for cleaning (*see* **Note 9**). Larvae are often "dirty" and cleaning the larvae by rinsing them in PBS facilitates preparation.
3. Transfer a few larvae to the other well filled with PBS, for dissection.
4. Tear the larva in half using two pairs of forceps **(Fig. 4C)**. With the exception of the genital disc, all imaginal discs are located in the anterior half of the larva.
5. Remove the posterior half and unnecessary tissue from the preparation well and discard it on a paper tissue placed next to the microscope.
6. Invert the anterior half in a way similar to inverting a sock. The easiest way of inversion is to hold the larva with one pair of forceps at its mouth hook and to use a second pair of forceps to push the anterior half of the larvae over the tip of the

Fig. 4. *(Continued)* of the multiwell plate. The first (l1) and second thoracic (l2) pairs of leg discs are attached to the brain. The eye-antennal disc (ed) is attached to the brain lobes through the optic nerve and to the mouth hook (mh). The wing disc (wd), haltere disc (hd), and third thoracic leg discs (l3) are attached to a secondary tracheal branch close to the anterior spiracle (asp). t, main tracheal branch.

forceps holding the mouth hook **(Fig. 4E)**. Now the inside of the body wall is on the outside and the imaginal discs are exposed to the medium.

7. Remove unnecessary tissue, such as the gut and fat tissue **(Fig. 4G–I)**. The imaginal discs are recognizable as transparent units attached to the trachea close to the anterior spiracles (third thoracic leg, wing, and haltere discs) and the brain (eye-antennal and remaining leg discs). The fat tissue is white and the gut has a yellowish appearance (*see* **Fig. 4G**). The eye-antennal disc is also attached to the mouth hook. If you do not need the eye antennal disc, tearing away the mouth hook is a convenient way to get rid of much of the superfluous tissue in one step. An example of an inverted larva with imaginal discs, freed from unnecessary tissues is shown in **Fig. 4I**.
8. Collect the inverted larvae, with the imaginal discs still attached, in a basket and immerse it in a well of the 24-well plate filled with PBS (*see* **Note 10**). From now on the imaginal disc must be kept in solution.

3.2. Fixation

For better handling of the imaginal discs, and to preserve the molecular architecture of the cell during treatment, the imaginal discs have to undergo a fixation step. Several fixatives are commonly used. In our hands, paraformaldehyde fixation works best. For other frequently used fixatives including formaldehyde (*see* **Note 2**).

1. Replace the PBS in the well containing the basket with 500 µL of the paraformaldehyde fixative and incubate for 30 min at room temperature (*see* **Note 11**). The PBS can be removed using a Pasteur pipet inserted between the basket and the wall of the well.

3.3. Immunostaining (see Note 12)

All the following incubation steps should be performed on a rocking plate at room temperature (*see* **Fig. 3**).

1. Replace the fixative with PBT and wash three times for 20 min with 500 µL of PBT. Triton-X-100 makes the cell membrane permeable to the antibody, allowing it to reach its epitope.
2. Incubate tissue with blocking solution for 30 min (*see* **Note 13**). Blocking reduces background staining by preventing unspecific protein–antibody interactions.
3. Incubate tissue with the primary antibody/ies or serum/a diluted in blocking solution for 90 min (*see* **Notes 14** and **15**).
4. Wash three times for 20 min with PBT. Washing removes unbound primary antibodies.
5. Incubate with the fluorochrome-conjugated secondary antibody/ies or serum/a for 60 min. Dilute secondary antibody/serum in blocking solution.
6. Wash once for 20 min with PBT.
7. Incubate in Hoechst 33258 staining solution for 4 min. This is an optional step. Hoechst 33258 (Sigma-Aldrich, Germany) stains DNA and thus, allows counting of cells and recognition of the shape of the imaginal discs.
8. Wash two times for 20 min with PBT.

3.4. Mounting of Imaginal Discs

Up to this step the imaginal discs are still attached to a portion of the larval epidermis, or other structures. In order to analyze them in detail under the microscope, they must be separated from the epidermis and residual larval tissue and mounted in a liquid medium on a microscope slide. This dissection is done under a stereo-microscope.

1. Transfer the imaginal discs still attached to the larval epidermis to a drop of mounting medium on a microscope slide.
2. Separate the imaginal discs from the larval epidermis and remaining tissue using a fine tungsten needle. A good landmark for the position of the wing and haltere disc, as well as the third thoracic leg disc, is the tracheal system: this group of imaginal discs is attached to a secondary tracheal branch located close to the anterior spiracle (*see* **Figs. 1** and **4** and **Notes 16** and **17**).
3. Remove larval epidermis and remaining tissue from the microscope slide with forceps (*see* **Note 18**).
4. Carefully add a cover slip onto the imaginal discs. Place the edge of a cover slip next to the drop of mounting medium. Using forceps, carefully lower the cover slip onto the imaginal discs.
5. Seal the edges of the cover slip with nail polish. To prevent movement of the cover slip, first add nail polish to the four corners of the cover slip and let it harden. Then add nail polish to the edges. Nail polish prevents the shearing of the cover slip when immersion lenses are used.
6. Microscope slides can be stored at 4°C for a few months; however, signals fade away slowly and thus, the best results are achieved if the microscope slides are analyzed under a fluorescence microscope shortly after mounting.

4. Notes

1. Forceps with fine tips are critical for dissecting larvae.
2. Another fixative often used is 1,4-piperazinediethane sulfonic acid-EGTA-NP-40 Fixative ***(5)***: add three parts Browers fix buffer to one part 8% formaldehyde. Browers fix buffer: 0.15 *M* 1,4-piperazinediethane sulfonic acid, 3 m*M* EGTA, 1.5% tergitol type NP-40 (NP-40) Sigma, Germany, adjust to pH 6.9 and store at 4°C.
3. Other protein solutions, for example, bovine serum albumin or goat serum, can be also used for blocking.
4. We routinely used fluorescin isothycyanate (FITC), Alexa568- and Cy5-coupled secondary antisera alone or in combinations. An exhaustive list of available fluorochromes can be found at http://flowcyt.salk.edu/fluo.html.
5. Alternative mounting medium: 80% glycerol, diluted in PBS, 4% n-propyl-gallate. n-propyl-gallate prevents photobleaching of the fluorochrome.
6. Try to avoid the combination of rat and mouse antibodies in multiple stainings, because the secondary sera often crossreact.
7. It is helpful to work with a black background to enhance the contrast between the background and the bright tissue of the larva during preparation. If you prepare in

three-well glass plates, use the black side of the dissecting microscope platform (*see* **Fig. 2A**). Alternatively, use a black preparation well plate, or prepare on black paper. Illuminate larvae from the side.

8. Other salines, such as Insect Ringers, are also suitable.
9. For some experiments, staging of the imaginal discs/larvae is necessary. The simplest way of staging is to let flies of the desired genotype lay eggs in a vial for a defined period (e.g., 8 h) and then rear the larvae for the required time at 25°C. However, this method achieves only crude staging, because many uncontrollable environmental and genetic factors (e.g., density of larva in the food or the genotype) determine the time of development of individual larva. A more precise, but more laborious method is described in **refs. *6*,*7***.
10. Alternatively, dissected larvae can also be collected in a test tube filled with PBS. However, exchange of solutions is more difficult in test tubes compared with baskets.
11. Some antibodies require a longer fixation time of the imaginal discs. In extreme cases, for example, for anti-Achaete antibody staining, overnight fixation is required.
12. An alternative to fluorescence immuno-labeling is DAB/HRP staining. In this case the secondary antibody is conjugated with HRP. HRP produces a brown or black precipitate generated by conversion of DAB. Whereas this staining method allows the observation of an expression pattern of a protein with a normal light microscope (*see* **Fig. 1G,H**), stainings involving multiple primary antibodies have usually a poorer quality and the resolution is not comparable with fluorescence immuno-labeling.

    For DAB/HRP staining, perform **steps 1–4** as described in **Subheading 3.3.**, then proceed as follows:

    a. Incubate tissue with HRP-conjugated secondary antibody or serum.
    b. Wash three times with PBT for 20 min.
    c. Stain with the DAB staining solution and observe the progress of the staining reaction under the dissection microscope.
    d. Stop the staining reaction, after sufficient staining, by replacing the staining solution with PBS. Inactivate DAB by mixing with the hypochlorite solution.
    e. Wash once with PBS.
    f. Transfer tissue through a series of 30, 50, and 70%, respectively, glycerol diluted in PBS for 20 min each dilution.
    g. Mount imaginal discs in 100% glycerol.
13. The blocking step can be extended up to 2 d. (over weekend) on a rocking plate at 4°C without any noticeable loss of staining quality.
14. Alternatively incubate overnight on a rocking plate at 4°C. This step can also be extended for up to 2 d. However, in our experience long incubation times often increase background staining.
15. Background staining can be also be reduced by preabsorbing the antibody with fixed imaginal discs, or embryos, overnight. This is especially suitable for antibodies directed against epitopes used in reporter assays and which are normally absent from the *Drosophila* proteome. An example is the β-Galactosidase antibody. Preincubation of the β-Galactosidase antisera/body reduces background staining

significantly. If the epitope is included in the proteome, one can use homozygous mutant embryos or embryonic stages, where the protein is not expressed for preabsorption.

16. Alternatively, the imaginal discs attached to the residual larval epidermis can be dissected in a PBS-filled well of the three-well glass plate and then transferred to a drop of mounting medium on a slide. Preparation in PBS has the advantage that the imaginal discs are more recognizable. Therefore, this alternative is better suited for the beginner. However, if many imaginal discs must be transferred, a considerable volume of PBS is transferred along with the imaginal discs. This increases the volume of liquid on the mounting slide and dilutes the mounting medium with its photobleaching protective substances. The volume can be reduced after transfer by removing superfluous liquid using a syringe with a thin hypodermic needle.
17. If younger and therefore, smaller imaginal discs are prepared, it is better to transfer them together with the structure they are attached to, such as the trachea in the cases of wing, haltere, and third thoracic leg imaginal discs. They can be identified later when the preparation is monitored using the microscope.
18. Alternatively, use forceps to transfer imaginal discs to a drop of fresh mounting medium on a second microscope slide.

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16

Imaging *Drosophila* Pupal Wing Morphogenesis

Anne-Kathrin Classen, Benoit Aigouy, Angela Giangrande, and Suzanne Eaton

Summary

Drosophila pupal (P) wing development entails a series of dynamic developmental events, such as epithelial and glial morphogenesis, that are of outstanding interest to cell biologists. Here, we first describe how to prepare P and prepupal (PP) wings for immunofluorescence microscopy. This protocol has been optimized to visualize wing epithelial architecture, such as polarized cortical domains of planar cell polarity proteins. We then provide a protocol to prepare pupae for whole mount live imaging of P wings. This procedure has allowed us to live-image glial cell migration and proliferation along wing sensory nerves.

Key Words: *Drosophila*; glial cell migration; immunofluorescence microscopy; live imaging; planar cell polarity; pupal wing.

1. Introduction

During pupal (P) metamorphosis a series of dramatic tissue rearrangements mediate the transformation of imaginal discs into adult fly structures. In the course of the first day of P development wing imaginal discs evaginate, increase their surface area by flattening, secrete and ecdyse a cuticle layer, undergo cell divisions, acquire a regular epithelial packing geometry, and grow hairs ***(1–4)***. In addition to these events that bring about epithelial morphogenesis, the wing gives rise to a sensory nervous system that is made up of peripheral neurons and their associated glial cells ***(5,6)***. During early P development glial cells proliferate and migrate extensively to completely cover the wing nerves ***(7,8)***. The timeline in **Fig. 1.** illustrates this sequence of events and the stages at which they occur. A detailed description of the wing morphology during these stages can be found in **refs. *4,9***. This information will aid the understanding of the issues raised in this chapter and allow you to develop a staging protocol for your specific application and your lab conditions.

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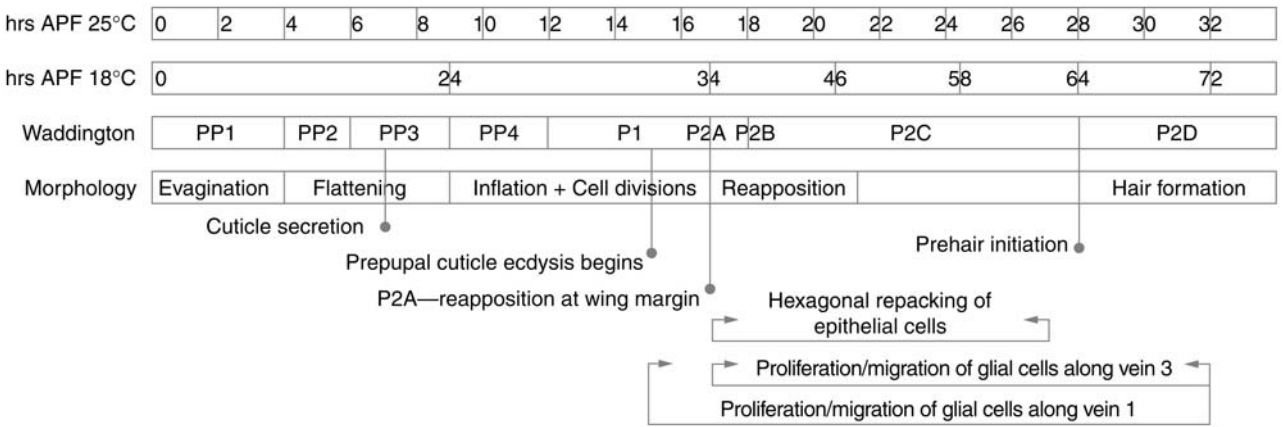

Fig. 1. Early P wing development. In this timeline we delineate morphological landmarks of P wing development, the associated Waddington's stages, and the times at which they occur at 18°C or 25°C temperatures. We use Waddington's staging terminology throughout this chapter. Development within the P case is divided into PP and P stages that are separated by a molt. During evagination, in the first PP stage (PP1), the dorsal and ventral halves of the wing pouch begin to adhere along their basal surfaces and the wing epithelium folds along the future wing margin to assume an approximately semicircular shape. In PP2, the tissue elongates and narrows until it resembles a thick cylinder three times as long as it is wide (at about 5 h APF at 25°C). During PP3 (6–9 h APF at 25°C), the wing cells flatten and the wing dramatically increases its surface area. Cuticle deposition begins at this time, and forms a chitinous sheath during PP4, when the wing becomes inflated and the dorsal and ventral surfaces move apart. This leaves the wing looking like a balloon. During inflation, a wave of cell divisions runs through the wing. Ecdysis of the cuticle—the shedding of the chitinous cuticle from the apical side of the wing epithelium—starts late in the first P stage (P1). Waddington subdivides the second P stage into substages **A–D**. In P2A, dorsal and ventral wing surfaces begin to reappose beginning at the wing margin, and the adhesion of the two basal surfaces spreads from the distal, anterior, and posterior ends of the wing during P2B and early P2C. During P2A–P2C the wing epithelium acquires a hexagonal cell packing geometry. P2D starts with the initiation of prehairs. Eventually hairs will cover the whole wing. Proliferation and migration of glial cells along vein 3 initiates in the beginning of P1 (by 15 h APF at 25°C). Migration, accompanied by proliferation of glial cells along vein 1 begins at approximately late P1 (by 17 h APF at 25°C). Glial cells cover the sensory nerves in vein 1 and 3 by mid P2D (32 h APF at 25°C). Abbreviations: APF, after puparium formation; Hrs APF 18°C, hours APF at 18°C; Hrs APF 25°C, hours APF at 25°C.

The dramatic events that occur during P wing development have posed interesting challenges to the curiosity of cell biologists. Some of the questions that have been addressed, so far, include integrin-mediated adhesion between epithelial cell sheets ***(10,11)***, vein cell fate specification by Notch signaling ***(12,13)***, establishment of wing hair planar cell polarity ***(3,14)***, and endocytic trafficking of E-cadherin during junctional remodeling ***(1)***.

The method section will first outline some general comments on staging P development (**Subheading 3.1.**). Then we will describe how prepupal (PP) (**Subheadings 3.2.** and **3.4.**) and P wings (**Subheadings 3.3.** and **3.4.**) are prepared for immunofluorescence microscopy. This protocol has been optimized in our lab to visualize polarized cortical domains of planar cell polarity proteins during PP and P stages ***(1)***. You may have to adapt some details to your specific application. In **Subheading 3.5.**, we will describe how a pupae is prepared to carry out live imaging on the P wing. This protocol has been successfully used to study the highly dynamic development of peripheral glial cells and their interactions with the sensory neurons ***(8)***, but may be used for other applications.

We will not discuss the process of imaging the samples at the microscope in this chapter as microscopy devices differ between labs. We successfully used laser scanning confocal microscopes built by Leica or Zeiss for immunofluorescence and live imaging.

2. Materials

*2.1. Immunofluorescence Microscopy (***see** *Subheadings 3.2.–3.4.)*

1. Fixatives.
   a. Fix 1: 8% paraformaldehyde (PFA) in phosphate-buffered saline (PBS)—prepare fresh or from 20% PFA stock stored at −20°C.
   b. Fix 2: 8% PFA, 200 m*M* sodium cacodylate, 100 m*M* sucrose, 40 m*M* potassium acetate, 10 m*M* EGTA (*see* **Note 1**).
2. PBS.
3. Washing solution PBT: PBT/PBT 0.01% TritonX-100/PBS (*see* **Note 2**).
4. Blocking solution PBTN: PBTN/PBTN 0.01% TritonX-100/PBS + 5% normal goat serum.
5. ProLong gold antifade mounting medium (Invitrogen, Karlsruhe, Germany) (*see* **Note 3**).
6. Microwell minitrays with lids (*see* **Note 4**)—60 wells, low profile, 10 µL well volume (Nalge Nunc, Wiesbaden, Germany).
7. Dumont forceps 55—tip measures: 0.05×0.02 mm^2, Fine Science Tools (*see* **Note 5**).
8. Round Petri dish lids (for dissection).
9. Watchmaker's glass dishes.

*2.2. Live Imaging (***see** *Subheading 3.5.)*

1. Glass bottom dishes—specialized Petri dishes for imaging (*see* **Note 6**).
2. 10S oil (Voltalef, PROLABO, Paris, France): store at room temperature in a dark vial.

3. A pair of spring scissors with small straight blades (8 cm long with 3 mm effective cutting edge, Fine Science Tools, Heidelberg, Germany).
4. Dissection forceps.
5. Tape (*see* **Note 7**).
6. Standard cover slips (*see* **Note 8**).
7. A stereo microscope with at least ×40 magnification.

3. Immunofluorescence Microscopy of PP and P Wings

3.1. Staging and Collecting Pupae

Even at the same temperature, the distinct developmental events outlined in **Fig. 1** can progress at different rates depending on the fly strain and on the specific lab conditions. Therefore, the careful establishment of a staging timeline under your lab conditions according to morphological landmarks (*see* **Fig. 1**) is recommended.

For staging, pick white prepupa (*see* **Note 9**) with a wet brush (*see* **Note 10**) off the wall of the fly culture flask and transfer to the wall of an Eppendorf tube. Note down the time and strain. Recover the pupa or prepupa at the desired stage by picking them off the Eppendorf tube wall with a wet brush.

*3.2. Dissecting and Fixing PP Wings (***see** *Note 11)*

1. Place a prepupa in 50 µL of PBS on the top of a Petri dish lid.
2. Gently make an incision using two forceps in the middle of the prepupa and separate the prepupa in two halves. Displace the posterior half.
3. Take the remaining gut and fat body out of the anterior half. Be careful not to pull on the white tissues (remaining larval epidermis) and the evaginating imaginal discs located in the head region.
4. Using one pair of forceps, pin down the prepupa in between the anterior spiracles. The ventral side of the prepupa needs to face up.
5. Slide the other pair of forceps (closed) along the ventral inside of the P case into the most anterior head region. The prepupa wings lie more dorsally on the lateral sides of the P case. Therefore, if you stay ventrally, you should not damage them when sliding the forceps past them into the most anterior head region.
6. Grab one of the two trachea near their attachment site to the anterior spiracle and slowly pull it back out posteriorly. Repeat with the wing on the other side. Each wing is attached to one tracheal branch and should therefore, ideally, be pulled out with it. By pulling on the trachea you will avoid having to touch the fragile wings directly (*see* **Note 12**).
7. Free the wings of surrounding tissue. Remove most of the debris from the drop and add 50 µL of fixative 1. Gently mix the liquid by sucking and expelling small volumes with the pipet. Leave the wings to fix in this droplet for 20 min (*see* **Note 13**).
8. Pick up the wings in a small drop between the two arms of the forceps and transfer them to a watchmaker's glass dish with a large volume of PBT. Wash them once with PBT using a P1000 pipet.
9. For staining and mounting follow the protocol in **Subheading 3.4.**

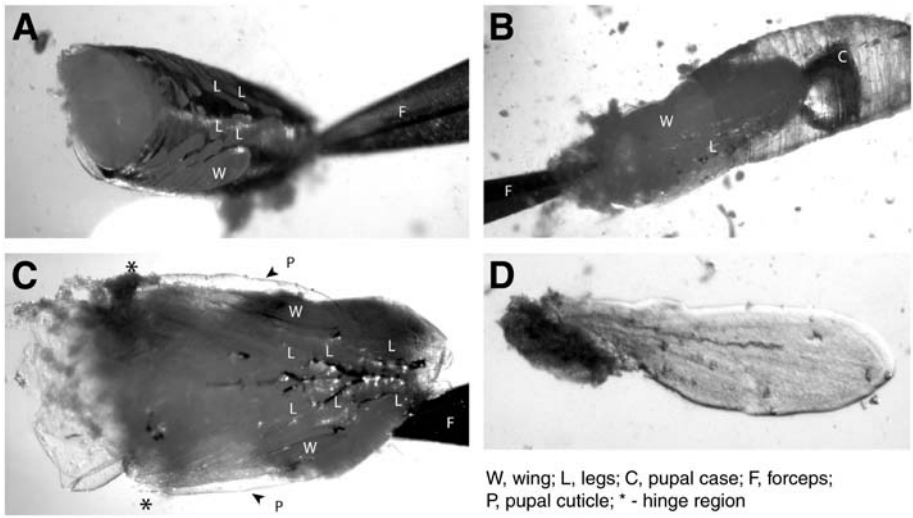

Fig. 2. Dissection for immunofluorescence microscopy. **(A)** The puparium operculum has been removed to expose the pupa's head. The pupa is fixed in position by pinning down the posterior spiracles with forceps. A small incision in the head cuticle has been made. You can see the outlines of wings and legs through the P case. **(B)** The pupa is pulled out of the P case using forceps by tugging on the thoracic P cuticle and fixing the P case at the posterior spiracles (*see* A). Image was taken viewing the lateral side of the pupa; the region where the wing and legs are located is labeled. **(C)** You can see the wing epithelium inside the translucent cuticle sac. Hold pupa in position, dorsal side down, with one pair of forceps. With the other pair of forceps make a little hole in the P cuticle by gently tugging on it near the wing hinge region. **(D)** Dissected wing (stage late P2C) with some excess hinge tissue. In all images anterior is to the left.

3.3. Dissecting and Fixing P Wings (see *Note 14*)

During the dissection it is important to allow for sufficient fixation in between the individual dissection steps. Therefore you can dissect 4 pupae in parallel, carrying out each dissection step on all 4 pupae before going on to the next dissection step. Each dissection step is represented by one numbered bullet point below.

1. Place four small droplets of fixative 2 onto the lid of a Petri dish and place one pupa into each one of them.
2. With one pair of forceps gently grab the posterior spiracles to hold the pupa and pin the forceps down onto the dish in order to fix the position of the pupa within the droplet. Fix the pupa into position like this during the subsequent steps (*see* **Note 15**). Take off the puparium operculum with the other forceps. Peel it off and remove enough P case to expose the head of the pupa. Make a little incision into the P head by gently poking the forceps into the head cuticle (*see* **Fig. 2A**).
3. Increase the size of the incision by sticking the closed forceps into the cuticle hole and gently releasing them to allow the forceps to open a little.

4. Move your forceps into the pupa through the head opening and remove some of the thoracic tissue inside the pupa. Repeat this step twice (*see* **Note 16**).
5. Carefully grab the ventral thoracic cuticle near the head incision and remove the P body from the case by gently tugging on the pupa (*see* **Fig. 2B** and **Note 17**).
6. Transfer the pupa to a fresh droplet of fixative solution.
7. Pin down the pupa at the posterior abdomen with one pair of forceps. With the other make a little hole into the translucent cuticle at the hinge region of the wing to allow the fixative to reach the wing epithelium (*see* **Fig. 2C** and **Note 18**).
8. Again, pin down the pupa at the posterior abdomen. Open the hole in the hinge cuticle a little bit more, so that the wing can fit through. Move your forceps into the hole and grab the hinge of the wing epithelium. Carefully pull the wing out of the cuticle sac (*see* **Fig. 2D**).
9. Free the wing of excess hinge tissue and debris. Pick up the wings in a small drop between the two arms of the forceps and transfer them to a watchmaker's glass dish with a large volume of PBT. Wash them once with PBT using a P1000 pipet.

3.4. Staining PP and P Wings

1. Always track your wings under the microscope. Exchange PBT in the watchmaker's glass dish with PBTN by using a P1000 pipet. Block wings for 20 min at room temperature.
2. Work all following steps with a P20-pipet; working volume is about 10–15 µL. Suck up the wings in a small volume into the yellow tip. Transfer the wings to microwell tray wells (*see* **Note 19**).
3. Make sure that for each of the following steps the wings are submerged in liquid; therefore, always leave a little bit of liquid in the wells when you exchange solutions. Take off the PBTN and then add the primary antibody diluted in PBTN to each well.
4. Incubate overnight on a gentle rocking platform at 4°C.
5. Take off the primary antibody/PBTN and wash with PBT (3X quickly followed by 3X for 10–15 min).
6. Block with PBTN for 20 min at room temperature.
7. Take off the PBTN and add the secondary antibody diluted in PBTN to each well.
8. Incubate for 3 h at room temperature on a gentle rocking platform.
9. Take off the secondary antibody/PBTN and wash with PBT (3X quickly and 3X for 10–15 min).
10. Wash once with PBS.
11. Take off PBS and add a few microliters of mounting medium into the wells. Mix the wings into the mounting medium by gently sucking them up and down in a small volume into the yellow tip (*see* **Note 20**).
12. Suck up the wings in a small volume into the yellow tip and transfer them into 12 µL of mounting medium placed on a microscope slide.
13. Spread out the drop at the edges and make sure that the wings lie flat. Slide an 18×18 mm^2 cover slip on them. Keep the slide at room temperature in the dark for 24 h to allow the mounting medium to solidify.

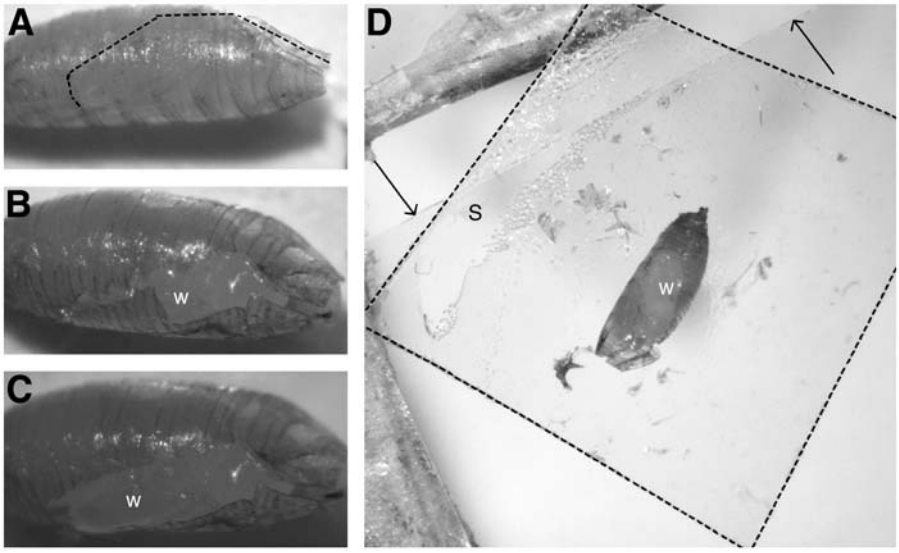

Fig. 3. Dissection for live imaging. **(A)** Once you have cut the puparium operculum, scale the P case surrounding the wing along the dashed line. **(B)** After scaling along one margin of the wing, now cut the P case on the ventral side of the animal close to the legs to release the wing. **(C)** The entire puparium case lying over the wing has been removed including additional puparium case above and below the wing (the equivalent of half a P leg width). **(D)** Tape the animal in a glass bottom dish with the wing facing the cover slip. S and dashed lines denote the region where the tape sticks to the cover slip. Arrows in (d) point to the cover slip edge. Image in (D) has been taken from below the glass bottom dish. In (A–C), anterior is to the right. In (D), anterior is south west. W indicates the P wing.

3.5. Live Imaging of Drosophila *P Wings (see Note 21)*

1. Recover the pupa to be dissected from the Eppendorf tube and place it on a facial tissue to dry it (*see* **Note 22**).
2. Cut a 5 cm long piece of tape.
3. Appose the 5 cm piece of tape onto the dried pupa.
4. From now on put the taped pupa under the objective of the dissection stereomicroscope. Place two fingers on the tape, one on the left and one on the right of the animal, to hold the tape and to reorient the pupa during dissection.
5. At this stage the pupa does not yet strongly adhere to the tape, so you can easily reposition it. Using your forceps, orient the pupa as indicated in **Fig. 3A**—the left lateral side of the pupa should be in contact with the tape. Then gently press with the closed forceps onto the puparium operculum and onto the posterior spiracle to increase the adhesion of the pupa to the tape (*see* **Note 23**).

6. Using a pair of fine scissors cut the puparium operculum just above the P head (*see* **Fig. 3A**). Slide one of the scissor blades in between the puparium case and the pupa, without injuring the pupa. The sharp side of the blade should face dorsally.
7. Use the sharp side of the blade to break and "scale" the P case surrounding the wing (*see* **Fig. 3B**). Do so by repeatedly tilting the blade up without closing the scissors. Try to follow the hollow folds surrounding the wing: start from the dorsal side of the wing hinge region and scale the puparium until you reach the distal tip of the wing. Scale the puparium case in this region as well. Reposition your scissors to the wing hinge region.
8. Place one of the two blades underneath the puparium case (which is now movable). Slide the blade ventrally (toward the legs) and progressively cut the puparium case along the ventrally lying wing margin until you reach the distal tip of the wing.
9. Remove additional P case surrounding the wing to expose the equivalent of half the width of a P leg below and above the wing (*see* **Fig. 3C** and **Note 24**).
10. Cover the exposed P wing with a small amount of 10S oil. To do so, dip a 20 μL yellow tip into a small drop of oil that has been placed in the middle of a cover slip. Remove the excess oil by rolling the yellow pipet tip over the oil-free area of the cover slip. Then, carefully touch the wing with the oily tip (*see* **Notes 25** and **26**).
11. Cut the tape (using normal scissors) at a 1 mm distance from the head and from the posterior spiracle of the animal and at a 3 mm distance from the dorsal and ventral sides of the pupa. Take this rectangle of tape using forceps and tape it in the glass bottom dish so that the wing of the animal faces the cover slip (as shown in **Fig. 3D**).
12. The pupa is now ready to be imaged (*see* **Notes 27** and **28**).

4. Notes

1. Especially to sensitively visualize polarized cortical PCP domains, it is important for the wing tissue to be fixed quickly. This improves the quality of staining and makes the dissection easier. Therefore, 8% PFA and the addition of Cacodylate is strongly recommended; they greatly speed up fixation.
2. In principle, only very low levels of detergent are required for immunolabeling of the P wing epithelium. Some antibodies may require higher levels of detergent. However, low detergent concentrations aid the preservation of membranous structures for imaging, such as small endocytic vesicles.
3. Other solidifying mounting media should work as well. The wings are extremely fragile and get easily destroyed or squished in nonsolid mounts.
4. Staining is carried out in miniwell-trays as they allow for easy tracking of wings under the dissection microscope throughout the protocol. Furthermore, only a minimal volume of diluted antibody solution (10 μL) is required.
5. Tungsten needles or sharp injection needles may be additional helpful tools for dissection.
6. Numerous glass bottom dishes are commercially available but make sure that the glass bottom dish you plan to buy is of appropriate size for your imaging devices. If you cannot find an appropriate glass bottom dish, you can easily make your own glass bottom dish by piercing a hole in the bottom of a plastic dish and sticking a cover slip below using double-sided tape.

7. Use the everyday tape that you find in general stores but take care that the tape you choose sticks well and that it is not difficult to cut with scissors.
8. The cover slips can be of any size but pay attention that they are dust free.
9. White prepupae are easy to recognize: they are immobile like pupae, they exhibit the characteristic shape of pupae but are white like larvae. Be careful to always pick white prepupae at the same stage. Consistency is crucial for good morphological concurrence because dramatic morphogenetic rearrangements may occur within 30 min.
10. Using a wet brush, instead of forceps, prevents damage to the pupae.
11. PP wings within the first hour after puparium formation can be dissected like larval imaginal discs. PP wings between 1 and 7 h after puparium formation (APF) at 25°C are dissected as described in **Subheading 3.2.**
12. If the wings do not remain attached to the trachea and stay inside the P case then you should try to carefully pull out the white tissues (evaginating imaginal discs, including wings, and larval epidermis) from the P case. At PP stage PP3, the PP body has already acquired a compact morphology, where the thoracic imaginal tissues have converged and fused. At this stage, you will be able to pull the entire anterior half of the pupa out of the P case in one step. The wings will lie at the side of the pupae and can be easily cut off from the rest of the pupae body for fixation.
13. The wings become extremely flimsy and fragile between 3 and 7 h APF at 25°C. Take care to never lift the wings out of the dissection droplet. They easily break and tangle up if they are removed from the droplet before fixation. Therefore, the fixative is added to the drop to a final concentration of 4% PFA—this stabilizes the tissue before transferring the wings for subsequent steps. If you feel that there is too much debris in the dissection droplet, then prefix the wings for 3 min (this is enough to stabilize the tissue) and then transfer them to a fresh droplet of fixative for the remainder of the incubation period.
14. In general, P wings from stage P2A–P2D (17–32 h APF at 25°C) can be prepared for immunofluorescence microscopy. PP cuticle ecdysis initiates in late P1. Thus, during P2A you will be able to peel the cuticle off the wing epithelium, thereby granting access of the antibody to the entire wing surface. However, as both wing blades have just started to appose at their basal sides the wing tissue is extremely fragile in P2A and P2B. P2C and P2D wing tissue is much more firm, and thus easier to dissect.
15. Alternatively, you can remove the posterior spiracles and stick one arm of your forceps into the ensuing hole in the P case and then close the forceps around the wall of the case. If you now also pin down the forceps onto the plastic dish at the same time you will be able to fix the pupa into a stable position.
16. Ideally, you should remove the remains of the larval salivary glands and gut (big green blob). Removing some of the thoracic tissue prevents it from being squeezed into the wing sac during subsequent steps. This often destroys the wings.
17. This may not work immediately because often the P cuticle sticks tightly to the P case. If this happens, then just gently tug on the pupa to allow the fixative to gain some access into the space between case and pupae. Give the fixative some time to

work by continuing with the other three pupae. Once you arrive back at the pupa it should be much easier to remove it from the case.

18. Especially in stage P2A the cuticle is still closely apposed to the wing epithelium. Once the fixative reaches the wing epithelium through the small hinge incision it will cause the tissue to dissociate from the cuticle. Therefore, it helps to repeat this step twice; in the second round you should try to increase the size of the hole at the hinge by tugging on the cuticle.
19. To prevent the wings from sticking to the tray plastic you can block the wells for 5 min with PBTN before transferring the wings into them. Collect 3–4 wings per well; too many stick to each other and are more difficult to track during the subsequent steps.
20. Avoid creating bubbles; the wings stick to them, thus it will be more difficult to mount the wings.
21. Wings can be live imaged starting from 13 to 14 h after puparium formation and until adulthood.
22. Wet pupae will not adhere to the tape properly and will be very difficult to dissect. To dry a pupa you can either let it for a couple of minutes on the facial tissue or if you cannot wait, gently roll them on the facial tissue using a dry brush.
23. The pupa now strongly adheres to the tape and you should not try to move the animal anymore or you may kill it.
24. Take much care to remove sharp pieces of the puparium case above and below the wing because they may damage the wing later on.
25. The small quantity of oil you have placed on the wing must not flow inside the puparium case; otherwise, this means that you have used too much oil. If you use too much oil the animal will die.
26. If you have damaged the wing with your scissors at any time during the dissection, or if you have forgotten to remove sharp fragments of cuticle you will see black spots (probably necrotic cells) appear on the wing (<3 h after dissection). You need to improve your dissection skills until you do not see these black spots anymore.
27. After the live imaging, the glass bottom dish can be cleaned with water and reused up to three times. The cover slip becomes more and more opaque after each use.
28. If you want to recover adults from the imaged pupae, place the pupae in a chamber with wet paper after imaging in order to prevent them from drying out. It is also important to cut as much tape as possible around the animal; otherwise, the fly will survive but get stuck on the tape soon after its eclosion.

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17

Microscopic Analysis of the Adult *Drosophila* Retina Using Semithin Plastic Sections

Konstantin Gaengel and Marek Mlodzik

Summary

The regular appearance and the repetitive nature of the *Drosophila* eye, consisting of several hundred identical multicellular units, the ommatidia, has long served as an invaluable experimental system to study cell–cell interactions, inductive signaling events, cell proliferation, programmed cell death, cell differentiation, cell organization, and planar cell polarity among others. Importantly, the eye is dispensable for viability and fertility of the fly and thus, it can easily be manipulated, making it an ideal target for genetic screens. This chapter described an essential technique in the analysis of different genotypes in the adult fly eye, and allows detailed analyses with single cell resolution.

Key Words: *Drosophila melanogaster*; eye; neurons; histology; lens; plastic sections; single cell resolution.

1. Introduction

The following protocol describes a method that allows the microscopic analysis of the adult *Drosophila* retina with a single cell resolution. Electron microscopy (EM) type fixation protocols and semithin plastic sections provide a very high preservation of tissue morphology and thus allow the identification of any of the eigth photoreceptor neurons (R-cells) and the analysis of their organization within the ommatidium. The protocol described here is a variation of and is based on the method described by Tomlinson and Ready ***(1,2)***. The resolution is high enough that with an adequate microscope each photoreceptor-neuron R-cell can be unambiguously identified by position, shape, and size of its respective light-harvesting organelle, the rhabdomere **(Fig. 1)**. Similarly, the pigment cells can also be identified **(Fig. 1)**. The only cell type in the adult retina that cannot be visualized by this technique are the lens secreting cone

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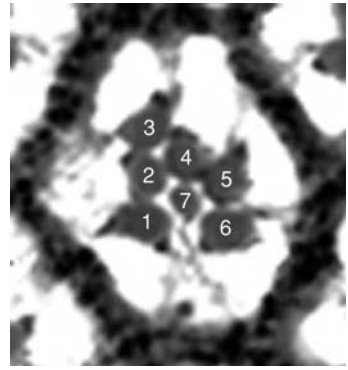

Fig. 1. Microscopic image of a tangential section of a single ommatidium. The optically insulating pigment cells (appearing in dark gray) define the hexagonal shape of the ommatidium. The photoreceptor rhabdomeres R1–R7 (visible as gray round structures numbered in white according to their identity) are arranged in an asymmetric trapezoid in the center of each ommatidium. Note that the rhabdomeres of the outer photoreceptors R1–R6 are larger in diameter than the central rhabdomere of the R7 cell. The rhabdomeres R1–R3 are aligned perpendicular to the equator, with R1 being more equatorial and R3 being more polar at the tip of the trapezoid. R4 lies posterior to R3 and is closer toward the equator. R5 and R6 form the posterior border of the trapezoid. The picture is taken with darkfield optics on a Zeiss Axioplan 2 scope.

cells, as they sit on top of the retina and thus the respective plastic sections only reveal lens material at the edges of a given section. In addition to an analysis on the cellular level, this method allows to depict the alignment and orientation of ommatidia with respect to the different axes of the eye and with respect to one another (e.g., original papers using this approach *see* **refs. *3*,*4***).

The protocol involves a fairly standard fixation of the tissue commonly used for EM-type preparations, but the embedding medium is a softer plastic resin, which is suited for semithin sections and light microscopy analysis. The whole process of preparing, embedding, and sectioning adult *Drosophila* eyes requires a multistep protocol with one overnight incubation before the "baking" of the plastic resin. From beginning (live adult flies) to end (analysis of sections in the microscope) the whole procedure takes 3 d. Eyes have to be dissected, fixed, dehydrated, and embedded, followed by hardening of the plastic resin, and finally the plastic blocks containing the eyes need to be prepared for sectioning (trimmed) and then sectioned.

The regular morphology of the adult *Drosophila* eye lends itself for easy analysis. It is a highly organized structure consisting of about 750–800 hexagonally shaped unit eyes, called ommatidia (**Figs. 1** and **2**). Each ommatidium is made up of 20 cells, including eight photoreceptor neurons (R-cells), four

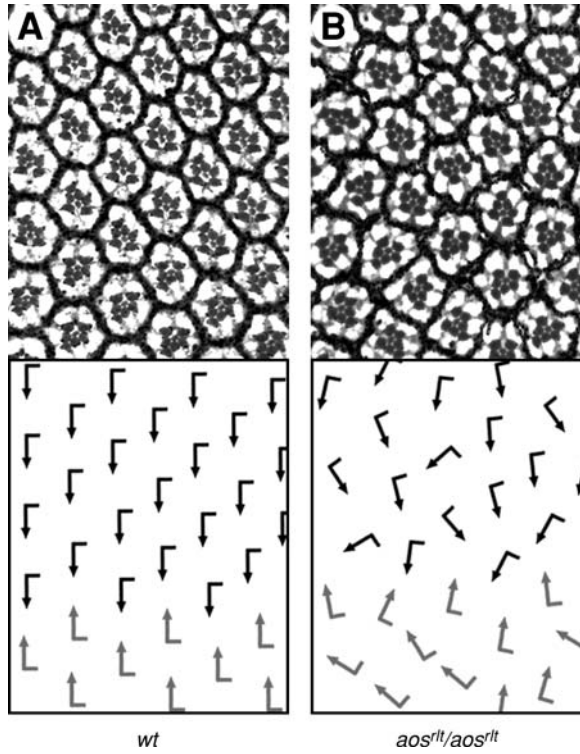

Fig. 2. Tangential sections through equatorial regions of adult Drosophila eyes Opposite chiral forms of ommatidia in the dorsal and ventral halves of the eye create two fields of mirror image symmetry that meet at the equator (the dorso-ventral midline). This is indicated below each section by black arrows (representing dorsal ommatidia) and gray arrows (representing ventral ommatidia), respectively. **(A)** In a wild-type eye the two chiral forms of ommatidia in the dorsal and ventral halfs are arranged 90° with respect to the equator (owing to a precise 90° rotation during development). **(B)** In contrast, flies homozygous mutant for the *aos*^{rlt} mutation, show a broad range of rotation angles. (Anterior is to the left and dorsal is up in all panels.)

lens-secreting cone cells, seven optically insulating pigment cells, and one mechanosensory bristle cell (**Fig. 1**; only 7 of the 8 R-cells are detected in any given plane of section as R7 sits on top of R8). Three different types of pigment cells are present in each ommatidium (red in **Fig. 1**; *see* **ref. *5*** for details). The primary pigment cells form the wall of the pseudocone chamber, which lies underneath the cornea, whereas the secondary and tertiary pigment cells define the hexagonal shape of the ommatidium **(Fig. 1)**. The rhabdomeres, membrane dense extensions of the photoreceptor neurons that contain the light-gathering rhodopsin proteins, are arranged in an asymmetric trapezoid within the center of each

ommatidium **(Fig. 1)**. The rhabdomeres of the outer R1–R6 cells are grouped around the inner rhabdomeres of R7 and R8 and have a noticeably larger diameter than the inner ones. Again, the R7 rhabdomere lies on top of the R8 rhabdomere, therefore only one of these rhabdomeres is visible in a single section (**Figs. 1** and **2**). Ommatidia display a different chirality in the dorsal and ventral halves of the eye and are arranged in two fields with mirror image symmetry around the dorso-ventral midline, called the equator **(Fig. 2)**. The rhabdomeres R1–R3 are aligned perpendicular to the equator, with R1 being most equatorial and R3 being most polar at the tip of the trapezoid (**Figs. 1** and **2**). The R4 rhabdomere lies just posterior to R3 and is noticeably closer toward the equator **(Fig. 1)**. R5 and R6 form the posterior border of the trapezoid with R5 being closer to the pole and R6 being closer to the equator **(Fig. 1)**. For an in depth review on structure and development of the *Drosophila* eye *see* **ref. *5***. These features make the eye an ideal model system to study fundamental biological processes such as cell cycle regulation, cell proliferation, cell fate induction, and cell differentiation, as well as cell death and the establishment of cellular polarity and organization (e.g., reviewed in **ref. *6***).

2. Materials and Solutions

2.1. General Equipment

1. Binocular dissection microscope: a high-end dissection microscope is needed at several steps in the protocol (we are used to the Zeiss Stemi 2000 or Stemi SV11 [http://www.zeiss.com], but other brands provide equivalent microscopes).
2. Light source: again several light sources are likely to work well. However, we recommend using a ring-light source/bulb that evenly illuminates the dissection pad and later also the trimming block. The very even light provided by the ring light minimizes interfering shadows and reflections, and thus is superior to other options. (We are using Schott KL1500 or Schott-Fostec LLC [http://www.us.schott.com], but we trust other suppliers have equivalent light sources.)
3. CO_2 equipped fly station as used for regular fly work and suitable fly pads: a CO_2-chamber that keeps the flies anesthetized. It is usually a plastic chamber with a porous surface on top, on which the flies are placed. It preferentially has a white (or light) surface on top. We are using a model manufactured at the European Molecular Biology Laboratory, but several fly supply companies have similar chambers.

2.2. Dissection

1. CO_2 station as described in **Subheading 2.1.3.** (various suppliers, country dependent).
2. Fly brush: no. 1 Pearl 308 Golden Takalon USA (http://www.pearlpaint.com) or similar.
3. Disposable scalpels: feather no. 11 (http://www.emsdiasum.com) or equivalent, we recommend to use fine scalpels that do not squeeze the eye or head structures owing to a wide blade.

2.3. Fixation and Embedding

For all chemicals we list our routine suppliers, it is likely that you might have different favorite suppliers with equivalent products:

1. 0.5 *M* Na_2HPO_4 (stock solution).
2. 0.5 *M* NaH_2PO_4 (stock solution).
3. 0.2 *M* Na-phosphate buffer pH 7.2. For 500 mL mix 136.8 mL of 0.5 *M* Na_2 HPO_4 with 63.2 mL of 0.5 *M* NaH_2PO_4 and add 300 mL of H_2O.
4. Fix solution: 2% glutaraldehyde solution in phosphate buffer. For 40 mL mix 10 mL of 8% glutaraldehyde solution with 20 mL of 0.2 *M* Na-phosphate buffer pH 7.2 and add 10 mL of H_2O. The fixation solution should be stored at 4°C and should not be used if older than 4 wk.
5. 4% OsO_4: polyscience Inc. (http://www.polysciences.com). To prepare *Osmium solution* (2% OsO_4 in phosphate buffer): Mix equal amounts of 4% OsO_4 (w/v) and 0.2 *M* Na-phosphate buffer pH 7.2; prepare fresh.
6. 8% Glutaraldehyde solution: e.g., Fluka no. 49627 (http://www.fluka.com).
7. Durcupan® ACM resin: Sigma no. D-0166 (http://www.sigmaaldrich.com). The Durcapan resin is based on the formula for Araldite resin and comes as four different components: A (epoxy resin), B (hardener), C (accelerator), and D (plasticizer). To prepare resin for eye embedding mix these components in the ratios indicated below in a plastic beaker using a stir bar at room temperature. Aliquot the resin in 20 mL plastic vials and store at −20°C until the day of use. Approximately 20 min before use thaw the frozen resin in a warm water containing beaker until it is completely liquid and is easily sucked-up into a plastic transfer pipet; do not let resin sit in warm water for too long as it might start to polymerize.

Components	Amount (g)
A	108
B	89
C	5
D	20

8. Conical tubes: 15-mL or 50-mL Falcon tubes (no. 352097 or no. 352098) were used to prepare the *Fix solution* and the *Osmium solution* (http://www.bdbiosciences.com).
9. Ethanol 200 proof (various suppliers: e.g., pharmaco no. 64-17-5).
10. Propylene oxide (various suppliers: e.g., Fisher no. 04332-1 [https://www1.fishesci.com]).
11. 20-mL Plastic vials for resin aliquots (various suppliers: e.g., Fisher no. 033374 [https://www1.fishersci.com]).
12. 1000-mL Plastic beaker (various suppliers: e.g., Nalgene no. 1201-1000 [http://www.nalgenelabware.com]).
13. Magnetic stir-bar.
14. 1.5-mL Microcentrifuge tubes (preferentially with flat lid that allows easy writing on).

15. Microcentrifuge: for example, Beckman Microfuge 18 (http://www.beckman.com) or equivalent.
16. Disposable Plastic transfer pipets (Pasteur pipet like) for resin work: for example, Samco Scientific Corporation no. 225, San Fernando, CA.
17. Pasteur pipets: 9 in. long.
18. Embedding molds: BEEM flat embedding molds, for example, from Electron Microscopy Science no. 70904-12 (http://www.emsdiasum.com).
19. Oven: an oven that can be heated up to 65–70°C for backing the resin.

2.4. Sectioning and Image Acquisition

1. Trimming block: these are equivalent to the stud holders provided by all EM manufacturers, they need to be able to firmly hold the stud that have the plastic block attached to it. Check with your local EM facility for equivalent holders.
2. Razor blades: Single edge Teflon® coated (e.g., Electron Microscopy Science no. 71970 [http://www.emsdiasum.com]). It is important that they are Teflon coated as other even more expensive blades that are not coated will leave scratches on the plastic resin during trimming and obscure the view of the eye. A good "look" at the embedded eye is essential to allow for adjustments of the trimming angle.
3. Microtome: any microtome that allows semithin sections (0.5–2 μm). We have used different microtomes at different institutions. Please check with your local EM facility or a neighboring laboratory with histology capabilities.
4. Diamond knife: "histo"-quality knife (EM quality is not needed). We have always used knives from Diatome. size 6, knife angle: 45° (http://www.emsdiasum.com/diatome).
5. Microscope slides: $25 \times 75 \times 1$ mm^3 (various suppliers: e.g., Corning Laboratory [http://www.corning.com/Lifesciences/us-canada/en]).
6. Cover slip glass: 24×60 mm^2 (various suppliers: e.g., Corning Laboratory [http://www.corning.com/Lifesciences/us-canada/en]).
7. Staining solution: dissolve 1% (w/v) toluidine blue and 1% (w/v) Borax in H_2O.
8. DPX mounting medium: Fluka no. 44581 (http://www.fluka.com or http://www. sigmaaldrich.com).
9. Microscope: Zeiss Axioplan2 or equivalent (http://www.zeiss.com).
10. Microscope lenses: 63×1.40 is essential and allows to capture almost an entire section of the eye (usually around 200 ommatidia), a ×100 is also very useful for detailed analysis of fewer ommatidia.
11. Microscope lens-oil: for example, Zeiss Immersol 518F (http://www.zeiss.com) or any equivalent oil supplied by your Microscope provider.
12. Image acquisition: ideally you will use a high-resolution digital color camera from your preferred source; we are using a Zeiss AxioCam with supplied acquisition software, and Adobe® Photoshop® for further processing (http://www.adobe.com/products/photoshop/main.html).
13. Computer: Apple Macintosh or PCs (whatever you prefer) that are capable of handling the camera acquisition software and Adobe Photoshop.

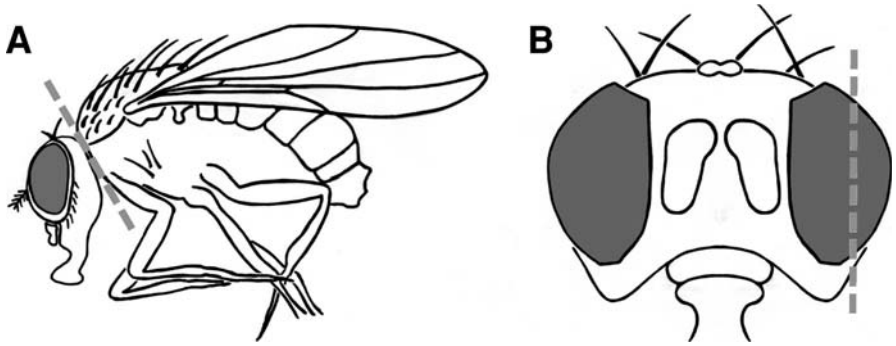

Fig. 3. Decapitating adult flies and preparing the heads for fixation: **(A)** Decapitate the flies with a fine scalpel (at the position indicated by the gray dashed line). Do not apply excessive pressure, which could potentially damage the eye you want to section later. **(B)** Next turn the heads on their base and carefully cut away about 1/2 of one eye (indicated by the gray dashed line) to allow the fix to penetrate the whole head structure and the remaining eye.

14. In addition, you will need a set of standard laboratory tools for pipeting, and a heating plate (to help evaporate excess liquid; *see* **Subheading 3.2.5.**), syringes with filters, ethanol proof marking pen, and so on. We trust these things are obvious and standard and thus not specifically listed.

3. Methods

These instructions assume that you are equipped for general fly handling, for example, source of CO_2 and associated requirements.

3.1. Embedding Procedure

1. Anaesthetize flies with CO_2 and decapitate them as outlined in **Fig. 3A**. One eye should be cut away to allow penetration of the *Fix solution* into the head structure **(Fig. 3B)**. It is suggested that for each genotype of interest you process at least 6 eyes and thus you should have 6 or more flies of the respective genotype ready on the "decapitation" fly pad, when you start the protocol.
2. Transfer the one-eyed heads (as outlined in **Fig. 3**) to a microcentrifuge tube (collect all heads of one genotype in one tube) containing 200 µL of *Fix solution* that is placed on ice, and incubate the eyes for 10–30 min. The eyes that were dissected first will be staying in this solution longer and so you can collect all eyes of the same genotype in this solution. To ensure that the "one eyed heads" sink into the solution, you can briefly centrifuge the microfuge tubes at the end of the dissection for 1 min or less at maximum speed in a tabletop microfuge.
3. Directly following centrifugation, add 200 µL of the *Osmium solution* to each sample and mix solutions carefully. Incubate for 30–60 min on ice in the fume hood.

Note that all handling and incubations involving Osmium have to be performed in a fume hood and on ice as Osmium is toxic and volatile (*see* **Note 1**).

4. Exchange the *Fix solution/Osmium solution* mix for *Osmium solution* only (200 µL), and incubate the samples for 1 h (up to 6 h is also fine) on ice. This fixation procedure is similar to protocols commonly used for EM samples. As the light-harvesting rhabdomeres of the photoreceptors are very membrane rich, this strong membrane fixation procedure is necessary.
5. Following the fixation remove the liquid (keep samples on ice in the same microfuge tubes) and start to dehydrate the eyes. This is best done in 10 min steps (again on ice). Use an ethanol series of the following concentrations: 30, 50, 70, 90, and 100%, respectively. Follow this with an additional step of fresh 100% ethanol at room temperature. It is recommended to keep samples in the fume hood all the time. We recommend to wash with a fairly large volume (~1 mL) and to leave the eyes always covered with a tiny amount of ethanol from the previous washing step when changing the solution. This prevents the eyes from getting exposed to oxygen and from drying out which can cause the eyes to collapse.
6. Follow the dehydration series with two 10 min washes in propylene oxide at room temperature to prepare the eyes for the resin.
7. Remove the propylene oxide and replace it with a 1:1 mix of propylene oxide and Durcupan resin. Note that this step needs to be done fairly quickly to avoid the eyes drying out completely; propylene oxide is highly volatile and so it will evaporate quickly; do not let the microfuge tubes stand around for longer periods after you have removed the propylene oxide and before you added the 1:1 mix. Incubate the samples in the 1:1 propylene oxide/Durcupan resin mix overnight at room temperature. Be sure to close the microcentrifuge tubes tightly to prevent the propylene oxide to evaporate and the resin to polymerize.
8. The next morning, replace the 1:1 propylene oxide/Durcupan resin mix with fresh pure resin and incubate the samples for about 2–4 h at room temperature. In these two steps the resin infiltrates the tissue samples and thus preserves the morphology of the retina. In the meantime pour resin into the embedding molds and incubate them at room temperature until the resin has reached a partially polymerized consistency (usually 2–4 h, *see* **Note 2**). Once Resin starts to thicken, transfer the eyes with a toothpick into molds. Carefully push them to the bottom of the mold and orient them as outlined in **Fig. 4A**. It is critical that the eye you will be sectioning faces toward the narrow end of the coffin shaped mold. Also do not place them too far away from the edge, as it will only increase the amount of plastic you will have to trim away later (*see* **Fig. 4B** for cartoon).
9. Bake the embedded eyes in the molds overnight at 70°C. The plastic will be completely polymerized the next morning and it is now safe to handle it with bare hands after that (like any household plastic ware).

3.2. Preparing Eye Sections and Microscopical Analysis

This part of the protocol describes how to prepare and analyze the plastic sections of the eyes. The thickness of the sections is not critical. Usually 0.5 µm

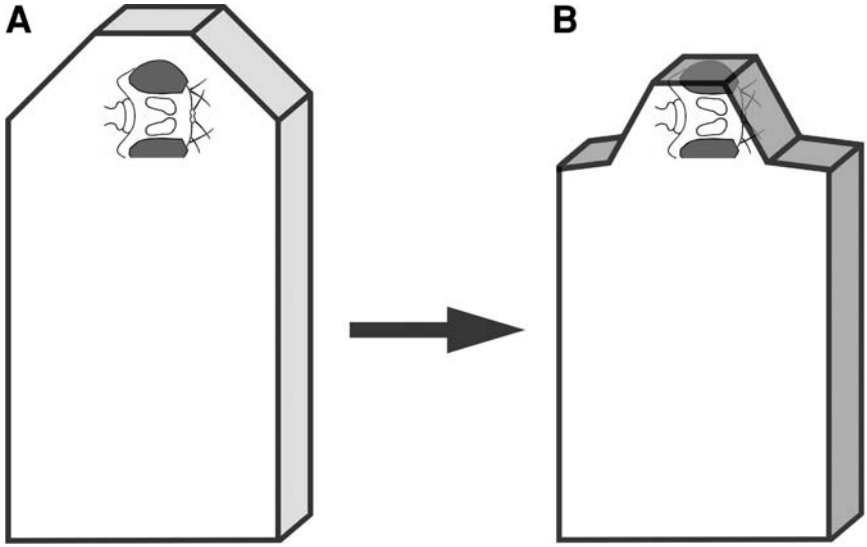

Fig. 4. Embedding and trimming: **(A)** Embed each eye separately in a suitable embedding mold. Carefully push the head to the bottom so that the base of the head is touching the bottom of the mold. Orient the eye you want to section so that it faces the narrow end of the coffin shaped mold and place it fairly close to the edge to decrease the amount of plastic you will have to trim away later. **(B)** After baking the plastic will have solidified, and you will have to trim the plastic block to the shape indicated. Using a Teflon-coated razor blade slice off a thin layer from the top first. This will leave a very smooth surface so that the eye can easily be seen through the plastic. Now start trimming of excess plastic laterally and from top in a series of thin slices. Do not cut too much at once. Trim away all plastic that does not contain eye material laterally and try to touch the surface of the lens (or even cut a bit into it) when taking the final slices of the top before taking it to the microtome.

sections have the best morphological appearance when analyzed in the microscope; however, sections of 1–2 μm are also fine (*see* **Note 3**).

1. Take the plastic blocks containing the eyes out of the mold. Usually the molds are a bit flexible so by bending them you can release the blocks and pop them out. Place a block in a microtome chuck (tighten it well, the eye should not move between trimming and sectioning) and attach the chuck to a chuck holder (*see* **Note 4**).
2. Place the holder-block unit under a dissection microscope, with preferentially a ring light source, and take the Teflon-coated razor blade. Slice off a thin layer from the top of the narrow end of the coffin-shaped plastic block (which should be oriented to face you). This will leave a very smooth surface that will allow you to see the eye clearly through the plastic. Now start trimming of excess plastic laterally and from top in a series of thin slices (*see* cartoon in **Fig. 4**). Do not cut away too much at

once; approach the eye from all directions with thin slices. Trim as close to the eye as you are comfortable with laterally and try to touch the surface of the lens (or even cut a bit into it) when taking the final slices of the top. You want to remove all plastic that does not contain eye material before taking it to the microtome (*see* **Note 5**).

3. When the "eye block" is ready, release the chuck with the plastic block from the holder and place it in the chuck holder on the microtome. Make sure that the eye has been well tightened early (*see* **step 1** of **Subheading 3.2.**), it should stay fixed once you started trimming (to facilitate the sectioning at the right angle).
4. Set the microtome to 0.5–1 μm thick sections and take tangential sections of the eyes with a Histo-quality diamond knife. The knife-holder unit is shaped like a little pool that needs to be filled with filtered water. Start sectioning.
5. As the sections come off the knife they will float on the water surface. Take a series of sections, about 20. Pick up the sections with either a small loop, or a flattened wooden stick (*see* **Note 6**) and transfer them on to a drop of filtered water on a subbed slide (*see* **Note 7**). Place the slide with the water drop and the sections on a heating plate to let the water evaporate. The section will then nicely settle on to the slide. You should plan to place multiple water drops sequentially on the slide side by side, which will allow you to place 6–8 water drops on each slide and thus you can place sections of more than one eye on a single slide (this is helpful in subsequent microscopic analyses and for easy comparisons).
6. Stain the section with Toluidine-blue to increase the contrast (optional). For this add the *staining solution* (*see* **Subheading 2.**) on each slide and heat the slides for 10–20 s on a heating plate that has been set to approx 80°C, rinse the slides well with running tap water and let them dry. Cover the sections with DPX-mounting medium (~45 μL) and a cover slip. Add small coins as a weight on top of the cover slip to achieve an evenly thin distribution of the mounting medium. After solidification of the DPX-mounting medium (usually about 30–60 min at room temperature) the eye sections are ready for analysis.
7. Take the slides with the section to a good light microscope (e.g., a Zeiss Axioplan2 microscope or equivalent) and first examine them at low magnification (×5 objective) using brightfield optics to see where the sections are located (as you placed them in discrete drops they will be clustered in groups rather then spread all over the slide). Add a drop of the immersion oil and analyze the section with a ×63 (or a ×100) objective using phase contrast or darkfield optics, or for well-stained sections regular brightfield optics will work as well (*see* **Fig. 2** for two examples of how the sections could look). Take images with your digital camera/software of choice and process them with your analysis software as appropriate (e.g., with Adobe® Photoshop®) (*see* **Note 8**).

4. Notes

1. Important safety note: OsO_4, propylene oxide and unpolymerized Durcupan resin should always be handled with extreme care. Vinyl gloves should be worn at all times when handling one of theses substances, for example, OsO_4 is a neurotoxin and Durcupan resin is carcinogenic when unpolymerized. All steps involving OsO_4

or propylene oxide must be carried out in a well-ventilated chemical fume hood. All items that become contaminated with OsO_4 should be sealed in an appropriate waste container according to institutional safety office guidelines. All items containing unpolymerized resin should be backed at 70°C overnight before being discarded.
2. It is recommended to test the polymerization stage of the resin after 2 h and beyond with a toothpick.
3. This is mentioned as not every microtome of the lower quality takes regular section of 0.5 μm and so do not worry if the section's thickness varies a bit.
4. Depending on the EM facilities at your institution, you will have probably several options of chucks and holders; all the once that we have tried at different institutions worked fine.
5. It will save you much time and also prevent that plastic surrounding the eye will later cover neighboring sections when they are transferred to microscope slides.
6. A wooden household barbecue stick or a better tooth pick that has been flattened at one end is ideal.
7. Having subbed slides (e.g., gelatin coated) helps to create a nice water-bubble through the increased water tension; and also the sections stick very well to a subbed slide once the water has evaporated.
8. Note that depending on the optical set up, the pigment granules in the pigment cells will look red or golden or greenish. Depending on the level of Toluidine blue staining and personal preferences either filter and optical setting of the microscope works fine.

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18

Fluorescent *In Situ* Hybridization Protocols in *Drosophila* Embryos and Tissues

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Summary

Fluorescent *in situ* hybridization is the standard method for visualizing the spatial distribution of RNA. Although traditional histochemical RNA detection methods suffered from limitations in resolution or sensitivity, the recent development of peroxidase-mediated tyramide signal amplification provides strikingly enhanced sensitivity and subcellular resolution. In this chapter, we describe optimized fluorescent *in situ* hybridization protocols for *Drosophila* embryos and tissues utilizing tyramide signal amplification, either for single genes or in a high-throughput format, which greatly increases the sensitivity, consistency, economy, and throughput of the procedure. We also describe variations of the method for RNA–RNA and RNA–protein codetection.

Key Words: *Drosophila melanogaster;* embryos and tissues; FISH; fluorescent *in situ* hybridization; RNA–protein costaining; single and double labeling; tyramide signal amplification.

1. Introduction

In situ hybridization in fixed tissues is the main method used for analyzing the spatial distribution of RNA, enabling the visualization of broad gene expression patterns, as well as subcellular localization properties ***(1)***. The method involves the recognition of the target RNA *in situ* through hybridization of a labeled antisense RNA probe. The most common detection strategy has been the use of digoxigenin (DIG)-labeled probes recognized by antibodies coupled to enzymes such as alkaline phosphatase (AP), which react with chromogenic substrates in order to reveal the distribution of the target RNA ***(2)***. Although this approach utilizes enzymatic amplification to increase staining sensitivity, the diffusibility of AP-generated dyes limits the resolution of the technique. Alternatively, the use of fluorescent *in situ* hybridization (FISH) presents several advantages, including the capacity to obtain clear views through thick samples, to reconstruct

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three-dimensional images using high-resolution microscopy techniques, and the ability to compare multiple overlapping signals with high resolution. Conventional FISH uses fluorochrome-conjugated probe labels or antibodies that provide nondiffusible signals ***(3,4)***, but are less sensitive as they lack an enzymatic signal amplification step. However, the use of tyramide signal amplification (TSA), involving the peroxidase-dependent complexing of fluorochrome-conjugated tyramides to molecules in the vicinity of the probe, provides a strong enzymatically amplified signal and strikingly improved subcellular resolution ***(5–7)***.

This chapter describes our optimized procedures for performing high-resolution FISH on *Drosophila* embryos and dissected tissues, either for a few genes or in a high-throughput format in 96-well microtiter plates. Instructions are given for the preparation of RNA probes, the fixation of embryos and tissues, and the hybridization and TSA-mediated detection of probes. Also described are variations of the procedure for RNA–RNA and RNA–protein costaining. These protocols have been optimized for economy, high-throughput, consistency, and sensitivity.

2. Materials

2.1. RNA Probe Preparation (see Note 1)

1. 1.5-mL Microcentrifuge tubes (Ultident, Saint-Laurent, QC, Canada; cat. no. 24-MCT-150-CS) or standard 96-well V-bottom microplates (Abgene, Rochester, NY; cat no. AB-1058).
2. RNAse-free water (Invitrogen Corp., Burlington, ON, Canada; cat. no. 10977-015).
3. T7, T3, or SP6 RNA polymerase (Fermentas Life Sciences, Burlington, ON, Canada; cat. nos. EP0101, EP0111, and EP0131) as appropriate.
4. 10X Transcription buffer (supplied with polymerases): 0.4 *M* Tris-HCl, pH 8.0, 60 m*M* $MgCl_2$, 100 m*M* dithiothreitol, and 20 m*M* spermidine.
5. DIG RNA-labeling Mix (Roche Applied Science, Laval, QC, Canada; cat. no. 11 277 073 910). Recommended for single FISH.
6. Biotin RNA-labeling mix (Roche Applied Science; cat. no. 11 685 597 910).
7. RNAguard (Amersham Biosciences, Piscataway, NJ; cat. no. 27-0816-01).
8. 3 *M* Sodium acetate.
9. Cold 100% ethanol.
10. Cold 70% ethanol.

2.2. Embryo Collection and Fixation

1. Chlorine bleach solution diluted 1:1 with water.
2. 20-mL Glass scintillation vials (Fisher Scientific Limited, Nepean, ON, Canada; cat. no. 03-337-15) or 1-L glass bottle.
3. 40% Formaldehyde solution (prepared on the day of fixing from paraformaldehyde): In scintillation vial, mix 0.92 g of paraformaldehyde in 2.5 mL of water containing 35 µL of 1 *N* KOH. Dissolve the paraformaldehyde by carefully heating the

solution on a stirring hot plate in a fume hood. Once the solution cools down, filter through a 0.45-µm filter and store at 4°C until ready for use. Scale up the recipe if a larger volume is required (*see* **Note 2**).
4. 1X Phosphate-buffered saline (PBS) solution.
5. Heptane.
6. Methanol.

2.3. Single FISH on Drosophila Embryos

2.3.1. Postfixation, Hybridization, and Posthybridization Washes

1. 5-mL Polypropylene tubes (Ultident; cat. no. 17-T415-2A), 1.5-mL and 0.5-mL microcentrifuge tubes, or 0.2-mL half-skirted 96-well polymerase chain reaction (PCR) plates (Abgene, Rochester, NY; cat. no. AB-0900).
2. Microplate sealing foil (Ultident; cat. no. 24-PCR-AS-200).
3. PBS-Tween (PBT) solution: 1X PBS and 0.1% Tween-20.
4. 40% Formaldehyde solution, freshly prepared (*see* **Subheading 2.2.**).
5. Proteinase K (20 mg/mL) (Sigma Aldrich, Oakville, ON, Canada; cat. no. P2308). Dissolve in double-distilled water and store aliquots (25–50 µL) at –20°C.
6. Glycine solution: 2 mg/mL glycine in PBT.
7. RNA hybridization solution: 50% formamide, 5X SSC, 100 µg/mL heparin, 100 µg/mL sonicated salmon sperm DNA, and 0.1% Tween-20. Filter through a 0.2-µm filter and store at –20°C (stable for several months).
8. Heating block(s) or water bath(s) adjustable to 56, 80, and 100°C, or PCR machine.

2.3.2. Development of FISH Signal

1. 1X PBS solution.
2. PBT solution: 1X PBS, 0.1% Tween-20.
3. PBS-tween-blocking agent (PBTB) solution: 1X PBS, 0.1% Tween-20, and 1% milk powder.
4. Detection of DIG-labeled probe.
   a. Biotinylated anti-DIG antibody followed by streptavidin-HRP (Molecular Probes, Eugene OR), recommended to obtain strongest signal: biotin-conjugated mouse monoclonal anti-DIG (1/400 dilution of a 1 mg/mL stock solution in PBTB; Jackson ImmunoResearch Laboratories Inc., West Grove, PA; cat. no. 200-062-156) *and* streptavidin-HRP conjugate (1/100 dilution of a 1 µg/mL stock solution in PBTB; Molecular Probes; cat. no. S991).
   b. HRP-conjugated anti-DIG antibodies, suitable for strongly expressed genes or for double-labeling experiments: HRP-conjugated mouse monoclonal anti-DIG (1/400 dilution of a 1 mg/mL stock solution in PBTB; Jackson ImmunoResearch Laboratories Inc.; cat. no. 200-032-156) *or* HRP-conjugated sheep monoclonal anti-DIG (1/500 dilution of stock solution in PBTB; Roche Applied Science; cat. no. 1 207 733).
5. TSA: Cy3 tyramide conjugates (1/50 dilution of stock solution in amplification buffer; Perkin Elmer Life Sciences, Boston, MA; cat. no. SAT704A) *or* Alexa Fluor 488 tyramide conjugate (1/50 dilution of stock solution in amplification buffer;

Molecular Probes; cat. no. T-20932). *See* **Note 3** for advice on when to use the reagents described in **steps 4** and **5**.

6. 100X 4′,6-diamidino-2-phenylindole (DAPI) solution (0.1 mg/mL in H_2O).

2.3.3. Storage, Mounting, and Viewing of Samples

1. Mountant: 70% glycerol, 2.5% 1,4-diazabicyclo [2.2.2.] Octane (DABCO, Sigma Aldrich; cat. no. D-2522). In light-shielded tube, add 1.25 g of DABCO crystals, 15 mL of 1X PBS, and 35 mL of glycerol and mix on rocking platform until the solution is homogeneous. Store at –20°C.
2. Microscope slides.
3. Cover slips (22×22 mm^2).
4. Fluorescence or confocal microscope.

2.4. Double FISH on Drosophila Embryos

1. Reagents for postfixation of embryos, probe hybridization, and mounting of samples as described in **Subheadings 2.3.1.** and **2.3.3.**
2. 1X PBS solution.
3. PBT solution: 1X PBS and 0.1% Tween-20.
4. PBTB solution: 1X PBS, 0.1% Tween-20, and 1% milk powder.
5. Quenching solution: 1X PBT and 1% H_2O_2.
6. Detection of DIG-labeled probe with HRP-conjugated antibodies: HRP-conjugated mouse monoclonal anti-DIG (1/400 dilution of a 1 mg/mL stock solution in PBTB; Jackson ImmunoResearch Laboratories Inc.; cat. no. 200-032-156) *or* HRP-conjugated sheep monoclonal anti-DIG (1/500 dilution of stock solution in PBTB; Roche Applied Science; cat. no. 1 207 733).
7. Detection of biotin-labeled probe: streptavidin-HRP conjugate (1/100 dilution of a 1 µg/mL stock solution in PBTB; Molecular Probes; cat. no. S991).
8. TSA: Cy3 tyramide conjugate (1/50 dilution of stock solution in amplification buffer; Perkin Elmer Life Sciences; cat. no. SAT704A). Alexa Fluor 488 tyramide conjugate (1/50 dilution of stock solution in amplification buffer; Molecular Probes; cat. no. T-20932). *See* **Note 3** for advice on when to use the reagents described in **steps 6–8**.

2.5. RNA–Protein Double Labeling

1. Reagents for postfixation of embryos, probe hybridization, detection of probes, and mounting of samples as described in **Subheadings 2.3.1.–2.3.3.**
2. Primary antibody directed against the protein of interest. To prevent antibody cross-detection, make sure that the species origin of this antibody differs from that of the anti-DIG antibody used to detect the FISH probe.
3. Select a fluorochrome-conjugated secondary antibody directed against the species of the primary antibody.

2.6. FISH on Dissected Tissues

1. 1.5-mL Microcentrifuge tubes.
2. 1X PBS solution.

3. 40% Formaldehyde solution, freshly prepared (*see* **Subheading 2.2.**).
4. PBT solution: 1X PBS and 0.1% Tween-20.
5. Fixation solution: 1X PBS and 4% formaldehyde.
6. For single or double FISH, prepare reagents for probe hybridization and detection as described in **Subheadings 2.3.1.**, **2.3.2.**, and/or **2.4.**

3. Methods

3.1. RNA Probe Preparation

1. Different strategies can be used to prepare template DNA for synthesizing antisense RNA probes by in vitro transcription. A gene segment of interest should first be cloned into an appropriate plasmid containing flanking bacteriophage promoter sequences (*T3*, *T7*, or *Sp6*). Then, the plasmid can either be linearized by restriction enzyme digestion or used as a template for PCR to generate an amplified gene fragment with promoter sequences on each extremity. The PCR based approach is particularly useful when templates for several genes are being prepared simultaneously, as most sequences can be amplified using universal primers that overlap the *T7*, *Sp6*, and/or *T3* sequences. Once the linearized DNA fragments or PCR products have been purified, either through traditional phenol/chloroform extraction combined with ethanol precipitation or agarose gel extraction, they can be used for in vitro transcription as detailed in **step 2**. Care should be taken to work in RNAse-free conditions. For most *Drosophila* genes, cDNA sequences cloned between flanking promoters are readily available in the *Drosophila* Gene Collections, and PCR protocols for the vectors used in these libraries have been described ***(8)***. For templates that are amplified in a 96-well plate format, the PCR products can be bulk purified by centrifugation using filter plates (Whatman Inc.; Clifton, NJ; cat. no. 7700-1303), concentrated by ethanol precipitation and centrifugation in V-bottom 96-well plates, and then resuspended in 15 µL of RNAse-free water.
2. RNA probes are prepared as described on the product sheets of their DIG and biotin RNA-labeling kits (Roche Applied Science). On ice, mix 0.5–1 µg linearized template DNA or PCR product, 2 µL DIG or biotin RNA-labeling mix, 2 µL 10X transcription buffer, 1 µL RNAguard (40 U/µL), 2 µL RNA polymerase (20 U/µL), and RNAse-free water to a final volume of 20 µL. Incubate at 37°C for 2–4 h.

   For PCR templates amplified and purified in 96-well format, probes can be bulk synthesized in V-bottom microplates in a total reaction volume of 10 µL. In each well, 5 µL of resuspended template is combined with 5 µL of premixed and prealiquoted transcription reaction mixture containing: 1 µL 10X transcription buffer, 0.5 µL DIG-labeling mix, 0.4 µL RNA polymerase (20 U/µL), 0.125 µL RNAguard (40 U/µL), and 3 µL RNAse-free water. Plates are then sealed with adhesive foil and incubated for 2–4 h at 37°C.
3. Once probe synthesis is completed, RNAse-free water is added to the reactions to bring the total volume up to 50 µL, then the probes are precipitated by adding 0.1 volumes 3 *M* sodium acetate and 2.5 volumes of cold 100% ethanol (*see* **Note 4**). Place at –70°C overnight, then spin and wash the pellets with cold 70% ethanol. After drying, resuspend the probe pellets in 50 µL RNAse-free water. Analyze and

quantify the run-off transcripts through conventional agarose gel electrophoresis and ethidium bromide staining. Store probes at –70°C.

3.2. Embryo Collection and Fixation

The following steps can be performed on a small or large scale depending on the size of the fly chambers used for embryo collection.

1. Prepare 40% formaldehyde stock solution just before embryo dechorionation.
2. Collect and rinse embryos using room temperature tap water and a collection sieve.
3. Dechorionate the collected embryos in a chlorine bleach solution for approx 90 s. As dechorionation proceeds, the embryos become clumpy and may tend to stick to the sides of the collection basket. Rinse the embryos immediately and thoroughly with fast flowing room temperature tap water or with embryo rinse solution (0.7% NaCl and 0.03% Triton X-100) to remove residual bleach.
4. For small collections (<250 µL settled embryos), transfer the embryos to a 20 mL glass scintillation vial containing a biphasic mixture of 8 mL heptane, 2 mL PBS, and 200 µL 40% formaldehyde. For large collections (>5 mL of settled embryos), transfer embryos to a 1-L bottle containing 300 mL heptane, 90 mL PBS, and 10 mL 40% formaldehyde. Shake for 20 min.
5. Using a Pasteur or serological pipet, eliminate the lower aqueous phase and most of the upper heptane phase, taking care not to draw up the embryos found at the interface. For small collections, transfer the embryos to a 1.5-mL microfuge tube containing 0.5 mL heptane and 0.5 mL methanol. For large collections, transfer embryos to a 500-mL bottle containing 100 mL heptane and 100 mL methanol. Devitellinize the embryos by shaking vigorously for 45 s until most of the embryos sink to the bottom. Carefully remove most of the heptane and add 1 or 100 mL of methanol, for small or large collections, respectively. Shake once more. All or most of the embryos should now sink to the bottom of the tube. Remove all of the liquid along with any unsettled embryos and rinse three times with methanol. Embryos can be pooled in polypropylene tubes and stored in methanol at –20°C for several months.

3.3. Single FISH on Drosophila Embryos

3.3.1. Postfixation, Hybridization, and Posthybridization Washes

Hybridizations can be performed in 1.5/0.5-mL microfuge tubes (50 µL settled embryos) or 0.2-mL PCR plates (10 µL settled embryos/well). The latter are particularly well suited for optimizing experimental conditions (i.e., antibody titrations) or when many samples are processed in one experiment. Using the recommended PCR plates, which can easily be cut into smaller sections when processing a few dozen samples, greatly facilitates sample manipulation and long-term storage. Make sure to seal plates appropriately with sealing foil for all incubations and washes (*see* **Note 5**). Unless otherwise indicated, the wash volumes used below are 1 mL *or* 150 µL for microfuge or PCR tubes,

respectively. If not, the appropriate volumes for each tube format are provided, separated by *or*.

1. Aliquot embrÏyos in tubes or plates (*see* **Note 6**).
2. Rinse the embryos once in methanol, once in a 1:1 mixture of methanol:PBT, and two times in PBT.
3. Postfix the embryos for 20 min in 4% formaldehyde (prepared by diluting fresh 40% formaldehyde 1/10 in PBT). Place tubes on a rocking platform or rotating mixer to ensure even fixation. If using PCR plates, secure plate in a vertical position to achieve more efficient mixing.
4. Wash embryos three times in PBT for 2 min each.
5. Prepare a working 3 µg/mL proteinase K solution from a 20 mg/mL stock by diluting in PBT. Add 500 *or* 100 µL of proteinase K solution to each embryo sample and incubate at room temperature for 13 min, or adjust the time according to the type of tissue (*see* **Note 7**). During this period, mix five to six times by gently rotating the tube once or twice or by jetting with a pipetman (Gilson, Inc., Middleton, WI). Transfer the embryos to ice and incubate for 1 h. This prolonged incubation on ice ensures uniform penetration and action of the protease.
6. Remove proteinase K solution and stop the digestion by performing a 2 min wash with a 2 mg/mL glycine solution with rocking. Repeat the glycine wash a second time.
7. Rinse embryos two times in PBT to remove the glycine.
8. Postfix the embryos again (as in **step 3**) for 20 min in 4% formaldehyde.
9. Wash embryos five times in PBT for 2 min each to remove all traces of fixative.
10. Rinse the embryos in a 1:1 mixture of PBT:RNA hybridization solution. Replace the mixture with 100% hybridization solution. At this point, the embryos can be stored for days/weeks at –20°C. If embryos were processed as a large batch (*see* **Note 5**), distribute embryos evenly into PCR plates using wide aperture tips, aiming for a final volume of 10 µL settled embryos/well. If 1.5-mL tubes were used up to this point, transfer embryos to 0.5-mL tubes. When ready to hybridize, proceed to **step 11**.
11. In a separate tube, boil 400 *or* 100 µL/sample of RNA hybridization solution at 100°C for 5 min, for 0.5- or 0.2-mL tubes, respectively. Cool on ice for at least 5 min. This freshly boiled hybridization solution will be used as the prehybridization solution.
12. Remove hybridization buffer from embryos. Add cooled prehybridization solution and place the embryos in a 56°C heat block/water bath. Incubate at 56°C for a minimum of 2 h.
13. Prepare probe solution by adding 50–100 ng of probe in 100 µL of hybridization solution, heat at 80°C for 3 min, and cool on ice for at least 5 min. The probe solution can be kept on ice until prehybridization is completed.
14. Remove the prehybridization solution and add the probe solution to the embryos. Incubate at 56°C for 12–16 h. This step is generally performed overnight.
15. Preheat all wash solutions to 56°C. Remove the probe solution and rinse the embryos once with 400 *or* 100 µL prewarmed hybridization buffer. Replace the rinse solution with another 400 *or* 100 µL prewarmed hybridization buffer and incubate at 56°C for 15 min.

16. Wash for 15 min each with 400 *or* 100 µL of 3:1, 1:1, and 1:3 mixtures of hybridization buffer:PBT.
17. Wash four times for 5 min each, with 400 or 100 µL prewarmed PBT, then cool embryos to room temperature.

3.3.2. Development of FISH Signal

Unless otherwise indicated, the wash volumes used below are 400 *or* 150 µL for 0.5-mL tubes or 0.2-mL PCR strips/plates, respectively. Antibody incubations and washes are performed in PBTB in order to reduce nonspecific staining (*see* **Note 8**).

1. Block embryos by incubating with PBTB for 10 min with constant mixing.
2. Incubate embryos with 300 *or* 100 µL of the appropriate anti-DIG antibody solution for 2 h. (*see* **Notes 3**).

   If an HRP-conjugated antibody is used in **step 2**, rinse embryos once with PBTB following the antibody incubation, then perform a nuclear counter stain by incubating for 10 min with a PBTB solution containing 1X DAPI, then proceed directly to **step 6**.
3. Perform six washes for 10 min each with PBTB.
4. Incubate embryos for 1 h with 200 *or* 75 µL of streptavidin-HRP solution (diluted 1/100 in PBTB).
5. Rinse embryos once with PBTB, then perform a nuclear counter stain by incubating for 10 min with a PBTB solution containing 1X DAPI.
6. Wash the embryos six times for 10 min each with PBTB, then once with PBT and two times with PBS for 5 min each.
7. Prepare 1/50 dilutions of the appropriate tyramide conjugate with the amplification buffer supplied in the tyramide kit (*see* **Note 3**). Remove the last PBS wash from the embryos, add 150 *or* 50 µL tyramide solution, and incubate in the dark at room temperature for 2 h with constant mixing.
8. Wash six times for 10 min each with PBS.

3.3.3. Storage, Mounting, and Viewing of Samples

1. Resuspend embryos in 200 *or* 100 µL of DABCO mountant. Allow the embryos to settle to the bottom of the tube (1–3 h or overnight at 4°C) before mounting (*see* **Note 9**). Embryos can be stored for months/years in microfuge tubes or PCR plates at 4°C in light-shielded receptacles.
2. Transfer approx 35-µL aliquot of embryos, by delicate resuspension using wide aperture tips, onto a clean slide and cover with a 22×22 mm^2 cover slip. Seal the edges with transparent nail polish. Slides can be stored for a few weeks at 4°C in the dark. In our experience, the DAPI stain tends to diffuse away after a few weeks on slides. Therefore, it is better to mount a fresh aliquot of embryos if samples are reanalyzed at a later date.
3. Analyze embryos by conventional fluorescence or confocal microscopy.

The stainings shown in **Fig. 1.** shows a comparison of *in situ* hybridization data obtained using TSA (A, B, C, E) vs conventional AP-based detection (C, F) *(8)*.

3.4. Double FISH on Drosophila Embryos

1. Generate two probes, each with a different label, as described in **Subheading 3.1.** (*see* **Note 1** for alternative/additional labels).
2. Collect and fix embryos as described in **Subheading 3.2.**
3. Perform the hybridization with both probes simultaneously; all other pre- and posthybridization washes are as described in **Subheading 3.3.1.**
4. Block embryos by incubating with PBTB for 10 min with constant mixing.
5. Incubate embryos with 300 *or* 100 µL of the appropriate HRP-conjugated anti-DIG antibody solution for 2 h. (*see* **Note 3**).
6. Wash the embryos six times for 10 min each with PBTB, then once with PBT and two times with PBS for 5 min each.
7. Prepare a 1/50 dilution of the first tyramide conjugate using the amplification buffer supplied in the tyramide kit (*see* **Note 3**). Remove the last PBS wash from the embryos, add 150 *or* 50 µL tyramide solution, and incubate in the dark at room temperature for 2 h with constant mixing. All of the following steps should be carried out in a light-shielded receptacle.
8. Wash six times for 10 min each with PBS.
9. Quench the first tyramide reaction by washing for 15 min with quenching solution (*see* **Note 10**). Wash two times with PBS and two times with PBT for 5 min each.
10. Block embryos with PBTB for 10 min as in **step 4**.
11. Incubate embryos for 1 h with 200 or 75 µL of streptavidin-HRP solution (diluted 1/100 in PBTB).
12. Rinse embryos once with PBTB, then perform a nuclear counter stain by incubating for 10 min with a PBTB solution containing 1X DAPI.
13. Wash the embryos six times for 10 min each with PBTB, then once with PBT and two times with PBS for 5 min each.
14. Prepare 1/50 dilutions of the second tyramide conjugate with the amplification buffer supplied in the tyramide kit (*see* **Note 3**). Add 150 *or* 50 µL tyramide solution and incubate for 2 h with constant mixing.
15. Wash six times for 10 min each with PBS.
16. Mount and view samples as described in **Subheading 3.3.3.**

Figure 2 shows an example of a double FISH staining for mRNAs encoded by the *CG1962* and *Canoe* genes.

3.5. RNA–Protein Double Labeling

1. Collect, process, and hybridize embryos essentially as described in **Subheading 3.2.** and **3.3.1.**; with the exception of the proteinase K step, which may have to be adapted for optimal immunostaining (*see* **Note 7**).
2. Take care to select noncrossreactive detection reagents (i.e., antibodies generated in different host species). Add the primary antibody against the protein of interest,

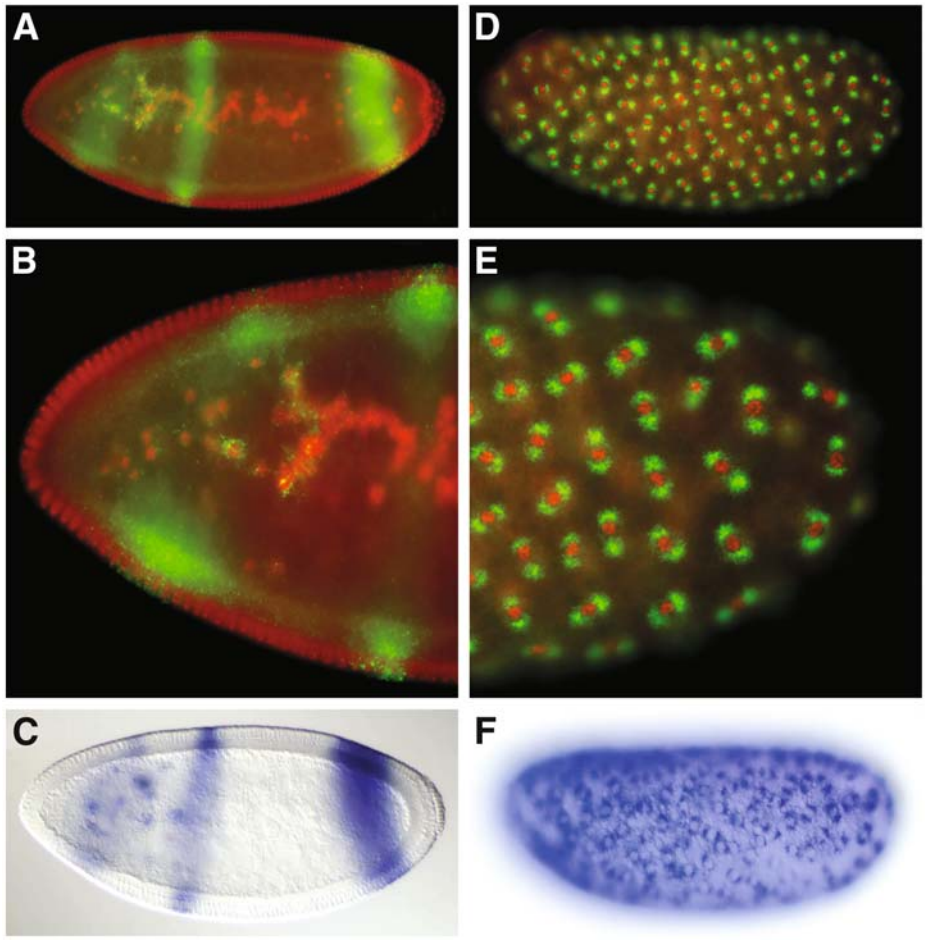

Fig. 1. Staining patterns obtained using DIG-labeled antisense probes for **(A–C)** *Hunchback* and **(D–F)** *CG1962* transcripts, either visualized using TSA **(A**,**B**,**D**,**E)** or conventional AP-based detection **(C**,**F)**. Tyramide stained embryos were processed through consecutive incubations with a biotinylated anti-DIG antibody, streptavidin-HRP and Cy3 tyramide, whereas nuclei were counterstained using DAPI. The Cy3 tyramide and DAPI signals were false colored in green and red respectively, as this coloring scheme provides better contrast. **(A–C)** Zygotic *Hunchback* gene expression is detected in stripes of peripheral blastoderm nuclei and in a subset of yolk nuclei. Tyramide detection enables the visualization of nuclear foci representing nascent zygotic transcripts, as well as cytoplasmic mRNA pools. **(D–F)** Transcripts of the *CG1962* gene demonstrate centrosome microtubule localization. AP-stained embryo images were obtained from the Berkeley *Drosophila* Genome Project *in situ* hybridization web resource ***(8)***.

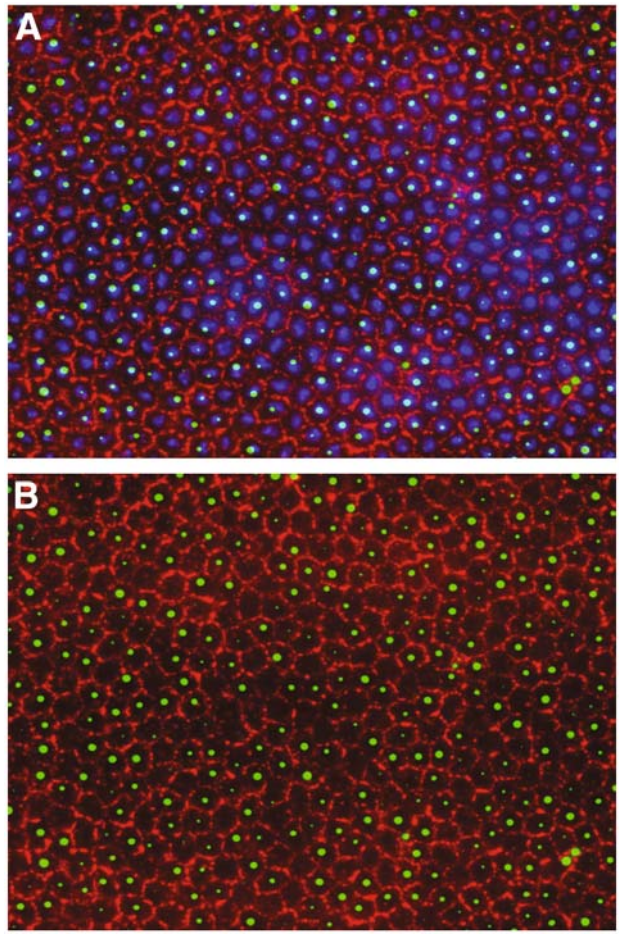

Fig. 2. Double FISH detection of *CG1962* and *Canoe* gene transcripts. **(A**,**B)** Hybridizations were performed using a biotinylated probe for CG1962, detected with streptavidin-HRP and Alexa 488 tyramide (green signal); and a DIG-labeled probe for Canoe, revealed using an HRP-conjugated sheep anti-DIG antibody and Cy3 tyramide (red signal). The image shown in (**A**) also shows the nuclei counterstained with DAPI (blue signal). *CG1962* mRNA localizes in foci that are localized above the nuclei, whereas *Canoe* gene transcripts are localized to membrane junctions.

along with the appropriate probe detection reagent; HRP- or biotin-conjugated anti-DIG antibodies, or streptavidin-HRP, for DIG- and biotin-labeled probes, respectively. Incubate embryos with 300 *or* 100 µL antibody solution (diluted in PBTB) for 2 h at room temperature with constant mixing.

3. Perform six washes for 10 min each with PBTB.

4. Add secondary detection reagents (fluorochrome-conjugated secondary antibodies and streptavidin-HRP). Perform incubations, washes, DAPI staining, and TSA reaction as in **Subheading 3.3.2.** (*see* **Note 11**).
5. Mount samples as described in **Subheading 3.3.3.**

3.6. FISH on Dissected Tissues

1. Dissect tissues, such as imaginal discs and salivary glands, in 1X PBS. Dissected tissues can be stored briefly on ice in a 1.5-mL microfuge tube containing PBS until enough tissue is obtained for analysis.
2. Remove the PBS and add 600 µL of fixation solution. Shake gently for 20 min.
3. Wash embryos five times in PBT for 2 min each to remove all traces of fixative.
4. Perform prehybridization, hybridization, antibody incubations, TSA reactions, and mounting of samples as described in **Subheadings 3.3.1.–3.3.3.**

4. Notes

1. The DIG and biotin labels described here can be substituted by or combined with many other labels, including fluorescein, dinitrophenyl, and a number of Alexa-conjugated nucleotides. These can be detected by a variety of commercially available antibodies and provide numerous possibilities for multilabeling experiments, as described by Kosman et al. *(7)*.
2. Preparing smaller batches of fresh formaldehyde solutions as needed ensures consistent and strong fixation of samples, whereas avoiding potential loss of activity that might occur with larger volumes of commercially available formaldehyde solutions kept in storage over long periods of time.
3. To obtain the strongest FISH signal, we recommend using the biotinylated anti-DIG antibody in combination with streptavidin-HRP, which provides an extra signal amplification step compared with the HRP-conjugated anti-DIG antibodies alone. However, although less sensitive, these directly conjugated antibodies are suitable for double FISH experiments, where biotin is used as a second probe label, or for RNA–protein codetection experiments, where antibody crossreactivity becomes a concern. Although we have mainly used Cy3 and Alexa 488 tyramide conjugates, which both give strong signals; there is a variety of additional fluor-conjugated tyramides available from Perkin Elmer Life Sciences and Molecular Probes. The amplification buffer supplied with the Perkin Elmer Life Sciences tyramide kits is in ready-to-use format. In contrast, when using Alexa tyramide conjugates from Molecular Probes, hydrogen peroxide supplied with the kit needs to be added to the amplification buffer (0.0015% final concentration) before use. The recommended antibody and tyramide dilutions found to be optimal in our laboratory might need to be optimized on a lab-by-lab basis owing to variability in research environments and product stocks.
4. We have found that removal of DNA templates by DNAseI treatment following the transcription reactions, as well as carbonate degradation of probes for increased tissue penetration, to be unnecessary and may risk reducing probe quality. After performing side-by-side comparisons, we opted for using sodium acetate instead of lithium chloride for probe precipitation, as it provided greater precipitation efficiency.

5. When intending to use PCR plates for hybridizations, it might be preferable to perform **steps 1–10** in **Subheading 3.3.1.** using 5-mL polypropylene tubes containing approx 300 µL settled embryos (one tube/quarter plate). This makes the manipulations easier at the proteinase K digestion step where delicate mixing is required and embryos can be aliquoted in PCR plates before starting the prehybridizations. When aliquoting the embryos into PCR plates, it is preferable to use a pipetman rather then a multichannel pipetor, as it is easier to maintain an even suspension of embryos by up and down pipeting in order to achieve equal embryo distribution in the plates. Take care to eliminate any air bubbles that may have formed under the embryo layer as these may damage the samples during the hybridization step. Once the embryos are aliquoted, multichannel pipetors are recommended for all subsequent washing, antibody incubation, and mounting steps. Furthermore, washes can be greatly facilitated by using an eight-well manifold connected to a vacuum pump to aspirate solutions.
6. When pipeting embryo/tissue samples, wide aperture tips should be used to avoid damaging the embryos. Wide aperture tips can be purchased from a variety of suppliers. If these are not available, simply cut off the ends of traditional tips.
7. Proteinase K digestion is an important parameter for optimal probe entry into the embryo or tissue of interest. Over digestion can disrupt tissue integrity and morphology, whereas under digestion can hinder even accessibility of the probe to the entire sample. Traditional protocols suggest a short incubation (1–5 min) at high proteinase K concentration (50 µg/mL); however, we have found that performing the digestions for a longer period of time at lower proteinase K concentrations, followed by a 1 h incubation on ice, significantly improves staining sensitivity and uniformity from embryo to embryo. When preparing new proteinase K stocks, or when working with new types of tissue (i.e., dissected tissues, mutant embryos that may be more delicate, and so on), we recommend titrating the concentration of proteinase K in order to find the optimal working concentration. Some tissues, such as dissected larval tissues, tend to be more sensitive to proteinase K digestion; as a result, we often omit the proteinase K step when dealing with such samples. It may also be necessary to reduce proteinase K levels when performing RNA–protein costaining experiments (*see* **Subheading 3.5.**), as over digestion may perturb epitope recognition.
8. The concentration of milk used in this protocol has been optimized for use with the antibodies described in **Subheading 2.3.2.** For other antibodies, it may be preferable to vary the concentration of milk or use alternate blocking reagents (i.e., bovine serum albumin or commercially available blocking solutions) to increase signal specificity.
9. We find that samples that have been precociously mounted often exhibit a hazy background appearance that dissipates a few hours after the mountant solution has been added to the embryos.
10. Although we have found quenching with 1% hydrogen peroxide to be satisfactory when performing double tyramide reactions, treatment with 0.01 *M* HCl for 10 min or heating at 70°C for 15 min have been suggested as alternative treatments for inactivating the first HRP reaction ***(6,9)***.

11. For RNA–protein costaining experiments; we have traditionally used secondary antibodies that are directly conjugated to a fluorogenic compound of interest. However, we have begun using TSA as a means of enhancing our immunostaining signal, through the use of HRP-conjugated secondary antibodies directed against the species of the primary antibody FC fragment, followed by TSA. We suggest testing each approach in parallel to determine which conditions work best on a case-by-case basis. Most of our secondary antibodies, including both fluor- and HRP-conjugated, were obtained from Jackson ImmunoResearch Laboratories Inc., and are recommended for multilabeling experiments.

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Linear RNA Amplification for the Production of Microarray Hybridization Probes

Ansgar Klebes and Thomas B. Kornberg

Summary

To understand *Drosophila* development and other genetically controlled processes, it is often desirable to identify differences in gene expression levels. An experimental approach to investigate these processes is to catalog the transcriptome by hybridization of mRNA to DNA microarrays. In these experiments mRNA-derived hybridization probes are produced and hybridized to an array of DNA spots on a solid support. The labeled cDNAs of the complex hybridization probe will bind to their complementary sequences and provide quantification of the relative concentration of the corresponding transcript in the starting material. However, such approaches are often limited by the scarcity of the experimental sample because standard methods of probe preparation require microgram quantities of mRNA template. Linear RNA amplification can alleviate such limitations to support the generation of microarray hybridization probes from a few 100 pg of mRNA. These smaller quantities can be isolated from a few 100 cells. Here, we present a linear amplification protocol designed to preserve both the relative abundance of transcripts as well as their sequence complexity.

Key Words: *Drosophila melanogaster*; expression profiling; in vitro transcription; linear RNA amplification; microarray; nucleic acid purification; reverse transcription; T7 RNA polymerase.

1. Introduction

Synthesizing probes for microarray hybridization requires microgram amounts of mRNA, quantities that might be difficult to obtain if the experimental protocol seeks a defined cell population, developmental stage, or mutant genetic background. Transcripts that are expressed in only a few cells might fall below the level of detection if the expressing cells are in low abundance. However, isolating the expressing cells might impose a practical limitation on the quantity of mRNA available for probe generation. In addition, there is an advantage to comparing cellular populations that are isolated from a single animal, even

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when larger quantities of mRNA can be readily isolated from many animals. Variability owing to differences in gender, genetic background, or developmental stage and technical artifacts that might arise from elongated periods of sample collection and manipulation, inevitably arise when materials from many animals are pooled. Linear RNA amplification is a practical method to make hybridization probes for determining expression profiles from small quantities of mRNA.

An amplification procedure for microarray hybridization must increase the quantity of RNA while preserving the relative abundance of transcripts in a high-complexity mRNA population. Polymerase chain reaction (PCR)-based methods produce high yields owing to exponential amplification, but they do not preserve relative levels. Eberwine and coworkers ***(1)*** first introduced a T7 RNA polymerase-based amplification protocol that applies linear amplification kinetics. The principle of this method is based on the conversion of the mRNA population to cDNAs (RT), the introduction of a T7 promoter sequence at one end of each cDNA, and amplification by multiple rounds of in vitro transcription (IVT). The equal probability that each promoter sequence can be bound by T7 RNA polymerase to initiate transcription serves to preserve the relative abundance of each transcript. T7-based linear amplification does not preserve relative transcript abundance perfectly, but most transcripts are amplified at comparable rates ***(2)***. By comparing RNA populations that had been amplified in parallel, we have found T7-based amplification to be highly reproducible and to make it possible to identify small differences in transcript levels.

In our experience, the combination of RT and IVT can yield greater than 1000-fold amplification. If this is insufficient, a second round of RT and IVT can result in an additional 100-fold amplification, yielding a total of 10^5–10^6-fold. A 10,000-fold amplification makes it possible to use starting material of a few 100 pg of mRNA. This corresponds to a few 100 cells of *Drosophila* tissue.

T7-based amplification relies on faithful RT, but shortening of the RT products and consequent loss of sequence complexity at both ends of the amplified RNA (aRNA) can occur. First strand cDNA synthesis can terminate before reaching the end of the mRNA template. The second strand cDNA can also terminate prematurely resulting in loss of 3′ sequences or if priming occurs from an internal location on the template, loss of 5′ sequences can result. We use an "anchor-primer" to increase the probability of synthesizing full-length second strands. This technique was pioneered by Wang and coworkers ***(3)*** who adapted the template switching technology (SMART, Clontech Laboratories Inc. Mountain View, CA). This method incorporates an anchor-primer that includes a $(dG)_3$ triplet. As the first strand cDNA was synthesized by moloney murine leukemia virus (M-MLV) reverse transcriptase, which adds a $(dC)_3$ triplet to the 3′ end of its products, the anchor-primer can anneal to the terminus of the first strand. In the second round of RT this anchor-primer is used to synthesize cDNA and thus preserves sequences at mRNA 5′ ends.

It is important to understand that the aRNA produced by linear amplification will be antisense, and that standard methods for making probes use reverse transcription (RT). Labeling probes will therefore result in sense copies. Use of sense probes is appropriate for cDNA microarrays, as they contain both sense and antisense strands. However, most oligonucleotide arrays represent the sense strand, so an antisense hybridization probe is required. Direct labeling of the aRNA (e.g., Universal Linkage System™ [ULS™] aRNA fluorescent-labeling kit, Kreatech Biotechnology, Amstadam, The Netherlands) can produce antisense probes.

The rationale underlying the linear amplification procedure is the incorporation of a T7 RNA polymerase promoter sequence. Synthesis of the first strand cDNA is initiated by a primer that includes both poly(dT) and a complementary T7 promoter sequence (labeled "T7" in **Fig. 1**). The second strand is primed from RNA fragments that are produced by ribonuclease (RNase H) (*see* **Notes 13** and **14**), resulting in the production of nicked cDNA strands. Nicks are sealed by DNA ligase (*see* **Note 12**) to provide an efficient template for T7 RNA polymerase. In second strand reaction of the first round of RT, the Ts-Amp-GGG anchor primer is designed to anneal to the 3′ $(dC)_3$ overhang that the MLV reverse transcriptase adds to the 3′ end of the first strand. The Ts-Amp anchor-primer is made double-stranded by T4 DNA polymerase before the first IVT (*see* **Note 15**). Following IVT, the aRNA copies will have the antisense orientation. As T7 RNA polymerase does not copy the T7 promoter sequence in the first IVT reaction, an oligo$(dT)_{24}$-T7 primer is included for second strand synthesis in the second round of RT. T4 DNA polymerase is used to fill in the complementary sequence. Because the first strand cDNA serves as template in the second IVT reaction, nicks in the second strand do not interfere with T7 RNA polymerase and sealing the nicks by DNA ligase is not necessary.

2. Materials

2.1. Preparation of Samples, and RNA Isolation

1. Phosphate-buffered saline (PBS) (10X stock): 1.37 *M* NaCl, 27 m*M* KCl, 100 m*M* Na_2HPO_4, 18 m*M* KH_2PO_4 (adjust to pH 7.4 with HCl); autoclave. Store at room temperature. For the 1X working solution dilute one part with nine parts water.
2. Two forceps (DuPont no. 5 or similar; [Fine Science Tools Inc., Foster City, CA]) and one pair of tungsten needles. (custom-made with tungsten wire [e.g., Electron Microscopy Sciences, Hartfield, PA]; and needle holder, [e.g., VWR International, West Chester, PA]).
3. Mini RNA isolation kit (Zymo Research, Orange, CA; cat. no. R1005).

2.2. First Round, RT

1. Speed vac concentrator (e.g., Concentrator 5301, [Eppendorf, Hamburg, Germany])
2. Thermocycler with heated lid (e.g., iCycler [Bio-Rad Laboratories Inc., Hercules, CA]).

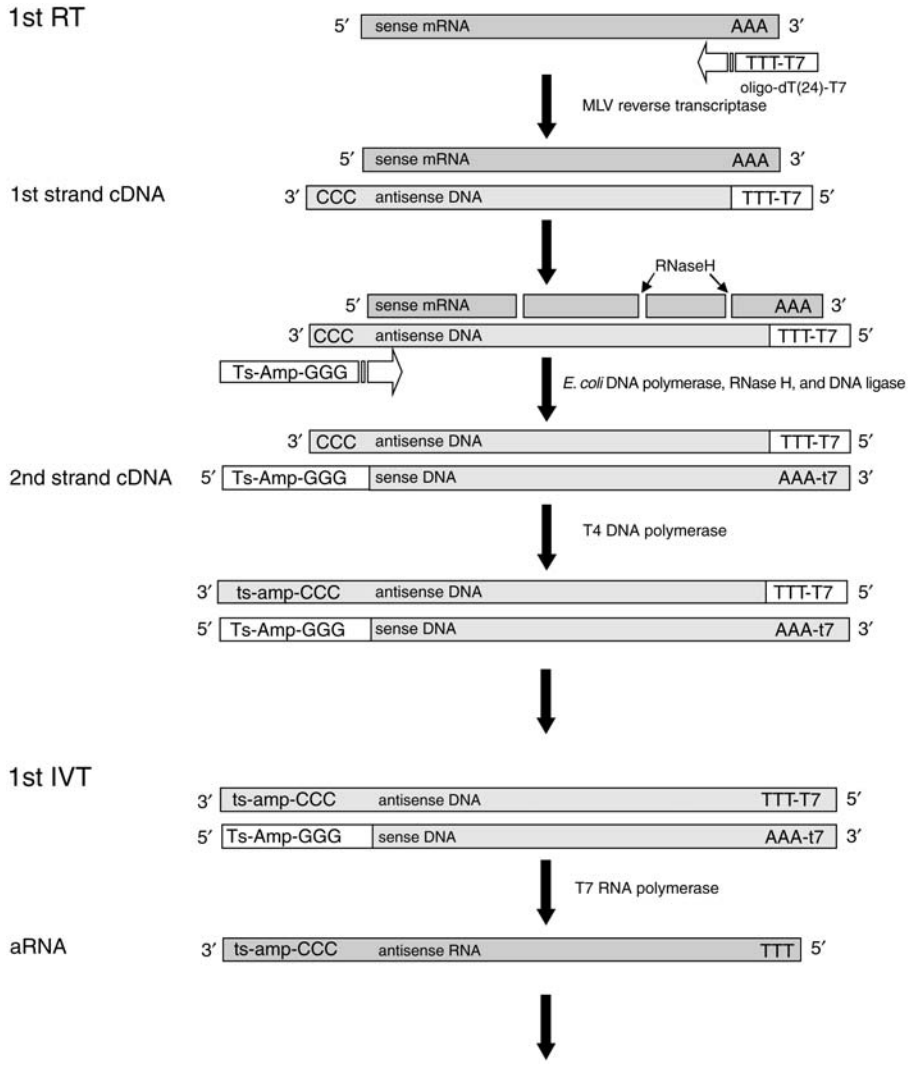

Fig. 1. Schematic representation of the linear amplification procedure. The first RT reaction is primed by the oligo-dT(24)-T7 primer, which includes a T7 RNA polymerase promoter sequence (T7). MLV reverse transcriptase produces a C overhang (CCC) at the 3′-end of the antisense cDNA strand. The Ts-Amp primer includes a complementary G triplet (GGG) and is incorporated into the second strand. The missing sequences at the end of each strand are filled-in by the T4 DNA polymerase. In the following IVT, T7 polymerase synthesizes multiple copies of each template strand, producing antisense RNA. As this aRNA contains the complementary ts-amp anchor sequence the Ts-Amp oligonucleotide can be used to prime the first strand synthesis of the second RT reaction. For the second strand synthesis the oligo-dT(24)-T7 primer is added to incorporate the

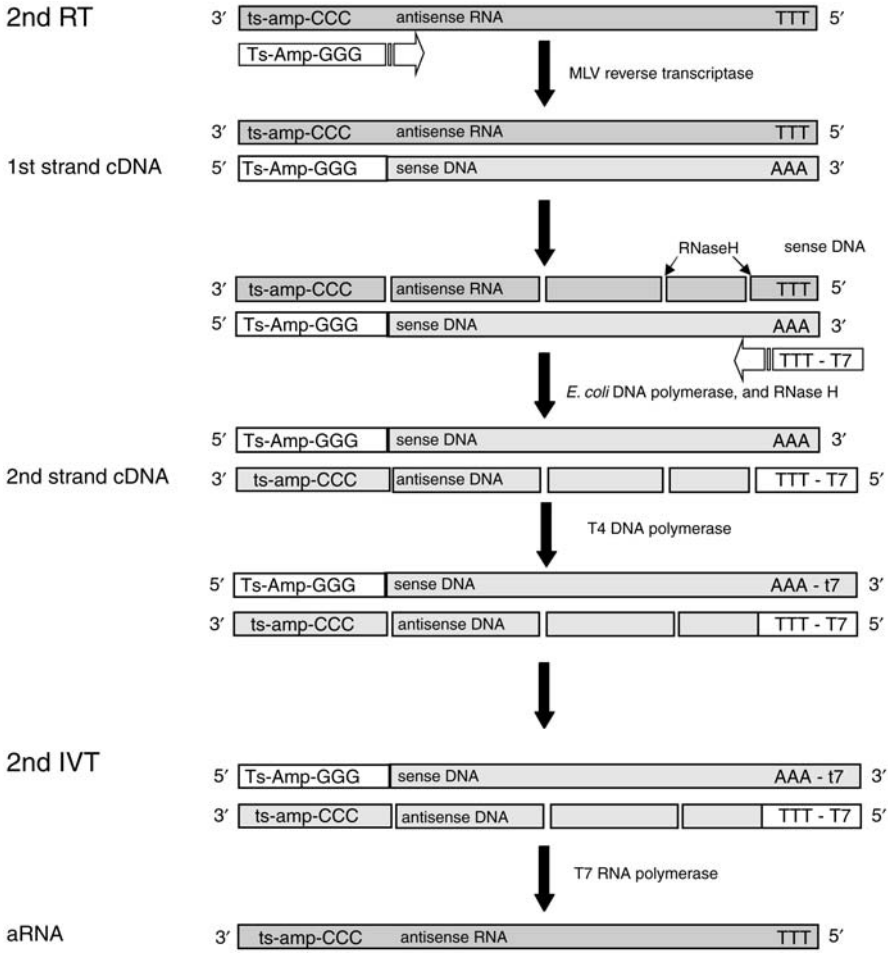

Fig. 1. *(Continued)* T7 promoter sequence. In the second IVT reaction the sense strand of the cDNA serves as template, which results in the synthesis of antisense RNA as the amplification product. Note that the poly(A)-tail of the mRNA and the stretch of 24 thymidine residues of the oligo-dT(24)-T7 primer are shown as triplets only (AAA and TTT, respectively).

3. Oligo-dT(24)-T7 primer (5′-GGCCAGTGAATTGTAATACGACTCACTATAG GGAGGCGGTTTTTTTTTTTTTTTTTTTTTTT-3′, high-pressure liquid chromato graphy purification is recommended).
4. Ts-Amp primer (5′-AAGCAGTGGTAACAACGCAGAGTACGCGGG-3′).
5. Deoxynucleotide 5′-triphosphate (dNTP) mix (e.g., [F. Hoffmann-La Roche Ltd., Basel, Switzerland], 10 m*M* each, PCR grade, cat. no.1969064).

6. T4gp32 (e.g., 13.8 mg/mL, USB, Cleveland, OH, cat. no. 74029Y).
7. RNasin (Promega Corporation, Madison, WI; cat. no.N251A).
8. Superscript III, reverse transcriptase, 5X reverse transcriptase buffer, dithiothreitol (DTT) (Invitrogen Corporation, Carlsbad, CA; cat. no. 18080-093).
9. 5X second strand buffer (Invitrogen, cat. no.10812-014).
10. *Escherichia coli* DNA ligase (Invitrogen, cat. no.180052-019).
11. *E. coli* DNA polymerase (Invitrogen, cat. no.18010-025).
12. *E. coli* RNase H (Invitrogen, cat. no.18021-071).
13. T4 DNA polymerase (Promega, cat. no.M421A).
14. DNA clean and concentrator-5 (Zymo Research, cat. no. D4003), includes DNA-binding buffer and wash buffer.

2.3. First Round, IVT

1. Megascript T7 transcription kit (Ambion Inc., Austin, TX; cat. no. 1334), includes T7 polymerase in an enzyme mix, nucleotide 5′-triphosphates (NTPs), 10X reaction buffer, and deoxyribonuclease (DNase).
2. 37°C air incubator or heat block with heated lid.
3. RNA clean-up Kit™-5 (Zymo-Research, cat. no. R1023), includes RNA-binding buffer and wash buffer.

2.4. Second Round, RT

The same reagents as in **Subheading 2.2.** First round, RT without *E. coli* DNA ligase.

2.5. Second Round, IVT

The same reagents as in **Subheading 2.3.** First round, IVT.

3. Methods

Two rounds of amplification require at least 2 d; the procedure can be interrupted at any step after nucleic acid purification. All reactions should be RNase-free (RNase-free glass and plasticware, RNase-free water [*see* **Note 4**], and so on, as well as RNase-free working conditions). Barrier pipet tips are used to limit contamination and low adhesive plasticware help to limit losses.

3.1. Preparation of Samples, and RNA Isolation

We have applied this protocol for the amplification of total-RNA isolated from *Drosophila* tissue culture cells and imaginal discs, and from larval organs such as brain, gut, salivary glands, or adult gonads ***(2,4,5)***. These tissues dissolve in the extraction buffer supplied with the RNA isolation mini kit (Zymo). If embryos (*see* **Note 1**) or whole larvae, pupae, or flies are used, it is necessary

to first homogenize in extraction buffer or grind in liquid nitrogen. The RNA purified with the Mini RNA isolation kit (Zymo) is high quality and can be eluted in a small volume of water (*see* **Note 2**).

1. Collect sample and wash well in PBS (*see* **Note 3**).
2. All subsequent work with RNA should be carried out under RNase-free working conditions (gloves, RNase-free plasticware and reagents, and so on).
3. The following protocol is according to the manufacturer's instructions (Mini RNA isolation kit, Zymo). Transfer sample into RNase-free 1.5-mL reaction tube filled with 200-µL extraction buffer, and dissolve tissue by vortexing (200 µL will work for most sample amounts that require RNA amplification).
4. Incubate on ice for 20 min and vortex after 10 min. Briefly spin down the tubes before opening the lid.
5. Add one volume ethanol (200 µL) and mix. Incubate another 10 min on ice.
6. Load the sample on a column that has been placed in a collection tube (the collection tube is provided in the Mini RNA isolation kit, [Zymo]; this and the following steps are at room temperature). To retain all solution we spin down the tube after we have loaded the column and add the remaining liquid.
7. Spin the column at maximum speed (10,000–14,000 rpm) for 30 s in a microfuge (e.g., centrifuge 5417 [Eppendorf]).
8. Add 200-µL wash buffer onto the column. Spin again and empty collection tube. Place column back into the collection tube and repeat the wash (200-µL washing buffer). Spin for 1 min, to completely dry the column. Remove column from collection tube and place in a fresh RNase-free 1.5-mL reaction tube.
9. For elution, pipet 8 µL of RNase-free water (*see* **Note 4**) on the matrix without punching it. Let incubate at room temperature for 2 min. Spin for 30 s at 10,000–14,000 rpm (rcf: 10,600–20,800*g*) in a microfuge. (Orient the open tube lid in the direction of the rotor rotation to prevent its breaking.) Repeat the elution with another 8 µL of RNase-free water.
10. Transfer the 16 µL total RNA solution into a thin-walled RNase-free PCR tube and reduce volume to 5 µL in a speed vac at room temperature. Avoid drying completely. If the sample has been dried to less than 5 µL, add RNase-free water to adjust the volume. If the sample has dried completely, add water, vortex, and incubate at 60°C to resuspend.

3.2. First Round, RT

3.2.1. Template Denaturation and Primer Annealing

1. Add 1 µL primer mix to the 5 µL total RNA. The primer mix contains dT(24)-T7 primer (20–50 ng/µL) and if two rounds of amplification will be performed, the Ts-Amp primer (250 ng/µL). The concentration of the dT(24)-T7 primer is critical and should not exceed 50 ng/µL (*see* **Note 5**). If only one round of amplification (RT and IVT) will be performed the Ts-Amp primer can be omitted.
2. Mix by pipeting up and down. Place in PCR machine and heat to 65°C for 10 min. Place on ice after the heating step (*see* **Note 6**).

3.2.2. Reverse Transcription

1. Add the following (*see* **Note 7**): 2 µL 5X first strand buffer (provided with Superscript III, Invitrogen).
2. 1 µL of 0.1 *M* DTT (provided with Superscript III, Invitrogen).
3. 1 µL dNTP-Mix (10 m*M* each, *see* **Note 8**).
4. 0.3 µL T4gp32 (13.8 mg/mL, USB, *see* **Note 9**).
5. 0.5 µL RNase inhibitor (e.g., RNasin, Promega).
6. 1 µL Superscript III (*see* **Note 10**).
7. Spin briefly and incubate at 50°C for 2 h.
8. Heat inactivate at 70°C for 10 min. Place on ice.

3.2.3. Second Strand Synthesis

1. Thaw second strand buffer (Invitrogen) on ice (*see* **Note 11**).
2. Prepare the mix on ice for a single reaction or as a master mix for multiple reactions.
3. 45.5 µL Water.
4. 15 µL 5X second strand buffer.
5. 1.5 µL dNTP-Mix (10 m*M* each).
6. 0.5 µL *E. coli* ligase (10 U/µL, Invitrogen, *see* **Note 12**).
7. 2 µL *E. coli* polymerase (10 U/µL, Invitrogen, *see* **Note 13**).
8. 0.5 µL *E. coli* RNase H (2 U/µL, Invitrogen, *see* **Note 14**).
9. Add these 65 µL to the first strand reaction, mix by pipeting up and down.
10. Incubate at 16°C for 2 h (*see* **Note 6**).
11. Add 2U T4 DNA polymerase (Promega, *see* **Note 15**).
12. Incubate at 16°C for 15 min.
13. Heat inactivate at 70°C for 15 min.

3.2.4. DNA Purification

The cDNA is purified using the DNA clean and concentrator-5 (Zymo Research) according to the manufacturer's instruction:

1. Add 200 µL (slightly more than two volumes) DNA-binding buffer, mix by pipeting up and down.
2. Transfer entire mix to a spin-column in a collection tube.
3. Centrifuge at 10,000–14,000 rpm (rcf: 10,600–20,800*g*) for 30 s.
4. Add 200 µL wash buffer. Centrifuge again for 30 s. Discard flow-through.
5. Repeat wash step.
6. Transfer the tube to a fresh RNase-free 1.5-mL reaction tube.
7. Add 8 µL RNase-free water directly on the matrix. Incubate for 2 min at room temperature.
8. Centrifuge for 30 s.
9. Repeat elution step with 8 µL RNase-free water.
10. Concentrate the cDNA to 8 µL using a speed vac.

3.3. First Round, IVT

3.3.1. In Vitro Transcription

Using the T7 Megascript Kit (Ambion, *see* **Note 16**):

1. Thaw the 10X reaction buffer at room temperature and vortex until any white precipitate has dissolved.
2. Thaw the nucleotides (ATP, GTP, CTP, and UTP) and prepare a NTP-Mix by adding equal amounts of each solution (75 m*M* each, *see* **Note 17**).
3. Add 2 µL 10X reaction buffer to the 8 µL purified cDNA.
4. Add 8 µL NTP-Mix from **step 2**.
5. Add 2 µL enzyme mix, mix by pipeting up and down.
6. Incubate at 37°C for 6 h (*see* **Note 18**).
7. Add 1 µL RNase-free DNase (provided with the T7 Megascript Kit).
8. Incubate at 37°C for 15 min.
9. Store at –70°C overnight or proceed further.

3.3.2. RNA Purification

Cleanup is performed with the RNA Clean-up Kit-5 (Zymo Research).

1. Add 200 µL (*see* **Note 19**) RNA-binding buffer, mix by pipeting up and down.
2. Transfer entire mix to a spin-column in a collection tube.
3. Centrifuge at 10,000–14,000 rpm (rcf: 10,600–20,800*g*) for 30 s.
4. Add 200 µL wash buffer. Centrifuge again for 30 s. Discard flow-through.
5. Repeat wash step.
6. Transfer the tube to a fresh RNase-free 1.5-mL reaction tube.
7. Add 8 µL RNase-free water directly on the matrix. Incubate for 2 min at room temperature.
8. Centrifuge for 30 s.
9. Repeat elution step with 8 µL RNase-free water.

An aliquot of the aRNA can be analyzed by electrophoresis in an agarose gel to assess concentration and quality (*see* **Note 20**).

3.4. Second Round, RT

If a second round of RT and IVT is to be performed, concentrate the aRNA to 4 µL using a speed vac.

The second round of RT is similar to the first round except for three significant differences:

- The first strand synthesis is primed with the Ts-Amp primer (or random hexanucleotides, *see* **Note 21**).
- The oligo-dT(24)-T7 primer is added to the second strand synthesis.
- No E. coli DNA ligase is added to the second strand synthesis (see Note 22).

3.4.1. Reverse Transcription

1. Add 1 µL Ts-Amp primer (1 mg/µL).
2. Add the following: 2 µL 5X first strand buffer (*see* **Note 7**).
3. 1 µL 0.1 *M* DTT (provided with Superscript III, Invitrogen).
4. 1 µL dNTP-Mix (10 m*M* each, *see* **Note 8**).
5. 0.3 µL T4gp32 (13.8 mg/mL, USB, *see* **Note 9**).
6. 0.5 µL RNase Inhibitor (e.g., RNasin, Promega).
7. 1 µL Superscript III (*see* **Note 10**).
8. Spin briefly and incubate at 50°C for 2 h.
9. Let the PCR machine cool down to 4°C or place the tubes on ice (*see* **Note 23**).

3.4.2. Second Strand Synthesis

1. Thaw second strand buffer (Invitrogen) on ice (*see* **Note 11**).
2. Add 1 µL oligo dT(24)-T7 primer (50 mg/µL, *see* **Note 5**).
3. Prepare the mix on ice for a single reaction or as a master mix for multiple reactions.
4. 45 µL Water.
5. 15 µL 5X second strand buffer.
6. 1.5 µL dNTP-Mix (10 m*M* each).
7. 2 µL *E. coli* polymerase (10 U/µL, Invitrogen, *see* **Note 13**).
8. 0.5 µL *E. coli* RNase H (2 U/µL, Invitrogen, *see* **Note 14**).
9. Add these 64 µL to the first strand reaction, mix by pipeting up and down.
10. Incubate at 16°C for 2 h (*see* **Note 6**).
11. Add 2 U T4 DNA polymerase (Promega, *see* **Note 15**).
12. Incubate at 16°C for 15 min.
13. Heat inactivate at 70°C for 15 min.

3.4.3. DNA Purification

The cDNA is purified using the DNA clean and concentrator-5 (Zymo Research) according to the manufacturer's instruction:

1. Add 200 µL (slightly more than two volumes) DNA-binding buffer, mix by pipeting up and down.
2. Transfer entire mix to a spin-column in a collection tube.
3. Centrifuge at 10,000–14,000 rpm (rcf: 10,600–20,800*g*) for 30 s.
4. Add 200 µL wash buffer. Centrifuge again for 30 s. Discard flow-through.
5. Repeat wash step.
6. Transfer the tube to a fresh RNase-free 1.5-mL reaction tube.
7. Add 8 µL RNase-free water directly on the matrix. Incubate for 2 min at room temperature.
8. Centrifuge for 30 s.
9. Repeat elution step with 8 µL RNase-free water.
10. Concentrate the cDNA to 8 µL using a speed vac.

3.5. Second Round, IVT

The IVT is performed with the T7 Megascript Kit (Ambion, *see* **Note 16**).

1. Thaw the 10X reaction buffer at room temperature and vortex well until any white precipitate has dissolved.
2. Thaw the nucleotides (ATP, GTP, CTP, and UTP) and prepare a NTP-Mix by adding equal amounts of each solution (75 m*M* each, *see* **Note 17**).
3. Add 2 µL 10X reaction buffer to the 8 µL purified cDNA.
4. Add 8 µL NTP-Mix from **step 2**.
5. Add 2 µL enzyme mix, mix by pipeting up and down.
6. Incubate at 37°C for 6 h (*see* **Note 18**).
7. Add 1 µL RNase-free DNase (provided with the T7 Megascript Kit).
8. Incubate at 37°C for 15 min.
9. Store at –70°C overnight or proceed further.

3.5.1. RNA Purification

Clean up is performed with the RNA Clean-up Kit-5 (Zymo Research). The amount of aRNA can exceed the capacity of the zymo spin-column (*see* **Note 24**). Typically, one-fourth of the total volume of the IVT reaction is processed per column.

1. Add 200 µL (*see* **Note 19**) RNA-binding buffer, mix by pipeting up and down.
2. Transfer entire mix to a spin-column in a collection tube.
3. Centrifuge at 10,000–14,000 rpm for 30 s.
4. Add 200 µL wash buffer. Centrifuge again for 30 s. Discard flow-through.
5. Repeat wash step.
6. Transfer the tube to a fresh RNase-free 1.5-mL reaction tube.
7. Add 8 µL RNase-free water directly on the matrix. Incubate for 2 min at room temperature.
8. Centrifuge for 30 s.
9. Repeat elution step with 8 µL RNase-free water.

The aRNA is now ready for quality control and labeling.

4. Notes

1. Embryos: dechorionate with 8% bleach for 3–5 min (or until chorion is no longer visible under the stereo-microscope) and wash several times with PBS in a 1.5-mL reaction tube. To collect the embryos at the bottom of the tube centrifuge at 8000 rpm (rcf: 6800*g*) for 3 min. Remove PBS and replace with 200 mL extraction buffer provided with the mini RNA isolation kit. A 1.5-mL plastic pestel (e.g., cat. no. 47747-358, VWR) is used to homogenize the embryos. Completely dissolve embryos. To increase the efficiency of the homogenization embryos can be concentrated at the bottom of the tube by centrifugation several times during the homogenization.

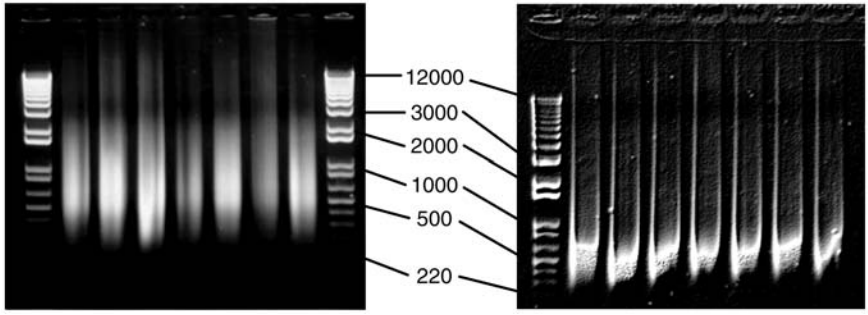

Fig. 2. Amplification products are dependent on primer concentration. **(A)** Product of seven independent amplification reactions of single imaginal discs using appropriate concentrations of $d(T)_{24}$-T7 primer. **(B)** Amplifications of imaginal discs with excess $d(T)_{24}$-T7 primer. Note that most products electrophorese at a rate similar to or faster than the 500 bp DNA marker, in this nondenaturing agarose gel. In contrast, the specific products in **Fig. 2A** are between 2 kb and 200 bp.

2. We have found that the mini RNA isolation kit (Zymo) works well with small amounts of RNA and allows elution of the RNA in a small volume of water. Other standard methods to isolate RNA such as the use of Trizol (Invitrogen) can also be used. If a Trizol- or phenol-based method is used, the RNA should be further purified with an affinity column such as RNA Clean-up Kit (Zymo Research) to remove traces of the organic solvent and to concentrate the sample. Larger volumes can also be concentrated in a speed vac (at room temperature to avoid RNA degradation).
3. Before the tissue is placed in the extraction buffer, it should be washed well in PBS to remove contaminating cells (e.g., fat body cells when larvae are dissected) and other materials. Wash tissue well by transferring into cold PBS in a watchmaker's glass on ice. A tungsten needle should be used to transfer tissue, because the use of forceps will result in the carry-over of large drops of PBS that might contain contaminating cells. Tissue is transferred by lifting from underneath and balancing it on the tip of the needle.
4. Nuclease-free water (e.g., Sigma-Aldrich, St. Louis, MO, cat. no. W4502) or sterile-filtered double-distilled water: Diethyl Pyrocarbonate (DEPC)-treated water was not used in order to avoid negative effects by traces of DEPC or products of its hydrolysis. The pH of the water should be around 7.0 or slightly acidic. In general, solutions that are used for RNA work should not be alkaline because this might result in alkaline hydrolysis of the phosphodiester bond in RNA.
5. The concentration of the $d(T)_{24}$-T7 primer is *critical*. **Figure 2** shows products from single imaginal disc amplifications. For the reactions in **Fig. 2A**, 50 ng/μL $d(T)_{24}$-T7 primer was used in the first RT and 100 ng/μL in the second RT. **Figure 2B** shows the amplification products when 200 ng/μL $d(T)_{24}$-T7 primer is used in both RTs. The excess of $d(T)_{24}$-T7 primer causes the amplification of unspecific product

at yields comparable with specific reactions. Unspecific product identified on these gels cannot be efficiently labeled.

6. The PCR machine is programmed to 4°C after the heating step. To add additional reagents, the tubes are removed and placed on ice. After adding all reagents, tubes are returned to the machine for subsequent cycles (not all PCR machines will allow this). An example PCR program:

65°C	10′
4°C	∞ (proceed manually)
50°C	2 h
(for the second round: 4°C)	∞ [proceed manually])
70°C	10′
4°C	∞ (proceed manually)
16°C	2 h
4°C	∞ (proceed manually)
16°C	15′
70°C	15′
4°C	∞ , end

7. If more than one reaction is performed, a master mix is prepared on ice.

Quantities per reaction	For X reactions (µL)
2 × X µL 5X first strand buffer	
1 × X µL 0.1 *M* DTT	
1 × X µL dNTP-Mix	
0.3 × X µL T4gp32	
0.5 × X µL RNase inhibitor	
1 × X µL Superscript III	

Add 5.8 µL to each reaction.

8. PCR-grade dNTPs are used.
9. T4 Gene 32 Protein is a single-stranded DNA- and RNA-binding protein that enhances yield and processivity in RNA amplification ***(6)***. This reagent is expensive at the recommended concentration (e.g., >10 µg/µL, USB, cat. no. 74029Y). Lower concentrations or reactions without Gene 32 Protein were tested and found to produce product without profound differences in yield or quality.
10. Superscript II and Superscript III from Invitrogen Inc. have been used with this protocol. Superscript III is an engineered version of the M-MLV reverse transcriptase with reduced RNase H activity and increased thermal stability. In principle, any other reverse transcriptase with similar properties should work. However, if a RT is used that has lower thermal stability (like Superscript II), the reaction temperature should be adjusted accordingly (e.g., 42°C for Superscript II).
11. The second strand buffer can be purchased at Invitrogen (cat. no. 10812-014) or prepared and stored as aliquots at –70°C (5X second strand buffer: 100 m*M* Tris-HCl [pH6.9], 23 m*M* $MgCl_2$, 450 m*M* KCl, 0.75 m*M* β-NAD^+, 50 m*M* $[NH_4]_2SO_4$).

12. *E. coli* ligase seals cohesive DNA ends and is active on nicked DNA. In contrast to T4 DNA ligase, this enzyme will not ligate blunt ends (if no polyethylene glycol or Ficoll is supplied). *E. coli* DNA ligase is not active on DNA–RNA or RNA–RNA hybrids; it requires NAD^+ and is active at 16°C.
13. DNA polymerase I from *E. coli* is a DNA-dependent DNA polymerase. The enzyme contains both 5′–3′ and 3′–5′ exonuclease activities. The 5′–3′ exonuclease activity enables the enzyme to synthesize DNA at nicks and can remove the RNA primer in DNA–RNA hybrids.
14. RNase H specifically degrades the RNA in RNA:DNA hybrids and will not degrade DNA or unhybridized RNA.
15. T4 DNA polymerase removes 3′ overhangs and fills-in 5′ overhang and is used to generate blunt ends. It does not have a 5′–3′ exonuclease.
16. RNase-free working conditions should be applied. Do not touch the tubes or any plasticware without gloves.
17. For a single reaction, 8 µL of a dNTP-Mix are required.
18. The incubation time can vary. However, within a series of experiments the incubation time should be constant to reduce variability. Uniform temperatures, such as in a hybridization oven, avoids condensation. Use of heat blocks that lack heated lids can result in condensation on tube lids. The tubes should be checked regularly and should be centrifuged briefly when condensation becomes detectable.
19. The recommended 10 volumes of RNA-binding buffer appear to improve binding to the matrix. The RNA-binding buffer may acquire a yellow tint, but this does not impact binding.
20. For rough assessment of the yield and quality, 2 µL of the 16 µL purified aRNA is electrophoresed on regular TAE or TBE agarose gels. These gels do not contain denaturing agents, but this quick method is sufficient to assess quality. The aRNA is determined to be of high quality if a long smear is detected (compare **Note 5**, **Fig. 2**) without bright signal in the low molecular range. Low molecular weight RNA is indicative of degradation.
21. The use of the Ts-Amp primer at this step is effective only if this primer has been added to the first RT reaction. Comparison of reactions that primed with Ts-Amp with reactions primed with random hexanucleotides detected no significant difference in yields.
22. No *E. coli* Ligase is added because in this round the first strand serves as the template for the IVT and nicks in the second strand will not interfere with the T7 polymerase processivity.
23. In contrast to the first round, a 4°C step is included in the program following the RT reaction (compare **Note 6**). This provides an interval to add oligo-dT(24)-T7 primer before continuing with the heat inactivation and denaturing step at 70°C.
24. If 1 ng of poly(A^+)-RNA is used as starting material, two rounds of amplification will yield more than 10 µg aRNA. A single imaginal disc generally yields approx 80–100 µg aRNA. The loading capacity of the Zymo spin-columns is 5 µg for the

RNA Clean-up Kit-5 (cat. no. R1023) and 25 µg for the RNA Clean-up Kit-25 (cat. no. R1025). In our experience, the RNA Clean-up Kit-5 columns can hold up to 30 µg. The unpurified in vitro reaction can be stored at –70°C for more than a year without noticeable loss of quality.

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An Overview of the Identification, Detection, and Functional Analysis of *Drosophila* MicroRNAs

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Summary

MicroRNAs (miRNAs), small noncoding RNAs that post-transcriptionally regulate gene expression, are one of the most abundant classes of gene regulators. Yet, little is known about the roles that specific miRNAs play in the development of multicellular organisms. *Drosophila* provides an excellent model system to explore the in vivo activities of particular miRNAs within the context of well-defined gene-expression programs that control the development of a complex organism. This chapter reviews the various approaches currently used to identify *Drosophila* miRNAs, detect their expression, determine their messenger RNA targets, and study their function.

Key Words: *Drosophila*; microRNA; miRNA; miRNA detection methods; miRNA target validation methods; pri-miRNA.

1. Introduction

MicroRNAs (miRNAs) are 21–25 nucleotide (nt), noncoding RNAs that post-transcriptionally regulate gene expression by basepairing with sequences in the 3′-untranslated regions (UTRs) of target messenger RNAs (mRNAs). Although only recently discovered, miRNAs constitute one of the most abundant classes of gene regulators. cDNA cloning, bioinformatic predictions, and expression analysis suggest that the human genome encodes as many as 1000 miRNAs, that this complement of genes is likely to regulate thousands of target mRNAs, and that a core set of 18 "ancient" miRNAs are phylogenetically conserved to the simplest metazoan genomes ***(1–3)***. Thus, miRNAs are clearly a critical component of the regulatory networks controlling gene expressions that are required for complex animals to develop normally. Yet, comparatively little is known about the specific roles that particular miRNAs play in the development of multicellular organisms.

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Drosophila, with its complex cell biology and sophisticated genetic techniques, offers a superior system to dissect the roles of miRNAs at the cellular, tissue, and organismal levels, and to identify the functional relationships between miRNAs and other genes. Furthermore, the fly's relatively simple genome appears to contain fewer cases of paralogous and hence, fewer redundant, *miRNA* genes than more complex vertebrate genomes. Indeed, genetic knockouts of *Drosophila* miRNAs display intriguing phenotypes indicating that their study will reveal new and perhaps unanticipated aspects of developmental biology. This chapter outlines the various methods that have been used to study the expression and function of *Drosophila* miRNAs and briefly summarizes the results of these studies.

2. Identification of *Drosophila* miRNAs

A miRNA is approx 21-nt RNA that is derived from a longer hairpin structure (~70 nt) by the processing activity of the enzyme Dicer and whose expression is validated either by its detection by Northern blot or its appearance in a cDNA library ***(4)***. To date, 78 *Drosophila* miRNAs have been identified ***(5–7)***. A complete list of these miRNA sequences and their genomic locations can be found at miRBase (http://microrna.sanger.ac.uk/sequences/). Of the 78 sequences, 48 are unique within the *Drosophila* genome. Furthermore, 62 are absolutely conserved between 12 *Drosophila* species whose genomes have been sequenced and 17 are phylogenetically conserved to humans ***(3)***. Multiple miRNAs can be derived from the same primary transcript and 41 miRNAs are contained within 13 different clusters that include between two and eight miRNAs. Developmental Northern blots representing the temporal expression profile of all *Drosophila* miRNAs can be found in a number of studies ***(5–9)***.

This section details the two main methods, molecular cloning and computational prediction, by which *Drosophila* miRNAs were identified. Indications are that additional miRNAs are yet to be found, and a current estimate predicts that the *Drosophila* genome contains approx 110 miRNA ***(7)***.

2.1. Molecular Cloning

Of the 78 known fly miRNAs, 68 have been molecularly cloned from a series of small RNA libraries made from S2 cells, staged embryos, 1st, 2nd, and 3rd instar larvae, pupae, adults, and testes ***(5,6)***. In this approach, total RNA is extracted and size is fractionated. The approx 18–25-nt fraction is isolated, ligated with linkers, reverse transcribed, cloned, and sequenced (for a detailed description of this technique, *see* **refs. *10*** and ***11)***. MiRNAs can represent just 2–10% of the sequences identified from such libraries, with the majority of sequences corresponding to degradation products of abundant or post-transcriptionally regulated RNAs (such as rRNAs, tRNAs, mRNAs, and so on). Furthermore, as the cloning frequency of a miRNA transcript is proportional to its abundance

compared with other miRNAs, extensive sequencing is required to identify rare miRNAs. Advances in cloning methodology have allowed the production of useful miRNA libraries from as little as 5 µg starting material. Thus, additional *Drosophila* miRNAs likely await identification from small RNA libraries generated from specific tissues and/or immunoaffinity purified miRNP complexes.

2.2. Computational Identification

A second method that has identified new miRNAs is based on the computational analysis of genomic sequences. A study applying this approach identified hairpin structures that were conserved between the *Drosophila melanogaster* and *D. pseudoobscura* genomes and displayed features characteristic of known miRNAs ***(7)***. The top set of 124 miRNA candidates contained 50 miRNAs identified by molecular cloning as well as 10 miRNAs not yet identified by cloning. This list might contain additional miRNAs because the sequence of a number of these candidates is conserved to more distant insects. Such candidates might represent rare miRNAs that are expressed at very low levels, in only a few cells, or only under specific stimuli and therefore, not detectable by Northern blot or molecular cloning.

Molecular cloning and computational identification offer complementary approaches to identifying and experimentally validating new miRNAs. Unlike molecular cloning, computational prediction is equally efficient for identifying abundant and rare miRNAs. Although, computational prediction does not accurately identify the 5′-end of predicted miRNAs ***(5)***, knowledge is currently essential for the accurate prediction of miRNA targets (*see* **Subheading 4.1.**). Furthermore, without improvements in algorithms to predict *bona fide* miRNAs, the task of validating miRNA candidates is daunting. However, the list of evolutionarily conserved hairpins generated from computational predictions will be an excellent resource to analyze sequence data from tissue-specific small RNA libraries.

3. Spatiotemporal Detection of *Drosophila* miRNAs

A first step toward understanding the in vivo function of *Drosophila* miRNAs is identifying where and when during development they are expressed. This section outlines methods for detecting the spatiotemporal expression patterns of miRNAs in *Drosophila* tissue. Other techniques for detecting miRNAs (i.e., miRNA microrarrays, reverse transcriptase-polymerase chain reaction, and so on) have been reviewed elsewhere ***(11)***.

3.1. In Vivo Detection of Pri-miRNAs

Hairpins containing mature, approx 21-nt miRNAs are derived from longer, primary transcripts, termed pri-miRNAs, through a processing step involving the

enzyme Drosha ***(12)***. Information about the spatiotemporal expression pattern of a mature, functional miRNA can therefore be gleaned from the expression profile of its pri-miRNA. The transcription start site, length, and intron/exon organization of most pri-miRNAs is not known; however, full-length pri-miRNA transcripts are generally not represented in libraries of cDNAs or expressed sequence tags, presumably because they are rapidly processed to produce mature miRNAs. Nevertheless, the embryonic expression profile of *pri-miR-10* was detected by *in situ* hybridization simply by using a probe antisense to a 1-kb genomic fragment that contains the 21-nt *miR-10* sequence ***(13)***. *Pri-miR-10* was detected in a distinct pattern of thoracic and abdominal cells in the *Drosophila* embryo.

Using this same *in situ* hybridization approach, a number of pri-miRNA expression profiles have been reported ***(14–17)***. Of particular note is a comprehensive study in which embryos were hybridized with probes to all known *Drosophil*a pri-miRNAs and a remarkable variety of dynamic expression profiles were cataloged for 38 pri-miRNAs ***(14)***. Interestingly, pri-miRNA probes detect a pair of nuclear dots, as opposed to the cytoplasmic signal detected by mRNA probes, indicating (1) the chromosomal locations where the pri-miRNAs are transcribed and (2) that pri-miRNA transcripts are rapidly processed. This processing of a mature miRNA from its pri-miRNA transcript may be regulated both spatially as well as temporally, as has been shown for *let-7* miRNAs during mouse development ***(18)***. Thus, a mature miRNA may be present and functional within only a subdomain of its overall pri-miRNA expression profile. Comparison of pri-miRNA expression patterns with mature miRNA expression profiles as detected with locked nucleic acid (LNA) oligos or miRNA "sensors" (*see* **Subheading 3.4.**) should indicate how prevalent post-transcriptional regulation of pri-miRNAs is in *Drosophila* **(Fig. 1)**.

3.2. Pri-miRNA Transcriptional Reporters

A transgenic approach has also been used to determine the expression pattern of the *miR-1* ***(15,16,19)***, *miR-278* ***(20)***, and the *miR-309/3/286/4/5/6-1/6-2/6-3* cluster ***(15)*** pri-miRNAs. In this approach, the expression patterns of transgenes containing enhancer/promoter fragments fused to reporters (green fluorescent protein [*GFP*] or *lacZ*) are analyzed. The 5′-UTRs of miRNAs can contain "mini-open reading frames" and consequently, if fused to a reporter, cause its' degradation by nonsense-mediated decay. Thus, if this approach is used, it would be prudent to map the pri-miRNA by 5′-RACE, fuse the reporter to the precise pri-miRNA transcriptional start site, and thereby avoid the inclusion of any pri-miRNA 5′-UTR. Indeed, there is evidence that some *Drosophila* miRNAs are produced from large genomic loci. For example, EP-elements inserted approx 50 kb away from *miR-278* can drive its expression ***(20,21)***.

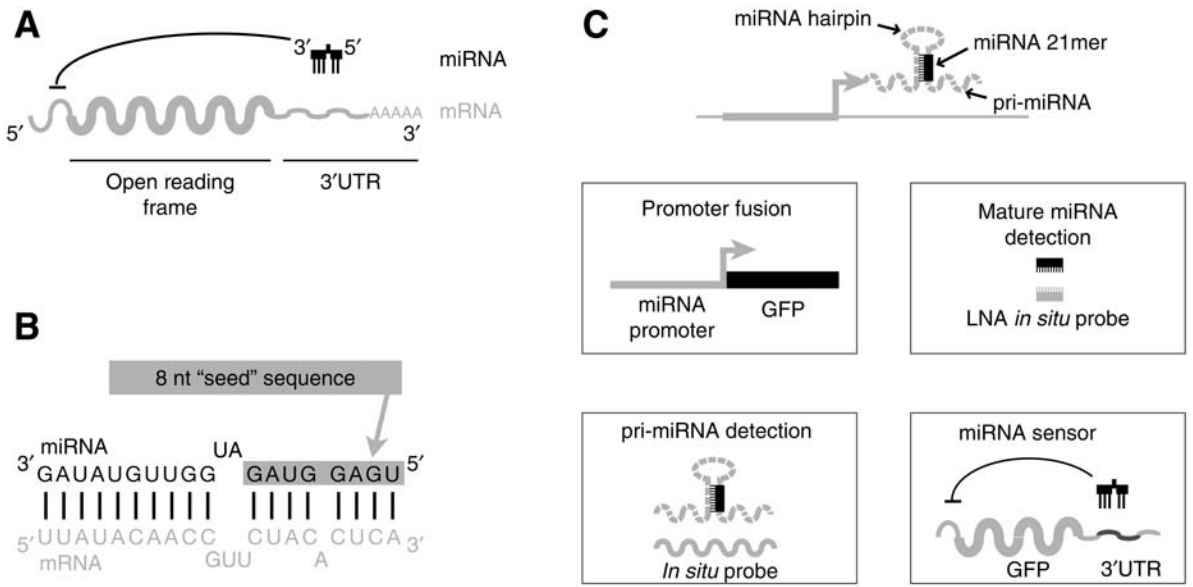

Fig. 1. Schematic of miRNA function and detection methods. **(A)** miRNAs repress gene expression through miRNA-binding sites located in the 3′-UTRs of target mRNAs. **(B)** Target recognition is mediated through the seed sequence, 6–8 nts at the 5′-end of a miRNA. **(C)** Spatiotemporal expression of miRNAs can be detected with miRNA promoter fusions, pri-miRNA *in situ* hybridization, mature miRNA *in situ* hybridization with LNA probes, and miRNA sensor transgenes.

3.3. In Vivo Detection of Mature miRNAs

The spatial expression patterns of mature, approx 21-nt miRNAs have been detected by *in situ* hybridization using LNA-modified DNA probes in a variety of animal tissues ***(22,23)***. LNA is a class of high-affinity RNA analogs that display a high hybridization affinity toward cRNA molecules. Thus, use of LNA-modified DNA probes solved the technical challenge of detecting the very short, processed forms of mature miRNAs. The expression patterns of two processed *Drosophila* miRNAs, *miR-1* ***(16)*** and *miR-7* ***(24)***, have been described. In both cases, digoxigenin-labeled LNA oligos detect the cytoplasmic staining representative of mature miRNA signal, as opposed to the nuclear signal detected by pri-miR probes. Mature *miR-1* is expressed in mesodermal and muscle cells during embryogenesis in a spatiotemporal pattern almost identical to that detected by *pri-miR-1* probes. However, *pri-miR-1* is detected at the cellular blastoderm stage whereas mature *miR-1* is not detected until gastrulation suggesting that processing of *miR-1* might be temporally regulated. Mature *miR-7* is expressed in the morphogenetic furrow as well as the posterior compartment of the larval eye imaginal disc in a pattern identical to a *miR-7* "sensor" ***(24)***. Standard *in situ* hybridization protocols can be followed when using LNA-modified DNA probes except that the hybridization temperature should be adjusted to 20–25°C below the T_m of the LNA oligo ***(22)***. Furthermore, digoxigenin-labeled LNA oligos should be purified with a Sephadex column (Amersham Biosciences, NJ) ***(22)***. LNA oligos are available from Exiqon (Vedbaek, Denmark).

3.4. miRNA Sensors

Although *in situ* hybridization detects the pattern where a specific pri-miRNA or mature miRNA is expressed, a transgenic approach reveals the specific pattern where a miRNA functions ***(25)***. A miRNA "sensor" transgene carries a reporter (e.g., GFP) under the control of a ubiquitous promoter (e.g.,β-tubulin) and a 3′-UTR (e.g., SV40) into which at least two tandem copies of miRNA complementary sequence have been inserted. Coexpression of the sensor mRNA and the miRNA leads to its destruction through RNA interference and thus loss of reporter expression. The expression pattern of this transgene is then compared with (1) its expression in the miRNA mutant background and/or (2) an analogous transgene containing a mutant or deleted miRNA complementary sites. To date, miRNA sensors have been successfully used to detect the expression of functional *bantam* ***(25)***, *miR-5* ***(26)***, *miR-6* ***(26)***, *miR-7* ***(24,26,27)***, *miR–277* ***(28)***, and *miR–278* ***(20,21,29)***.

4. miRNA Targets

MiRNAs regulate gene expression post-transcriptionally, either by modulating the translation of target mRNAs or by mediating their degradation. Identification of

the targets of a particular miRNA is a critical step toward understanding miRNAs function. As known miRNAs regulate gene expression by incomplete basepairing with near complementary sequences within the 3′-UTRs of mRNAs, targets of particular miRNAs should, in principle, be predictable based on the miRNA sequence. This section summarizes the methods used to predict targets of *Drosophila* miRNAs as well as the assays currently used to test these target predictions.

4.1. Identification of miRNA Targets

Seven groups have reported algorithms designed to predict mRNA targets of *Drosophila* miRNAs (*see* **Table 1**) ***(17,27,30–36)***. Six of the groups ***(17,27,30–35)*** used a similar overall strategy consisting of three main steps: (1) assemble a database containing 3′-UTRs encoded by the *D. melanogaster* genome; (2) query this database for sequences complementary to a particular miRNA; (3) determine whether the identified putative miRNA-binding sites are conserved in the orthologous 3′-UTRs of *D. pseudoobscura* and/or more distantly related insects. The main difference between these six studies is how the queried sequence is defined, for example, the number of noncomplementary basepairings allowed between miRNA and mRNA, whether deletions or gaps are allowed in either sequence, whether G:U basepairing is allowed, and so on (for a more detailed analysis of these variables, *see* **refs. *37*** and ***38***). The fifth group ***(36)*** ignored evolutionary conservation but instead took the secondary structure of the 3′-UTR into account: putative binding sites in portions of the 3′-UTR that displayed secondary structure were penalized. For each miRNA, most algorithms produce ranked lists of putative targets based on the number and quality of binding sites their 3′-UTRs contain (*see* **Table 1** for locations of previously computed target predictions). However, Burgler et al. ***(30)*** simply report a collection of 60 *Drosophila* genes that are likely to be regulated by a subset of miRNAs. A few of these algorithms are also available to search user-supplied sequences for putative miRNA-binding sites (*see* **Table 1**).

4.2. Validation of Predicted Targets by miRNA Overexpression

Two main experimental methods have been used to test whether predicted targets contain functional miRNA-binding sites. Both methods rely on the overexpression of the targeting miRNA, either in cultured S2 cells ***(17,19,30,36)*** or in whole animals ***(6,19,20,24–26,29,37)***. In the first method, target 3′-UTRs are fused to the luciferase open reading frame and transfected into S2 cells. Luciferase levels are compared between cells in which a miRNA-expressing construct has either been cotransfected or not and then normalized relative to a second reporter expressed in both cell populations. Most studies conclude that a 3′-UTR contain functional miRNA-binding sites if reporter levels are reduced two- to fivefold ***(17,19,30,36)***.

Table 1
***Drosophila* miRNA Target Predictions**

Algorithm	Website	References
Lists of previously computed target predictions		
MiRanda	http://www.microrna.org/drosophila/targetsv2.html http://microrna.sanger.ac.uk/targets/v3/	***32***
Pictar	http://pictar.bio.nyu.edu/	***33,35***
EMBL	http://www.russell.embl-heidelberg.de/miRNAs/	***17,27***
RNAhybrid	http://www.techfak.uni-bielefeld.de/persons/marc/mirna/targets/drosophila	***34***
Ref.27	http://tavazoielab.princeton.edu/mirnas/	***31***
Moving Targets	No website available (*see* reference for predicted targets)	***30***
Padgett Lab	No website available (*see* reference for predicted targets)	***36***
Software available for target prediction with user-supplied sequence		
MiRanda	http://www.microrna.org//miranda.html	***32***
RNAhybrid	http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html	***34***
DIANA-microT	http://diana.pcbi.upenn.edu/cgi-bin/micro_t.cgi	
Moving Targets	Available on DVD on request	***30***

The second method assays for miRNA:mRNA interaction in whole animals using two transgenes: (1) a reporter transgene in which GFP is fused to the putatively targeted 3′-UTR and expressed throughout the animal under the control of a ubiquitous promoter and (2) a UAS transgene to ectopically express the miRNA in a specific location. MiRNA hairpins can be inserted within the 3′-UTR of dsRed because they are processed efficiently and dsRed can be used to monitor miRNA expression ***(27)***. A 3′-UTR is considered a validated target if reporter levels are reduced in cells in which the targeting miRNA is ectopically expressed relative to neighboring cells where the miRNA is not ectopically expressed ***(6,19,20,24–26,29,37)***.

Variations of this second method have been used in a number of studies in which the effects of ectopic miRNA expression are assayed in other ways. For example, *bantam* regulation of *head involution defective* ***(25)*** and *miR-278* regulation of *expanded* ***(20)*** were examined in cells in which both miRNA and target gene were ectopically expressed. In both cases *in situ* hybridization and antibody staining revealed that target transcripts were expressed but target proteins were not detectable, validating both genes as targets of miRNA-mediated post-transcriptional repression. Two other studies examined the effects of ectopic *miR-1* ***(19)*** and *iab-4* ***(37)*** expression on the endogenous protein levels of putative targets

Delta and Ultrabithorax protein, respectively. In both cases, ectopic miRNA expression resulted in reduced protein levels of the targets in imaginal disc cells examined, and furthermore, resulted in phenotypes (wing vein thickening, homeotic transformation of the haltere) consistent with known phenotypes associated with loss of these proteins.

Experimental analysis testing putative miRNA-binding sites has defined the minimal sequence required to constitute a functional miRNA-binding site. Brennecke et al. ***(29)*** found that basepairing between target and the 6–8 nt sequence at the 5′-end of a miRNA, termed the "seed," was sufficient to mediate target downregulation. Thus, at least two classes of functional miRNA-binding sites exist; one class possesses perfect complementarity to the 5′-end of the targeting miRNA with little complementarity to its 3′-end whereas the second class has reduced complementarity to the 5′-end of the targeting miRNA and compensatory complementarity to its 3′-end. The utility of "seed" sequences was presaged by a series of molecular genetic analyses of a large set of Notch target genes ***(38,39)*** that were noted to contain within their 3′-UTRs three different 6–7-nt motifs, termed the GY-box, the Brd-box, and the K-box, which mediate negative post-transcriptional regulation. Subsequent analysis has demonstrated that these sequences are complementary to the seed sequences of known miRNAs ***(40)*** and indeed function as miRNA-binding sites ***(26,27,29)***.

A recent analysis ***(17)*** collated the number of confirmed 3′-UTRs containing functional miRNA-binding sites validated by methods involving miRNA overexpression (of 133 3′-UTRs tested, 71 contained functional miRNA-binding sites). This finding was then used to assess the success rate of the various target prediction algorithms; three algorithms (Pictar, Moving Targets, and the European Molecular Biology Laboratory [EMBL] predictions) were identified as very accurate (88–94%) in predicting functional miRNA-binding sites ***(17)***. It was furthermore reported that genes containing known miRNA-binding sites generally have longer 3′-UTRs than genes devoid of miRNA-binding sites, usually contain binding sites for multiple miRNAs, and are much more likely to be involved in developmental processes than in basic processes common to all cells.

4.3. Validation of Predicted Targets by miRNA Depletion

Although miRNA overexpression studies indicate that many genes contain functional miRNA-binding sites ***(17,29)***, additional analysis is required to determine whether and in what contexts validated binding sites are biologically relevant. For example, studies examining the effects of *mir-7* overexpression ***(26,27)*** have clearly shown that (1) GY-box motifs within some Notch target genes function as *miR-7*-binding sites and (2) *miR-7* overexpression results in wing notching, a phenotype consistent with reduced Notch pathway signaling. However, *mir-7* mutant flies do not display phenotypes associated with elevated Notch pathway

signaling ***(24)***. Not surprisingly, GY-box containing Notch target genes are known to contain functional binding sites for multiple miRNAs, including *miR-2*, *miR-4*, *miR-6*, *miR-11*, and *miR-79* ***(26,27,40)***, and thus might be regulated redundantly by these miRNAs in vivo. This example illustrates that there is a difference between whether a 3′-UTR contains a functional binding site for a particular miRNA and whether that particular miRNA is required to regulate the expression of the 3′-UTR-containing gene in vivo. Further evaluation of this distinction will come from studies that rely on endogenous levels of miRNAs to test the downregulation of predicted targets. For example, a recent study of the targets of the *Caenorhabditis elegans* miRNA *lsy-6* found that none of the top 13 predicted target 3′-UTRs mediated the downregulation of a reporter transgene when expressed in the particular cells where *lsy-6* is known to function ***(41)***. Genetic analysis of miRNA loss-of-function mutations will ultimately provide the strongest validation for target predictions: if a miRNA is required to repress a target mRNA in vivo, then loss of the gene encoding the mRNA should suppress phenotypes associated with a miRNA loss-of-function mutation (which are presumably caused by misexpression of that mRNA).

To date, two studies in *Drosophila* have used miRNA depletion to validate putative targets. First, expression of a GFP reporter containing the *yan* 3′-UTR was derepressed in a *miR-7* mutant background ***(24)***. Interestingly, *yan* was not reported as a putative *miR-7* target by any of the computational predictions; rather, it was tested as a result of the analysis of *miR-7* expression pattern and the similarity between the *yan* loss-of-function and *miR-7* overexpression phenotypes. Second, a series of GFP reporters containing the *hid*, *reaper*, *grim*, and *sickle* 3′-UTRs were derepressed in embryos in which members of the *miR-2* family (*miR-2/6/11/13/308*) were depleted by injection of 2′ *O*-methyl (2OM) modified oligoribonucleotides (*see* **ref. *8*** and **Subheading 5.2.2.**).

5. Phenotypic Analysis of miRNAs

The function of miRNAs can be determined by examining the phenotypic consequences resulting from the overexpression and/or depletion of miRNA activity. Because miRNA overexpression has largely been used to validate putative miRNA-binding sites, studies analyzing the phenotypic consequences of ectopic miRNA expression are covered in the previous section. This section summarizes the current understanding of miRNA functions resulting from the analysis of (1) pan-miRNA mutant phenotypes caused by mutations that disrupt the miRNA biogenesis pathway and (2) phenotypes caused by depletion of individual miRNAs.

5.1. Phenotypes Associated With a General Loss of miRNAs

The *Drosophila* genome encodes two Dicer orthologs, Dcr-1 and Dcr-2. Mutations in *dcr-1* disrupts the processing of known miRNAs and result in

profound affects on the development of both somatic and germline lineages ***(42)***. Detailed analysis of *dcr-1* function in the germline revealed that *dcr-1* regulates the rate of cell division specifically of germline stem cells in both males and females, but does not play a role in the maintenance of germline stem cell fate ***(43)***. Similarly, mutations in *loquacious*, the gene encoding the *Drosophila* homolog of the human dicer-interacting protein human immunodeficiency virus transactivating response RNA-binding protein, as well as *r3d1-l*, the gene encoding a novel dsRNA-binding protein that physically interacts with Dcr-1, cause reductions in miRNA processing and result in defects in germline stem cells ***(28,44)***. Finally, in addition to its affect on early events in germline development, *dcr-1* may control additional events later in oogenesis; mature *dcr-1* mutant oocytes are ventralized and abnormally small ***(45)***. A proteomics comparison of wild-type and *dcr-1* mutant oocytes identified a set of 22 proteins, which are upregulated in the *dcr-1* mutant and whose translation might be directly regulated by miRNAs ***(45)***.

5.2. Phenotyes Associated With Loss of Individual miRNAs

The two methods used to study particular miRNA loss-of-function phenotypes, genetic knockouts, and antisense-oligo-mediated depletion, are covered here.

5.2.1. miRNA Mutants

Genetic loss-of-function mutations in five miRNAs have been reported to date ***(16,19–21,24,25,46)***. Two miRNA loci, *bantam* ***(47)*** and *miR-278* ***(20,21)***, were identified from modular misexpression P-element screens designed to detect genes that, when ectopically expressed, cause cellular overgrowth. Deletions removing *bantam* were subsequently generated by P-element mobilization ***(25)***. Strikingly, *bantam* levels correlate with rates of cell proliferation; *bantam* mutant larvae display decreased proliferation of larval tissues, lack imaginal discs, and die shortly after entering pupation. As *bantam* overexpression is associated with the inhibition of apoptosis and the repression of endogenous Hid protein levels, the decreased proliferation of *bantam* mutant cells might be the result of a concomitant increase in apoptosis caused by ectopic Hid expression.

Unlike *bantam*, the P-elements used to overexpress *miR-278* were located 45–50 kb away from *miR-278* and could not be used to generate loss-of-function mutations. Two groups isolated null mutations of *miR-278* by independent methods: point mutants were isolated from a genetic screen for revertants of the *miR-278* overexpression phenotype ***(21)*** and a gene knockout was generated using ends-out homologous recombination ***(20)***. Homozygous *miR-278* mutants survive to adulthood and, although wild-type in size, weigh approx 50% as much as age-matched wild-type adults owing to increases in their insulin levels ***(20)***. These elevated insulin levels may, in part, be resulting from misexpression of *expanded* because (1) *expanded* contains functional *miR-278*-binding sites and

(2) ectopic expression of *expanded* in a *miR-278*-expression pattern phenocopies the leanness phenotype of *miR-278* loss-of-function mutants ***(20)***. The molecular mechanism by which *expanded* affects insulin levels awaits further study.

Like *bantam* and *miR-278*, P-elements affecting *miR-14* were identified from a forward genetic screen, designed to identify loss-of-function mutations that enhance cell death phenotypes caused by the ectopic expression of the proapoptotic protein Reaper ***(48)***. As increases in *miR-14* levels suppress cell death caused by multiple stimuli, *miR-14* functions in part to regulate cell death. Interestingly, *mir-14* mutants are viable and fertile, although they are stress sensitive and display a reduced life-span ***(48)***.

Two groups took a reverse genetic approach to study the function of *miR-1* ***(16,19)***, a miRNA whose sequence and muscle expression pattern are conserved to vertebrates. Loss-of-function mutations disrupting the *miR-1* locus were generated, either by ends-in homologous recombination resulting in a 54-bp deletion ***(16)*** or by recombination between P-elements resulting in a 31-kb deletion ***(19)***. Most *miR-1* mutants survive embryogenesis, die as 2nd instar larvae, and can be rescued to adulthood by muscle-specific expression from a *UAS-miR-1* transgene. Strikingly, larval growth triggers *miR-1* associated paralysis and death; starved first instar *miR-1* mutant larvae are essentially normal ***(16)***. Thus, *miR-1* is not essential for the formation or physiological function of the larval musculature, but is required for the dramatic postmitotic growth of larval muscle.

MiR-7, another *Drosophila* miRNA whose sequence is conserved to vertebrates, was also the subject of a reverse-genetics approach ***(24)***. *MiR-7* is expressed in differentiating photoreceptor cells and misexpression of *miR-7* in progenitor cells, where the ETS-domain transcription factor Yan is expressed, causes the ectopic differentiation of photoreceptors. Evidence indicates this phenotype is caused by the repression of *yan* directly by *mir-7*: *miR-7* overexpression phenocopies *yan* loss-of-function phenotypes, endogenous Yan levels are reduced in *miR-7* expressing cells, the *yan* 3′-UTR contains functional *miR-7*-binding sites, and Yan protein persist in *miR-7* mutant cells. Because Yan represses *miR-7* transcription, Yan and *miR-7* form a reciprocal negative feedback loop assuring the normal differentiation of photoreceptors. Interestingly, mutants homozygous for a *mir-7* deletion, generated by P-element excision, are viable and do not display defects in photoreceptor differentiation suggesting that there are additional redundant features within this feedback loop ***(24)***.

5.2.2. 2OM Antisense Oligoribonucleotide Injections

The sequence-specific depletion of miRNA activity by 2OM modified oligoribonucleotides (2OM-ORNs) provides a powerful complement to traditional genetic approaches in determining the in vivo function of miRNAs. 2OM-ORNs are resistant to degradation in cellular extracts and presumably affect miRNA activity by disrupting the interaction between miRNAs and their targets ***(49)***. A recent study

systematically assessed the function of all known embryonically expressed miRNAs using 2OM-ORNs ***(8)***. Of 46 embryonically expressed miRNAs tested, depletion of 25 show readily discernible defects: for example, loss of *miR-9* disrupts cellularization, loss of *miR-31* disrupts segmentation, loss of *miR-310* disrupts dorsal closure, and loss of *miR-2*, *miR-13*, and *miR-6* results in increased apoptosis. Substantial evidence indicates that the phenotypes resulting from 2OM-ORN injection are caused by loss of specific miRNA function: depletion of miRNA family members that share 5′-seed sequences results in similar but distinct phenotypes; injection of 2OM-ORNs encoding scrambled or sense miRNA sequences have no effect; increased miRNA expression from a transgene rescues the phenotype associated with depletion of that miRNA. Indeed, in most cases, phenotypes resulting from miRNA depletion either by 2OM-ORN injection or genetic knockout match each other. Homozygous *miR-7* ***(24)***, *miR-14* ***(48)***, and *bantam* ***(25)*** mutants proceed normally through embryonic development and similarly, 2OM-ORN injection of these three miRNAs results in normal embryos. However, results differ in the case of *miR-1*. Depletion of *miR-1* by 2OM-ORN injection results in a highly penetrant arrest at cellularization ***(8)***, a stage before the appearance of pri- and mature *miR-1* expression ***(16)***. In contrast, most *miR-1* mutants proceed through embryogenesis and die as larvae ***(16,19)***. Thus, injection of 2OM-ORNs antisense to *miR-1* result in neomorphic phenotypes not associated with depletion of *miR-1*. This discrepancy indicates that additional, interesting biological consequences of 2OM-ORN injection remain to be discovered.

6. Final Thoughts

A recent study proposed a general model for miRNA function based on the expression patterns of *Drosophila* miRNAs as well as the expression patterns of target mRNAs containing validated miRNA-binding sites ***(17)***. MiRNAs and their targets are usually expressed in neighboring tissues. Conversely, mRNAs expressed in the same tissue as a miRNA tend not to contain binding sites for it. Such mutually exclusive expression suggests that miRNAs likely function to reinforce developmental gene-expression programs, thus supporting cell-lineage decisions and ensuring tissue identity. This is a compelling model and one that is consistent with functional data from miRNA mutants. Nevertheless, given the number of *Drosophila* miRNAs and the variety of their collective expression patterns, miRNAs are likely involved in an unanticipated range of diverse biological processes. Indeed, much exciting work remains to achieve a comprehensive understanding of the roles that *Drosophila* miRNAs play.

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21

Extraction and Immunoblotting of Proteins From Embryos

Andreas Wodarz

Summary

For many decades, *Drosophila* has been used as a model system primarily for studies in the fields of genetics and developmental biology. Relatively little attention has been given to the potential of *Drosophila* as a model system for biochemistry. However, *Drosophila* embryos as a source for biochemical material offer some unique advantages as compared with cultured cells or tissue samples. For instance, mutant *Drosophila* embryos can be sorted before protein extraction and compared with wild-type embryos by using green fluorescent protein-marked balancer chromosomes. Studies of this kind can give important information on the effect of a mutation on the biochemical properties of a protein, which cannot be obtained in experiments using cultured cells or conventional tissue samples ***(1,2)***. Transgenic *Drosophila* embryos expressing a tagged version of a protein can be used to isolate and identify interaction partners of the tagged protein from a whole organism rather than from a specific cell line that expresses only a limited set of genes ***(3,4)***. Thus, it is the combination of genetics and transgenic approaches that offers unique opportunities for biochemical studies in the fruit fly. In this chapter, I describe methods to extract proteins under denaturing and nondenaturing conditions from embryos, and to perform sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunoblotting, and coimmunoprecipitation.

Key Words: Coimmunoprecipitation; *Drosophila melanogaster*; immunoblotting; protein extraction; SDS-PAGE; stripping and reprobing of blots.

1. Introduction

Drosophila embryos are easy to collect and protein extracts can be made from quantities ranging from a single embryo up to several 100 g of embryos ***(5,6)***. The protein extraction procedure differs depending on the experiments intended with the extract. Following protein extraction, the extracts can either be directly analyzed by immunoblotting or can be subjected to protein fractionation, enzyme assays, coimmunoprecipitation experiments, and so on.

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2. Materials

2.1. Collection of Embryos

1. Nipagin (methyl 4-hydroxybenzoate) solution: 100 g nipagin, 700 mL 96% ethanol pro analysi, and 300 mL water. Mix until nipagin has completely dissolved. Store at room temperature.
2. Apple juice agar plates: 40 g agar–agar, 17 g sucrose, 340 mL apple juice, 1 L water, and 20 mL nipagin solution. Mix agar–agar, sucrose, water, and apple juice and heat in microwave until everything is dissolved. Cool down to 60°C and add nipagin solution. Pour into Petri dishes on level surface. Let the agar cool down until it is solid. Apple juice agar plates can be stored at 4°C.
3. Yeast paste: mix bakers yeast with a small amount of water until it has a cream cheese-like consistency. Fill into a 20-mL syringe and store at 4°C.
4. 5% Sodium hypochlorite (*see* **Note 1**).
5. Egg collection cages. We make them from plastic cups that fit snugly on a Petri dish with apple juice agar. It is important to poke numerous small holes into the plastic to allow the exchange of air.
6. Nylon mesh, 80 µm mesh opening (Small Parts Inc., Miami Lakes, FL)
7. Funnel with wire mesh that holds back flies and larvae.
8. Vacuum flask with bottle-top filter unit (**Fig. 1**).
9. Paint brush.

2.2. Extraction of Proteins Under Denaturing Conditions (see Note 2)

1. Sodium dodecyl sulfate (SDS) sample buffer (2X): 100 m*M* Tris-HCl (pH 6.8), 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200 m*M* β-mercaptoethanol. Store at –20°C.
2. Dounce homogenizer (Fisher Scientific, Pittsburgh, PA). The size of the homogenizer depends on the volume of the embryo suspension to be homogenized.
3. Microhomogenizer with disposable plastic pestles, battery-driven (Kleinfeld Labortechnik, Gehrden, Germany). This homogenizer can be used for the homogenization of small samples in an Eppendorf tube.

2.3. Extraction of Proteins Under Nondenaturing Conditions

1. Lysis buffer: 1% Triton X-100, 50 m*M* Tris-HCl (pH 8.0), 150 m*M* NaCl, and protease inhibitor cocktail (Roche, Mannheim).
2. Dounce homogenizer. The size of the homogenizer depends on the volume of the embryo suspension to be homogenized.
3. Bradford protein assay solution.

2.4. SDS-Polyacrylamide Gel Electrophoresis

1. 30% Acrylamide/*bis* (29:1) (*see* **Note 3**).
2. 1 *M* Tris-HCl (pH 8.8).
3. 1 *M* Tris-HCl (pH 6.8).
4. 20% SDS.

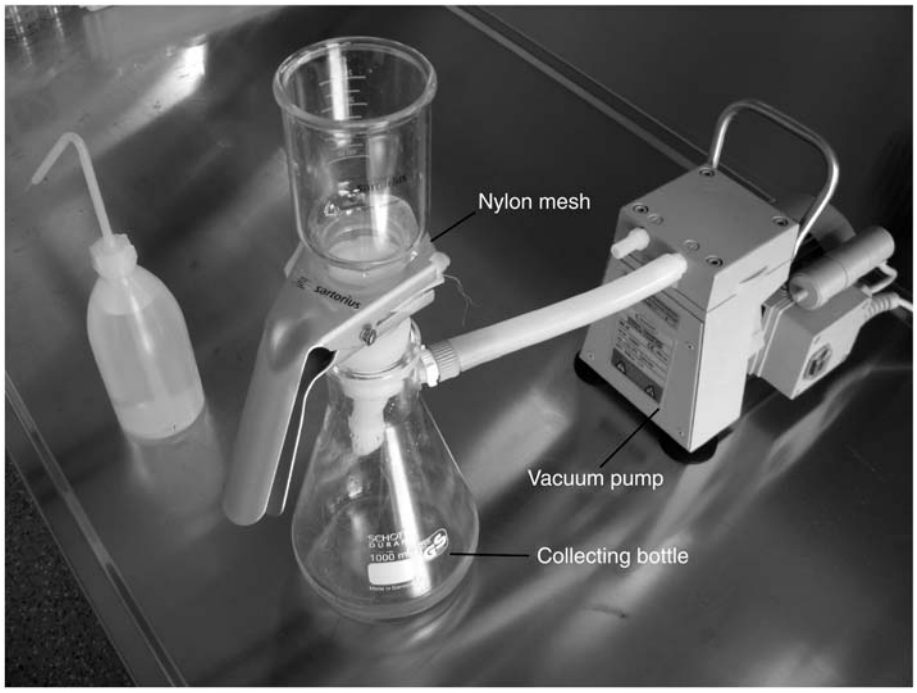

Fig. 1. The vacuum unit for collecting and washing embryos.

5. 10% Ammonium persulfate (APS). Store in aliquots at −20°C.
6. N,N,N,N′-Tetramethyl-ethylenediamine (TEMED). Store at 4°C.
7. Isopropanol.
8. Running buffer (10X): 250 m*M* Tris, 1.92 *M* glycine, and 1% SDS.
9. Prestained molecular-weight markers.
10. Hamilton syringe (Hamilton, Bonaduz, Switzerland).

2.5. Immunoblotting

1. Western transfer buffer: 25 m*M* Tris, 192 m*M* glycine, and 20% methanol. Prepare 10X stock solution with 250 m*M* Tris and 1.92 *M* glycine. Dilute 1 part of 10X stock solution with seven parts of water before adding two parts of methanol.
2. Nitrocellulose transfer membrane (0.45 μm) (PROTRAN, Schleicher and Schuell Dassel, Germany).
3. Gel blotting paper (Whatman, Schleicher and Schuell).
4. Tris-buffered saline with Tween (TBS-T): 20 m*M* Tris-HCl (pH 8.0), 150 m*M* NaCl, and 0.2% Tween-20. Prepare from 10X TBS stock solution (200 m*M* Tris-HCl (pH 8.0), and 1.5 *M* NaCl), add Tween-20 separately.
5. Blocking buffer: 3% (w/v) nonfat dry milk and 1% (w/v) bovine serum albumin fraction V in TBS-T.

6. Ponceau S staining solution (Bio-Rad, Munich, Germany).
7. Primary antibody.
8. 2% Sodium azide (*see* **Note 4**).
9. Sealable plastic bags (Rische, Herfurth, Germany).
10. Secondary antibody that binds to primary antibody, conjugated with horseradish peroxidase (HRP [Jackson Immunoresearch, Dianova, Hamburg]).
11. BM chemiluminescence blotting substrate peroxidase (Roche).
12. Saran wrap (Johnson, Racine, WI).
13. X-ray film (Fuji, Düsseldorf, Germany).

2.6. Stripping and Reprobing Blots

1. Stripping buffer: 62.5 m*M* Tris-HCl (pH 6.7) and 2% SDS. Store at room temperature. Add β-mercaptoethanol to 100 m*M* after warming the buffer to working temperature of 60°C (*see* **Note 5**).
2. Wash buffer (TBS-T): 20 m*M* Tris-HCl (pH 8.0), 150 m*M* NaCl, and 0.2% Tween-20. Prepare from 10X TBS stock solution, add Tween-20 separately.

2.7. Coimmunoprecipitation of Proteins

1. Lysis buffer: 1% Triton X-100, 50 m*M* Tris-HCl (pH 8.0), 150 m*M* NaCl, and protease inhibitors (Roche).
2. Protein A agarose beads (Roche).
3. 2-mL Syringe with 23-gauge needle.

3. Methods

The methods outlined here are only a starting point for biochemical experiments using *Drosophila* embryos as the source for protein extraction. More advanced methods like protein fractionation, enzymatic assays, or affinity purification of protein complexes may require different buffer conditions and protocols and are beyond the scope of this chapter. In general, it is highly recommended to try out several alternative lysis buffers and homogenization procedures to determine the optimal conditions for working with your protein of interest.

3.1. Collection of Embryos

1. Streak a thin layer of yeast paste onto the apple juice agar plates.
2. Anesthetize an appropriate number of flies and transfer them to an egg collection cage. Place an apple juice agar plate at the bottom of the cage and allow the flies to lay eggs onto the agar plate. The length of the egg collection period can be varied between 30 min and up to 20 h, depending on the desired developmental stage of the embryos.
3. Remove the agar plate from the cage.
4. Add tap water from a squeeze bottle to the agar plate and resuspend the eggs that got stuck in the yeast paste with a paintbrush.
5. Transfer the slurry of eggs and yeast to a vacuum filter unit (Sartorius, Göttingen, Germany) covered with a nylon mesh (mesh size 80 μm) that holds back eggs but

lets yeast cells pass **(Fig. 1)**. The transfer can either be done by pouring or by using a disposable transfer pipet. If there are dead flies or larvae on the agar plate, use a funnel with a wire mesh for the transfer to the filter unit.
6. Wash the eggs on the nylon mesh in the filter unit extensively with tap water by turning on the vacuum to remove yeast cells that are stuck to the eggs.
7. Turn off the vacuum and cover the eggs with sodium hypochlorite for 5 min to remove the chorion.
8. Turn the vacuum on again and wash the embryos extensively with tap water to remove the sodium hypochlorite.
9. Put the nylon mesh with the embryos onto a paper towel to remove excess water and transfer the embryos to a dounce homogenizer (*see* **Note 6**) with a paint brush.

3.2. Extraction of Proteins Under Denaturing Conditions

1. Transfer the dechorionated embryos to a cooled dounce homogenizer in an ice bath (*see* **Note 7**).
2. Add an appropriate volume of 2X SDS sample buffer to the embryos. As a rule of thumb, the volume of the SDS sample buffer should be about five times the volume of the packed embryos.
3. Homogenize the embryos in SDS sample buffer by 5–10 strokes with a tight fitting pestle.
4. Allow the foam to settle and transfer the homogenate to one or more Eppendorf tubes (*see* **Note 8**).
5. Heat the embryo extract to 100°C in a boiling water bath or in a heat block for 5 min (*see* **Note 9**).
6. Cool the extract briefly on ice and spin in a tabletop centrifuge (Eppendorf centrifuge 5417c [Eppendorf, Hamburg, Germany]) at maximum speed (20,000*g*) for 5 min.
7. Load an aliquot of the supernatant into a well of an SDS polyacrylamide gel (*see* **Note 10**).

3.3. Extraction of Proteins Under Nondenaturing Conditions

1. Transfer the dechorionated embryos to a cooled dounce homogenizer in an ice bath (*see* **Note 7**).
2. Add an appropriate volume of lysis buffer to the embryos. As a rule of thumb, the volume of the lysis buffer should be about five times the volume of the packed embryos.
3. Homogenize the embryos in lysis buffer by 5–10 strokes with a tight fitting pestle.
4. Transfer the homogenate to one or more Eppendorf tubes.
5. Spin the homogenate in a tabletop centrifuge cooled to 4°C at maximum speed (20,000*g*) for 10 min.
6. Transfer the supernatant to a new Eppendorf tube (*see* **Note 11**). At this point, you can freeze the extract at −80°C for future use.
7. Determine the protein concentration of the supernatant by measuring the OD_{595} after mixing an aliquot of the supernatant with Bradford reagent. For details on the protocol see the manual of the manufacturer.

8. For loading a well of an SDS polyacrylamide minigel, take an aliquot of the supernatant containing 10–50 µg of total protein and mix it with an equal volume of 2X SDS sample buffer. The total volume should not exceed 20 µL if you use a minigel for SDS-polyacrylamide gel electrophoresis.
9. Heat the sample to 100°C in a boiling water bath or in a heat block for 5 min (*see* **Note 9**).
10. Cool the sample briefly on ice and spin in a tabletop centrifuge at maximum speed (20,000*g*) for 5 s.
11. Load the whole sample into a well of an SDS polyacrylamide minigel.

3.4. SDS-Polyacrylamide Gel Electrophoresis

1. The volumes given in this protocol are optimized for the Mini Protean electrophoresis system (Bio-Rad) with 1-mm spacers but can easily be adjusted to other gel sizes. Make sure that the glass plates for the gels are thoroughly cleaned and rinsed extensively with distilled water.
2. To prepare a 10% separating minigel, mix the following: 2.5 mL 30% acrylamide/*bis* (29:1), 2.8 mL 1 *M* Tris-HCl (pH 8.8), 38 µL 20% SDS, 2.1 mL water, 30 µL 10% APS, and 8 µL TEMED. Pour the gel immediately after mixing the reagents, leaving enough space for the stacking gel. Overlay the gel with isopropanol to get a smooth upper edge of the gel. The gel should polymerize within 30 min (*see* **Note 12**). To prepare gels with different percentages of acrylamide, adjust the volumes of 30% acrylamide/*bis* (29:1) and water accordingly.
3. When the gel is completely polymerized, pour off the isopropanol and remove residual isopropanol with a paper towel.
4. Prepare the stacking gel by mixing the following: 310 µL 30% acrylamide/*bis* (29:1), 235 µL 1 *M* Tris-HCl (pH 6.8), 10 µL 20% SDS, 1.3 mL water, 10 µL 10% APS, and 5 µL TEMED. Pour the stacking gel on top of the separating gel and insert the comb, making sure that no air bubbles get stuck at the base of the comb. The stacking gel should polymerize within 30 min (*see* **Note 13**).
5. Prepare the running buffer by diluting 100 mL of 10X running buffer with 900 mL of water in a measuring cylinder. Cover with parafilm and invert to mix.
6. Mount the polymerized gel in the electrophoresis apparatus. Fill the upper and lower chambers of the apparatus with running buffer. Make sure that the lower edge of the separating gel is well submerged in the buffer in the lower chamber. The level of the running buffer in the upper chamber should be at least 1 cm above the edge of the stacking gel.
7. Pull the comb and rinse the wells carefully with running buffer, using a syringe with a 22-gauge needle.
8. Load the wells with your samples using a Hamilton syringe. Alternatively, you can also use an automatic pipet with a pointed tip for loading the gel. Include one well for the prestained molecular-weight marker.
9. Connect the electrophoresis apparatus to a power supply and run the gel at 200 V. The gel run will be completed after approx 1 h.

3.5. Immunoblotting

1. Cut the nitrocellulose membrane and the gel blotting paper to the size of the separating gel.
2. Wet the nitrocellulose membrane by floating on water for 2–3 min (*see* **Note 14**).
3. Submerge the nitrocellulose membrane in water for 1 min.
4. Incubate the nitrocellulose filter in Western transfer buffer for 2 min.
5. Remove the gel from the electrophoresis apparatus and separate the glass plates carefully. Cut the gel at the border between the stacking gel and the separating gel with a razor blade and discard the stacking gel.
6. Assemble the blot in the following order in a tray with Western transfer buffer:
   - Foam pad.
   - Two sheets of gel blotting paper.
   - SDS gel (separating gel only).
   - Nitrocellulose membrane.
   - Two sheets of gel blotting paper.
   - Foam pad.

   During this procedure, the whole sandwich should be covered by buffer.
7. Insert the sandwich into the gel holder of the blotting apparatus.
8. Place the gel holder into the transfer chamber filled with Western transfer buffer: transfer is from (–) to (+), the gel points to the (–) pole, the nitrocellulose filter to the (+) pole.
9. Transfer for 1 h at 100 V in the cold room at 4°C (or use BioIce cooling unit filled with ice [Bio-Rad]).
10. Disassemble the sandwich, place the nitrocellulose filter in a tray with water.
11. Discard water, add Ponceau S staining solution for 1 min.
12. Remove Ponceau S staining solution (can be reused many times) and wash the blot two to three times in water. Protein bands should be clearly visible now (*see* **Note 15**).
13. Block the filter in blocking buffer for 30 min.
14. Dilute the primary antibody to the desired concentration in blocking buffer (2–3 mL of antibody solution is sufficient for a minigel) (*see* **Note 16**).
15. Transfer the blocked filter into a plastic bag with antibody solution. Make sure to remove air bubbles before sealing by streaking the bag along a vertical surface with the opening of the bag pointing upward. Incubate the nitrocellulose filter with the primary antibody for 2–3 h at room temperature or overnight at 4°C.
16. Transfer the filter to a tray and wash the filter three to four times (5 min each) in TBS-T on a rocking platform.
17. Prepare the secondary antibody solution in blocking buffer. For enhanced chemiluminescence detection use HRP-conjugated secondary antibodies at 1:10,000. About 10–20 mL antibody solution is sufficient for a filter from a minigel.
18. Incubate the filter in secondary antibody solution for 1 h at room temperature in a tray on a rocking platform.
19. Wash the filter three to four times in TBS-T on a rocking platform (5 min each). During the first wash, prepare the BM chemiluminescence blotting substrate (Roche). Three milliliters are sufficient for a filter from a minigel.

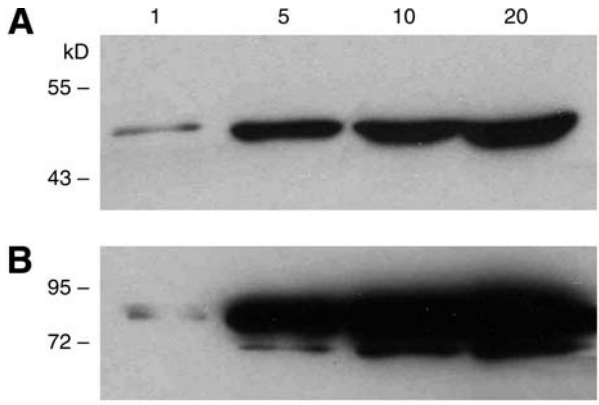

Fig. 2. Western blotting from single embryo extracts. Extracts prepared according to **Subheading 3.2.** from 1, 5, 10, and 20 embryos were run on a 10% SDS polyacrylamide gel and blotted with antibodies against actin **(A)** and atypical protein kinase C **(B)**. Note that even the extract of a single embryo contains sufficient amounts of protein to detect a band on a Western blot.

20. Incubate the filter in BM chemiluminescence blotting substrate for 1 min.
21. Wrap the filter in Saran wrap and expose to X-ray film for 10 s to several min, depending on the intensity of the chemiluminescence signal **(Fig. 2)** (*see* **Note 17**).

3.6. Stripping and Reprobing Blots

1. Blots can be stripped of bound antibodies and can subsequently be reprobed with different antibodies (*see* **Note 18**).
2. Heat stripping buffer to 60°C and add β-mercaptoethanol to 100 m*M* and the blot in a tray. Use at least 20 mL of stripping buffer for each blot. Agitate the tray gently on a rocking platform for 30 min (*see* **Note 5**).
3. After stripping wash the blot extensively in wash buffer. Use at least 100 mL of wash buffer and wash three times for 10 min each.
4. The blot can now be blocked again in blocking buffer and can be incubated with a primary antibody as described in **Subheading 3.5.**

3.7. Coimmunoprecipitation of Proteins

1. Transfer an aliquot of a protein extract containing 500 µg–2 mg of total protein that was prepared under nondenaturing conditions (*see* **Subheading 3.3.**) to an Eppendorf tube. The volume of the sample should be between 500 µL and 1 mL. If necessary, the volume of the sample can be adjusted to the desired volume by adding ice cold lysis buffer containing protease inhibitors.
2. Preincubate the protein extract with 30 µL of protein A agarose beads for 2 h at 4°C on a rocking platform. This step should eliminate proteins that bind unspecifically to the protein A agarose beads.

3. Spin down the protein A agarose beads in a table top centrifuge at maximum speed (20,000*g*) for 2 min at 4°C.
4. Transfer the supernatant to a fresh tube and discard the tube with the pellet.
5. Add the antibody against your protein of interest to the supernatant (*see* **Note 19**). Incubate at 4°C on a rocking platform for 4 h or overnight.
6. Add 30 µL of protein A agarose beads and incubate for 2 h on a rocking platform at 4°C.
7. Collect the beads by centrifugation at maximum speed (20,000*g*) in a tabletop centrifuge at 4°C for 15 s (*see* **Note 20**).
8. Remove the supernatant as completely as possible by aspiration with a 23-gauge needle attached to a syringe or a vacuum pump. The needle should be inserted directly into the beads.
9. Wash the beads three times with ice cold lysis buffer and aspirate the supernatant each time as described in **step 8** of **Subheading 3.7.** (*see* **Note 21**).
10. After the last wash step, add 20 µL of 2X SDS sample buffer to the beads.
11. Heat the sample to 100°C in a boiling water bath or in a heat block for 5 min (*see* **Note 9**).
12. Cool the sample briefly on ice and spin in a tabletop centrifuge at maximum speed (20,000*g*) for 15 s.
13. Load the whole supernatant of the sample into a well of an SDS polyacrylamide minigel using a Hamilton syringe.

4. Notes

1. Wear a labcoat and gloves when handling sodium hypochlorite to avoid bleaching of your clothes.
2. This procedure can be used if you simply want to detect a protein on a Western blot. The SDS sample buffer has the advantage that it solubilizes many proteins that are insoluble under milder conditions, for example, in a lysis buffer containing Triton X-100. As the proteins are completely denatured by the SDS in the sample buffer, this method is not suitable for coimmunoprecipitation or any kind of enzymatic assay.
3. Acrylamide is a neurotoxin! Always wear gloves when handling acrylamide and avoid exposure to skin.
4. Sodium azide is highly toxic! Always wear gloves when handling solutions containing sodium azide.
5. β-mercaptoethanol has a very unpleasant smell. Use a container with a tight fitting lid and do the stripping under a fume hood.
6. If you have only a small amount of embryos (even the protein content of a single embryo might be sufficient for a Western blot), transfer the embryos to an Eppendorf tube and use a battery driven microhomogenizer instead of a Dounce homogenizer.
7. The preparation and subsequent handling of protein extracts should always be performed on ice to minimize the activity of proteases.
8. At this step the protein extracts can be frozen at −20°C for future use.
9. It is very useful to secure the lids of the Eppendorf tubes with little clamps to avoid popping of the lids and evaporation of the sample during the boiling.

10. The determination of the protein concentration of samples containing high concentrations of SDS is not very reliable with most standard assays, including the Bradford method. Thus, the volume of the sample to be loaded onto the gel either has to be determined empirically, or special detection reagents suitable for measuring samples with high SDS content have to be used.
11. For some applications (e.g., purification of proteins by chromatography or fractionation by sucrose gradient centrifugation) it is recommended to spin the supernatant at 100,000*g* for 1 h at 4°C in an ultracentrifuge to pellet all insoluble proteins.
12. To determine when the gel has completely polymerized, it is useful to keep the remainder of the mixed gel solution in a beaker. As soon as this liquid has hardened, the gel should also have polymerized.
13. Depending on the temperature in the room, the stacking gel can polymerize quite rapidly. It is therefore important to pour the gel immediately after adding APS and TEMED.
14. This step is important to fill the pores of the filter with water by capillary forces. If the filter is submerged right away, the pores may remain filled with air, which can result in poor transfer efficiency.
15. The Ponceau S staining is important to check the gel run and the transfer quality. For documentation purposes, the filter can be photographed at this time. If you do not use prestained molecular weight markers, you can mark the bands of the conventional marker with a ball pen at this step. The Ponceau S staining is fully reversible and will disappear after incubation in blocking buffer.
16. If you have only a small amount of a precious antibody, you can reuse the antibody solution many times. In this case, add sodium azide to a final concentration of 0.01% to prevent growth of bacteria or fungi in the solution.
17. It is very important to take several different exposure times for each blot. If you have no clue about the chemiluminescence intensity of your blot, start with a 10 s exposure. While you develop the first film, expose a second film for a longer time. Once you have checked the result of the first exposure, you can decide whether you need additional exposures for different lengths of time. A quantitative comparison of the intensity of different bands on the same blot can only be performed if the bands are still gray and not black already.
18. If you want to reprobe the blot with an antibody raised in a species different from the one used in the first experiment, you often do not even need to strip the blot. Instead, you can destroy the activity of the HRP enzyme conjugated to the secondary antibody used in the first incubation by blocking the blot in blocking buffer containing 0.01% sodium azide. Dilute the alternative primary antibody in blocking buffer containing 0.01% sodium azide and proceed according to **Subheading 3.5.**, from **step 15** onwards.
19. As a rule of thumb, take 1–5 µL of a crude serum or 10–100 µL of a hybridoma supernatant containing a monoclonal antibody *(7)*. The optimal volume depends on the antibody titer of the serum or supernatant and on the abundance of the protein to be immunoprecipitated in the protein extract.

20. It is sufficient to accelerate the centrifuge until it has just reached maximum speed before stopping it again.
21. In each wash step, mix the beads with the wash buffer by gently inverting the tube until all the beads are in suspension again. Do not vortex the beads, as weak protein–protein interactions may not survive this treatment.

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Purification of *Drosophila* Protein Complexes for Mass Spectrometry

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Summary

Drosophila melanogaster is one of the best characterized model systems for genetic analysis. Protein biochemical methods have lagged behind for quite some time but meanwhile have reached a state where protein interaction networks can be elucidated at a similar speed and accuracy as genetic interactions. Therefore, *Drosophila* now offers the advantages of both genetic and biochemical approaches.

Here, we present a basic method for the purification of the endogenous Par-6/aPKC protein complex, which plays a central role in orchestrating asymmetric cell divisions in the developing nervous system of *Drosophila*. The procedure can be subdivided into the following steps: acquisition of sufficient starting material, complex stabilization by crosslinking (optional), purification of the protein complex by immunoprecipitation, separation of the isolated material on a polyacrylamide gel, sample preparation for mass spectrometry, and sample analysis. The protocol can easily be adapted to different affinity-tagged or endogenous protein complexes of interest.

Key Words: Asymmetric cell division; atypical protein kinase C (aPKC); crosslinking; *Drosophila melanogaster*; immunoprecipitation; mass spectrometry; partitioning-defective 6 (Par-6); protein complex purification; protein–protein interaction.

1. Introduction

Drosophila melanogaster has proven to be an ideal model system for the genetic analysis of developmental processes for decades. In recent years, however, the methods for purification and identification of protein complexes have technically advanced at such a pace that they now are ideally suited to complement the genetic approaches for identifying pathways and interaction networks in flies. Proteomics in *Drosophila* has been enabled by the combination of the following technologies: the introduction of affinity tags for protein purification,

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the generation of transgenic flies, and the improvement of mass spectrometric methods. The following protocol describes, as an example, the isolation of a protein complex that plays a central role in asymmetric cell division, the Par-6/aPKC protein complex (for review, *see* **ref. *1***).

2. Materials

2.1. Embryo Extract Preparation

1. Apple juice plates: dissolve 70 g agar (high gel-strength powder research grade, Serva, Heidelberg, Germany in 3 L of water and let the solution cool down to about 60°C. Separately dissolve 100 g household sugar in 1 L of apple juice at 60°C in a water bath and add 40 mL of a 15% (w/v) methyl-4-hydroxybenzoate (methyl paraben, Sigma, St. Louis, MO) in ethanol solution (*see* **Note 1**). Mix the apple juice solution with the agar solution and pour into 90-mm Petri dishes.
2. Yeast paste: mix dry active yeast with water so that the mixture has a consistency like peanut butter.
3. Fly collection cages with flies: at least 2 d before the experiment put about eight bottles of young flies (2–3 d old) per cage (9 cm diameter, 16 cm height, *see* **Note 2**). Feed the flies twice a day with yeast paste streaked out in a thin layer on an apple juice plate.
4. Embryo wash buffer: 120 m*M* NaCl and 0.1% (v/v) Triton X-100.
5. 50% (v/v) Household bleach.
6. Embryo collection net, 24 mm diameter, 74-µm mesh size (Netwell insert, Corning, Acton, MA).
7. 100X Complete protease inhibitor mix (CPIM, Roche, Mannheim, Germany): dissolve one CPIM tablet in 500 µL of water and store at –20°C.
8. 100X Phenylmethylsulfonylfluoride solution (PMSF): 100 m*M* PMSF in ethanol. Store in aliquots at –20°C (*see* **Note 3**).
9. 10X Extraction buffer: 250 m*M* Tris-HCl, pH 8.0 (*see* **Note 4**), 275 m*M* NaCl, 200 m*M* KCl, 250 m*M* sucrose, 100 m*M* ethylenediamine-tetraacetic acid (EDTA), and 100 m*M* ethylene glycol-*bis*(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) (*see* **Note 5**).
10. Lysis buffer: 25 m*M* Tris-HCl, pH 8.0, 27.5 m*M* NaCl, 20 m*M* KCl, 25 m*M* sucrose, 10 m*M* EDTA, 10 m*M* EGTA, 0.5% (v/v) Nonidet P 40 Substitute (NP-40), and 10% (v/v) glycerol. Immediately before use, add 1 m*M* DL-dithiothreitol (DTT), 1X CPIM, and 1X PMSF (*see* **Note 6**).
11. Crosslinking buffer: 50 m*M* HEPES-KOH, pH 8.5 (*see* **Note 7**), 27.5 m*M* NaCl, 20 m*M* KCl, and 25 m*M* sucrose.
12. 2 mL Potter-Evehjem tissue grinder (cat. no. 358029, Wheaton Science Products, Millville, NJ).

2.2. Crosslinking of Embryo Extract

1. Dimethylpimelimidate (DMP) solution: 40 m*M* DMP in crosslinking buffer. Prepare freshly because DMP quickly hydrolyzes.
2. Stop buffer: 1 *M* Tris-HCl, pH 8.0.

2.3. Immunoprecipitation of Protein Complexes

1. Rabbit anti-Par-6 antibody, affinity purified ***(2)***.
2. Protein A Sepharose CL-4B (GE Healthcare, Freiburg, Germany).
3. Low-salt buffer: 100 m*M* NaCl in lysis buffer.
4. High-salt buffer: 500 m*M* NaCl in lysis buffer.
5. Urea buffer: 500 m*M* urea in lysis buffer.

2.4. SDS-Polyacrylamide Gel Electrophoresis

1. 4X NuPAGE lithium dodecyl sulfate (LDS) sample buffer (Invitrogen, Carlsbad, CA).
2. 10X NuPAGE reducing agent (Invitrogen).
3. 20X NuPAGE 3-(N-Morpholino)propanesulfonic acid (MOPS) sodium dodecyl sulfate (SDS) running buffer (Invitrogen).
4. NuPAGE antioxidant (Invitrogen).
5. Precast 4–12% gradient gel (NuPAGE Novex *Bis*-Tris, Invitrogen).
6. XCell SureLock Novex Mini-Cell (Invitrogen).
7. Molecular weight marker (e.g., silver stain SDS-poly acrylamide gel electrophoresis molecular weight standard mixture, Sigma).

2.5. Western Blotting

1. Hybond-enhanced chemiluminescent (ECL) nitrocellulose membrane (GE Healthcare).
2. 3MM Chromatography paper (Whatman Schleicher & Schuell, Dassel, Germany).
3. Blotting buffer: 25 m*M* Tris-base, 192 m*M* glycine, 20% (v/v) methanol, and 0.1% (w/v) SDS. This buffer can be reused several times.
4. Hoefer TE 22 tank transfer unit (Hoefer, San Francisco, CA).
5. 10X Phosphate-buffered saline (10X PBS): 1.37 *M* NaCl, 27 m*M* KCl, 43 m*M* Na_2HPO_4, and 14 m*M* KH_2PO_4, pH 7.4 (*see* **Note 8**).
6. PBS: 137 m*M* NaCl, 2.7 m*M* KCl, 4.3 m*M* Na_2HPO_4, and 1.4 m*M* KH_2PO_4, pH 7.4.
7. PBS-T: 0.1% (v/v) Tween-20 in PBS.
8. Ponceau S solution: 0.1% (w/v) Ponceau S in 5% (v/v) acetic acid.
9. Blocking buffer: 5% (w/v) nonfat dry milk powder in PBS.
10. Primary antibody: rabbit anti-Par-6 ***(2)***, affinity purified, dilute 1:1000 in blocking buffer.
11. Secondary antibody: donkey antirabbit immunoglobulin (IgG), conjugated to horseradish peroxidase (GE Healthcare); use 1:5000 in blocking buffer.
12. ECL Plus reagent (GE Healthcare) and Hyperfilm ECL (GE Healthcare).
13. ECL Plus solution: Mix solutions A and B in a ration of 40:1 (e.g., 1 mL solution A, 25 µL solution B) immediately before use.

2.6. Silver Staining

1. It is recommended that all steps be performed in clean, white plastic containers to best observe the developing silver gel bands. The size of the tray should allow complete immersion of the gel.
2. To rapidly remove solutions from the gel a water-jet vacuum pump can be used.

3. All chemicals should be of analytical grade. Unless stated otherwise, all solutions should be prepared with water that has a resistivity of 18.2 MW·cm.
4. Fix solution: 40% (v/v) ethanol and 10% (v/v) acetic acid.
5. Wash solution: 30% (v/v) ethanol.
6. Sensitizing solution: 0.02% (w/v) sodium thiosulfate ($Na_2S_2O_3$). Prepare freshly to ensure sensitive and reproducible stainings.
7. Silver solution: 0.1% (w/v) silver nitrate ($AgNO_3$). Keep the solution at 4°C in the dark.
8. Developing solution: 3% (w/v) sodium carbonate (Na_2CO_3), 0.05% (v/v) of a 37% formaldehyde solution (*see* **Note 9**). Prepare freshly.
9. Stop solution: 5% (v/v) acetic acid.
10. Storage solution: 1% (v/v) acetic acid.

2.7. Sample Preparation for Mass Spectrometry

2.7.1. In Solution Digestion

1. Reduction buffer: 3 m*M* DTT in 50 m*M* NH_4HCO_3. Prepare freshly.
2. Alkylation buffer: 15 m*M* iodacetamide (IAA) in 50 m*M* NH_4HCO_3. Prepare immediately before use.
3. Trypsin solution: 100 ng/µL trypsin (Trypsin Gold, Mass Spectrometry Grade, Promega, Mannheim, Germany) in 50 m*M* acetic acid. Store at –80°C. Limit the number of freeze–thaw cycles to less than five.
4. Digestion solution: immediately before use, dilute the trypsin solution 1:1 in 50 m*M* NH_4HCO_3.
5. Trifluor acetic acid (TFA) solution: 10% (v/v) TFA.

2.7.2. Gel Band Digestion

1. Wash buffer 1: 50 m*M* NH_4HCO_3 (*see* **Note 10**).
2. Wash buffer 2: 50% (v/v) acetonitrile and 50% (v/v) wash buffer 1.
3. Reduction buffer: 10 m*M* DTT in 50 m*M* NH_4HCO_3. Prepare freshly.
4. Alkylation buffer: 5 m*M* IAA in 50 m*M* NH_4HCO_3. Prepare immediately before use.
5. Trypsin solution: 100 ng/µL trypsin (Trypsin Gold, Mass Spectrometry Grade, Promega) in 50 m*M* acetic acid. Store at –80°C. Limit the number of freeze–thaw cycles to five.
6. Digestion solution: immediately before use, dilute the trypsin solution 1:8 in 50 m*M* NH_4HCO_3.
7. Extraction solution: 5% (v/v) formic acid.

3. Methods

3.1. Embryo Extract Preparation

1. Collect embryos of the desired stage on an apple juice plate. For a 3–6 h collection, put an apple juice plate with a bit of yeast paste onto a fly cage. After 3 h take off the plate and incubate it for another 3 h at 25°C (*see* **Note 11**).
2. To dechorionate the embryos cover the apple juice plate containing the embryos with 50% (v/v) household bleach and suspend the embryos carefully with a brush. Dechorionate for 2–3 min.

3. Transfer the embryos into an embryo collection net (*see* **Note 12**) and wash them extensively with water and twice with embryo wash buffer.
4. Transfer embryos in embryo wash buffer from the collection net into a clean glass homogenizer, let the embryos settle, and remove the embryo wash buffer.
5. Wash the embryos once with precooled crosslinking buffer (*see* **Note 13**). From now on everything should be done on ice with precooled solutions.
6. Add 1 mL of crosslinking buffer (*see* **Note 13**) supplemented with 1 m*M* DTT, 1X CPIM, 1X PMSF (for up to 500 µL of settled embryo volume, for bigger volumes take two volumes of crosslinking buffer (*see* **Note 13**) per volume of embryos), and homogenize the embryos by moving a tight fitting piston slowly up and down 5–10 times.
7. Transfer the homogenate into a microcentrifuge tube and centrifuge with 1200*g* at 4°C for 5 min in order to remove debris.
8. Transfer the supernatant into a new tube and centrifuge again like before.
9. Transfer the supernatant into a fresh tube. Depending on the intracellular localization of the protein complex, differential centrifugation steps can be applied to enrich the protein complex of interest.

3.2. Crosslinking of Embryo Extract (Optional Step, See *Note 14)*

1. Crosslink reaction: Add the DMP solution to the embryo extract to a final concentration of 20 m*M* DMP (*see* **Note 15**) and incubate on a roller wheel at 25°C for 30 min.
2. Stop crosslink: add 100 µL of 1 *M* Tris-HCl, pH 8.0 and incubate at 25°C on a roller wheel for 15 min (*see* **Note 16**).
3. Adjust the crosslinked embryo extract to 0.5% NP-40 and 10% (v/v) glycerol (final concentration) before immunoprecipitation (IP).
4. The crosslinked embryo extract can be frozen down by snap-freezing in liquid nitrogen and stored at –80°C for future use.

3.3. IP of Protein Complexes

This protocol uses a rabbit anti-Par-6 antibody against the endogenous, untagged Par-6 protein. The use of tagged proteins can significantly simplify the purification. For example, 3xFLAG, 6xmyc, or TAP-tagged proteins have been used successfully (e.g., *see* **refs. *3*** and ***4***).

1. Wash the required amount of protein A beads three times with 1 mL of lysis buffer. Prepare a 50% slurry of protein A beads in lysis buffer.
2. Add 60 µL of washed protein A bead slurry to the lysate for preabsorbption (for up to 500 µL of settled embryo volume). Incubate on a roller wheel at 4°C for 30 min.
3. Preclear the lysate by centrifugation at 15,000*g* at 4°C for 15 min (*see* **Note 17**). Transfer the supernatant into a fresh vial and take a sample of the input. If the peptide against which the antibody has been raised is available, do a control IP with a 100-fold molar excess of peptide to antibody. This will serve as a control for unspecific binding.

4. Add 10 µL of rabbit anti-Par-6 to the lysate (*see* **Note 18**) and incubate on a roller wheel at 4°C for at least 2 h. It is possible to extend this incubation over night at the risk of increasing the background of nonspecific binding.
5. Add 60 µL of washed protein A bead slurry. Incubate on a roller wheel at 4°C for at least 1 h.
6. Spin down the protein A beads at 1000*g* at 4°C for 1 min. Take a sample of the unbound fraction (optional) and remove the supernatant.
7. Wash the beads twice with 500 µL of lysis buffer for 5 min.
8. Wash the beads twice with 500 µL of low salt buffer for 5 min. Take a sample of the first wash supernatant.
9. Wash the beads twice with 500 µL of high-salt buffer for 5 min. Take a sample of the first wash supernatant.
10. Wash the beads twice with 10 m*M* Tris-HCl, pH 7.5 to remove detergents that would later interfere with mass spectrometry. Completely remove the supernatant (*see* **Note 19**).
11. Elute the bound protein complexes with 30 µL of 0.1 *M* glycine, pH 2.0. Save the eluate and repeat the elution once. Pool the eluates. The samples can be frozen down by snap-freezing in liquid nitrogen and stored at –80°C for future use.

3.4. SDS-Polyacrylamide Gel Electrophoresis

These instructions assume the use of a XCell SureLock Novex Mini-Cell (Invitrogen) gel system. For silver staining followed by mass spectrometric analysis it is highly recommended to use precast gels to reduce the risk of contamination.

1. Add the required amount of 4X NuPAGE LDS sample buffer and 10X NuPAGE reducing agent to the sample and heat at 70°C for 10 min to denature the proteins.
2. Assemble the 4–12% precast gel into the gel system. Remove the comb from the gel and wash the gel slots with running buffer. Fill the upper chamber with 200 mL of 1X NuPAGE MOPS SDS running buffer containing 500 µL NuPAGE antioxidant and the lower chamber with about 700 mL of 1X NuPAGE MOPS SDS running buffer.
3. Remove the comb from the gel and wash the gel slots with running buffer.
4. Load the protein samples and an appropriate marker into the gel slots. Make sure to use different marker (concentration) for silver staining and for Western blots.
5. Run the gel at 200 V constant for about 50 min until the gel front has reached the bottom of the gel.

3.5. Western Blotting

Western blotting is used to verify that the protein complex of interest has successfully been immunoprecipitated. If some components of the complex are already known and antibodies are available it should be checked whether the complex has stayed intact during IP. In case a crosslinking approach has been chosen the optimization of the crosslink reaction conditions

(crosslinker concentration, reaction time-course) should be monitored by Western blotting. This protocol assumes the use of a Hoefer TE 22 tank transfer unit but it can easily be adapted to other wet or semidry blotting systems.

1. Cut a Hybond-ECL nitrocellulose membrane (GE Healthcare) and four pieces of 3MM paper a bit larger than the size of the separating gel and equilibrate them in blotting buffer (*see* **Note 20**).
2. Assemble two pieces of 3MM paper, the nitrocellulose membrane, the separating gel, and two pieces of 3MM paper (in this order) into a sandwich structure between the two sponges of the transfer cassette. Carefully avoid or remove air bubbles between the layers (*see* **Note 21**).
3. Place the cassette into the transfer tank filled with blotting buffer so that the membrane is oriented toward the anode and the gel toward the cathode. Blot for 1 h at 100 V (with maximum 400 mA) while cooling with tap water and mixing the blotting buffer with a magnetic stir-bar.
4. After the transfer is completed briefly rinse the membrane with water, stain it with Ponceau S solution for 2 min, and destain in 2% (v/v) acetic acid for several minutes. Label the marker bands with a pen. Make a photocopy or scan the stained membrane for documentation.
5. If necessary cut the membrane with a razor blade on solid support. Do not forget to label the membrane (pieces).
6. Incubate the membrane in blocking buffer for at least 30 min at room temperature on a rocking platform.
7. Discard the blocking buffer and replace it with the primary antibody diluted in blocking solution while shaking/rocking. Incubate for at least 2 h at room temperature or overnight at 4°C (*see* **Note 22**).
8. Wash the membrane three times for at least 5 min in PBS.
9. Incubate the membrane with the secondary antibody diluted 1:5000 in blocking buffer for at least 30 min at room temperature.
10. Wash three times for at least 5 min in PBS and once with PBS-T.
11. Place the membrane on a piece of parafilm (American National Can, Menasha, WI) and remove excess liquid. Distribute ECL Plus solution on the nitrocellulose membrane so that its entire surface is covered (about 0.1 mL/cm^2 of membrane). Incubate for 5 min at room temperature, then remove excess liquid.
12. Put the membrane into a sheet protector and remove air bubbles. Expose to a Hybond ECL hyperfilm for 1 min or longer depending on the intensity of the signal and then develop the film immediately. On the basis of the observed signal intensity adjust the exposure time for a second film. Make sure that the membrane does not dry out during exposure as this will lead to a high background signal.

3.6. Silver Staining (Modified From Ref. 5)

1. Transfer the polyacrylamide gel into a gel tray containing 100 mL of fix solution and incubate for 1 h at room temperature with agitation (*see* **Note 23**).

2. Wash the gel two times for 20 min with 100 mL of wash solution, followed by 20 min with 100 mL of water (*see* **Note 24**).
3. To sensitize the gel for the silver staining reaction incubate it for 1 min in 100 mL of sensitizing solution with agitation (*see* **Note 25**).
4. Rinse the gel three times with 100 mL of water for 20 s (*see* **Note 26**).
5. Impregnate the gel in 100 mL of silver solution at 4°C for 20 min with agitation (*see* **Note 27**).
6. Rinse the gel three times with 100 mL of water for about 20 s. Transfer the gel into a clean gel container.
7. Wash the gel in 100 mL of water for 1 min (*see* **Note 28**).
8. Develop the gel in 100 mL of developing solution with agitation. Carefully observe the appearance of bands (*see* **Note 29**).
9. As soon as the staining is sufficiently intense, rinse the gel briefly with water and incubate it in 100 mL of stop solution for 10 min with agitation (*see* **Note 30**).
10. Wash the gel three times for 10 min in 100 mL of water with agitation.
11. Use a scanner to document the stained gel. Store the gel in storage solution at 4°C for further processing (*see* **Note 31**).

3.7. Sample Preparation for Mass Spectrometry

3.7.1. In Solution Digestion

1. Prepare a clean working space (e.g., under a lamina flow) and carefully clean all required devices with ethanol (*see* **Note 32**).
2. Determine the pH of the protein samples and adjust it, if necessary, to pH 8 with 1 *M* Tris-HCl, pH 8.5. If possible try to determine the protein concentration of the sample.
3. Reduction of disulfide bonds: for each 100 µL of sample volume add 2 µL of reduction buffer. For sample volumes below 100 µL add 2 µL of reduction buffer. Incubate for 30 min at 56°C.
4. Alkylation of reduced cysteine residues: for each 100 µL of sample volume add 2 µL of alkylation buffer and incubate in the dark for 30 min at room temperature. For sample volumes below 100 µL add 2 µL of alkylation buffer (*see* **Note 33**).
5. Stop the alkylation reaction by adding 10 µL of reduction buffer for each 100 µL of sample volume.
6. Digestion: add 4 µL of digestion solution to the sample and incubate at 37°C for about 6 h. Then add another 4 µL of digestion solution and incubate at 37°C overnight.
7. Add 10 µL of TFA solution to stop the digestion. Verify that the pH is acidic and store the sample at 4°C.

3.7.2. Gel Band Digestion

1. Prepare a clean working space (e.g., under a lamina flow) and carefully clean all required devices.
2. Rinse the gel briefly with water and put it on a clean glass plate onto an illuminator. Excise bands of interest with a clean scalpel. Try to cut as close to the bands as possible. Cut out proper control bands as well.

3. Transfer the excised band on a clean slide. Chop the band into pieces of about 1×1 mm^2 and transfer the gel particles into a 0.5-mL microcentrifuge tube with a tweezers.
4. Wash the gel particles with 200 µL of wash buffer 1 for at least 10 min at room temperature on a shaker.
5. Wash the gel particles with 200 µL of wash buffer 2 for at least 10 min at room temperature. Repeat both washing steps with wash buffer 1 and 2 once more.
6. Shrink the gel particles: add 100 µL of acetonitrile and shake for 5 min at room temperature. Completely remove the supernatant.
7. Reduction of the disulfide bonds: add 100 µL of reduction buffer to the gel particles and incubate for 30 min at 56°C. Completely remove the supernatant.
8. Alkylation of reduced cysteine residues: add 200 µL of alkylation buffer and incubate in the dark for 30 min at room temperature (*see* **Note 33**).
9. Wash the gel particles again (as described in **steps 4** and **5**).
10. Shrink and dry gel particles: add 100 µL of acetonitrile and shake for 5 min at room temperature. Completely remove the supernatant. Dry gel particles in the speed vac (Concentrator 5301, Eppendorf, Hamburg, Germany) (*see* **Note 34**).
11. Add 20 µL of digestion solution to the dried gel particles. Incubate at 4°C for 5 min. Then remove the remaining digestion solution that has not been soaked up by the gel particles.
12. Add 20 µL of wash buffer 1 and in-gel digest to the samples at 37°C overnight.
13. On the next morning pipet the supernatant with a gel loader tip (*see* **Note 35**) into a 0.2-mL low-absorbing polymerase chain reaction (PCR) tube (*see* **Note 36**). Store the tube at 4°C to prevent further digestion.
14. Add 20 µL of extraction solution to the gel particles and sonicate for 10 min in an ice-cooled water bath. Pipet the supernatant with a gel loader tip into the PCR tube.
15. Add 20 µL of extraction solution to the gel particles and sonicate for 10 min in an ice-cooled water bath. Pipet supernatant with a gel loader tip into PCR tube. The tube should now contain about 60 µL of peptide solution. Store at –80°C until starting the mass spectrometry.

4. Notes

1. Methyl paraben inhibits the growth of yeasts and molds.
2. The density of flies in the cage should not exceed six flies per cm^2 of cage surface area. To increase the surface that can be occupied by the flies, one or two folded paper filters can be put into the cage. The flies need about 2 d to adapt to the cage conditions and become most productive in terms of egg laying. Exchange the collection cage at least every second day for a clean one—otherwise the flies will start to lay eggs onto the walls of the cage instead of the apple juice plate and larvae will crawl into the agar. The cages should be cleaned without using detergents as this can interfere with egg laying.
3. PMSF is toxic and unstable in aqueous solution.
4. Tris(hydroxymethyl)aminomethane.
5. 10X Extraction buffer is used for making lysis buffer, low salt, high salt, and urea buffer.

6. Depending on the protein complex of interest certain modifications should be made to the lysis buffer. If complex stability depends on, for example, Ca^{2+}- or Zn^{2+}-ions, EGTA or EDTA should be omitted, respectively. If phosphatases have to be inhibited, pyrophosphate (2 m*M*), fluoride (20 m*M*), and *ortho*-vanadate (2 m*M*) should be added. Changing the NP-40 concentration can sometimes improve the result.
7. 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid. HEPES is light sensitive and should be stored in the dark.
8. 10X PBS is used for making PBS, PBS-T, and blocking buffer.
9. A 37% solution of formaldehyde in water is commonly referred to as formalin. Formaldehyde is toxic and volatile.
10. Always prepare all NH_4HCO_3 containing buffers freshly, as NH_3 and CO_2 are volatile.
11. Adjust the time window to the developmental process and protein of interest. For investigating central nervous system neuroblasts, embryos of 3–6 h after egg deposition are optimal. If protein expression is induced by heat shock, the duration of heat shock and recovery phase have to be determined empirically—30 min heat shock at 37°C and 1 h recovery at 25°C are good starting points. A precollection on an apple juice plate 1–2 h before the experiment can be done to improve the staging of the embryos.
12. The transfer of embryos is best done with a cut blue pipet tip that has been rinsed with embryo wash buffer to prevent embryos sticking to the plastic.
13. If the crosslinking will be omitted, use lysis buffer instead of crosslinking buffer.
14. In order to stabilize labile or transient protein–protein interactions, the fly embryo extract can be crosslinked with different crosslinking reagents (e.g., DMP, or disuccinimidyl suberate [DSS]) before IP. DMP and DSS are membrane permeable, homobifunctional crosslinkers that react specifically with primary amino groups. DMP is a water soluble imidoester crosslinker that has a spacer length of 9.2 Å. DSS is an amine-reactive *N*-hydroxysuccinimide ester with a spacer length of 11.4 Å. The spacer length restricts possible crosslinks to amino groups that fall in this range. Besides stabilizing transient interactions, crosslinking allows the application of more stringent washing conditions during IP, as the complex can no longer dissociate. However, compared with noncrosslinked bands, crosslinked bands generally show a lower coverage of protein sequence in mass spectrometry.
15. The crosslinker concentration has to be adjusted empirically for each protein complex. Always include a control reaction without crosslinker.
16. Alternatively, ethanolamine can be used to quench the amine reactive groups of the crosslinker.
17. This step is to remove any material that sticks to the protein A beads unspecifically.
18. The amount of antibody required for IP has to be determined empirically. A good starting point is to assume an average specific IgG concentration in serum of 50 μ*M*. The amount of protein A beads has to be adjusted accordingly. The binding capacity of protein A beads is about 20 mg IgG/mL of packed bead volume. When using mouse IgG antibodies, protein G beads might give better results. In order to prevent antibodies from being eluted from the protein A beads together with the immunoprecipitated proteins, they can be crosslinked to the beads before IP. However, this can interfere with epitope recognition for some antibodies.

19. A 28-gauge syringe needle can be used to remove the last traces of buffer.
20. If Western blots are done repeatedly it is advisable to prepare (or buy) a stock of properly sized membranes and 3MM papers.
21. A 15-mL Falcon tube (BD Biosciences, Bedford, MA) can be used for this purpose.
22. Make sure that the whole membrane is equally covered with the antibody solution.
23. Fixation prevents the diffusion of the separated proteins, removes interfering substances, and is very important for a clear background of the stained gel. The fixation can also be done overnight.
24. Washing is important to remove the acetic acid because of the acid lability of thiosulfate in **step 3**.
25. For **steps 3–7** the indicated times should be observed exactly in order to ensure reproducible image development. Thiosulfate increases the sensitivity of the staining and generates high contrast. Longer incubation than 1 min result in increased background staining.
26. Excess thiosulfate has to be removed as it would lead to surface staining by Ag_2S.
27. After $AgNO_3$ impregnation, gels may look slightly yellowish owing to the pretreatment with thiosulfate.
28. The wash-steps are done to remove excess $AgNO_3$ from the gel surface and the gel container.
29. The duration required for band development depends on the kind of protein and its concentration in the band. The intensity of the bands increases continuously. If the gel is developed for more than 10 min, a yellowish background may appear.
30. The bands can continue to darken somewhat after stopping.
31. The stained gel can be stored at 4°C for a few weeks with only minor losses of image quality. The detection limit for this protocol is in the low femtomole range.
32. Human keratin is the most prevalent sample contamination.
33. IAA is sensitive to light and it is very toxic.
34. As soon as the gel pieces have dried they start to "jump" when the microcentrifuge tube is tipped.
35. The use of gel loader tips ensures that no gel pieces are transferred by accident.
36. The use of low-absorbing material is recommended in order to prevent any loss of peptides and to ensure the utmost sensitivity of the method.

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23

Mass Production of *Drosophila* Embryos and Chromatographic Purification of Native Protein Complexes

Natascha Kunert and Alexander Brehm

Summary

The purification of native protein complexes requires the availability of sufficient amounts of starting material. *Drosophila melanogaster* embryos have proven to be a rich source for nuclear protein complexes. Here we describe establishment and maintenance of a fly facility for the production of large amounts of embryos, protocols for the production of nuclear extracts, and a scheme for the chromatographic purification of a nuclear multisubunit protein complex.

Key Words: Chromatography; *Drosophila melanogaster*; embryo; nuclear extract; protein complex; protein purification.

1. Introduction

Drosophila melanogaster has long served as a model for the genetic analysis of metazoan development. More recently, the fruit fly has also become popular with biochemists attempting to purify and analyze native nuclear protein complexes.

Many protein biochemists aiming to purify nuclear protein complexes from higher eukaryotes make use of mammalian cell lines. A number of well-established protocols exist that describe the production of nuclear extracts from cultured cells such as HeLa or HEK293, many of which are based on the protocol first published by Dignam and Roeder in 1983 ***(1)***. The availability of sufficient amounts of nuclear extract is one of the most crucial bottlenecks that can impede the successful purification of native protein complexes. As a consequence, large amounts of cells have to be cultured and this takes both time, and owing to the costs of medium and supplements, considerable amounts of money.

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The fruit fly provides an attractive alternative model system. Fly embryos can be produced in large quantities with comparative ease ***(2)***. Here we describe how to set up and maintain a large-scale fly facility that can house up to half a million flies and can produce up to half a kg of dechorionated embryos within a few days. Importantly, large-scale fly embryo production can be achieved at a fraction of the cost required to generate an equivalent amount of cultured mammalian cells. An additional advantage of *Drosophila* over mammalian cell lines is the fact that protein families tend to be smaller than the corresponding protein families in mammals. This reduces the problem of redundancy and minimizes the number of related protein complexes that must be separated during fractionation.

The core of a large-scale fly facility is a "fly chamber," which provides the required temperature and humidity. Flies are kept in this chamber in 12–15 special cages, which can house between 25 and 50,000 flies each. Every 2 wk the fly generation has to be renewed. A tight schedule needs to be implemented to ensure that fly food and larval boxes are prepared, flies hatch, cages are seeded, and embryos are collected at the right times to keep the facility running smoothly. Large-scale fly facilities have been established in several laboratories, including those of Peter Becker (Munich), Carl Wu (NIH), Jim Kadonaga and Robert Tjian (Berkeley), and Peter Verrijzer (Rotterdam). We have recently established a fly facility at the Institute for Molecular Biology and Tumour Research in Marburg. In doing this and in writing this chapter we have heavily drawn on the invaluable experience we have gained in the lab of Peter Becker.

For extract preparation embryos can be collected over a period of 3 d. This results in the accumulation of 250–500 g of embryos. Embryos are dechorionated and broken up in a continuous flow homogenizer (LSC LH-21 steady flow homogenizer, Yamato, Orangeburg, NY). Nuclei are purified by centrifugation and then lysed with ammonium sulfate. Chromatin and debris are separated from the soluble nuclear extract by ultracentrifugation. The nuclear extract is then concentrated by ammonium sulfate precipitation and dialyzed. A 3-d embryo collection will typically give 30–60 mL of nuclear extract at a protein concentration of 10–20 mg/mL.

Nuclear extracts can be fractionated by classical chromatography and nuclear protein complexes can be purified to homogeneity provided they are sufficiently abundant and stable. Here we describe the purification of a Retinoblastoma protein containing multisubunit protein complex as an example.

2. Materials

2.1. Establishment and Maintenance of a Large-Scale Fly Facility

1. Fly stock: wild-type flies, *D. melanogaster* Oregon R.
2. Fly chamber: a chamber that is large enough to keep a minimum of 12 large fly cages and larval boxes (*see* **Note 1**). The chamber is fitted with a thermostat-controlled heating and cooling system to maintain a temperature of approx 25°C. A humidifier

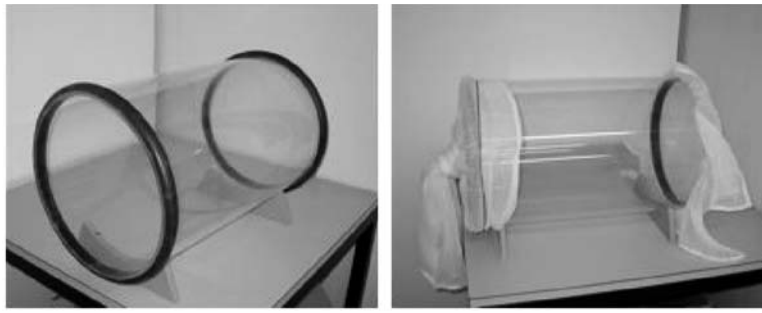

Fig. 1. Fly cage before (left) and after (right) fitting it with curtains. Note stands (gray) and rubber rings (black). For details *see* text.

keeps the relative humidity at approx 50%. The electric lighting operates in a circadian ryhthm to provide the chamber with an automated day–night cycle.

3. Fly cage: acrylic glass or plexiglass cylinder (inside diameter: 300 mm; length: 480 mm) on two stands **(Fig. 1)**. Each end is fitted with a rubber ring. The back end is closed with a piece of curtain by fixing it with an elastic rubber band to the cage (the rubber ring prevents the rubber band from sliding off). A cylindrical "curtain tube" (diameter: 300 mm; length: 500 mm) is attached to the front end of the cage in a similar manner. The curtain tube serves as an opening to populate the cages with flies or to exchange the agar plates. It is closed with a knot to keep flies from escaping. A minimum of 12 fly cages is required.
4. Larval box: a square plastic box (length: 150 mm; height: 130 mm) with a tightly closing lid that can withstand freezing to –20°C. A 120×120 mm^2 square is cut out of the lid and replaced by wire gauze. The gauze is bolted to the remaining plastic frame.
5. Anesthetic unit for carbon dioxide narcosis of flies: a source of carbon dioxide, a manometer, and a handheld dispenser for controlling CO_2 flow.
6. Nonstandard laboratory apparatuses: gas cooker, 50 L cooking pot with lid, adequate wire whisk, dough scrapers, kitchen machine with dough hooks, cleaning brushes for tubs, washbasin with an inner diameter of 600 mm, and a washing machine to clean the curtain tubes of the fly cages.

2.2. Fly Food

1. Fly food (yeast paste): 500 g dry yeast (e.g., Fermipan rot from Uniferm, Werne, Germany); propionic acid. The yeast paste can be stored in a beaker at 4°C for up to 2 wk (*see* **Note 2**).
2. Agar trays: *see* **Subheading 2.3.1.**

2.3. Drosophila *Embryo Collection*

2.3.1. Agar Trays for Embryo Collection

1. Agar–agar (Probio GmbH, Eggenstein, Germany).
2. Apple juice (100% juice without citric acid, ascorbic acid, or insect repellents) (*see* **Note 3**).

3. Treacle (Bauck GmbH, Rosche, Germany).
4. Methyl-4-hydroxy-benzoate.
5. 225 × 175 × 17 mm^3 styrofoam trays (Margret Link GmbH, Nürtingen, Germany).

2.3.2. Three Sieve Embryo Collection Apparatus

1. Sieve 200 × 50 mm^2, 125 µ*M*, and Deutsches Institut für Normung (DIN) ISO 3310-1 (VWR International GmbH, Darmstadt, Germany).
2. Sieve 200 × 50 mm^2, 355 µ*M*, DIN ISO 3310-1.
3. Sieve 200 × 50 mm^2, 710 µ*M*, DIN ISO 3310-1.

2.4. Larval Boxes

2.4.1. Larval Food

1. Ortho-phosphoric acid (Merck, Darmstadt, Germany).
2. Propionic acid (Merck).
3. Sugar.
4. Brewer's yeast (pulverized and inactivated barm, Volk, Germany) (*see* **Note 4**).
5. Methyl-4-hydroxy-benzoate (Fluka, Seelze, Germany).

2.4.2. Cellulose Paper

1. Unbleached, 400 × 600 mm^2 cellulose paper sheets (VWR International GmbH).

2.5. Nuclear Extract From Drosophila Embryos

2.5.1. Buffers

1. Embryo wash solution: 0.7% NaCl and 0.04% Tween-20.
2. Nuclear extract buffer I (NXI) (filter sterilized): 15 m*M* HEPES, pH 7.6, 10 m*M* KCl, 5 m*M* $MgCl_2$, 0.5 m*M* ethylene glycol tetraacetic acid (EGTA), 0.1 m*M* ethylene diamine tetraacetic acid (EDTA), 350 m*M* sucrose, 1 m*M* dithiothreitol (DTT), 0.2 m*M* phenylmethanesulphonylfluoride (PMSF), and 1 m*M* sodium metabisulfite.
3. NXII buffer (filter sterilized): 15 m*M* HEPES, pH 7.6, 110 m*M* KCl, 2 m*M* $MgCl_2$, 0.1 m*M* EDTA, 1 m*M* DTT, 0.2 m*M* PMSF, and 1 m*M* sodium metabisulfite.
4. NXIII buffer (filter sterilized): 20% glycerol, 25 m*M* HEPES, pH 7.6, 100 m*M* KCl, 12.5 m*M* $MgCl_2$, 0.1 m*M* EDTA, 1 m*M* DTT, 0.2 m*M* PMSF, and 1 m*M* sodium metabisulfite (*see* **Note 5**).

2.5.2. Tubing

1. Dialysis tubing: Spectrapor 1, MWCO 6000–8000 (Carl Roth GmbH, Karlsruhe, Germany).

2.5.3. Nonstandard Laboratory Apparatuses

1. LSC LH-21 or LH-22 steady-flow homogenizer (Yamato, Orangeburg, NY).
2. Miracloth (Calbiochem-Novabiochem Corporation, La Jolla, CA).

2.6. Chromatographic Purification of Native Protein Complexes From Drosophila Embryos

2.6.1. Buffers

1. Qx buffers (filter sterilized): 10% glycerol, 20 m*M* Tris-HCl, pH 8.0, *x* m*M* KCl, 1 m*M* $MgCl_2$, 1 m*M* DTT, 0.2 m*M* PMSF, 1 m*M* sodium metabisulfite. "*x*" denotes concentration of KCl in m*M*. Q100 buffer contains 100 m*M* KCl and Q450 buffer contains 450 m*M* KCl.
2. Bx buffers (filter sterilized): 10% glycerol, 25 m*M* HEPES, pH 7.6, *x* m*M* KCl, 1 m*M* $MgCl_2$, 1 m*M* DTT, 0.2 m*M* PMSF, 1 m*M* sodium metabisulfite. "*x*" denotes concentration of KCl in m*M*. B100 buffer contains 100 m*M* KCl and B250 buffer contains 250 m*M* KCl.
3. EX300 buffer (filter sterilized): 10% glycerol, 10 m*M* HEPES, pH 7.6, 300 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.5 m*M* EGTA, 10 m*M* β-glycerophosphate, 1 m*M* DTT, 0.2 m*M* PMSF, and 1 m*M* sodium metabisulfite.

2.6.2. Tubings

1. Dialysis tubing: Spectrapor 1, MWCO 6000–8000 (Carl Roth GmbH).

2.6.3. Chromatography

1. ÄKTA fast protein liquid chromatography (FPLC) and purifier chromatography systems (GE Healthcare, Munich, Germany; cat. no. 18-1900-26).
2. Q Sepharose resin (GE Healthcare; cat. no. 17-0510-01).
3. XK columns (GE Healthcare; cat. no. 18-8773-01).
4. 5-mL Q Sepharose HiTrap HP column. (GE Healthcare; cat. no. 17-1153-01)
5. Superose 6 10/300 GL (GE Healthcare; cat. no. 17-5172-01).
6. Hydroxyapatite Bio-Gel HT gel (Bio-Rad Laboratories, Munich, Germany; cat. no. 130-0150).
7. BioBiorex 70 resin (Bio-Rad Laboratories).
8. StrataClean resin (Stratagene, Amsterdam, The Netherlands; cat. no. 400714).

3. Methods

3.1. Establishment and Maintenance of a Large-Scale Fruit Fly Facility

3.1.1. General Considerations

In order to run an efficient large-scale fly facility, it is advisable to strictly follow the 14-d schedule detailed in **Table 1**. The flies are populated in cages that are kept in a humified, temperature-controlled room with an automated day–night circle. One fly cage can accommodate between 25,000 and 50,000 flies.

On the first day (a Friday is recommended), fly cages are seeded (*see* **Subheading 3.1.2.**). Flies are then fed with yeast paste on agar trays (*see* **Subheading 3.1.3.**) until day 7–10 depending on demand and on how well

Table 1
In Order to Run an Efficient Large-scale Fly Facility, It is Advisable to Strictly follow the 14-d Schedule Displayed in this Table.

	Fr	Sa	Su	Mo	Tu	Wd	Th	Fr	Sa	Su	Mo	Tu	Wd	Th
Seed fly cages	■													
Feed flies	■	■	■	■	■	■	■	▒	▒	▒				
Set up larval boxes					■									
Collect embryos			■	■	■	■	■	▒	▒	▒				
Clean fly cages											▒	▒	▒	▒
Clean larval boxes	▒	▒	▒	▒										

Black boxes denote days when the specified action should be taken; gray boxes denote days when the specified action could be taken. For example, the cleaning of fly cages can be performed on any one of the 4 d marked in gray. For details *see* text.

flies are laying. Embryos can be collected (*see* **Subheading 3.1.4.**) beginning with day 3 (*see* **Note 6**) until days 7–10. Collected embryos can be stored for up to 72 h at 4°C to keep them fresh and to arrest development before using them for extract preparation. Cleaning of larval boxes (*see* **Subheading 3.1.6.**) is carried out between days 1 and 4. To set up the next generation, new larval boxes are set up (*see* **Subheading 3.1.5.**) on day 5. After fly feeding is discontinued (between days 7 and 10) and flies are dead, the cages are cleaned (*see* **Subheading 3.1.7.**). New fly cages are populated with hatched adult flies (*see* **Subheading 3.1.2.**) on day 14 (*see* **Note 7**).

Tidiness is important: used agar trays and larval boxes are frozen at –20°C to kill excess flies and embryos. After each feeding session escapees need to be removed using a vacuum cleaner. The walls and floor of the fly chamber as well as shelves and other surfaces need to be cleaned at regular intervals. Similar rules apply to the areas outside the fly chamber where flies and fly food are handled. It is crucial to remove any potential food source (yeast paste, apple juice, and agar plates) to avoid a reproduction of uncaged flies.

3.1.2. Seeding of Fly Cages

1. Plexiglass cages to be populated with flies are provided with clean curtain tubes that are fixed to the cage by flexible rubber bands.

2. Larval boxes are removed from the fly chamber and flies are anaesthetized by a steady stream of carbon dioxide applied through the wire gauze until the flies are motionless.
3. By shaking and tapping the box, flies are collected in one corner. The box is opened at this corner and the flies are poured through a wide funnel into a preweighed Erlenmeyer flask. About 25–30 g of flies are then transferred to an empty cage (*see* **Note 8**).

3.1.3. Feeding of Flies

1. Preparation of fly food (yeast paste): Using a kitchen machine with a dough hook, 500 g dry yeast, 4.7 mL propionic acid, and 835 mL deionized water are mixed until a homogenous, peanut butter-like paste is produced.
2. Preparation of agar trays for embryo collection: 500 g agar is dissolved under continous stirring in 11.5 L of boiling water. After cooling to 80°C, 5 L of apple juice, 750 mL of treacle, and 420 mL of 10% methyl-4-hydroxy-benzoate are added. After extensive mixing, about 80 mL are poured on styrofoam trays. This will give 200 agar trays. Trays are cooled to allow agar to harden, piled up, put in plastic bags, and stored at 4°C.
3. Agar trays are prepared by drying the surface of the agar with a tissue paper and a portion of yeast paste is added to the center of the tray. Plates should be at room temperature when put into the fly cage.
4. The curtain tubes of the fly cages are unknotted. After about 2 min, flies who have fallen or been caught inside the curtain tube will have crawled up the sleeve (toward the light) and back into the cage. A new agar tray is carefully placed into the cage. The old one is removed after shaking off the flies and covering it with an empty tray used as a lid. Before complete removal from the curtain tube, the remaining flies on the outside of the tray are removed or squashed by tapping the curtain against the tray.
5. The curtain tubes of the fly cages are reclosed by making a tight knot, and the fly room is vacuumed to prevent the escape of those flies that have managed to get out of the cage into the lab. All surfaces are cleaned, and any potential fly food is removed.

3.1.4. Collecting Drosophila Embryos

1. The three sieve embryo collection apparatus is arranged in a sink by piling up the sieves in such an order that steel meshes get finer toward the bottom.
2. An agar tray with *Drosophila* embryos is placed into the upper sieve. Remaining yeast paste, dead flies, and embryos are rinsed off with cold tap water and gentle strokes with a paint brush.
3. The top sieve will hold back dead flies, the second sieve will hold back fly parts (wings, heads, and so on). Embryos are washed through the upper two sieves and collected in the bottom sieve, which has the finest steel mesh. Yeast paste washes through all three sieves.

3.1.5. Seeding of Larval Boxes

1. Preparation of larval food for one larval box: 250 mL water, 3 mL ortho-phosphoric acid, 0.5 mL propionic acid, 37.5 g sugar, 56.5 g brewer's yeast, and 0.75 g

methyl-4-hydroxybenzoate are thoroughly mixed in a plastic beaker on a magnetic stirrer until the suspension is homogeneous.
2. Preparation of cellulose paper for one larval box: to avoid mold contamination, nine sheets of unbleached cellulose paper are piled up, bagged, autoclaved, and dried.
3. Clean plastic boxes with wire gauze lids (larval boxes) are filled with a stack of nine sheets of unbleached autoclaved cellulose paper that is folded twice.
4. About 325 mL larval food is poured into each plastic box, thoroughly wetting most of the pulp. It will take time for the slurry to be soaked up completely.
5. Meanwhile, three halves of round filter paper per larval box are spread out on a few sheets of cellulose paper.
6. Embryos from five agar trays are collected with distilled tap water and a paint brush in a three sieve embryo collection apparatus. Extensive washing is required to remove residual yeast paste.
7. Using a squeeze bottle, embryos are rinsed from the third sieve with 70% ethanol into a measuring cylinder, and carefully stirred for 10 min.
8. By using a disposable 3-mL plastic pipet with a cutoff tip, these embryos are evenly spread over the filter paper halves. Large clumps of embryos are avoided.
9. After the cellulose paper under the filter papers has soaked up the ethanol, two filter paper halves are transferred to the larval boxes and carefully pressed on the surface of the cellulose paper sheets.
10. The wire gauze lids of the plastic boxes are closed carefully. Boxes are placed side-by-side on the highest shelf in the fly room.

3.1.6. Cleaning of Larval Boxes

1. After seeding of fly cages, used larval boxes are stored at –20°C for at least 12 h to kill remaining flies.
2. After thawing, the content of the plastic boxes is removed. Empty boxes and wire gauze lids are incubated in water with detergent.
3. Boxes and gauze lids are thoroughly cleaned with a tub brush, detergent, and running water.

3.1.7. Cleaning of Fly Cages

Flies die, if they are not fed for 2 d. Fly cages need to be cleaned thoroughly between the fly generations.

1. Curtain tubes and back covers from all cages are removed, washed at 60°C in a washing machine, and spin-dried.
2. Remaining flies are shaken off the curtains.
3. Fly cages are thorougly cleaned with a cleaning tub brush, detergent, and running water.

3.2. Preparation of Nuclear Extract From Drosophila *Embryos*

Several protocols for preparation of nuclear extract from fly embryos have been established over the years ***(2–6)***. Many of these are similar to the protocol presented here.

Drosophila embryos should be between 0 and 12 h old. The collected embryos can be stored for up to 72 h at 4°C. *Drosophila* embryo development will be arrested at 4°C.

1. Embryos are collected with distilled tap water and a paint brush in a three sieve embryo collection apparatus. Extensive washing is required to remove residual yeast from fly food.
2. Collected embryos are dried by placing tissue papers under the sieve with the finest steel mesh (*see* **Note 9**), then transfered into a beaker, soaked in bleach solution (tap water, 3% sodium hypochlorite), and stirred for 3 min.
3. Dechorionated embryos are quickly transfered into the sieve with the finest mesh and quickly rinsed with 1 L embryo wash solution. They are then washed with a high power stream of tap water for a further 5 min. This will mechanically remove chorion membrane from embryos. To achieve a water stream with sufficient power a plastic tubing is attached to the tap and its opening is narrowed by squeezing during the washing procedure.
4. After transferring the embryos into a preweighed, chilled 800-mL beaker, the weight of the embryos is determined. *Note*: all following steps are carried out at 4°C in a cold room.
5. About 2 mL of NXI buffer per gram dechorionated embryos are added, the mixture is passed six times through a Yamato LSC LH-21 homogenizer (*see* **Note 10**) set to 1000 rpm to disrupt embryos. The homogenate is then passed quickly through a funnel with a single layer of miracloth. Gentle pressure can be applied to force the homogenate through the cloth but care must be taken to avoid rupture.
6. The homogenizer, all used glassware, and the debris left behind in the miracloth is then rinsed with additional 2 mL of NXI buffer per dechorionated embryos. NXI buffer is added to the filtrate to give a final volume (V) of 5 mL per gram of dechorionated embryos.
7. The nuclei are pelleted by centrifugation in a Sorvall RC5B with a precooled GSA rotor at 8000 rpm (6552*g*) for 15 min. The supernatant is carefully decanted without disturbing the loose pellet, and the white lipid layer remaining on the wall of each centrifuge tube is removed with a tissue paper.
8. The pellet consists of two layers: a brownish layer on top which contains the nuclei and a yellowish layer below which contains yolk. The nuclei are carefully resuspended in 1 mL of NXII buffer per gram of embryos. Resuspension of the yolk pellet must be avoided. The nuclear pellet is further resuspended by two strokes in a glass dounce homogenizer (Dounce Tissue Grinder, Wheaton/Fisher Scientific GmbH, Schwerte, Germany; cat. no. 357546) fitted with a B pestle.
9. The suspension is transferred into centrifuge tubes fitting a 45Ti-rotor. The V in each centrifuge tube is determined. 1/10 V of 4 *M* $(NH_4)_2SO_4$ (*see* **Note 11**) is added to each centrifuge tube and the contents are rapidly mixed by inverting the tube several times. The nuclei should lyse and the solution should become viscous.
10. The centrifuge tubes are rotated for 20 min at 4°C, then centrifuged in a 45Ti-rotor at 35,000 rpm (182,777*g*) for at least 1 h (*see* **Note 12**).

11. The supernatant is carefully removed with a pipet by plunging the tip of the pipet below the upper white lipid layer and sucking steadily (*see* **Note 13**). The supernatant is transferred into a glass beaker.
12. For each mL of supernatant 0.3 g of finely ground $(NH_4)_2SO_4$ is added while the mixture is stirred at 4°C. It is important to add the ammonium sulfate in small portions over a period of 5 min to ensure a gradual increase of concentration.
13. After 5 min of additional stirring, the mixture is centrifuged in a Sorvall RC5B with a precooled SS-34 rotor at 15,000 rpm (26,892*g*) for 20 min. The supernatant is discarded. The remaining wet protein pellet can be stored for up to 1 wk at 4°C.
14. The protein precipitate is resuspended in 0.2 mL of NXIII buffer per gram dechorionated embryos by pipeting up and down. The precipitate will not go into solution easily and resuspension can take several minutes.
15. The extract is dialyzed against 2 L of NXIII buffer, until the conductivity is equal to that of Q100 buffer. This usually takes about 4 h. To minimize protein precipitation during dialysis, overnight dialysis is not recommended.
16. Precipitated proteins are pelleted by centrifugation in a SS-34 rotor for 5 min at 9000 rpm (9681*g*). The resulting supernatant is frozen (as "nuclear extract") in liquid nitrogen and can be stored at –80°C for up to 6 mo.

3.3. Chromatographic Purification of Native Protein Complexes From Drosophila Embryos

The protocols to be used to purify native protein complexes from *Drosophila* embryo nuclear extracts obviously heavily depend on the nature and properties of the complex in question. As an example, we will describe in the following section the purification of the *Drosophila R*BF, *E*2F, and *M*yb-interacting proteins (dREAM) complex ***(7,8)***. The dREAM complex contains *Drosophila* homologs of the Retinoblastoma tumor suppressor protein and functions to regulate gene transcription. All steps are performed in the coldroom. Columns are run using a FPLC chromatography system.

3.3.1. Preparation of Starting Material

1. Nuclear extract is prepared from 300 g dechorionated embryos (*see* **Subheading 3.2.**).
2. Extract is dialyzed against 2 L of Q100 buffer for 2 h. Q100 buffer is replaced and dialysis is continued for another 2 h.
3. Precipitated proteins are pelleted by centrifugation in a SS-34 rotor for 5 min at 9000 rpm (9681*g*).

3.3.2. Anion Exchange Chromatography—Step Elution

1. The supernatant is applied at a flow rate of 1 mL/min to 70 mL Q Sepharose fast flow anion exchange resin (GE Healthcare; cat. no. 17-0510-01) in a XK 26/40 column (GE Healthcare; cat. no. 18-8768-01) (*see* **Note 14**). The running buffer is Q100. The flowthrough is collected.
2. The column is washed with five column V (CV) Q100 (1 mL/min).

3. Proteins are eluted with Q450 buffer and protein-containing eluates are combined (*see* **Note 15**). These eluates contain dREAM complex (*see* **Note 16**).
4. Presence of dREAM complex subunits in the 450 m*M* KCl eluate is confirmed by Western blotting input extract, flowthrough, and eluate with antibodies recognizing dREAM subunits.
5. The 450 m*M* eluate is dialyzed against 2 L of B100 buffer for 2 h. B100 buffer is replaced and dialysis is continued for another 2 h.

3.3.3. Cation Exchange Chromatography—Step Elution

1. The sample is then applied to 25 mL Biorex 70 cation exchange resin (Bio-Rad Laboratories) in a XK 16/20 column (GE Healthcare; cat. no. 18-8773-01) at a flow rate of 1 mL/min. The running buffer is B100. The flowthrough is collected.
2. The column is washed with 5 CVs B100 buffer (1 mL/min).
3. Proteins are eluted by stepwise increasing the KCl concentration in the running buffer to 250 m*M*, 500 m*M*, and finally 1000 m*M*. dREAM complex elutes with the 250 m*M* fraction.
4. Presence of dREAM complex subunits in the 250 m*M* KCl eluate is confirmed by Western blotting input extract, flowthrough, and eluates with antibodies recognizing dREAM subunits.
5. The 250 m*M* eluate is dialyzed against 2 L of Q100 buffer for 2 h. Q100 buffer is replaced and dialysis is continued for another 2 h.

3.3.4. Anion Exchange Chro matography—Linear Gradient

1. The sample is applied to a 5 mL Q Sepharose HiTrap high-performance anion exchange column at 0.5 mL/min.
2. The column is washed with 5 CV Q100 (0.5 mL/min).
3. The column is resolved with a linear gradient from Q100 to Q500 over 25 CV. Fractions of 2.5 mL are collected.
4. Fractions containing dREAM complex are identified by Western blotting and pooled. dREAM complex elutes in one peak at a KCl concentration of approx 400 m*M*.

3.3.5. Hydroxyl Apatite Chromatography

1. Pooled fractions are directly loaded onto a 0.8 mL hydroxyl apatite column at a flow rate of 0.2 mL/min.
2. The column is washed with 2 CV Q400 containing 10 m*M* phosphate.
3. Proteins are eluted with a linear phosphate gradient ranging from 10 to 500 m*M* phosphate over 25 CV. Fractions of 0.5 mL are collected.
4. Fractions containing dREAM complex are identified by Western blotting.

3.3.6. Gel Filtration Chromatography

1. 0.2 mL of the peak fraction are applied to a Superose 6 10/300 GL column pre-equilibrated with buffer EX300.
2. The column is resolved at a flow rate of 0.2 mL/min and 0.5 mL fractions are collected. 0.25 mL of each fraction is precipitated with StrataClean resin, separated by

sodium dodecyl sulphate polyacrylamide electrophoresis and analyzed by Western blotting, Coomassie (Colloidal Blue Staining Kit, Invitrogen Corporation, Karlsruhe, Germany; cat. no. LC6025) or silver staining. The remainder is frozen in liquid nitrogen and stored at –70°C.

4. Notes

1. It is not necessary to obtain a custom-built fly chamber. Existing chambers can be modified. We are currently using a retrofitted cold room (height: 2, 360 mm; length: 3300 mm; width: 2400 mm).
2. If yeast paste is too wet then flies landing on it get stuck and die.
3. Presence of citric acid or ascorbic acid can upset the digestion of the flies and severely limit embryo yield.
4. Pulverized yeast is highly volatile. Special care must be taken to avoid inhalation. The use of face masks is recommended.
5. NXIII buffer is supplemented with proteinase inhibitors aprotinin, leupeptin, and pepstatin (final concentration: 10 µg/mL) immediately before use.
6. Flies produce only few embryos during the first 2 d after being transferred from larval boxes into cages.
7. It usually takes 12 d for flies to hatch in the larval boxes. They should be transferred into cages within 2 d of hatching.
8. For obvious reasons it is important to work rapidly to avoid flies waking up before transfer into the cage. If flies wake up in the Erlenmeyer flask they will start crawling up the walls. A gentle stream of CO_2 is then directed into the flask (CO_2 is heavier than air and sinks to the bottom), the flask is then knocked on the table a few times, so that flies are falling off the wall into the CO_2 cushion below.
9. Alternatively, embryos can be dried by transferring them into a Buechner funnel fitted with a paper filter and applying a gentle vacuum.
10. This is a motor driven continuous flow homogenizer consisting of a serrated teflon pestle within a glass cylinder. James Kadonaga has suggested that if a Yamato homogenizer is not available, homogenization could be carried out with six to eight strokes in a motorized Potter-Elvehjem homogenizer with a serrated Teflon pestle and a glass vessel. In this case it may be necessary to homogenize further using a 40-mL Wheaton Dounce homogenizer with a B pestle (www.bio.com/protocol-stools/protocol.jhtml?id=p9052).
11. The ammonium sulfate solution has a pH of 7.9 and should be stored at room temperature to avoid precipitation.
12. An extended spin for up to 2 h improves the stability of the upper white lipid layer, making it easier to remove the liquid whereas leaving the lipid film behind.
13. Lipids can interfere with the subsequent ammonium sulfate precipitation and it is therefore important that lipids are not transfered.
14. All columns are equilibrated with their respective running buffer before use.
15. This step serves to remove most nucleic acids, which remain bound to Q Sepharose at 450 m*M* KCl. The column can be restored by washing with 5 CV Q1000 and 5 CV Q100.

16. Column eluates can be frozen in liquid nitrogen and stored at –70°C. On rethawing samples on ice they should be passed through a 0.45-µm filter unit before applying them to the next column to remove protein precipitates.

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Flow Cytometric Analysis of *Drosophila* Cells

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Summary

Flow cytometry is a powerful technique that allows the researcher to measure fluorescence emissions on a per-cell basis, at multiple wavelengths, in populations of thousands of cells. In this chapter, we outline the use of flow cytometry for the analysis of cells from *Drosophila's* imaginal discs, which are developing epithelial organs that give rise to, but not exclusively, the wings, eyes, and legs of the adult. A variety of classical and transgenic genetic methods can be used to mark cells (e.g., mutant, or overexpressing a gene, or in a particular compartment) in these organs with green fluorescent protein (GFP), which is readily detected by flow cytometry. After dissecting an organ out of the animal and dissociating it into single cells, a flow cytometer can be used to assay the size, DNA content, and other parameters in GFP-marked experimental cells as well as GFP-negative control cells from the same sample. Specific marked cell populations can also be physically sorted, and then used in diverse biochemical assays. This chapter includes protocols for isolation and dissociation of larval imaginal discs and pupal appendages for flow cytometry, and as well as for flow cytometric acquisition and analysis. In addition, we present protocols for performing flow cytometry on fixed or live-cultured *Drosophila* S2 cells.

Key Words: Cell cycle; cell growth; dissociation; *Drosophila*; FACS; Flow cytometry; Hoechst 33342; imaginal disc; live cells; papain; GFP; propidium iodide; pupa; S2 cells; Schneider cells; trypsin.

1. Introduction

The most common application of flow cytometry has been to measure cellular DNA content in conjunction with fluorescent markers for specific gene products or processes such as DNA replication, mitochondrial function, or apoptosis. In the flow cytometer, cells pass through a chamber in a liquid stream where they are exposed to laser light of defined wavelengths, and their emitted fluorescence at defined wavelengths is detected and recorded. Flow cytometers also measure light scatter, and this data can be used to evaluate cell size and shape. However, light scatter is useful only for assaying relative cell sizes and cellular membrane complexity. If

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absolute cell volume measurements are required, an instrument that measures cell volume on the basis of electrical conductivity such as a Coulter Counter (Beckman Coulter Inc., Fullerton, CA) should be used. Flow cytometers capable of fluorescence-activated cell sorting (FACS) are commonly used to separate and collect cells marked with a specific fluorescent properties from mixed populations of cells using electrostatic deflection off charged plates. Cells purified in this way can then be cultured or used in biochemical assays. Flow cytometry has been applied in *Drosophila* not only to tissue culture cells (e.g., S2, Kc, Cl8), but also to cells or nuclei isolated from living animals. Protocols are available for performing flow cytometry on nuclei isolated from embryos ***(1)*** and ovaries ***(2,3)***, for epithelial cells isolated from larval imaginal discs ***(4)***, and for hemocytes isolated from the hemolymph of larvae and adults ***(5)***. In principle, any tissue that can be cleanly dissociated into single cells or nuclei can be analyzed by flow cytometry, although the technique is best applied when large numbers of cells or nuclei (>4000/sample) are available. Recently, flow cytometers have become available that can even analyze and sort whole *Drosophila* embryos and 1st instar larvae based on their size or expression of a fluorescent marker ***(6)***. In this chapter, we include protocols appropriate for larval imaginal discs and pupal appendages (wings, eyes, and legs), and for cultured S2 cells.

The availability of hundreds of fluorescently tagged proteins in transgenic flies has made flow cytometry a very powerful technique for in vivo studies. Green, yellow, and red fluorescent proteins (GFP, YFP, and RFP, respectively) and fusion proteins containing these sequences are readily scored and quantified by flow cytometers, allowing many applications. Using the Gal4/ upstream activating sequence (UAS) transgenic system ***(7)***, a combination of *UAS-GFP* with UAS-linked transgenes can be coexpressed in clones of cells or tissue-specific regions of an organ ***(4,8)***. The GFP-marked cells can then be distinguished from normal, GFP-negative cells by the flow cytometer, and analyzed or sorted accordingly. This technique can also be applied to acquire data on mutant cells in genetic mosaics generated by the Flp/FRT ***(9)*** or MARCM ***(10)*** mitotic recombination systems. When the Flp/FRT system is used, homozygous mutant cells are usually marked by the lack of GFP expression ***(11)***, whereas with the MARCM system mutant cells are positively marked ***(12)***. Flow cytometry has also been used to compare cells from different regions of an organ, in cases where the Gal4/UAS system is used to mark a particular region (e.g., dorsal or posterior compartments) with GFP. We find that it is best to use tissues in which an equal number of cells are affected (mutant or expressing a particular gene) and are normal (wild-type) (e.g., two samples with identical genotypes, but at different developmental stages, or two samples with different genotypes). This gives the researcher an internal control population of cells, which is important because it reduces both variation between biological samples, and the variation that occurs between separate data collection sessions (e.g., daily alignment is not exact and therefore it is difficult to obtain alignment identical to that of previous sessions). We recommend using internal control cells whenever possible.

The general outline of a typical flow cytometry experiment using imaginal discs is as follows: first, the researcher chooses a genetic design that will generate adequate numbers of animals containing cells of interest (e.g., mutant, or overexpressing a particular gene) and control cells (e.g., wild-type) that are differentially marked with GFP. The crosses are then established and eggs are collected for a short period from the parental generation, to assure good synchrony in the developing experimental progeny. Newly hatched larvae are seeded at low density (30–50/vial) into vials and allowed to develop to the stage desired. The animals may be genotyped using visible markers (e.g., GFP-marked balancers). When conducting mosaic analysis, clones of cells marked by GFP (or lack thereof) are induced by heat shock treatment at various developmental stages. When the animals reach the desired developmental stage, 10–40 imaginal discs from each sample of interest are dissected out. The discs are dissociated into single cells using a solution containing trypsin or papain, a vital DNA stain (e.g., Hoechst 33342) and perhaps a stain for viability (e.g., propidium iodide) are added, and the cells are then processed through the flow cytometer. The dissociation protocol included here is for the analysis of live cells that have not been fixed or subjected to centrifugation, and is optimized to work well for experiments in which small numbers of animals of different genotypes will be assessed for changes in cell cycle progression or cell size. Once the animals are dissected, the entire procedure must be completed promptly, before the inevitable lysis of the cells. This works well for larval imaginal discs, and pupal wings (PW) or eyes up to 36 h after puparium formation, at which point there is too much pupal cuticle in the differentiating organs to allow efficient cell dissociation. The cells are analyzed using a multiparameter flow cytometer that is capable of detecting forward and side scatter (SSC), GFP, and the fluorochromes aforementioned. The data is then processed using software tools to exclude debris, dying cells, and clumps, which allows the investigator to focus on the cells of interest. Relative changes in cell size and cell cycle progression can be detected within a sample if there is an internal control population present or against other samples if an external control is analyzed within the same session.

The protocol for cultured S2 cells we include here is for preparing live or fixed cells for flow cytometric analysis. Flow cytometry can, in principle, also be used for live or fixed cells stained with fluorescent-conjugated antibodies or other fluorescent markers for virtually any gene product or cellular process. However, a protocol for fixed imaginal disc cells is not currently available.

2. Materials

2.1. Fly Husbandry and Embryo Collection

1. *Collection container:* standard round bottom polypropylene bottle 64-mm diameter bottom × 103-mm height (Applied Scientific Drosophila Products). With a medium gauge needle, poke enough holes to allow ample airflow.
2. *Grape plates:* 75 g bacto agar, 3 L dH_2O, 1 L grape juice, 100 g sucrose, and 6 g methyl paraben (methyl p-hydroxybenzoate).

Mix the agar and H_2O in appropriate flasks and autoclave for 45 min. Meanwhile, stir 100 g sucrose and 1 L grape juice on low heat until the sucrose is dissolved and the flask is warm to the touch. Then add 6 g methyl paraben (heat sensitive and only poorly water-soluble). Let the autoclaved agar cool until the flask is warm to the touch, and then mix in the juice mixture. Aliquot approx 4 mL of media into the lids of 35×10 mm^2 Petri dishes (BD Biosciences, San Jose, CA). Grape plates can be stored in an airtight container at 4°C for several weeks. Before use, warm the grape plates to room temperature for collecting embryos.

3. *Active yeast:* dry active Baker's yeast (MP Biomedical, LLC, Solon, OH). Make fresh paste by mixing sterile H_2O and yeast together. The consistency should not be runny, as this will decrease the area available for egg laying.
4. Incubator with proper humidity.
5. *Vials of fruit fly food:* a recipe of choice suitable for the particular experimental requirements. Otherwise, using any standard recipe, for example, Bloom Food (http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/bloomfood.htm) that supports normal and consistent developmental timing is suitable. Plug the vials with cotton.

2.2. Incubation and Heat Shock

1. Incubators with proper humidity.
2. For heat shock treatment use either an incubator, culture room, or water bath set at 37ºC.
3. Ultraviolet (UV) dissecting microscope (Leica Microsystems, Wetzlar, Hessen, Germany).

2.3. Dissection

1. *20% (w/v) Sucrose:* 200 g sucrose, 100 mL 10X phosphate-buffered saline (PBS), and 400 mL dH_2O. Stir well, bring up to 1 L, and filter sterilize.
2. *10X PBS (1 L):* 80 g NaCl, 2 g KCl, 6.1 g Na_2HPO_4 (anhydrous), 2 g KH_2PO_4, pH 7.3, and dH_2O. Mix well and filter sterilize.
3. *Forceps:* Dumont Inox no. 5 Biologie tip forceps (Manufactures des Outils Dumont S.A., Montignez, Aargau, Switzerland).
4. *Clear glass dish:* volume capacity 3 mL. Avoid using plastic dishes as imaginal disc cells readily adhere to plastic.
5. *20-µL Micropipet tip:* precoat the plastic tip by pipeting several pieces of larval fat body up and down several times.
6. Dissecting microscope with visible light source (Leica Microsystems).

2.4. Tissue Dissociation and DNA Staining

1. *10X PBS (1 L):* 80 g NaCl, 2 g KCl, 6.1 g Na_2HPO_4 (anhydrous), 2 g KH_2PO_4, pH 7.3, and dH_2O. Mix well and filter sterilize.
2. *1000X Hoechst 33342:* 0.5 mg/ml Hoechst 33342 (Acros Organics, NJ) 100% dimethyl sulfoxide. Store at 4°C protected from light.
3. *Tube for BD Biosciences flow cytometers:* round bottom 6-mL polystyrene tube with cap, 12×75 mm^2 (BD Biosciences). If using a cytometer made by a different company, use the tube required for that cytometer.

4. *Trypsin solution (22 mL):* add 2 mL of 10X PBS and 22 µL of 1000X Hoechst 33342 to 20 mL of 10X trypsin-ethylenediaminetetraacetic acid (EDTA) solution (Sigma, St. Louis, MO). Aliquot 0.5 mL trypsin solution into 6-mL polystyrene tubes. The tubes may be stored at –20°C protected from light until ready for use. Thaw to room temperature before adding dissected tissues.
5. *Agitation:* Clay Adams Nutator Mixer, BD Diagnostic Systems (VWR LabShop, Batavia, IL). Set all other mixers or shakers on low speed.
6. Standard pencil with double-sided tape applied lengthwise. This provides a secure and elevated position for the 6-mL tubes while mixing.

2.5. S2 Cells

2.5.1. Harvesting and DNA Staining of Live S2 Cells

1. 1X Hoechst 33342 in 1X PBS (0.5 mL per sample).
2. Tube for BD Biosciences flow cytometers: round bottom 6-mL polystyrene tube with cap, 12×75 mm^2 (BD Biosciences). If using a cytometer made by a different company, use the tube required for that cytometer.

2.5.2. Harvesting and Fixation of S2 Cells

1. *Sterile PBS:* Dulbecco's phosphate-buffered saline without calcium chloride and without magnesium chloride (Gibco, Carlsbad, CA).
2. Centrifuge with swinging bucket rotor.
3. *95% Ethanol:* filter-sterilized 95% (v/v) ethanol in dH_2O.

2.5.3. DNA Staining of Fixed S2 Cells

1. *20X Propidium iodide:* 0.5 mg/mL propidium iodide in 38 m*M* sodium citrate.
2. *40X RNAse A:* 100 µg/mL DNAse-free RNAse A, 10 m*M* PIPES, pH 6.8, 2 m*M* $MgCl_2$, 0.1 *M* NaCl, 0.25 m*M* EDTA, and 0.2% Triton X-100.
3. Centrifuge with swinging bucket rotor.
4. Tube for BD Biosciences flow cytometers: round bottom 6-mL polystyrene tube with cap, 12×75 mm^2 (BD Biosciences). If using a cytometer made by a different company, use the tube required for that cytometer.

2.6. Flow Cytometry

2.6.1. Acquisition

2.6.1.1. Hardware and Parameters

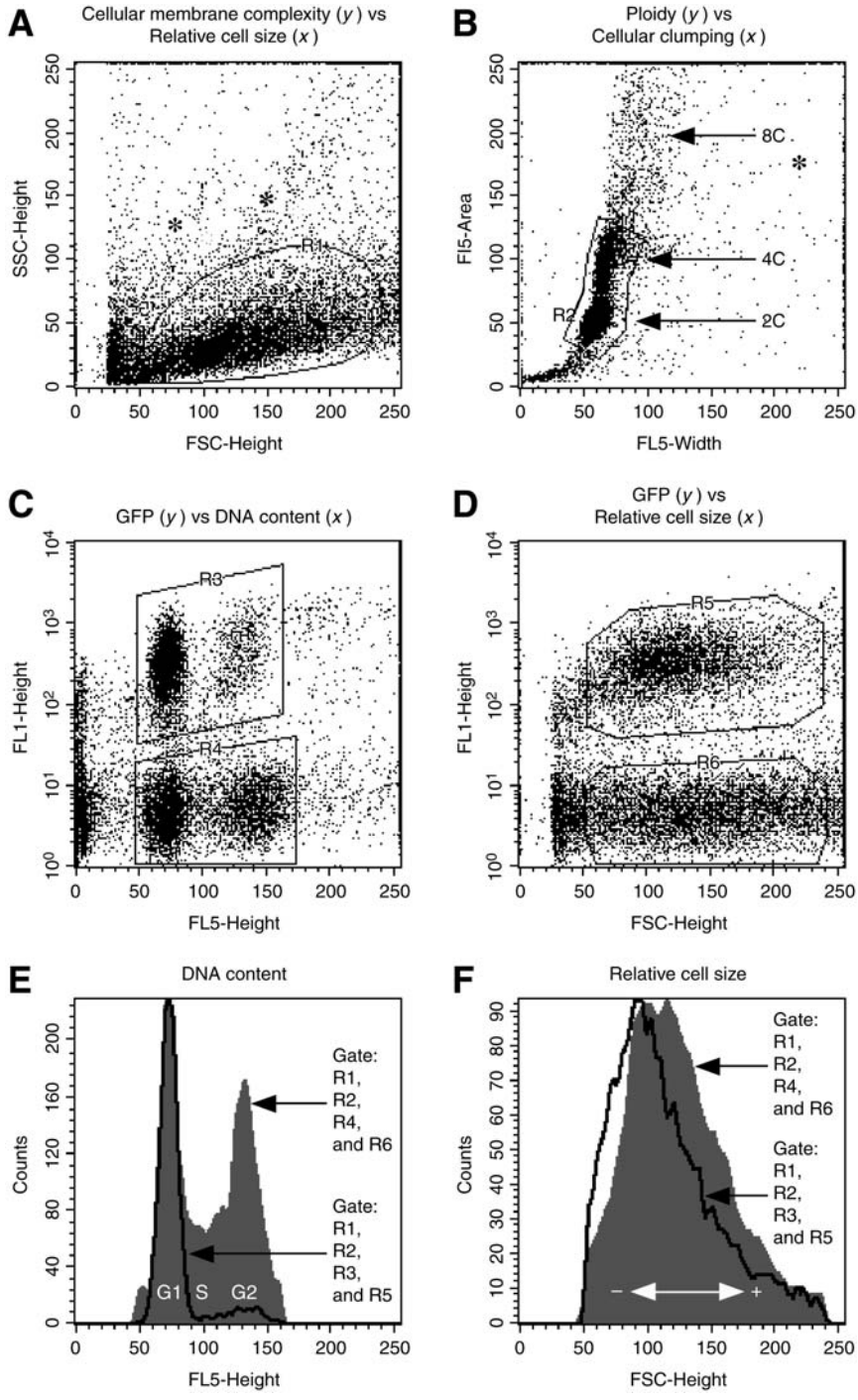
1. There are many multiparameter flow cytometers available that are capable of measuring DNA content and relative cell size. The critical requirement is that the flow cytometer must be able to excite and detect the fluorescence of both Hoechst 33342 and GFP. The FACS Vantage (which can sort cells) and LSR 1 (which cannot sort cells) by BD Biosciences (San Jose, CA) both fulfill this requirement and were used to establish these methods. The following instructions are for these BD Biosciences' cytometers and software, but the same principles should apply generally.

2. BD Biosciences provides online technical resources for possible configurations of lasers, filters, mirrors, and detectors for the FACS Vantage and LSR 1.
3. Hoechst 33342 detection uses a UV source (355-nm laser on the FACS vantage and 325-nm laser on the LSR) and detection through a 424/44 bandpass filter. GFP detection uses a 488-nm laser and detection through a 530/30 bandpass filter. Propidium iodide uses a 488-nm laser and detection through a 630/30 bandpass filter. Both instruments are setup with the 488-nm laser as the primary beam and the UV as a secondary beam.
4. *Required parameters:* height, width, and area of fluorescence emission signal for either Hoechst 33342 (live cells) or propidium iodide (fixed S2 cells), height of fluorescence emission signal for GFP, and height of forward and side scatter (SSC) of visible light. Both the FACS vantage and LSR 1 support eight parameters total.
5. *Nomenclature:* forward scatter (FSC); a parameter measuring light scattered less than 10°. FSC measures relative cell size. SSC; light scattered at a 90° angle. This measurement is related to the internal granularity or complexity of cellular membranes combined. FL stands for fluorescence. Assign FL1 for GFP, FL4 or FL5 for Hoechst 33342, and FL3 for propidium iodide. Height (H) is the maximum amplitude of the emission signal. Width (W) is the duration of the signal or time of flight and can be considered a measure of cellular or nuclear clumping. Area (A) is the area of the signal.

2.6.1.2. Software and Template Design

1. Using Cell Quest (BD Biosciences) software, create an acquisition template with dot plots with the following parameters (*y*- and *x*-axes, respectively): SSC height (SSC-H) vs FSC height (FSC-H), GFP height (FL1-H) vs Hoechst 33342 height (FL4/5-H), Hoechst 33342 area (FL4/5-A) vs Hoechst 33342 width (FL4/5-W), FL1-H vs FSC-H, and for live/dead exclusion, include FL5-4/H vs propidium iodide height (FL3-H).

Fig. 1. *(Opposite page)* An example acquistion/analysis template for acquiring and analyzing differences between live dissociated cells expressing GFP and cells not expressing GFP. Regions are drawn to include the populations of interest **(A–D)** and after combining specific regions, called gates, relative changes in DNA content and cell size can be assessed **(E,F)**. **(A)** Region 1 (R1) is drawn and placed to include most of the events that exhibit similar SSC and FSC values. The asterisks indicate two populations with different SSC values and therefore no attempt to include them is necessary. **(B)** R2 is drawn and placed to include cells with only 2C and 4C (a full diploid complement of chromosomes and after replication, respectively) DNA content, as there is no apparent change in ploidy of these diploid cells (verified by the plot). Nonetheless, the region can be extended to detect potential effects in ploidy. The asterisk indicates where a clump of four cells with 2C DNA content would likely be versus a single cell with 8C content indicated by the upper arrow as clumped cells exhibit a longer duration of signal. **(C)** R3 and R4 are drawn and placed to distinguish GFP-positive from GFP-negative cells with respect to DNA content. **(D)** R5 and R6 are drawn and placed to distinguish GFP-positive from GFP-negative cells with respect to relative cell size. **(E)** Comparing data using appropriate gates is useful for detecting differences in DNA content. Cell cycle phases gap 1 (G_1), synthesis (S), and gap 2 (G_2) can be further quantified with other software. **(F)** Using the same gates as in **E**, differences in relative cell size for the two populations can be visualized.


A
Cellular membrane complexity (y) vs
Relative cell size (x)
SSC-Height
FSC-Height
R1
B
Ploidy (y) vs
Cellular clumping (x)
Fl5-Area
FL5-Width
8C
4C
2C
R2
C
GFP (y) vs DNA content (x)
FL1-Height
FL5-Height
R3
R4
D
GFP (y) vs
Relative cell size (x)
FL1-Height
FSC-Height
R5
R6
E
DNA content
Counts
FL5-Height
Gate:
R1,
R2,
R4,
and R6
Gate:
R1,
R2,
R3,
and R5
G1
S
G2
F
Relative cell size
Counts
FSC-Height
Gate:
R1,
R2,
R4,
and R6
Gate:
R1,
R2,
R3,
and R5
–
+

2. As seen in **Fig. 1A–D**, plotting SSC and FSC together is useful for isolating a homogeneous population of cells and for viewing their size distribution. A degraded cell will have different light scattering properties than will an intact cell of the same cell-type, owing to the higher organizational complexity of the total membrane content of the former. Furthermore, cellular debris causes less light scatter than do intact cells, and conversely clumps of cells cause more. Plotting Hoechst 33342 signal area and width together is useful for analyzing ploidy, while excluding spurious signals that arise from clumps of cells or nuclei. If experimental cells exhibit an effect in ploidy, then a region can be drawn to include these cells of interest. Plotting GFP and DNA content together is useful for distinguishing populations of cells that have GFP proteins from those that do not. Such cells can also be simultaneously analyzed for differences in DNA content. Plotting GFP and FSC together is useful for viewing differences in relative cell size between GFP-positive and negative cells.
3. With the polygon tool, draw one or more regions of interest for each dot plot. These regions, if saved, can serve as a reference area for tuning the cytometer for future experiments.
4. Create a "Gate list" that includes regions individually and combined. The gating function is a critical tool to display, assess the quality, and further analyze the data.
5. Create histograms of FSC-H and of FL4/5-H. Apply appropriate gates generated in **step 4** to view the relative cell sizes and DNA content while acquiring data as seen in **Fig. 1E**,**F**.
6. For fixed S2 cells, modify the acquisition template by omitting all FL*4*/5-containing dot plots and histograms. Instead, include FSC-H vs FL3-H, and FL3-A vs FL3-W dot plots and a FL3-H histogram. FL1 is also not a necessary parameter when GFP is not being used to mark cells.
7. Save acquisition templates and instrument settings for future use.

2.6.2. Data Analysis

1. *Analysis software:* Cell Quest or Cell Quest Pro (BD Biosciences). Use or reproduce the appropriate acquisition template to analyze data. Save analysis templates for future use.
2. *Quantification of DNA content:* MultiCycle AV (Phoenix Flow Systems, San Diego, CA).

3. Methods

3.1. Fly Husbandry and Embryo Collection

1. Optimize the size of the cross to obtain a sufficient number of GFP-marked or -unmarked cells. **Table 1** provides a suggested number of organs needed per flow analysis for four different genetic conditions. Depending on the genetic technique, take into account the proportion of experimental cells generated and the developmental timing. For example, there is a smaller fraction of glassmultimer reporter *(GMR)-Gal4* expressing cells in the eye imaginal disc (EID) from a mid-L3 larva than in a wandering L3 larva, therefore dissecting more EIDs from younger larvae is necessary not only because they are smaller, but also because the proportion of experimental cells is less. The number of discs listed

Table 1
Suggested Number of *Drosophila* Organs Needed for Flow Cytometric Analysis

Developmental timing	G.O.F. clones (*Flp/Gal4*)	L.O.F. clones (*Flp/FRT*)	*Engrailed-Gal4*	*GMR-Gal4*
Early L3	40 WID	Unknown	50 WID	n/a
Mid L3	20 WID	80 WID	30 WID	40 EID
Wandering L3	10 WID	60 WID	20 WID	20 EID
0–6 h APF	10 PW	Unknown	20 PW	15 PE
6–12 h APF	15 PW	Unknown	20 PW	15 PE
12–24 h APF	30 PW	Unknown	30 PW	15 PE
24–36 h APF	40 PW[a]	Unknown	40 PW[a]	10 PE
36–48 h APF	50 PW[a]	Unknown	50 PW[a]	10 PE

G.O.F., gain of function; L.O.F., loss of function; APF, after pupae formation; WID, wing imaginal disc; PW, pupal wing; EID, eye imaginal disc; PE, pupal eye; PW[a], usually requires longer than 4 h of dissociation; n/a = not applicable.

under gain of function (G.O.F) clones, for example, Flp/Gal4 method in the table, assumes half of the total tissue is made up of experimental cells. The number of discs listed under loss of function (L.O.F) clones, for example, Flp/FRT system assumes one quarter of the total tissue is made up of experimental cells. Proper heat shock conditions should be determined for each genetic condition.

2. Apply a thin layer of yeast paste in the middle of the grape plate.
3. Collect embryos for ≤2 h inside a collection polypropylene bottle. The shorter the collection, the more developmentally synchronous the larvae are.
4. Carefully take off any excess yeast paste to make it easier to find, count, and transfer the larvae.
5. Store seeded grape plates in clean Petri dishes, cover to avoid drying out of the embryos.
6. Incubate the embryos at 25°C.
7. Transfer equal numbers of larvae into vials within 2 h of hatching. Depending on the food quality and health of the larvae, the optimal range of the number of larvae per vial is between 30 and 50.

3.2. Incubation and Heat Shock

1. Incubate the larvae at 25°C for the duration desired. If appropriate, apply a 37°C heat shock to the larvae to generate mosaic clones. Optimal heat shock timing to obtain an equal number of experimental and control cells should be predetermined. **Table 2** provides heat shock conditions for G.O.F or L.O.F mosaic analyses.
2. If necessary, use a UV dissecting microscope to confirm that the GFP patterns or intensity levels in the larvae are correct.
3. Set aside larvae or pupae with correct genotypes and appropriate GFP levels for dissection.

Table 2
Suggested Heat Shock Conditions for G.O.F or L.O.F Mosaic Analyses

Method	Heat shock at	37°C Incubator (min)	37°C Water bath (min)	Harvest at
G.O.F. clones (Flp/Gal4): *hsFlp^{122}; Actin > CD2 > Gal4/+; UAS-GFP/UAS-GeneX*	72 h AED	60–90	20–30	112 h AED
L.O.F. clones (Flp/FRT): *hsFlp^{122}; FRT 42D, Ubiquitin-GFP/FRT 42D, geneX; +*	60 h AED	90–120	30–40	112 h AED

AED, after egg deposition; G.O.F., gain-of-function; L.O.F., loss of function.

3.3. Dissection

The following three steps are specifically for larval imaginal discs. Dissected pupal organs are treated identically thereafter.

1. Gently transfer larvae to a clear glass dish with 1X PBS and dissect immediately. If necessary, use 20% sucrose to float larvae out of the food and gently wash them in 1X PBS several times.
2. With a dissecting microscope and ample light, use two Dumont Inox no. 5 forceps to tear the larva in half. If the imaginal discs of interest are eye-antennae, leg, wing, haltere, or labial, then gently invert the anterior half. Carefully remove the fat body and gut, because they can impair the view and because they can fall apart and contaminate the sample.
3. Transfer the inverted, clean carcass to a new glass dish with fresh 1X PBS. After identifying the appropriate disc, pluck it off by gently pulling the disc away from attachment points. To avoid damage, do not squeeze the disc. Accumulate the imaginal discs in a pile at one side of the dish away from the carcasses and other stray tissues. For PWs, take off the cuticle because it can both clog the cytometer filters and it also prevents efficient dissociation.
4. Coat a 20-µL micropipet tip with left over disposed carcass (specifically the fat body) from the first glass dish, as described in **step 2**. For pupal organs, cut some of the coated tip off to accommodate their larger size. Do not forget to coat tips, otherwise the entire sample might stick to the tip. Do not use the same tip for different samples.

5. Using a coated tip, carefully transfer the discs by gently pipeting up the pile of tissues and then slowly dispensing the discs to a new glass dish with fresh 1X PBS. Repeat this if there is a significant amount of debris carried over.
6. Do not take more than 1 h to dissect each sample. The faster tissues can be dissected without damage, the more accurate the results will be.

3.4. Tissue Dissociation and DNA Staining

1. With a coated 20-μL micropipet tip, transfer all of the discs with as little volume as possible (e.g., 20 discs in ≤15 μL 1X PBS) into a 0.5 mL of trypsin solution in a 6-mL tube (specific for the cytometer). Trypsin, a serine endopeptidase, catalyzes the cleavage of peptide bonds on the carboxyl side of either arginine or lysine except when those residues are followed by a proline (*see* **Note 1**). A large proportion of cells will not survive this treatment, so compensate for this loss when planning the experiment especially when sorting cells (*see* **Note 2**). Dying cells with permeabilized membranes can also be detected by flow cytometry by addition of propidium iodide to the trypsin solution (*see* **Notes 3** and **4**).
2. If dissociating a large number of organs, divide the sample into several tubes of trypsin solution rather than placing them all in a single tube. Otherwise, dissociation will take longer and therefore increase any toxic trypsin- and dye-induced effects on cell cycle phasing and cell growth. Combine the contents from the multiple tubes just before running the cells through the cytometer. Adding more trypsin solution to the tube is fine as long as the organs can still be agitated well.
3. Place the tube on a Nutator Mixer platform to mix it, with the top end of the tube slightly elevated so that the contents do not flow into the cap during mixing. A pencil with double-sided tape applied lengthwise can be used to elevate and secure the tubes. Additional brief and intermittent shaking of the sample by hand can be effective, but do not vortex, as this damages the cells. Restrict fluid movement to a minimum in order to minimize the number of cells sticking to the tube walls, while still allowing some flow to aid in the dissociation.
4. Incubate 1–4 h on the mixer at room temperature with gentle agitation until no tissue fragments are visible (gently shake the tube while holding up to the light to check this). There is significant cell lysis of larval imaginal discs after 4 h of incubation at room temperature, therefore optimizing the duration and strength of the dissociation reaction is critical for finding a satisfactory balance between quick dissociation and retention of healthy intact cells.
5. For one sample only (e.g., the control sample), dissect extra organs to provide enough cells to calibrate the cytometer.

3.5. Fixed S2 Cells

3.5.1. Harvesting and DNA Staining of Live S2 Cells

1. Gently pipet S2 cells up and down several times to dislodge attached cells from the culture dish.
2. Transfer 0.5–3 million cells into a clear 1.5-mL microfuge tube. This is more than sufficient for flow cytometric analyses while still producing a visible pellet, which makes it easier to transfer cells in **step 4**.

3. Allow the cells to settle to the bottom of the tube while the tube is on ice (~60–90 min). Do not spin the cells to avoid clumping.
4. With a sterile tip, pipet 20 µL of cells from the pellet.
5. Transfer the cells to a 6-mL tube with 0.5 mL 1X Hoechst 33342 in 1X PBS.
6. Allow 20 min for the Hoechst 33342 to be taken up (at room temperature).
7. Run the samples through the cytometer as soon as possible.

3.5.2. Harvesting and Fixation of S2 Cell

(Modified N. Dyson lab protocol, Massachusetts General Hospital Cancer Center, Charlestown, Massachusetts.)

1. Gently pipet S2 cells up and down several times to dislodge attached cells from the culture dish.
2. Transfer 0.5–3 million cells into a 15-mL conical tube. This is more than sufficient for flow cytometric analysis, but harvesting an excessive number of cells allows for other analyses and compensates for cells lost from the fixation procedure.
3. Spin the cells for 5 min at 1000 rpm (300*g*) at 4°C. Take off the media.
4. Using a glass pipet, resuspend the cells in 5 mL cold sterile 1X PBS.
5. Spin the cells for 5 min at 300*g* at 4°C. Take off the 1X PBS.
6. Resuspend the cells in 0.5 mL cold sterile 1X PBS.
7. Vortex the cells at medium speed while slowly adding 5 mL of 95% ethanol.
8. The fixed S2 cells may be stored for up to 2 wk at 4°C.

3.5.3. DNA Staining of S2 Cells

1. The day before flow analysis, transfer a desired number of the fixed cells into a 15-mL conical tube.
2. Spin the cells for 5 min at 1000 rpm (200*g*), take off the ethanol, and then resuspend the cells in 3 mL sterile 1X PBS.
3. Let the cells sit for 5 min to allow them to rehydrate.
4. Spin the cells for 5 min at 300*g*, take off the PBS, and then resuspend the cells in 1 mL sterile 1X PBS.
5. Add RNAse A and propidium iodide to a 1X final concentration and store the cells at 4°C overnight. It is critical to degrade RNA, as propidium iodide binds to both RNA and DNA.
6. Warm samples to room temperature and transfer the total volume to a fresh 6-mL tube suitable for flow cytometric analysis. While running the cells through the flow cytometer, vortex samples intermittently to prevent clogging of the cytometer filters.

3.6. Flow Cytometry

3.6.1. Acquisition

1. Proper calibration of the cytometer with DAPI-stained chicken embryonic nuclei and multichannel calibration beads is necessary before data acquisition. Check the fluid levels and adjust the flow to a low initial speed and pressure.
2. Open a previously designed acquisition template appropriate for the experiment (e.g., live cells or fixed cells).

3. Connect the computer to the cytometer and enter the appropriate instrument settings for either live cells or fixed S2 cells. If conducting an experiment for the first time, save the instrument settings after **step 4** for future use.
4. In setup mode, run a control sample to tune the instrument settings by adjusting the voltage and gain, and choosing the appropriate scale (log scale if experimental cells are GFP-positive and linear scale if GFP negative) so that a specific population lies within the designated regions within the dot plot and within the histogram plots.
5. Activate the "Parameter Description" page and assign a root file name, a starting file number, and a data storage location. The parameters listed at the bottom of the "Parameter Description" page must match the parameters in the instrument settings or an error in acquiring the data will occur.
6. Because machine performance varies from day-to-day, instrument settings will need to be optimized daily. This variance is one of the reasons one should not directly compare samples measured on different days or machines.
7. If there are samples containing cells with known or predicted differences in cell size, fine-tune the FSC-H gain setting using both a control sample and the sample with larger or smaller cells. For a cell to be measured, it must lie within the dot plots. Therefore, if a large proportion of cells are off-scale, the FSC information will be incomplete and no quantitative comparisons should be made. Furthermore, if the cells are positioned too close to the debris, it is difficult to distinguish the two populations.
8. If there are multiple samples to compare directly to each other, do not change the instrument settings until all of the samples have been measured. Otherwise, no comparisons between the samples can be made.
9. In acquisition mode, load a sample and run the cells less than 350 cells per second with a "Sample Differential" less than 1 PSI. Running the cells through too quickly will decrease the amount of time for excitation and detection and therefore lower the accuracy of the data.
10. If possible, collect 20,000 experimental cells. The minimum number of intact cells is 4000 for both cell cycle and cell size analysis. Output plots become smoother as increasing numbers of cells are analyzed.
11. Save the data and unload the sample. Make certain the data was properly stored as it is irretrievable once the acquisition of another sample has begun. An option exists which will automatically store the data after a specified number (e.g., 20,000) of postgated events have been measured.
12. Between samples, wash the sample intake tubing with 1X PBS (filter sterilized). Shake the next sample by hand to resuspend the sample before loading it onto the stage.

3.6.2. Data Analysis

1. Open the data through an analysis template that includes relevant plots, regions, and gates. Remember to use an appropriate template for live dissociated or live S2 cells (stained with Hoechst 33342) and for fixed S2 cells (stained with propidium iodide) as those cells were measured differently.
2. Cross-reference regions to each other. For example, to assure a region is in the proper location and proper shape, view the cells or events (particles the cytometer is able to detect and measure) of interest inside the region within other dot plots.

Optimally, those cells will lie mostly within the other regions drawn. This is done using the "Format Dot Plot" function. Reduce or expand the size of the region to obtain a stringent fit.

3. Repeat this for all regions. The contamination of data from debris, dying or dead cells, and clumps of cells or nuclei, is greatly reduced when the regions are properly placed and shaped and when an appropriate combination of regions are applied.
4. **Figure 2** shows a single data set plotted as a function of a variety of pairs of parameters. As seen in **Fig. 2D–F**, gating a region in one dot plot might appear to exclude all irrelevant events, but in fact, some of the included events are outliers when plotted according to a different pair of parameters. Conversely, as seen in **Fig. 2A–C**,**G–I**, cells that are outliers in one dot plot are included in regions within other dot plots. Therefore, cross-referencing regions to each other and combining them is critical for obtaining meaningful results.
5. Histogram plots are then useful for analyzing the data. The software's histogram "Overlay" function allows comparison of different populations of interest within the same plots as seen in **Fig. 1E**,**F**.
6. Relative cell size differences between the experimental population and the internal control population can be represented by a ratio of the FSC mean of those two populations. The software provides the mean and other statistical information for the "Region list" and "Gate list." The units for the parameters are arbitrary as the parameters are not absolute measurements.
7. Quantify cell cycle phasing with MultiCycle AV software or other available software. Refrain from quantifying data that contain less than 4000 relevant (postgated) events or data collected after poor cytometer alignment or that show poor dissociation or excessive cell death. Otherwise, the fit between the data and the modeling program will be poor.

4. Notes

1. Papain, a cysteine protease, is also effective in dissociating imaginal tissues and generating spherical-shaped cells. The following papain solution provides similar results as the trypsin solution after 2 h of incubation of 20 late 3rd instar wing imaginal discs (WID) with gentle agitation at room temperature.
   Papain solution (20 mL): 1X Earle's Balanced Salt Solution without Phenol Red, 44 mg $NaHCO_3$, 1 m*M* L-cysteine, 0.5 m*M* EDTA, 1X Hoechst 33342, and 80 U papain from papaya latex (Sigma), pH > 7.0. Aliquot 0.5 mL of Papain solution into 6-mL tubes. The tubes may be stored at −20°C protected from light until ready for use. Thaw to room temperature before adding dissected tissues.
2. When sorting cells, try to minimize deleterious factors. Dissociated cells cannot endure for long the lack of nutrition and normal cell contact, prolonged exposure to proteases and nucleic acid stains, the fluid pressure in the cytometer, and the cytometer's electrical charge that directs the cells to an appropriate tube. Most of the cells will be lysed after they are sorted, making it difficult to do further image analyses on individual cells. However, nucleic acids and protein can be harvested. In this case, it is best to sort the sample directly into Trizol (Invitrogen, Carlsbad, CA) or any appropriate preservation solution.

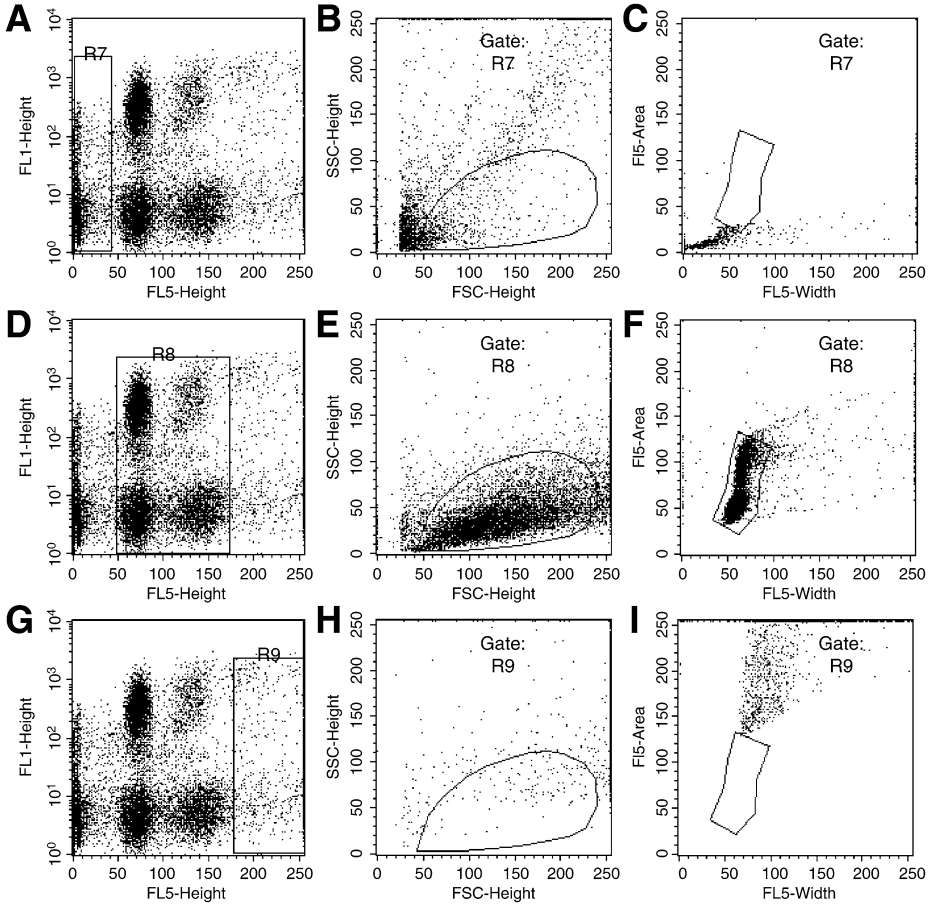


Fig. 2. A GFP and DNA content dot plot is analyzed using three regions. The regions are plotted according to two other pairs of parameters in order to illustrate that (1) gating on GFP and DNA content is extremely effective in identifying informative events; and (2) even after gating on GFP and DNA content, it is possible to further enrich for informative events by also gating on cellular membrane complexity vs relative cell size and ploidy vs cellular clumping. **A–C** demonstrate that gating on low FL5-H signal, R7, **(A)** includes events that are uninformative as determined when R7 is plotted according to cellular membrane complexity vs relative cell size **(B)** or ploidy vs cellular clumping **(C)**. **G–I** illustrate the same point for high FL5-H signal using R9. Conversely, **D–F** illustrate that, although the majority of events with defined DNA content using R8 **(D)** are informative, one can improve the quality of the data by additional gating on cellular membrane complexity vs relative cell size **(E)**, and ploidy vs cellular clumping **(F)**.

3. Propidium iodide can be added to the trypsin solution to obtain live/dead exclusion information. Add 2.5 µL of 1 mg/mL propidium iodide in 38 m*M* sodium citrate per 0.5 mL trypsin solution. Adjust the *acquisition* template to detect both propidium iodide and Hoechst 33342 fluorescence emission signals.
4. Both Hoechst 33342, a lipophilic *bis*-benzimide that binds to adenine thymine-rich regions in the minor groove of DNA, and propidium idodide, a membrane-impermeant dye that intercalates nucleic acid molecules nonspecifically, are toxic to cells ***(13)***. Omitting these stains from the trypsin solution until the last 20–30 min of dissociation might increase the yield of total intact cells and better preserve the integrity of nucleic acids for any subsequent manipulations. Also, cold temperature inhibits Hoechst 33342 uptake and therefore, it is not recommended to store samples on ice before flow cytometry.

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Drosophila Cell Lines as Model Systems and as an Experimental Tool

Buzz Baum and Lucy Cherbas

Summary

Given the power of *Drosophila* genetics, it may seem surprising to discover that many fly researchers are turning to *Drosophila* cell culture as an experimental system. However, as we will show in this chapter, there are many benefits to be gained by using cell lines as a complement to studies in a tissue and developmental context in the fly. Moreover, one can argue that *Drosophila* cell culture, in itself, provides an excellent model system for the study of many fundamental questions in molecular and cellular biology. In this review, we offer a summary of techniques that should be useful to researchers in the *Drosophila* community working with fly cell lines. These include techniques for growing and maintaining cell lines, transient and stable transfection, RNA interference, imaging, immunostaining, fluorescence-activated cell sorting, and for the isolation of RNA and protein from fly cells.

Key Words: Cell culture; cell lines; *Drosophila*; imaging; Kc; RNAi; RNAi screens; S2; S2R$^+$; stable transformants; transfection.

1. Introduction

Advantages of using *Drosophila* cell lines include the following:

1. Cell lines are relatively homogeneous, their behavior is stable over time, and they can be frozen and thawed decades later.
2. Over 100 fly cell lines have been generated. These exhibit a diverse range of behaviors and most are readily available from the Drosophila Genomics Resource Center (DGRC) stock center.
3. The synchronous cellular and biochemical response of the population can be followed immediately after the addition of a stimulus, whether this be a signaling molecule like insulin or ecdysone ***(1,2)***, a drug ***(3)***, or a pathogen ***(4)***.
4. Transgenes can be easily and quickly introduced into cells in culture, making this a useful system in which to characterize transgene function, for example, before the generation of fly transformants.

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5. RNA interference (RNAi) is simple, cheap, and effective in fly cell culture (5,6).
6. A monolayer of flat cells in culture can be easily imaged at a high spatial and temporal resolution ***(7,8)***.
7. Cells in culture can be adapted for use in a wide variety of large-scale RNAi screens, which for many purposes can be used in place of classical genetic screens (*see* Chapter 8).

In most cases, protocols for fly cell culture follow those developed for mammalian cell lines (*see* **Note 1**). However, by using fly cell lines instead of mammalian cell culture, drosophilists can make use of reagents developed for work in flies, and results and tools from cell culture studies can then be reapplied in vivo. In addition, RNAi is more effective and cheaper in fly cell culture than it is in mammalian cells, and is less affected by the problems of genetic redundancy, which can confound the analysis in mammalian cells. Finally, fly cells can be grown at room temperature, under atmospheric CO_2 levels, making handling and imaging relatively simple compared with mammalian cell culture (*see* **Note 1**).

For description of methods, including protocols for the generation of cell lines from primary embryonic cultures, we refer readers to the excellent guides written by Schneider in 1972 and Echalier in 1997 ***(9)***. We also suggest the following websites, in which more information about fly cell lines and protocols can be found: *dgrc.cgb.indiana.edu, flight.licr.org*, *and flyrnai.org*.

1.1. Identifying a Suitable Cell Line

More than 100 cell lines have been established from *Drosophila* embryos and larvae over the last 40 yr. The first of these, including the commonly used S2 and Kc lines ***(10–12)***, were generated from spontaneously immortalized cells in cultures of mechanically dissociated embryos. In subsequent years, similar methods were used to derive cell lines from the early embryos of genetically defined fly stocks ***(11,13–15)***. Interestingly, these embryonic lines all share characteristics that suggest that they are derived from immortalized hematopoietic cells. This idea is supported by the similarities of these lines to mbn-2 and mbn-3 lines that were established from primary cultures of mutant embryos carrying blood cell tumors ***(16)***. Furthermore, the $S2R^+$ cell line, an isolate of the S2 cell line ***(17)***, bears a striking resemblance in form and behavior to larval hemocytes isolated by bleeding larvae.

In the 1980s, two laboratories devised techniques for establishing cell lines from late larval imaginal discs. The Miyake laboratory established a series of lines from protease-digested wing, antennal, and haltere discs ***(18)*** and subsequently applied the same method to establish cell lines from the late larval central

nervous system (CNS) ***(19)***. Independently, the Milner laboratory established a series of lines from protease-digested wing and leg discs ***(20)***, which require the presence of a fly extract for growth (*see* **Note 2**). One of these, the "Clone.8" line, has been widely used, in part because it has been shown to retain several intact tissue-specific growth factor signaling cascades ***(21–23)***. Interestingly, these imaginal disc and CNS lines exhibit much greater variation in their forms and expression patterns than embryonic cell lines (Cherbas, unpublished observations). Unfortunately, we will have to wait for the characterization of a true epithelial *Drosophila* cell line.

Although some of these lines, such as S2, Kc, mbn-2, and Clone.8, have been widely used in the community, there was, until recently, little attention focused on other cell lines. This situation is now likely to change with the assembly of a large collection of lines at the DGRC, which acts as a distribution center, and a source of detailed information about cell lines *http://dgrc.cgb.indiana.edu.* In general, cells within a given cell line are reasonably homogeneous, even though many of them have never been cloned. This suggests that the population of cells in each line is derived from the same ancestral cell.

1.2. Varied Uses of Drosophila *Cell Lines as Research Tools to Study Gene Products and Pathways*

If an appropriate cell line is not available for a particular study, other avenues are available to a researcher (*see* **Note 3**). First, it is possible to derive a new cell line from a mutant fly background of choice ***(9)***. In this way, it is possible to generate a cell line with a genetically defined background that is more homogeneous in its properties than a line made by stable transformation (*see* **Subheading 3.7.3.**). However, the approach is laborious, and given the vagaries of primary cell immortalization, it is hard to predict how the new cell line will behave. More commonly, an existing cell line is modified for a specific purpose by the expression of a transgene or RNAi construct. Although this approach is usually used to study the properties of the transgene or its product directly, transgenes can be used to alter the behavior of a cell line. A good example of this is the use of ectopic *Daughterless* and *Nautilus* expression to switch the fate of S2 cells from a blood cell lineage to a muscle phenotype ***(24)***.

However, in the simplest of cases, fly cell lines are used as a source of large quantities of material for biochemical experimentation ***(2)*** or as "test tubes" to express large amounts of transgene products for biochemical analysis. For a survey of this approach *see* **ref. *25***. In addition, fly cells can be used to dissect the function of transgenes at a cell biological level, for example, to assess homotypic and heterotypic interactions between cell surface receptors such as delta and Notch in S2 cells, which do not normally express these proteins ***(26,27)***.

1.3. Fly Cell Lines as Model Systems

Given the ease of cell biology and biochemistry in cell culture, and the ability to use RNAi to silence the expression of one or more individual genes in fly cell culture ***(5,6)***, cell lines can also be used as model systems in which to study fundamental aspects of molecular or cellular biology. Examples of this include the many studies that have used *Drosophila* cell lines to delineate growth factor signaling pathways. In some cases, for example, dissection of the ecdysone response, cells lines can be identified that have an intact signal–response and an easy way of visualizing the output of signaling ***(2,28)***. In cases where an appropriate cell line or output cannot be identified, missing parts of the pathway can be reconstituted and functionally interacting genes can be identified using RNAi. In the simplest of cases, a vector carrying a reporter (e.g., luciferase) downstream of the test enhancer is introduced into cells (together with a control vector) usually by transient transfection, as a measure of pathway activity ***(21,23)***. This approach can be taken further, by engineering a synthetic pathway in a cell line of interest. A striking recent example of this type of approach was the introduction of a transcription factor unique to mammalian cells into fly cells. A *Drosophila* RNAi screen was then used to identify conserved genes that regulate its transcriptional activity in both fly and human cells ***(29)***. Importantly, RNAi can also be used to identify interacting pathway components in a signaling cascade of interest by, for example, using RNAi to deregulate signaling to generate a strong phenotype, and then carrying out a modifier screen to identify double-stranded RNAs (dsRNAs) that enhance or suppress this effect ***(7)***.

Many studies have used Kc, S2, S2R$^+$, and mbn-2 as models for the study of blood cell immunity in *Drosophila* ***(30)***. These cell lines share many of the properties of *Drosophila* hemocytes observed in vivo, and in primary cultures of larval hemocytes, including immune signaling ***(31)***. Taking advantage of this fact, several studies have used these cell lines as subjects for RNAi screens to identify the genes and signaling pathways involved in *Drosophila* blood cell immunity; screening for genes that regulate antibacterial gene expression on exposure to a bacterial antigen ***(32)***, or the phagocytosis of bacteria ***(4)***, yeast ***(33)***, or dying cells. For most of the other cell lines, the precise in vivo counterparts are not known. Nevertheless, these lines can still be used to address fundamental questions in biology: such as the mechanisms underlying cytoskeletal dynamics ***(8,34)***, the movement of mitochondria ***(35)***, and cell cycle control ***(36,37)***—frequently with the help of fluorescent-tagged marker proteins ***(8,35)***. As more researchers in fly community turn to fly cell culture and more cell lines are characterized, we expect that a greater variety of cell behaviors will become amenable to this approach. For example, the CNS-derived BG2-c2 and BG3-c1 cell lines exhibit robust motility and can be used for the study of cell motility and migration (Wei Bai and Baum, unpublished data).

2. Materials

2.1. Cell Lines

Over 100 *Drosophila* cell lines are now available from the DGRC, *dgrc.cgb.indiana.edu*. S2 cells are also available from Invitrogen and the American Type Culture Collection, and other cell lines can be obtained from individual research labs. Information on many of these lines, including images and microarray data, are available at the DGRC website *dgrc.cgb.indiana.edu* and at *flight.licr.org*.

2.2. Media and Serum

1. Schneider's ***(38)***, D-22 and M3 (also called Shields and Sang medium) ***(39)*** media are the most commonly used for growing *Drosophila* cell lines. They are available in powder and liquid form (*see* **Note 4**) from commercial sources (e.g., Sigma Aldrich and US Biological), and are stable for approx 6 mo. They require the addition of heat-inactivated fetal calf serum (at concentrations specific to the cell line), and some cell lines require additional media supplements. Requirements of individual lines may be found on the DGRC website (*dgrc.cgb. indiana.edu*).
2. M3 + BPYE is a modification of M3 that is similar in composition and properties to Schneider's medium ***(40)***. To make M3 + BPYE, add 1 g yeast extract and 2.5 g bacto-peptone to each liter of M3.
3. Defined commercial serum-free media, such as HyQ, CCM3 (HyClone) and InsectExpress (PAA Laboratories), can also be used to grow some of the more robust lines (*see* **Note 5**).
4. Heat-inactivated fetal calf serum (10%) is required for the growth of most cell lines. Some, such as Kc and S2, grow well in virtually all lots of serum; but many of the imaginal disc and CNS lines are much more finicky. We recommend that users test serum samples before ordering in bulk. Once it has been confirmed that a particular lot of serum supports the growth of cell lines of interest over a period of weeks (*see* **Note 6**), enough should be purchased to last for several years. Serum should be stored at –20°C or lower. Before use, serum should be thawed completely and heat-treated at 56°C for 30–60 min (to inactivate complement). Heat-treated serum can be stored at 4°C for several weeks.

2.3. Additional Reagents for Work With Fly Cell Lines

1. Fly extract is required for the growth of several cell lines, in either M3 or Schneider's medium. It is not available from any commercial source; a protocol for its preparation is given in **Subheading 3.3.**
2. Sterile 1X phosphate-buffered saline (PBS): 120 m*M* NaCl in phosphate buffer at pH 7.4. This can be made up directly and autoclaved or filter sterilized, and is available from numerous commercial sources as a powder or liquid. It is useful when working with fixed *Drosophila* cells (e.g., in antibody staining protocols). For handling living *Drosophila* cells (e.g., washing cells before the extraction of RNA or protein), use "*Drosophila* PBS" (120 m*M* NaCl in phosphate buffer at pH 6.7).

3. 100X Penicillin-streptomycin stock (10,000 U/mL penicillin-G and 10,000 µg/mL streptomycin) is available from numerous commercial sources and is an optional addition to medium that can be used to limit bacterial infection (*see* **Note 7**). Store in 5-mL aliquots at –20°C, and add one to each 500 mL of medium.
4. Trypsin-ethylenediaminetetraacetic acid (EDTA): 0.05% Trypsin, 0.53 m*M* EDTA. This can be used to remove strongly adherent cells from the substrate. It is available as a liquid from many vendors and should be stored at 4°C. It is advisable to adjust the pH to 6.7 before use. Versene (Invitrogen) can be used as a nonenzymatic alternative, but is rarely as effective as Trypsin-EDTA.
5. Insulin is required for the growth of some cell lines (*see* **Note 8**). Human or bovine insulin perform fine and are usually used at 1–10 µg/mL. Buy as liquid or powder. For powder, dissolve 10 mg in 0.5 mL of 0.01 *N* HCl then dilute in 20 mL of medium. Filter-sterilize to make aliquots of 1 mg/mL stock (do not repeatedly freeze–thaw).
6. 200X Sodium azide: 10% NaN_3 in double-distilled (dd)H_2O. This reagent is used to maintain sterility in some stock solutions; it should not be used in cell cultures. It is extremely toxic, so take care with powder when making up stock. As it is unstable, it is best kept at 4°C or over long periods in frozen aliquots.
7. Dimethylsulfoxide (DMSO) is available from multiple vendors and should be kept in the dark. When in use, care should be taken to avoid contamination.
8. Trypan blue: 0.4% solution in 1X *Drosophila* PBS. This is a vital dye. It is useful as a stain for dead cells during cell counting, and is available from many commercial sources.
9. Pluronic F68 (Sigma) can be used to protect cells in spinner flasks from mechanical damage, at approx 0.05% ***(41)***.

2.4. Vessels and Other Equipment for Cell Culture Work

1. Tissue culture plastic-ware (*see* **Table 1** for volumes for each vessel). This is available from all standard laboratory suppliers. Be sure to use plastic-ware designed for tissue culture, not microbiological plastic-ware. For imaging cells in flasks and dishes, a thin base is required (even if using long working distance lenses). Cells often behave differently in plates from different manufacturers. For use of 96- and 384-well plates in fluorescent imaging, we recommend black clear bottom plates from Costar-Corning.
2. Glass bottom dishes. These provide better optics than plastic for high-resolution cell biological studies. For live imaging of individual cells in culture, we recommend using cover-glass bottom dishes (MatTek). It is also possible to grow cells on glass chamber slides (Labtek II), but cell behavior on glass can be variable and this type of vessel has been known to leak. High quality glass 96- or 384-well plates are available from MMI AG Scientific instruments for high throughput work.
3. Spinner flasks. These are useful for large-volume cultures (100 mL to several liters), and should be used with a special stirring motor designed for steady rotation at low speeds to minimize the generation of heat. Both the flasks and stirrers are available from several manufacturers (*see* **Note 9**).

Table 1
Recommended Volumes for Cell Culture in Commonly Used Plates and Flasks

Type of plate	Volume of cell suspension
384-well plate	0.04 mL/well
96-well plate	0.1 mL/well
48-well plate	0.3 mL/well
24-well plate	0.5 mL/well
12-well plate	1 mL/well
6-well plate	2 mL/well
35-mm plate	1 mL
60-mm plate	4 mL
100-mm plate	10 mL
150-mm plate	25 mL
25-cm^2 T flask	5 mL
75-cm^2 T flask	15 mL

4. Wands to remove liquid from plates. For high throughput work in 96- or 384-well plates we recommend the purchase of 12/24-well wands (V&P Scientific or equivalent). The wand needs to be adjusted with tape at either end so that it just falls short of the bottom of the multiwell plate to avoid drying the well, when attached to a pump.
5. Multichannel pipets. These are available from many vendors.
6. Hemocytometers. For the accurate determination of cell density they must be used together with special thick glass cover slips (normally sold with the hemocytometer). As an alternative, cell number can be measured using an automated CASY or Coulter counter.
7. Plate-sealing tape. For sealing 384-well plates we recommend thermowell aluminium sealing tape (Corning) or equivalent. Make sure the seal is airtight.
8. Homogenizer. Any homogenizer capable of breaking open flies is adequate for the preparation of fly extract or genomic DNA.
9. Heated water bath for the treatment of fetal calf serum and for the preparation of fly extract. Copper sulfate or another antifungal agent can be added to limit fungal growth.
10. Filters. Different 0.22-µm pore size filter units are available for the sterilization of different volumes of liquid (Millipore). For small volumes, attach a sterile filter unit (various manufacturers) to a hypodermic syringe.
11. Cryovials. These are available from a variety of manufacturers. A fine marker pen is required to ensure labeling is clear and permanent.
12. Cylindrical Dewar flask with foam stopper or an isopropanol cell freezing container (various manufacturers) or a polystyrene box that can be tightly sealed.

2.5. Major Equipment for Cell Culture Work

1. Refrigerated incubator, capable of maintaining 25°C. CO_2 should not be used.
2. Laminar flow hood (*see* **Note 10**).

3. Pump, tubing, and sterile trap for removing liquid medium (*see* **Note 11**).
4. Inverted compound microscope (*see* **Note 12**).
5. Inverted fluorescent time-lapse microscope for high-throughput imaging work. Long working distance lenses are required if cells are to be imaged through thick substrates. To automate image-based screens in 384-well plates, the microscope needs to have an automated *z*-axis, shutter, turret for filter-cubes or filter wheel, a robotic stage, and an appropriate screening software platform (e.g., MetaExpress from Molecular Devices).
6. Benchtop centrifuge that can take 384/96-well plates, 50- and/or 15-mL tubes. Cells should be spun down at 1500*g*.
7. Microfuge for making RNA and protein extracts.
8. Robot to speed up liquid handling (the WellMate from Matrix Technologies Corporation Ltd., the multidrop dispenser from Thermo Electron Corporation or equivalent). Several heads should be purchased and each dedicated to cell plating, fixation, or staining.
9. –80°C freezer.
10. Liquid nitrogen tanks. These need to be monitored and filled up on a regular basis. Ideally, a copy of cell stocks should be stored in a backup tank to protect against catastrophic accidents.
11. Thermal cycler (polymerase chain reaction [PCR] machine).
12. 37°C incubator, water bath, or heated block for T7 transcription reaction.

2.6. Transfection

Standard liposome-based transfection reagents can be used. The authors have found that the following reagents work well with *Drosophila* cell lines; we expect that many other commercially available reagents will work equally well:

1. FuGENE HD or FuGENE 6 (Roche Diagnostics).
2. Effectene (Qiagen).
3. Cellfectin (Invitrogen).

2.7. Selection of Stably Transformed Cells

The following reagents are widely used for the selection of stably transformed *Drosophila* lines:

1. Methotrexate (Sigma) (L-amethopterin). Store the stock solution (4×10^{-4} *M* in 10 m*M* Na_2CO_3) at –20°C protected from light. After dilution in medium, store at 4°C protected from light for no more than 2 wk (*see* **Note 13**). The stock solution may be frozen and thawed many times, and is stable for years. Use at a final concentration of 2×10^{-7} *M*.
2. Hygromycin B. Invitrogen recommends using it at 200–300 µg/mL.
3. α-Amanitin. Dissolve in H_2O at 1 mg/mL, and store the stock solution at –20°C. Use at a final concentration of 5–10 µg/mL.
4. Blasticidin S. Invitrogen recommend using it at approx 5 µg/mL.

2.8. Useful Vectors for Stable Transformation and Transfection

A variety of vectors can be used for transient expression in *Drosophila* cells; many of which are available from the DGRC. Most are based on the strong constitutive *Actin5C* promoter (*see* **Note 14**). An extensive set of GATEWAY (Invitrogen) vectors is also available from the Murphy lab or the DGRC. These were designed by the Murphy lab to facilitate the generation of epitope-tagged fusion proteins for use in *Drosophila*, using either the constitutive *Actin5C* promoter or the inducible *hsp27* promoter: *http://www.ciwemb.edu/labs/murphy/Gateway%20vectors*. A variety of additional fluorescent labels and epitope tags are available in vectors made in many laboratories. An Mt-Gal4 or Actin5C-Gal4 plasmid (available from the DGRC) can be used to drive the expression of a gene inserted behind a UAS enhancer in cell culture ***(42)*** as a quick test of its function prior to use in the germline transformation of flies ***(43)***. pFastBac *Actin5C* is useful for transient expression through baculovirus infection (constructed by Aaron Straight, available from the DGRC).

For stable transformation, it is often desirable to use a regulated promoter for the expression of a transgene. Promoters for this purpose are discussed in **Subheading 3.7.3.**

The following plasmids can be used for selection of stable transformants, using the selection reagents listed in **Subheading 2.7.** (*see* **Note 15**).

1. Methotrexate resistance: pHGCO, pHCO ***(44)***, p8HCO ***(27)***, and actDHFR ***(45)***. P8HCO and actDHFR are available from DGRC (*see* **Note 15**).
2. Hygromycin B resistance: pCoHygro ***(46)*** is available from Invitrogen.
3. α-Amanitin resistance: pPC4 ***(47)*** is available from the DGRC.
4. Blastocidin S resistance: pCoBlast is available from Invitrogen.

2.9. Fixing, Staining, and Cell Imaging

1. To generate clean cover slips, wash circular 13 mm glass cover slips overnight in a 50:50 mix of methanol and chloroform. Rinse-sterilize in 70% ethanol and dry in a laminar flow hood (*see* **Note 16**).
2. Concanavalin A (Sigma). Store as a 0.5 mg/mL stock at –20°C. Before use, dry down 100 µL of a diluted 15 µg/mL solution (1.5 µg) onto each 13-mm cover slip.
3. Vitronectin. Store as a 5 µg/mL solution at 4°C. Use 100 µL for each 13-mm cover slip and do not dry (*see* **Note 17**).
4. Formaldehyde (4%) in 1X PBS. We recommend the 16% electron microscope grade stock solution (Polysciences). Store stock at room temperature, then make up a 4% solution in 1X PBS before use. This can be kept at room temperature or at 4°C for a limited period of several weeks.
5. 10X PBS (liquid) or PBS powder (widely available). Make up with ddH_2O and autoclave or filter sterilize.
6. Triton X-100 (many vendors). Store at room temperature as a 10% stock solution in H_2O.

7. Bovine serum albumin (BSA). This is available from many vendors as a relatively high-purity solid (typically >97%) or in solution, and should be stored at 4°C. BSA can be kept as 10% solution in 0.05% NaN_3 at 4°C. Before using, check by eye for contamination.
8. Fluorescent-labeled phalloidin (Sigma or Molecular Probes). Store in methanol at –20°C. As methanol interferes with actin filament staining, allow most of the methanol to evaporate before adding it to the staining solution.
9. Mounting medium. We recommend Fluorsave reagent (Calbiochem), which rapidly solidifies on use.

2.10. RNAi

1. Genomic DNA can be made using a variety of standard protocols or kits. For each reaction use approx 30 flies or a pellet of approx 10^7 cultured cells.
2. Taq DNA polymerase or Hot-start Taq (Fermentas). We recommend Hot-start Taq for the amplification of large numbers of samples in parallel.
3. T7 Megascript kit (Ambion).
4. PCR 96-well cleanup plate and vacuum manifold (Millipore).
5. 0.1% Diethylpyrocarbonate (DEPC)-treated water. Add DEPC to water, mix overnight, and then autoclave 20 min to destroy DEPC by causing hydrolysis of DEPC. Note that DEPC is very toxic.
6. TE: 10 m*M* Tris, pH 8.0 and 1 m*M* EDTA, pH 8.0.
7. A dedicated RNase-free agarose gel tank, box, and combs. It is also possible to remove most of the RNase from equipment already in use by scrubbing it clean with detergent and rinsing with autoclaved water. RNase inhibitory sprays can also be purchased from a variety of vendors and used to help eliminate residual RNAse activity.
8. RNase-free sample loading buffer.

2.11. RNA and Protein Extraction

1. TRIzol (Invitrogen) for RNA isolation. The isolation protocol requires chloroform and isopropanol.
2. RIPA buffer: 50 m*M* Tris or HEPES (pH 8.0), 150 m*M* NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulphate, 5 m*M* EDTA. Add a cocktail of standard inhibitors immediately before use (1 m*M* PMSF, 5 µg/mL aprotinin, 5 µg/mL leupeptin, and 5 µg/mL pepstatin).
3. Laemmli sample buffer: 0.2 *M* Tris, pH 6.8, 8% sodium dodecyl sulfate, 40% glycerol, 5% β-mercapto-ethanol, 0.004% bromophenol blue. Store as 2–4X stock at 4°C.

3. Methods

3.1. General Guidelines for Sterility

The following comments are intended to help readers who are not experienced with tissue culture. Sterility requirements are much more stringent than those for bacterial culture because contaminating bacteria or fungus are

likely to grow faster than fly cells in the rich cell culture media. All operations should therefore be carried out in a sterile laminar flow hood. Glassware should be sterilized by autoclaving or by baking in a sterilizing oven. Tissue culture medium is not chemically stable in an autoclave and should therefore be sterilized by filtration (0.22-μm pore size). If possible, tissue culture should be carried out in a clean room separated from areas where bacterial or yeast are used or where there is heavy human traffic; a good general guideline is to keep tissue culture hoods separated from the fly room by two doors.

Keep unnecessary objects out of the hood in order to minimize distortions of the airflow pattern and avoid sudden, fast movements. However, it is perfectly acceptable to leave bottles of sterile medium open for prolonged periods of time. Wipe up minor spillages immediately and swab the surface of the hood, hands/gloves with 70% ethanol on a regular basis, for example, just before and just after each session of use. Either keep hoods running continuously or allow sufficient time for the airflow to stabilize before use.

The inclusion of antibiotics in culture medium is not necessary, but some workers prefer to include a standard penicillin-streptomycin cocktail (*see* **Note 7**). We know of no cases where mycoplasm has been found in *Drosophila* tissue cultures, probably because mycoplasm does not grow well at temperatures used for fly cell culture. If in doubt, a simple commercial PCR-based assay can be used to test for infection. Fly cells can carry fly-specific viruses, so care should be taken not to mix medium from different cultures.

3.2. Preparation of Fetal Calf Serum

1. Thaw a bottle of serum, and bring it to room temperature.
2. Place the bottle in a water bath at 56°C; there should be enough water in the bath to reach the level of top of the serum in the bottle (*see* **Note 18**).
3. Incubate for 30–60 min, occasionally swirling the contents of the bottle.
4. Store treated serum at 4°C for up to a few months, or in aliquots at –20°C for longer periods.

3.3. Preparation of Fly Extract

1. Collect adult flies in a 50-mL plastic disposable centrifuge tube and place in a –20°C freezer for at least 45 min. Flies can be used once they are quiescent, or stored at –20°C for future use (*see* **Note 19**).
2. Weigh the flies and transfer them into a glass homogenizer, together with 6.8 mL medium/gm of flies. Homogenize on ice; a single pass of the plunger is usually sufficient.
3. Spin the homogenate at 1500*g* at 4°C for 15 min. Decant the supernatant into fresh tubes. Discard the pellet.
4. Incubate the supernatant at 60°C for 5 min to inactivate tyrosinase.

5. Spin at 1500*g* at 4°C for 90 min. Collect the supernatant—this is the fly extract.
6. Filter-sterilize the extract through a 0.22-µm filter. Store 2.5-mL aliquots at –20°C; each aliquot is appropriate for 100 mL of tissue culture medium.

3.4. General Guidelines for Maintaining Cells

The following instructions should be taken as rough guidelines; actual growth conditions vary somewhat from cell line to cell line.

1. Most cells prefer to be maintained in the range 10^6–10^7 cells/mL. The concentration of cells can be estimated using a hemocytometer or a cell counter.
2. Split cell lines at a density of approx 10^7/mL. For robust fast-growing cell lines, for example, Kc and S2 cells, dilute cells 1–10 with fresh medium before dispensing them into new plates. Most other fly cell lines are sensitive to low cell density and should only be diluted two- to threefold at each passage (*see* **Note 20**).
3. Most *Drosophila* cell lines adhere loosely to the substrate. Because of this, they can be easily dislodged using a flow of medium from a pipet or, for cells grown in a sealed flask, by banging the bottom of the vessel with the palm of one's hand. Cell lines that adhere more strongly to the substrate can be removed using a cell scraper or by blowing medium at close range. Both procedures lead to some mechanical damage. As an alternative, Trypsin-EDTA can be used to loosen cells from the substrate by cleaving integrins *(7)*. For this procedure, starting with a culture growing in a 25-cm^2 flask:
   a. Bang the flask to remove the top layers of cells.
   b. Wash the cell monolayer once with 2 mL sterile 1X *Drosophila* PBS or trypsin-EDTA to remove serum-containing medium (which contains an inhibitor of trypsin).
   c. Replace with 5 mL trypsin-EDTA and incubate for 5–15 min at room temperature, watching continuously to catch cells as they visibly round up.
   d. Taking care not to dislodge cells, gently replace the trypsin-EDTA with serum-containing medium to inactivate trypsin activity. Then resuspend cells in 5 mL medium and dispense cells into a fresh flask.
4. With the exception of a few lines made from temperature-sensitive mutant fly stocks, all *Drosophila* cell lines should be grown at approx 25°C, with air as the gas phase. Liquid depth should be kept low to ensure adequate aeration (*see* **Table 1**). In order to prevent the plates from drying out, it is necessary to keep them in a humid environment; either by placing plates in sealed plastic food containers or by placing a trough of water at the bottom of the incubator, supplemented with an antifungal agent, for example, copper sulfate, to maintain sterility (*see* **Note 18**).
5. *Drosophila* cell lines can be grown in any standard tissue-culture grade plastic vessel. Petri dishes can be used instead of T flasks, and are much less expensive. *See* **Table 1** for suggested volumes of cell culture for different types of vessel.
6. Some *Drosophila* cell lines, including Kc, S2, and S3 cells, can be grown successfully in spinner flasks (*see* **Note 21**). When using spinner flasks, it is important to keep the volume below 50% of the calibrated volume of the spinner flask, to ensure

adequate aeration. Pluronic-F68 can be added to help protect cells from mechanical damage and to reduce clumping.

7. Cultures of *Drosophila* cell lines occasionally become sick—often without explanation. Bad signs to watch out for include changes in form, size, adhesive properties, or the accumulation of phase bright "vacuoles." Once significant changes have occurred, it is rarely possible to rescue the culture, so discard the cells and start a fresh culture from a frozen stock (*see* **Subheading 3.6.**).
8. As cell lines change over time, it is very important that new *Drosophila* cell lines be quickly amplified and frozen down for future use. Cells should then be thrown out and rethawed every few months to limit changes in the properties of the line.

3.5. Preparation of Frozen Stocks for Storage

When properly frozen, cell lines can be stored indefinitely in liquid nitrogen or for several months at –80°C. The freezing protocol relies on slow freezing (–1°C/h) and DMSO to prevent ice crystals forming in cells. As DMSO is toxic to cells when metabolized, efforts must be made to minimize the time exposed to DMSO at room temperature, for example, by working quickly and/or by keeping cells on ice.

1. Resuspend a healthy culture of cells at midexponential growth (~5×10^6 cells/mL) in the medium in which they are growing; spin in a centrifuge at 1500*g* to pellet cells.
2. Discard the supernatant and resuspend the cell pellet by gently pipeting up and down in "freezing medium" (10% DMSO in complete medium or 10% DMSO in fetal calf serum) at 4°C, at an appropriate concentration.
3. Dispense the cell suspension into 0.5–1-mL aliquots in cryovials, each of which should contain sufficient cells to populate a new flask (~1×10^7 cells).
4. Place the cryovials in a Dewar flask or freezing chamber (Nalgene) at 4°C. Cap securely, and place in a –80°C freezer.
5. After 2–3 d, remove the cryovials and place them in a liquid nitrogen container (*see* **Note 22**).
6. Thaw one ampule to test the quality of the frozen stock (*see* **Note 23**).

3.6. Thawing Cells From a Frozen Stock

1. Prepare 5 mL of the appropriate medium.
2. Remove an ampule from the liquid nitrogen, and allow the contents to thaw, partially. *Optional*: decontaminate the outside of tube with 70% ethanol, making sure not to get ethanol into the ampule.
3. Using a sterile Pasteur pipet, add a few drops of medium to the ampule to accelerate thawing. Pipet up and down, and transfer the mixture to a fresh flask or tube. If there is any frozen material left, repeat the procedure until the entire contents of the vial are transferred.
4.
    a. Allow cells to settle on the surface of a T25 flask for 1–2 h. Carefully remove the medium from the flask with a sterile pipet, being careful not to disturb the

cells on the surface. Replace with 5 mL of fresh medium. Place the flask in a 25°C incubator overnight. The next day, remove the medium again and replace it with fresh medium (*see* **Note 23**).

b. Alternatively, place thawed cells directly into a 15 mL tube, spin down (1500*g* for 4 min), resuspend in fresh medium, and transfer to a fresh flask (*see* **Note 24**).

5. Allow the cells to recover before use. In general it takes 5–10 doublings for cells to return to normal growth.

3.7. Introducing Transgenes Into Fly Cells

A variety of methods can be used to introduce exogenous DNA into fly cells in culture, including viral infection and transfection. All have some associated toxicity (*see* **Note 25**).

Calcium phosphate/DNA precipitates and electroporation have been successfully used to introduce DNA into S2 and Kc cells. Protocols for both procedures are available from previous reviews ***(25,48)*** and will not be replicated here. Modern lipid-based transfection reagents can be used to achieve higher transfection efficiency with minimal toxicity. This is particularly important for the transfection of imaginal disc and CNS lines, which can otherwise prove difficult. Good results can be achieved using a number of kits (including Fugene 6, Fugene HD, Effectene, and Cellfectin), following manufacturers' recommended protocols. Other reagents are likely to work equally well (Baum, Osswalt, and Cherbas, unpublished). The efficiency of transfection can reach 20–50% for embryonic lines, but it is always much lower for imaginal disc and CNS lines.

Higher rates of DNA delivery can be achieved using baculoviral infection. The efficiency of infection that can be achieved using a standard titre viral stock varies between cell types, but is typically high (>50%) even in CNS-derived lines that are relatively refractory to transfection (Baum, unpublished). On the down side, there is some toxicity associated with infection and, because the virus does not replicate in *Drosophila* cells, the technique cannot be used to generate lines with stable transgene expression. Once generated, a single high-titre stock of baculovirus can be used for thousands of experiments and, once filter-sterilized, can be kept for more than 1 yr at 4°C, before it needs to be reamplified. Moreover, the ease and low cost of infection mean that the technique can be used to introduce transgenes in a high-throughput format, for example, following an RNAi screen.

3.7.1. Baculovirus Infection

When using baculovirus to introduce and express a transgene of interest in fly cells, a modified baculovirus vector is required that carries the gene of interest under the control of a *Drosophila* promoter, in place of the polyhedron promoter. Protocols and reagents from Invitrogen can be used to generate viral particles. Infection then relies on the fact that baculovirus can enter cells from many species,

even though this does not result in a productive infection (*see* **Note 26**) ***(49)***. Infection provides an easy and efficient method to drive transient gene expression, although some cytotoxicity is observed approx 48 h after infection.

1. Clone DNA into the pFastBac *Actin5C* plasmid—available from the DGRC (constructed by Straight, cited in **ref. *3***).
2. Transform the DNA into DH10Bac bacteria (from an Invitrogen baculovirus expression kit) for recombination into the baculovirus genome.
3. Transfect purified baculovirus DNA into insect Sf9 cells.
4. After 3 d growth at 25°C, check for transgene expression in the Sf9 cells (e.g., using GFP).
5. Filter-sterilize the medium containing infectious viral particles (using a 0.22-µm pore size). The viral titre can be determined using a plaque assay, and can be increased using a second round of viral infection in insect Sf9 cells (available from Invitrogen and a number of other vendors).
6. To initiate infection, simply add the filtered medium to *Drosophila* cells in culture (viral titre needs to be optimized).
7. Assay gene expression after 8–24 h.

3.7.2. Strategies for Transfection: Transient Transfection

In transient expression experiments, one or more plasmids are introduced into cells in culture, and the effects are analyzed within a relatively short period of time, usually limited to hours or days. This obviates the need for inducible promoters (*see* **Subheading 3.7.3.**) but precludes cell selection using plasmid-borne markers. As a result, the majority of cells do not usually carry the transgene and expression is very heterogeneous in those that do. In principle, it should be possible to reduce the heterogeneity using a fluorescence-activated cell sorting (FACS) or magnetic sorting protocol and a suitable transfection marker in the plasmid mix, but we are not aware of these techniques having been successfully used in *Drosophila* cell lines.

During transfection, a single cell usually takes up a large number of plasmid molecules (*see* **Note 27**). Therefore, it is possible to introduce multiple transgenes into the same cell by mixing plasmids at the start of the procedure. This approach has been routinely used to cotransfect as many as four or five plasmids by electroporation ***(50)***, and seems to work well for many transfection procedures (Cherbas, unpublished). In experiments that involve the cotransfection of GFP and dsRed expression plasmids into $S2R^+$ cells using liposome-based reagents, the rate of cotransfection is typically more than 95%, but less than 100% (Baum, unpublished). Because of this, care should be taken when carrying out cell biological experiments that rely on the use of a "reporter plasmid" (e.g., *pActin5C-GFP*) to mark cells that express an unmarked "test plasmid." To increase the chance that every cell that has the "reporter plasmid" also expresses the gene of interest to approx 100%, we suggest mixing 10% of the "reporter plasmid" with 90% of the "test plasmid."

Protocol for the introduction of plasmid DNA into fly cells in a well of a four-well dish or 24-well plate using Fugene HD.

1. Add 3 µL Fugene HD to 47 µL serum-free media in a 1.5 mL microfuge tube. Mix by pipeting up and down three times and leave for 5 min at room temperature.
2. Add 1 µg of plasmid, pipet up and down three times, and incubate for a further approx 2 h.
3. Plate 3×10^5 cells into each well of the plate in 300 µL serum-free medium. Allow time (~60 min) for cells to attach to the substrate.
4. Gently add the mix of 50 µL Fugene/DNA to cells.
5. 1 h later add a further 200 µL of serum-containing medium.
6. The following morning replace the medium with 500 µL fresh medium containing 10% serum.
7. Harvest cells 24–48 h after transfection.

3.7.3. Strategies for Transfection: Stable Transfection

The effects of "transient expression" can be assessed in a cell population within a few hours or days. Alternatively, following transfection, one can select cells in which the exogenous DNA has been stably incorporated into the genome. This takes time, but enables the establishment of a population of stably transformed cells. The selective reagents and the corresponding resistance plasmids required for this procedure are listed in **Subheadings 2.7.** and **2.8.**

To isolate a stable transformant, a selectable marker is included in the cotransfection mix (*see* **Note 27**). Cells are then left for 2 d after transfection to allow for the expression of the selectable marker; after which time the appropriate selective agent can be added to the medium to kill nontransfected cells. For most purposes, we suggest using methotrexate as a selective agent, because it has the advantages of a low price and relatively low toxicity in humans. Some selectable marker plasmids are sufficiently strong that a single copy per cell is sufficient to give resistance; this is true of actDHRF (methotrexate resistance) and pPC4 (amanitin resistance). Because of this, these plasmids can be used to construct P-element vectors for P-element transposition in cell culture, yielding a single plasmid copy per transformed cell ***(45)***. Although the question has not been carefully tested, we would expect genes introduced into cells by P-element transposition to be expressed in a relatively stable and uniform manner.

As high level of expression of an exogenous protein is often cytotoxic, transgenes are best expressed from an inducible promoter when generating stable cell lines (*see* **Note 28**). The two most commonly used inducible promoters are the *Metallothionein* promoter (induced by 1 m*M* $CuSO_4$) and the *Hsp70* promoter (induced by 1–2 h heat-shock at 37°C). Neither heat-shock nor exposure to copper is physiologically neutral, but we recommend the copper induction system as giving fewer side effects. Copper sulfate usually induces a 5–30-fold increase

in transgene expression within 12–24 h (the kinetics of induction depend on the stability of the expressed RNA and protein). Several vectors for expression from a *Metallothionein* promoter were made by Bunch ***(51)***, some of which are available from the DGRC; additional vectors are available from Invitrogen. Other inducible systems that have been shown to work in flies (e.g., the Tetracyclin-regulated Tet off and Tet on systems and the Gal4-ER system ***[52]***) have not to our knowledge been used in fly cell culture.

3.8. Generating Lines With Homogeneous Gene Expression

For most experiments, it is sufficient to select a population of stably transformed cells following transfection. In such a population, all cells will carry the resistance marker and the vast majority will carry the transgene plasmid. However, transgene expression in the population is usually highly heterogeneous (*see* **Note 29**). In addition, if the expression of a transgene has a deleterious effect on cell proliferation or growth, bulk selection will enrich the population for cells that have lost the plasmid or which express the transgene at very low levels. Fortunately, methods are available to minimize these problems:

Variation in transgene expression can be reduced, although not eliminated by cloning the transformed cells (*see* **Note 29**). This is best done soon after transfection, to maximize the possibility of isolating independently transformed clones. It is then possible to choose a suitable clone (e.g., low basal expression and high induction ratio). Protocols for cloning S2 and Kc cells can be found in previous reviews ***(25,40,48)***. Although cell lines that grow more slowly can be expected to be more difficult to clone, the Milner and the Miyake laboratories have succeeded in cloning imaginal disc and CNS lines ***(19,53)***.

Second, cell lines with stable transgene expression can be generated from the embryos of P-element transformed flies. In this way, Karpova et al. ***(54)*** generated a new embryonic cell line from flies that, following exon trapping, expresses GFP from an endogenous promoter. The resulting cells express GFP at a uniform and physiologically normal level (Debec, personal communication). This approach is time-consuming, but has the great advantage of producing a line that carries the transgene at a defined locus.

Additional techniques have been developed for targeted mutagenesis and for the targeted insertion of transgenes in mammalian cells and in flies. Such techniques, including the use of phage or yeast recombinases (e.g., cre/lox or FLP/FRT) and BAC recombineering ***(55)***, offer great promise but have yet to be exploited in *Drosophila* cell culture.

3.9. Introducing dsRNA Into Fly Cells

In many eukaryotes, dsRNA is able to target homologous messenger RNAs (mRNAs) for degradation leading to a loss-of-function phenotype, through

a process termed dsRNA-mediated interference or RNAi ***(56)***. dsRNA-induced gene silencing occurs in fly cell lines following the simple addition of 300–1500 bp pieces of dsRNA to the culture medium ***(5)***. The dsRNA enters the cells through an endocytotic route ***(57)*** and is processed by DICER into small 21–23 bp siRNAs. These are incorporated into the RISC complex, which catalyzes the cleavage of target mRNAs. The resulting ability to combine loss-of-function genetics in fly cell culture with more traditional genetics in flies has proved extremely powerful ***(33)***.

In a typical RNAi experiment, a 500–1000 bp genomic fragment of exonic sequence is amplified using oligonucleotides with flanking T7 sites. This PCR product is then used as a template for T7 RNA polymerase, generating a dsRNA product. dsRNA (30 µg/mL) is then added to the culture medium and 3–7 d later, depending on protein stability and the assay used, a more than 90% target-specific reduction in gene expression is observed in close to 100% of cells (*see* **Note 30**). By simultaneously adding multiple dsRNAs to cells, it is also possible to target two to three genes in the same population of cells, for modifier screens or for studying gene epistasis ***(7)***. It is vital that off-target effects be ruled out before it is concluded that any observed phenotype is the result of gene-specific RNAi. This can be done by simply verifying the result using a second dsRNA that targets a nonoverlapping region of the same gene or by rescuing the phenotype using a transgene that is not targeted by the dsRNA (e.g., using a dsRNA that targets the 5′- or 3′-UTR and rescuing with the ORF or with the equivalent gene from a related species).

In what follows, we provide a general procedure for synthesizing dsRNA, a general procedure for introduction of RNAi into fly cells, and two more detailed, specific protocols for individual applications.

*3.9.1. dsRNA Synthesis (*see ***Note 31****)*

To synthesize dsRNA for gene silencing: Design two pairs of oligonucleotides that can be used to amplify two nonoverlapping 500–1000 bp stretches of genomic DNA homologous to the target mRNA of interest. Oligos should carry flanking T7 polymerase-binding sites (taatacgactcactataggg) for the subsequent transcription reaction. We recommend using a primer-design program that has been developed by the Boutros lab for this purpose, at: *http://e-rnai.dkfz.de.*

1. For each primer pair, use a standard PCR reaction to amplify the desired fragment from genomic DNA.
2. Transcribe the unpurified PCR product in both directions using T7 RNA polymerase. We use the Megascript kit (Ambion), following the manufacturer's protocol. A 16-h reaction at 37°C produces approx 100 µg of dsRNA. Yields are proportional to the concentration of PCR product used in the reaction.

Table 2
Appropriate Scale of Culture for Different Experiments

Plate size	Serum-free medium added (µL)	Serum-containing medium added (µL)	dsRNA in well (µg)	Assay
6-well	500	1500	15	Western blot
12-well	250	750	7.5	Western blot
24-well	125	300	3.75	FACS/cell count
96-well	40	120	1.2	Microscopy
384-well	10	40	0.3	Microscopy

3. The majority of RNA is usually double stranded by this stage, but strand annealing can be facilitated by warming the mix to 65°C for 10 min in a water bath. The water bath is then switched off so that the mix slowly cools to room temperature.
4. dsRNA can be used as it is or purified. Purify by filtration using a Millipore PCR cleanup plate, the Qiagen's RNAeasy kit or Ambion's NucAway Spin Columns and resuspend dsRNA in RNAse-free water or TE buffer (pH 8.0) and store at –20°C.
5. Estimate the concentration by monitoring absorbance at 260 nm. The extinction coefficient for dsRNA is similar to that for dsDNA.
6. The quality of the dsRNA should be checked on an agarose gel; using dsDNA or dsRNA standards to determine the size and approximate concentration of the dsRNA.

3.9.2. Introducing dsRNA Into Cultured Fly Cells

The scale of an RNAi experiment will depend on the type of assay being used (*see* **Table 2**). Typically, cells are suspended in the appropriate medium without serum and mixed with dsRNA to give a final concentration of 15–30 µg/mL (*see* **Note 32**) ***(5,7)***. Cells are then plated into tissue culture dishes and incubated at 25°C for 30–60 min. Subsequently, three volumes of complete medium are added and cells grow for 4–7 d at 25°C, splitting when necessary.

3.9.3. Protocol: RNAi in S2R$^+$ Cells in 24-Well Dishes for Microscopy

1. Bang a small T25 flask of confluent adherent S2R$^+$ cells growing in approx 5 mL of medium to remove top cells, leaving the adherent monolayer intact (*see* **Note 33**).
2. Rinse adherent monolayer with 1X *Drosophila* PBS or trypsin-EDTA, and incubate in fresh trypsin-EDTA at room temperature for 5–10 min until cells begin to round up.
3. Remove trypsin-containing medium with care using a pipet and replace with full serum medium. Bang the flask to remove cells.
4. Count cells using hemocytometer (50:50 in Trypan blue, optional) or an automatic cell counter (Casy or Coulter counter), then spin and resuspend cells in serum-free medium at a concentration of 2×10^6 cells/mL. Two T25 flasks contain more than enough cells for 24 equivalent experiments.

5. Add 2×10^5 cells (100 µL) to 3 µL of dsRNA (at 1 µg/µL in ddH_2O) in a 1.5-mL microfuge tube, and transfer the mix as a single droplet to the center of a 24-well plate to ensure formation of a confluent cell monolayer.
6. After allowing 30 min for dsRNA uptake, add 300 µL of complete medium. Seal the dish with parafilm to avoid dehydration and incubate for 4–7 d at 25°C, if necessary splitting cells into two new plates when overgrown.
7. For imaging, remove old medium and gently resuspend cells in 100 µL fresh medium using a pipetor with disposable plastic tip.
8. Replate cells onto clean, serum-treated 13-mm cover slips (*see* **Subheading 3.10.1.**) and process as discussed in **Subheading 3.10.3.**

*3.9.4. Protocol: High Throughput Cell-Based RNAi Screens in 384-Well Plates (*see ***Note 32****)*

1. Add 3 µL of dsRNA in water at 0.1 µg/µL to each well of a clear bottom 384-well plate. Seal wells with sealing tape, and freeze at –80°C. Before use, thaw plate and spin down dsRNA by brief spin at 1500*g*.
2. Wash the tubing of the automated liquid dispenser handler with:
   a. 20 mL of 70% ethanol; leave for 10 min.
   b. 50 mL ddH_2O.
   c. 25 mL of the appropriate serum-free medium (M3 or Schneider's).
   d. Leave medium in tubing to prevent precipitate forming in drying droplets.
3. Count cells using a hemocytometer or cell counter (cell number is critical, and varies with the cell line), and resuspend them in serum-free medium at an appropriate concentration: 2×10^6 cells/mL for $S2R^+$, Kc, and S2 cells.
4. Plate 10 µL cells into each well of a thawed 384-well plate using liquid handler or a multichannel pipet. At this point each well contains 3 µL of dsRNA plus 10 µL of cell suspension.
5. Spin plate in centrifuge for 10 s at 1500*g*. Use microscope to check that cells cover between 30% and 50% of the well surface.
6. Incubate for 30 min at room temperature to allow cells to take up dsRNA.
7. Add 30 µL complete medium (containing serum) to each well using the liquid handler (three passes of 10 µL is usually more gentle on cells than one pass of 30 µL).
8. Seal the plate with parafilm and incubate at 25°C. Clean liquid handler with 50 mL ddH_20, sterilize with 70% ethanol and allow to air-dry.
9. Optional: after 3 d add another 30 µL fresh medium to each well and incubate for another 2 d at 25°C (*see* **Note 34**). If necessary, at this stage dilute and replate fast growing cells into two fresh plates. In general, however, replating compromises well-to-well reproducibility.
10. After 3–5 d (*see* **Note 35**), remove medium from top left hand corner of each well using a 24-well "wand" (*see* **Note 36**).
11. Gently wash the monolayer with 30 µL 1X *Drosophila* PBS (three passes of 10 µL).
12. Replace PBS with 30 µL 4% formaldehyde in 1X PBS, and incubate for 10 min at room temperature.

13. Wash twice with 30 µL 1X PBS containing 0.1% TritonX-100 (PBST) and seal the plate with foil. At this stage, plates can be processed for staining or stored at 4°C for weeks without significant antigen degradation.
14. For staining, block with 20 µL 5% BSA in 1X PBST for 1 h, then add primary antibody in 1% BSA 1X PBST and leave overnight at 4°C. Wash three times with PBST. Add fluorescent secondary antibody in 1X PBST for more than 1 h at room temperature. Wash once with 1X PBST, then label DNA with DAPI solution (*see* **Note 37**) for 30 min at room temperature. Finally wash twice in PBST containing 0.05% sodium azide and seal plate. Cells may be imaged when convenient; they are stable for several weeks at this point.

Methods have been developed for carrying out RNAi screens on glass cover slips in a high throughput format ***(58)***. This approach has the advantage of reduced cost, increased throughput, excellent imaging, and uniform processing between samples. At present, this technology is not in widespread use, because the spotting and imaging techniques require specialized equipment and are hard to perfect, but this approach promises to become more widespread in the coming years.

3.10. Fly Cell Imaging

Following RNAi treatment or transfection, cells can be replated for live-cell imaging or for fixation followed by imaging. For optimal high-resolution imaging, it is important to be able to image a monolayer of flat cells through thin, optically clear glass using a high magnification oil objective. Nevertheless, for most purposes, satisfactory images of cells can be obtained through the thin plastic optical bottom of 96 or 384 plates using long working distance ×20 and ×40 lenses.

3.10.1. Getting Fly Cells to Adhere to a Surface

When optimizing imaging, the goal is to get fly cells in culture to spread on the substrate. Most fly cell lines adhere on tissue culture plastic, probably as the result of integrin-mediated adhesion ***(7)***; however, good imaging is best achieved using lines which spread well on the substrate. A favorite line for this purpose is the $S2R^+$ line ***(17)***, an isolate of S2 cells, which combines good spreading behavior with ease of culture. For optimal high-resolution imaging, it is better to image flat cells through glass. This is not trivial as most fly cell lines, including $S2R^+$ cells, adhere poorly to untreated glass or to glass surfaces in multiwell dishes. This problem is often compounded by impurities found on most commercial glass. To induce cell spreading for imaging the following procedures can be used:

1. For high-throughout imaging, clear bottom black plastic 384/96-well plates can be used. Cells should be grown as a flat monolayer, with 10,000–50,000 cells in each 384 well. Most cell lines maintain adhesion over the course of a 5-d RNAi experiment in these plates, enabling medium-resolution imaging using long working distance ×20 or ×40 lenses. If, after a few days, cells come loose, they usually readhere

several hours after replating in a fresh 384-well plate. For high-throughput imaging, DAPI-stained nuclei are used to aid automated focusing (*see* **Note 37**). Cells in fresh or serum-free medium also adhere reasonably well to the glass bottom 384/96-well plates we have tested.

2. Most fly cell lines adhere quite well to clean glass when placed in serum-free medium. Use the cleaning procedure described in **Subheading 2.9.**
3. The plant lectin, concanavilin A (ConA), can be used to induce cells to flatten on a surface through its interactions with glycosylated surface proteins ***(8)***. For this procedure, place 300 µL of a 50 µg/mL solution of ConA in ddH_2O on a 13-mm coverslip in a dish, seal to prevent evaporation and incubate at 37°C for several hours or 25°C overnight. Rinse the cover slip once with ddH_2O, then allow cells 1–2 h to settle and adhere to the substrate. Because this type of adhesion is unregulated, there are a variety of artifacts associated with this treatment. In particular, cells on ConA fail to undergo complete cytokinesis, making the procedure unsuitable for long time-course experiments.
4. Fly cell lines bind to extracellular matrix proteins through integrin-mediated adhesion ***(7)***. Therefore, by coating a two-dimensional surface with extracellular matrix components, cells can be induced to spread. The favored substrates for adhesion and spreading include extracellular matrix components from fetal calf serum and mammalian vitronectin (fly cells do not adhere well to fibronectin). Typically, a drop of 100% serum or a 5 µg/mL solution of vitronectin/serum is placed on a cleaned 13-mm cover slip for 2 h at 37°C or overnight at 4°C. The solution is then removed (*see* **Note 17**) and the cover slip washed in 1X PBS. Cells are then allowed to attach for 1–12 h before fixation. Alternatively, for high-resolution live-cell imaging, cells are viewed from below through coverglass bottom dishes (MatTek) on an inverted fluorescent microscope, using a ×60 or ×100 oil lens. This enables inhibitors or growth factors to be easily added from above during imaging (*see* **Note 38**).

3.10.2. Watching Fly Cells Move In Vitro

In order to observe cell migration in vitro, cell-substrate adhesion must be of intermediate strength; such that cells can adhere strongly enough to spread, but not so strongly that they cannot release their tails. Although few studies of fly cell migration in vitro have been published, rapid motility is a characteristic of ecdysone-treated Kc cells ***(59)*** and of many CNS-derived fly cell lines, including BG3-c1 and BG2-c2, which move at speeds of several microns per minute on a variety of substrates (Wei-Bai and Baum, unpublished). Motility is most easily studied in coverglass bottom dishes, so that compounds or growth factors can be introduced to perturb cell behavior.

3.10.3. Fixing Cell Lines for Immunofluorescence

Several fixation procedures can be used to preserve the subcellular structure of cells in culture. Protocols need to be optimized for each antigen and antibody used. For example, to ensure a good preservation of microtubules, we fix in methanol at

–20°C. However, this precludes visualization of the actin cytoskeleton using labeled phalloidin, which is best observed after fixation in 4% formaldehyde in 1X PBS or in a "cytoskeletal buffer" ***(3)***. In addition, we have used trichloroacetic acid fixation (10% trichloroacetic acid in 1X PBS for 10 min) for phospho-protein antigens that cannot be imaged well following formaldehyde fixation. Users are encouraged to optimize the procedure, experimenting with a variety of fixation protocols from the mammalian literature, beginning with 4% formaldehyde in 1X PBS, which works for most purposes. A typical formaldehyde fixation procedure is given below. All steps can be carried out on a cover slip in 100 µL drops of liquid. The cover slip is placed on a piece of Parafilm to prevent liquid spreading. Special care must be taken not to allow cells to dry during the washing or fixation steps.

1. Clean a 13-mm cover slip as described in **Subheading 2.9.**
2. Pretreat with a 100 µL drop of serum (2 h, 37°C), as described in **Subheading 3.10.1.**, and wash once with 1X PBS.
3. Use a micropipetor to plate 100 µL cells in a droplet on the cover slip and leave to adhere for 1–2 h.
4. Rinse cells once in 1X PBS, then fix in 4% formaldehyde in 1X PBS for 10 min.
5. Wash cells two to three times with 1X PBST (PBS with 0.1% Triton X-100) to permeabilize.
6. Block for 1 h at room temperature with 5% BSA in 1X PBS (horse or goat serum can be included as an additional blocking agent).
7. Incubate overnight at 4°C with the primary antibody at appropriate dilution in PBST with 1% BSA.
8. Wash three times with 1X PBST.
9. Incubate for more than 1 h with labeled secondary antibody (1:250) in 1X PBST with 1% BSA (add labeled phalloidin/DAPI if required).
10. Wash three times with 1X PBST.
11. Invert glass cover slip onto a drop of 30 µL mounting medium that contains antifade on a glass slide, being careful not to trap air-bubbles. Store in the dark at 4°C. Image in the following days or weeks.

3.11. Measuring Fly Cell DNA Content and Cell Size

To prepare fly cells for FACS analysis or to determine cell number and cell size using a Coulter or CASY counter, follow standard protocols (*see* Chapter 24). For the analysis of cell number/size or for FACS sorting, use live cells in the buffer suggested by the machine manufacturers. When isolating cells, avoid treatments that will lead to mechanical cell damage.

3.11.1. Protocol for the Analysis of DNA Content and Cell Size

1. Harvest more than 10^5 cells, centrifuge in a 15-mL tube, and wash with 1X *Drosophila* PBS.
2. Remove PBS and fix in 1 mL cold 70% ethanol (kept at –20°C). Add the ethanol drop-wise to the cell pellet while vortexing to minimize clumping and to ensure an

even fixation. Leave overnight at 4°C (samples that are tightly sealed can be kept in 70% ethanol for weeks or months).
3. Spin cells at 1500*g* for 5–10 min and wash in 1X PBS. Be careful to avoid cell loss when discarding supernatant.
4. Resuspend cells in 500 µL 1X PBS containing RNAse (DNase-free) and propidium iodide (~50 µg/mL each). Allow sufficient time for the complete degradation of RNA—usually overnight.
5. Analyze by flow cytometry. For specific procedures, see the manager of your flow cytometry facility. It is recommended that RNAi be used to generate controls with elevated G1 or G2 peaks (CDK4 or Dp1 and Cdc25 RNAi, respectively).

3.12. Isolating RNA and Protein From Cells

3.12.1. RNA Preparations

The following protocol, based on the manufacturer's instructions supplied with TRIzol, can be used to prepare RNA from fly cell lines, suitable for use in microarray hybridizations. Fifty milliliter of cells at 5×10^6 cells/mL yields approx 1 mg of total RNA by this procedure.

1. Pellet cells from 50 mL of culture by centrifugation.
2. Resuspend/wash in 5 mL 1X *Drosophila* PBS; transfer to a 15-mL centrifuge tube.
3. Pellet cells by centrifugation, remove supernatant and lyse by repetitive pipeting in TRIzol reagent (0.75 mL per 5–10×10^7 cells).
4. Incubate at room temperature for 5 min.
5. Add 0.2 mL of chloroform per 0.75 mL of TRIzol used. Shake vigorously for 15 s, then incubate at room temperature for 2–5 min.
6. Divide the sample into two microfuge tubes and centrifuge for 15 min at 4°C at less than 12,000*g*.
7. Transfer the top (aqueous) phase to two clean tubes and precipitate the RNA by adding 0.5 mL of isopropanol per 0.75 mL of TRIzol used. Mix by inverting the tubes and incubate samples at room temperature for 10 min.
8. Centrifuge for 10 min at 4°C at less than 12,000*g*.
9. Remove supernatant and wash the RNA pellet once with 75% ethanol: add 1 mL per 0.75 mL of TRIzol used, vortex briefly, and centrifuge less than 7500*g* for 5 min at 4°C.
10. Carefully remove all visible liquid. Air-dry the pellet for approx 10 min. Do not dry completely—dry the pellet until it loses its translucent quality.
11. Dissolve in RNase-free (DEPC-treated) water.
12. Incubate at 55–60°C for 10 min or 30°C overnight to dissolve the RNA completely.
13. Store RNA at –80°C.

3.12.2. Protein Extracts

Standard *Drosophila* protocols can be used to make cytoplasmic protein extracts from fly cells (*see* Chapter 21). About 1×10^7 cells yield sufficient protein for several lanes on a Western blot. Typically, cells from each well of a 12-well

dish are lysed in 100 µL standard RIPA buffer (*see* **Subheading 2.11.**). Nuclei and cell debris are then removed by centrifugation in a microfuge (~13,000 rpm for 15 min), protein concentration is calculated, and extracts are denatured by boiling in sample buffer. Alternatively, to freeze cell state, for example, for the analysis of phosphorylation status, cells can be lysed directly in boiling sample buffer.

4. Notes

1. There are several reasons to consider using mammalian cell lines as an alternative to fly cell lines. First, up until now most fly cell culture work has been pioneered by a relatively small number of labs. As a result, there are only a very few well-characterized fly cell lines and, as yet, no lines with epithelial characteristics. Second, most fly cell lines cannot be grown in a defined medium, and fly cell lines other than S2 and Kc lines are difficult to maintain for long periods, are not easily cloned, and can be troublesome when freezing and thawing. Third, there are far fewer reagents, such as antibodies and growth factors, available for use in *Drosophila* cell culture than there are for mammalian cell culture. Finally, there is no fly cell equivalent of embryonic stem cells that can be used to follow different pathways of cell and tissue differentiation in cell culture.
2. Clone.8 lines are available that can be grown free from Fly extract (Martin Milner personal communication).
3. As an alternative to the generation of cell lines, researchers can use primary cultures from an appropriate fly stock. Cells can be isolated from embryos in significant numbers, for example, from embryos expressing GFP protein, a membrane antigen (e.g., CD8), or a selectable marker. Cells from a specific lineage can then be purified using FACS or using magnetic beads ***(60)***, or by selection for resistance to the corresponding toxin. The use of primary cells is limited by the fact that they are less uniform than cells in a permanent line, and are developmentally unstable, typically undergoing differentiation over a period of hours after their isolation ***(61,62)***.
4. Make up medium from powder according to manufacturer's directions and filter-sterilize (0.22-µm pore-size). Powdered medium is generally less expensive than liquid medium if it is made up in large quantity (e.g., 10-L batches), and making media up from scratch is cheap but time-consuming. As large filter-units are expensive, low volume users usually buy medium in a ready-to-use form.
5. A few *Drosophila* lines, including S2 and Kc cells, can be grown in a variety of serum-free media, such as HyQ, CCM3 ***(63)***, and PAA. The use of a serum-free medium avoids the costs associated with serum and has the important benefit of avoiding serum variability. However, some of the advantages of a defined medium are lost, because the compositions of commercial media are proprietary. Most *Drosophila* lines grow poorly if at all in these media.
6. Most vendors are willing to hold a specified number of bottles of serum for a few weeks while the customer tests a small sample. Problems with most of the serum may not show up for a month or more in some of the slow-growing lines, for example, CNS lines. Unfortunately, vendors may not be willing to hold serum for that long.

7. Routine use of antibiotics is unnecessary and can even be the cause of problems—by delaying the time before an infection becomes visible. The presence of penicillin/streptomycin in media can also reduce transfection efficiencies. Our recommended strategy is to omit antibiotics in routine culture and to accept the occasional contaminated plate. For situations in which a single bacterium would lead to a major loss of time or materials (e.g., 1 L cultures grown in spinner flasks), include penicillin/streptomycin. If good sterile technique is maintained, contamination is rarely an issue. Viral infection (e.g., C-virus) may be a problem in fly cell culture, but is not easily identified or remedied.
8. Be cautious about changing insulin concentrations from those recommended for a particular cell line. Insulin activates Ras and PI3K signaling to promote cell survival ***(1)***, but can also induce cell cycle arrest, senescence, and cell death (Baum and Cherbas, unpublished observations), as observed in vivo ***(64)***.
9. To sterilize, fill with water (a volume larger than the volume of cell suspension to be used), autoclave, allow to cool at room temperature overnight, and autoclave again. Pour off the water just before using. The water helps leach out chemical contaminants from the glass. Autoclaving twice will kill the rare fungal spore that is activated but not killed by autoclaving once.
10. As *Drosophila* cell cultures are not thought to carry vectors of human disease, it is not necessary to use a tissue culture cabinet of the type required for mammalian cell cultures. Simple laminar flow hoods are less expensive and easier to use than tissue culture cabinets. Be aware that these hoods maintain good sterility in the working area while providing no protection to the experimenter. Use a tissue culture cabinet if your experiment involves materials that may be hazardous to humans, such as baculovirus (*see* **Note 26**).
11. Bleach can give off noxious fumes when mixed with fly cell culture medium. For this reason, we recommend using nonbleach equivalents for decontamination.
12. An inverted microscope makes it possible to focus on the bottom surface of a dish or flask to observe an undisturbed cell culture. Alternatively, a water immersion lens can be used to observe cells growing in culture on an upright microscope.
13. When methotrexate degrades it becomes toxic even to methotrexate-resistant cells. Therefore, do not use methotrexate that has been stored at 4°C for more than 2 wk, and always protect methotrexate from light.
14. As data accumulates from microarray and other detailed expression studies, it becomes increasingly apparent that there is no such thing as a truly constitutive promoter; this is a problem that must be faced in designing controls for reporter assays. *Actin5C* is not expressed at equal levels in all cells at all times; furthermore, the short (300 bp) promoter used in cell-line vectors is expressed poorly in many cells in which the endogenous gene is expressed strongly (Cherbas, unpublished observations). Nonetheless, the short *Act5C* promoter can be used as a strong promoter in most if not all cell lines.
15. It is neither necessary nor desirable to combine the selectable marker and expression construct into a single plasmid for most transformation techniques. *See* **Note 29** for discussion of this point.

pHGCO and pHCO have very similar properties and were made by Bourouis and Jarry for methotrexate selection. p8HCO is a transfer of the insert of pHCO from pBR322 into pUC8; its properties are identical to pHCO except that its yield in plasmid preps is at least an order of magnitude higher. Methotrexate resistance requires at least 5–10 copies of this plasmid per transformed cell ***(65)***. By contrast, a single copy of actDHFR is sufficient to confer methotrexate resistance. actDHFR is p8HCO with a strong Act5C promoter substituted in place of the original, relatively weak copia promoter.

16. Alternative cleaning procedures include acetone, followed by 70% ethanol and flaming. Acid (nitric or hydrochloric) can also be used to remove more stubborn surface impurities.
17. Vitronectin is expensive and can be stored at 4°C and reused a number of times.
18. To prevent a water bath acting as a source of contamination, place the bottle of serum in a glass beaker of clean water, and place the beaker in the water bath. Copper sulfate or other antifungal agents can be added to the water in the bath, but must not be allowed to contaminate the serum.
19. This protocol is slightly modified by the DGRC (http://dgrc.cgb.indiana.edu/files/tissue-culture-medium-additions.pdf) from that of the Milner lab (http://biology.st-andrews.ac.uk/sites/flycell/flyextract.html). Care should be taken to keep the homogenate cold, as tyrosinase is activated during homogenization and melanization can ruin the extract.
20. The time between transfers varies widely from line to line, as does the optimal regimen for splitting cells. We typically dilute Kc cells 10-fold every 2–3 d, whereas slow-growing CNS lines should be diluted two- to threefold every 10–14 d. Do not let cells overgrow and do not split to below 30% confluence. Always transfer cells into new plates; do not reuse the old plate. When freezing cells, each vial should have sufficient cells to allow for a rapid recovery, equivalent to newly split cells. For Kc or S2 cells, for example, we suggest freezing approx 2×10^7 cells from a single plate in 2.5 mL of freezing medium in 5×0.5 mL aliquots.
21. Lines that require substrate adhesion for growth (such as most disc and CNS lines) are likely to grow poorly in suspension, without using the type of microparticles that have been developed for the substrate-independent growth of mammalian cell lines. We use antibiotics in spinner flasks, because although contamination is rare, when it occurs in a spinner flask, it results in the loss of a large volume of cell culture. Penicillin and streptomycin are completely ineffective against fungi contaminants and largely ineffective against mycoplasm.
22. Seal cryovials tightly. If liquid nitrogen enters the tubes, they are liable to explode when removed from the tank; hence the need for safety goggles during thawing. Keep in mind that it can be difficult to search for a specimen at liquid nitrogen temperatures. Be sure that you keep an accurate catalog of frozen stocks and their locations, and that all sample labels are easily visible. Depending on the conformation of your liquid nitrogen freezer, this may involve labeling boxes or canes, and labeling the individual cryovials on their tops and sides, with a good permanent marker and/or using color-coded lids.

23. On thawing, make sure that the number of cells is adequate, that a large proportion of the cells are normal in appearance. In our experience, if cells do not adhere to the substrate in the first few hours after thawing, they are unlikely to recover.
24. The purpose of the changes of medium is to remove as much DMSO as possible. Centrifugation is likely to cause more cell breakage than decanting the medium from the flask, but it enables DMSO to be removed quickly and completely.
25. As a rough guide, we offer the following anecdotal observations on toxicity: Following treatment with calcium phosphate-DNA precipitates, Kc and S2 cells appear somewhat unhealthy, and we have observed differences in the gene expression response of mock-transfected cells and untreated cells ***(2)***. Electroporation seems to lead to the rapid death and lysis of some cells, but the cells remaining survive and grow. Some lipid-based reagents are mildly toxic; the level of toxicity depends on the amount and type of reagent, and the cell line. It is therefore advisable to remove the transfection reagent after a day. Liposome-based reagents frequently exhibit significant variation between batches, so it is advisable to use a batch that has been tested and optimized.
26. Care should be taken when using baculovirus, as the virus can enter mammalian cells, even though it is then unable to replicate. Baculovirus cannot productively infect *Drosophila* cells, but infection has some associated toxicity over long periods. Because of this, we recommend limiting the use of baculovirus to transient expression experiments, where expression is assayed after 8–24 h.
27. In general, the ratio of plasmids in a transformed cell will be equivalent to the ratio of plasmids in the mixture used for transfection. Given the difficulties of predicting transgene toxicity, we find it useful to transfect with several different plasmid mixtures, in which the ratio of selectable marker to transgene expression plasmid(s) is varied, in order to increase the likelihood of obtaining a transformed line with the desired properties (Cherbas, unpublished).
28. Constitutive promoters can be used for stable transformation if, like Wingless in S2 cells, they have no effect on their host cells (Nusse, personal communication). One can also use inducible promoters in their inactive state to maintain low levels of expression to avoid the detrimental effects of high-level gene expression. For example, cytoskeletal structures can be viewed using the GFP-lines made in the Rogers and Vale laboratories (Rogers, personal communication) without induction.
29. Most transformation techniques lead to the formation of long tandem arrays of the exogenous DNA ***(63)***. It is the presence of these long arrays that leads to high levels of transgene expression. Although the arrays are generally stable in the absence of selection, an occasional event, presumably homologous recombination within the array, may lead to a drastic loss in the number of copies of the transgenes. For this reason, we recommend maintaining selection conditions whenever the cells are grown. Transgene expression varies widely between cells in the population. Even after cloning, there is significant variation in a population, probably because of low-frequency recombinations which affect the level of expression without rendering cells sensitive to selection.
30. When cells are soaked in 30 μg/mL dsRNA, enough dsRNA enters cells in the first 30 min to induce a robust RNAi response. For these reasons, there is no reason to

avoid changing the medium in the hours and days following the addition of dsRNA ***(5)***. However, gene-silencing can be enhanced by repeating the RNAi treatment several days into the experiment. For some cell types, especially imaginal disc cells such as Clone.8 cells ***(21)***, the entry of dsRNA into cells is rate-limiting at normal dsRNA concentrations. In these cases, liposome-mediated transfection can be used in place of simple dsRNA soaking. This procedure appears to enhance RNAi in all cell types, so that by adding less than 3 μg/mL of dsRNA to a normal transfection mix one can induce a robust gene silencing response in close to 100% of cells. In general, the inclusion of serum in medium during dsRNA treatment reduces the efficiency of RNAi. However, some CNS lines require the presence of 2% serum during the dsRNA treatment if they are to remain viable.

31. Because RNase is ubiquitous and ssRNA is unstable compared with DNA, care must be made at all steps in handling RNA. We recommend wearing gloves, cleaning the bench before working with RNA, and using certified RNAse-free plasticware, autoclaved or DEPC-treated water and dedicated RNase-free gel tanks. Nevertheless, dsRNA is relatively stable compared with ssRNA. It can be stored for long periods at –20°C or colder. However, repeated freezing and thawing should be avoided.
32. For procedures to transfect dsRNA into disc and CNS cells in a 384-well plate we recommend the FlyRNAi website: *http://flyrnai.org/all_protocols*. Briefly, add transfection mix to 30 ng dsRNA, incubate for 15–30 min, then add 2×10^5 cells and incubate for 5 d at 25°C.
33. $S2R^+$ cells grow as a confluent monolayer. As cells divide, this gives rise to a top layer of cells. In general, both cells from both bottom and top layers can be used. However, care should be taken not to repeatedly isolate and use the top cells, as this will select for changes in the cell population over time. Trypsin-EDTA should be used to replate the bottom layer every 5–7 d, to prevent them from undergoing senescence. Trypsin treatment can have a rejuvenating effect on cells.
34. Too much media in a flask or well can compromise cell viability.
35. RNAi or dsRNA-mediated interference induces a reduction in gene expression and should not be considered equivalent to a complete loss of function. In our experience, most genes (even those that code for abundant stable proteins such as histone or actin) can be functionally silenced using RNAi in *Drosophila* cell culture. We have noticed that some genes with a high catalytic activity (e.g., PI3K) can prove somewhat refractory to RNAi. Following the induction of RNAi, the levels of a target protein decrease gradually over time. As a result, in $S2R^+$, S2, or Kc cells, RNAi-induced phenotypes usually first appear after 2–3 d (e.g., IAP or cdc25). For most genes the loss of function phenotypes then peak at about 5 d, although for some stable proteins (e.g., myosin II and Arp3) the response can peak at 7 d. Because of this, phenotypes will vary at different times following dsRNA treatment. The silencing induced by dsRNA soaking is transient so that protein levels recover after approximately more than 9 d in $S2R^+$, S2, and Kc cells. To maintain gene silencing over longer periods, dsRNA hairpin expression constructs can be used to induce permanent gene silencing. RNAi can be used in CNS and imaginal disc lines, but the efficiency of the RNAi-induced silencing is reduced.

36. Extreme care must be taken when processing cells in plates. Ideally, to avoid perturbing the cell monolayer, all operations should be performed in the same corner of the square 384 well. Liquid must be added gently, and when removing liquid, a small volume should remain so that cells do not dry out. This can be achieved by winding tape around the part of the wand that rests on the top of the plate, raising the prongs above the bottom of each well.
37. DAPI-stained nuclei provide a good signal for automated focusing and for subsequent automated image analysis *(7)*. However, many systems now include a laser-based automated focusing system that finds the air/plastic or plastic/sample interface, avoiding the need for a generic stain. This is not without its problems, as these systems can confuse top and bottom surfaces of the plate.
38. An advantage of working with cells in culture is the ease with which an entire population of cells can be simultaneously exposed to stimulants and drugs, for example, insulin to perturb growth, ecdysone to induce differentiation, or microtubule poisons to arrest the cell cycle. Care has to be taken to control the effects of the carrier (e.g., DMSO).

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Appendix

Phenotypic Markers in *Drosophila*

Jennifer Childress and Georg Halder

From: *Methods in Molecular Biology:* Drosophila: *Methods and Protocols*
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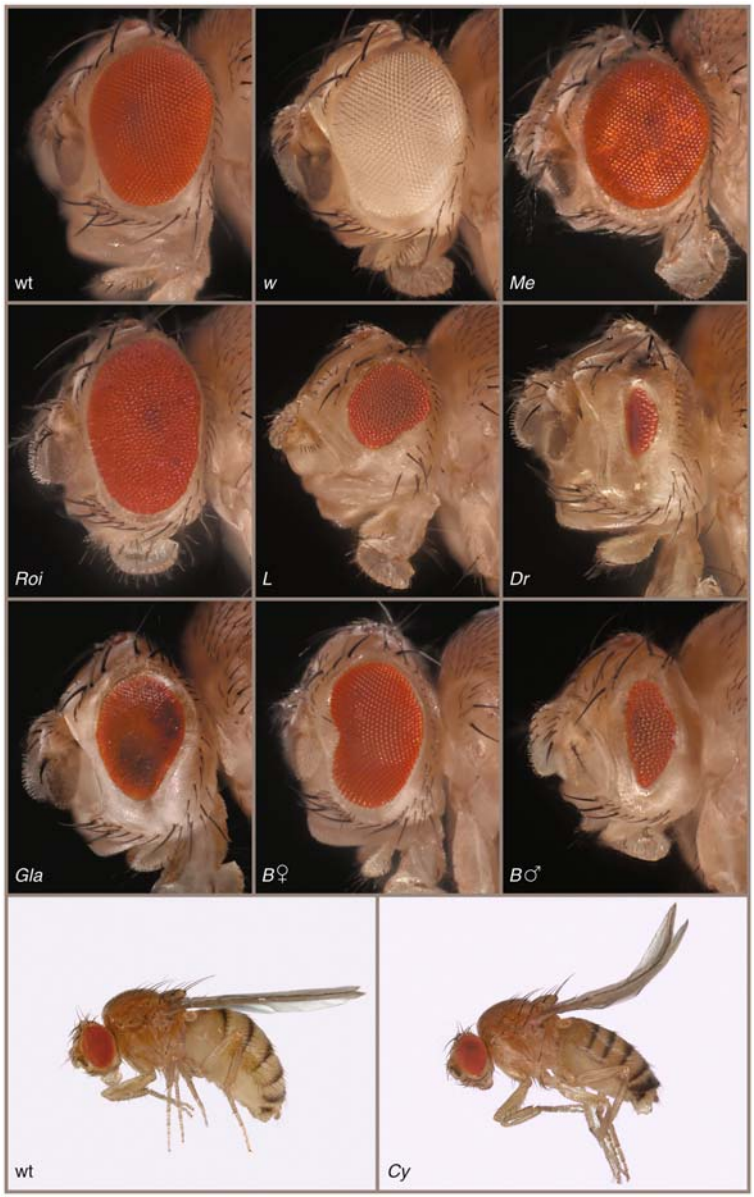

Phenotypic markers in *Drosophila*: wt, wild type; *w, white; Me, Moiré; Roi, Roughoid; L, Lobe; Dr, Drop; Gla, Glazed; B, Bar; Cy, Curly; Hu, Humeral; y, yellow; e, ebony; sn, singed; Sb, Stubble; Sco, Scutoid; Tb, Tubby; Bc, Black cells; Sp, Sternopleural; Ser, Serrate; Ubx, Ultrabithorax.* Photographs were taken on a Leica Z16 using the In-Focus software from Meyer Instruments, Houston, Texas. Graphic production by Leisa McCord.


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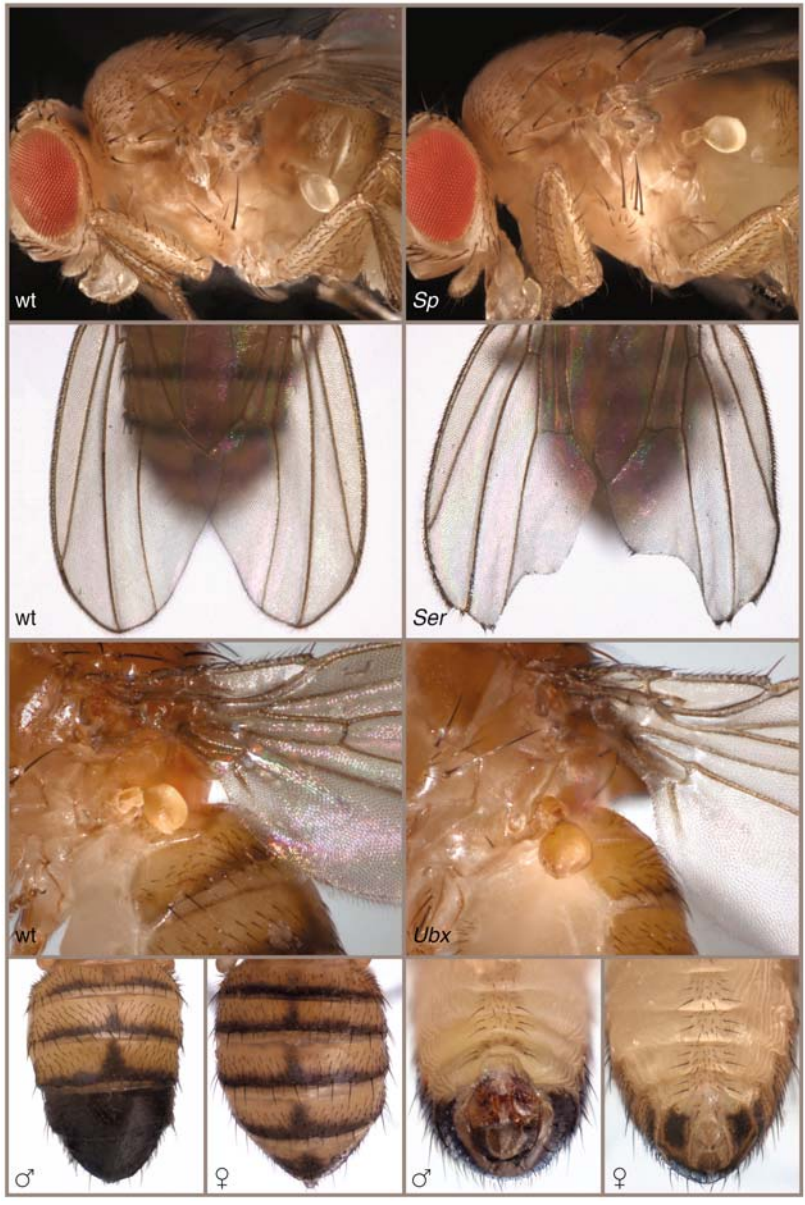
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