A rough guide to *Drosophila* mating schemes (lite version 5.2) [1]

This document is one part of a *Drosophila* genetics training package, the entire strategy of which is described in detail elsewhere [54].

Content

[1] Updated versions of this document can be downloaded @ dx.doi.org/10.6084/m9.figshare.156395 and a full version for intense training @ dx.doi.org/10.6084/m9.figshare.106631

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1. Why work with the fruitfly *Drosophila melanogaster*?

More than a century ago, the fruitfly *Drosophila melanogaster* was introduced as the invertebrate model organism that founded the field of classical genetics. It has been argued that *Drosophila* was kept in a number of laboratories as a cheap model for student projects since, as an omnipresent follower of human culture, it was easy to obtain and maintain in laboratories. This was useful in times of neo-Darwinism (the study of Darwinian evolution with Mendelian genetics), especially for an experimentalist like Thomas H. Morgan who wanted to reproduce evolution in the laboratory to understand how organisms undergo the changes that can then be selected for during evolution [13,36] [1]. It was the serendipitous discovery of the *white* mutation during mass breeding experiments, and the recognition of its linkage to the X chromosome by T.H. Morgan [2,42] which kick-started the systematic use of the fly for genetic research, essentially fuelled by Morgan's students Sturtevant and Bridges [36]. Already 3 years later the first chromosome map was published [62].

Building on the sophisticated fly genetics gained during the early decades, research during the second half of the 20th century gradually turned flies into a powerful "boundary object" linking genetics to other biological disciplines [34]. Thus, fly genetics was systematically applied to the study of development, physiology, behaviour and evolution, generating new understanding of the principal genetic and molecular mechanisms underpinning biology, many being conserved with higher animals and humans [2&3]. Notably, it has been estimated that "...about 75% of known human disease genes have a recognisable match in the genome of fruit flies" [53], and this evolutionary conservation reaches through to higher organisation levels. For example, fly and human organs often share fundamental functions and principles of organisation [4], thus providing fantastic opportunities to discover concepts and mechanisms, with a high potential to impact on the biomedical sciences [9]. It is therefore not surprising that *Drosophila* is the insect behind seven Nobel laureates (Box 1).

Box 1. Nobel prizes for work on *Drosophila*

1933	Thomas Hunt Morgan - *The role played by chromosomes in heredity*
1946	Hermann Joseph Muller - *The production of mutations by means of X-ray irradiation*
1995	Edward B. Lewis, Christiane Nüsslein-Volhard, Eric F. Wieschaus - *The genetic control of early embryonic development*
2004	Richard Axel - *Odour receptors and the organisation of the olfactory system*
2011	Jules A. Hoffmann - *The activation of innate immunity*

This said, *Drosophila* is not a "mini human", and the questions to be addressed need to be chosen wisely and concern problems that are fundamental enough to deliver understanding that can be translated into mammalian or human contexts. Furthermore, *Drosophila* and its worm counterpart *Caenorhabditis elegans* are no longer the only "boundary objects", and vertebrate models including frogs, chicks, zebrafish, mice or human stem cells and cell lines have become much more amenable to genetic manipulation and research, especially following the advent of CRISPR/Cas9 technologies. However, the importance of genetic invertebrate model organisms is undiminished, simply because of their efficiency and continuous high-speed technology development, which maintain them as powerhouses for unravelling concepts and fundamental understanding of basic biology. Thus, *Drosophila* is nowadays often used as a "test tube" to screen for and investigate genetic components of disease-relevant processes or pathways, or to unravel their cellular and molecular mechanisms, covering a wide range of disease mechanisms including cancer, metabolism, neurodegeneration and even neurotoxicological effects [32,44,51,52,65]. The most important practical advantages available for this research are listed in the following [5&6]:

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[1] Films explaining the history & importance of *Drosophila* research: droso4schools.wordpress.com/why-fly/#Movies

[2] Articles about the history of fly research: www.flyfacility.ls.manchester.ac.uk/forthepublic/outreachresources/#History

[3] Informative lay descriptions of fly research: www.flyfacility.ls.manchester.ac.uk/forthepublic/whythefly/#Lay

[4] For a comparison of human and fly organs see: droso4schools.wordpress.com/organs

[5] See also the "Why fly?" page: droso4schools.wordpress.com/why-fly

[6] Overviews of *Drosophila* as a model: www.flyfacility.ls.manchester.ac.uk/forthepublic/outreachresources/#Model

- Fruit flies are **easy and cheap to keep**. High numbers of different fly stocks can be kept in a handful of laboratory trays, thus facilitating high-throughput experiments and stock management (section 3).
- A fruit fly generation takes about 10 days (Fig.1), thus fly **research progresses rapidly** and pedigrees over several generations can be easily planned and monitored.
- The fly **genome is of lower redundancy** than in higher organisms, i.e. only one or very few genes often code for members of one protein class. In contrast, higher organisms tend to have more paralogous genes encoding closely related proteins that tend to display functional redundancy and complicate loss-of-function analyses.
- A particular strength of *Drosophila* is the possibility to perform **unbiased screens** for genes, that regulate or mediate biological processes of interest, often referred to as forward genetics (Fig. 2; Box 2). Highly efficient and versatile screening strategies have been developed that can be adapted to the experimenter's needs [11,26,30,57,60]. Similarly, *Drosophila* can be used to screen for drugs or toxic effects of substances [52]
- **Virtually every gene of *Drosophila* is amenable** to targeted manipulations, ideal to perform reverse genetics (Box 2). For this, **experimental strategy development** in *Drosophila* always has been at the forefront of contemporary science, providing powerful investigative means at all levels of biology [23,46], including genomics, classical and population genetics, molecular biology, physiology and development and even behaviour. For an example of the breadth of strategies to investigate he function of a single gene, see this review on the investigation of *short stop* (*shot*) [28].
- In this way, a **huge body of knowledge and rich resources** have been accumulated, enormously facilitated by the highly collaborative spirit of the fly community dating back to the early days of *Drosophila* genetics. Originally this culture of generous exchange of materials and information was realised through an informal newsletter (*Drosophila* Information Service) [35,36][1] and has nowadays been further elaborated into **well organised databases** and **stock centres** which provide easy access to knowledge, fly strains and materials. Importantly, all this information is well integrated and curated in **FlyBase** (flybase.org) the central point of reference for fly researchers worldwide [40,59] [2]. A collection of all fly-specific resources and infrastructure which so enormously facilitate research, has been put together to facilitate access: www.flyfacility.ls.manchester.ac.uk/flylinks.
- Experimental manipulations and observations of cells and tissues ***in vivo*** are relatively easy. Thus, organs are of relatively low complexity and size, and can usually be studied live or via straightforward fixation and staining protocols in the whole organism. Only in exceptional cases are these experiments subject to legal requirements or procedures, thus enormously facilitating the fast implementation of experimental ideas. Furthermore, there is a "parallel universe" of complementary *Drosophila* research in **cell culture**. Firstly, an impressive number of *Drosophila* cell lines is readily available (dgrc.cgb.indiana.edu/cells/Catalog), of which especially S2 cells have achieved considerable recognition beyond the community of fly researchers [16,17]. Secondly, primary cell cultures (cells directly harvested from the organism) are well established, especially for neurons and haemocytes [50,56], and offer important complementary readouts amenable to the full range of versatile *Drosophila* genetics.

Finally, it should be mentioned that the vast knowledge of biology gained in *Drosophila*, is not only enormously important for research, but it also provides unique opportunities to teach younger generations in schools. *Drosophila* is the conceptually best understood organism we have, providing countless opportunities to teach curriculum-relevant understanding in school biology lessons, shoulder-to-shoulder with human examples and spiced up with easy to perform, insightful and memorable classroom experiments with living organisms reflecting real science, hence relevance. The detailed rationale, strategy and increasing pool of resources for *Drosophila* as a modern teaching tool are explained on a recent blog (poppi62.wordpress.com/2015/08/28/school-flies). Notably, teaching *Drosophila* in schools is only one way to promote the general awareness of

[1] All issues of the legendary *Drosophila* Information Service can be browsed here: www.ou.edu/journals/dis/

[2] For an easy guide to FlyBase see: http://flybase.org/static_pages/docs/pubs/FlyBase_workshop_2009.pdf

fruit flies as important pillars in the process of scientific discovery, and a comprehensive overview of communication strategies is given elsewhere [45]. Clearly, there is a need to stay alert and communicate the importance of *Drosophila* research at all levels. As M. Brookes quite rightly points out in his book about *Drosophila*: "*... we seem reluctant to accept that this tiny creature can teach us anything, let alone anything about ourselves"* [13]. Therefore, if you are faced with the question of WHY you work with FLIES, make use of the arguments given above.

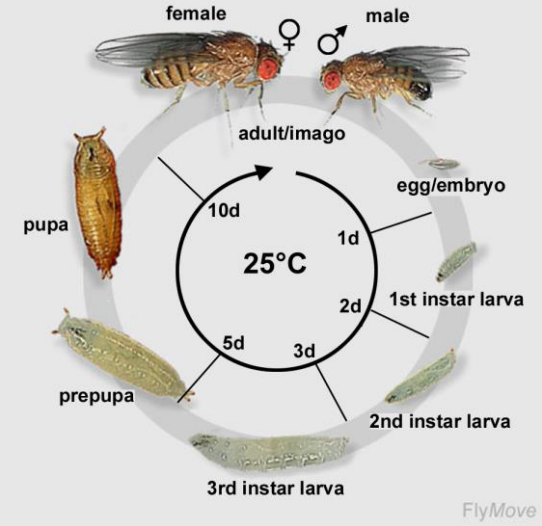


Figure 1. The life cycle of *Drosophila melanogaster* [1]

Fertilised females store sperm in their *receptaculum seminis* for the fertilisation of hundreds of eggs to be laid over several days. At 25°C embryonic development lasts for ~21hr. The hatched larvae (1st instar) take 2 days to molt into 2nd then 3rd instar larvae. 3rd instar larvae continue feeding for one more day (foraging stage) before they leave their food source and migrate away (wandering stage) and eventually pupariate (prepupa then pupa). During the pupal stages, all organs degenerate (histolysis) and restructure into their adult shapes (metamorphosis). 10d after egg-lay, adult flies emerge from the pupal case. After eclosure, males require up to 8 hr to mature sexually, which can be capitalised on for virgin female collection (section 3). The times mentioned here need to be doubled when flies are raised at 18°C [3]. Image modified from FlyMove [66].

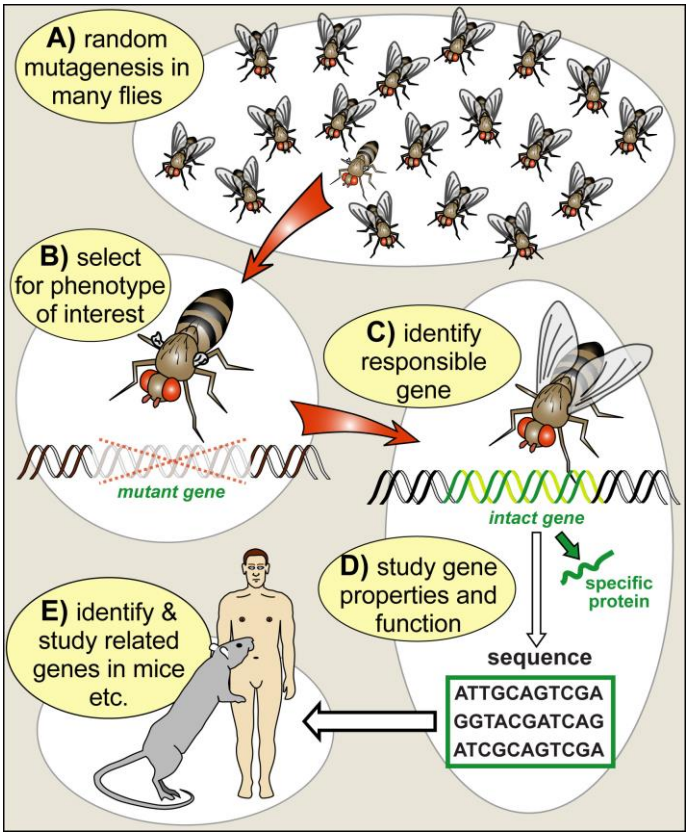


Figure 2 A typical flow diagram of how genetic screens in *Drosophila* contribute to research

A) To induce random mutations, large numbers of flies are treated chemically (e.g. using EMS, ethyl methanesulfonate - **highly carcinogenic!**), manipulated genetically (e.g. through P-element mutagenesis; section 5.1) or exposed to irradiation (e.g. applying X-ray). Other unbiased approaches are screens with large collections of transgenic *RNAi* lines to systematically knock down genes one by one (section 5.2d) or with EP-line collections to systematically over-express genes (section 5.2c). **B)** The essential task is to select those mutant or genetically manipulated animals that display phenotypes representing defects in the biological processes to be investigated. **C)** The responsible gene is either indicated by the specific RNAi- or EP-line inducing the phenotype, or can be identified using classical genetic or molecular strategies to map newly induced mutations to defined genes within the fly genome (Fig. 12B and section 6). **D)** Once the gene is identified, its nature and normal function can be studied. **E)** Vertebrate or human homologues of *Drosophila* genes are usually known (listed under "Orthologs" in FlyBase) and their principal mechanisms are often well conserved. Therefore, capitalising on knowledge from fly research, they can be studied with informed, focussed experiments in vertebrate/ mammalian model organisms, or human patients can be screened for mutations in these genes.

[1] For a computer game about the *Drosophila* life cycle: scratch.mit.edu/projects/74443210

Box 2. Concepts for genetic research: forward *versus* reverse genetics & LOF *versus* GOF

Gene manipulations are generally employed to serve two principal strategies: forward and reverse genetics [58]. FORWARD GENETICS is the approach to identify the genes that are responsible for a particular biological process or function. In *Drosophila,* this is usually performed through using unbiased large-scale screens for genetic aberrations that disturb the process/function in question, and the subsequent identification of the genes affected through these aberrations (Fig. 2). REVERSE GENETICS is the approach to unravel the functions behind specific genes of interest, for example when trying to understand molecular mechanisms or functions of genes known to cause human disease (using the fly as a "test tube"). For this, loss- or gain-of-function (LOF, GOF) approaches are employed, using existing mutant alleles and a wide range of transgenic fly lines that are often readily available (Box 3).

GOF approaches attempt to obtain functional information by creating conditions where the gene is excessively or ectopically expressed or its function exaggerated. This can be achieved through classical GOF mutant alleles (section 4.1.2) or through targeted expression of genes, either of their wild type alleles or of constitutive active versions (section 5).

LOF approaches attempt to eliminate a gene's function partially or completely. This can be achieved by employing classical LOF mutant alleles (section 4.1.2), transposable element insertions (existing for virtually all gene loci; section 5.2b-d), knock-down of genes using RNA interference strategies (readily available as transgenic lines for virtually every gene; section 5.2f), the targeted expression of dominant-negative constructs (e.g. catalytically dead versions of enzymes titrating out the function of the endogenous healthy enzyme), or the use of targeted expression of single domain antibodies (Box 3). Furthermore, there are constantly improving strategies for the manipulation of genes *in situ*, i.e. in their chromosomal location.

2. The importance of genetic mating schemes

Daily life in a fly laboratory requires performing classical genetic crosses. In these crosses, mutant or genetically modified flies are used (Box 3). These different fly variants are the bread-and-butter of fly research, providing the tools by which genes are manipulated or visualised in action in order to investigate their function. The art of *Drosophila* genetics is to use these tools, not only in isolation but often combined in the same flies. This combinatorial genetic approach significantly enhances the information that can be extracted.

For example, you investigate a certain gene called *Mef2*. You have isolated a candidate mutation in this gene which, when present in two copies in embryos, correlates with aberrant muscle development. You hypothesise that this phenotype is caused by loss of *Mef2* function. A standard approach to prove this hypothesis is to carry out "rescue experiments" by adding back a wild type copy of the gene into the mutant background, analogous to gene therapy. For this, you will need to clone the *Mef2* gene and generate transgenic fly lines for the targeted expression of *Mef2* (section 5.1). To perform the actual experiment, you now need to bring the *Mef2* transgenic construct into *Mef2* mutant individuals. This last step requires classical genetic crosses and the careful design of genetic mating schemes.

These mating schemes are a key prerequisite for successful *Drosophila* research. The rules underpinning these schemes are simple. However, they often require thinking ahead for several generations, comparable to planning your moves during a game of chess. To enable you to design such mating schemes, this manual will provide you with the key rules of the game and explain the main parameters that need to be considered.

3. Handling flies in the laboratory

3.1. Keeping flies

Before starting the theoretical part, it is necessary to give a brief insight into the practical aspects of fly husbandry and how the genetic crosses are performed. This should allow you to imagine the actual "fly pushing" work required to execute the mating schemes designed on the drawing board.

As indicated in Box 3, many different fly stocks are available for fly work. *Drosophila* research laboratories usually maintain considerable numbers of stocks relevant to their projects (Fig. 3A). But always be aware that stock keeping is work intense since you deal with live animals which need to be cared for like pets! Therefore, you should have a good reason for keeping stocks.

For example, they may be unique (in this case also consider to send them on to stock centres or interested colleagues for back-up), or you may want to have them readily available to be able to kick-start practical work on experimental ideas that arise through daily discussion and thought. Always consider that most stocks can be ordered from public or commercial stock centres (FlyBase / Links / Stock Collections) or by sending requests to colleagues all over the world, most of whom are willing to freely share fly stocks, especially when they are already published in scientific journals. Note that new flies coming into the laboratory should be properly filed and kept in quarantine under observation for a couple of generations in order to exclude diseases or parasites they may carry. Fly stocks are kept in small vials (Fig. 3B) containing food (the main ingredients of which are corn flour, glucose, yeast and agar)[1] and they can easily be transferred to fresh vials for maintenance. These vials are usually stored on trays in rooms or incubators (Fig. 3A) which are temperature-controlled since temperature influences the developmental time of flies (Fig. 1).

Box 3. Fly stocks available for *Drosophila* research

1. Flies with classical genetic tools, in particular..
   - ..classical loss- & gain-of-function mutations (incl. marker mutations) or deficiencies (section 4.1b)
   - ..chromosomal rearrangements (duplications, inversions, translocations etc.) [3,27]
   - .. balancer chromosomes (section 4.3; Box 4)
2. Flies with transgenic constructs encoding a range of products (section 5.2) including..
   - ..wildtype or mutant versions of genes (including dominant negative constructs) from *Drosophila* or other organisms
   - ..whole chromosomal fragments for rescue, gain-of-function or targeted mutagenesis experiments [63,64]
   - ..reporter genes (encoding ß-Gal, fluorescent proteins, calcium indicators, pH indicators etc.) fused to gene-specific or inducible promoters, or under the control of position-specific activating elements at their chromosomal insertion site (section 5.2a-c)
   - ..exogenous transcription factors (e.g. Gal4, tTA, LexA) with known expression patterns to induce targeted expression of a gene of choice (section 5.2d)
   - ..small interfering RNAs to knock down gene expression (section 5.2f)
   - ..single-domain antibodies against endogenous proteins [38] or designed into anti-GFP nanobodies for the targeted degradation of GFP-tagged proteins [15]
   - ..recombinases (e.g. flippase, ϕC31) or their recombination target sites (e.g. FRT, attP) at specific chromosomal locations; they are jointly used for site-directed insertion of transgenes (section 5.1) or to generate mosaics of mutant cells in the germline or in somatic tissues (section 5.2e)
   - ..genetically encoded toxins (e.g. ricin, tetanus toxin), cell death inducers (e.g. *hid*, *DTS*; Box 4), optogenetic tools (e.g. channel rhodopsin) [31] or other physiological tools (e.g. Kir channels, Shibire^{ts}) for the analysis and/or experimental manipulation of cells

- **Stock keeping** is usually done at 18°C (generation time of about 1 month). It is good practice to keep one young and one two week older vial of each stock. Every fortnight, freshly hatched flies from the month-old vial are flipped into a fresh vial, whilst the two-week-old vial should have produced larvae and serves as back-up. Such a routine allows you to spot any problems on time, such as infections (mites, mould, bacteria, viral infections) [3], the need to add water (if the food is too dry and coming away from the wall) or to reduce humidity (if vials are too moist so that fungus accumulates and/or flies get stuck in the food and at walls).
- **Experiments** with flies tend to take place at room temperature or at certain conventional temperatures, such as 25°C for well-timed experiments or 29°C to speed up development or enhance targeted gene expression with the *Gal4/UAS* system (section 5.2).

[1] fly media recipies: flystocks.bio.indiana.edu/Fly_Work/media-recipes/media-recipes.htm

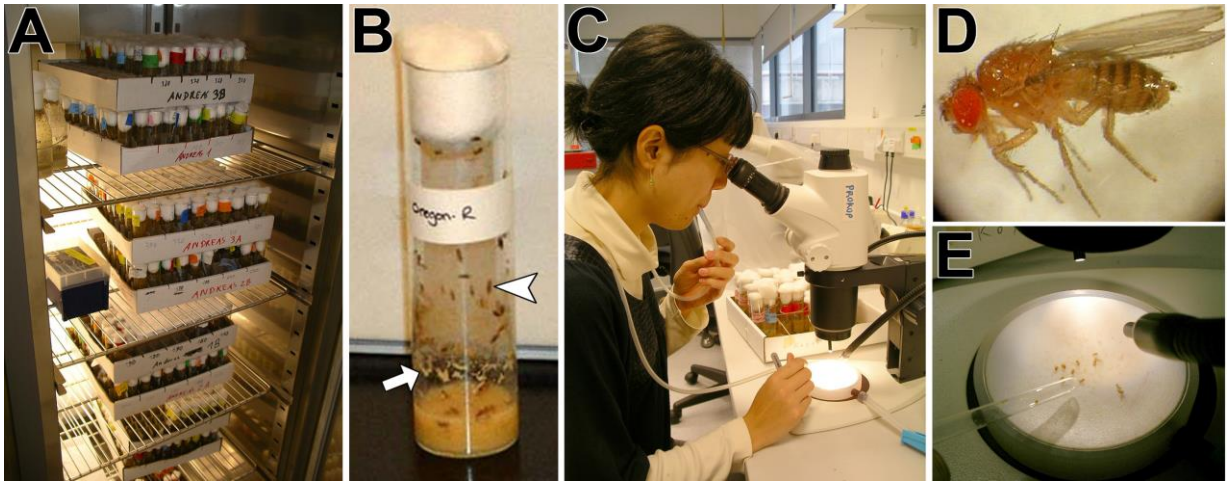


Figure 3. Maintaining and handling flies in the laboratory

A) Fly stocks are stored in large numbers on trays in temperature controlled rooms/incubators[1] (the trays shown here each hold two copies of 50 stocks). **B)** Each fly stock is kept in glass or plastic vials which contain food at the bottom and are closed with foam, cellulose acetate, paper plugs or cotton wool. Larvae live in the food and, at the wandering stage, climb up the walls (white arrow) where they subsequently puparíate (white arrow head). **C-E)** To score for genetic markers and select virgins and males of the desired phenotypes, flies are immobilised on CO_2-dispensing porous pads (E), visualised under a dissecting scope (C, D) and then discarded into a morgue or transferred to fresh vials via a paint brush, forceps or pooter / aspirator[2] (C, E). For further information on how a typical fly laboratory is organised see other sources [3,4,5,61][3].

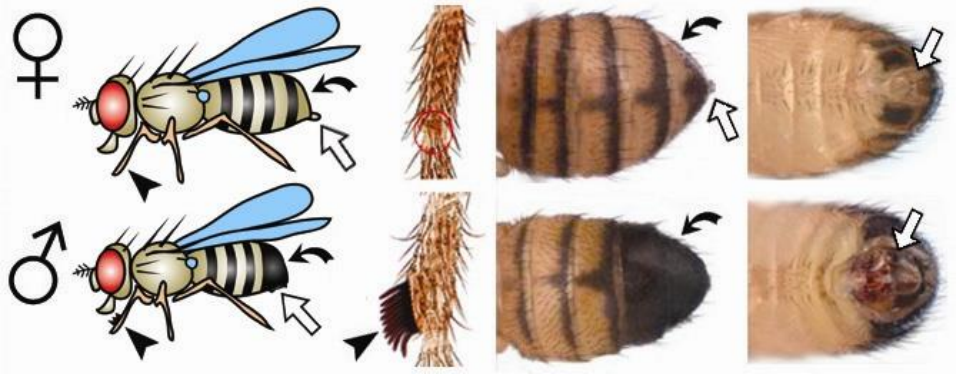


Figure 4. Criteria for gender selection

Images show females (top) and males (bottom): lateral whole body view (1st column), a magnified view of the front legs (2nd column), dorsal view (3rd column) and ventral view (4th column) of the abdomen. Only males display sex combs on the first pair of legs (black arrow heads). Females are slightly larger and display dark separated stripes at the posterior tip of their abdomen, which are merged in males (curved arrows). Anal plates (white arrows) are darker and more complex in males and display a pin-like extension in females. The abdomen and anal plate are still pale in freshly eclosed males and can be mistaken as female indicators at first sight. Photos are modified from [1] and [18]. During a very short period after eclosion, flies display a visible dark greenish spot in their abdomen (*meconium*; not shown) which can be taken as a secure indicator of female virginity even if fertile males are present.

3.2. Performing crosses

To perform crosses, females and males carrying the appropriate genotypes are carefully selected and transferred into one vial for mating (Fig. 3). Some aspects need consideration:

- **Males** and **females** need to be distinguished using the criteria explained in Figure 4.

[1] Incubators need to be fly-proof: copper is aggressively corroded in the presence of flies and should be replaced by stainless steel or needs at least to be well protected (e.g. thoroughly coated with resin).

[2] for use and construction of pooters see: ndownloader.figshare.com/files/3306824

[3] detailed stock-keeping instructions: flystocks.bio.indiana.edu/Fly_Work/culturing.htm

- Selected females have to be **virgin**, i.e. selected before they are randomly fertilised by sibling males in their vial of origin. To select virgins, choose vials containing many dark mature pupae from which adult flies are expected to eclose. To start the selection procedure, discard all flies from the vial and thoroughly check that all eclosed flies (including those that transiently stick to the food or walls) have been removed or otherwise eliminated. The key rationale of this procedure is that freshly eclosed males remain sterile for a period of several hours and will not court females. Hence, after clearing vials, all females eclosed within this period will be virgin. This period lasts for 5-8 hrs at 25°C, about double the time at 18°C, and considerably longer at even lower temperatures (we use 11°C to maintain crosses up to two days for subsequent virgin collection). Therefore, a typical routine for virgin collection is to keep vials **overnight** at low temperatures (ideally below 18°C) and harvest virgins first thing in the **morning**. During the day, they are kept at higher temperatures (to enhance yield) and harvested again around **lunchtime** and **early evening**, before moving them back to lower temperature for the night.
- Flies have to be selected for the right **phenotypic markers**. When designing a **mating scheme**, combinations of markers need to be wisely chosen so that the correct genotypes of both sexes can be unequivocally recognised at each step of the scheme (often from parallel crosses). Phenotypic markers will be explained in section 4.2, and the rules how to choose them will become clear from later sections.

In general, more female flies are used in a cross than male flies (unless males are expected to be of low fitness), with two thirds being female as a reasonable approximation. In general, consider that di- and trihybrid crosses (see example in Fig. 6) will have a low yield of the required offspring and that the numbers of flies available for crosses in a complex mating scheme may gradually reduce from generation to generation. Complex mating schemes should therefore be initiated with large volume crosses, and special measures need to be taken if mating schemes involve genetic combinations that render flies morbid.

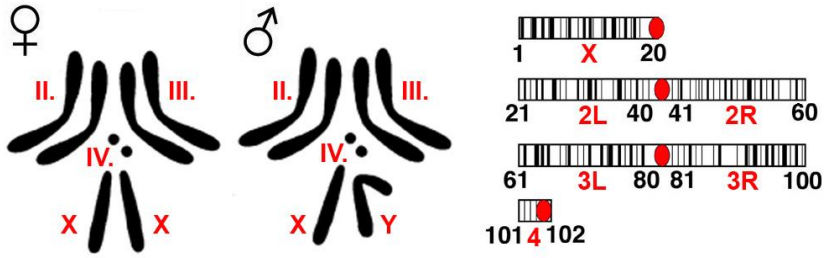


Figure 5. *Drosophila* chromosomes

Cytological images of mitotic *Drosophila* chromosomes. **Left**: Female and male cells contain pairs of heterosomes (X, Y) and three autosomal chromosomes. **Right**: Schematic illustration of *Drosophila* salivary gland polytene chromosomes which display a reproducible banding pattern which can be used for the cytogenetic mapping of gene loci (black numbers); 2nd and 3rd chromosomes are subdivided into a left (L) and right (R) arm, divided by the centrosome (red dot). Detailed descriptions of *Drosophila* chromosomes can be found elsewhere [29].

4. How to design a mating scheme

4.1. Genetic rules

In order to design mating schemes for *Drosophila*, the typical rules of classical genetics can be applied. These rules are briefly summarised here and described in greater depth elsewhere [3,27].

4.1.1. Law of segregation

Drosophila is diploid, i.e. has two homologous sets of chromosomes, and all genes exist in two copies (except X-chromosomal genes in males; Fig. 5). By convention, homologous alleles are separated by a slash or horizontal line(s) (Fig. 6, Box 5). According to the first law of Mendel (**law of segregation**), one gene copy is inherited from each parent or, *vice versa*, the two copies of a gene are separated during meiosis and only one copy is passed on to each offspring (Fig. 6). **Non-disjunction** events are rare exceptions in which both copies pass to one gamete.

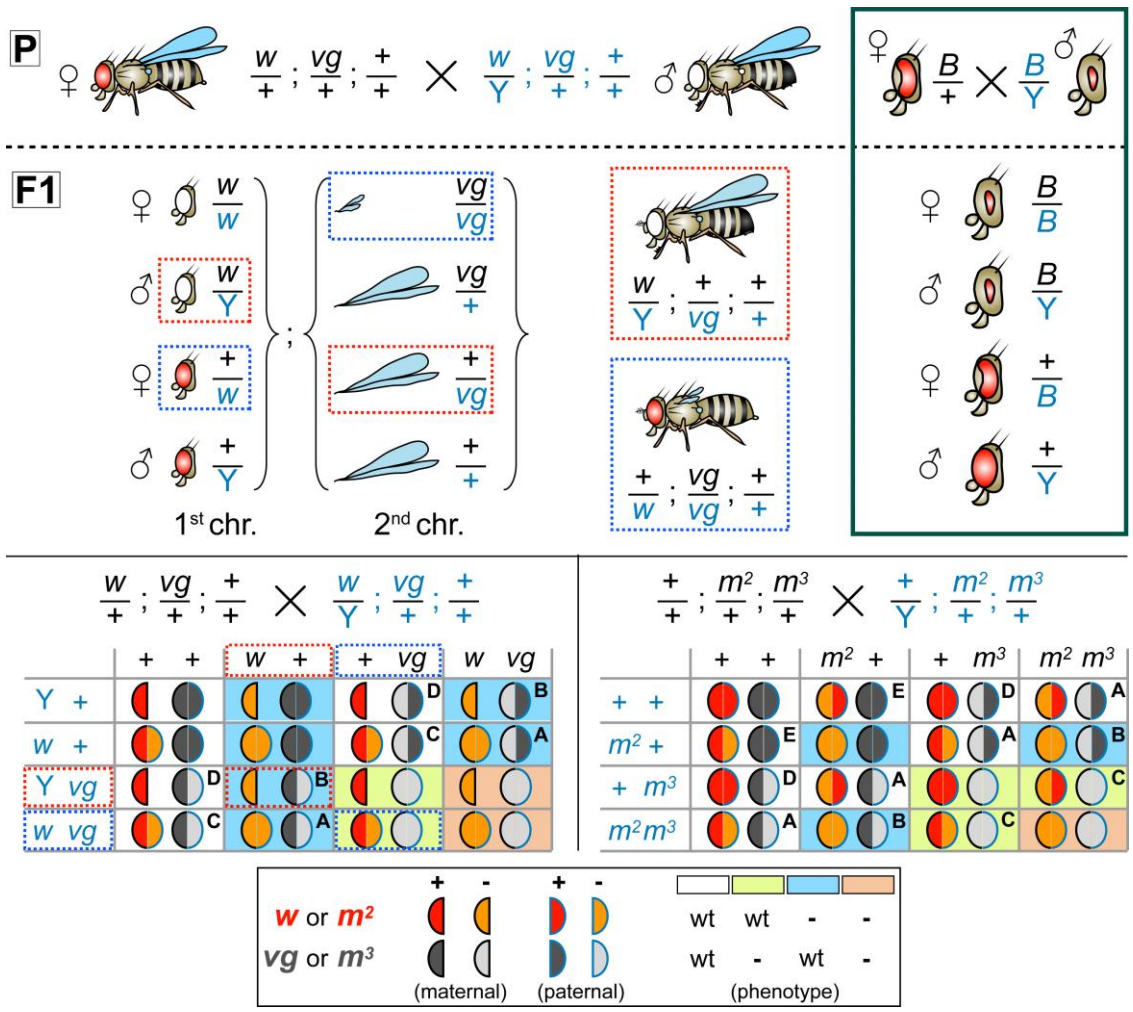


Figure 6. Independent assortment of alleles & comparison of recessive and dominant inheritance

Top: Two examples of dihybrid crosses with recessive mutant alleles on the heterosomal 1st/X chromosome and autosomal 2nd chromosome; mothers of the parental (P) generation are heterozygous on both chromosomes and males are hemizygous on first and heterozyous on 2nd chromosome; **emboxed on the right:** a cross with an X-chromosomal dominant/intermediate allele for comparison. Homologous alleles are separated by a horizontal line; maternal alleles are shown in black, paternal ones in blue. Mutant alleles are *w* (*white*; white eyes), *vg* (*vestigial*; reduced wings), *B* (*Bar*; reduced eyes); phenotypes are indicated by fly diagrams (compare Fig. 9). When comparing inheritance of the eye marker mutations *w* (left) and *B* (right), it becomes apparent that the allele assortments (i.e. genotypes) are the same, but the heterozygous *B* mutant females show an intermediate eye phenotype. **Bottom:** Punnett square of the above cross (left) compared to another cross (right) where parents are heterozygous for recessive mutant alleles on the second (m^2) and third chromosome (m^3). Red and blue stippled boxes in the curly bracket scheme and Punnett square show the same examples of two possible offspring.

Further explanations: In the first offspring/filial generation (F1) each chromosome has undergone independent assortment of alleles (demarcated by curly brackets) and can be considered like in a monohybrid cross, with the 1st chromosome showing a gentoypic / phenotypic distribution of 1:1:1:1 / 2:2, and the 2nd chromosome displaying a genotypic / phenotypic distribution of 1:2:1 / 3:1. Each of the outcomes for one chromosome can be combined with any of the outcomes of the other chromosome resulting in 4 x 3 = 12 genotypic combinations (listed in the left Punnett square; 4 duplicates indicated by letters) and 6:6:2:2 phenotypic distributions (6 white : 6 blue : 2 yellow : 2 pink fields in the left Punnett square). If the two recessive alleles are on the 2nd and 3rd chromosome (Punnett square on the right), the "Y" is replaced by "+". In that case, there would be 3 x 3 = 9 genotypic combinations (1 quadruplicate and 4 duplicates indicated by letters) and a phenotypic distribution of 9:3:3:1 (9 white : 3 blue : 3 yellow : 1 pink fields in the Punnett square). Note that the Punnett square reflects the numerical outcome of this cross in its full complexity, whereas the curly bracket strategy is only qualitative and therefore easier to interpret for the purpose of mating scheme design (Box 5). Punnett squares become substantially more complex in trihybrid crosses (Appendix 2).

4.1.2. Alleles[1]

Genes exist in different alleles. Most **loss-of-function** mutant alleles (**hypo-** or **amorphic/null**) are **recessive**. Their phenotypes are not expressed in heterozygous (-/+) but only in homozygous animals (-/-), i.e. the wildtype allele mostly compensates for the functional loss of one gene copy (see *w*, *vg* or *e* in Fig. 6). Loss-of-function mutant alleles can also be **dominant**. For example, phenotypes are observed in animals heterozygous for *Ultrabithorax* (*Ubx*/+), *Polycomb* (*Pc*/+), or Notch (*N*/+) loss-of-function alleles, i.e. the wildtype allele is insufficient to compensate for loss of one functional gene copy (**haplo-insufficiency**). Dominant alleles can also be **gain-of-function**, usually caused by over-expression of a gene product (**hypermorph** or "dominant negative" **antimorph**) or by ectopic expression or activation of a gene product, potentially conveying novel gene functions (**neomorph**). For example, *BarH1* over-expression in the eye causes kidney-shaped eyes in Bar^{1}/+ individuals (Fig. 6) [37], ectopic *Antp* expression in antennae the antenna-to-leg transformations in $Antp^{73b}$/+ (Fig. 9) [25], and *Krüppel* mis-expression the reduced eyes in If^{1}/+ animals (Fig. 9) [14]. Dominant alleles may display **intermediate** inheritance showing a stepwise increase in phenotype strength from heterozygous to homozygous animals. Thus, the eyes of heterozygous flies (B^{1}/+) are kidney-shaped, whereas they display a stronger slit-shaped phenotype in homo- (*B*/*B*) or hemizygous (*B*/Y) flies (Fig. 6). Animals carrying the loss-of-function mutant allele $abd\text{-}A^{MX1}$ in heterozygosis are viable and show a weak dominant cell proliferation phenotype, whereas homozygous animals are lethal and show a strong cell proliferation phenotype [49]. Note, that the phenotype distribution in pedigrees involving dominant mutant alleles differs from those with recessive mutant alleles (Fig. 6). Also note that the existence of dominant and recessive alleles has impacted on gene names (capitalisation of the first letter), which can be confusing or even misleading[2]. As a further matter of complication, a **phenotype** you observe may not always be caused by the gene or mutant allele you believe to study, but a whole range of potential independent factors in the background of your fly stock/cross might modify the strength or quality of the observed phenotype, or be causing the whole phenotype all together. Be aware of this and carry out appropriate control experiments before drawing hasty conclusions.

4.1.3. Independent assortment of chromosomes

Drosophila has one pair of sex chromosomes (heterosomes: X/X or X/Y) and three pairs of autosomes (Fig. 5). Usually, non-homologous chromosomes behave as individual entities during meiosis and are written separated by semicolon in crossing schemes (Fig. 6, Box 5). According to the second law of Mendel (**law of independent assortment**), they assort independently of one another during gamete formation, leading to a high number of possible genotypes (Fig. 6). A good strategy to deal with this complexity during mating scheme design is to define selection criteria for each chromosome independently (curly brackets in Fig. 6; see Box 5). The **4th chromosome** harbours very few genes and its genetics slightly differs from other chromosomes [27]. It plays a negligible role in routine fly work and will therefore not be considered here.

4.1.4. Linkage groups and recombination

Genes located on the same chromosome are considered a **linkage group** that tends to segregate jointly during meiosis. However, when homologous chromosomes are physically paired during meiotic prophase (**synapsis**), the process of **intra-chromosomal recombination (crossing-over)** can lead to exchange of genetic material between homologous chromosomes (Fig. 7; note, that recombination does **not occur on the 4th chromosome**). As a rule of thumb, the recombination frequency increases with distance between gene loci, but non-uniformly across the chromosome arms (**map expansion/compression**). Frequencies are usually high in the middle of chromosome arms and low in regions adjacent to heterochromatin-rich telomeres and centromeres. Recombination frequencies have been used to generate spatial chromosomal maps of gene loci (recombination maps), defining 1% chance of crossing-over between two loci as 1 map unit (or centimorgan, cM) [27][3]. 50% is the maximum detectable crossing-over frequency because crossing-over is happening at the 4-strand stage; only 2 strands are involved in any one event and exchange

[1] see also http://en.wikipedia.org/wiki/Muller's_morphs

[2] for rules see FlyBase / Help / Nomenclature

[3] The first ever linkage map [62]: www.esp.org/foundations/genetics/classical/holdings/s/ahs-13.pdf

between sister chromatids produces no observable changes. If two genes are 50 cMs apart then they are equivalent to being unlinked (due to the increase in multiple crossing-over events occurring between them). If the location of two loci is known relative to the cytogenetic map, their position on the recombination map can be roughly estimated and the recombination frequency between them deduced (Fig. 7B). For mating schemes, recombination can be a threat as well as an intended outcome:

- There are two key remedies to prevent unwanted recombination during mating schemes. The first strategy is to use **balancer chromosomes** (section 4.3). The second strategy is to take advantage of the recombination rule. The **recombination rule** states that there is **no crossing-over in *Drosophila* males** (Fig. 7). The reason for this is not clear.
- In other occasions it can be the intended outcome of a mating scheme to **recombine mutations onto the same chromosome**. For example, in reverse to what is shown in Fig. 7, you may want to combine the *rosy* (*ry*) and *ebony* (*e*) mutations from different fly stocks onto one chromosome in order to perform studies of *ry,e* double-mutant flies. A typical mating scheme for this task is explained in Appendix 1.

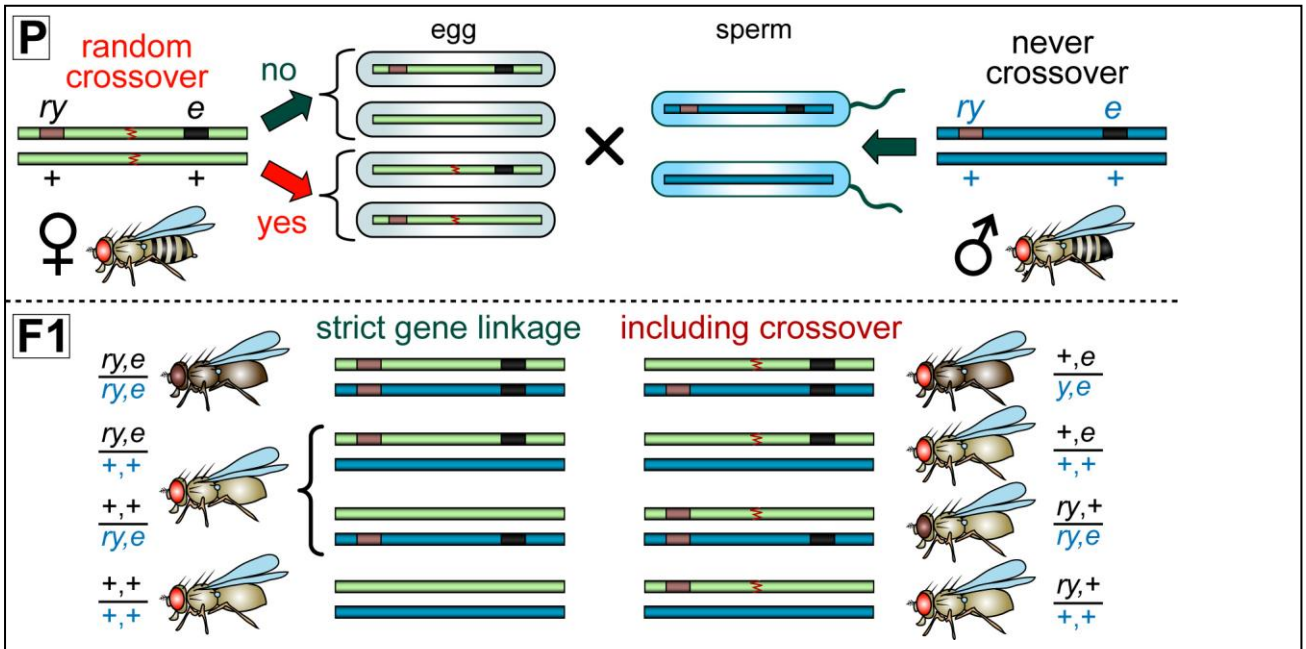


Figure 7. Inheritance of genes on the same chromosome (linked genes)

(**P**) A cross between flies heterozygous for viable recessive mutations of the 3rd chromosomal loci *rosy* (*ry*; brownish eyes, 87D9-87D9) and *ebony* (*e*; black body colour, 93C7-93D1); female chromosomes are shown in beige, male in blue. According to the law of segregation, homologous chromosomes are distributed to different gametes (egg and sperm) during gametogenesis. Male chromosomes do not undergo crossing-over. In females, crossing-overs are possible (red zigzag lines), and recombination between any pair of genes may (yes) or may not (no) occur (at a frequency dependent on their location and distance apart), thus increasing the number of different genotypes. In the first filial generation (**F1**), three potential genotypes and two potential phenotypes would have been expected in the absence of recombination (strict gene linkage); this number is increased to 7 genotypes and 4 phenotypes when including crossing-over.

4.2. Marker mutations

The anatomy of the fly is highly reproducible with regard to features such as the sizes and positions of bristles, the sizes and shapes of eyes, wings and halteres, or the patterns of wing veins (Fig. 8). Many mutations have been isolated affecting these anatomical landmarks in characteristic ways and described with great detail [39][1]. On the one hand these mutations can be used to study biological processes underlying body patterning and development (by addressing what the mutant

[1] available on FlyBase at the bottom of "Summary Information" for genes that were listed in the red book

phenotypes reveal about the normal gene function). On the other hand these mutations provide important markers to be used during genetic crosses and, hence, for mating scheme design. A few marker mutations commonly used for fly work are illustrated in Fig. 9.

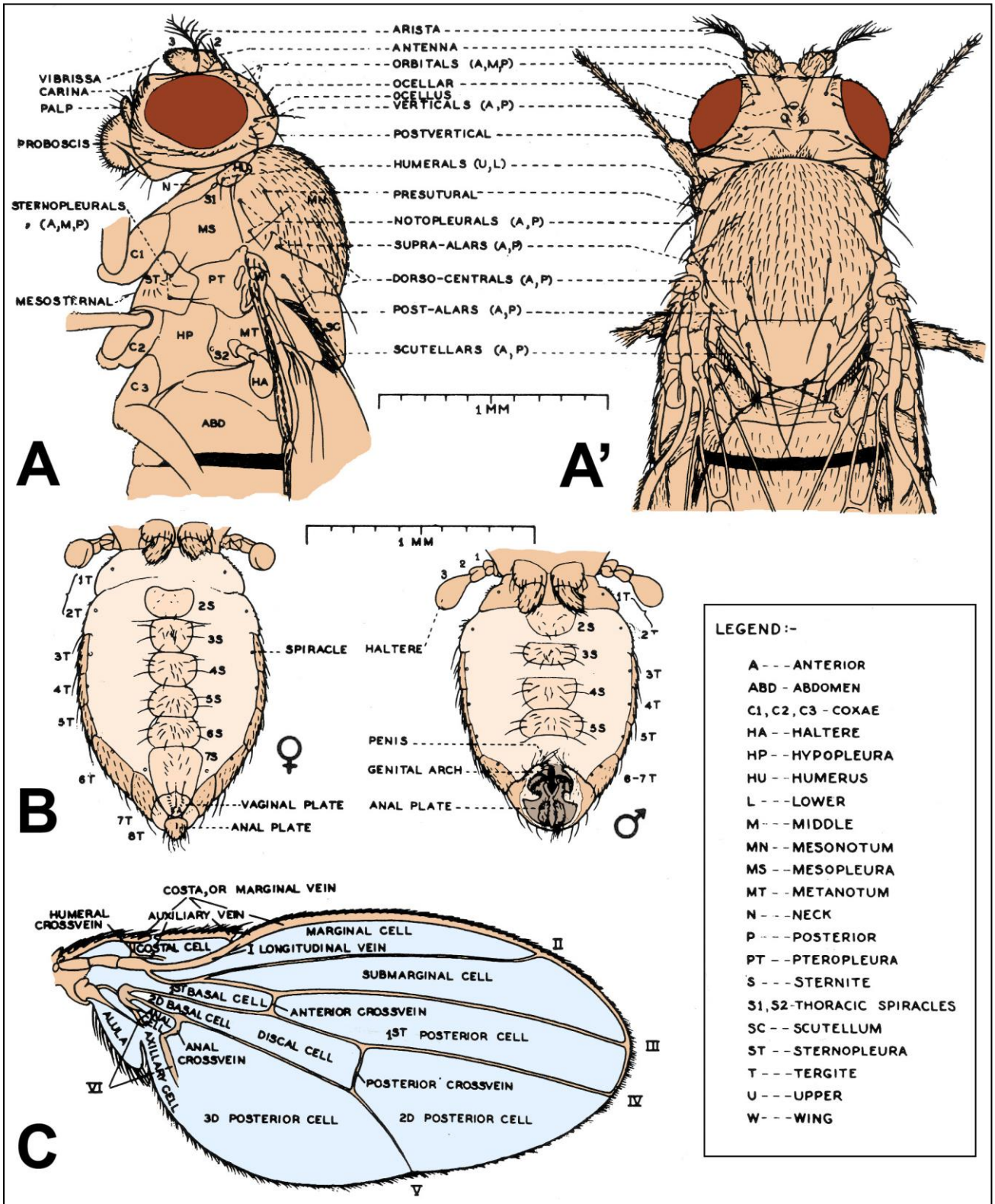


Figure 8. Anatomy of adult *Drosophila*

Lateral (**A**) and dorsal (**A'**) view of the head and thorax region of an imago; body parts and bristles are indicated. **B**) Ventral views of a female (left) and male (right) abdomen; note differences of the anal plate in B which provide easy markers to determine gender (Fig. 4). **C**) Morphology of the wing and its characteristic veins. Image modified from [12].

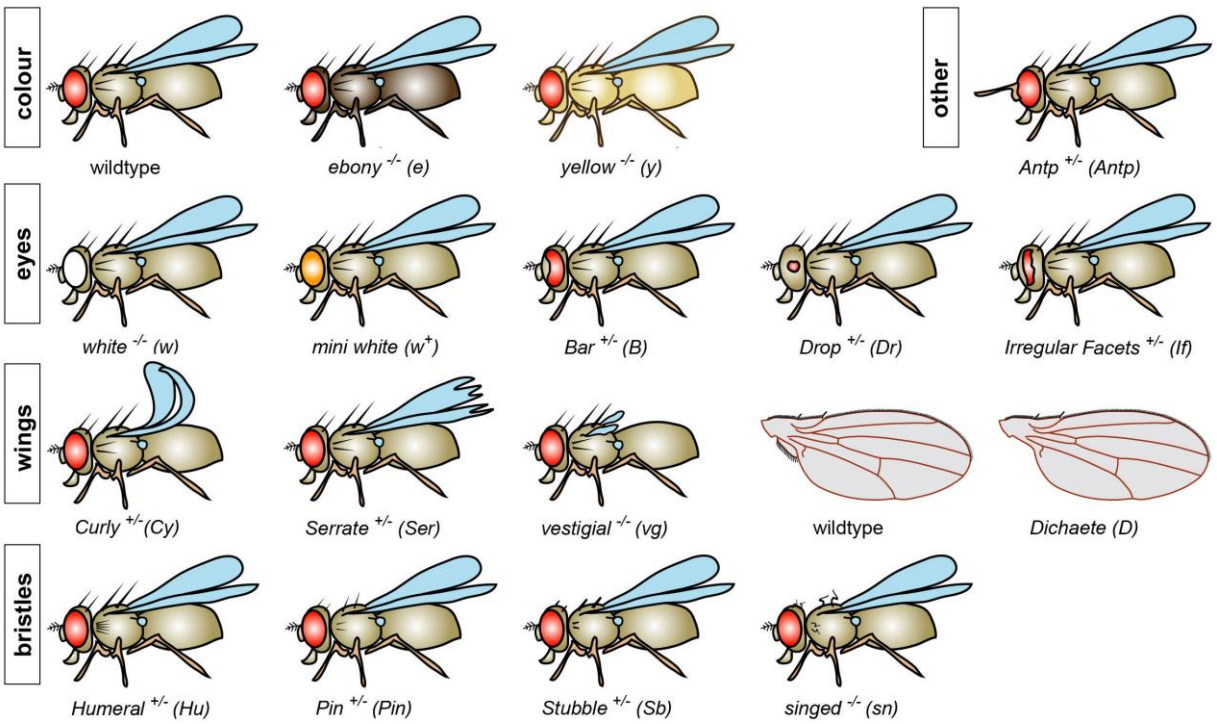


Figure 9. Examples of typical marker mutations used during genetic crosses

Mutations are grouped by body colour (top), eye markers (2nd row), wing markers (3rd row), bristle markers (bottom row), and "other" markers (top right). Explanations in alphabetic order:

- *Antennapedia*^{73b} (dominant; 3rd; antenna-to-leg transformation)
- *Bar*^{1} (dominant; 1st; kidney shaped eyes in heterozygosis, slit-shaped eyes in homo-/hemizygosis)
- *Curly* (dominant; 2nd; curled-up wings; phenotype can be weak at lower temperatures, such as 18ºC)
- *Dichaete* (dominant; 3rd; lack of alula, wings spread out)
- *Drop* (dominant; 3rd; small drop-shaped eyes)
- *ebony* (recessive; 3rd chromosome; dark body colour)
- *Humeral* (dominant; 3rd; *Antennapedia* allele, increased numbers of humeral bristles)
- *Irregular Facets* (dominant; 2nd; slit-shaped eyes)
- *mini-white* (dominant in *white* mutant background, recessive in wildtype background; any chromosome; hypomorphic allele commonly used as marker on transposable elements)
- *Pin* (dominant; 2nd; short pointed bristles)
- *Serrate* (dominant; 3rd; serrated wing tips)
- *singed* (recessive; 1st; curled bristles)
- *Stubble* (dominant; 3rd; short, blunt bristles)
- *vestigial* (recessive; 2nd; reduced wings)
- *white* (recessive: 1st; white eye colour)
- *yellow* (recessive; 1st; yellowish body colour)

Photos of flies carrying marker mutations have been published elsewhere [18,19] [1].

4.3. Balancer chromosomes

Balancer chromosomes (often referred to as "balancers") are chromosomes which carry multiple overlapping inversions through which the relative positions of genes have been significantly rearranged (Fig. 10A) [3]. Balancer chromosomes are essential for the maintenance of mutant fly stocks as well as for mating scheme design, and their key properties are explained here:

- Balancer chromosomes segregate normally during meiosis, but they **suppress recombination** with a normal sequence chromosome and the products of any recombination that does occur are lethal due to duplications and deletions of chromosome fragments (**aneuploidy** of chromosome fragments). The cytological order of breakpoints for each balancer is listed on the

[1] see also images available on FlyBase or as an App, or download the poster "Learning to Fly".

Bloomington site (Balancers / Inversion breakpoints present on balancers) and shown as pictograms in THE ATLAS [19], nicely illustrating the weak spots where balancers are prone to recombination (asterisk in Fig. 10A). Further recombination events occurring upon long-term stock keeping were reported recently [41].

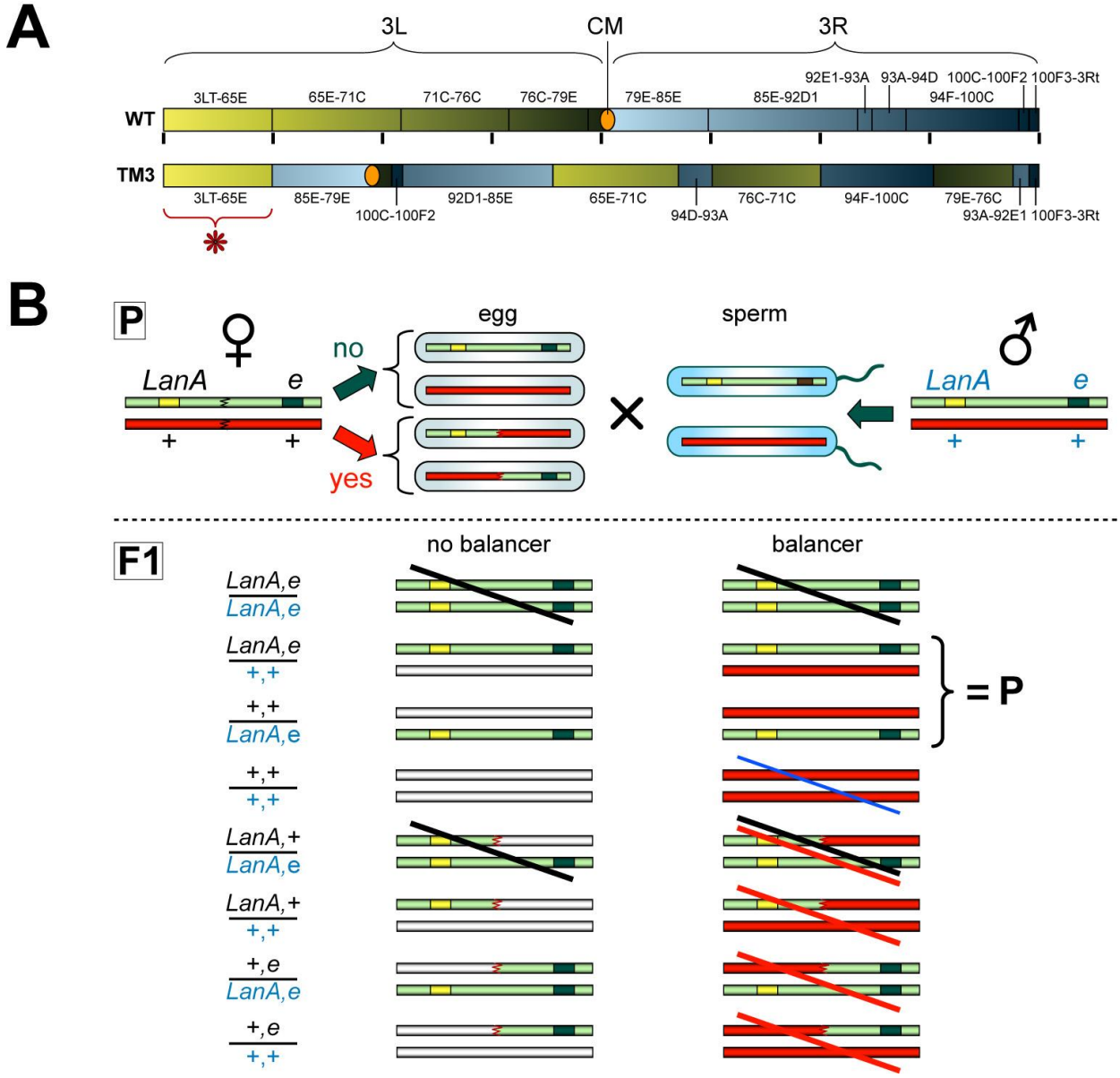


Figure 10. The use of balancers in stock maintenance

A) Chromosome 3 of wildtype (WT; left arm, green gradient; CM, centromere; right arm, turquoise gradient) compared to a TM3 balancer chromosome. Chromosomal rearrangements of TM3 are shown as boxes which correspond to analogous boxes on the wildtype chromosome and display the original colour-code and cytogenetic location in their new orientation (data taken from the Bloomington site: Balancers / Inversion breakpoints present on balancers). The asterisk indicates a non-rearranged region prone to recombination. **B)** A cross of two parents (**P**) heterozygous for *LamininA* (*LanA*; a homozygous embryonic lethal mutation on 3L, 65A8-65A9) and the recessive and viable marker mutation *e* (*ebony*, dark body colour; 3R, 93C7-93D1). Both mutations are on the 3rd chromosome and kept over a balancer. The mutant chromosome is shown in light green, the balancer chromosome in red, paternal alleles in blue, maternal in black. The first filial generation (**F1**) is shown on the right. It is compared to a parallel cross (left) where the balancer was replaced by a wildtype chromosome (white). In the parallel cross, only the two combinations containing *LanA* in homozygosis are lethal (black strikethrough). Out of 6 viable combinations, only two are identical to the parents. In the cross with balancers, also the homozygous balancer constellation is eliminated (blue strikethrough) as well as all combinations involving recombination (red strikethrough). Only the combinations identical to the parental genotype are viable, ideal for stock maintenance.

Box 4. Examples of balancer chromosomes

Numerous balancer stocks are available from *Drosophila* stock centres (e.g. Bloomington / Balancers); typical standard balancers are listed below, most marker mutations are explained in Fig. 9:

- ***FM7a*** (*1st multiply-inverted 7a*) - X chromosome - typical markers: *y, w^a, sn, B^1*
- ***FM7c*** (*1^{st} multiply-marked 7c*) - X chromosome - typical markers: *y*, *sc*, *w*, *oc*, *ptg*, *B^1*
- ***CyO*** (*Curly derivative of Oster*) - 2^{nd} chromosome - typical markers: *Cy* (*Curly*), *dp* (*dumpy*; bumpy notum), *pr* (*purple*; eye colour), *cn^2* (*cinnabar;* eye colour)
- ***SM6a*** (*2nd multiply-inverted 6a*) - 2^{nd} chromosome - typical markers: *al*, *Cy*, *dp*, *cn*, *sp*
- ***TM3*** (*3rd multiply-inverted 3)* - 3^{rd} chromosome - typical markers: *Sb, Ubx^{bx-34e},* (*bithorax*; larger halteres) *e, Ser*
- ***TM6B*** (*3rd multiply-inverted 6B*) - 3^{rd} chromosome - typical markers: *$Antp^{Hu}$, e, Tb* (*Tubby*; physically shortened 3^{rd} instar larvae and pupae)

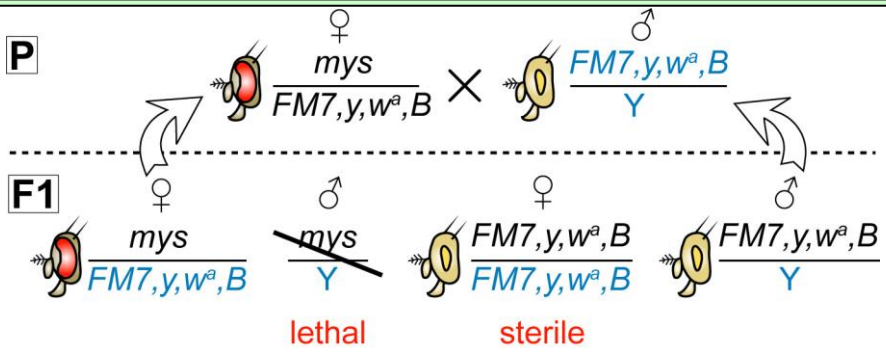


Figure 11. First chromosome balancer, FM7

A stable stock carrying a recessive, homozygous lethal allele of myospheroid (*mys*) balanced over the *FM7* chromosome carrying the following marker mutations: recessive *y* (yellow body colour), recessive *w^a* (bright orange eyes), dominant *Bar^1* (reduced eyes; Fig. 6). In the F1 generation, hemizygous *mys* mutant males die as embryos, females homozygous for FM7 are viable but sterile. Therefore, only the parental genotypes contribute to subsequent generations, thus maintaining the *mys* mutant stock.

- As a further essential property, balancer chromosomes in homozygosis are either **lethal** or they cause **sterility** so that no offspring is produced by these animals (explained in Figs. 10B & 11).
- The third key feature of balancer chromosomes is the **presence of dominant and recessive marker mutations**. Through their dominant marker mutations, balancer chromosomes are easy to follow in mating schemes.

Together these properties are essential for stock maintenance, since they eliminate all genotypes that differ from the parental combination (Fig. 10B). First chromosomal balancers (*FM7, first multiply-inverted 7*) are usually viable in homo- or hemizygosis, but carry recessive mutations such as *sn^{X2}* and *lz^s* that cause **female sterility** in homozygosis. The principal outcome for stock maintenance is the same (Fig. 11). The third key feature of balancer chromosomes is the **presence of dominant and recessive marker mutations**. Through their dominant marker mutations, balancer chromosomes are easy to follow in mating schemes. For example, by making sure that a recessive mutant allele of interest is always kept over dominantly marked balancers, the presence of this allele can be "negatively traced" over the various generations of a mating scheme - especially since recombination with the balancer chromosomes can be excluded. Examples of balancer chromosomes are listed in Box 4. Note that the **4^{th} chromosome** does not require balancers since it does not display recombination.

5. Transgenic flies

5.1. Generating transgenic fly lines

Transgenic flies have become a key resource for *Drosophila* genetics with many important applications (see below). Accordingly, transgenic animals are omnipresent in mating schemes, and it is important to understand their principal nature and some of their applications. The generation of transgenic fly lines is based on the use of **transposable elements/transposons**. Transposable

elements are virus-like DNA fragments that insert into the genome fairly randomly, where they can be maintained in position over many generations, replicate like endogenous genes and follow Mendelian rules of inheritance. There are ~100 types of natural transposons in *Drosophila melanogaster* and thousands of insertions per individual genome [33]. Transposons encode specialised enzymes called **transposases** which catalyse mobilisation of the transposons into other genomic locations, either through excision/re-integration or through replication (Fig.12A). In *Drosophila*, the most frequently used class of transposon is the **P-element** which will be dealt with primarily in this manual. For the purpose of transgenesis, transposons are **modified genetically**. The transposase gene is removed and replaced by the genes the experimenter wants to introduce into the fly genome (e.g. lacZ in Fig. 12B), in addition to marker genes and genes/motifs for the selective cloning of the transposable element in bacteria, as well as further potential features enhancing the use of these constructs (section 5.2).

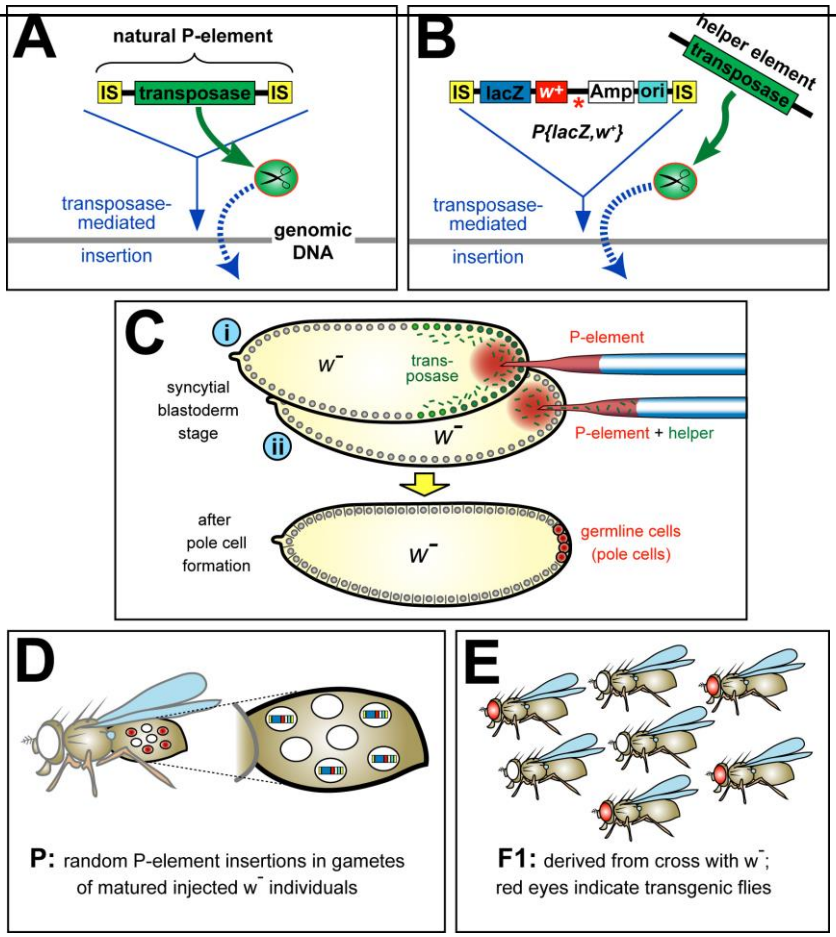


Figure 12. Using P-elements to generate transgenic insertions

A) The insertion of natural P-elements into the genome (grey line) requires two key features: firstly, flanking IS motifs (insertion sequences) mediating stem-loop conformation important for the insertion process (blue arrow); secondly, catalytic transposase activity (scissors and dashed blue arrow), and this enzyme is encoded by the P-element itself. **B)** *P{lacZ,w+}* is a classic example of an engineered P-element used for transgenesis where the transposase gene is replaced by: the *lacZ* from *E. coli* (dark blue box) as reporter gene, a *mini-white* gene (red box) as selection marker in F1 (see F), an antibiotic resistance gene (e.g. ampicillin; white box) and an origin of replication (ori; grey box). Once a fly strain with a stable genomic P-element insertion is established, the exact insertion site can be easily mapped. **C-E)** Making transgenic flies: P-element solution (red) is injected into the posterior pole of early embryos (C); transposase is either (i) expressed in the embryo or (ii) co-injected with the P-element solution in form of helper elements which lack IS motifs (D) and will therefore not insert but disappear during subsequent cell divisions. P-elements become randomly inserted into the genome of posterior pole cells (D) which will differentiate into egg/sperm cells when the injected individuals mature into w^- adults (E). When these adults are crossed to other w^- animals, the transgenic individuals amongst the F1 offspring can be selected by their red eye colour (F), encoded by the *mini-white* gene marker on the inserted P-element (B, D). Note that P-element insertions are still heterozygous in these F1 animals.

To introduce purpose-tailored transposons into the fly genome, they are **injected** into early embryos at the syncytial blastoderm stage. Injection has to take place at the posterior pole where the pole cells will form, which are the precursors of sperm and egg cells (Fig. 12) [6]. If successfully integrated into the genome of some pole cells, the injected transposons will give rise to transgenic offspring. To catalyse genomic insertion of these P-elements, injections are performed in the presence of transposases, through using transgenic fly lines **expressing transposase** in the germline, or co-injecting **helper elements** (Fig. 12C, D). Transgenic transposases are crossed out in the next generation, helper elements don't integrate into the genome and get lost subsequently. Through this disappearance of the enzymatic transposase activity, successful P-element insertions are stabilised and can be maintained as stocks.

Using genetic tricks, existing P-element insertions can be mobilised to produce excisions and transpositions into new chromosomal locations. P-element mobilisation is used for a number of reasons. For example, random P-element insertions into genes can disrupt their functions and provide new mutant alleles for these genes (**P-element mutagenesis**) [30]. In other approaches, reporter genes on P-elements (e.g. *lacZ*, Gal4 or GFP) are used to interrogate the genome for gene expression patterns (**enhancer/gene/protein trap screens**; details in section 5.2.). Mobilisation of mapped P-element insertions can also be used to induce deletions at their insertion sites. For these approaches, countless mapped transposable element insertions are readily available for most gene loci, which are carefully listed in FlyBase and the Berkeley *Drosophila* Genome Project (BDGP) [7].

5.2 Important classes of transgenic lines

There is a great variety of transgenic fly lines (Box 3) and their nomenclature is complex (see FlyBase / Help / Nomenclature). This nomenclature takes into consideration the respective class of transposon, the molecular components it contains including dominant markers, the insertion site and other unique identifiers. Here we use a "light" version of this nomenclature (Figs. 12 and 13), with **P** indicating P-element as the vector, information **between curly brackets** naming the key transgenic components including ***w*$^{+}$** as the dominant marker, and further information **behind brackets** may indicate the gene locus of insertion. Usually further identifiers in superscript are required to unequivocally describe each individual insertion line but will not be considered here. In the following some important classes of transgenic lines will be explained.

a. Enhancer/reporter construct lines (Fig. 13 A): Enhancers are gene regulatory elements which induce/facilitate the transcriptional activation at gene promoters, in some cases acting over distances of several kilo bases. Usually enhancers act on the promoters of endogenous genes in their region, but they can also activate the promoters on transgenic constructs. Therefore, to identify and characterise enhancers in non-coding regulatory regions of genes, genomic fragments containing these regions can be cloned in front of a P-element promoter (which alone is too weak to initiate gene expression) fused to a reporter gene (e.g. GFP or *lacZ*/ß-Gal from *E. coli*). Transgenic animals carrying these constructs can then be analysed for the spatiotemporal expression pattern of the reporter gene as a readout for enhancer activity, and also be used as genetic tools to label certain tissues for experimental purposes.

b. Enhancer trap lines (Fig. 13 B): The P-element promoter alone is too weak to initiate gene expression of fused reporter genes. Therefore, transposable elements carrying such a P-element promoter fusion construct will display reporter gene expression only if inserted in a genomic site which lies within the activity range of endogenous enhancers. By generating many random insertions of such P-elements, the genome can therefore be screened for enhancers which are active in specific tissues at certain stages. Such activity often indicates the presence of genes which are expressed and therefore potentially relevant in these tissues. This procedure is referred to as an **enhancer trap screen** [8]. Since P-element insertions frequently affect the function of genes at their insertion site (stippled red T in Fig. 13 B), they can be used for systematic **P-element mutagenesis screens** [30] (see also Fig. 2). Once P-induced insertions have been generated, reporter gene patterns may reveal when and where the gene is active (Fig. 13 B), and efficient cloning strategies can be used to map the insertion and identify the targeted gene.

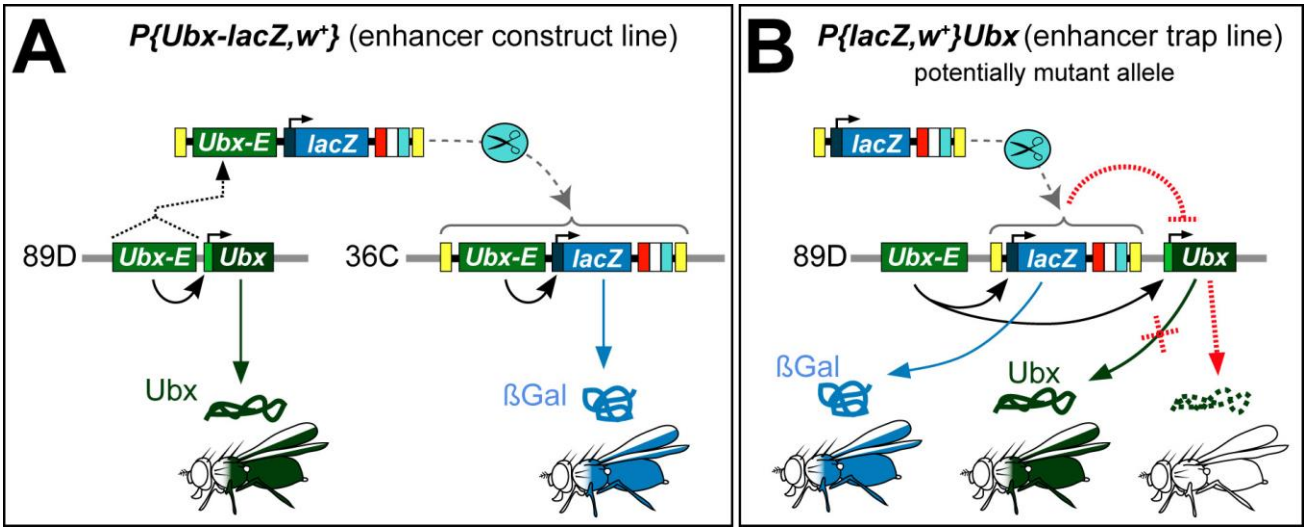


Figure 13. Enhancer trap and enhancer/reporter lines

A) *P{Ubx-lacZ,w+}* illustrating an enhancer/reporter line. An enhancer element that usually activates the promoter of the *Ubx* gene at cytogenetic map position 89D (light green box with right pointing arrow) is cloned (stippled black line) into a P-element, next to a *lacZ* reporter gene with a basal promoter (dark box with right pointing arrow) that alone is insufficient to drive *lacZ* expression. After genomic insertion (scissors; here at cytogenetic map position 36C), *Ubx-E* activates (black arrow) transcription of the basal promoter in a *Ubx*-like pattern translating into a *Ubx*-like ßGal expression pattern in the transgenic flies (blue). **B)** *P{lacZ,w+}Ubx* illustrating an enhancer trap line. A P-element (curly bracket; colour code as in Fig. 12) carrying *lacZ* with a basal promoter is inserted in the *Ubx* gene locus at 89D. The endogenous *Ubx-E* activates expression of the *lacZ* gene on the P-element (blue in fly). Note that the inserted P-element may disrupt (red stippled T) expression or function of the endogenous gene (red stippled X), thus generating a mutant allele (red stippled arrow).

c. Gal4/*UAS* lines: Gal4 is a transcription factor from yeast that activates genes downstream of *UAS* (upstream activating sequence) enhancer elements. Gal4 does not exist endogenously in flies and does not act on any endogenous loci in the fly genome. Very many transgenic ***Gal4* fly lines** have been and are still being generated. To illustrate this point, the simple search term "Gal4" produces almost 6000 hits representing individual fly stocks at the Bloomington Stock Centre. Of these, numerous *Gal4* lines are readily available that display Gal4 expression in different tissues or cells at specific developmental stages (Fig. 14 a, b). By simply crossing Gal4-expressing flies to ***UAS* construct lines** (Fig. 14 c, d) or **enhancer-promoter (EP) lines** [55] (Fig. 14 e), the genes downstream of *UAS* enhancers are being activated. *UAS*-linked genes can be of very different nature including reporters, different isoforms of fly genes (or of other species), optogenetic or physiological tools, small interfering RNAs or cytotoxins (Box 3). Once crossed to a Gal4 line, the offspring will display expression of these *UAS*-coupled genes in the chosen Gal4 pattern. This provides an impressively versatile and powerful system for experimentation, the spatiotemporal pattern of which can be further refined through technical improvements [21,24,47]. A further important feature of the Gal4/UAS system is that its **expression strength can be decreased/increased** by keeping Gal4/UAS individuals at lower (e.g. 18ºC)/higher (e.g. 29ºC) temperatures.

d. *RNAi* lines: Application of RNA interference strategies in flies has become a powerful alternative to the use of mutant alleles. As one key advantage, fly lines carrying *UAS-RNAi* constructs (available for virtually every gene) [22,43] allow the targeted knock-down of specific genes in a reproducible tissue or set of cells, often at distinct stages of development. This approach can therefore overcome problems caused by systemic loss of gene function, such as early lethality (often impeding analyses at postembryonic stages) or complex aberrations of whole tissues that can be difficult to interpret. However, the use of RNAi lines needs to be well controlled. Demonstration of reduced protein or RNA levels of the targeted gene is not sufficient, since phenotypes can still be due to additional **off-target effects** (i.e. knock-down of independent gene functions). Therefore, it is advised to use more than one independent RNAi line targeting different regions of the gene. Other proof of specificity can come from

enhancement of the knock-down phenotype in the presence of one mutant copy of the targeted gene or, *vice versa*, suppression of the knock-down phenotype through co-expression of a rescue construct for the targeted gene (using the degenerate code to protect rescue RNA from knock-down).

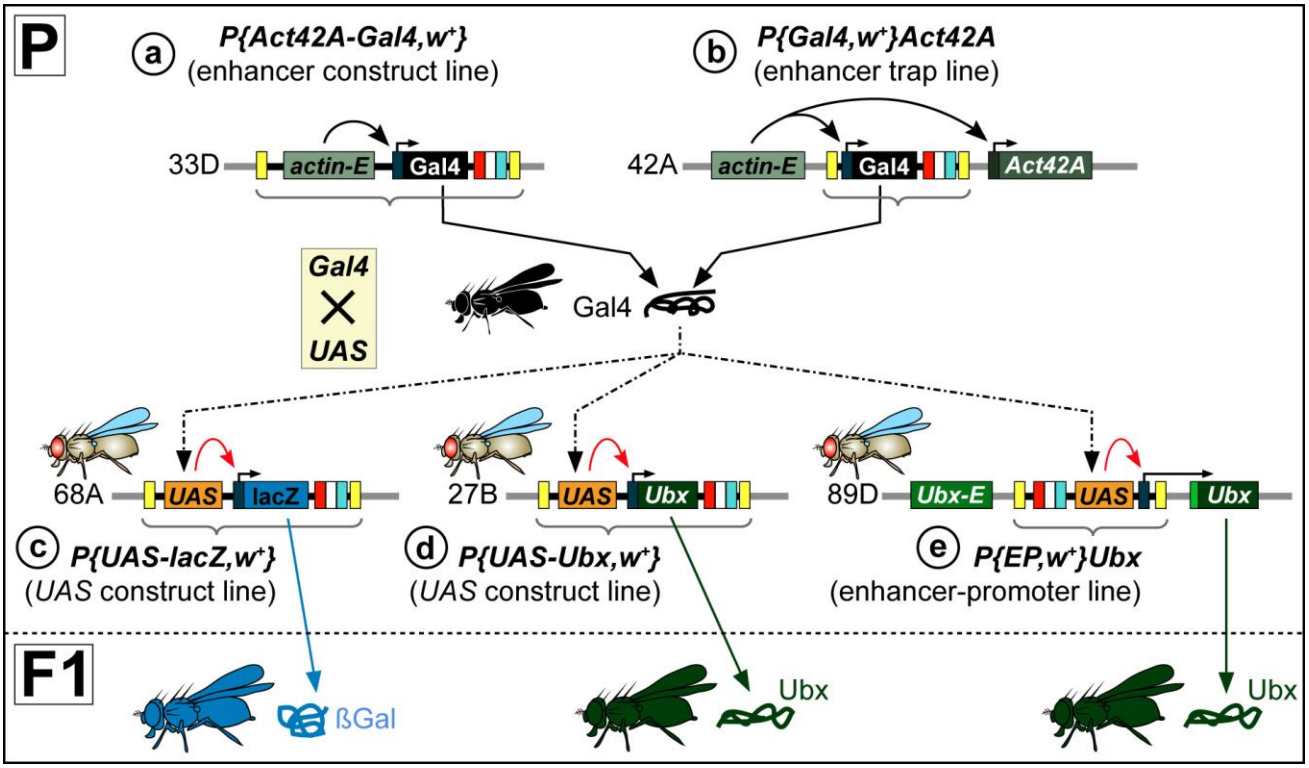


Figure 14. The versatile Gal4/*UAS* system for targeted gene expression

The Gal4/UAS system is a two component system where flies carrying *Gal4*-expressing constructs (**a, b**) are crossed to flies carrying *UAS*-constructs (**c-e**). In animals of the daughter generation (**F1**), Gal4 (black knotted line) binds and activates *UAS* enhancers (dotted-stippled lines), so that the pattern in which Gal4 is expressed (here ubiquitously in the fly) will determine the expression pattern of any genes downstream of the *UAS* enhancer (here ß-Gal or Ubx). The two components can be freely combined providing a versatile system of targeted gene expression. For example, *Gal4*-expressing constructs can be enhancer construct lines (**a**) or enhancer trap lines (**b**). The shown Gal4 lines are analogous to those in Fig. 12 with some modifications: P-elements carry *Gal4* instead of *lacZ*, the enhancer trap line is inserted into the ubiquitously expressed *Act42A* actin gene at cytogenetic map position 42A, and the enhancer element is the *Act42A* enhancer (*actin-E*) which activates expression of Gal4 ubiquitously in the fly (black). Three examples of *UAS* lines are shown: **c**) *P{UAS-lacZ,w+}* carries a *UAS* enhancer in front of the *lacZ* reporter gene; **d**) *P{UAS-Ubx,w+}* carries the *UAS* enhancer in front of the *Ubx* gene; **e**) *P{EP,w+}Ubx* is an enhancer-promoter (EP) line with a random insertion into the *Ubx* locus at 89D (analogous to enhancer trap line in Fig. 12 A); P-elements of EP lines carry an *UAS* enhancer plus basal promoter which, on Gal4 binding, jointly activate genes that lie downstream of their random insertion sites (here the *Ubx* gene).

6. Classical strategies for the mapping of mutant alleles or transgenic constructs

You may encounter situations in which the location of a mutant allele or P-element insertion is not known, for example after having conducted a chemical or X-ray mutagenesis (Fig. 2) or when using a P-element line of unknown origin (unfortunately not a rare experience). To map such mutant alleles, a step-wise strategy can be applied to determine the chromosome, the region on the chromosome and, eventually, the actual gene locus. Nowadays, mapping can often be achieved through molecular strategies, such as **plasmid rescue** (Fig. 12 B), **inverse or splinkerette PCR** [48] or **high-throughput genome sequencing** [10]. However, classical genetic strategies remain important and are briefly summarised here.

a. Determining the chromosome: You hold a viable *P{lacZ,w+}* line in the laboratory that serves as an excellent reporter for your tissue of interest, but it is not known on which chromosome

the P-element is inserted. To determine the chromosome of insertion you can use a simple two-generation cross using a w^- mutant double-balancer stock (Fig. 15).

b. Meiotic mapping: During meiosis, recombination occurs between homologous chromosomes and the frequency of recombination between two loci on the same chromosome provides a measure of their distance apart (section 4.1.4). To make efficient use of this strategy, **multi marker chromosomes** have been generated that carry four or more marker mutations on the same chromosome (Bloomington / Mapping stocks / Meiotic mapping). Each marker provides an independent reference point, and they can be assessed jointly in the same set of crosses, thus informing you about the approximate location of your mutation [11,27]. Note that multi-marker chromosomes can also be used to generate recombinant chromosomes where other strategies might fail. For example, recombining a mutation onto a chromosome that already carries two or more mutations, or making recombinant chromosomes with homozygous viable mutations is made far easier with multi-marker chromosomes.

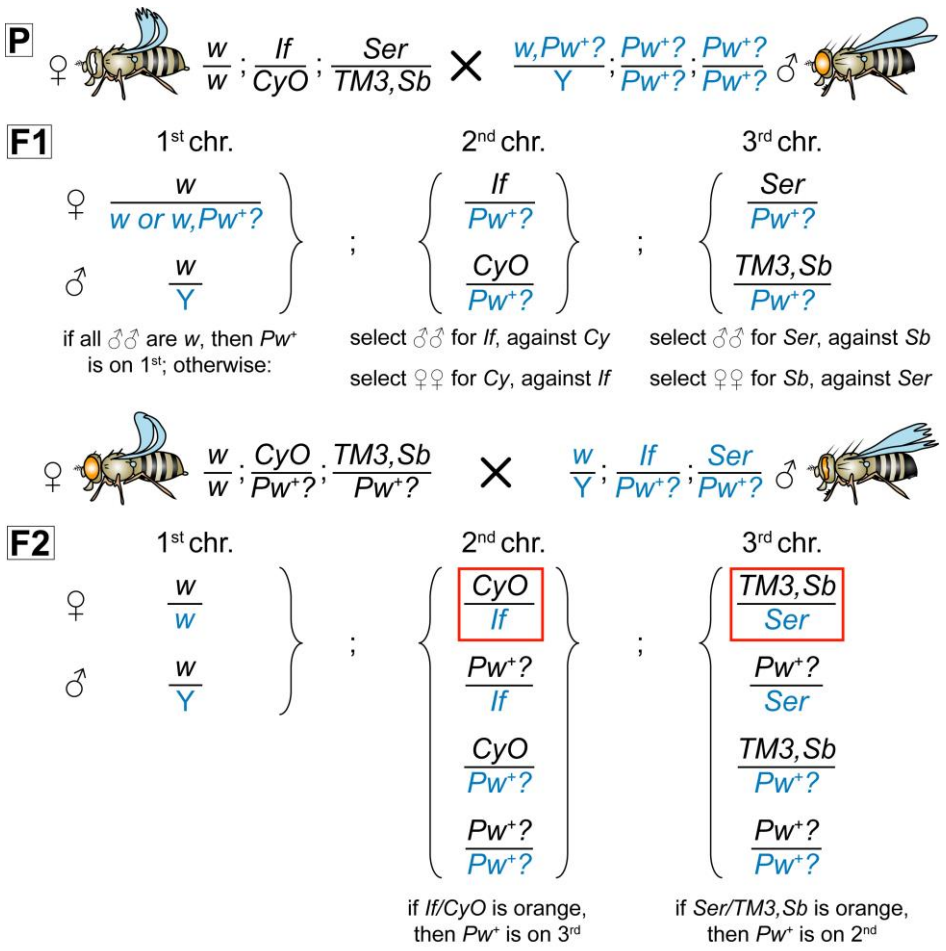


Figure 15. Determining the chromosome of insertion of a P-element

A homozygous viable transgenic fly line carries a *P{lacZ,w+}* insertion on either 1st, 2nd or 3rd chromosome (*Pw+?*). **P**) To determine the chromosome of insertion, males of this line (paternal chromosomes in blue) are crossed to a *white* mutant double-balancer line carrying balancers on both 2nd and 3rd chromosome (note, that the same can be done in two parallel crosses to single balancer stocks carrying balancers on only 2nd and only 3rd; try it out!). **F1**) In the first filial generation potential X chromosome insertions can be determined; if X is excluded, complementary chromosome combinations are selected for a second cross; males can be used for the dominant marker combination (*If* and *Ser*) since recombination is excluded by default in males (section 4.1.4.), whereas recombination in the females is suppressed through using the balancers (*CyO* and *TM3*). **F2**) In the second filial generation, potential 2nd or 3rd chromosomal insertions can be determined (note that helpful stocks for follow-up crosses can be selected at this stage, e.g. *If/CyO;Pw+/Pw+* would facilitate future combinations of the P-element insertion with a mutation on the 2nd chromosome); if *w/w;If/CyO;Ser/TM3,Sb* flies in F2 are still orange, you have a rare event in which your insertion is on the 4th or the Y chromosome.

c. Deletion mapping: Deficiencies are chromosomal aberrations in which genomic regions containing one, few or many genetic loci are deleted. Large collections of balanced deficiencies are available through stock centres (e.g. Bloomington / Deficiencies) and listed in FlyBase. Using improved technology the Bloomington Deficiency Kit now covers 98.4% of the euchromatic genome [20]. These deficiencies provide a rich resource to map genes through classical complementation testing. For this, you cross your mutant to deficiencies of the region determined by meiotic mapping. If your mutation crossed to the deficiency displays its known phenotype (e.g. lethality) you can infer that the gene of interest is uncovered by this deficiency (**hemizygous constellation**). Note that, when dealing with lethal mutations, only 25% of your offspring are expected to carry the phenotype, so you look for presence/absence of balancer-free animals in F1 (Fig. 6). Absence of the phenotype excludes the group of genes uncovered by the deficiency. By using various deficiencies in the area, the mapping of the gene can be further refined (Fig. 16).

d. Complementation tests with known loss-of-function mutant alleles: Once the location of your gene has been narrowed down by deletion mapping, you can cross your mutation to available loss-of-function mutations for the genes in this area, basically following the same strategy as for deletion mapping. Presence of the phenotype indicates that the mutations are alleles of the same gene (**hetero-allelic constellation**). Absence of the phenotype suggests that these alleles belong to different genes (**trans-heterozygous constellation**).

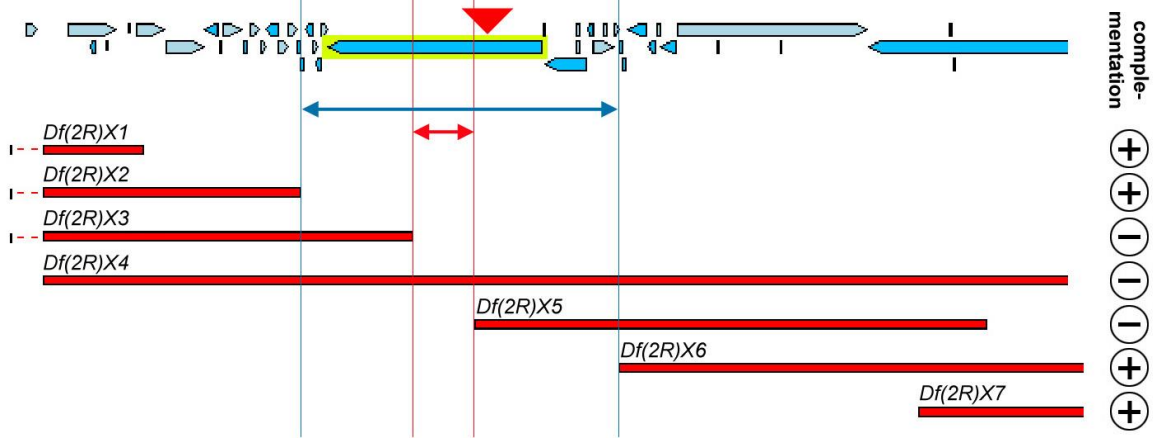


Figure 16. Deletion mapping

A mutation (red triangle) in the yellow highlighted gene locus has been mapped (e.g. through meiotic mapping) to a region of the right arm of chromosome 2 (2R). To refine its mapping, the mutant allele is crossed to deficiencies (Df) that have their breakpoints in this region (red bars indicate the deleted chromosomal region for each deficiency). Closest breakpoints of deficiencies that complement the mutation (+) indicate the region in which the gene is located (blue double-arrow). Closest breakpoints of non-complementing deficiencies (-) may lie within the gene in question and, in this example, clearly identify the mutated gene (red double-arrow).

7. Concluding remarks and next steps (Powerpoint presentation)

You should now have gained the key knowledge and terminology required to design mating schemes for *Drosophila* and to function in a fly laboratory. However, the information given is still basic and requires that you further explore the details behind the various aspects mentioned here. For this, some literature has been provided throughout the text. Should there be mistakes, passages that are hard to understand or information that is missing or wrong, please, be so kind to let me know (Andreas.Prokop@manchester.ac.uk).

To apply and consolidate your knowledge you can now download and study the PowerPoint presentation "*Roote+Prokop-SupplMat-3.ppt*" (shar.es/YcX2f). The presentation briefly reiterates the principal features of meiosis *versus* mitosis and the key rules of fly genetics. You will then be confronted with a standard crossing task, and the presentation will lead you step-by-step through

the solution of this task, illustrating how the rules of *Drosophila* genetics are applied and explaining the various strategic considerations and decisions that have to be taken. Make use of this opportunity to test your knowledge by making your own suggestions first, before being presented with a possible solution. To answer queries, revisit this manual, thus consolidating your knowledge.

8. Acknowledgements

I am most grateful to Casey Bergman, Ian Dworkin, Sanjai Patel and John Roote for comments and feedback on this manual. I would like to thank figshare.com for providing the facilities to upload and manage this document so easily.

Box 5. How to design mating schemes (illustrated in Figs. 6 and 15)

- write 'X' between maternal and paternal genotypes to indicate the crossing step
- genes on the same chromosome may be separated by comma, and also the names of balancer chromosomes may be separated by comma from the list of their markers (e.g. *TM3,Sb,e*)
- genes on homologous/sister chromosomes are separated by a slash or horizontal lines (usually one, sometimes two)
- genes on different chromosomes are separated by a semicolon
- always write chromosomes in their order (1^{st} ; 2^{nd} ; 3^{rd}); to avoid confusion indicate wildtype chromosomes as "+" (e.g. *y/Y ; + ; Sb/+*); note, that the 4^{th} chromosome is mentioned only in the relatively rare occasions that 4^{th} chromosomal loci are involved in the cross
- the first chromosome represents the sex chromosome; always assign a Y chromosome to the male of a cross (see Fig. 6); note that the Y chromosome is sometimes indicated by a horizontal line with a check on its right side (⟝)
- especially as a beginner, stick to a routine order, such as...
  - ...the female genotype is always shown on the left side, male on right
  - ...the maternal chromosomes (inherited from mother) are shown above, paternal chromosomes (grey) below the separating line
- especially as a beginner, always write down all possible combinations resulting from a cross; carefully assign phenotypes to each genotype, define selection criteria and check whether these criteria unequivocally identify the genotype you are after
- to keep this task manageable, use curly brackets for chromosome separation and assess each chromosome individually (Fig. 6). At the end, cross-check whether criteria might clash (for example, a *mini-white* marker on the second chromosome only works as a selection criterion if the first chromosome is homo- or hemizygous for *white*)
- always make sure that you avoid unwanted recombination events by using balancer chromosomes and/or the recombination rules (no crossing-over in males or on the 4th chromosome). If recombination is the task of your cross, make sure you use females during the crossing-over step (usually in F1).
- be aware of fly nomenclature which can be confusing, especially with respect to capitalisation and the indication of whether an allele is recessive, dominant, loss- or gain-of-function (Box 3). Be aware that you understand the nature of the involved alleles, since dominant alleles behave differently to recessive ones in a cross (Fig. 6)
- The nomenclature of transposable elements or chromosomal aberrations can be tedious. To work more efficiently, feel free to use your own unequivocal short hand during the task. For example, "*P{UAS-lacZ,w$^+$}*" and "*P{eve-Gal4,w$^+$}*" could be shortened to "PUw$^+$" and "PGw$^+$".

9. References

1. Ahuja A, Singh RS (2008) Variation and evolution of male sex combs in *Drosophila*: nature of selection response and theories of genetic variation for sexual traits. Genetics 179: 503-509.
2. Allchin D (1997) Thomas Hunt Morgan & the white-eyed mutant. In: Hagen JB, Allchin D, Singer F, editors. Doing Biology (chapter 5): Benjamin Cummings.
3. Ashburner M, Golic KG, Hawley RS (2005) *Drosophila*: a laboratory handbook. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. 1409 p.
4. Ashburner M, Roote J (2007) Culture of *Drosophila*: the laboratory setup. CSH Protoc 2007: pdb ip34.

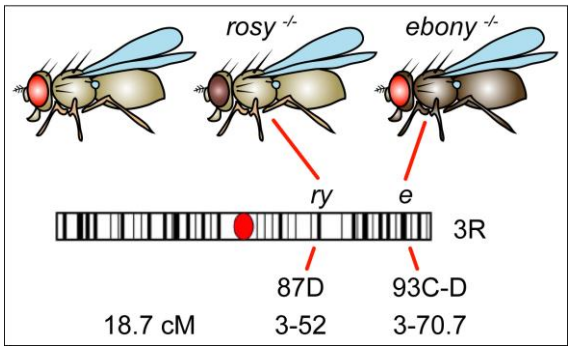
5. Ashburner M, Roote J (2007) Maintenance of a *Drosophila* laboratory: general procedures. CSH Protoc 2007: pdb ip35.
6. Bachmann A, Knust E (2008) The use of P-element transposons to generate transgenic flies. In: Dahmann C, editor. *Drosophila* Methods and Protocols. 2008/07/22 ed: Humana Press. pp. 61-77.
7. Bellen HJ, Levis RW, He Y, Carlson JW, Evans-Holm M, et al. (2011) The *Drosophila* gene disruption project: progress using transposons with distinctive site specificities. Genetics 188: 731-743.
8. Bellen HJ, O'Kane CJ, Wilson C, Grossniklaus U, Pearson RK, et al. (1989) P-element-mediated enhancer detection: a versatile method to study development in Drosophila. Genes Dev 3: 1288-1300.
9. Bellen HJ, Tong C, Tsuda H (2010) 100 years of *Drosophila* research and its impact on vertebrate neuroscience: a history lesson for the future. Nat Rev Neurosci 11: 514-522.
10. Blumenstiel JP, Noll AC, Griffiths JA, Perera AG, Walton KN, et al. (2009) Identification of EMS-induced mutations in *Drosophila melanogaster* by whole-genome sequencing. Genetics 182: 25-32.
11. Bökel C (2008) EMS screens : from mutagenesis to screening and mapping. In: Dahmann C, editor. Drosophila Methods and Protocols. 2008/07/22 ed: Humana Press. pp. 119-138.
12. Bridges CB (1936) D.I.S. work sheet no. 1: external structure of *Drosophila*. *Drosophila* Information Service 6: 76.
13. Brookes M (2001/2002) Fly: The Unsung Hero of Twentieth-Century Science: Ecco/Phoenix.
14. Carrera P, Abrell S, Kerber B, Walldorf U, Preiss A, et al. (1998) A modifier screen in the eye reveals control genes for Krüppel activity in the *Drosophila* embryo. Proc Natl Acad Sci U S A 95: 10779-10784.
15. Caussinus E, Kanca O, Affolter M (2012) Fluorescent fusion protein knockout mediated by anti-GFP nanobody. Nat Struct Mol Biol 19: 117-121.
16. Cherbas L, Gong L (2014) Cell lines. Methods 68: 74-81.
17. Cherbas L, Hackney J, Gong L, Salzer C, Mauser E, et al. (2015) Tools for targeted genome engineering of established *Drosophila* cell lines. Genetics.
18. Childress J, Halder G (2008) Appendix: Phenotypic Markers in *Drosophila*. In: Dahmann C, editor. *Drosophila* Methods and Protocols: Humana Press. pp. 27-44.
19. Chyb S, Gompel N (2013) Atlas of *Drosophila* morphology: wild-type and classical mutants: Academic Press. 200 p.
20. Cook RK, Christensen SJ, Deal JA, Coburn RA, Deal ME, et al. (2012) The generation of chromosomal deletions to provide extensive coverage and subdivision of the *Drosophila melanogaster* genome. Genome Biol 13: R21.
21. Diegelmann S, Bate M, Landgraf M (2008) Gateway cloning vectors for the LexA-based binary expression system in *Drosophila*. Fly (Austin) 2: 236-239.
22. Dietzl G, Chen D, Schnorrer F, Su KC, Barinova Y, et al. (2007) A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. Nature 448: 151-156.
23. Ejsmont R, Hassan B (2014) The little fly that could: wizardry and artistry of *Drosophila* genomics. Genes 5: 385-414.
24. Elliott DA, Brand AH (2008) The GAL4 system: a versatile system for the expression of genes. In: Dahmann C, editor. *Drosophila* Methods and Protocols. 2008/07/22 ed: Humana Press. pp. 79-95.
25. Emerald BS, Cohen SM (2004) Spatial and temporal regulation of the homeotic selector gene *Antennapedia* is required for the establishment of leg identity in *Drosophila*. Dev Biol 267: 462-472.
26. Giacomotto J, Segalat L (2010) High-throughput screening and small animal models, where are we? Br J Pharmacol 160: 204-216.
27. Greenspan RJ (2004) Fly pushing: The theory and practice of *Drosophila* genetics. Cold Spring Harbor New York: Cold Spring Harbor Laboratory Press. 183 p.
28. Hahn I, Ronshaugen M, Sánchez-Soriano N, Prokop A (2015) Functional and genetic analysis of spectraplakins in *Drosophila*. In: Wilson KL, Sonnenberg A, editors. Methods in enzymology (Vol 569): Intermediate filament associated proteins. Oxford, UK: Elsevier. pp. in press. [PDF]
29. Henderson DS (2004) The chromosomes of *Drosophila melanogaster*. Methods Mol Biol 247: 1-43.
30. Hummel T, Klambt C (2008) P-element mutagenesis. In: Dahmann C, editor. *Drosophila* Methods and Protocols. 2008/07/22 ed: Humana Press. pp. 97-117.
31. Inagaki HK, Jung Y, Hoopfer ED, Wong AM, Mishra N, et al. (2013) Optogenetic control of *Drosophila* using a red-shifted channelrhodopsin reveals experience-dependent influences on courtship. Nat Meth advance online publication.
32. Jaiswal M, Sandoval H, Zhang K, Bayat V, Bellen HJ (2012) Probing mechanisms that underlie human neurodegenerative disease in *Drosophila*. Annu Rev Genet 46: 371-396.

33. Kaminker J, Bergman C, Kronmiller B, Carlson J, Svirskas R, et al. (2002) The transposable elements of the *Drosophila melanogaster* euchromatin: a genomics perspective. Genome Biology 3: research0084.0081 - 0084.0020.
34. Keller EF (1996) *Drosophila* embryos as transitional objects: the work of Donald Poulson and Christiane Nusslein-Volhard. Hist Stud Phys Biol Sci 26: 313-346.
35. Kelty CM (2012) This is not an article: Model organism newsletters and the question of `open science'. BioSocieties 7: 140-168.
36. Kohler RE (1994) Lords of the fly. *Drosophila* genetics and the experimental life. Chicago, London: The University of Chicago Press. 321 p.
37. Kojima T, Ishimaru S, Higashijima S, Takayama E, Akimaru H, et al. (1991) Identification of a different-type homeobox gene, BarH1, possibly causing Bar (B) and Om(1D) mutations in *Drosophila*. Proc Natl Acad Sci U S A 88: 4343-4347.
38. Layalle S, Volovitch M, Mugat B, Bonneaud N, Parmentier ML, et al. (2011) Engrailed homeoprotein acts as a signaling molecule in the developing fly. Development 138: 2315-2323.
39. Lindsley DL, Zimm GG (1992) The genome of *Drosophila melanogaster*: Academic Press. 1133 p.
40. Matthews KA, Kaufman TC, Gelbart WM (2005) Research resources for *Drosophila*: the expanding universe. Nat Rev Genet 6: 179-193.
41. Miller DE, Cook KR, Yeganehkazemi N, Smith CB, Cockrell AJ, et al. (2015) Rare recombination events generate sequence diversity among balancer chromosomes in *Drosophila melanogaster*. arXiv: arXiv:1511.04997 [q-bio.GN].
42. Morgan TH (1910) Sex limited inheritance in *Drosophila*. Science 32: 120-122.
43. Ni JQ, Liu LP, Binari R, Hardy R, Shim HS, et al. (2009) A *Drosophila* resource of transgenic RNAi lines for neurogenetics. Genetics 182: 1089-1100.
44. Pastor-Pareja JC, Xu T (2013) Dissecting social cell biology and tumors using *Drosophila* genetics. Annu Rev Genet 47: 51-74.
45. Patel S, Prokop A (2015) How to develop objective-driven comprehensive science outreach initiatives aiming at multiple audiences. bioRxiv 10.1101/023838.
46. Perrimon N (2014) *Drosophila* developmental biology methods. Methods 68: 1.
47. Pfeiffer BD, Ngo T-TB, Hibbard KL, Murphy C, Jenett A, et al. (2010) Refinement of Tools for Targeted Gene Expression in *Drosophila*. Genetics 186: 735-755.
48. Potter CJ, Luo L (2010) Splinkerette PCR for mapping transposable elements in Drosophila. PLoS One 5: e10168.
49. Prokop A, Bray S, Harrison E, Technau GM (1998) Homeotic regulation of segment-specific differences in neuroblast numbers and proliferation in the *Drosophila* central nervous system. Mech Dev 74: 99-110.
50. Prokop A, Küppers-Munther B, Sánchez-Soriano N (2012) Using primary neuron cultures of *Drosophila* to analyse neuronal circuit formation and function. In: Hassan BA, editor. The making and un-making of neuronal circuits in *Drosophila*. New York: Humana Press. pp. 225-247.
51. Rajan A, Perrimon N (2013) Of flies and men: insights on organismal metabolism from fruit flies. BMC Biol 11: 38.
52. Rand MD (2010) Drosophotoxicology: the growing potential for *Drosophila* in neurotoxicology. Neurotoxicol Teratol 32: 74-83.
53. Reiter LT, Potocki L, Chien S, Gribskov M, Bier E (2001) A systematic analysis of human disease-associated gene sequences in *Drosophila melanogaster*. Genome Res 11: 1114-1125.
54. Roote J, Prokop A (2013) How to design a genetic mating scheme: a basic training package for *Drosophila* genetics. G3 (Bethesda) 3: 353-358.
55. Rørth P (1996) A modular misexpression screen in *Drosophila* detecting tissue-specific phenotypes. Proc Natl Acad Sci U S A 93: 12418-12422.
56. Sampson C, Williams M (2012) Protocol for *ex vivo* incubation of *Drosophila* primary post-embryonic haemocytes for real-time analyses. In: Rivero F, editor. Rho GTPases: Springer New York. pp. 359-367.
57. St Johnston D (2002) The art and design of genetic screens: *Drosophila melanogaster*. Nat Rev Genet 3: 176-188.
58. St Johnston D (2013) Using mutants, knockdowns, and transgenesis to investigate gene function in Drosophila. Wiley Interdisciplinary Reviews: Developmental Biology 2: 587-613.
59. St. Pierre SE, Ponting L, Stefancsik R, McQuilton P, Consortium tF (2014) FlyBase 102—advanced approaches to interrogating FlyBase. Nucleic Acids Research 42: D780-D788.

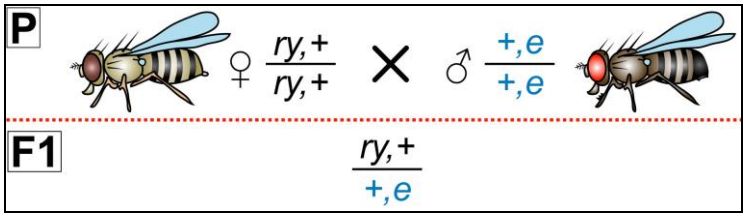
60. Steinbrink S, Boutros M (2008) RNAi screening in cultured Drosophila cells. In: Dahmann C, editor. *Drosophila* Methods and Protocols. 2008/07/22 ed: Humana Press. pp. 139-153.
61. Stocker H, Gallant P (2008) Getting started: an overview on raising and handling *Drosophila*. In: Dahmann C, editor. *Drosophila* Methods and Protocols. 2008/07/22 ed: Humana Press. pp. 27-44.
62. Sturtevant AH (1913) The linear arrangement of six sex-linked factors in *Drosophila*, as shown by their mode of association. J Exp Zool 14: 43-59.
63. Venken KJ, Carlson JW, Schulze KL, Pan H, He Y, et al. (2009) Versatile P[acman] BAC libraries for transgenesis studies in *Drosophila melanogaster*. Nat Methods 6: 431-434.
64. Venken KJ, Popodi E, Holtzman SL, Schulze KL, Park S, et al. (2010) A molecularly defined duplication set for the X chromosome of *Drosophila melanogaster*. Genetics 186: 1111-1125.
65. Wangler MF, Yamamoto S, Bellen HJ (2015) Fruit flies in biomedical research. Genetics 199: 639-653.
66. Weigmann K, Klapper R, Strasser T, Rickert C, Technau G, et al. (2003) FlyMove - a new way to look at development of *Drosophila*. Trends Genet 19: 310-311.

Appendix. A recombination scheme

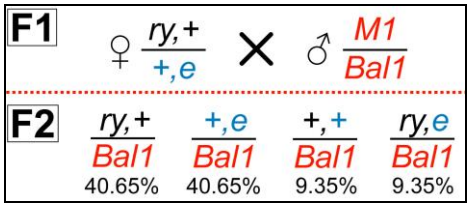
You want to recombine mutant alleles of the viable, recessive, 3rd chromosomal loci *rosy* (*ry*; dark brown eyes) and *ebony* (*e*; black body colour) onto one chromosome. According to FlyBase, *ry* localises to recombination map position 3-52, and *e* to 3-70.7. Hence, they lie 18.7cM apart, indicating that slightly less than 1 in 5 oocytes will carry the desired recombination event.



For this, you start by crossing *ry* females with *e* males or *vice versa* (**P**, parental cross). In the first filial generation (**F1**), all flies are trans-heterozygous (*ry,+/+,e*). Note that the different fly stocks used in this cross will be colour-coded to allow you to easily trace the origin of each chromosome.



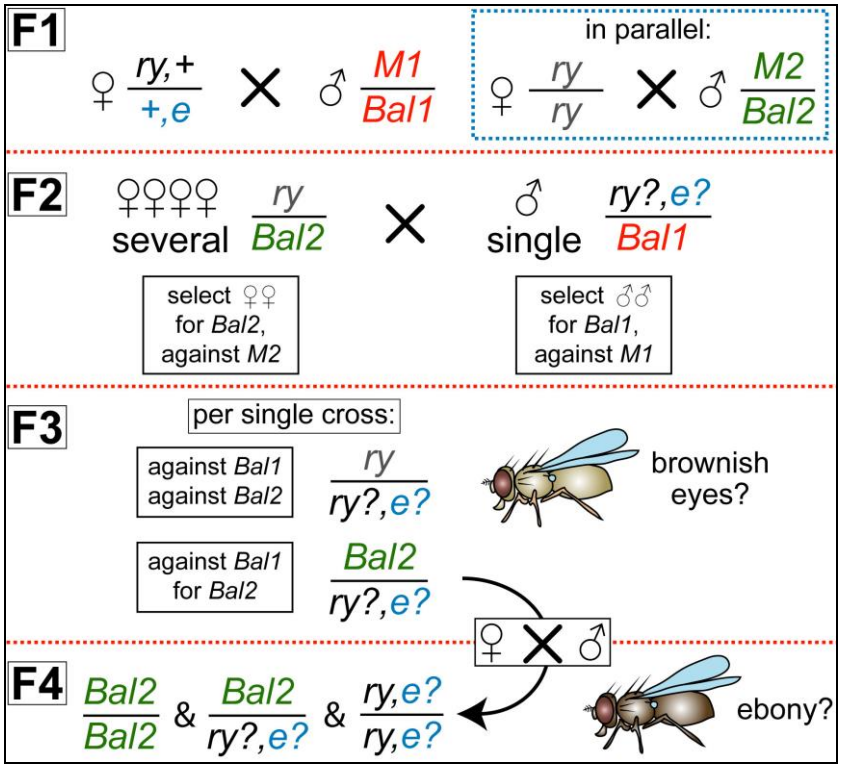
According to the recombination rule, you need to take females so that recombination can occur. Note that crossing-over during oogenesis in these females occurs at random, i.e. their eggs which give rise to the second filial generation (**F2**) represent a cocktail of recombination events with a statistical likelihood of 18.7% as calculated above. Note that only half of the tested animals carry the first marker *ry*, out of which only 18.7% display the wanted recombination. Therefore, 9.35% of the single F2 individuals carry a recombinant chromosome with both markers, and 9.35% a recombinant chromosome with wildtype alleles of both markers. The key task is to **identify and isolate these recombination events through a step-wise process**.



In the first step, recombination events need to be "stabilised" to prevent further recombination. For this, F1 females are crossed to a balancer stock carrying a balancer chromosome (Bal1) over a dominantly marked chromosome (M1; sections 4.2. and 4.3). In the third filial generation (F3), you determine whether one of the markers (here *ry*) is present (remember that, according to the law of segregation, only 50% of balanced F2 individuals carry *ry*). To determine the presence of *ry*, you cross F2 animals back to a *ry* mutant stock. Two important issues need to be considered here.

- Firstly, each individual in F2 is the result of an individual recombination event in its mother's germline. Therefore, **single animals** need to be tested for the presence of *ry*. For practical reasons, use single males since they can fertilise several females and therefore have a higher likelihood to generate enough offspring.
- Secondly, you have to cross back to *ry* mutant flies, but need to be able to distinguish your recombinant chromosome from the *ry* chromosome of the back-cross. For this, cross the *ry* stock to a balancer stock (*Bal2*) that can be distinguished from *Bal1*.

In **F3**, use simple selection to separate out two groups of flies: non-balanced flies allow you to determine whether flies have brownish eyes (i.e. carry *ry* on their potentially recombinant chromosome). If this is the case, flies carrying *Bal2* over the potentially recombinant paternal chromosome (rather than the *ry* chromosome of their mothers) can be used to establish a stable fly stock. The fourth filial generation (**F4**) emerging from these newly established fly stocks will contain non-balanced animals (*ry* and *e* are viable mutations). Stocks in which non-balanced flies have brownish eyes and dark body colour bear the desired recombinant chromosome and will be kept, the rest discarded.



For consideration:

- To have a statistical chance of isolating recombination events, more than 10 single crosses in F2 should be used to match the 9.35% chance of obtaining a recombinant.
- The example of *ry* and *e* represents an unusual case, since they are common marker mutations that are found on several balancer chromosomes (section 4.3.). Using balancers with these markers would allow you to immediately identify the presence of the desired mutations on the potentially recombinant chromosomes. Try it yourself.