A rough guide to *Drosophila* mating schemes (version 2.2)

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*, as an omnipresent follower of human culture, was easy to obtain and maintain in laboratories, and that it was kept in many laboratories as a cheap model for student projects suitable in times of neo-Darwinism (the study of Darwinian evolution with Mendelian genetics) [1]. Several laboratories started using the fly for their main research, but it was the serendipitous discovery of the white mutation and recognition of its linkage to the X chromosome in 1910 by T.H. Morgan which kick-started the systematic use of the fly for genetic research, essentially fuelled by Morgan's graduate students Sturtevant and Bridges [1,7]. 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 [10]. Thus, fly genetics was systematically applied to the study of development, physiology and behaviour, generating new understanding of the principal genetic and molecular mechanisms underpinning biology, many being conserved with higher animals and humans [7,10,11,12,13,14,15]. Notably, it has been estimated that "...about 75% of known human disease genes have a recognisable match in the genome of fruit flies" [19]. Therefore, besides remaining a powerhouse for unravelling concepts and fundamental understanding of basic biology, *Drosophila* is nowadays often used as a "test tube" to screen for genetic components of disease-relevant processes or pathways, or to unravel their cellular and molecular mechanisms, covering a wide range of disease mechanisms including neurodegeneration and even neurotoxicology [20,21,22,23]. It is therefore not surprising that *Drosophila* is the insect behind six Nobel laureates (Box 1).

**Box 1. Nobel prizes for work on Drosophila** (www.nobelprize.org/nobel_prizes/medicine/laureates/)

<table>
<thead>
<tr>
<th>Year</th>
<th>Laureate(s)</th>
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<tbody>
<tr>
<td>1933</td>
<td>Thomas Hunt Morgan - <em>the role played by chromosomes in heredity</em></td>
</tr>
<tr>
<td>1946</td>
<td>Hermann Joseph Muller - <em>the production of mutations by means of X-ray irradiation</em></td>
</tr>
<tr>
<td>1995</td>
<td>Edward B. Lewis, Christiane Nüsslein-Volhard, Eric F. Wieschaus - <em>the genetic control of early embryonic development</em></td>
</tr>
<tr>
<td>2011</td>
<td>Jules A. Hoffmann - <em>the activation of innate immunity</em></td>
</tr>
</tbody>
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*Drosophila*'s enormous success originates from the numerous practical advantages this tiny insect and the community of fly researchers have to offer to the experimenter. The most important advantages are briefly listed below (and tongue-in-cheek here):

1. Updated versions of this document can be downloaded @ dx.doi.org/10.6084/m9.figshare.106631
2. Dan Lindley (2008) *Drosophila* genetics - The first 25 years @ hstalks.com/?t=BL0341788
3. Informative lay descriptions of fly research can be found on the Wellcome Trust Blog:
The portrait of a fly (Part 2) - wellcometrust.wordpress.com/2012/11/23/the-portrait-of-a-fly-part-2-fly-on-the-wall/
• 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. Pedigrees over several generations can be easily planned and carried out in a few months.

Figure 1. The life cycle of Drosophila melanogaster

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. Newly eclosed males require up to 8 hr to mature sexually, facilitating the collection of virgin females (section 3). The times mentioned here need to be doubled when flies are raised at 18°C [3]. Image modified from FlyMove [24].

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 manipulated chemically (e.g. using EMS, ethyl methanesulfonate - highly carcinogenic!), genetically (e.g. through P-element mutagenesis; section 5.1) or with 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 5.2e) 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 pinpointed by the specific RNAi- or EP-line inducing the phenotype, or classical genetic or molecular strategies are used 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) Using the gene’s sequence in data base searches (capitalising on the existing sequences of total genomes) homologous genes in higher vertebrates or humans are identified. Based on knowledge derived from fly research and the empirical assumption that principal mechanisms are often conserved, informed and focussed experiments can be carried out on these genes in vertebrate/mammalian model organisms, or human patients can be screened for mutations in these genes.

• The fly genome is of low redundancy, i.e. only one or very few genes code for members of the same protein class. In contrast, higher organisms usually have several paralogous genes coding for 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 strategies have been developed that can be adapted to the experimenter’s needs [25,26,27,28,29].

• Virtually every gene of *Drosophila* is amenable to targeted manipulations through a wide range of available genetic strategies and tools, ideal to perform reverse genetics (Box 2) [5,9,16,17,18,30,31,32,33,34,35,36].

• Experimental manipulations and observations of cells and tissues are relatively easy. Thus, organs are of low complexity and size, and can often be studied live or via straightforward fixation and staining protocols in the whole organism. These experiments are usually not subject to legal requirements or formal procedures.

• More than a century of fly work has produced a huge body of knowledge and a rich resource of genetic tools. Well organised databases and stock centres provide easy access to both knowledge and genetic tools [38,39]. Furthermore, the highly collaborative spirit of the fly community that has prevailed since the early days of fly research [1], enormously facilitates research through generous exchange of materials and information.

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

Two principal classes of manipulation are usually employed to study gene function. **LOSS-OF-FUNCTION** (LOF) approaches attempt to eliminate gene function partially or completely, for example by employing LOF mutant alleles (section 4.1.2), knock-down of genes using RNA interference strategies (section 5.2.e), the targeted expression of dominant-negative constructs (e.g. catalytically dead versions of enzymes titrating out the function of the endogenous healthy enzyme), or transgenic expression of single-domain antibodies [4]. **GAIN-OF-FUNCTION** (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 targeted over-expression of genes, either of their wild type alleles or of constitutive active versions (section 5), or by using GOF mutant alleles (section 4.1.2).

Gene manipulations are generally employed to serve two principal strategies. **FORWARD GENETICS** is the approach to identify the gene(s) that are responsible for a particular biological process or function in an organism. In *Drosophila* this is usually performed through using unbiased large-scale LOF or GOF screens to identify genes that can disturb the process/function in question (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, LOF or GOF approaches are employed, using mutant alleles or genetic tools that are often readily available or can be generated. The generation of transgenic tools is daily routine in most fly laboratories (section 5.1). Also manipulations of genes *in situ*, i.e. in their chromosomal location, can be achieved through various strategies, such as

- classical mutagenesis strategies to generate candidate alleles that are then selected over suitable deficiencies uncovering the targeted gene locus (section 6c)
- generation of targeted deletions at the gene locus through mobilising local P-elements (section 5.1)
- targeted manipulations of the gene locus through genomic engineering using recombinase-based strategies [9], TALEN strategies (transcription activator-like effector nuclease) [16] or CRISPR (clustered regularly interspaced palindromic repeats) strategies [17,18].

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.

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Box 3. Fly stocks available for Drosophila research comprise...

1. flies carrying classical loss- and gain-of-function mutations or deficiencies (section 4.1.b)
2. flies with chromosomal rearrangements (duplications, inversions, translocations etc.) [2,3]
3. flies with balancer chromosomes (section 4.3)
4. flies with transgenic constructs encoding a range of products (section 4.4.) including:
   - reporter genes, such as lacZ or fluorescent proteins, fused to gene-specific or inducible promoters, or under the control of position-specific activating elements at their chromosomal insertion site
   - wildtype or mutant versions of genes from Drosophila or other organisms
   - exogenous transcription factors (e.g. Gal4, tTA) with known expression patterns to induce targeted expression of a gene of choice
   - small interfering RNAs to knock down gene expression
   - recombinases (e.g. flippase, φC31) or their recombination target sites at specific chromosomal locations (e.g. FRT or attP); they are jointly used for site-directed insertion of transgenes or to generate mosaics of mutant cells in the germline or in somatic tissues
   - genetically encoded toxins (e.g. ricin, tetanus toxin), optogenetic tools (e.g. channel rhodopsin, Ca²⁺ indicators) or other physiological tools (e.g. Kir channels, Shibire²³) for the analysis or experimental manipulation of cells
   - whole chromosomal fragments for rescue, gain-of-function or targeted mutagenesis experiments [5,6]

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. Yet, 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. How to handle flies in the laboratory

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 groups usually store in their laboratories considerable numbers of stocks relevant to their projects (Fig. 3A). In this way stocks are readily available to kick-start practical work on experimental ideas that arise through daily discussion and thought. Other stocks can be ordered from public or commercial stock centres (FlyBase / Resources / Stock Collections) or by sending requests to colleagues all over the world most of whom are willing to freely share fly stocks once published in scientific journals. Note, that new flies coming into the laboratory should be kept in quarantine and observed for a couple of generations in order to exclude diseases or parasites they may carry. Fly stocks are kept in small vials containing larval food and they can easily be transferred to fresh vials for maintenance (Fig. 3B). These vials are usually stored in trays in temperature-controlled rooms or incubators (Fig. 3A). As indicated in Fig. 1, temperature influences the developmental time of flies.

Figure 3. Maintaining and handling flies in the laboratory

A) Large numbers of different fly stocks are stored in trays in temperature controlled rooms or incubators\(^1\) (the trays shown here each hold two copies of 50 stocks). B) Each fly stock is kept in glass or plastic vials containing food, the main ingredients of which are corn flour, glucose, yeast and agar. The vials are closed with foam, cellulose acetate, paper plugs or with cotton wool. Larvae live in the food. When reaching the wandering stage they climb up the walls (white arrow) where they subsequently pupariate (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 aspirator (pooter) (C, E).

Tip 1. Keeping information about laboratory stocks

Work in a fly laboratory involves constant influx of new fly stocks, but only a small percentage of these will eventually be kept in your stock collection. Follow good practice by making it your routine to instantly document the essential information for each incoming stock in a dedicated folder or data sheet/base before it gets lost and forgotten in daily routine:

1. Keep the full original genetic description and any other information you may find on vials or accompanying notes (e.g. stock centre references or any other seemingly meaningless numbers). Note that genetic descriptions you are given by the donor may be incomplete, and your accessory notes may provide unique identifiers for this fly stock when communicating with the donor laboratory.
2. Note down the donor laboratory/person and contact. You will need this information for further enquiries and acknowledgements in future publications.
3. When introducing stocks into your collection, transfer the above information into the accompanying data base/sheet. Make sure there is a proper genetic description, a clearly assigned shorthand for daily use, info on the donor and the key reference publication. This information will be most useful when writing up your project and for people succeeding you in the laboratory.

- **Stock keeping** is usually done at 18°C (generation time of about 1 month). Be aware that you deal with live animals that need to be cared for like pets! 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 a 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) or to reduce humidity (if vials are too moist).
- **Experiments** with flies tend to take place at room temperature or at certain conventional temperatures, such as 25°C for well timed experiments (Fig. 1) or 29°C to speed up development or enhance targeted gene expression with the Gal4/UAS system (section 5.2).

\(^1\) Incubators need to be fly-proof: copper is aggressively corroded in the presence of flies and either needs to be well protected (e.g. coated with resin) or should be avoided and replaced by stainless steel.
To perform crosses, females and males that carry the appropriate genotypes are carefully selected. Some aspects need consideration:

- **Males and females** need to be distinguished using the criteria explained in Figure 4.
- 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 at low temperatures overnight (ideally below 18°C) and harvest virgins first thing in the morning. During the day, they are kept at higher temperatures (to enhance the 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 mating scheme (often from parallel crosses). Genetic markers will be explained in section 4.2., and the rules how to choose them will become clear from later sections.

To select them for gender and phenotypic markers, freshly eclosed flies are tipped from their vial onto a porous pad dispensing CO₂. CO₂ acts as a narcotic and is not harmful if exposure is kept to a few minutes. Flies can be easily inspected on this pad under a dissection microscope (Fig. 3C-E). Selected flies are added to fresh standard vials properly labelled with gender and genotype (Fig. 3B) and kept at standard temperature (room temperature or 25°C). Remaining flies are disposed of in a fly morgue (usually a bottle containing 70% alcohol) and never returned to their vials of origin. Some further considerations are explained in the box "Tip 2".

In general, more female flies are used in a cross than male flies, with two thirds being female as a reasonable approximation (unless males are expected to be of low fitness due to the mutations they carry). Also, if gender choice is an option and one of the stocks/genotypes to be
used is morbid, choose the more vital stock/genotype for virgin collection. In general, consider that di- and trihybrid crosses (see example in Fig. 6) and crosses with mutant combinations that affect viability will have a very low yield of the required offspring and have to be initiated by large volume crosses. Consequently, expect that the volume of flies available for crosses in a complex mating scheme may gradually reduce from generation to generation. Also be aware that certain genotypes may cause flies to eclose later or earlier than others. For example, males carrying the balancer chromosome FM7 in hemizygosis (over a Y chromosome) may eclose days after their female siblings carrying the same balancer in heterozygosis (over an X chromosome; see Fig. 10). Finally, fly strains may be carrying bacterial or viral diseases or they can be infected with fungi or mites [3]. These conditions can pose a threat to the feasibility of mating schemes. The best prophylaxis is careful and regular husbandry of your fly stocks.

Especially in complex mating schemes with complex marker combinations, a safe way of selecting the right animals for your next cross is to merely separate males from females into distinct vials during your daily routine. Only when enough animals have been collected, perform the marker selection in one single session. This mode is safer and less time-consuming, especially for the inexperienced fly pusher or when various crosses are running in parallel and keeping an overview becomes a challenge.

**Tip 2. How to perform counts on experimental crosses**

Certain experiments demand that you quantitatively assess the relative abundance of the various geno-/phenotypes emerging from a cross, for example when carrying out meiotic mapping experiments (section 6b). In another scenario, a mutant allele may cause death in some of the individuals of a relevant genotype but not in all of them (semi- or sub-lethal allele). To determine the degree of lethality (as one possible measure for the strength of your mutant allele), you need to perform geno-/phenotypic counts of homozygous mutant versus heterozygous/balanced animals.

1. To achieve accuracy of results and not bias the outcome, you need to make sure that **vials are not over-populated with larvae of the F1 generation** (i.e. the individuals that will be assessed as adults). Over-population tends to disadvantage morbid individuals, enhancing their lethality above usual proportion.
   a. Make sure you transfer parents to new vials when sufficient eggs have been laid (within a time frame of 1 day to 1 week, depending on fertility of stocks used and number of parental flies).
   b. Monitor crosses for start of eclosure, then score crosses daily, otherwise sick eclosed progeny may get stuck and lost in the wet food.
   c. Weakest animals tend to hatch late, therefore continue scoring for as long as possible. However, be aware that F2 flies start emerging after about 19 days in a modestly populated tube at 25ºC (Fig. 1). When this point is reached cease counting and discard the tube.

2. When scoring a large number of flies, arrange them into a line across the plate and pull out one phenotypic class at a time. In this way you only have 2 piles of flies on the plate at any one time.

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 [2,3].

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. The two copies of a gene are separated during meiosis and only one copy is passed on to each offspring (Fig. 6). Rare exceptions to this in which both copies pass to one gamete are termed non-disjunction events.
4.1.2. Alleles

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 (Pcl+), 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+/+ individuals (Fig. 6) [43], ectopic Antp expression in antennae the antenna-to-leg transformations in AntpT3b/+ (Fig. 9) [44], and Krüppel mis-expression the reduced eyes in Ifl/+ animals (Fig. 9) [45]. 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'/+) 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-AMK1 in heterozygosis are viable and show a weak dominant cell proliferation phenotype, whereas homozygous animals are lethal and show a strong cell proliferation phenotype [8]. 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 (Box 4).

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 [2]. It plays a negligible role in routine fly work and will therefore not be considered here.

1 see also http://en.wikipedia.org/wiki/Muller's_morphs
Figure 6. Independent assortment of alleles & comparison of recessive and dominant inheritance

Two examples of crosses between heterozygous parents (P) involving recessive alleles (top left) and a dominant allele (green box top right) are shown. 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 are identical, yet only the heterozygous \( B \) mutant females show an intermediate eye phenotype.

The left example is a dihybrid cross involving mutant alleles on X and 2\(^{nd} \) chromosomes (separated by semicolons). In the first offspring/filial generation (F1) each chromosome has undergone independent assortment of alleles (demarcated by curly brackets) and each of the four possible outcomes per chromosome can be combined with any of the outcomes of the other two chromosomes resulting in \( 4 \times 4 = 16 \) combinations. In case of two autosomal genes, the phenotypic distribution would be 9:3:3:1 (homogeneously coloured fields in the Punnett square), as compared to 3:1 in a monohybrid cross (only one of 4 animals displays \( vg \) phenotype). However, since \( w \) is X-chromosomal, the phenotypic distribution here is 6:6:2:2 (indicated by hatched fields in Punnett square). The Punnett square lists all possible combinations (symbols explained on the right); red and blue stippled boxes show the same examples of two possible offspring in both the curly bracket scheme and the Punnett square. Note that the Punnett square reflects the numerical outcome of this cross in its full complexity, whereas the curly bracket strategy only qualitatively reflects potential combinations and is easier to interpret for the purpose of mating scheme design (Box 5). The complexity of Punnett squares become even more obvious when dealing with trihybrid crosses (Appendix 2).

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 4\(^{th} \) 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). Therefore, frequencies are 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) [2]. 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 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 and bottom of Box 4).

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 but might relate to the observation that, although reductional divisions occur and haploid gametes are produced, the type of genes expressed in male meiosis "is much more similar to mitosis than to female meiosis" [46].

- 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.
Box 4. Gene descriptors and locators

- *Drosophila* genes have different descriptors: name, symbol, synonyms, the annotation symbol and the FlyBase ID. As an example, go to the FlyBase home page flybase.org. In the "Quick Search" box click on the "Data Type" tab, select "Data Class / genes" and type "shot" into the text field. This will direct you to the gene page where you will find a full description of the gene short stop including various identifiers and locators in the top section and further synonyms in the second last bottom section [e.g. kakapo/kak, groovin/ogr, kopup/kop, (2)CA4]. The naming of genes and chromosomal aberrations follows clear rules (FlyBase / Documents / Nomenclature), and a few are summarised here:

- The names of *Drosophila* genes (and their associated short forms or symbols) reflect the classical (and certainly most human) way to describe a gene or marker mutation. They are most commonly used in daily life and publications and tend to reflect the mutant phenotypes of genes - often in very creative ways (e.g. faint sausage, ether-a-gogo, couch potato). For example, white loss-of-function mutations cause white eyes, indicating that white gene function is normally required for red eye colour. However, not everybody has followed this tradition when naming genes. Furthermore, mutations of genes which were identified as homologues to known mouse or human genes tend to be named after their mammalian relatives. Note that genes encoding products of similar molecular function may be given names/symbol with identical prefixes (usually indicating the protein class) and unique suffixes (usually referring to a gene's cytogenetic location; e.g. Actin-5C, Actin-42A, Actin-57B). For an entertaining radio feature about fly names listen to www.bbc.co.uk/programmes/b00lyfy1.

- As illustrated by the shot example, genes have often been called differently by independent researchers (Synonyms & Secondary IDs), and these names come with their independent symbols. FlyBase usually follows the rule that the first published name for a mutation of a gene (usually not the wildtype locus or protein) becomes official, but a searchable list of all synonymous names is maintained. In any case, FlyBase is your key point of reference and you are advised to use their official naming.

- The annotation symbol (CG number, the Computed Gene identifier) originates from the genome sequencing projects and has only been assigned to genetic loci that have been identified as genes. For example, Cy.Curly is a mutation of unknown molecular nature and has therefore no CG number. CG numbers are primarily used if no other name has been given yet.

- The FlyBase ID (FBgn = FlyBase gene) is the only unique identifier available for both annotated genes and non-annotated marker mutations. It is often the prime reference during database searches.

- As a general convention, genes/symbols that were FIRST named after recessive mutant alleles (section 4.1.2) start with lower case, those FIRST identified by dominant alleles are capitalised. For example, abd-A is lower case due to its original classification as a recessive gene, although subsequent analyses have revealed dominant loss-of-function mutant phenotypes [8]. Capitalisation can be confusing, since identical symbols starting with either upper or lower case represent different gene or marker names (e.g. syn/synrome versus Syn/Synapsin). Furthermore, genes named after vertebrate homologues are capitalised, regardless of whether their mutant alleles are dominant or recessive (e.g. Nrx-IV/Neurexin IV or Syn/Synapsin).

- Be aware that short hand for mutant alleles in daily use can differ. For example, "w+;+" or "w" or "w" or "w/w" all mean the same thing, i.e. a white mutant fly. Whereas the first two versions do not discriminate gender, the fourth option clearly indicates a female.

- Note that genes and their mutant alleles are usually italicised, whereas proteins are written in plain and often capitalised (the shot gene, the shotp200 mutant allele, the Shot protein).

- The genomic location of a gene is given in up to 4 ways: the chromosome (arm), cytogenetic map position (both Fig. 5), the sequence location within the fully sequenced *Drosophila* genome and, for marker mutations, also the recombination map position (e.g. the shot gene is on chromosome arm 2R, in cytogenetic map position 50C6-50C9, in sequence location 2R:9,751,742..9,829,615 corresponding to the recombination map position 2-68). Use the "Map Conversion Table" (importable in Excel) for determining recombination map positions of other genes (section 4.1.4).

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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 [47].
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 specific ways [49]. On the one hand these mutations can be used to study biological processes underlying body patterning and development (by addressing what the mutant phenotypes reveals 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 10. The use of balancers in stock maintenance
A cross of two parents (P) heterozygous for the homozygous embryonic lethal mutation lamininA (lanA; 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 orange, the balancer chromosome in beige, parental 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.

4.3. Balancer chromosomes
Balancer chromosomes are essential for the maintenance of mutant fly stocks as well as for mating scheme design [3]. Balancer chromosomes carry multiple inversions through which the relative positions of genes have been significantly rearranged. 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 Bloomington site and shown as pictograms in THE ATLAS [48], nicely illustrating the weak spots where balancers are prone to recombination. In addition, most balancer chromosomes are lethal in homozygosis. Together these properties are essential for stock maintenance, since they eliminate all genotypes that differ from the parental combination (Fig. 10). First chromosomal balancers (FM7, first multiply-inverted 7) are usually viable in homo- or hemizygosis, but carry recessive mutations such as snr and lc that cause female sterility in homozygosis. The positive effect 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. The following balancer chromosomes are commonly used (for mentioned markers refer to Fig. 9; also see Tip 3):

- **FM7a** (1st multiply-inverted 7a) - X chromosome
typical markers: y, w\(^a\), sn, B\(^I\)
- **FM7c** (1st multiply-marked 7c) – X chromosome
typical markers: y, sc, w, oc, ptg, B\(^I\)
- **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
frequent markers: Antp\(^{Hu}\), e, Tb (Tubby; physically shortened 3\(^{rd}\) instar larvae and pupae)

Note that the 4\(^{th}\) chromosome does not require balancers since it does not display recombination. Instead the \(ci^D\) mutant allele is used to maintain stocks with lethal/sterile mutations of genes on the 4\(^{th}\) chromosome; \(ci^D\) is a recessive lethal, dominant marker mutation caused by a chromosome rearrangement that led to a fusion protein encoded by the *cubitus interruptus* and pan genes.

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 Bar1 (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.

5. Transgenic flies

5.1. Generating transgenic fly lines

Transgenic flies have become a hub of *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 where they are replicated like endogenous genes and can therefore be maintained in that position over many generations. Natural transposons encode specialised enzymes called transposases. Transposases 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 mainly dealt with in this manual. For the purpose of transgenesis, transposons are modified genetically. The transposase gene is removed and replaced by those genes the
experimenter wants to introduce into the fly genome. Furthermore, they contain marker genes and genes/motifs for the selective cloning of the P-element in bacteria (Fig. 12B).

To introduce purpose-tailored transposons into the fly genome, they are injected into the posterior pole of early embryos where they are incorporated into newly forming pole cells (Fig. 12) [50]. Pole cells are the precursors of sperm and egg cells that will then give rise to a certain percentage of transgenic offspring. To catalyse the insertion of these P-elements in the pole cell genome, transposase-encoding helper elements are co-injected with them (or transgenic fly lines are used that display targeted expression of transposase specifically in the germline). Helper elements can themselves not insert/replicate and will gradually disappear when pole cells and their progeny proliferate (Fig. 12D). Through this disappearance of the enzymatic activity, successful P-element insertions are stabilised and can be maintained as stocks. Generating transgenic fly lines through transposon/helper element injection requires technical expertise and specialised equipment such as micromanipulators and glass needle pullers. It is often considered more economical to outsource this task to specialised companies (of which there are a number existing worldwide), instead of establishing and maintaining this capacity in individual laboratories.

Existing P-element insertions can be mobilised to produce excisions and transpositions into new chromosomal locations. For this, a stock carrying the stable transposase-encoding insertion P(\(\Delta\)2-3) is crossed with P-element-carrying flies to induce transposition. In the next generation, P(\(\Delta\)2-3) is crossed out again to stabilise any newly generated P-element insertions [28]. 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) [28]. 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. This can occur through a process called imprecise excision where the P-element may remove genetic material either side of the insertion site. Deletions can also be generated through homologous recombination, a strategy that removes the genomic sequence between two adjacent P-element insertions [32]. For these latter approaches, several transposable element insertions for most gene loci are readily available, which are carefully listed in FlyBase and the Berkeley Drosophila Genome Project (BDGP) [51].

### Tip 3. Special balancer chromosomes

Numerous balancer stocks with interesting features are available from Drosophila stock centres (e.g. Bloomington / Balancers). A few are listed here:

- **extra features** on balancers can make your life easier:
  - most 1st and 2nd chromosomal balancers carry the same dominant marker (B and Cy, respectively); additional dominant markers, such as Star/S* on CyO or Lobe/L* on SM1, can be helpful to distinguish paternal and maternal balancers, e.g. in back-crosses
  - balancers may carry I(2)DTS, a temperature-sensitive cell-lethal locus; by elevating the temperature during development, the individuals carrying this balancer are automatically eliminated, thus enriching for animals homozygous for the non-balancer chromosome
  - green/blue balancers carry constructs expressing GFP or βGal, ideal to select against balanced animals also in embryos, larvae or pupae - live or in fixed/stained preparations

- **multiple-balancer stocks** carry balancers on more than one chromosome, ideal to cross together and keep mutations/markers on different chromosomes (see also Fig. 15)

- **translocation balancer stocks** also carry two balancers, but these act as one balancer across different chromosomes; large fragments have been exchanged between these balancers [e.g. T(2;3)CyO-TM3] causing lethality in animals that do not inherit both of them

- **compound-X or attached-X chromosomes** [e.g. C(1)DX] are not true balancers but can be used in similar ways; they consist of two X chromosomes fused together so that they do not segregate during meiosis and are jointly passed on to one gamete. Females are C(1)DX/Y because they inherit the attached X from their mothers and a Y from their fathers; males are XY, i.e. only males pass on the non-attached X chromosome, ideal for maintaining dominant female sterile mutations; C(1)DXIX females are lethal or sterile.
Figure 12. Using P-elements to generate and map 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 activity of transposase (scissors and dashed blue arrow), an enzyme 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 gene of E. coli (dark blue box), a mini-white gene as selection marker (see F; red box), an antibiotic resistance gene (e.g. to 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 mapped: genomic DNA from these flies is extracted and then digested using defined restriction sites in the P-element (e.g. EcoR1; red asterisk) and random sites for the same enzyme (light blue box) in the nearby genome (grey letters); the obtained restriction fragment contains the C-terminal part of the P-element permitting selective cloning along with the adjacent genomic sequence; the obtained gene sequence can be blasted against the fly genome to map its precise position (box with blue letters) and deduce the cytogenetic map position (box with green letters; see Box 3). C-F) Making transgenic flies: a mix of P-elements and helper element (red) is injected into the posterior pole of early embryos, where they become incorporated into the genome of pole cells (C), the precursors of the gametes in the adult; the helper elements encode transposase which catalyses the insertion of all genetic material flanked by IS motifs (D), but they lack IS motifs themselves, i.e. they fail to insert and replicate but are diluted out during subsequent cell divisions; injected individuals mature into w' adults that carry random P-element insertions in their gametes (E); after a cross to a w' stock, only transgenic offspring display red eyes (due to the mini-white gene on the P-element) and can be selected (F).
A number of problems with P-elements have been identified and led to improved strategies. For example, P-elements have size limitations for the DNA inserts they can successfully insert into the fly genome. Fragment sizes can be significantly increased through the use of BAC (Bacterial Artificial Chromosome) technology, which allows whole genomic loci of greater than 100 kb to be used for transgenesis [5,52]. Another problem is the so called position effect, which results in identical P-element constructs having different levels of expression according to their genomic insertion sites. Such position effects are due to the fact that each genomic locus displays its individual transcriptional base-level and degree of chromosomal condensation. This problem can be circumvented by using site-directed integration of transposons into known genomic positions. For example, ФС31 integrase (as an alternative to the P-transposase) promotes recombination between attP and attB motifs. Consequently, when attB-bearing transposons are injected into ФС31-expressing fly strains carrying attP sites at defined genomic locations, a high percentage of transposons will insert only at the defined attP site [53]. ФС31-mediated recombination can also be used to engineer genes or genomic regions within their natural chromosomal location (genomic engineering) [9]. Finally, P-elements display a pronounced non-random insertion spectrum (insertion hot & cold spots), meaning that certain classes of transposons are biased to insert in certain regions of the genome avoiding others, or show preferential insertion in 5' regulatory rather than coding regions of genes. This can be advantageous in some cases, but primarily poses a problem in particular for genome-wide genetic screens (Fig. 2). To circumvent this problem and complement existing P-element collections, a number of alternative vectors with different or less pronounced preferences are available, such as the lepidopteran piggyBac or the Minos transposon [28,51].

5.2 Important classes of P-element lines

There is a great variety of transgenic fly lines (Box 3) and their nomenclature is complex (see FlyBase / Documents / 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.

- **Enhancer/reporter construct lines (Fig. 13 A):** In order to study regulatory non-coding regions of genes, genomic fragments containing these regions can be cloned in front of a reporter gene (e.g. lacZ from E. coli) fused to a P-element promoter which alone does not initiate gene expression. Regulatory regions of genes contain enhancers, regulatory activators of gene transcription which may act over distances of several kilo bases to facilitate transcriptional initiation at gene promoters. Usually they act on promoters of endogenous genes in their region, but also on P-element promoter of transgenic constructs. Transgenic fly strains with these constructs can be used to analyse the spatiotemporal expression pattern of βGal (the lacZ product), thus revealing tissue- or stage-specific enhancers regulating the transcription of specific genes. Once lines with unique expression patterns have been generated, they may as well be used as powerful genetic tools. For example, enhancer/reporter construct lines carrying target sequences for a certain transcription factor may represent excellent reporters reflecting the activity status of that specific transcription factor under experimental conditions.

- **Enhancer trap lines (Fig. 13 B):** If a P-element carrying lacZ behind a P-element promoter is inserted within the activity range of endogenous enhancers, lacZ expression can be induced by these enhancers, often reflecting (aspects of) neighbouring genes' expression patterns. This strategy has been used to systematically search for genes which are expressed (and therefore potentially relevant) in specific tissues. This procedure is referred to as an enhancer trap screen [54]. Since P-element insertions frequently affect the function of genes at their

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1 P[acman] clones of genes or genomic regions can be found at pacmanfly.org and are distributed at bapac.chori.org
insertion site (stippled red T in Fig. 13 B), they can be used for systematic P-element mutagenesis screens (see Fig. 2) [28]. Once P-induced insertions have been generated, lacZ staining 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 (Fig. 12 B). Transposon-based screens have been carried out with various technical modifications. For example, protein trap screens select for insertions of specifically engineered transposons into introns of genes (within or next to their coding regions). These transposons carry sequences coding for protein tags (e.g. GFP) flanked by splice acceptor and donor sites. During the natural splicing of the host gene, this tag sequence gets incorporated into the splice product, thus fusing the tag to the endogenous protein. Many protein trap lines are listed in FlyBase displaying fluorescent versions of endogenous proteins, allowing their natural expression and localisation patterns to be studied [36,55].

Figure 13. Enhancer trap and enhancer/reporter lines

A) \( P\{Ubx\text{-}lacZ,w'\} \) (enhancer construct line)

B) \( P\{lacZ,w'\}Ubx \) (enhancer trap line) potentially mutant allele

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 alone. 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 [56] (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 such as the use of Gal80 (a Gal4 repressor), dual binary systems or Split Gal4 [31].

**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 are crossed to flies carrying UAS-constructs (inset). 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 ßGal 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: these 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) activating 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).

**d. FRT lines**: FRT (FLP recognition target) sites are specifically targeted by the yeast FLP recombinase (“flippase”). The FLP/FRT system is widely used in Drosophila as an inducible recombination system that has mostly replaced former X-ray based strategies [33,57]. It is used to excise genetic material (to activate/inactivate genes or markers) or to cause somatic recombination between homologous chromosomes, an event that would normally only occur during meiosis (Fig. 7). Somatic recombination requires specific insertions of FRT-bearing P-elements close to the centromere of both homologous chromosomes. At these FRT sites, FLP will catalyse breakage and exchange of the homologous chromosome arms which can distribute into different cells in subsequent cell divisions. When starting from heterozygous individuals, this method can produce mosaic tissues with homozygous clones of cells surrounded by heterozygous cells [33]. Somatic recombination is used for MARCM (Mosaic Analysis with a Repressible Cell Marker) analysis studying the behaviour of single mutant cells or cell groups in normal or mutant tissue [58] (Fig. 15). Another important application is the generation of germline clones in female gonads (Fig. 16). Germline clones are an efficient strategy to generate maternally mutant embryos, i.e. to circumvent the problem of
maternal product [59]. Thus, mothers heterozygous for a homozygous lethal/sterile mutation may deposit maternal product, consisting in mRNAs and/or proteins of their healthy gene copy, in oocytes. Perdurance of his maternal product into embryonic or even postembryonic stages may mask mutant phenotypes of homozygous mutant (zygotic mutant) offspring, posing a problem for mutant analyses. Through using the antimorphic, dominant female sterile ovo<sup>D1</sup> or ovo<sup>D2</sup> alleles, germline clone analyses positively select for successful recombination events and have become highly efficient [59].

**Figure 15.** Clonal analysis using MARCM:

A) MARCM scheme: Activation of UAS-GFP through a tissue-specific Gal4 driver (blue arrow) is suppressed (grey T) by the Gal4 inhibitor Gal80 present in heterozygosis on a chromosome with an FRT site; the homologous chromosome carries a mutation of interest and an equivalent FRT site; activation of Flippase (Flp) causes somatic recombination at these FRT sites (red arrows); in a subsequent mitosis and cell division the mutant allele may assort into one common daughter cell and become homozygous (m/m) creating a parallel twin clone (+/+) that carries both the wildtype allele and Gal80 in homozygosis. Subsequent divisions multiply cells with these genotypes embedded in heterozygous tissue.

B) C) D) Illustration for the use of MARCM in research: B) Image of a normal wing imaginal disc at the late larval stage displaying blue marker gene expression along the antero-posterior compartment boundary.

C) In larvae carrying a particular mutation in homozygosis (m/m), wing discs express the marker gene throughout and are under-grown and aberrant to a degree that no sensible conclusions can be made about the gene's function. D) Small MARCM clones do not disturb the overall morphology of the wing disc and allow the study of mutant cells unequivocally identifiable by their GFP-expression (green outline). In this example, m/m mutant cells away from the compartment boundary display ectopic expression of the blue marker gene, suggesting that the wildtype function of the gene behind m is to negatively regulate expression of the marker gene.

**e. 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) [34] allow the targeted knock-down of specific genes in a reproducible tissue or set of cells, often at distinct stages of development. Like analyses using mutant clones (section 5.2d), 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...
A targeted gene (ideally carrying a mutation that does not affect its function but makes it immune to the knock-down construct).

![Figure 16. Maternal gene product and germline clones](image)

**A)** Many genes are expressed in the female germline and gene product in the form of mRNA or protein (blue) is deposited in oocytes but not in sperm (white), often perduring into early embryonic stages or even larval stages and beyond (depending on the half life of the particular mRNA and/or protein). **B)** If mutant alleles of such genes are homozygous lethal, heterozygous parents \((m/+)^*\) have to be used in crosses to generate homozygous mutant F1 individuals \((m/m)^*\); these mutant individuals display maternal gene product (derived from their mother's wildtype copy of the gene) that may mask mutant phenotypes especially at earlier stages of development. **C)** Heterozygosis for \(ovo^d\) causes cell-autonomous elimination of female germline cells; through FRT-mediated recombination in \(m/ovo^d\) mothers, homozygous oocytes can be generated that are no longer eliminated and are the only eggs being laid by these females [59]. Homozygous mutant embryos developing from these eggs lack both maternal function and zygotic function, whereas heterozygous embryos display zygotic expression of the wildtype allele starting earliest at \(-3.5\) hr after fertilisation at \(25^\circ C\) (embryonic stage 8) [60].

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 by molecular strategies, such as plasmid rescue (Fig. 12 B), inverse or splinkerette PCR [61] or high-throughput genome sequencing [62]. 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. 17).

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 [2,27]. 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.
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 [63]. 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. 18).

![Figure 17. Determining the chromosome of insertion of a P-element](image)

A homozygous viable transgenic fly 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; make sure that males are used for the dominant marker combination (If and Ser) to prevent unwanted recombination (section 4.1.4.), whereas recombination in the females is excluded by 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+/w 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.
Figure 18. Deletion mapping

A mutation (red triangle) in the yellow highlighted gene locus is roughly mapped 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).

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).

However, be aware that the nature of a gene may be complex and lead to false interpretations of your complementation analysis:

- Genes may display transvection, a phenomenon where different homozygous mutant alleles affecting different areas of the same gene may complement each other [64].
- Genes can be nested, i.e. complete genes can be lying within introns of another gene, or they may map to the complementary strand of DNA at the same locus.
- Coding regions of genes may be separate, but they may share the same enhancers.
- Finally, non-coding RNAs are encoded by independent loci that may often be considered to represent genes themselves. These loci have important gene regulatory functions and can complicate the analyses of other genes in their vicinity.

To circumvent some of these problems, other strategies are available. For example, collections of UAS-RNAi fly lines (section 5.2e) can be used to systematically knock down the functions of genes in the area of interest. This strategy only works if your mutation has phenotypes characteristic enough to be unequivocally identifiable upon gene knock-down. Furthermore, important clarification can often be obtained from the detailed transcriptional profiles displayed for every gene on FlyBase (at the bottom of the "Expression/Regulation" view in GBrowse).

7. Concluding remarks

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

1 nice example: [http://biobabel.wordpress.com/2012/05/30/a-dual-purpose-ra-and-hox-regulation/](http://biobabel.wordpress.com/2012/05/30/a-dual-purpose-ra-and-hox-regulation/)
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).

Box 5. How to design mating schemes (illustrated in Figs. 6 and 17)

- 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 (1st; 2nd; 3rd); to avoid confusion indicate wildtype chromosomes as "+" (e.g. y/Y; +; Sb/+); note, that the 4th chromosome is mentioned only in the relatively rare occasions that 4th 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


Appendix 1. A recombination scheme

You want to recombine mutant alleles of the viable, recessive, 3rd chromosomal loci r osy (ry; dark brown eyes) and e bony (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,+/+). 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
**Appendix 2. A trihybrid cross**

Example of a trihybrid cross between heterozygous parents (P, top) involving recessive alleles on X, 2nd and 3rd chromosomes (separated by semicolons). 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), \( e \) (ebony; dark body colour); phenotypes are indicated by fly diagrams (compare Fig. 9). In the first offspring/filial generation (F1) each chromosome has undergone independent assortment of alleles (demarcated by curly brackets) and each of the four possible outcomes per chromosome can be combined with any of the outcomes of the other two chromosomes resulting in \( 4 \times 4 \times 4 = 64 \) combinations. The Punnett square at the bottom systematically lists all possible combinations (different phenotype classes are colour-coded and display a 18:18:6:6:6:6:2:2 distribution; symbols are explained at the bottom). Red and blue stippled boxes show the same examples of two possible offspring in both the curly bracket scheme and the Punnett square. Note that the Punnett square reflects the numerical outcome of this cross in its full complexity, whereas the curly bracket strategy only qualitatively reflects potential combinations and is easier to interpret for the purpose of mating scheme design (Box 5).