A rough guide to *Drosophila* mating schemes (light version 2.1)[1]

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 [1,3]. 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 [4]. 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 [3-9]. Notably, it has been estimated that "...about 75% of known human disease genes have a recognisable match in the genome of fruit flies" [10]. Therefore, *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 diseases including neurodegeneration [11-13][2]. 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/)

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*
2011	Jules A. Hoffmann - *the activation of innate immunity*

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 here:

- 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.

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[1] The full version can be downloaded @ dx.doi.org/10.6084/m9.figshare.106631

[2] Informative lay descriptions of fly research can be found on the Wellcome Trust Blog:
The portrait of a fly (Part 1) - wellcometrust.wordpress.com/2012/11/20/feature-the-portrait-of-a-fly-part-1/
The portrait of a fly (Part 2) - wellcometrust.wordpress.com/2012/11/23/the-portrait-of-a-fly-part-2-fly-on-the-wall/

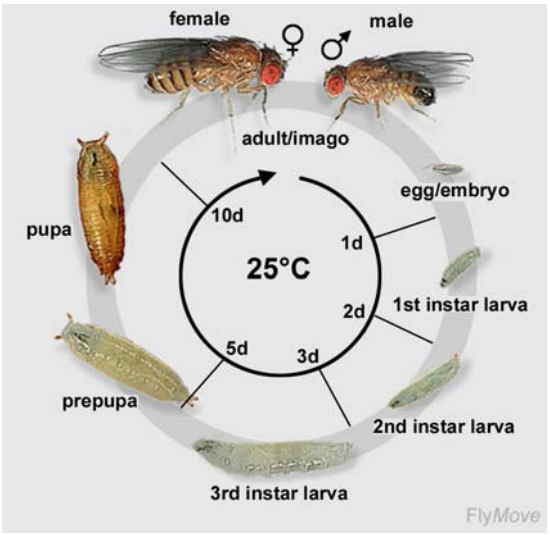

Figure 1. The life cycle of *Drosophila melanogaster*

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 [14]. Image modified from FlyMove [15].

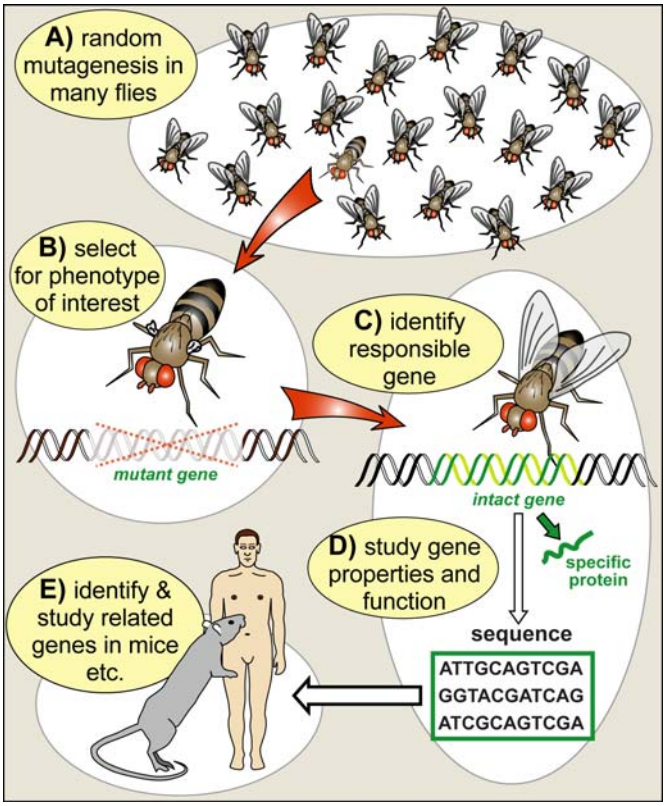

Figure 2 A typical flow diagram of how genetic screens in *Drosophila* contribute to research

A) Random mutations are induced in large numbers of flies. **B)** The essential task is to select those mutant animals that display phenotypes relating to the biological processes to be investigated. **C)** The responsible gene can be identified using classical genetic or molecular strategies. **D)** Once the gene is known, 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). Highly efficient and versatile strategies have been developed that can be adapted to the experimenter's needs [16-20].
- 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 [21-28][1].

[1] for overviews of *Drosophila* genetics see http://www.scribd.com/doc/6125010/Drosophila-as-a-Model-Organism and [29] (http://highered.mcgraw-hill.com/sites/007352526x/student_view0/genetic_portrait_chapters_a-e.html)

- 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 [30,31]. 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 5.2e), 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 [2]. 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 through the use of 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).

2. The importance of genetic mating schemes

Daily life in a fly laboratory requires performing classical genetic crosses. In these crosses, flies are used that carry gene mutations, chromosomal aberrations or transgenic constructs. 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 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.

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. 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.

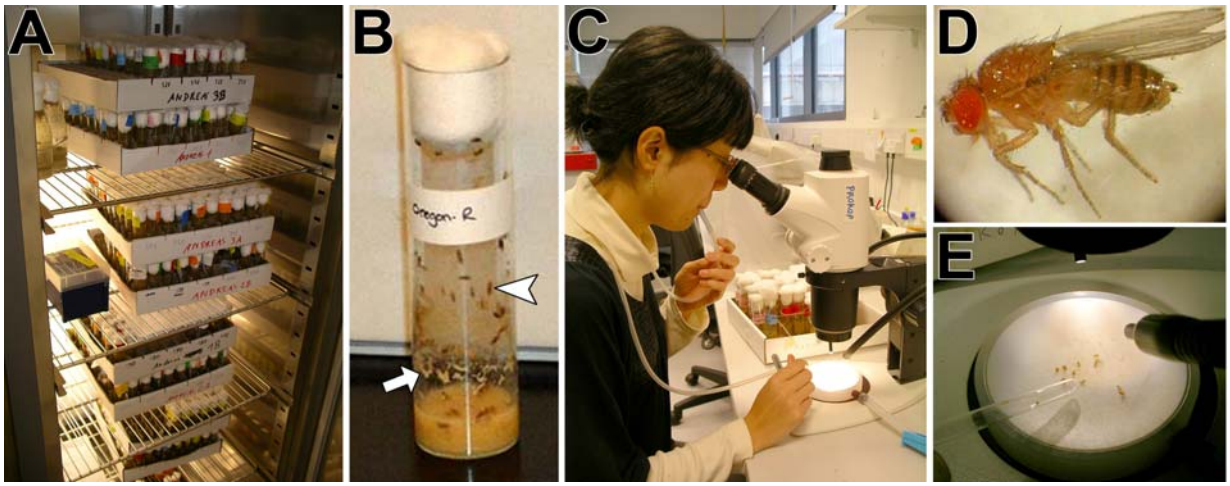

Figure 3. Maintaining and handling flies in the laboratory

A) Large numbers of different fly stocks are stored in trays in temperature controlled rooms or incubators (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 eventually disposed of into a morgue or transferred to fresh vials using a paint brush, forceps or aspirator (pooter) (C, E).

- **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) [14], 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 4.4.2).

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 Fig. 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 CO_2. CO_2 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.

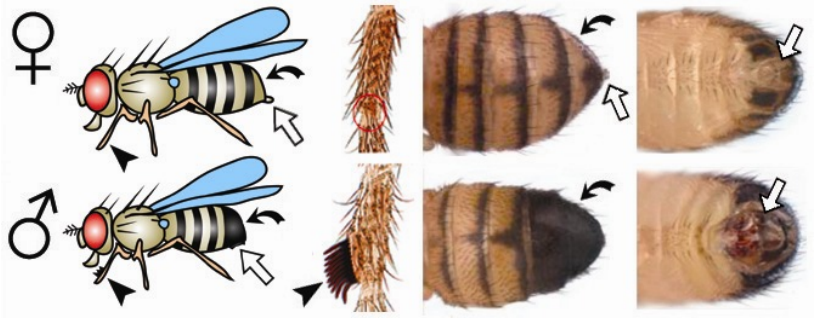

Figure 4. Criteria for gender selection

Images show females (top) and males (bottom): lateral whole body view (left), a magnified view of the front legs (2nd column), dorsal view (3rd column) and ventral view (right) 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 [32] and [33]. During a very short period after eclosion, females display a visible dark greenish spot in their abdomen (*meconium*; not shown) which is a secure indicator of virginity even if fertile males are present.

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 [14].

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.

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 are described in greater depth elsewhere [14,34].

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). 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.

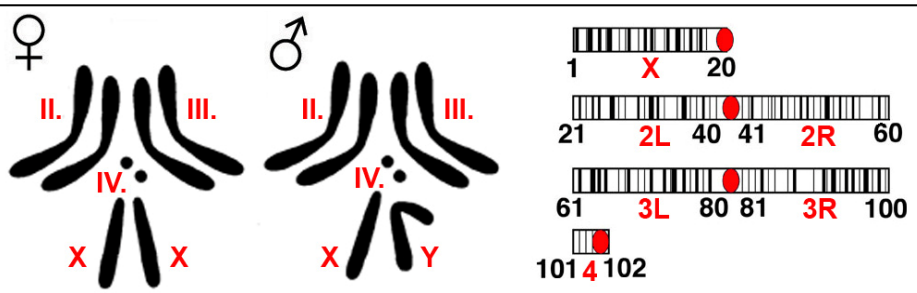

Figure 5. *Drosophila* chromosomes

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 used for the cytogenetic mapping of gene loci (black numbers; see FlyBase / Tools / Genomic/Map Tools / Chromosome Maps for detailed microscopic images); 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 [35].

4.1.2. Alleles[1]

Genes exist in different alleles. Classifications of these alleles are complex and will not be explained in greater detail here (but see the link at the bottom of the page). To simplify matters, we will deal here with two principal allele classes. The phenotypes of **recessive alleles** (names not capitalised) are not visible 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). The phenotypes of **dominant alleles** (names capitalised) are apparent in heterozygous animals (-/+; see Bar^1/+ individuals in Fig. 6). Dominant alleles are often lethal in homozygosis, but they may display **intermediate** inheritance showing a stepwise increase in phenotype strength from heterozygous to homozygous animals. For example, 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).

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

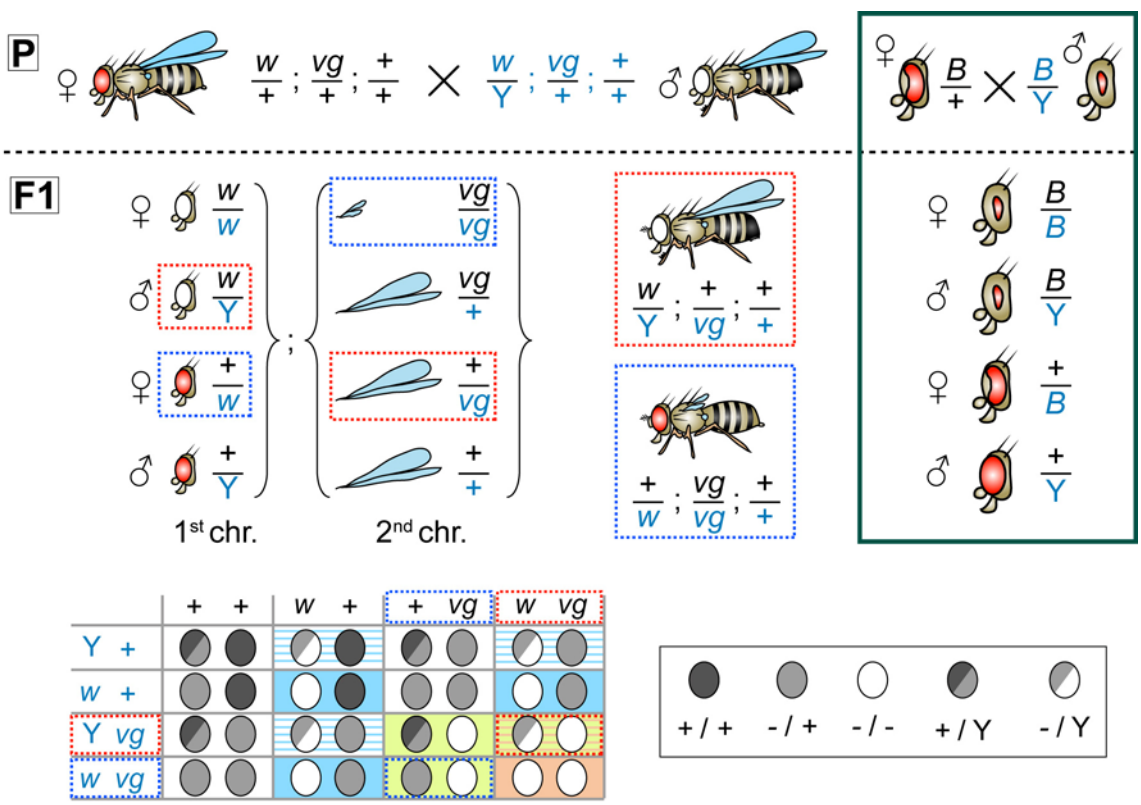

Figure 6. Independent assortment of alleles & comparison of recessive and dominant inheritance

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 2nd 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 x 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 3).

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 3). 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 3). The **4th chromosome** harbours very few genes and its genetics slightly differs from other chromosomes [34]. It plays a negligible role in routine fly work and will therefore not be considered here.

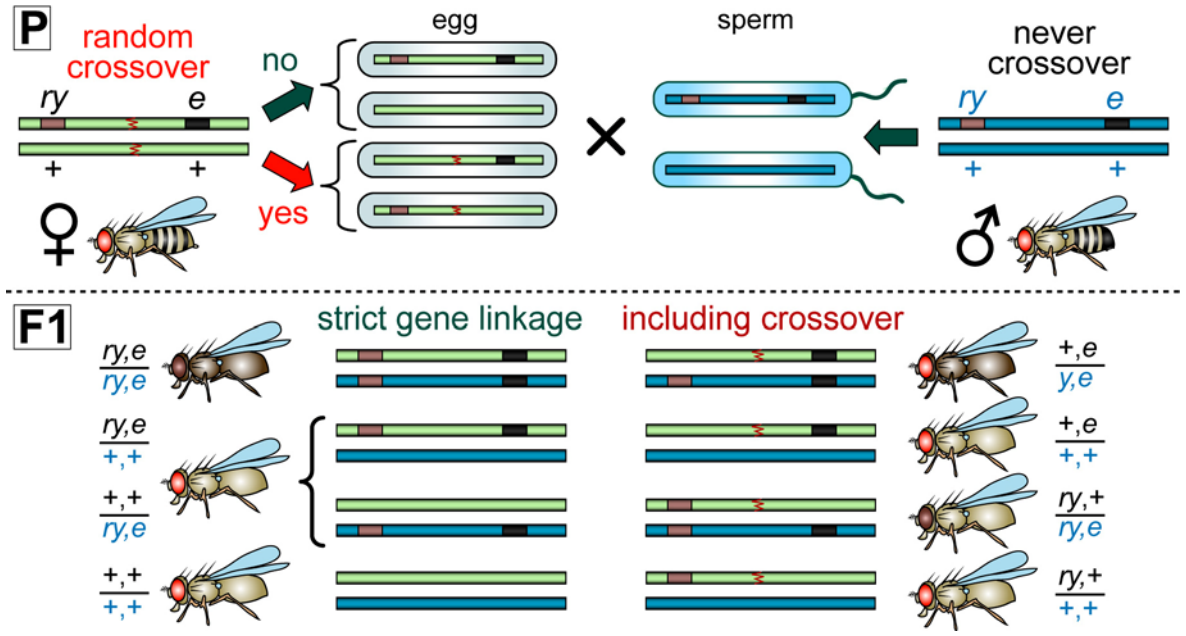

Figure 7. Inheritance of genes on the same chromosome (linked genes)

(**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.

4.1.4. Linkage groups and recombination

Genes located on the same chromosome are considered a **linkage group** that tends to segregate jointly during meiosis. However, through the process of **intra-chromosomal recombination (crossing-over)** when homologous chromosomes are physically paired (**synapsis**) during meiotic prophase, exchange of genetic material occurs between homologous chromosomes, except the 4th chromosome (Fig. 7). 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 the design of 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).
- 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.

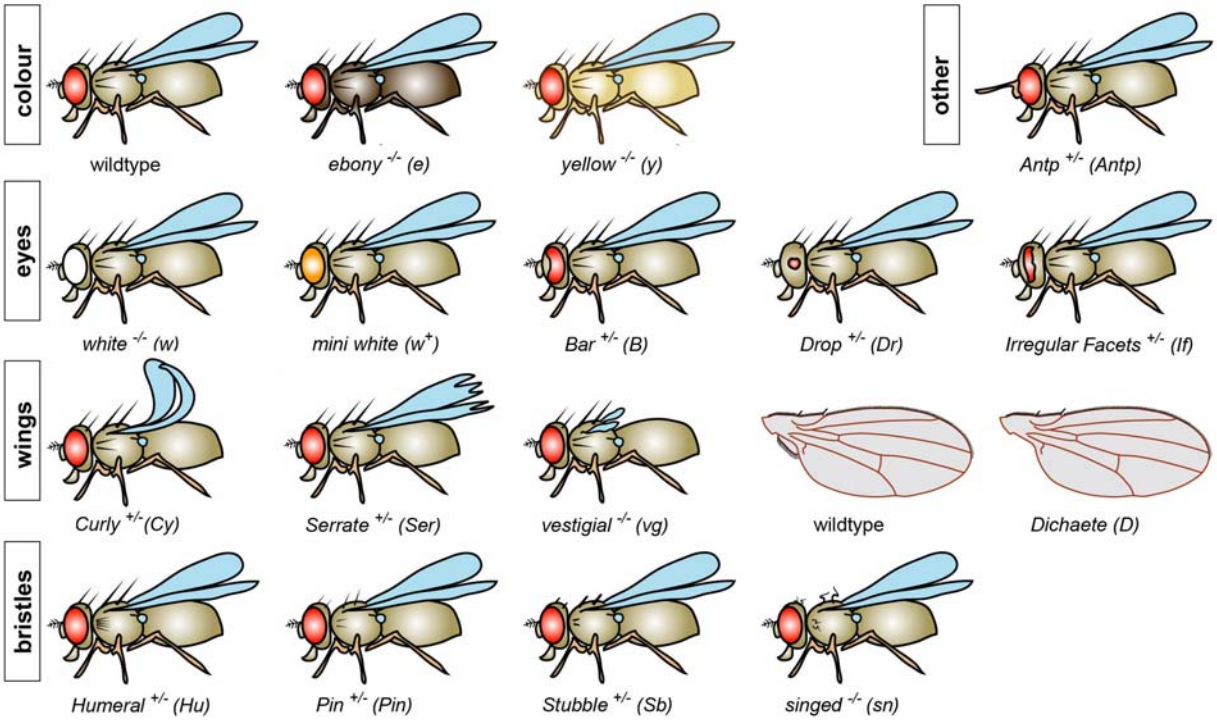

Figure 8. Examples of typical marker mutations used during genetic crosses

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)
- *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 these marker mutations have been published elsewhere [33,36][1].

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 [37][2]. 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

[1] or download the poster "Learning to Fly": http://onlinelibrary.wiley.com/journal/10.1002/%28ISSN%291526-968X/homepage/free_posters.htm

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

genetic crosses and, hence, for mating scheme design. A few marker mutations commonly used for fly work are illustrated in Fig. 8.

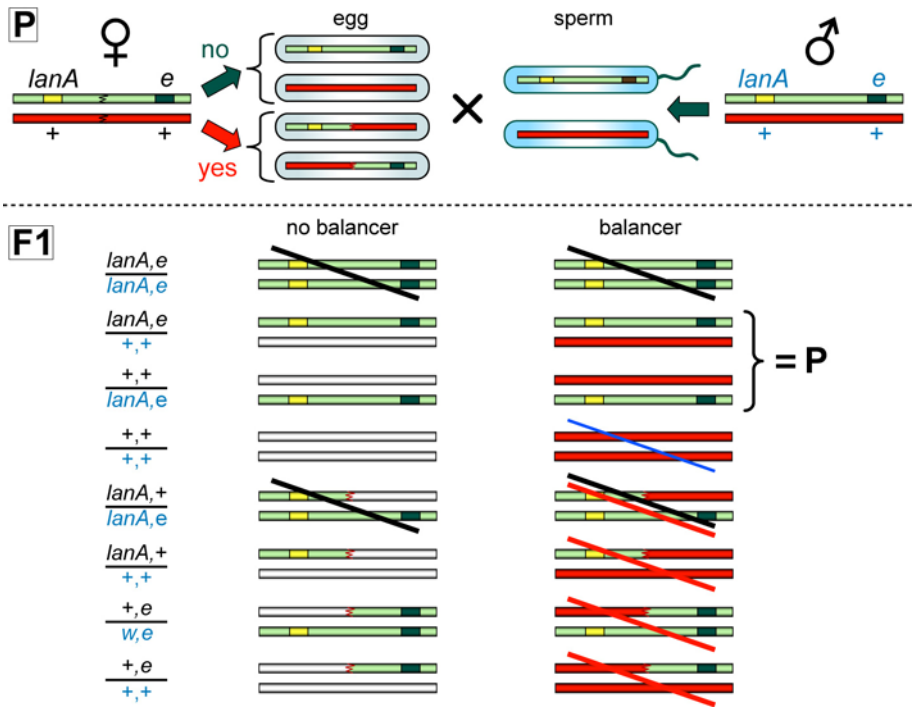

Figure 9. The use of balancers in stock maintenance

A cross of two parents (**P**) heterozygous for the homozygous embryonic lethal mutation *lamininA* (*lanA*) and the recessive and viable marker mutation *e* (*ebony*, dark body colour). 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 [14]. 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. 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. 9). 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 positive effect for stock maintenance is the same (Fig. 10). 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. 8):

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

Note that the **4th chromosome** does not require balancers since it does not display recombination.

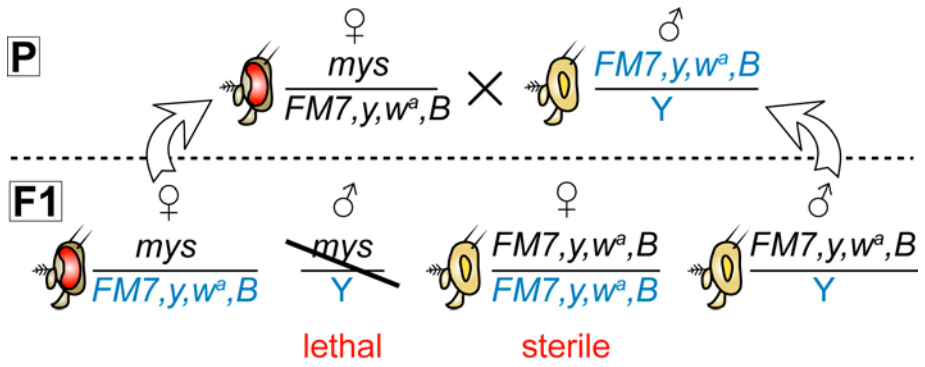

Figure 10. First chromosome balancer, FM7

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.

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.11A). 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. 11B).

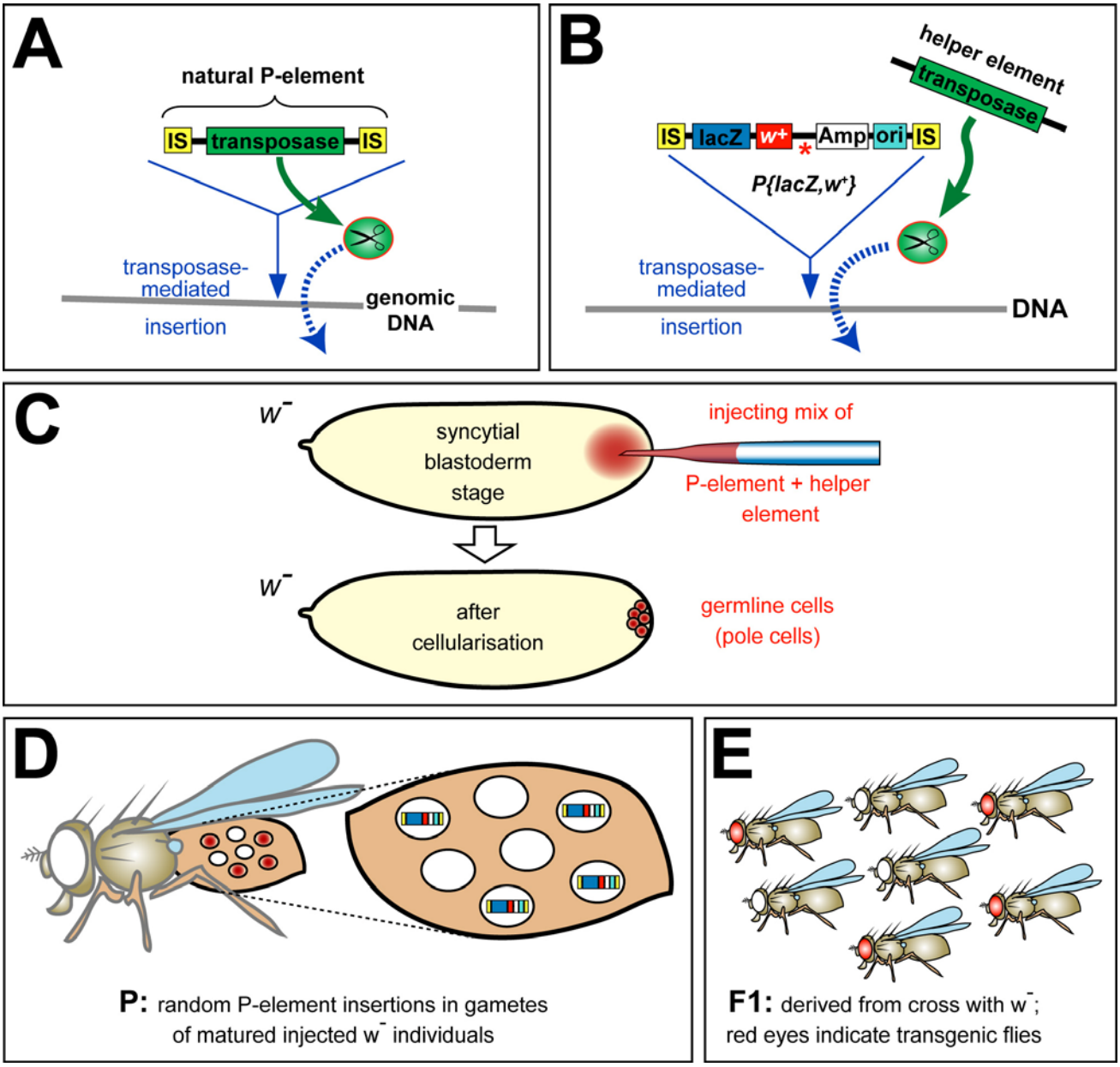

Figure 11. Using P-elements to generate and map transgenic insertions

A) The insertion of natural P-elements (blue arrow) into the genome (grey line) requires flanking IS motifs (insertion sequences) as substrate for the enzymatic activity of transposase (scissors and dashed blue arrow). **B)** *P{lacZ,w+}* is an engineered P-element used for transgenesis. Its 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). **B-D)** 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 (B), but they lack IS motifs themselves, i.e. fail to insert and replicate but are diluted out during subsequent cell divisions; injected individuals mature into *w-* adults with random P-element insertions in their gametes (D); 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 (E).

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. 11) [38]. 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. 11D). Through this disappearance of the enzymatic 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. This 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**) [19]. 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 4.4.2.). Transposable elements inserted near or in genes are readily available for almost every chromosomal locus [39]. They can be used to generate new mutant alleles of genes.

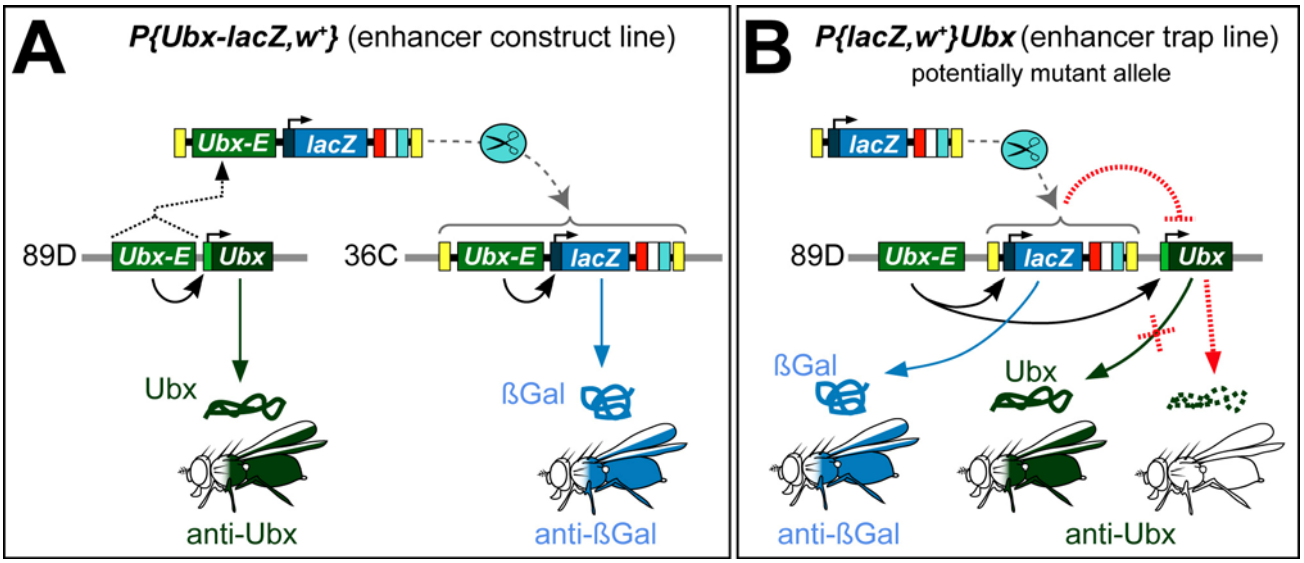

Figure 12. Enhancer trap and enhancer/reporter lines

A) *P{Ubx-lacZ,w+}* illustrating an enhancer/reporter line. A transcription 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; *Ubx-E* is cloned 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. 11) 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).

5.2 Important classes of P-element lines

There is a great variety of transgenic fly lines 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. 11 and 12), 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: In order to study regulatory regions of genes, genomic fragments containing primarily non-coding regions of these genes can be cloned in front of a reporter gene (e.g. *lacZ* from *E. coli*; Fig. 12 A). Transgenic fly strains with these constructs

are generated and used to analyse the spatiotemporal expression pattern of βGal (the *lacZ* product). Through this, tissue- or stage-specific enhancers regulating the transcription of specific genes can be identified and studied. 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.

b. Enhancer trap lines: Enhancers are regulatory activators of gene transcription. They may act over distances of several kilo bases. If a *lacZ*-bearing P-element (which alone does not display lacZ expression) is inserted within the activity range of enhancers, lacZ expression can be induced by these enhancers, often reflecting (aspects of) neighbouring genes' expression patterns (Fig. 12 B). 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** [40]. Since P-element insertions frequently affect the function of genes at their insertion site (stippled red T in Fig. 12 B), they can be used for systematic **P-element mutagenesis screens** (see Fig. 2) [19]. Once P-induced insertions have been generated, lacZ staining patterns may reveal when and where the gene is active (Fig. 12 B), and efficient cloning strategies can be used to map the insertion and identify the targeted gene (Fig. 11 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 [28,41].

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. 13 a, b). By simply crossing Gal4-expressing flies to ***UAS* lines** (Fig. 13 c, d), 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. 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 [23].

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) [26] allow the targeted knock-down of specific genes in a reproducible tissue or set of cells, often at a 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 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 (ideally carrying a mutation that does not affect its function but makes it immune to the knock-down construct).

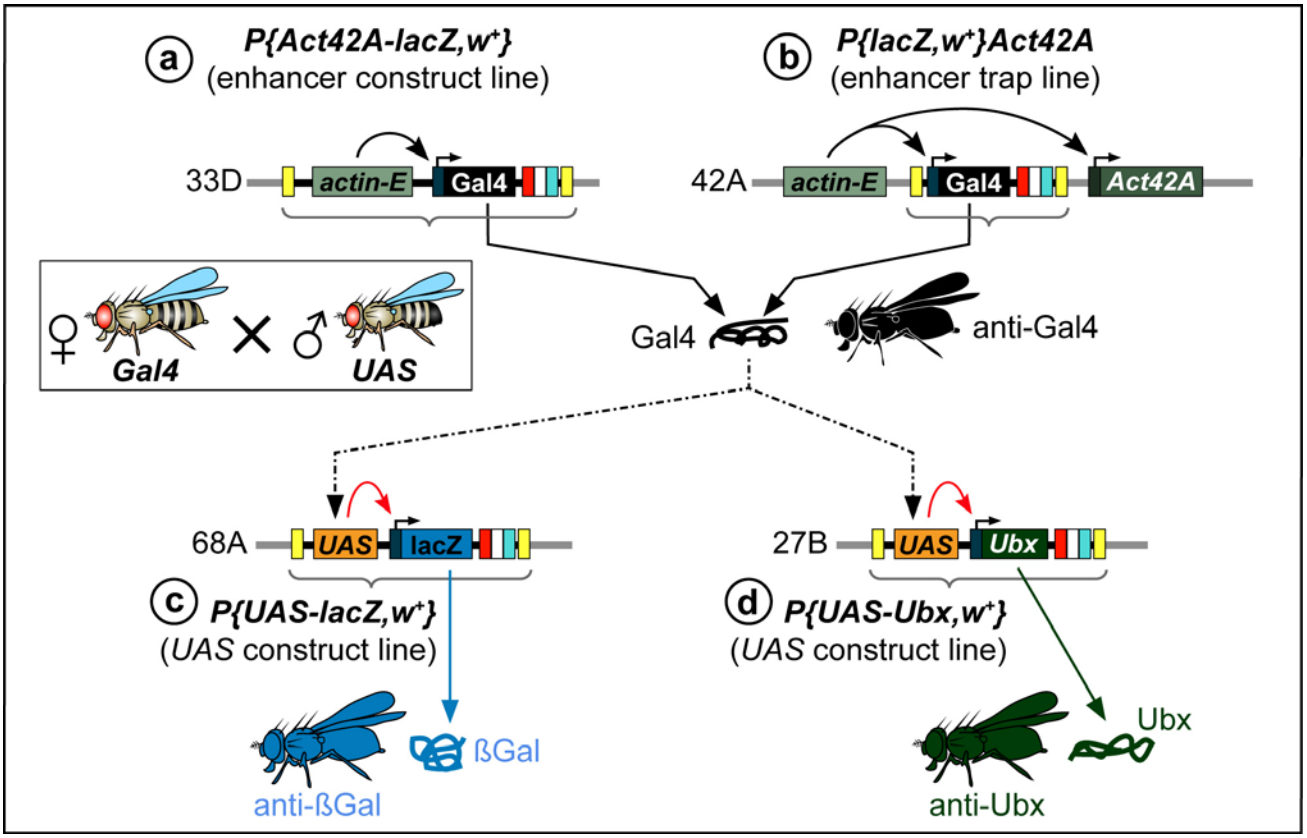

Figure 13. The versatile Gal4/*UAS* system for targeted gene expression

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). Two 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.

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 [42,43]. 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. 14).

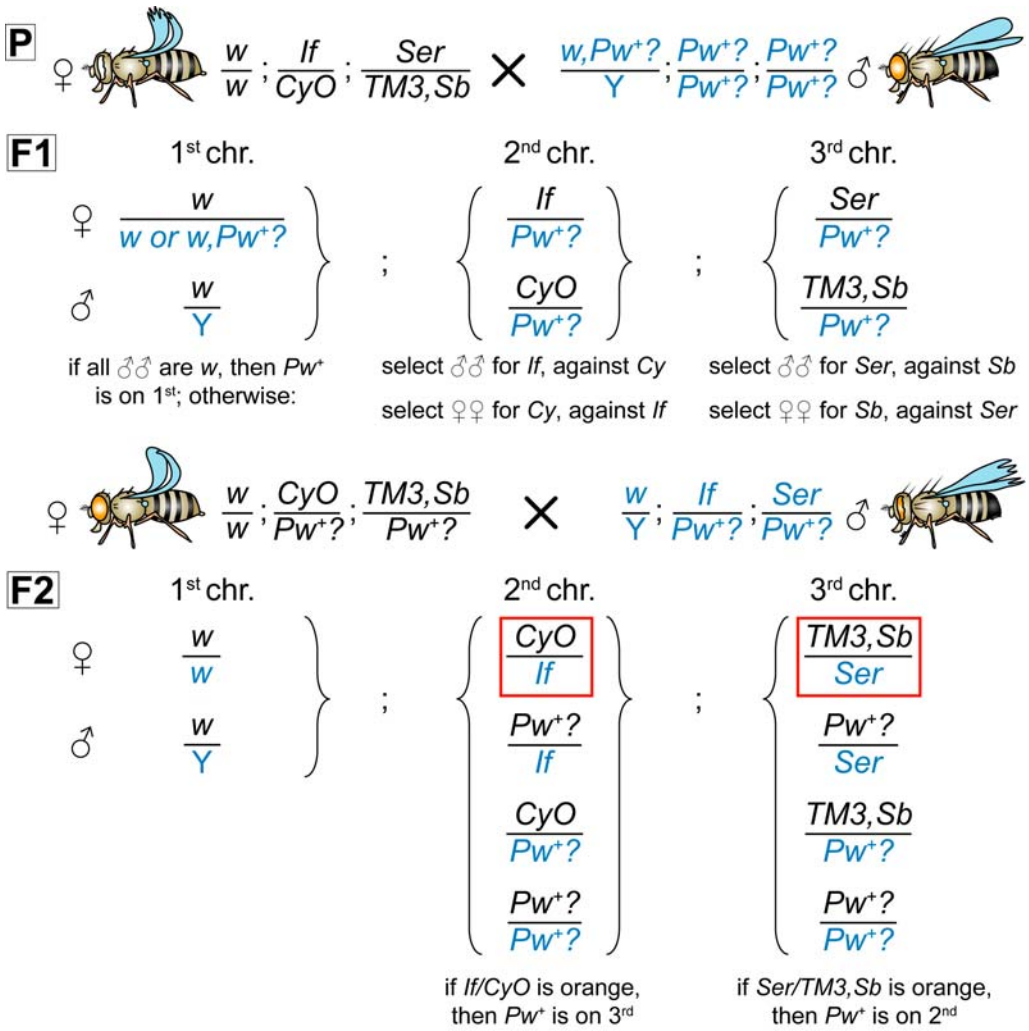

Figure 14. Determining the chromosome of insertion of a P-element

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. **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; 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.

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 [18,34]. 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.

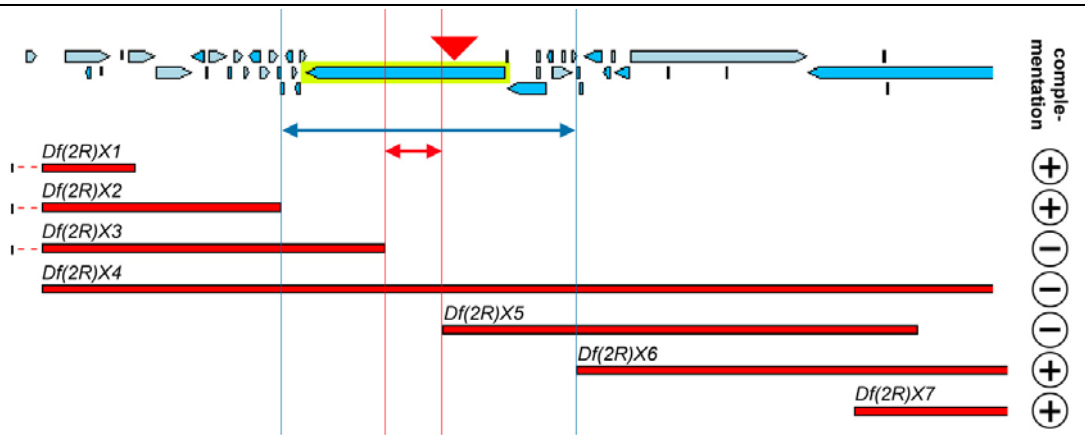

Figure 15. Deletion mapping

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).

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 listed in FlyBase and are available through stock centres (e.g. Bloomington / Deficiencies). Using improved technology the Bloomington Deficiency Kit now covers 98.4% of the euchromatic genome [44]. 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**). However, be aware that, when dealing with lethal mutations, only 25% of your offspring are expected to carry the phenotype, so you look for 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. 15).

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**).

7. An example of a mating scheme (Powerpoint presentation)

You can now apply and consolidate your knowledge acquired from this manual by downloading and studying the Powerpoint presentation "*Roote+Prokop-SupplMat-3.ppt*" (http://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 laboratory task in which a homozygous viable P-element insertion on the second chromosome has to be recombined with a second chromosomal, recessive, homozygous lethal mutation. To perform this task, two separate stocks carrying the mutation and the P-element insertion, respectively, and a third fly stock with different balancer chromosomes are given. The presentation leads through the solution of this task step by step, illustrating and explaining the various strategic considerations and decisions that have to be taken and how the rules of *Drosophila* genetics are applied. At each step of the mating scheme you will be prompted to make your own suggestions, before being presented with a possible solution. Make use of this opportunity to test your knowledge. Revisit this manual to answer queries. This is a good

strategy to consolidate your knowledge. As you will see, the presentation includes an example of a dead-end solution, demonstrating how trial and error and creative and flexible solution seeking usually lead to optimal cross design.

8. 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 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, please, be so kind to let me know (Andreas.Prokop@manchester.ac.uk).

Box 3. How to design mating schemes (illustrated in Figs. 6 and 15)

- write 'X' between two 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...
    - o ...the female genotype is always shown on the left side, male on right
    - o ...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$^+$".

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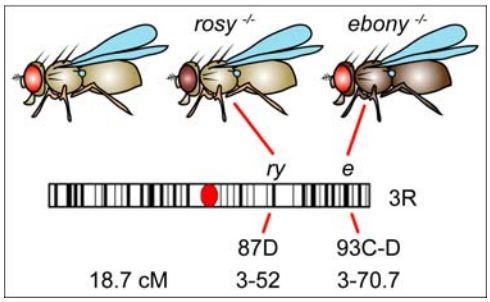
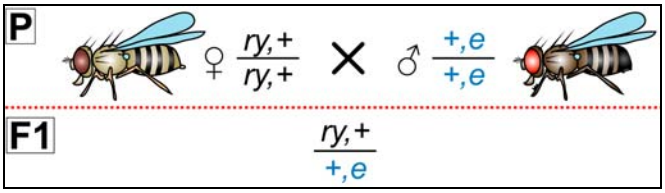
Appendix 1. A recombination scheme

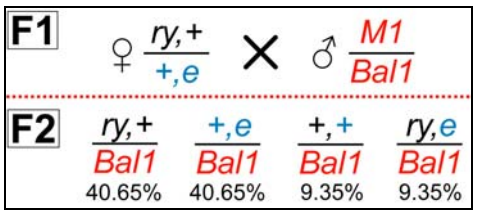
You want to recombine mutant alleles of the viable, recessive, 3rd chromosomal loci *rosy* (*ry*; dark brown eyes) and *ebony* (*e*; black body colour) onto one chromosome. According to FlyBase, *ry* localises to recombination map position 3-52, and *e* to 3-70.7. Hence, they lie 18.7cM apart, indicating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



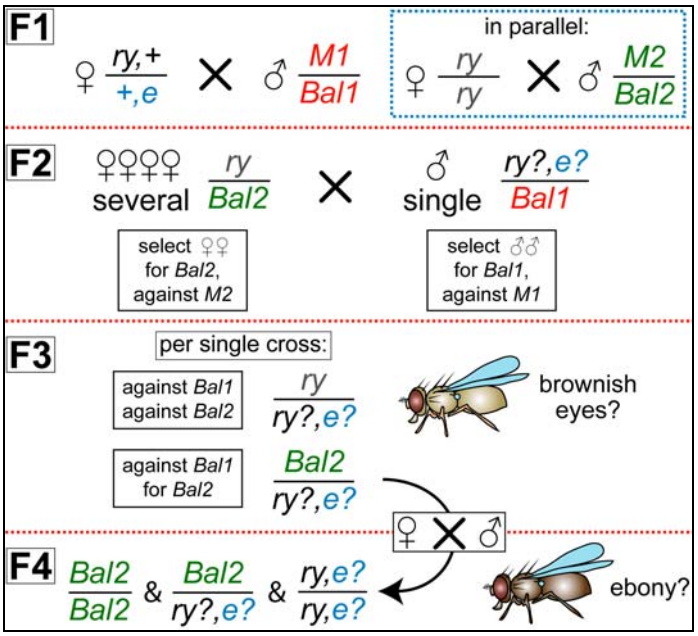
According to the recombination rule, you need to take females so that recombination can occur. Note that crossing-over during oogenesis in these females occurs at random, i.e. their eggs which give rise to the second filial generation (**F2**) represent a cocktail of recombination events with a statistical 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.