Mosaic Analysis in *Drosophila*

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ABSTRACT Since the founding of *Drosophila* genetics by Thomas Hunt Morgan and his colleagues over 100 years ago, the experimental induction of mosaicism has featured prominently in its recognition as an unsurpassed genetic model organism. The use of genetic mosaics has facilitated the discovery of a wide variety of developmental processes, identified specific cell lineages, allowed the study of recessive embryonic lethal mutations, and demonstrated the existence of cell competition. Here, we discuss how genetic mosaicism in *Drosophila* became an invaluable research tool that revolutionized developmental biology. We describe the prevailing methods used to produce mosaic animals, and highlight advantages and disadvantages of each genetic system. We cover methods ranging from simple "twin-spot" analysis to more sophisticated systems of multicolor labeling.

KEYWORDS *Drosophila*; mosaicism; Flp/FRT; FlyBook

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Genetic mosaics are individuals composed of cells with at least two different genotypes. Over the past century, the phenomenon of mosaicism has been extensively studied by geneticists and has become a vital tool in numerous aspects of biology. In nature, mosaics occur in all multicellular organisms and generally arise from a mutation within a single cell, acquired during development. Mosaicism can be "silent" and cause no apparent harm to the individual (Martincorena and Campbell 2015), but is also associated with numerous human pathologies. Cancers arise due to mosaicism, and the mosaic complexity of tumors can increase during their malignant progression. Trisomy syndromes such as Turner and Klinefelter Syndromes also result from mosaicism, as do some milder forms of Down's Syndrome (Niikawa and Kajii 1984; De 2011).

Mosaicism was harnessed as a research tool soon after *Drosophila* was established as a genetic model, and has since become indispensable for studies of the biology of development, physiology, and disease. The experimental creation of genetic mosaics has permitted the study of essential genes, delineated cell lineages, helped distinguish between cell autonomous and nonautonomous gene function, and revealed how genetically distinct populations of cells can interact with each other. In this chapter, our goal is to provide an overview of different ways in which genetic mosaics have been used and of some of the techniques that allow their generation. While this review discusses many methods for generating mosaics, it is not exhaustive. We hope that our discussion will prompt readers to probe the literature for additional approaches, and perhaps spur the creation of new ways to use mosaic analysis in *Drosophila* research.

Natural Origins of Mosaicism

Mosaics can arise spontaneously from abnormal mitotic events and the persistence of the resulting mutant cell's progeny, yielding a clone of mutant cells. Mutations that give rise to mosaic tissues can originate from improperly repaired DNA double-strand breaks (DSBs) caused by environmental factors including ionizing radiation (UV light and X-ray) and a variety of chemicals, including nicotine (Sachs *et al.* 1992; De 2011). DSBs are repaired by two different cellular mechanisms: NHEJ (Nonhomologous End Joining) (Moore and Haber 1996; Lieber *et al.* 2003) and Homology-Directed Repair (HDR). NHEJ is a common repair mechanism in somatic cells (Gloor *et al.* 2000; Min *et al.* 2004) in which the two broken DNA ends are joined without restoring the original sequence. By contrast, HDR usually reestablishes the original sequence. In somatic cells, HDR relies on an identical sister chromatid, and thus is limited to G2 phase of the cell cycle (Johnson and Jasin 2000). However, homologous recombination is not always a perfect process, and inaccurate repair can result in the formation of a genetic mosaic. Whether this has consequences for the animal depends upon the nature of the mutation and whether it is propagated.

Mosaicism can also arise from ring-X chromosomes, where the two arms of the X chromosome have fused together to form a ring. This defect can arise spontaneously or, more often, after induction of DNA damage by ionizing radiation (Baker 1957). The ring-X chromosome is unstable and consequently is easily lost or broken during mitotic divisions, which promotes the formation of gynandromorphs (Box 1). In addition, activation of mobile elements, such as the L1 retrotransposons that are transiently activated in preimplantation mammalian embryos, can lead to mosaicism. Their mobilization can lead to genomic diversity and heterogeneous cell populations (van den Hurk *et al.* 2007; De 2011).

The prevalence of mosaicism increases in aging tissues, as reported for normal human skin (Martincorena and Campbell 2015). Indeed, the extent of natural mosaicism may be underestimated, due to lack of an associated physical marker or because less vigorous cells might be lost from tissues due to competitive interactions with other cells.

Experimental Induction of Mosaics: A Brief History

Artificial induction of genetic mosaics has long been of interest to experimental biologists. Although the transplantation of nuclei or cells from one embryo to another can generate chimeric animals, this method is infrequently used because

Box 1 Basic definitions

Mosaic animals: organisms composed of two or more genotypically different cells.
Gynandromorphs: from the Greek words *gyne* and *andro* (female and male, respectively). A sexual form of mosaicism in which the individual is composed of both male and female parts.
Mitotic recombination: recombination that occurs between sister chromatids in somatic cells before the mitotic division, producing two daughter cells with different genotypes.
Clone: a population of genetically identical cells that originates from a single ancestral cell.
Twin spot clones: two individual and genetically different populations of cells that originate from the same mitotic recombination event (Figure 1A).
***trans*-chromosomal recombination:** recombination occurring between two different chromosomes; also known as mitotic recombination (Figure 1A).
***cis*-chromosomal recombination:** recombination occurring between FRT sites within the same chromosome; also known as FLP-out recombination (Figure 1B).
FLP-out clones: A method for inducing clones of cells via FLP-FRT-mediated intrachromosomal recombination (Figure 1B).

it is technically demanding, and because clonal analysis after transplantation is often complicated by significant variation in the timing of donor incorporation into the host environment (Lawrence and Johnston 1986). The use of genetic manipulation to induce mosaic tissues was developed in Thomas Hunt Morgan's famous Fly Room at Columbia University in the early 20th century. Indeed, the numerous seminal discoveries that arose from the work in Morgan's lab at Columbia and subsequently at CalTech completely transformed genetic approaches to experimental biology.

While at Columbia, Morgan and his colleagues noticed the occasional presence of gynandromorphs and mosaics among the lab's stocks of mutant flies. Gynandromorphs (Box 1), named from the Greek words *gyne* and *andro* (female and male, respectively), are mosaics composed of male and female parts. Morgan determined that they originated via the mitotic loss of one X chromosome during embryonic development (Morgan 1914). Further studies indicated that the point in development at which the chromosome is lost determines the extent of mosaicism in the animal. If loss of the X occurs during the first embryonic divisions, much of the animal will be mosaic and develop with bilateral dimorphism, where one half of the fly is XX with female characteristics, while the other half receives a single X chromosome (X0) and is therefore male. Gynandromorphism also occurs in humans, and a partial or complete loss of the X chromosome is a cause of Turner Syndrome, in which most patients are genetic mosaics composed of both XX and X0 cells (Berkovitz *et al.* 1983).

In 1926, Lilian Morgan (wife of T. H. Morgan), observed the first ring-X chromosome in *Drosophila* (Morgan 1926). Due to its extreme instability, the ring-X is most often lost in the first few embryonic nuclear divisions, resulting in very large clones. This property made the ring-X chromosome quite useful for mosaic studies, and led to its extensive use in lineage-tracing experiments to identify tissue- and stage-specific developmental histories. The ring-X technique was the preferred method for fate mapping of the blastoderm embryo and was also used for studies of sex differentiation (Janning 1978; Gilbert 2000). However, ring-X studies are restricted to X-linked mutations, and controlling chromosome loss is difficult, making its usefulness in experiments somewhat limited (Dang and Perrimon 1992).

Around the same time, Hermann Joseph Muller—who had previously worked with T.H. Morgan—demonstrated that X-rays cause genetic mutations in the offspring of exposed flies, allowing the generation of large numbers of mosaics (Muller 1927, 1928). Muller was awarded the Nobel Prize in 1946 "for the discovery of the production of mutations by means of X-ray irradiation." Later, Charlotte Auerbach discovered that chemicals also act as mutagens in flies, and observed that chemically-induced mosaics were generated at higher frequency than with X-rays (Auerbach 1946; Auerbach and Robson 1946; Beale 1993).

Early in their studies at Columbia, T. H. Morgan and his colleagues observed that the inheritance of some sex chromosome traits was linked (Morgan 1910). They also noticed that linked traits could sometimes separate, suggesting that crossover between chromosomes occurs (Morgan 1911). They postulated that the crossover frequency was related to how closely the genes were physically linked (Morgan *et al.* 1915). These observations led his student, Alfred Sturtevant, to produce the first genetic linkage map, which located genes within a sequence of physical positions on the chromosome (Sturtevant 1913). Later, Curt Stern discovered mosaic flies that carried adjacent clones with bristles that were mutant for either *y* or *singed (sn)*. Using Sturtevant's linkage map of the X chromosome region that contained both of these genes, Stern deduced that the two adjacent clones resulted from a mitotic crossover (Stern 1936; Becker 1978). Stern's insightful finding corresponded to what we now define as twin spots: two individual populations of cells that originate from the same mitotic recombination event (Stern 1936) (Box 1).

Following the crucial discovery of somatic crossover in *Drosophila*, forcing mitotic crossover between sister chromatids

Box 2 Cell autonomous markers commonly used in mosaic analysis

yellow [y]: Body color marker. The *y* gene controls the pigmentation of the larval (visible only in L3) and adult cuticle, and the pigmentation of the larval mouth-hooks. *y* flies lack the wild-type dark pigmentation and appear amber in color.
forked [f]: Bristle and trichome marker. *f* mutants affect the shape and length of microchaetae, macrochaetae, and trichomes.
***singed* [*sn*]:** Bristle marker. *sn* flies are characterized by deformed (shorter, gnarled, or wavy) macrochaetae.
white [w]: Eye color marker. *w* flies lack the pteridine red pigments that compose the adult eye. The protein is thought to be a membrane-bound transporter of the pteridine pigment precursors. *w* was the first *Drosophila* mutant to be identified by Morgan.
multiple wing hairs [mwh]: Trichome marker. *mwh* is involved in the organization of hairs and binds to F-actin polymers to exert its function. *mwh* flies show an increase in trichome number from one to three. In addition, they are reduced in length and mis-oriented. *mwh* is widely used as a cell marker for clonal analysis in the wing blade.
javelin [jv]: Bristle and trichome marker. Bristles and trichomes appear cylindrical and enlarged before the tip. It is cell autonomous and has been used to mark epidermal clones.
pawn [pwn]: Bristle and trichome marker. Clones of cells marked with *pwn* have truncated bristles with pale tips and pin-shaped trichomes with basal spurs and thin, transparent hairs.
CD2: CD2 is a T-cell surface antigen precursor in rats (*Rattus norvegicus)* that is widely used in *Drosophila* FLP-out cassettes. Since CD2 is a foreign sequence in the *Drosophila* genome, it can be used to label clones with commercial anti-CD2 antibodies. In the FLP-out cassette, *CD2* is positioned between two FRT sites, allowing the excision after FLP-induced recombination (Basler and Struhl 1994).
Fluorescent proteins: GFP, RFP, mCherry, and YFP are among the commonly used markers for larvae and pupae. They are useful for marking cell clones and are visible under a fluorescent microscope. New generation fluorescent markers are also available, such as sf-GFP, BFP, and tdTomato. They broaden the spectrum of possible colors that can be used in combination with each other to mark different clonal populations. Fluorescent markers can also be targeted to specific compartments within a cell (*e.g.*, mito-GFP labels mitochondria, mCD8-GFP, and myr-GFP labels the plasma membrane). Many of these markers can be obtained via stock centers (*e.g.*, http://flystocks.bio.indiana.edu/Browse/GFP/GFP_markers.php).
β-**galactosidase:** β-galactosidase is an enzyme that hydrolyses β-galactosides into monosaccharides. The bacterial *lacZ* gene is widely used as a cell marker in *Drosophila*; its expression can be detected by detection of its enzymatic activity or by its presence in cells using specific antibodies.

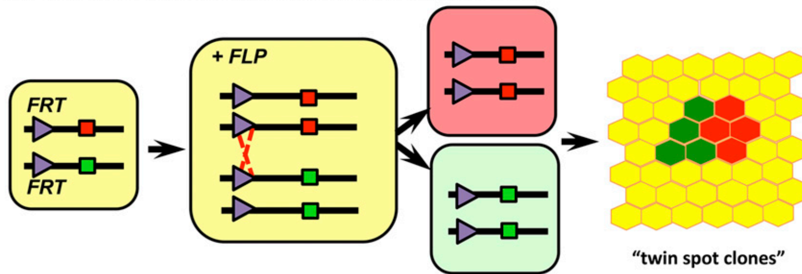
of different genotypes—primarily by causing DSBs with γ-irradiation—became an important and widely used experimental strategy to generate genetically distinct cells in the same tissue. Indeed, the twin-spot technique was used by numerous researchers to identify cell lineages in imaginal discs, the epithelial organs that give rise to the appendages of the adult fly. Thereafter, mosaic analysis became synonymous with clonal analysis and remains a critical research tool.

Today, a plethora of methods exists for generating mosaics in research. The development of each method stems from the initial work carried out by Mueller, Morgan, and colleagues. As was the case in the original Fly Room, the central motor for the development of new mosaic-generation techniques is problem solving to overcome thorny, seemingly intractable experimental complications. As a result, the method of choice will vary according to the problem the researcher wishes to solve. In the following sections, we discuss several methods that are currently used to generate mosaic tissues, their uses, their advantages, and their limitations. Importantly, through the combination and intersection of multiple mosaic generation systems, there is room for considerable creativity in tailoring an experiment for a specific problem.

Recombination and Clonal Analysis in Research

A critical requirement of clonal analysis is the ability to detect the mutant clones with nonlethal and easily detectable cell markers. In the adult *Drosophila*, cuticle markers such as *y*, *sn*, *forked* (*f*), and *multiple wing hairs* (*mwh*) are commonly used to recognize clones in wings, legs, thorax, and the abdomen (Box 2). An ideal marker should have the following characteristics: it should not be toxic or influence the cell in which is expressed; it must be cell autonomous, to guarantee that a marked cell is also a cell that has recombined; and the protein should have short perdurance (Box 2). A commonly used eye marker is the *white* (*w*) gene, which produces a transporter belonging to the ABC transporter superfamily (ABCG family) and acts cell autonomously. This transporter is used to import the red eye pigment that gives wild-type flies their red eye pigmentation. When *w* clones are generated, white and red patches can be seen in the adult *Drosophila* eye. In the embryonic, larval, and pupal stages, common clonal markers include expression of the bacterial *lacZ* gene and GFP or other fluorescent proteins (Box 2).

A major advantage of clonal analysis is that it permits functional studies of genetic mutations that would cause



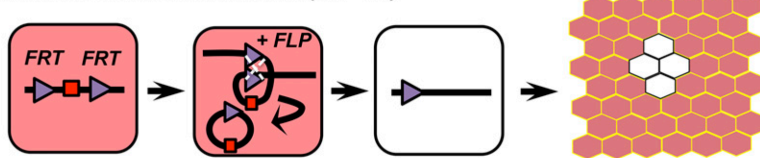


Figure 1 *trans*-chromosomal and *cis*-chromosomal recombination. (A) *trans*-chromosomal recombination is mediated by the presence of FRT sites on distinct chromosomes. Expression of FLP promotes recombination between the FRT sites specifically during the G2 phase of the cell cycle, generating two individual cells with distinct genotypes after the mitotic event. Mitotic descendants of the two original cells are generated, producing sibling clones ("twin spots") when X segregation occurs (see text for details). In epithelia, sibling clones generally stay in close proximity. In the scheme, distinct chromosomes carry two independent markers (red and green boxes). After recombination occurs, the two original cells will give rise to two differently-labeled clones (red and green twin-spot clones). Because it relies on sister chromatid exchange, *trans*-chromosomal recombination can only occur in mitotically active cells and is also known as Mitotic Recombination. (B) *cis*-chromosomal recombination occurs when FRT sites are present on the same chromosome arm and oriented in the same direction. FLP recognizes FRTs and causes recombination with the consequent excision of the DNA sequence (generally called an FRT cassette) contained within the two FRTs. This cassette contains a cell marker (red box). Thus, a cell with a different genotype is generated in a background of cells with the original genotype. The excision–recombination event is heritable, thus all of the progeny of the recombined cell will inherit the "FLP-out" genotype that loses the red marker (white clone in a background of red cells). Notably, this type of recombination can occur in any stage of the cell cycle.

lethality in whole animals. For example, systemic loss of most of the important growth and pattern-regulating genes causes early embryonic lethality, impeding their functional characterization *in vivo*. The generation of clones of cells mutant for such genes enabled the characterization of the gene's function and the ordering of epistatic relationships.

In addition, whether a particular gene functions in a cell autonomous or noncell autonomous manner can be addressed through clonal analysis. For example, a clone mutant for the morphogen Dpp may not show a phenotype, due to the presence of Dpp secreted by nearby wild-type or heterozygous cells; whereas clones homozygous for a mutation in the Dpp receptor Thickvein (Tkv), and thus unable to transduce a Dpp signal, will be subject to increased apoptosis (Burke and Basler 1996). Such experiments demonstrated that Dpp functions noncell autonomously, while Tkv functions cell autonomously.

Clonal analysis in *Drosophila* continues to be an extremely powerful tool for geneticists. It enabled, and still enables, seminal discoveries in developmental biology and growth control, many of which served as the foundation for charting the most important patterning and growth-regulatory pathways, such as the Wg/Wnt, Dpp/BMP/TGF-β, and Hippo signal transduction pathways that are conserved in mammals (Spencer *et al.* 1982; Simcox *et al.* 1989; Justice *et al.* 1995; Xu *et al.* 1995; Wu *et al.* 2003).

Site-specific recombination

The great promise of clonal analysis for research studies was nonetheless hindered by the inefficient and nonspecific method of using ionizing radiation to induce genetic recombination. While irradiation of mutant strains coupled with identifiable markers generated clones that could be recognized and studied, it was also mutagenic itself, often causing tissue damage throughout the organism.

A solution to the problems associated with irradiation came from the development of *P*-element-mediated germ line transformation in *Drosophila* (Rubin and Spradling 1982), which permitted researchers to insert genes from other organisms into flies. This advance, along with the identification of the bacteriophage Cre/loxP (Hamilton and Abremski 1984) and the yeast FLP/FRT (Andrews *et al.* 1985; Sadowski 1995) site-specific recombinase systems, spurred experiments that demonstrated that these recombinases function in flies. FLP/FRT-mediated recombination was shown to be highly efficient in flies (Golic and Lindquist 1989; Golic 1991), but the Cre/loxP system proved to be less efficient and somewhat toxic in flies (Siegal and Hartl 1996; Heidmann and Lehner 2001). The FLP/FRT recombination system thus quickly became the method of choice in *Drosophila*, making site-specific recombination between homologous chromosomes much more user-friendly and significantly modernizing the generation of mosaics.

The beauty of the FLP/FRT system is that recombination in flies is now inducible, site-specific, and highly efficient. FLP is a sequence-specific recombinase that mediates recombination by binding two conserved, short FRT sequences, and catalyzing cleavage and chromosomal exchange (Senecoff and Cox 1986; Ma *et al.* 2007). Due to its ease and utility, the FLP/FRT system has undergone many innovations since it was first introduced into flies and remains the premier tool for generating mosaics in *Drosophila* research.

Depending upon the position and orientation of FRT sites in DNA, FLP/FRT-directed activity leads to recombination or to DNA excision and rejoining (Golic and Lindquist 1989; Golic 1991). When positioned at the same location on each sister chromatid, the FRT site will catalyze *trans*-chromosomal exchange (Box 1 and Figure 1A). Recombination occurs between the two chromosomes specifically during the G2 phase of the

Box 3 Weblinks for useful Bloomington *Drosophila* Stock Center pages for making mosaic tissues

FLP stocks
- Main FLP page: contains all generally useful FLP stocks
- Defined FLP variants: lines carrying known FLP variants
- CoinFLP: lines for expressing GAL4 and lexA in clones
- FINGR system stocks: enhancer trap FLP lines

FRT and FRT cassette stocks
- Main FRT page: lines carrying generally useful FRT-bearing chromosomes
- FRT cassettes: lines carrying FLP recombinase-removable FRT cassettes
- Dominant female sterile technique: lines for assessing gene function in the female germ line by clonal analysis using "ovo[D]" stocks.

Non-FLP/FRT recombination systems
- Alternate recombinase page: non-FLP/FRT systems, including B2, B3, KD and Cre
- B3RT cassettes: B3 recombinase-removable B3RT cassettes
- KDRT cassettes: KD recombinase-removable KDRT cassettes

cell cycle when sister chromatids are paired. Conversely, when two FRT sites are positioned in the same orientation on the same chromosome, FLP catalyzes *cis*-recombination and excision of the sequences between the two FRTs (Figure 1B).

Control of FLP expression

Since the inauguration of FLP/FRT as a tool in *Drosophila*, a variety of FLP transgenes have been created to precisely control the expression of FLP both temporally and spatially (Box 3). The heat shock promoter *hsp70^P* is commonly used to control pulses of FLP expression in response to modulations in temperature. Under this promoter, FLP is not expressed at 18° and remains low at 25°, but is highly expressed above 30° (Golic and Lindquist 1989; Solomon *et al.* 1991). Although the heat shock promoter induces FLP throughout the entire organism, recombination frequency and clone induction can be controlled easily by manipulating the duration of heat shock, the temperature (*e.g.*, 30 or 37°), or the stage of development. A heat shock early in development, when there are few cells, will yield a few clones that will be able to grow fairly large; a heat shock at a late stage, when tissues can have thousands of cells, will result in numerous, small clones (Struhl and Basler 1993). Recombination efficiency can also be varied by the method of heat delivery: for instance, a water bath tends to be more efficient at heat transmission than an air incubator, and therefore a shorter heat shock is required to achieve the same result (Golic 1993). Moreover, the expression of FLP can be restricted spatially by coupling it to tissue-specific promoters. For example, *eyeless-FLP*, which allows clones to be generated specifically in cells where the *eyeless* promoter is normally active, has been particularly useful because mutant eyes can be generated while the rest of the animal remains wild-type and thus viable (Newsome *et al.* 2000). Expressing FLP under GAL4/UAS (upstream activating sequence) control (see Figure 2) is another way to increase the flexibility of FLP-mediated recombination.

Below, we discuss FLP/FRT-mediated *cis*- *vs.* *trans*-recombination (also respectively called intra- or interchromosomal recombination), in separate sections for clarity.

Trans-Recombination

FLP/FRT has been used extensively in *trans*-chromosomal recombination to analyze recessive embryonic or larval lethal mutations, and in experiments where the focus of action of a mutation—the question of cell autonomy—needed to be addressed. The majority of *Drosophila* genes can be studied by clonal analysis because FRTs have been integrated via *P*-element insertions close to the centromere of each major chromosome arm (Chou and Perrimon 1992; Xu and Rubin 1993), and fly strains with these integrations are readily available at stock centers. Placement of the FRTs near the centromere allows FLP-induced recombination to occur along each arm, carrying along with it any distally-located mutant gene. To generate clones via FLP-FRT-mediated *trans*-recombination, FRT sites should be located at the same position on two homologous chromosomes (Figure 1A). FLP recognizes the FRT sequences on sister chromatids during G2 phase of the cell cycle and mediates recombination. Chromosome segregation during mitosis then results in two recombinant daughter cells of two different genotypes (Figure 1A). Notably, chromatid segregation can occur in one of two ways: X segregation, where the recombinant chromosomes segregate into the two daughter cells, giving rise to twin spots; and Z segregation, where they segregate to the same cell. In this latter case, the genotype of each daughter remains the same as the parental cell and thus they are indistinguishable. In *Drosophila*, X segregation occurs at a higher frequency than Z segregation (Pimpinelli and Ripoll 1986; Blair 2003), increasing the likelihood that twin spots will be recovered after recombination; however, the possibility of Z segregation should be kept in mind.

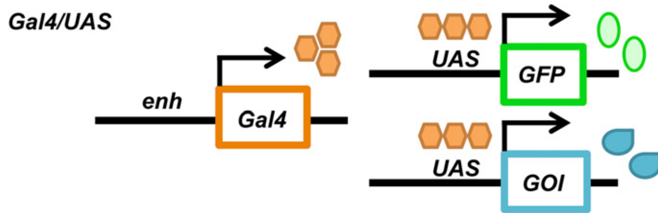


Figure 2 *The Gal4/UAS* system. Flies carry an *enhancer-Gal4*, *UAS-GFP*, and *UAS-GOI* (gene of interest) transgenes. When the enhancer is active, Gal4 is expressed and binds the Upstream Activating Sequence (UAS), leading to the expression of both GFP and the gene of interest (GOI).

Twin-spot clones

The generation of twin-spot clones remains an important example of a method that involves *trans*-chromosomal recombination. A widely used refinement of the twin-spot method is to use *Drosophila* strains in which a fluorescent gene under control of a ubiquitously expressed promoter (*e.g.*, ubi^{P}*-GFP*) is combined with an FRT chromosome and used in *trans* to the FRT labeled with (or without) another marker. Such lines are widely available in the community, making it possible to study clonal behavior in all stages of development (Box 3). As an example, crossing flies that carry a mutation of gene X on an FRT 40A chromosome to flies expressing $hsp70^{P}$*-FLP* and carrying ubi^{P}*-GFP* on an FRT40A chromosome that is wild-type for X, will yield progeny that are *trans*-heterozygous for the X mutation and for ubi^{P}*-GFP*. In mitotically active cells, when a heat shock is applied, FLP is expressed, resulting in recombination between the two FRTs (Figure 1A). After chromosome segregation, two genotypically distinct daughter cells are generated. One cell will be homozygous mutant and lack GFP expression, distinguishing it from its sister cell that possesses two copies of ubi^{P}*-GFP* (in Figure 1A, illustrated as red *vs.* green cells). Cell proliferation then generates “twin” marked clones of cells of each genotype that are easily identified by GFP expression. As illustrated in Figure 1A, the recombinant, double ubi^{P}*-GFP*-positive cells (green) can also be distinguished from nonrecombinant cells that retain one copy of ubi^{P}*-GFP* by the different intensity of the fluorescent signal (yellow).

The power of the twin-spot method lies in the fact that an internal control clone is always generated, in the same event, next to the mutant clone. Twin spots allow a direct and reliable comparison between mutant and wild-type clones, and the size of the wild-type twin can serve as a reference for comparison with the homozygous mutant clones. Studies of growth regulation have long relied on the twin-spot method, as clone size provides insight into how a mutation affects cell and tissue growth (Neufeld *et al.* 1998; Johnston *et al.* 1999). When mutant clones are smaller than their wild-type twin clones the gene likely functions as a positive growth regulator. Conversely, the gene is probably a negative growth regulator when the mutant clones are larger than their wild-type twins. The use of mosaic analysis as the basis of genetic screens was critical for the identification of new growth regulators that ultimately elucidated the Hippo growth regulatory pathway (Moberg *et al.* 2001; Tapon *et al.* 2001, 2002; Wu *et al.* 2003; Huang *et al.* 2005).

Twin-spot generator

Clone-marking methods such as ubi^{P}*-GFP* were improvements over adult cuticle markers, and allowed analysis of positively marked clones *in situ* during growth of imaginal discs and other tissues. However, a disadvantage was that one of the twin clones contained no marker—was negatively marked—after recombination. Such negatively-marked clones can be difficult to distinguish, especially in highly folded tissues. An ingenious FLP/FRT-mediated strategy that independently labels each sibling clone was developed by Griffin *et al.* who named it “Twin Spot Generator” (TSG) (Griffin *et al.* 2009). The TSG method utilizes chromosomes carrying hybrid sequences encoding partial, complementary fluorescent proteins [*e.g.*, the C-terminus of enhanced GFP (EGFP) and the N-terminus of monomeric red fluorescent protein (RFP)1], flanking an FRT site placed where an intron is normally located. Mitotic recombination is required for the hybrid coding sequences to be reconstructed, transcribed, and spliced to produce fully functional proteins, thus generating sibling clones marked with either GFP or RFP. Since chromosomal crossover is required to link the hybrid coding sequences, all nonrecombinant cells remain unlabeled (Griffin *et al.* 2009). An advantage of this method is that due to the fluorescent proteins, it permits immediate detection of clones, thus allowing clones to be followed from the first cell division after clone induction.

Mitotic clones in the female germ line

The generation of mitotic clones in the female germ line allows the genetic analysis of the maternal role of genes in oogenesis and embryogenesis, but recessive lethal and dominant female-sterile (DFS) mutations cause arrest of early embryogenesis in females, rendering them unable to lay eggs (Wieschaus 1980; Garcia-Bellido and Robbins 1983; Perrimon and Gans 1983). Chou and Perrimon took advantage of the X-linked DFS ovo^{D1} mutant to devise a way to study the role of maternal gene products (Chou and Perrimon 1992). Since males are unaffected by ovo^{D1} mutations, they are used as carriers of the mutation, thus by coupling the ovo^{D1} mutation to an FRT-bearing X chromosome germ line clones could be generated for studying the maternal effect of homozygous lethal mutations. In the “FLP-DFS” technique, males carrying the ovo^{D1} FRT^{101} chromosome are crossed with females carrying the FRT^{101} linked to a recessive lethal zygotic mutation (*m*). Once $hsp70^{P}$-FLP expression is induced by a brief heat shock, recombination between the FRT sites generates homozygous ovo^{D1} daughter cells, which die, and also homozygous *m* mutant daughter cells that survive and produce germ line clones. Nonrecombinant heterozygous ovo^{D1} mutant cells also die because of the mutation. This powerful tool has been used to study the effect of essential maternal genes for the development of the oocyte and embryo, and allowed genetic

screens that would identify X-linked genes required for these early stages of development (Chou and Perrimon 1992). Later, the FLP-DFS technique was extended to the autosomes through insertion of a *P*-element containing the *ovo*^{D1} mutation on each autosomal chromosome arm, greatly expanding its versatility (Chou and Perrimon 1996).

The Minute technique

Minute mutants constitute a class of ~50 mutations that affect ribosomal protein genes (Marygold *et al.* 2007). Most *Minutes* (*M*) lead to developmental delay and thin bristle defects in heterozygous mutants ($M^{+/-}$), whereas $M^{-/-}$ is cell lethal. Clones of $M^{+/+}$ (*i.e.*, wild-type) cells generated in heterozygous $M^{+/-}$ animals have a significant growth advantage and can take over large territories within the tissue.

This property was exploited by Antonio Garcia-Bellido and his students in their studies of Engrailed function, and led to the seminal discovery of developmental compartments, the fundamental units of development (Garcia-Bellido *et al.* 1973, 1976). Using markers such as *y* and *f* to mark $M^{+/-}$ cells in mosaic animals, Gines Morata and Pedro Ripoll, then PhD students in the Garcia-Bellido laboratory, observed that the $M^{+/-}$ cells were eliminated from the tissue due to active competition with $M^{+/+}$ cells (Morata and Ripoll 1975). They named this phenomenon "cell competition" and experiments from several groups ensued to investigate the properties of the competitive elimination of cells (Morata and Ripoll 1975; Simpson 1979) [discussed in detail in Alpar and Johnston (2017)].

$M^{+/-}$ mutants have also been used in what is known as the "Minute technique." In cases where a mutation results in very small or no clones, an elegant trick is to put it in a $M^{+/-}$ background. This gives the mutant cells a relative growth advantage, and can allow weak but otherwise viable cells to survive and form a clone. Generation of *Notch (N)* mutant clones serves as an illustrative example. Cells homozygous for *N* mutations do not survive. However when a *N* mutation is combined with $M^{+/+}$ in a $M^{+/-}$ background, the *N*+ $M^{+/-}$ cells are at a disadvantage, allowing the *N* mutant cells to survive and form clones for analysis (Portin 1980).

The mosaic analysis with a repressible cell marker (MARCM) system

The need for a positive cell label to trace cell lineages and at the same time provide a means for manipulating gene expression in those cells led to the invention of MARCM (Lee and Luo 1999, 2001). MARCM employs the Gal4/UAS system (Figure 2) and also the Gal4 repressor Gal80 (Lue *et al.* 1987; Suster *et al.* 2004), to specifically mark one of the sibling clones of a recombination event while leaving the remaining cells unmarked (Figure 3). Gal4, a yeast transcriptional activator whose activity is inhibited by Gal80, functions in *Drosophila* as a transcriptional activator of any gene positioned downstream of UAS elements (Fischer *et al.* 1988) and is extensively used for conditional gene expression (Brand and Perrimon 1993) (Figure 2). The advantage of MARCM over a typical twin-spot analysis is that it allows expression of a Gal4-regulated (UAS) transgene only within the marked clone, while the transgene is repressed in all other cells.

A typical genetic set up for a MARCM experiment consists of flies carrying an inducible FLP, a *Gal4* transgene driven by a promoter, such as *actin*, a *UAS-GFP* (or any other fluorescent marker), and FRT chromosomes heterozygous for a mutation in a gene of interest. The presence of a constitutively expressed *Gal80* distal to one of the FRTs will determine which sibling clone will express *UAS-GFP* (Figure 3A). The *FLP*, *Gal4*, and *UAS-GFP* are generally located on other chromosomes, to remain unlinked to the FRTs. Upon induction of FLP expression, the two FRT chromosomes recombine. As mitotic recombination requires exchange between sister chromatids during the G2 phase of the cell cycle, MARCM is limited to proliferating cells. Chromosomal segregation then generates two different daughter cells: one cell expresses two copies of Gal80, thereby inhibiting Gal4 activity and keeping the cell unlabeled (Figure 3A'). The other daughter cell does not express Gal80 and consequently Gal4 is active and drives expression of the marker. Placement of *Gal80* on the FRT chromosome that carries the mutation of interest prevents *UAS-GFP* expression in homozygous mutant clones, but allows *GFP* expression in the homozygous wild-type clones. Likewise, placement of *Gal80* on the wild-type FRT chromosome yields *GFP*-expressing homozygous mutant clones (Figure 3A").

The MARCM system lends itself well to powerful variations. It is easily adapted for clonal expression of a UAS-regulated transgene, either as a way to overexpress a particular gene or to knock it down with a UAS-RNAi (RNA interference). In addition, by adding a constitutively expressed or inducible marker, such as another fluorescent protein or *CD2*, to the *Gal80*-carrying FRT chromosome, both sibling clones resulting from a recombination can be positively marked (de la Cova *et al.* 2004). Positive marking of both sibling clones allows direct comparison of differences in their size and shape, for example, which has been illuminating in studies of growth and cell competition (de la Cova *et al.* 2004, 2014); a double repressor method, called twin-spot MARCM, was also developed to allow developmental tracking of single neurons and distinct neuronal populations (Yu *et al.* 2009). Here, loss of either one or the other of the two repressors by a daughter cell allows for differential marking. These methods have proven to be effective tools for a variety of different research questions in larval and adult tissues.

Notes on the use of Gal80 in mosaics

The combined use of Gal80 and the Gal4/UAS system is an extremely useful addition to the *Drosophila* genetic toolkit, especially when temporal control of gene expression is an important aspect of mosaic generation. In the context of the MARCM technique, temporal control of Gal80 (and thus Gal4-regulated gene expression) can be achieved by regulating FLP induction. However, in this case, functional repression by Gal80 can be only released—but not induced—in a

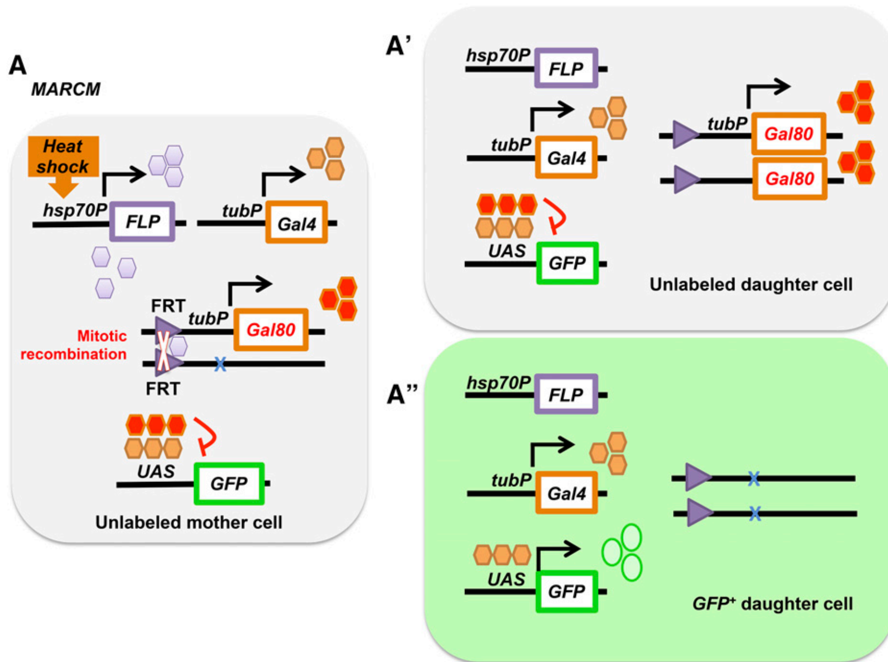


Figure 3 The MARCM system. (A) The mother cell carries *hsp70FLP* and *Gal4* coding sequences under the control of two different promoters, a *UAS-GFP* to mark cells and FRT sites on the two homologous chromosomes that carry either the mutation of interest (depicted by a blue X) or *Gal80*. Gal4 cannot drive GFP expression because of the inhibitory activity of Gal80. Two daughter cells are generated after heat shock induction and FLP-mediated recombination. (A') One cell contains two copies of *Gal80* and consequently has no GFP expression. (A") The sibling cell does not inherit *Gal80* and consequently Gal4 is transcriptional active, driving the expression of the marker UAS-GFP. Thus, homozygous mutant cells are labeled, whereas wild-type and heterozygous mutant cells remain unlabeled. MARCM, mosaic analysis with a repressible cell marker; UAS, upstream activating sequence.

temporally controlled manner, and only in the daughter cells in which copies of the *Gal80* gene have not been inherited. Gal80 is also useful in FLP-out experiments, since control of its induction is possible. In one variation of the FLP-out method, Gal80 can be constitutively turned on or off in FLP-expressing cells using $tub^P > stop > Gal80$ (FLP-in) or $tub^P > Gal80 >$ (FLP-out) (Zecca and Struhl 2002; Gordon and Scott 2009; Bohm *et al.* 2010). Temporal regulation can also be achieved by linking Gal80 to promoters that are active at a specific stage of development. Still, inherent in these uses of Gal80 is its perdurance, which prevents rapid release of Gal4 inhibition. Gal80 can perdure as long as 40 hr after recombination in MARCM experiments in imaginal discs (de la Cova *et al.* 2004), so experiments need to be designed with this in mind. A widely used method for rapid temporal modulation of Gal4 activity is through a temperature-sensitive *Gal80* allele ($Gal80^{ts}$) that is active and represses Gal4 at 18° but is inactive at 29° (McGuire *et al.* 2004). This feature allows fairly tight and reversible temporal control over overexpression or knockdown experiments by a simple shift of the incubation temperature from 18 to 29° at any stage of development.

Cis-Recombination

The FLP-out technique

In the 1990s, a significant innovation arose from the use of the FLP/FRT system in the fly to recombine DNA sequences in *cis*. When FRT sites are oriented in the same direction and located at two distinct locations within the same chromosome or segment of DNA (Figure 1B), FLP catalyzes the excision of the DNA in between the FRT sites. This technique, known as "FLP-out," is widely used to generate clones of cells—without a requirement for a specific cell cycle phase—that express a gene or RNAi at a specific time or in a specific tissue. The FLP-out method was developed by Gary Struhl and Konrad Basler as a way to test the idea that Wingless (Wg) functions as a pattern-organizing morphogen in the wing disc (Struhl and Basler 1993). They took advantage of the knowledge that when two FRTs are placed as direct repeats on the same DNA segment, FLP will catalyze the excision of the region between them and joining of the resulting DNA ends (Golic and Lindquist 1989) (Figure 1B). The original constructs were designed so that the *actin5C* promoter (act^P) was separated from the *wg* coding sequence by a FRT-flanked "STOP cassette," consisting of the *Draf* gene (used as an inert spacer) followed by transcriptional termination sequences to prevent expression of *wg* (Struhl and Basler 1993). A short, heat shock-induced pulse of $hsp70^P$-driven *FLP* expression led to excision of the STOP cassette and allowed expression of the *wg* gene. Since the FLP-out event is heritable, after cell division each of the cell's progeny also expressed *wg*. This technique allowed Struhl and Basler to demonstrate that when expressed in a clone, *wg* "organized" the nearby tissue, non-autonomously patterning cell fates (Struhl and Basler 1993). Later, the method was adapted for similar studies of Hedgehog (Hh), and the FRT sites were shortened to make them slightly less efficient, giving additional control to the FLP-out process (Senecoff *et al.* 1988; Basler and Struhl 1994).

The FLP-out method was subsequently combined with the *Gal4/UAS* system to make it broadly useful (Pignoni and Zipursky 1997). The FLP-out Gal4 system (Pignoni and Zipursky 1997) allows the induced clonal expression of *Gal4* and, accordingly, expression of any UAS-regulated transgene (Figure 4). The typical FLP-out cassette consists of a ubiquitously expressed promoter (*e.g.*, act^P), a cell marker [a commonly used one is the rat CD2 coding sequence (Dunin-Borkowski and

Brown 1995)] followed by a transcription termination (STOP) sequence; this is then flanked by FRT sequences (annotated $act^P > CD2^{STOP} > Gal4$, where > denotes FRT)] (Box 2). In the absence of FLP activity, CD2 is expressed under the control of act^P, but the STOP sequence prevents expression of Gal4 (Figure 4). In the presence of FLP, stochastic FRT-mediated recombination and excision of the $CD2^{STOP}$ cassette occurs, allowing *Gal4* and *UAS-GFP* expression (Figure 4). The *Gal4/UAS-GFP*-expressing cell and its progeny are permanently labeled by GFP expression, providing a positive marker of the clone. These clones also lack expression of CD2, providing a negative clonal marker, whereas all cells that have not undergone recombination still express it (Figure 4).

The FLP-out Gal4 system has become an extremely powerful and avidly used tool for conditional, heritable regulation of gene expression in clones in *Drosophila*. In addition to UAS-GFP, the presence of other UAS-regulated transgenes in the animal allows gene expression to be manipulated in the cells within the clones. Control of FLP expression by the $hsp70^P$, which can be expressed in all cells, allows clone number to be easily optimized for different tissues, as described above. However, it is possible to restrict clone generation to certain tissues by putting FLP expression under the control of tissue-specific enhancers. Gene expression can also be restricted from specific tissues by including the Gal4 inhibitor Gal80 under the control of a tissue-specific enhancer. A further useful innovation of Gal4-regulated expression is Split-Gal4 (Luan *et al.* 2006), in which the Gal4 coding sequence is split into two modules, with each fused to a leucine zipper (Zip+ or Zip−) motif, which will create strong heterodimers with each other [for example, Gal4 DNA-binding domain (DBD)-Zip- and Gal4 transcription activation (TA) domain-Zip+ fusions]. Each Gal4 domain-Zip fusion is expressed under the control of a different promoter/enhancer, thus fully functional Gal4 is reconstituted only when the modules' expression overlaps and they heterodimerize via their Zip− and Zip+ motifs. The Split-Gal4 system can be a powerful way to restrict gene expression to exquisitely refined regions. However, a disadvantage of this system is that existing Gal4 driver strains cannot be used. Making Split-Gal4 strains is potentially a time consuming endeavor as each must be derived *de novo*. Moreover, with the addition of motifs to enhance transcriptional activation (*e.g.*, VP16 and p65), the Gal80-binding region was eliminated in many Split-Gal4 TA-Zip fusions, making these unresponsive to Gal80 suppression and limiting their versatility (Luan *et al.* 2006; Pfeiffer *et al.* 2010). A way around the latter problem was introduced with the "Killer Zipper" (KZip+) system (Dolan *et al.* 2017), which consists of a dominant-negative repressor of Split-Gal4 activity that is expressed under the control of a third promoter/enhancer . Expression of KZip+ (Gal4DBD-Zip+) in cells containing Split-Gal4 components effectively prevents reconstitution of functional Split-Gal4 due to dominant homodimerization of KZip+ with the Gal4DBD-Zip- module; the Gal4DBD homodimers can also swamp out UAS binding by functional Split-Gal4 heterodimers. The former caveat was addressed with a Split-LexA system called G-MARET (Gal4-based Mosaic-inducible And Reporter-exchangeable Enhancer Trap), which makes use of existing Gal4 driver lines (Yagi *et al.* 2010). In G-MARET, any existing Gal4 reporter can be switched to a LexA TA, and as a consequence, genetic mosaics can be generated that are composed of cells expressing either Gal4 or LexA TA in a tissue-specific manner. Further details of the Split-Gal4 and Split-LexA methods can be found in the original publications.

FLP-out modifications and alternative recombinase enzymes

Over the years, the FLP-out method has been modified in several ways that extend its uses. Prominent examples are the integration of FLP-out technology with the Q and LexA/lexO systems. The Q system is a binary expression system from the *Neurospora qa* (Q) gene cluster (Potter *et al.* 2010), in which a DNA element (*e.g.*, gene-X) is transcriptionally regulated by the activity of a QUAS sequence. When a promoter drives the expression of QF, a transcription factor that binds QUAS, the transcription of a QUAS-responder gene is promoted. QS, a repressor of QF activity, can also be expressed, via the activity of a different promoter. The Q system can be used with or instead of the Gal4/UAS system. Similarly, the LexA transcriptional system (Yagi *et al.* 2010) takes advantage of the bacterial transcriptional activator LexA, which binds the *LexA operator* sequence (*lexO*) to drive the transcription of a gene of interest. The Gal4/UAS, QF/QUAS, and LexA/lexO systems can be combined to generate, mark, and manipulate different populations of cells within the same animal, greatly expanding the repertoire of uses for the FLP-out system.

Construction of mutant variants of the FRT sequence (*e.g.*, FRT3, in which minor alterations in the spacer element eliminate cross-reactivity with FRT) have provided increased control over FLP-mediated recombination (Schlake and Bode 1994). Furthermore, in addition to FLP, several other site-specific yeast recombinases have been adapted for targeted recombination in *Drosophila* (Nern *et al.* 2011). These recombinases include KD from *Kluyveromyces drosophilarum*, R from *Zygosaccharomyces rouxii*, B2 from *Z. bailii*, and B3 from *Z. bisporus* (Araki *et al.* 1985; Toh-e and Utatsu 1985; Chen *et al.* 1986; Utatsu *et al.* 1987). Each of these recombinases recognizes a unique and specific sequence (KDRT, RSRT, B2RT, and B3RT, respectively) and catalyzes recombination at high frequency. They have low toxicity in flies and, importantly, do not cross-react with FLP or each other (Nern *et al.* 2011). The recombinases can thus be coexpressed, permitting orthogonal manipulation of multiple recombinases and recognition target sites. For example, FLP/FRT and KD/KDRT pairs can be combined in the animal and used with tissue- or cell-specific Gal4 drivers to sequentially refine expression in particular cell lineages (Awasaki *et al.* 2014; Nern *et al.* 2015). The principles of such toolkit intersection underlies some of the most powerful multicolor cell-labeling techniques.

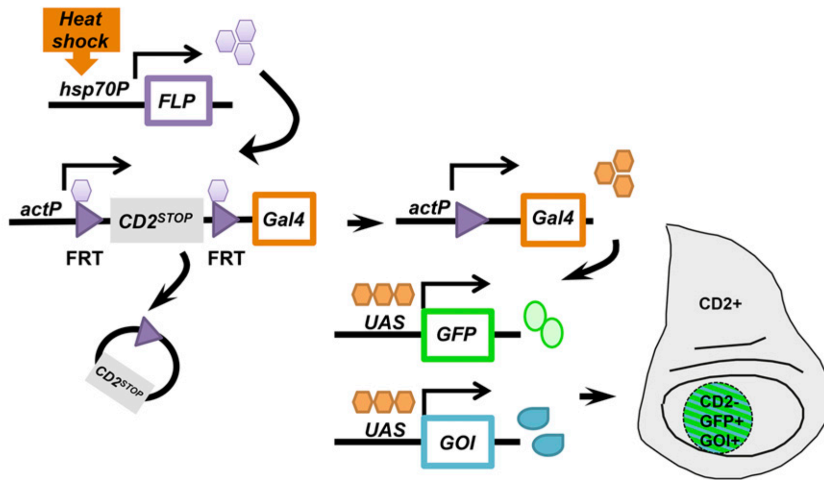


Figure 4 The FLP-out Gal4 technique. Schematics of the genetics of the offspring of a cross: the animal carries a *hsp70-FLP*, a transgene containing the *actin* promoter followed by a FRT-flanked cassette containing the coding sequence of the marker protein CD2; downstream of the FRT cassette is the *Gal4* coding sequence. *UAS-GFP* and *UAS-GOI* are also present in the genome. After heat shock, FLP expression is induced and it causes recombination between FRT sequences and excision of the CD2 cassette. Consequently the *Gal4* coding sequence comes under the control of the *actin* promoter, and Gal4 is expressed. Gal4 binds UAS driving the expression of both GFP and the GOI. This generates clones of cells overexpressing the GOI that are marked by GFP and by the absence of CD2. GOI, gene of interest; UAS, upstream activating sequence.

"Memory" experiments and G-TRACE

Drosophilists can take advantage of hundreds of Gal4 lines for targeted gene expression, where Gal4 is expressed under the control of either known or unknown tissue- or region-specific enhancer sequences (Brand and Perrimon 1993; Duffy 2002) (see, for example, the Gal4 section of the Bloomington *Drosophila* Stock Center, http://flystocks.bio.indiana.edu/). However, a major caveat to their use is that gene expression regulated by these enhancers can vary both temporally and spatially depending on the tissue or stage of development. This can be problematic if gene expression in a specific tissue or at a specific time in development is desired. After the FLP-out system was developed, it was quickly appreciated that it could be used to track enhancer expression dynamics and identify cell lineages during development (Ito *et al.* 1997; Weigmann and Cohen 1999; Jung *et al.* 2005; Wu and Johnston 2010). As an example, combining *ptc-Gal4*, an enhancer trap insertion that is expressed in all cells that express the *patched* gene, with a *UAS-FLP* and the $act^P > CD2^{STOP} > Gal4$ FLP-out cassette, will permanently label every cell that ever expresses *ptc*, past or present, effectively preserving a memory map of *ptc-Gal4* spatial expression. Memory experiments provide a visual record of where different enhancers are expressed during development.

Despite the utility of such memory experiments in providing a snapshot of ongoing and transient expression patterns from a given Gal4 line, they do not differentiate past Gal4 expression from present, real-time Gal4 expression. To address the problem of temporal information, the University of California, Los Angeles Undergraduate Research Consortium in Functional Genomics developed G-TRACE (Evans *et al.* 2009), a labeling method that combines real-time analysis with the memory marking system to provide information about temporal and spatial expression of a Gal4 driver (Figure 5). G-TRACE is based on the original memory methods using enhancer-Gal4 lines, but adds a second level of control. First, Gal4 binds *UAS-RFP* and *UAS-FLP* and drives their expression simultaneously. FLP then excises the FRT-flanked transcriptional STOP cassette that separates the *ubi* promoter from the *GFP* open reading frame (Figure 5). This event allows GFP expression to be maintained in all progeny of the cell that underwent FLP-mediated recombination. Conversely, RFP is only expressed in those cells that express Gal4 at the moment of visualization. G-TRACE analysis therefore reveals both the current and previous expression patterns of individual enhancers, with the fluorescent "color code" defined as follows: red marks cells in which the promoter is active in real-time; yellow identifies both real-time expression and lineage expression, meaning that the cell expresses the gene of interest at the moment of the analysis and in an antecedent time; and green identifies cells that previously expressed the gene of interest, providing lineage-tracing information (Evans *et al.* 2009).

CoinFLP

The need to conduct genetic mosaic screens in which a reliable ratio of mutant to nonmutant cells would be generated drove the development of the CoinFLP method (Bosch *et al.* 2015). This method makes use of a FLP-out cassette containing alternating canonical FRT and FRT3 sites, which are chosen randomly by the FLP recombinase to yield one of two outcomes (Bosch *et al.* 2015). Since each FRT type is capable of recombining with its own type but not a different type, recombination is mutually exclusive: excision of sequences will only occur between canonical FRT sites or between FRT3 sites.

In CoinFLP, FLP recombinase is expressed under the control of a tissue-specific enhancer (*e.g.*, *ey-FLP* or *Ubx-FLP*). Within the FLP-out module, a STOP cassette is placed between a FRT site and a FRT3 site, followed by another FRT and another FRT3, and finally by the *Gal4* coding sequence. When FLP is expressed, recombination that occurs between the FRT sites generates a cell that expresses a Gal4 and any UAS-regulated gene of interest, while recombination that occurs between the FRT3 sites generates a cell that does not express Gal4 and thus no transgenes. Due to the arrangement of FRT and FRT3 sites, when FRT3 sites recombine the intervening FRT site is excised and subsequent FLP/FRT-mediated events are prevented. The

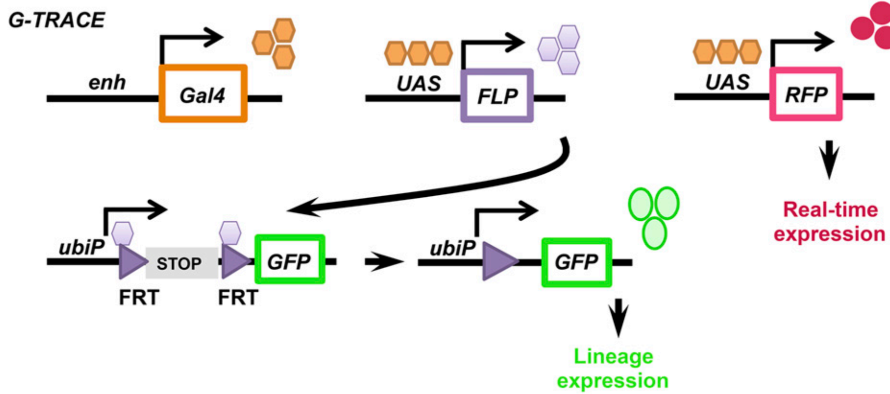


Figure 5 G-TRACE. This method establishes a "memory" of expression from the *enhancer-Gal4* throughout the life history of the animal, and is useful for determining where an enhancer is expressed through all stages of development. The genetic toolbox of the G-TRACE method is shown. Gal4 is expressed under the control of an enhancer of choice. When the enhancer is active, Gal4 is expressed and drives the expression of both *UAS-RFP* and *UAS-FLP*. FLP then mediates recombination between the FRTs and excision of the STOP cassette in a FLP-out reaction. Consequently, *UAS-GFP* is constitutively expressed under the control of a ubiquitously active promoter (*e.g.*, the ubiquitin promoter) in that cell and all of its progeny. RFP, red fluorescent protein; UAS, upstream activating sequence.

combination of the stochastic and mutually exclusive nature of the FLP recognition of FRT sites makes recombination similar to the 50–50 chance of a "coin toss" (Bosch *et al.* 2015). The tissue-specific expression of FLP allows clones to be restricted to certain tissues and, depending upon the onset of expression, can lead to large clones amenable to screens using gene overexpression or knockdown.

An even more powerful tool, based on the CoinFLP system, is CoinFLP-LexGAD/Gal4 (Bosch *et al.* 2015). Here, the STOP cassette is followed downstream by one canonical FRT site, the *LexGAD* coding sequence, one FRT3 site, and the Gal4 coding sequence. After differential recombination, daughter cells will express either LexGAD or Gal4: the former will bind to LexAop sequences driving the expression of downstream sequences, similarly to the Gal4/UAS system. The ladder binds the UAS driving downstream expression. One daughter cell will express a fluorescent marker whereas the other cell will express a different fluorescent marker. Furthermore, the combination of the CoinFLP-LexGAD/Gal4 system with the GFP Reconstitution Across Synaptic Partners system (Feinberg *et al.* 2008; Gordon and Scott 2009) allows the visualization of the boundary between two clonal populations: in addition to the specific fluorescent marker, both LexAop and UAS drive the expression of a different portion of a "split" GFP, where the two components reconstitute at the interface between the two populations (Bosch *et al.* 2015).

Colorful mosaics: Brainbow, Raeppli, TIE-DYE, and MultiColor FlpOut (MCFO)

Tracking cell lineages over long periods of time can be extremely challenging. MARCM permits a variety of strategies to label individual groups of cells with different markers; however, only a portion of cells of a given tissue can be labeled with these techniques, and information about the unlabeled cells is not easily deduced. Several new strategies have been devised to surmount this problem.

The first such labeling tool reported, Brainbow, is a stochastic site-specific recombination-based system in which different fluorescent proteins are deployed depending on selection of a series of loxP sites (Livet *et al.* 2007). This technique and variants of it have been extensively used in mammalian models to differentially label multiple cell lineages simultaneously in a variety of tissues. *Drosophila* versions of Brainbow were subsequently developed, including Flybow (Hadjieconomou *et al.* 2011) and dBrainbow (Hampel *et al.* 2011). Both methods were created using modifications of the original Brainbow strategy, and are compatible with intersectional strategies that use traditional FLP/FRT and Gal4-, Q-, and LexA-based systems for even greater sensitivity and control (Hadjieconomou *et al.* 2011).

TIE-DYE (Worley *et al.* 2013) can be considered the first whole-tissue-labeling tool, since it will successfully mark most cells in a target tissue or organ (Figure 6). TIE-DYE takes advantage of FLP-out methodology and is comprised of an inducible FLP and three constructs, each with an independent FRT-flanked STOP cassette that is preceded by a ubiquitously active promoter (ubi^P, tub^P, and act^P are commonly used) and followed by the coding sequence of *GFP*, *lacZ*, or *Gal4*. *FLP*-induced recombination leads to the excision of the STOP cassette, allowing *GFP*, *lacZ*, and *Gal4* to be expressed. These constructs can be combined with *UAS-RFP* and UAS responder transgenes of interest, leading to generation of clones of cells expressing *GFP*, *lacZ*, *Gal4*, or combinations of these cell markers (Figure 6, B and C). Gal4 activates expression of a cell marker (RFP) and also potentially of a gene of interest (GOI). Overexpression or downregulation of a GOI is therefore marked in red. As with traditional FLP/FRT technology, the longer the heat shock, the more excisions are generated and therefore more combinations will be possible. A benefit of this technique is that it permits estimation of growth rates in different regions of tissues in conjunction with genetically-induced perturbations (Figure 6, B and C).

Another appealing whole-tissue-labeling tool is Raeppli (Kanca *et al.* 2014), which generates a combination of fluorescent markers (Figure 7). The combinatorial complexity depends on the number of used copies of the Raeppli construct present in the genome. Similar to TIE-DYE, Raeppli allows simultaneous analysis of perturbed and nonperturbed regions of the tissue. Whereas TIE-DYE requires fixation and thus is not suitable for live imaging, visualization of Raeppli

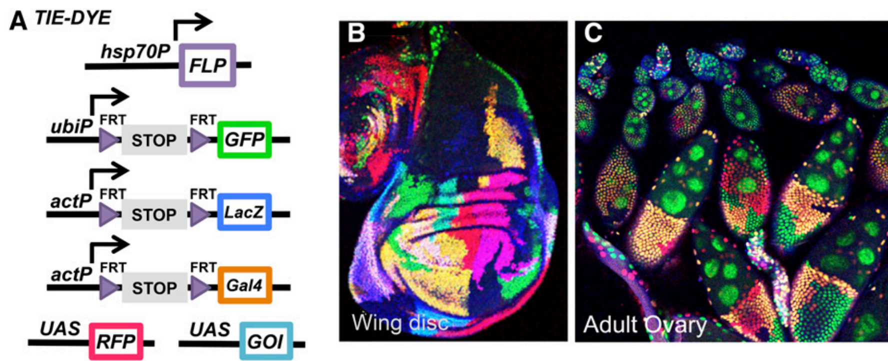


Figure 6 *TIE-DYE*. (A) In TIE-DYE, heat shock-driven expression of FLP mediates excision of three different STOP cassettes, allowing the expression of *Gal4*, *GFP*, *lacZ*, or a combination of those, depending on the severity and duration of the heat shock. Gal4 expression enables the activation of the UAS transgenes: *UAS-RFP* and *UAS-GOI*. Clones overexpressing the GOI are marked with RFP. If the transgene is an RNAi construct then its expression will result in the downregulation of the GOI. (B and C) The images show examples of clones in wing discs (B) and ovaries (C), taken from Worley *et al.* (2013). GOI, gene of interest; RFP, red fluorescent protein; RNAi, RNA interference; UAS, upstream activating sequence.

clones does not require fixation and staining. The efficiency of recombination is also higher than that observed with TIE-DYE.

Figure 7A depicts a simplified scheme of the Raeppli construct and its activation. Activation requires several sequential steps. First, FLP activity within a cell mediates excision of a stop cassette that prevents *integrase* expression (Figure 7B). The expression of *integrase*, driven by the activity of the full *hsp70*^{P}, then causes recombination between the attB and one of the attP sites. Following this event, the *integrase* is excised together with the coding sequence of one or more fluorescent proteins (Figure 7B). When Gal4 is expressed, UAS sequences allow expression of one of the fluorescent protein genes, each of which is followed by a stop codon. The progeny of each individual cell inherits and maintains the ability to express this same fluorescent protein, creating several individual clones marked with different colors (Figure 7C). LexA can also be used to induce the expression of one fluorescent protein gene, since it binds the LexO sequence that promotes downstream transcription (Yagi *et al.* 2010). In addition, because either Gal4 or LexA can be used, Raeppli works well in combination with other genetic manipulations. Finally, Raeppli's real power is that it is a combinatorial method: the use of multiple copies increases the number of color combinations that can be generated. For example, two copies of the Raeppli constructs produce 10 different color combinations, and it is also possible to use three copies.

Another tool for stochastic multicolor labeling of neurons, MCFO, uses smGFP ("Spaghetti Monster GFP") as a labeling tool that, like Brainbow, Flybow, and dBrainbow, takes advantage of the FLP/FRT system. SmGFP is a mutant, nonfluorescent form of GFP that retains the solubility, stability, and low toxicity of small fluorescent proteins while eliminating their problematic broad excitation and emission spectra (Viswanathan *et al.* 2015). smGFP essentially operates as a brightly fluorescent hyper-antigenic-labeling tool, by serving as a scaffold for multiple copies of a single-epitope tag that can be detected with commercially available antibodies (*e.g.*, HA, FLAG, MYC, V5, or OLLAS). The MCFO system is comprised of a FLP-out cassette controlling expression of smGFP (*>STOP > smGFP*) under control of 10× UAS, combined with a cell-type or tissue specific Gal4 driver. In the presence of FLP, the FLP-out cassette is excised, and Gal4/UAS activates expression of smGFP (which is directed to the membrane by a myristoylation signal). The combination of differentially-tagged smGFPs can be used in combination to obtain multicolor FLP-out to reveal cell shape and position, and allows tracking of neurons during development (Nern *et al.* 2015), but in principle could be useful in a variety of cellular contexts.

Additional Tools for Conditional Mutant Clonal Analysis

FLP/FRT-mediated recombination in all of its forms has clearly enhanced the *Drosophila* experimental toolkit, and provides numerous efficient ways to produce mosaic tissues. Even so, under some conditions using mitotic recombination to generate clones can be problematic. Since mitotic recombination requires DNA replication and chromosome segregation, it is restricted to mitotically-active cells. Furthermore, not all genes are readily analyzed by *trans*-recombination using the existing chromosomal FRT sites, which are located close to the centromere (thus allowing for most of the genes on a given chromosome to undergo recombination with its sister chromatid). Any gene that lies between the centromere and the FRT site, as well as genes located on the fourth chromosome, which contains no FRTs, cannot be analyzed. Three new FLP/FRT-based conditional gene disruption methods have been developed to circumvent these problems.

Mutant Analysis by Rescue Gene Excision (MARGE)

The MARGE technique is a newly developed tool for mosaic analysis that avoids these problems (Zhou *et al.* 2016). MARGE is an intrachromosomal recombination-based method in which an FRT-flanked FLP-out transgenic cassette rescues a homozygous mutation. The FRT-delimited cassette, which consists of the DNA sequence that rescues the homozygous mutation and a ubiquitously expressed fluorescent marker, is excised upon induction of FLP. Since MARGE does not require cell proliferation, this technique allows the study of the effect of mutations on already differentiated cells. In principle, the effect of mutations in any gene in the genome could be studied, even

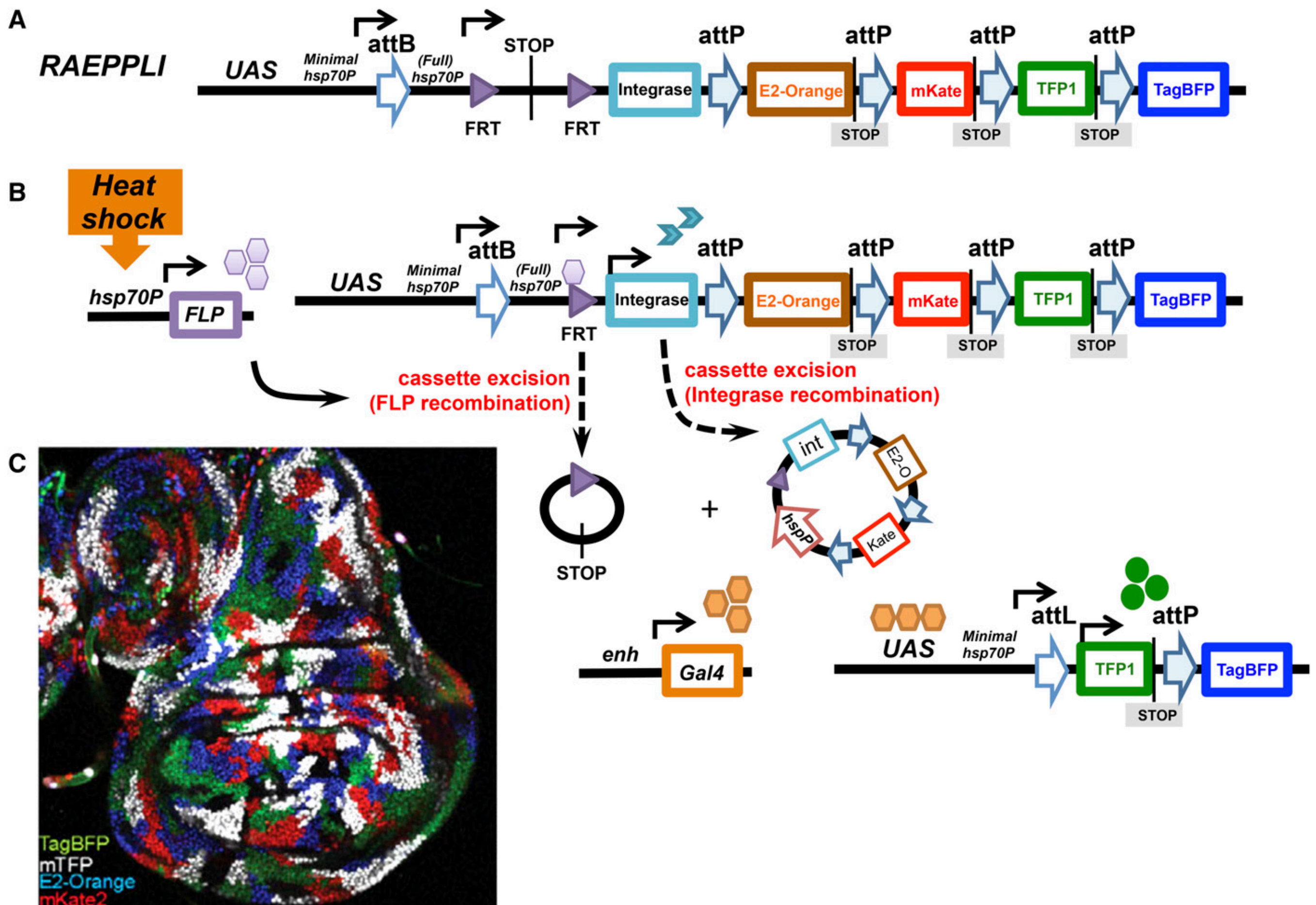


Figure 7 The Raeppli system. (A) A simplified version of the Raeppli construct is depicted. The UAS is followed by a minimal *hsp70* promoter, an *attB* site, a full *hsp70* promoter, and one FRT-flanked STOP cassette. Downstream, the *integrase* coding sequence precedes a series of four different fluorescent protein genes (*E2-Orange*, *mKate*, *TFP1*, and *TagBFP*) interspersed by attP sites. cassette. Downstream, the *integrase* coding sequence precedes a series of four different fluorescent protein genes (*E2-Orange*, *mKate*, *TFP1*, and *TagBFP*) interspersed by attP sites. (B) Upon heat shock, FLP-mediated recombination excises the STOP cassette, leaving *integrase* under the control of the (full) *hsp70* promoter. Integrase protein actively recombines *attB* and *attP* sites. Different fluorescent protein genes become expressed when Gal4 is present and are governed by the specificity of the genomic enhancer used. In the example, *E2-orange* and *mKate* are excised and TFP1 is expressed, becoming the cell marker. TagBFP is not expressed because of the presence of a stop signal downstream of the TFP1 coding sequence. The coding sequence of each fluorescent gene is followed by a stop codon. (C) An image of a Raeppli wing disc labeled with four markers, taken from Kanca *et al.* (2014). UAS, upstream activating sequence.

those located on the highly heterochromatic fourth chromosome, simply by inserting a rescuing cassette into the fly genome.

FlpStop

An equally useful method takes advantage of the almost 3000 intron Minos-mediated integration cassette (MiMIC) insertions in >1800 distinct genes produced by the Gene Disruption Project (http://flypush.imgen.bcm.tmc.edu/pscreen/index.php) to engineer conditional mutant alleles. "FlpStop" is a small construct designed to integrate into MiMIC insertions via φC31 integration and disrupt gene function in a cell type-specific manner with transcriptional and translational blocks (Fisher *et al.* 2017). The gene disruptive construct consists of a "FLEx-switch" convertible FRT-flanked cassette (Schnutgen *et al.* 2003) that contains transcriptional terminator sequences and a splice acceptor and stop codon (SA-STOP). Cell-type specificity is achieved with UAS sequences in front of the cassette, subjecting it to Gal4 regulation.

In contrast to FLP-out cassettes, in which FRT sites are in the same orientation (>STOP>) and embedded sequences are excised, the FlpStop cassette contains FRT sites that are in the opposite orientation (>STOP<), taking advantage of the ability of FLP to catalyze inversion of DNA sequences between inverted FRT sites (Golic and Lindquist 1989). Once the FlpStop cassette is integrated into a MiMIC insertion in the proper orientation, upon expression of FLP it is conditionally inverted ("flexibly switched") via FLP-FRT-mediated recombination, locking in the disruptive element and creating a loss-of-function allele (Fisher *et al.* 2017). Cells that also express Gal4 will express UAS-tdTomato, thus positively labeling the cells in which the cassette has been inverted. A real advantage of FlpStop is that both disrupting (D) and nondisrupting (ND) alleles can be made. When FLP is under control of a promoter such as *hsp70*^{P}, this

inventive method can also be used to generate conditional alleles: when put in *trans* to a null allele of the gene of interest and heat shocked at appropriate times, D alleles can be reverted to ND to rescue the mutant and yield information about genetic sufficiency, and ND can be reverted to D, inducing a mutant and allowing tests for genetic necessity.

Flip-Flop

Like FlpStop, the Flip-Flop strategy (Nagarkar-Jaiswal *et al.* 2017) allows mosaic analysis in postmitotic cells via the inversion of a Flip-Flop cassette that is induced by FLP activity. As described for FlpStop, the Flip-Flop cassette is integrated into a MiMIC insertion site and, when the cassette is in the disruptive orientation, generates mutant cells marked with mCherry. An advantageous feature of Flip-Flop is that it also marks wild-type cells with EGFP. The module containing the EGFP coding sequence has the opposite orientation of the module containing the mCherry coding sequence. The inversion of the Flip-Flop cassette is therefore marked with the loss of EGFP and the expression of mCherry. This method thus provides the added advantage of a broadly useful way to tag endogenous proteins in combination with the generation of mutant cells, independently marked, for clonal analysis (Nagarkar-Jaiswal *et al.* 2017).

Summary

Genetic mosaics provide an extremely powerful way to manipulate gene function and expression without adversely affecting the overall health or viability of the animal. We have discussed a variety of types of mosaicism and how mosaicism was harnessed to become an extraordinary experimental tool. Even after a century of its use in *Drosophila*, genetic mosaics remain an influential and extensively used technique in flies and, more recently, in mammalian model systems. As outlined above, each method for generating genetic mosaics was generally tailored to achieve a certain experimental purpose, but with intersectional approaches to combine different systems, exquisite sensitivity can be attained. The continuing explosion of new and innovative methods to generate and use genetic mosaics is a strong testament to its power as a research tool in biology.

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