Advanced Techniques for Cell Lineage Labelling in Drosophila

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Article Contents

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The ability to mark and genetically manipulate clonally related cells in live organisms is invaluable for investigating the mechanisms of tissue development, homeostasis and repair. A wide variety of techniques have been developed in *Drosophila melanogaster* for this purpose. These cell lineage labelling techniques range from simple methods for randomly marking cells to complex schemes for differentially labelling and genetically altering specific cells or more than one clone at a time. For example, coupled MARCM makes it possible to simultaneously label both halves of a cell lineage with positive markers; FINGR uses a combinatorial approach, using Gal4 and Gal80, to provide finer spatial control over clone induction; Flybow and Drosophila Brainbow increase the resolution and efficiency of clonal analysis through multicolour labelling; and G-trace differentially marks cells that currently express a driver from cells that expressed the driver in the past. These labelling techniques each have their own advantages and disadvantages. But together they create a powerful arsenal of tools for the study of many diverse topics in tissue biology.

Introduction

Lineage tracing has been a key approach to understanding tissue development, homeostasis and repair. The objective in lineage tracing is to establish the patterns of division and specification that a progenitor and its daughters undergo *in vivo*. If cells can be visualised in a live specimen or morphologically identified in a fixed specimen, then the lineage can be traced directly. For example, the entire cellular lineage of the *Caenorhabditis elegans* embryo was mapped by following the result of each cell division in live embryos by transmitted light microscopy (Sulston *et al*., 1983). When this is not possible investigators often apply lineage labelling, a technique which allows populations of clonally related cells to be identified. By analysing the patterns of labelled cells at multiple time points, it is often possible to identify the original progenitor of the labelled population and infer the position, birth order, and fate of each of the daughter cells during development. In addition, most lineage labelling methods also allow for genetic manipulations of the labelled cells, which makes it possible to determine the function of individual genes within specific cell types or lineages. The extensive array of genetic tools currently available for lineage labelling in *Drosophila melanogaster* is unmatched. Here we review the development of these techniques, with emphasis on a new generation of methods that allow for more sophisticated genetic manipulations and multicolour labelling. We also discuss the limitations of the current methods and how they may be overcome in the future. **See also**: *Drosophila* as a Model for Human Diseases

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Early Techniques for Lineage Labelling in Drosophila

Lineage labelling requires the ability to confer a cell of interest with a heritable marker. The earliest techniques relied on either the injection of soluable markers into developing tissues or endogenous genetically encoded markers. Locally injected markers included enzymes, such as horse radish perioxidase (HRP) (Technau and Campos-Ortega, 1987), and dyes, such as DiI (1,1′,di-octadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate) (Bossing and Technau, 1994). These markers are retained in daughter cells following mitosis, however they are diluted with each division so the signal becomes weak and nonhomogenous in mature clones. In addition, the microinjection of the marker can cause significant tissue damage. Alternatively, homozygous viable alleles of genes that cause recognisable phenotypes, such as hair (e.g. *multiple wing hairs*, *singed*), pigment (e.g. *ebony*, *yellow*), or eye colour (e.g. *white*) variations, were used as markers. Genetic markers have the advantage that they are heritable, and thus do not dilute with each cell division. However, each marker can only be visualised in tissues that expressed the phenotype and many

do not clearly delineate the boundary of clones (e.g. a hair marker could not distinguish a border in a region where there are no hairs) or are noncell-autonomous (e.g. *yellow* can be nonautonomous over a short distance) (Hannah, 1953). In addition, introducing these markers into specific cells was not trivial, requiring cell transplation (Illmensee, 1972) or the induction of rare genetic errors, such as chromosome missegregation during mitosis (Stern, 1936).

A particularly dramatic version of this method was the study of gynandromorphs, which occur when a blastocyst in a female (XX) embryo loses one X chromosome, becoming male (XO) (Janning, 1978). The result is a mosaic animal that contains both female and male cells. Gynandromorphs are normally extremely rare, but certain genetic backgrounds produce a significantly higher frequency of gynandromorphs. Studies of gynandromorphs used hair and cuticle markers on the X chromosome to track the XO cells and construct morphogenetic maps that depict cell lineage based on how frequently the two were separated by a mosaic border.

Mosaicism can also be achieved through mitotic recombination (Golic and Lindquist, 1989; Stern, 1936). Early methods used X-ray irradiation to induce double-stranded breaks, which were repaired through recombination between homologous chromosomes during mitosis. Following mitotic recombination, the portion of the chromosome distal to the site of recombination becomes susceptible to incorrect segregation. Thus, a cell that is heterozygous for a genetic marker distal to the site of recombination might divide to form one daughter that contains two copies of the marker and one that contains none. Following recombination, each daughter and all of its respective progeny will form a clone that is genetically distinct from the rest of the tissue. **See also**: Homologous Genetic Recombination in Eukaryotes

With these early methods for lineage tracing, experimenters had to choose between accurate cellular resolution and control in timing and cell-targeting of markers (afforded by injected markers) and sustained heritability (afforded by genetic markers). In the modern era, these issues have been overcome through the use of various genetic tools, including transgenic markers, such as LacZ or green fluorescent protein (GFP) to label cells, and control mechanisms, such as Gal4/Upstream Activating Sequence (UAS) or the Flippase (Flp)/FLP Recognition Target (FRT). **See also**: Genetic Engineering: Reporter Genes; Green Fluorescent Protein (GFP)

The New Genetic Tool Kit for Lineage Labelling

Transgenic cell markers

The ideal marker for fate mapping is cell autonomous, allows a cell to be visually distinguished from other cells, and genetically heritable. A number of transgenic markers have been developed that meet all of these requirements. They can be colorimetric (e.g. β-galactosidase), luminescent (e.g. luciferase) or fluorescent (e.g. GFP), for easy visualisation. Although luciferase and βgal are more quantitative, fluorescent markers tend to be the easiest to use and most versatile for lineage labelling (Arnone and Dmochowski, 2004). A major advantage of fluorescent markers is the ability to express and visualise them in live cells. **Table 1** lists the various fluorescent markers that have already been incorporated into Drosophila stocks and are publicly available. The most commonly used fluorescent markers are derivatives of GFP that differ in their photostability, brightness, and excitation/emission properties. Another commonly used fluorescent protein is the Red Fluorescent Protein (RFP) from the reef coral, Discosoma striata, and multiple variations of RFP have also been derived (Shaner *et al.*, 2005).

Table 1 Fluorescent reporters available in drosophila

Color of Fluorescence	Protein	Origin
Green	EGFP	*Aequoria victoria*
	Emerald	–
Yellow	EYFP	*Aequoria victoria*
	Venus	*Aequoria victoria*
Blue	ECFP	*Aequoria victoria*
	Cerulean	*Aequoria victoria*
Red	RFP	*Discosoma striata*
	mCherry	*Discosoma striata*
	Red Stinger	*Discosoma striata*
	Tomato	*Discosoma striata*
Green → Red	Kaede	*Trachyphyllia geoffroyi*
	Eos	*Lobophyllia hemprichii*
Green → None	*Dronpa*	*Pectiniidae* sp.

Recently, photoactivateable fluorescent markers, which are fluorescent proteins that change their spectral properties upon exposure to certain wavelengths of light (Lukyanov *et al.*, 2005), have been incorporated into Drosophila lineage tracing systems. These are useful because they allow the exact cell of interest, or even a specific subcellular compartment, to be marked at a precise moment in time. Furthermore, because fluorescence is activated by fluorescent light rather than mitotic recombination, cells do not need to be mitotic or be within the expression pattern of a known driver in order to be marked. The first photoconvertible molecules used in Drosophila, caged fluorescein (Vincent and O'Farrell, 1992) and DiI (Bossing and Technau, 1994), were injected into early embryos. Now photoactivateable fluorescent proteins are

available in transgenic Drosophila lines, including Kaede (Grueber *et al*., 2007), Eos (Mavrakis *et al*., 2009) and Dronpa (Ando *et al*., 2004). Kaede and Eos have similar spectral properties – they irreversibly convert from red to green fluorescence upon exposure to ultraviolet (UV) light. Kaede tends to be brighter, but Eos is smaller and more amenable to protein tagging. In contrast, Dronpa reversibly converts from a green to a nonfluorescent form upon exposure to blue light. One drawback of using photoconversion to label cells is that only mature proteins will be converted. Thus, the label will diminish with protein turnover and dilute with cell division.

Regulated gene expression

There are many tools in Drosophila for expressing a gene of interest in the temporal and spatial patterns of a different gene. The expression of a transgene can be controlled by either placing the transgene with a minimal promoter near an endogenous enhancer (called an 'enhancer trap') or by cloning a specific enhancer into the transgenic construct upstream of the transgene. The promoter regions of housekeeping genes such as ubiquitin, actin and tubulin, are commonly used to achieve broad, constitutive expression. Tissue-specific expression is often driven by the promoter of selector genes, such as *eyeless* (*ey*) expressed in the eye, or *nanos* (*nos*) expressed in the germline. Although we are currently limited to expressing genes in patterns derived from expression patterns that occur naturally, there is little shortage of naturally occurring patterns, which can range from ubiquitous expression to single cell expression. Furthermore, enhancers can be split or combined through various techniques described below to create either coarser or finer expression patterns.

Currently our temporal control over gene expression is much greater than our spatial control, as a result of inducible expression systems. The promoter of the heat-shock protein, *hsp70*, activates gene expression in response to a heat shock (McGarry and Lindquist, 1985). Expression occurs in every cell and ceases shortly after the temperature returns to normal. The promoter for metallothionine has also been used as an inducible promoter (Bunch *et al*., 1988). Alternatively, temporal control can be achieved through the addition of a regulatory domain. For example, in the Pswitch system, Gal4, a common transcriptional activator (see below), is fused to the human progesterone receptor ligand-binding domain. Basal Gal4 activity is suppressed in this chimeric protein but becomes activated in the presence of the antiprogestin, RU486, which can be administered through feeding (Roman *et al*., 2001).

Binary systems

A significant strength of Drosophila genetics is the way in which regulated gene expression has been streamlined through the use of binary systems. Binary systems are comprised of a transcriptional activator and its deoxyribonucleic acid (DNA)-binding site. Typically, a designated regulatory sequence drives expression of the transcriptional activator (referred to as a 'driver'), which, in turn, drives activation of a coding sequence that is downstream of its DNA-binding site. Thus the components of the binary system serve as molecular adaptors, driving expression of the coding sequence through the regulatory sequence. Libraries of drivers and binding site-linked-coding sequence can be created independently and then systematically combined. Binary systems offer several additional advantages over directly linking a regulatory element to a coding DNA sequence including the ability to amplify the gene expression level with multiple activator-binding sites and the ability to use additional regulators, such as repressors or small molecules, to control gene expression (Brand and Perrimon, 1993). However, foreign DNA is a target for epigenetic silencing and expression from a binary system does not always faithfully recapitulate the expression pattern of the transcriptional activator (Skora and Spradling, 2010).

To be used effectively, the components of a binary system cannot be naturally present in the organism being studied. The binary systems currently used in Drosophila are Gal4/UAS, lexA and the Q-system, adapted from Yeast, Bacteria and Neurospora, respectively.

Gal4/UAS

The most common binary system used in Drosophila is the Gal4/UAS system adapted from yeast (Brand and Perrimon, 1993; Fischer *et al*., 1988). The Gal4 protein contains a DNA-binding domain that binds to a UAS. Upon binding, Gal4 recruits ribonucleic acid (RNA) polymerase, thereby activating transcription of downstream sequences. Brand and Perrimon showed that Gal4 retains these functions when expressed in Drosophila. However, the original UAS (sometimes denoted UASt) has a reduced efficiency in the germline and a modified UAS (UASp) was developed that is more responsive in germ cells (Rørth, 1998).

Vast collections of lines have been established in which Gal4 is randomly inserted downstream of various endogenous Drosophila enhancers. In each enhancer trap line, Gal4 is expressed in a unique temporal and spatial pattern that is determined by the enhancer. Similarly there are vast collections in which UAS is upstream from various Drosophila genes, reporters, RNAi or miRNA constructs, or other transgenes. The complementary libraries can then be systematically combined without the need for making additional stocks.

Furthermore, a number of tools have been developed for refined control over the spatial and temporal patterns of Gal4-driven expression. The Gal4 locus in yeast contains an inhibitor of Gal4-driven gene expression, Gal80, that acts by binding to Gal4 and preventing the recruitment of the transcriptional machinery (Ma and Ptashne, 1987). This has also been incorporated in Drosophila and can be expressed to prevent Gal4-driven expression in a region

(Lee and Luo, 1999). Expression of a temperature-sensitive form of Gal80 grants further temporal control over Gal4-driven expression – preventing expression in a region until a shift to the restrictive temperature (McGuire *et al*., 2003). In the Split Gal4 technique, the Gal 4 driver is replaced by two 'hemidrivers' (Luan *et al*., 2006). Each hemidriver consists of one of the two functional domains, the DNA-binding domain and the activation domain, fused to heterodimerising leucine zippers. In cells where both domains are expressed, the domains associate through the leucine zippers, reconstituting a functional Gal4.

Other binary systems

Several additional binary systems have been developed that complement the Gal4/UAS system. The availability of multiple binary systems allows independent modulation of different genes at the same time as well as another method for driving gene expression in intersectional patterns.

The bacterial transcriptional regulatory system for regulating the SOS response to stress, was adapted as an alternate binary system in Drosophila (Szüts and Bienz, 2000). In this system the regulatory factor, LexA, is a transcriptional repressor. In normal conditions LexA represses transcription by binding to DNA-binding sites upstream of a set of stress-response genes. Stress triggers the expression of RecA, a protease that degrades LexA, thereby allowing expression of the genes. Taking advantage of the modular nature of transcriptional regulators, Brent and Ptashne combined the DNA-binding domain of LexA and the Gal4 transcriptional activation domain (GAD) to create a chimeric activator that has the site recognition and specificity of LexA (LexA:GAD) (Brent and Ptashne, 1985), but, like Gal4, is suppressed by Gal80.

The Q system, adapted from the qa gene cluster in Neurospora crassa, includes the qa-1F (QF) transcriptional activator, its 16 bp recognition sequence, QUAS, and a repressor of QF activity, qa-1S (QS) (Potter *et al*., 2010). The Q system functions similarly to Gal4/UAS but with somewhat lower efficiency.

The Q and lexA systems were developed much more recently and large libraries of Q and LexA stocks have not yet been created. However, a new Gal4 enhancer trap construct, known as InSITE, includes a tool for exchanging the coding sequence expressed by the trapped enhancer through a genetic cross thereby making it easy to convert an existing library rather than having to create an entirely new library (Gohl *et al*., 2011). Stocks have already been created for switching to have the trapped enhancer drive a Gal80, split Gal4, LexA or QF. Likewise, the latest generation of constructs used by the Drosophila Knockout Consortium contains a phiC31 site (see below) that facilitates efficient site-specific recombination and thus can be converted into enhancer traps (Venken *et al*., 2011).

Site-specific recombination

DNA recombination can be used to mark a cell or to make a cell mutant. The presence of enzymes in nature that catalyse recombination at certain sites in the DNA provides a way for gaining control over the process. Site-specific recombinases from yeast and bacteriophage have been introduced into Drosophila to drive the induction of recombination at high frequency, at defined sites in the genome, and in defined tissues. **See also**: Transposases and Integrases

FLP/FRT

FLP/FRT is part of a mechanism in the 2 μm plasmid of yeast that maintains high copy numbers of the plasmid. The enzyme and its recognition sites were introduced into Drosophila by Golic and Lindquist (1989) for creating mosaic animals. FLP is an Integrase family site-specific recombinase that induces recombination at defined inverted repeat sequences known as FRT sites. The minimal FRT site is 34 bp – comprised of 13 bp inverted repeats on either side of an asymmetric sequence or 'spacer'. FLP binds an FRT site, dimerises with another FRT-bound FLP, making a synaptic complex between the two FRT sites, and catalyses a recombination event between them (Branda and Dymecki, 2004). The asymmetry of the spacer imposes directionality on the FRT sites, and enzyme-mediated recombination only occurs between FRT sites in a specific orientation. Recombination between two FRT sites in *cis* pointing in the same direction results in excision of the intervening sequence, whereas recombination between two FRT sites in *cis* pointing in opposite directions results in inversion of the intervening sequence. Recombination between two FRT sites in *trans* on separate chromosomes pointing in the same direction results in exchange of the distal sequences between the chromosomes. Each of these is used in the different lineage tracing systems.

Several considerations should be taken into account when using FLP/FRT. First, although flipping can be catalysed in either mitotic or nonmitotic cells, the use of FLP/FRT in various lineage labelling systems is often dependent on the cell cycle (Beumer and Pimpinelli, 1998) for either bringing FRT sites in close proximity (i.e. from chromosome alignment during mitosis) or segregating away parts of the DNA (i.e. from cytokinesis) as described below. Second, FLP is not 100% efficient (Golic and Lindquist, 1989). This is, in fact, desirable for most lineage tracing experiments, where the frequency of recombination must be low enough so that clones are distinguishable. The extent of mosaicism is influenced by the levels of FLP, and this can be controlled through the choice of promoter. Promoters are available that lead to constitutive expression in certain tissues (e.g. eyFLP, ubxFLP) and near complete mosaicism, whereas others are inducible (e.g. hsFLP). With hsFLP, the level of expression is dependent on the severity and duration of the heat shock (Golic and Lindquist, 1989). Brief heat shocks at lower temperatures result in lower levels of expression whereas long heat shocks at higher temperatures cause higher expression levels. Importantly, hsFLP can be 'leaky', causing the induction of flipping in

the absence of heat shock. Thus, when making inferences based on the time of clone induction, it is important to include a 'no-heat-shock' control in order to determine the rate of background clone induction. Furthermore, leakiness can also occur in the germline, making stocks that contain both hsFLP and FRT sites unstable over multiple generations.

Cre/lox

Cre from bacteriophage P1 catalyses recombination between loxP sites. Cre is in the same family of site-specific recombinases as FLP, therefore its function and mechanism of action is analogous (Branda and Dymecki, 2004). Likewise, loxP sites have the same inverted repeat and spacer structure as FRT sites. Cre/lox is more efficient than FLP/FRT in mammalian cells (Nakano *et al*., 2001), and is more commonly used in mammalian genetics. However, Cre appears to be toxic to Drosophila cells in some cases (Nakano *et al*., 2001), though these effects can be mitigated by reducing the expression levels (Heidmann and Lehner, 2001).

phiC31

phiC31 is a site-specific recombinase from Streptomyces bacteriophage (Groth and Olivares, 2000). Unlike FLP and Cre, phiC31 mediates recombination between two different DNA sequence sites – attB and attP. Furthermore, recombination causes the sites to be altered – attL and attR – such that it cannot be reversed. In Drosophila, this recombinase system has been predominantly used to create a transgene 'docking' system (Bateman and Lee, 2006; Groth *et al*., 2004). Thus, transgenes can be reproducibly inserted into the same location in the genome, eliminating variation in transgene expression due to chromosome effects or regulatory elements.

Lineage Tracing Systems

The tools described above have been combined in many clever ways to express markers in specific cells of interest and at the desired time. We now describe the various lineage tracing systems that are currently in use.

FLP/FRT-based mitotic recombination

Mitotic recombination is still a popular method for lineage labelling, however, FLP/FRT has replaced irradiation as the method of inducing mitotic recombination. Standardised FRT sites are used that are very close to the centromere to cause homozygosity in the maximum number of loci on that chromosome arm (Xu and Rubin, 1993). Fluorescent protein transgenes (e.g. Ubi-GFP), inserted distal to the FRT sites, are used as markers. Following mitotic recombination, one daughter cell will lose the marker, becoming 'negatively marked' (**Figure 1**).

Many genetic mutations are publicly available on FRT chromosomes, including over 1200 stocks in the Bruin Collection (Call *et al*., 2007). Thus, the marked clone can also be made homozygous mutant for a gene of interest. This type of mosaic analysis makes it possible to examine gene function in specific tissues and at specific times during development, even when the allele is recessive lethal for the organism. In addition, it is also possible to determine which cells or cell types within a complex tissue must be mutant in order for the phenotype to be expressed. Mutations that cause a phenotype in the mutant cell(s) are 'autonomous' whereas those that cause phenotypes in wild type cells are 'nonautonomous'.

Because the negatively marked clone system requires that a marker be asymmetrically segregated during mitosis, only dividing cell populations can be labelled with this method. In addition, clones may not be visible immediately after the first cell division because of marker perdurance. GFP is useful in this regard, because it has a relatively short half-life.

Positive labelling systems

Tubulin-LacZ

In this system the ubiquitous tubulin promoter and a transgene, lacZ, are placed in trans at identical locations (60B) on homologous chromosomes (Harrison and Perrimon, 1993). In this arrangement there is no expression of the reporter. Mitotic recombination between the FRT sites of homologues positions the promoter upstream of the reporter on one chromosome, which results in constitutive expression of the reporter in one of the daughter cells (**Figure 2**). For lineage tracing, sparse clones are typically created through hsFLP. Although this system is typically

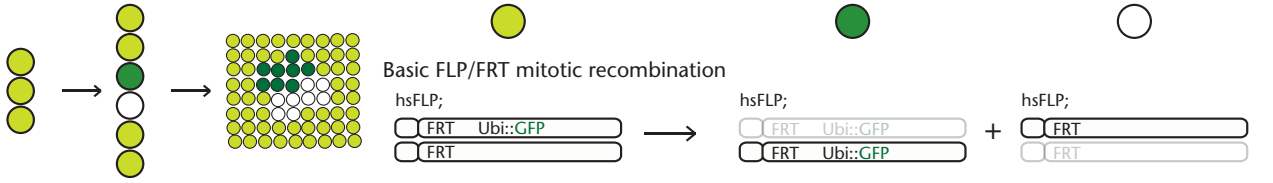

Figure 1 *Negative labelling systems*: FLP/FRT-based mitotic recombination system for generating negatively labelled clones in a positively labelled background. Colours signify the genotype of cells, as indicated by the cartoon cell above each genotype. The chromosome arms that undergo mitotic recombination are illustrated for a parent cell and its daughters. In the daughter cells, grey indicates an unrecombined chromosome. Unrecombined cells (light green), homozygous GFP-positive cells (dark green), homozygous negatively labelled cells (white). Note that, in practice, there is often no visible difference in fluorescence from one versus two copies of GFP (light green and dark green).

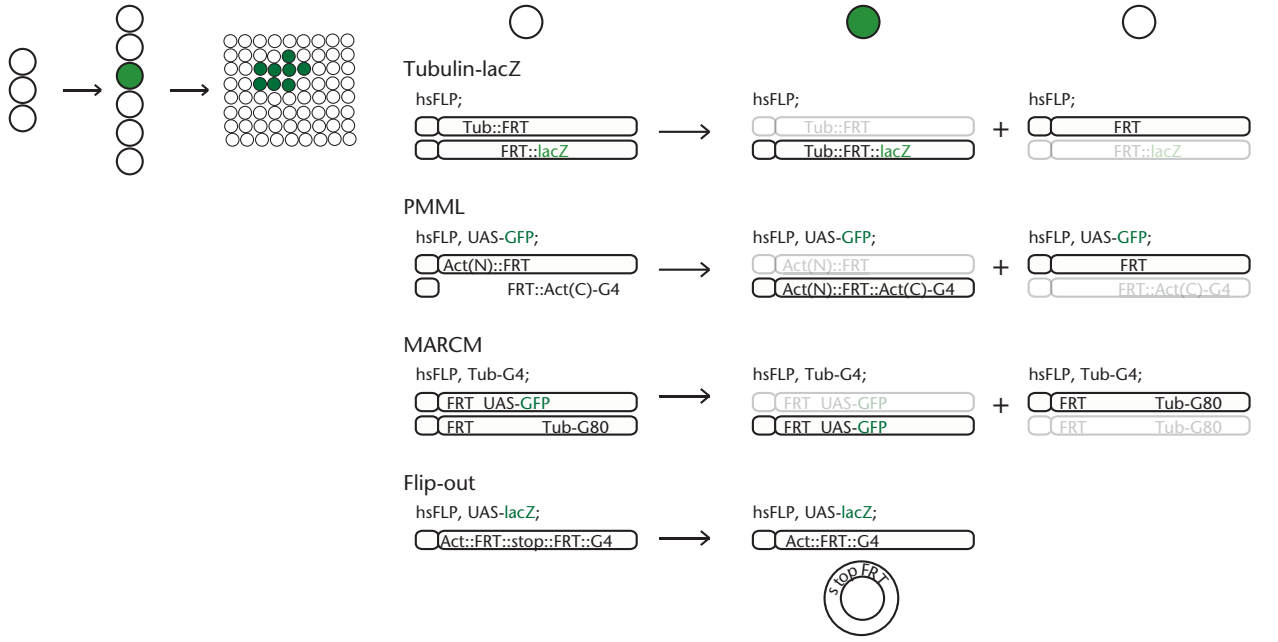

Figure 2 *Positive labelling systems*: FLP/FRT-based mitotic recombination systems for generating positively labelled clones in a negatively labelled background. The chromosome arms that undergo mitotic recombination are illustrated for a parent cell and its daughters. In the daughter cells, grey indicates an unrecombined chromosome. In the PMML method the *Actin5c* promoter is divided into *N*-(Act(N)) and *C*-(Act(C))terminal halves. Unrecombined cells and daughter cells that do not express a label (white), daughter cells expressing the corresponding label for each method (green).

used to study mitotic lineages, nonmitotic cells can also be labelled. Thus, the presence of label should not be taken as evidence of cell division (Fox and Spradling, 2009).

Positively marked mosaic lineage (PMML)

Similar to the Tub-LacZ system, PMML uses mitotic recombination between homologues to reconstitute a transgene in one of the daughters – in this case an Actin5c Gal4 driver (Kirilly *et al*., 2005) (**Figure 2**). The functional transgene then drives the expression of a reporter, UAS-GFP, marking that cell and its lineage. In addition, other UAS-transgenes can be included, allowing a gene of interest to be overexpressed or knocked-down in the marked clone. Unlike mosaic analysis with repressible cell marker (MARCM) (described below), activation of UAS-transgenes does not require a transcriptional repressor to turn over, and thus occurs immediately after clone induction. In its current form, however, this system cannot be readily used for phenotypic analysis of homozygous mutant alleles.

MARCM

The MARCM technique takes advantage of the Gal4 transcriptional repressor, Gal80 (Lee and Luo, 1999). Initially Gal80 inhibits expression of a Gal4-driven reporter. Mitotic recombination causes both copies of Gal80 to be segregated into one of the daughters. Consequently, the reporter is turned on in the other daughter, marking the cell (**Figure 2**). The disadvantage of MARCM is that, due to the perdurance of Gal80, it takes at least 48 h to ensure that clones are marked. However, the strength that has made MARCM very popular is that it is designed to allow both mutant and overexpression analysis of the marked clone. This makes it possible to study genetic interactions, such as testing for the rescue of a mutant clone phenotype by overexpression of a transgene.

Flip-out

'Flip-out' refers to the excision of a DNA sequence by FLP-induced mitotic recombination between FRT sites that are on the same chromosome, flanking the sequence (**Figure 2**). Because the FRT sites are in *cis*, flip-out recombination does not require that homologues align (as in mitotic recombination) and recombination is generally very efficient, even in nonmitotic cells. Pignoni and Zipursky (1997) combined the flip-out method with Gal4/UAS by creating a transgene in which the ubiquitous Actin5c promoter is separated from Gal4 by a 'flip-out cassette'. The flip-out cassette consists of CD2, a spacer gene containing a transcriptional stop site, flanked by FRT sites. Excision of the cassette removes the transcriptional stop and activates transcription of Gal4 under Actin5c. Gal4 then activates expression of a UAS-driven marker, as well as any other UAS-controlled transgenes that can be added for genetic analysis.

Dual-labelling systems

Several dual marking strategies have been developed that make it possible to simultaneously label each daughter of a cell division with a distinct marker. This increases the

number of cell lineages that can be analysed in a tissue, while maintaining the precision of the lineage data. The major disadvantage shared by all of these systems is that they involve so many separate transgenes that it is difficult to conduct any concurrent genetic analysis.

Dual-marked mitotic clones

In the dual-marked mitotic clone system, lacZ and GFP are each used to mark one of the arms of homologous chromosome that undergo mitotic recombination (Nystul and Spradling, 2007) (**Figure 3**). Thus, an unrecombined cell will bear both markers and can be differentiated from each of the two recombined classes of cells, which will bear only one of the two markers.

Twin-spot MARCM

In Twin-spot MARCM, each chromosome arm that will undergo mitotic recombination bears a marker (GFP or RFP) and a repressor for the marker on the opposite homologous chromosome arm (miRNA to RFP or GFP, respectively) (Yu *et al.*, 2009). Recombination isolates each marker away from its repressor, resulting in expression of that marker. Thus, each of the two daughter cells will express one of the markers (**Figure 3**). A major advantage of this system is that the initial cell is unlabelled whereas the products of mitotic recombination are distinctly labelled. Thus, the labelled clones stand out and are more easily distinguished against an unmarked background.

Coupled MARCM

Coupled MARCM combines the Gal4/UAS and Q systems to label clones (Potter *et al.*, 2010). Each binary system drives the expression of a separate marker, however expression is initially blocked by the presence of repressors for each binary system in the heterozygous cell. Mitotic recombination segregates away one of the repressors in each cell, turning on expression through the corresponding binary system (**Figure 3**). When marker expression is driven

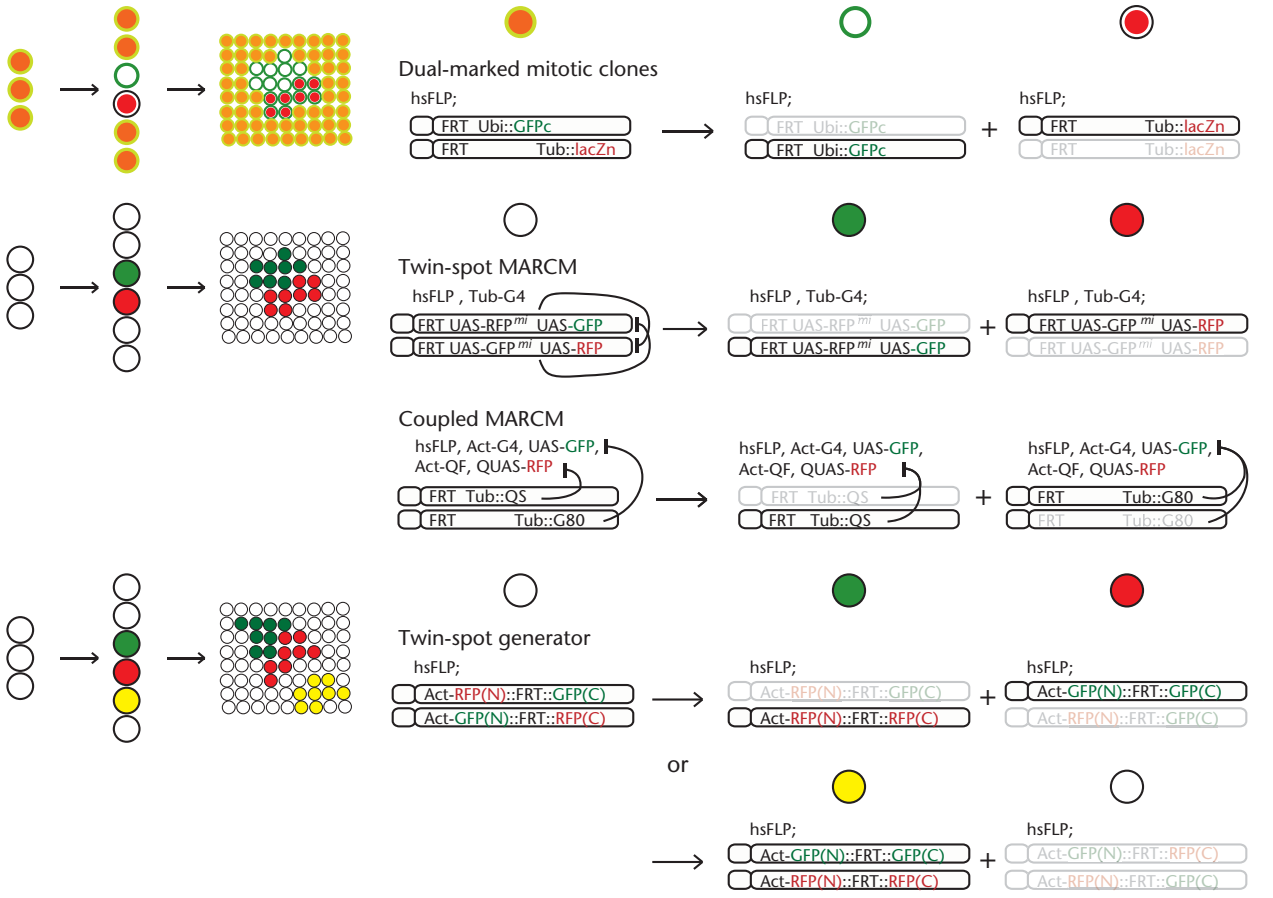

Figure 3 *Dual labelling systems*: FLP/FRT-based mitotic recombination systems for generating clones in which the two halves of a lineage are labelled with different markers. The chromosome arms that undergo mitotic recombination are illustrated for a parent cell and its daughters. For dual-marked mitotic clones, unrecombined cells with cytoplasmic GFP (GFPc) and nuclear LacZ (lacZn) are red with a green border, daughter cells with only GFPc are white with a green border, and daughter cells with only lacZn are red with a black border. For other methods, unlabelled, unrecombined cells are white, daughter cells expressing a GFP label are green, daughter cells expressing an RFP label are red, and daughter cells expressing both GFP and RFP labels are yellow. In Twin-spot MARCM, *mi* stands for miRNA. In the Twin Spot Generator method, both types of segregation that lead to labelling and are depicted. (*N*) and (*C*) refer to the corresponding terminal half of GFP or RFP.

through ubiquitous promoters (i.e. Act-Gal4 and Act-QF), the labelling pattern is similar to that of Twin-spot MARCM and the Twin-spot Generator (described next) – each daughter after a mitotic recombination is distinctly labelled. Finer clone patterns can also be made by driving marker expression through more specific promoters (as described below).

Twin-spot generator

In this system, the *N*- and *C*-terminal halves of two separate reporter transgenes, GFP and RFP, were swapped and an FRT site was placed at the junction (Griffin *et al*., 2009). The two hybrid, nonfunctional reporters are placed in trans. Mitotic recombination between the FRT sites rejoins the *N*- and *C*-terminal halves of each reporter, thereby restoring reporter function. Depending on how the restored reporters segregate, either a red daughter cell (containing one functional RFP) and a green daughter cell (containing one functional GFP) will be produced, or a yellow daughter cell (containing one functional RFP and one functional GFP) and a nonlabelled daughter cell (containing no functional transgenes) will be produced. Thus, in this system, clones can bear one of three distinct marks (**Figure 3**).

Rainbow labelling systems

The strategy of labelling more than one clone at a time was taken a step further with the advent of Brainbow in mouse (Livet *et al*., 2007). Brainbow causes stochastic expression of 3 or more fluorescent proteins of different colours. Cells can therefore be labelled uniquely by any one of these 'primary' colours or any colour resulting from expression of combinations of the fluorescent proteins. Thus, in one sample, up to 90 cells can be tracked, each identified by a unique colour. There are currently two variations of this technique in flies – Flybow and Drosophila Brainbow (Hadjieconomou *et al*., 2011; Hampel *et al*., 2011).

Flybow 1.0 and 1.1

Flybow uses a variant of FLP/FRT, mFLP5/mFRT71, to stochastically drive inversion and excision events that place a single fluorescent protein downstream of a UAS (Hadjieconomou *et al*., 2011). These variants allow FLP/FRT to be used concurrently with the Flybow system. In Flybow 1.0, before recombination, mCherry is under the control of a UAS promoter. Following recombination, mCherry is switched with another marker, Cerulean, by a single inversion event. In Flybow 1.1, a combination of two possible inversion events and two excision events places one of four fluorescent proteins downstream of the UAS (**Figure 4**). Inversion events are reversible, thus, the system remains susceptible to further recombination (and colour switching) in Flybow 1.0, and only becomes refractory to recombination in Flybow 1.1 after one of the two excision events occurs. At that point a single marker becomes fixed behind the UAS and thereafter the cell and its lineage become stably marked. Three of the fluorophores used in Flybow 1.1 are exceptionally bright and thus useful for live imaging; the fourth marker, Cerulean, is dim and best visualised by immunofluorescence.

Drosophila Brainbow

Drosophila Brainbow uses Cre and three sets of mutually exclusive LoxP sites (Hampel *et al*., 2011). One of three possible excision events will place one of three markers downstream of UAS, and after a single excision event, the genotype is stabilised (**Figure 4**). Two copies of this Brainbow construct can be used to increase the number of unique labels from three to six. Brainbow was designed to allow discrimination of each of these six labels through epitope

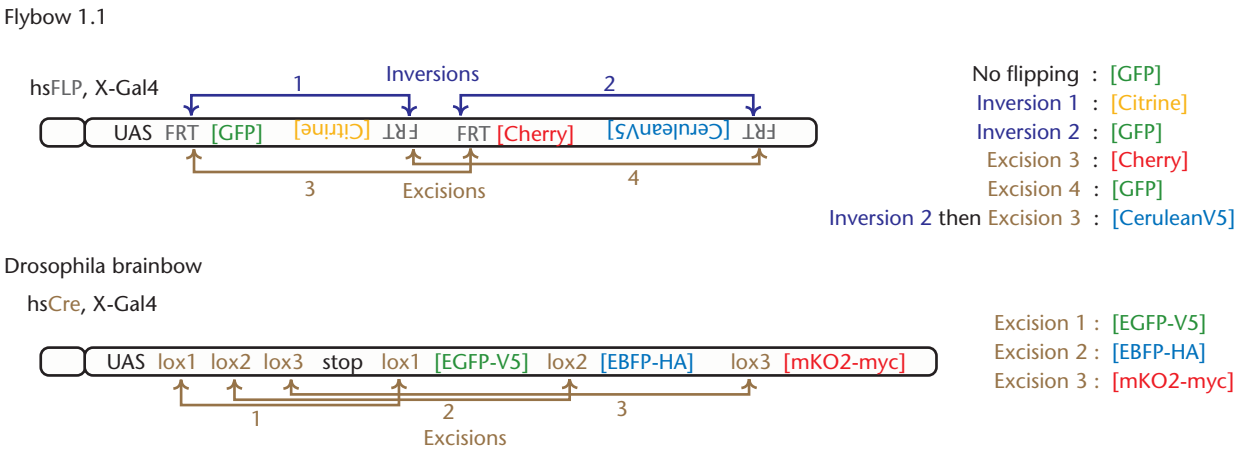

Figure 4 *Rainbow labelling systems*: Mitotic recombination systems for generating clones with multicoloured markers. Components of the systems are depicted on the left. Upside-down lettering indicates that a coding sequence or binding site is in the opposite orientation on the chromosome. Recombination between binding sites with the same orientation results in an excision, and recombination between binding sites with opposite orientations results in an inversion. All possible inversions or excisions are indicated on the cartoons, and the marker that would be expressed as a result of each is listed on the right. In Flybow, Cerulean is only expressed if inversion 2 is followed by excision 3. Flybow uses a variant of FLP/FRT, indicated by grey lettering.

staining, however, two of the markers are fluorescent proteins that can be visualised live.

The use of Cre in this system allows concurrent and independent use of FLP/FRT. However, a major limitation of this system is that Cre must be expressed at high enough levels to induce recombination but low enough levels to avoid toxicity. Hampel *et al*. used hsCre, which expresses at high levels early in development, even in the absence of heat shock. This causes large clones to be induced, which decreases lineage resolution.

Targeted labelling systems

All of the labelling systems discussed thus far involve the use of hsFLP to create clones randomly. This is not ideal for the labelling of a specific small subset of cells because conditions that leads to labelling of the specific cells at a sufficient frequency, but restricts clone induction to levels that are sparse enough for individual clones to be identified as separate, may be difficult or impossible to identify. Similarly, in genetic studies, inducing clones in only the region of interest can avoid systemic effects of the manipulations on the animal.

An alternative is to use nonubiquitous promoters to restrict the expression of flippase and, thereby, labelling to a subpopulation of interest. This can be accomplished with a UAS-driven FLP or with a FLP that is directly conjugated to a cell- or tissue-specific enhancer trap. To this end, a collection of enhancer-trap FLP (ET-FLP) lines were recently established by Bohm *et al*. (2010). Similarly, in each of the MARCM systems – MARCM, Twin-spot-MARCM and Coupled MARCM – a more specific Gal4 driver can be used to visualise only the clone region that is within the Gal4 expression domain. In this case, any concurrent overexpression would also be limited to the region of interest, however, mutant clones would still be created throughout the lineage of the original recombined cell.

Gal4/Gal80 intersectional expression

Two systems have been developed in which Gal80 is used to limit the region in which clones can be marked (**Figure 5**). Gordon and Scott (2009) placed a Gal80 between the FRT sites in a flip-out cassette. Clones lacking Gal80 (through hsFLP) are created throughout the animal, however, only the subset of cells within these clones that express Gal4 can express a UAS transgene.

Conversely, Bohm *et al*. (2010) created a construct in which Gal80-expressing clones are created by flip-out throughout the animal. Only cells that express Gal4 but are not part of a clone can express a UAS transgene. This technique, ET-FLP-induced intersectional GAL80/GAL4 repression (FINGR), is especially useful for studying wild type cell behaviour in a mutant background.

Binary MARCM

Binary MARCM makes use of both the Gal4- and LexA:GAD-drivers (Lai and Lee, 2006). Because LexA:GAD contains the Gal4 activation domain (Brent and Ptashne, 1985), both systems are susceptible to the influence of Gal80. Thus, clones that lose Gal80 through mitotic recombination will express either the Gal4-driven marker (GFP), or the LexA:GAD-driven marker (RFP), or both, depending on which expression domain(s) the clone resides within. This leads to the creation of clones that are labelled green if they reside in Gal4-expression domain, red if they reside within the LexA expression domain, and yellow if they reside in the intersection between the two expression domains.

Flybow 2.0

Rainbow labelling, in both Flybow and Drosophila Brainbow, can be restricted to a region through the use of a specific Gal4 driver. However, a separate Flybow construct was made for intersectional studies with a second expression pattern (Hadjieconomou *et al*., 2011). The Flybow 2.0 construct contains an extra canonical FLP-based flip-out cassette between the UAS and the various markers (**Figure 5**). The Flybow labelling can only be 'activated' once this cassette is excised. Thus, rainbow labelling will only occur in regions where flip-out is activated by FLP-expression, and the Flybow construct marker (which is UAS-controlled) is activated by Gal4 expression.

G-trace

A caveat to the use of Gal4 drivers for lineage labelling is that the label may not be permanent. If a cell naturally ceases to express the driver, then the label will be lost. The G-trace system allows the tracing of all cells that currently express or ever previously expressed a Gal4 driver (Evans *et al*., 2009). Gal4 driven expression of a UAS-marker labels all cells that currently express the driver, whereas Gal4 driven expression of UAS-FLP drives a flip-out event that permanently marks that cell (**Figure 5**). Thus, expression of the flip-out marker is maintained in the absence of further Gal4 expression.

Summary and Conclusions

One of the first goals in studying a tissue or cell is to identify the progenitor(s) that gave rise to it and determine the signals that are required to develop and maintain its normal function. Lineage labelling has been a key technique in answering these, as well as several other questions. Lineage labelling is routinely used to identify cell populations and their developmental origins and to study the molecular mechanisms that contribute to their function. In addition, lineage labelling is currently the 'gold standard' method for unambiguously identifying a cell as a multipotent stem cell. And lineage labelling methods are being used to generate maps of the neuronal circuitry of the brain.

These efforts are feasible because of the advances in the tools and techniques for lineage labelling in Drosophila. It is currently possible to noninvasively label cells with stable and genetically heritable fluorescent markers. The label can

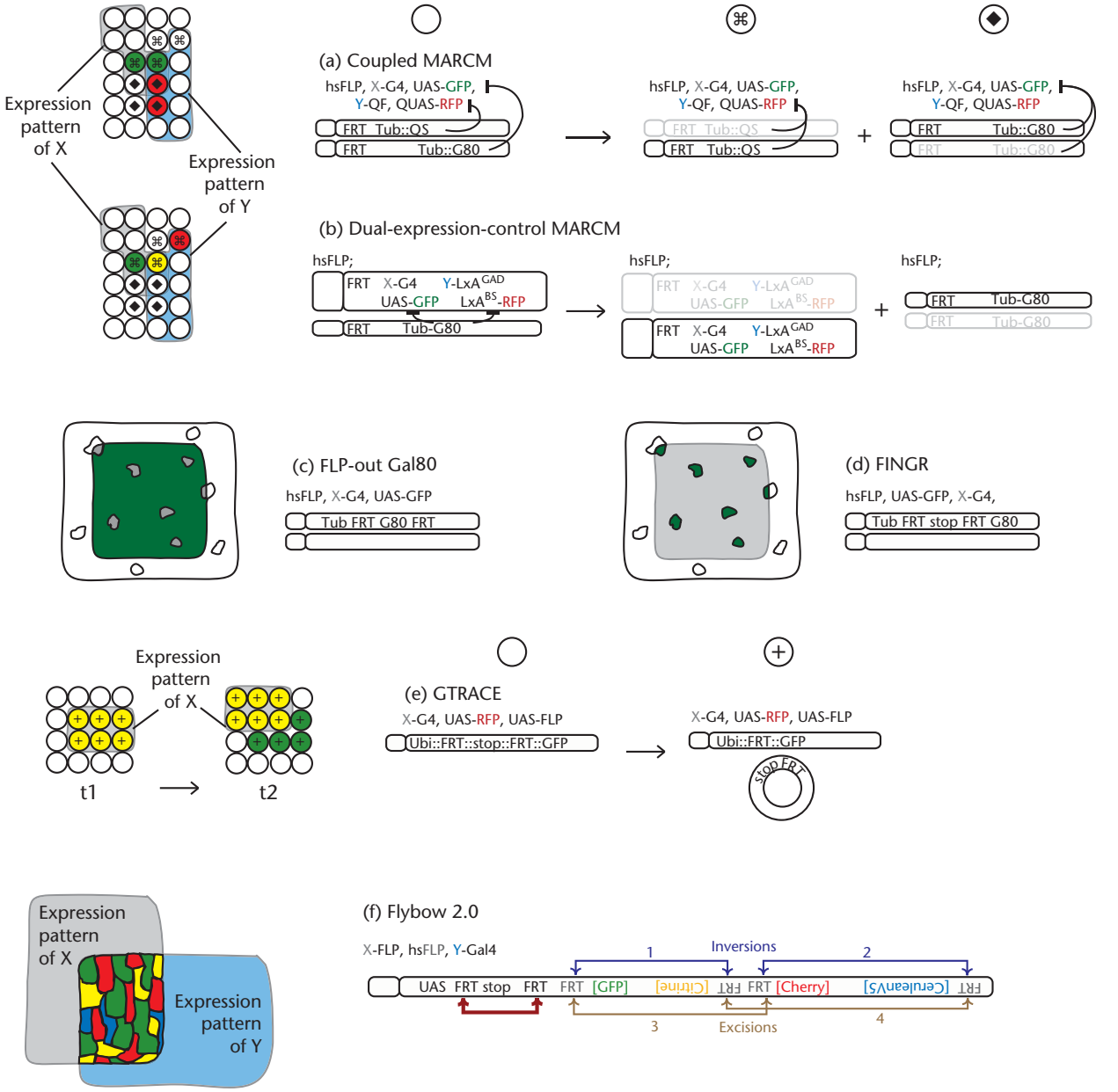

Figure 5 *Targeted labelling systems*: Mitotic recombination systems that use driver expression patterns to more specifically target labelling. (a, b) The genetic components of the system are shown on the right and labelling patterns, based on the genotype and expression domain of the driver(s), are shown on the left. Colours signify the label expressed by a cell: GFP (green), RFP (red) or both (yellow). No symbol indicates an unrecombined parent cell; the cloverleaf and diamond symbols identify the cells in each of the two halves of the lineage of the parent cell that underwent recombination. The cloverleaf and diamond patterns are shown above their corresponding genotypes in the diagram on the right. The grey 'X' or blue 'Y' areas indicate the driver expression domains. *Abbreviations*: LexA fused with the Gal4 activation domain (LxA:GAD), LexA-binding sites (LxABS). (c, d) The overall clonal patterns in a tissue, rather than cellular details, are shown for FLP-out Gal80 and FINGR systems. Clones are outlined, grey area denotes the Gal4 expression domain, and areas with GFP-expression are green. (e) For the GTRACE method, the change in the labelling pattern is shown for two time points is shown for a tissue in which a population of Gal4-expressing cells divide to produce some Gal4 nonexpressing cells. Although the lineage relationship between specific cells cannot be determined with this method, the population of green cells must have derived from the population of yellow cells, and all cells derived from the population of Gal4-expressing cells must be either green or yellow (all Gal4-expressing cells and their progeny are indicated with a+). Colours are the same as in (a) and (b). (f) Flybow 2.0 is a variant on Flybow that only undergoes rainbow labelling in the region defined by the intersection of a Gal4 and FLP expression pattern. Components are similar to Flybow 1.1 except for the addition of a flip-out cassette (red arrows). Colour coding is the same as for Flybow1.1. Black and grey lettering distinguish canonical from variant FLP/FRTs, respectively.

be introduced at any point in development. Cells can be randomly labelled or labels can be specifically targeted to tissues or cells where a known gene is expressed. Multiple cells can be uniquely labelled at the same time and in the same sample, allowing complex cell interactions to be studied. Furthermore, labelled cells can be genetically manipulated to examine developmental mechanisms.

There are still some technical caveats to the current tools of lineage labelling that we discussed. In some cases, there is a lag in marker detectability, for example, due to perdurance of the Gal80 inhibitor in MARCM (Lee and Luo, 1999). The leaky expression of hsFLP in some labelling systems can lead to incorrect interpretation of results (Fox and Spradling, 2009). Some of the genetic tools of lineage labelling are slightly toxic to cells, such as Cre (Siegal and Hartl, 1996), or require stressful treatments, such as heat shock-induced Flp. Many of the lineage labelling systems discussed are difficult, in their current form, to combine with genetic analysis. Furthermore, it is still very difficult to label small subsets of cells for which there is no known driver or marker. Although the current systems are effective for charting proliferation patterns and identifying the cell types that arise from a certain progenitor, a distinct weakness remains in being able to track cell movements.

However, these limitations are likely to be mitigated by future iterations of the lineage labelling systems. The repertoire of available promoters is expanding as well as the number of methods for taking advantage of combinatorial expression patterns. And as part of a newer trend, several groups are beginning to use the genetic labelling techniques in conjunction with advances in methods of culturing tissues and imaging to directly observe developing cells through extended live imaging in previously inaccessible tissues. These live imaging studies have already led to many surprising revelations. The next few years should be an exciting time as more results from other lineage labelling studies continue to appear. **See also**: Methods for Live Microscopy of *Drosophila* Spermatocytes

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