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Power tools for gene expression and clonal analysis in *Drosophila*

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Abstract

The development of two-component expression systems in *Drosophila melanogaster*, one of the most powerful genetic models, has allowed the precise manipulation of gene function in specific cell populations. These expression systems, in combination with site-specific recombination approaches, have also led to the development of new methods for clonal lineage analysis. We present a hands-on user guide to the techniques and approaches that have greatly increased resolution of genetic analysis in the fly, with a special focus on their application for lineage analysis. Our intention is to provide guidance and suggestions regarding which genetic tools are most suitable for addressing different developmental questions.

Advances in the genetic technologies available to scientists studying *Drosophila* have allowed precise spatiotemporal manipulations of gene expression for cell labeling, gene-function analysis or cell-lineage tracing. Since the development and first use of the GAL4–upstream activating sequence (UAS) for transgene expression[1], numerous methods have been developed using alternative expression systems as well as site-specific recombinases to manipulate and label cell populations very precisely.

In this Review, we discuss recent technological developments that have greatly expanded the genetic toolkit available to scientists using *Drosophila* as an animal model. We start by discussing how the GAL4-*UAS* system has inspired sophisticated and versatile expression systems—such as the split-GAL4 system[2] and QF-*QUAS* system[3]—that allow for complex genetic manipulations. In the second part of this Review, we explore clonal analysis techniques partly inspired by the development of mosaic analysis with a repressible cell marker (MARCM)[4] and discuss powerful methods to analyze and manipulate tissues and neural networks with an exquisite resolution. We discuss new techniques for clonal analysis and gene manipulation, such as twin-spot generator (TSG)[4,5], twin-spot MARCM (TS-MARCM)[6] and Gal4 technique for real-time and clonal expression (G-TRACE)[7], and recently developed multicolor labeling schemes inspired by the mouse Brainbow technique[8–10]. Although most of the work to date has focused on the application of these methods in the fly nervous system, the tools themselves can be applied to any desired tissue. The availability of cell type–specific or tissue-specific drivers is the only limitation when applying these techniques to non-neuronal systems. We provide here a hands-on user guide to the genetic toolkit available to Drosophilists, make specific recommendations on use and provide guidance for the selection of tools for specific experiments.

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Techniques for the control of gene expression

Binary expression systems: GAL4-*UAS*, LexA-*lexAop* and QF-*QUAS* systems

The GAL4-*UAS* system is the workhorse of *Drosophila* genetics, and few papers are currently published that do not use it. The GAL4-*UAS* system is a binary expression system consisting of two main components: the yeast GAL4 transcriptional activator expressed in a specific pattern and a transgene under the control of a *UAS* promoter that is largely silent in the absence of GAL4 (ref. 1) (Fig. 1a). Additionally, the GAL4-*UAS* system is repressible by the GAL80 protein[11] (Fig. 1a and Tables 1 and 2). GAL4 can be used to drive effector-gene expression in two different ways: defined promoter elements can be used to express GAL4 in a specific pattern, or GAL4 can be randomly inserted into the genome such that it 'reports' the *cis*-regulatory architecture of the insertion location and is expressed in a reproducible pattern. Thousands of these GAL4-promoter fusions and enhancer trap insertion lines have been generated and are publically available (see ref. 12 for one example). The GAL4-*UAS* system can be used for cell- or tissue-specific genetic mutant rescue, gene overexpression, RNA interference screens and many other applications, and has been extensively used for developmental studies in tissues such as the central nervous system, retina and muscle.

The utility of the GAL4-*UAS* system spawned the generation of a second independent binary expression system for *Drosophila*, LexA-*lexAop*[13]. LexA binds to and activates the lexA operator (*lexAop*). The LexA-*lexAop* system uses the LexA DNA-binding domain from a bacterial transcription factor that can be linked to the GAL4 activation domain or the VP16 activation domain, a strong activation domain from the herpes simplex virus[14]. This adds the flexibility of having a GAL80-repressible (GAL4 activation domain) or independent (VP16) LexA driver, depending on one's needs (Tables 1 and 2). The GAL4 system is the default expression system for most studies owing to the abundance of characterized lines. The LexA system is most often used in combination with GAL4, for instance, when one requires GAL80-independent expression or when one desires high levels of expression, as the VP16 activation domain attached to the LexA DNA-binding domain is one of the strongest known to date.

The availability of GAL4-*UAS* and LexA-*lexAop* systems allows *Drosophila* biologists to simultaneously perform two manipulations of gene expression *in vivo.* Owing to the ubiquitous use of GAL4-*UAS*, the LexA-*lexAop* system is primarily used in experiments that already use the former and require multiple genetic manipulations (Table 1). For example, one could use LexA-*lexAop* and GAL4-*UAS* systems to check whether two reporters are expressed in the same or different cells. One can use GAL4 to report the expression of one gene by driving GFP expression and LexA to report expression of another gene by driving RFP expression. The LexA-*lexAop* system can also be used to label the fate of a given cell while using GAL4-*UAS* to drive siRNA expression in the precursor of this cell. The LexA-*lexAop* system is only limited by the availability of enhancer trap and promoter fusion lines, but this limitation is soon to be overcome with the generation of new lines and the development of swappable GAL4 lines discussed below.

The QF-*QUAS* system, based on a cluster of regulatory genes from *Neurospora crassa* (Table 2), is the newest binary expression system available in flies[3]. Conceptually identical to the repressible binary GAL4-*UAS* system, the QF-*QUAS* system uses the *QUAS* promoter, the transcriptional activator QF and its specific repressor called QS (Fig. 1a). Additionally, the QF-*QUAS* system is regulated by the small molecule quinic acid, which relieves QS-mediated repression when provided in the fly's food. It may be advantageous to use the QF-*QUAS* system when low basal transgene expression is required in the absence of the *trans*-activator because the QF-*QUAS* system has been shown to be less leaky than the

GAL4-*UAS* system. The QF-*QUAS* system may also be used in combination with both GAL4-*UAS* and LexA-*lexAop* to label multiple unrelated lineages. Finally, the QF-*QUAS* system is preferred for analyzing the temporal requirement of a gene in a fly behavior that may be temperature sensitive as the system can be activated by the addition of quinic acid in the diet rather than by temperature as in the temperature-sensitive GAL80 mutant, $GAL80^{ts}$ (see below). Currently there are few drivers available for the QF-*QUAS* system, and only time will determine the usefulness of this system.

Ternary expression systems: GAL80, split GAL4 and split LexA

The GAL80 repressor of GAL4 can be used with the GAL4-*UAS* system (or the LexA-*lexAop* system) for additional control, for example, in intersectional strategies of transgene expression[15–17] (Fig. 1b). GAL80 represses activation of GAL4 by acting specifically through the GAL4 activation domain. It can be used to restrict transgene expression both spatially and temporally. For spatial control, GAL80 can be fused to a given promoter to repress GAL4 activity in a specific region or tissue. For temporal control, one can use the temperature sensitive mutant $GAL80^{ts}$, which is active at 18 °C but does not repress GAL4 at 29 °C or higher temperatures. Alternatively, one can use recombinases to temporally control the expression of GAL80, as we will discuss below. This three-component system (GAL4-*UAS*, transgene and GAL80) is the basis of two powerful technologies: MARCM (discussed below) and temporal and regional gene expression targeting (TARGET)[18,19]. TARGET uses $GAL80^{ts}$, which allows a high degree of temporal control, making it ideally suited when one wants to determine the exact temporal requirements of transgene expression for a specific developmental program or behavior (Table 1). For example, TARGET was used to determine the acute role of the adenylyl cyclase rutabaga in memory formation in mushroom bodies[18].

Temporal regulation of the GAL4-*UAS* system can also be achieved through alternate techniques, such as using drug-inducible GAL4 variants. GAL4 chimeras with the estrogen receptor (GAL4-ER) can be induced by estradiol[20]. In GeneSwitch, both the progesterone receptor and p65, the transcriptional activation domain of the human Nf-kappa B gene[21], were fused to GAL4, allowing induction by the steroid RU486 (mifepristone). However, although these systems add temporal control, their disadvantage is that they both require feeding the ligand to the flies, which slows activation and makes the off kinetics slow after the ligand has been removed from the diet. TARGET is thus a preferred system over GeneSwitch or GAL4-ER when tight temporal control of transgene expression is required.

The modular nature of the GAL4 transcription factor allows for independent expression of the GAL4 DNA-binding domain and the GAL4 activation domain. GAL4 DNA-binding domain and GAL4 activation domain transgenes fused to sequences encoding leucine zipper dimerization motifs cannot result in activation of transcription when expressed individually, but they reconstitute a functional transcriptional activator when they are expressed together (Fig. 1c). This technology, known as ‘split GAL4’, allows for the restricted expression of transgenes by intersectional methods[2] (Fig. 1b and Table 1). Once again, this technique has been modified to generate both GAL80-repressible and independent expression systems by the replacement of the GAL4 activation domain with the VP16 activation domain fused to a leucine zipper. One drawback of the split-GAL4 system is that it requires that new hemi-lines be generated *de novo*, thus not allowing the use of the wealth of existing GAL4 enhancer trap and promoter fusions. This limitation has been overcome with the split-LexA technology discussed below, which uses a split-molecule ternary system that is compatible with existing GAL4 lines. Furthermore, the ability to exchange effector cassettes in new transgenes or enhancer traps will alleviate this problem[22].

The split LexA is conceptually similar to the split GAL4 (ref. 23). Split LexA also consists of separate LexA DNA-binding domain and activation domains (VP16) fused to leucine zippers that are expressed under the control of different promoters. Only in cells in which both promoters are active is the *LexA :: VP16* functional effector reconstituted (Fig. 1c). A tremendous advantage of split LexA is that it can use pre-existing GAL4 lines to drive expression of part of the *LexA :: VP16* effector, by placing either the LexA DNA-binding domain or the activation domain under a *UAS* promoter. The other hemidriver has to be generated *de novo*, but the same lines used for split-GAL4 activation domain or VP16 can be used because the leucine zipper dimerization domain is the same. Therefore, this technique can be used to refine the expression of previously existing GAL4 lines through intersectional methods. For instance, a group of neurons expressing a GAL4 line can be further restricted to only cholinergic neurons by crossing this line to *UAS*-split LexA DNA-binding domain gene and to a split-VP16 transgene expressed under the promoter of the choline acetyl transferase gene (*ChAT*).

Flp-out

Another technology that allows for the spatial and temporal restriction of transgene expression relies on the use of the yeast site-specific recombinase, flipase (Flp) and its recognition target sequence (*FRT*)[24,25] (Table 2). This technique, called Flp-out, uses transgenes that are silenced by a transcriptional stop signal flanked by *FRT* sites that can be removed by the expression of Flp to activate the gene of interest[25–27]. The 'Flp-out' technology can be combined with a binary expression system such as GAL4-*UAS* for additional layers of control. A Flp-out cassette can be placed between a *UAS* promoter and a transgene of interest (for example, in the construct *UAS :: FRT-stop-FRT-CD8 :: GFP*). Flp can be induced by heat-shock if a heat-shock promoter is used to drive its expression (*hs-FLP*) to provide temporal control, or it can be placed under the control of a defined promoter to provide spatial control (for example, *ey-FLP* to restrict expression of the eye imaginal disc). The latter case is again an intersectional method of transgene restriction; the transgene is only expressed in cells in which both the GAL4 and the Flp are expressed (Fig. 1b). Flp can also be used to repress GAL4 with GAL80 (Flp-in) or to relieve GAL80 repression (Flp-out). For example, one can use Flp to repress or relieve GAL4 expression at a specific time during the fly's development using the constructs *tubP::FRT-stop-FRT-GAL80* or *tubP::FRT-GAL80-FRT-stop*, respectively (*tubP* is the tubulin 1 alpha promoter). This technique can also be combined with LexA-*lexAop*, split GAL4 and split LexA to restrict the expression of a driver by intersectional methods, allowing for a previously unachievable control of transgene expression. For example, one could use three partially overlapping promoters to drive expression of each of the two parts of split GAL4 or split LexA and Flp. These three effectors will restrict the expression of a transgene—controlled by a *UAS* (split Gal4) or *lexAop* (split LexA) promoter driving a Flp-out cassette—to only those cells in which all three promoters are active.

Swappable enhancer trap lines

To gain an even greater control of gene expression to dissect biological processes, a modular expression system has recently been developed[22]. Integrase swappable *in vivo* targeting element (InSITE) is a convertible genetic platform that allows for the replacement of *GAL4* in newly generated promoter fusions or enhancer trap lines by any effector gene of interest (*GAL80, LexA, QF* and others) while preserving the expression pattern of the original construct. This technology has the added advantage that the swap can be performed efficiently *in vivo* without additional cloning. The system is compatible with split systems and intersectional methodologies described above, and, owing to its modular nature, new genetic tools can be easily incorporated into the system. The platform uses three different *Drosophila*-compatible recombinase systems (Flp-*FRT*, Cre recombinase–*Lox* and φC31

integrase–*attP/attB*; Table 2) to mediate the swap events by recombinase-mediated cassette exchange (Fig. 2 and Table 1)[22]. The InSITE system is ideal when one needs to drive different effector molecules in the given pattern of an enhancer trap or promoter fusion. One drawback of this platform is the relatively low rate of transgenesis compared to other strategies using similar site-specific genomic insertion methods.

Expression controlled by defined promoter fragments

A complementary system that allows for spatially and temporally controlled transgene expression is based on using promoter-fusion constructs. In an enormous effort, Gerald M. Rubin's group at Howard Hughes Medical Institute, Janelia Farm campus cloned over 5,000 genomic fragments from intergenic and intronic regions from 925 genes known to display neural expression into a system that allows for efficient site-specific genomic insertion[28]. The genes 'bashed' in this manner encode transcription factors, neuropeptides, ion channels, transporters and receptors with the purpose of focusing expression in the adult fly brain. Rather than using transposable elements (such as *P* elements) to insert the constructs at random locations in the genome, these investigators used the φC31 site-specific integration system (Table 2). In this system, vectors containing an *attP* site can be inserted with very high efficiency at a given genomic location that contains an *attB* site through recombination. This eliminates position effects and ensures that the vector is inserted at a specific chromosomal landing site. The authors found that over half of the fragments drive unique expression in 10–200 cells in the central brain. This work yielded a collection of lines in which transgene expression can be restricted to small subsets of neurons for manipulation and dissection of the neural circuitry. The entire collection of lines covers most *Drosophila* neurons. An advantage of this system is that the expression patterns generated by the defined genomic fragments can be used to drive expression of any effector molecule (that is, *GAL80*, *LexA*, *FLP* and others). In this way, these lines used singly or in combination could allow for the controlled expression of any effector molecule in any neuronal subset (Table 1). These lines and the InSITE lines are complementary and could be used to target specific neuron populations. Although only few lines using these systems are currently available, large libraries of InSITE-ready lines are being generated and the library from Janelia farm is expected to be publicly available in the near future.

Clonal analysis and gene manipulation

The MARCM technique

The development of MARCM[4] constituted a seminal advancement that led to the emergence of methods that allow imaging the architecture of the *Drosophila* nervous system and studying gene function governing its development at the single-cell level. Although these linage techniques have been most commonly applied to the fly nervous system, they are applicable for studying the lineage relationships between cell types in any system. MARCM allows one to positively label single cells or groups of cells related by lineage, to generate homozygous mutations and to express a gene of choice—all of this together or independently in the same clone. In its simplest implementation, MARCM is a lineage tracing system that has replaced the use of the classical Golgi staining used over 100 years ago by Ramón y Cajal and others for the study of neural systems[29–32]. Because labeled cells are the progeny of a common cell (neuroblast in the case of the nervous system), by studying multiple clones one can infer the lineage relationships in complex neuronal networks[4,33–35], contributing to attempts to reconstruct the whole fly brain circuitry.

In addition to the use of MARCM for lineage tracing, another application of the technique arises from its ability to positively mark clones of mutant cells over an unlabeled background. Analyzing the behavior of mutant neurons for a particular gene in an otherwise

wild-type background allows studying the role of that gene in cell-fate specification, axonal path finding and so on without confounding effects from the cellular environment or from developmental expression of the gene[4,36–38].

The basic premise of MARCM is generation of clones homozygous for a mutant chromosome using the heat shock–inducible Flp-*FRT* system. In the heterozygous and homozygous wild-type tissue, a *GAL80* transgene[11] under the control of a ubiquitous promoter represses GAL4 activity and prevents expression of a membrane associated reporter (*UAS-CD8*::*GFP*). *FRT* sites are placed proximal to the mutation in one chromosome arm and proximal to GAL80 in the homologous chromosome arm (Fig. 3 and Table 3). Heat shock–induced mitotic recombination generates homozygous mutant clones that have lost the repressive GAL80 and are thus labeled by the expression of GFP. GFP can be visualized in all mutant clones if it is driven by a ubiquitous GAL4 driver or in only a subset of the mutant cells when using a specific *GAL4* driver. MARCM has been used to study the behavior of single homozygous mutant cells in wild-type tissue, as reported for the short stop gene involved in axonal pathfinding in the mushroom bodies[4] or the *Drosophila* small GTPase RhoA, required to regulate neuroblast proliferation and dendritic morphogenesis[36]. Making homozygous mutant clones in a wild-type background is essential when studying the function of genes that are critical early in development or when determining the cell autonomy of gene function.

MARCM also allows one to simply label a single cell, sister cells or multicellular clones depending on the timing of the heat shock that induced recombination between the *FRT*-containing chromosomes (Fig. 4a). The ability to induce the heat shock at a specific stage is useful to define patterns of neurogenesis. For instance, correlation between lineage and birth time of neurons has allowed scientists to define the sequential generation of different neuron types in the mushroom bodies[39] or how the lineage and birth time of projection neurons match their targets (glomeruli) in the olfactory system[33]. The MARCM technique has also proven very useful outside the nervous system. MARCM was used to show that a multipotent lineage of self-renewing gastric stem cells exists in the *Drosophila* gut that gives rise to the acid-secreting copper cells of the midgut and the endocrine cells of the stomach[40]. Additionally, MARCM analysis was used to show that the gene *Zfh1* is involved in the development of the neuromuscular junction in the larva[41]. Finally, MARCM can be used to identify new genes involved in a developmental process by mutagenizing chromosomes carrying *FRT* sequences and examining mosaic flies as was done to study tracheal cell migration during *Drosophila* air sac morphogenesis[42]. By this method one can limit the mutant tissue to that being studied and examine mutations that are nonviable.

The Q system can also be used for a new form of Q-MARCM that can be used simultaneously with MARCM (Fig. 3a and Table 3). In ‘independent double MARCM’ the GAL80 and QS repressors are placed on different chromosome arms, whereas in ‘coupled MARCM’ they are placed on the same chromosome arms in *trans*. In coupled MARCM, sister cells from the same recombination event are independently labeled in different colors, whereas in independent double MARCM the offspring of two independent recombination events are labeled in different colors.

Two-color techniques (TSG and TS-MARCM) represent a substantial improvement over simple MARCM, which make them even more suitable for lineage analysis.

Recent elaborations on the MARCM technique: twin–spot generator

The power and versatility of the MARCM technique has been improved by a second generation of techniques. These use the Flp-*FRT* mediated recombination to switch on two different cell markers that label each of the two daughter cells of a precursor cell (Figs. 3b

and 4b and Table 3). They permit the analysis of clones and their twin spot, thus providing a deeper analysis of cell lineage while maintaining the ability to mutate genes and to express various genes in the same clone.

The TSG is an adaptation of the mouse mosaic analysis with double markers (known as MADM)[43]. In the TSG, the two halves of two fluorescent marker genes, whose expression is driven ubiquitously by the *Act5C* promoter, are split by an *FRT* site. One hybrid consists of the sequence encoding the N terminus of GFP[44] and the C terminus of RFP[45] and the other is its complementary gene (sequences encoding the N terminus of RFP and the C terminus of GFP) (Fig. 3b). They are placed at the exact same location on homologous chromosomes and are not expressed because neither GFP nor RFP is functional (Fig. 3b). Heat shock–induced Flp mitotic recombination reconstitutes the GFP and RFP markers and at the same time segregates them into the two daughter cells and their progeny (Fig. 4b). Validation of the system in the optic lobe has suggested symmetrical division of neuroblasts in the medulla primordium (ref. 5 and A.d.V.R. and C.D., unpublished data).

An advantage of the TSG is that immediate reconstitution of the markers allows for their expression faster than in MARCM, in which the GAL80 repressor protein can persist for a brief period of time. This technique is very useful to study how the progeny of stem cells are distributed in a tissue during development, allowing the description of patterns of cell division and migration. TSG has been tested in the eye, antennal, wing and leg imaginal discs. In the latter, clonal separation of twin spots (sister cell clones) indicates that cell migration occurs[44]. In the nervous system the use of TSG has been limited because of a lack of membrane-tethered markers, which are necessary to visualize neuronal arborizations. Recently, a TSG version with membrane-bound markers has become available (R. Griffin and N. Perrimon; personal communication). One drawback of the TSG system is that it uses a pan-cellular actin 5 (*Act5*) promoter that cannot be replaced by other drivers. This particularity can limit its use in tissues in which *Act5* expression is weak. In addition, RFP and GFP can be expressed together in the same cell as a result of nonmitotic recombination between *FRT* sites, which can interfere when performing lineage analysis. These problems are overcome with the TS-MARCM technique.

Recent elaborations on the MARCM technique: twin–spot MARCM

Like the TSG, the TS-MARCM labels both progeny of a cell division in different colors[46] (Fig. 4b and Table 3). TS-MARCM uses two membrane-bound fluorescent markers encoded by the constructs *UAS-CD8::GFP* and *UAS-CD2::RFP*, which are placed on two homologous *FRT*-containing chromosomes[6] (Fig. 4b). Expression of each of these genes is suppressed in *trans* by *UAS*-driven micro-RNA transgenes placed on the homologous chromosomes. Therefore, one chromosome carries *UAS-GFP* and *UAS-CD2-miR* (a microRNA targeting *CD2*) and the other *UAS-RFP* and *UAS-GFP-miR* (a microRNA targeting *GFP*) (Fig. 3b). Heat shock-induced mitotic recombination between the *FRT* sites segregates the microRNA suppressors from their target genes, enabling the expression of the markers under the control of a GAL4 driver that can be present on a different chromosome. Each daughter cell and its progeny will remain labeled with one of the markers (Fig. 4b).

The TS-MARCM is an efficient and powerful system for labeling the progeny of a given cell, for example, the neural progeny of a neuroblast clone. It can be applied to study cell lineages in a given area, for example, using pan-neuronal drivers, but it also allows for the use of specific GAL4 lines to focus the study on restricted cell types. TS-MARCM also allows for the study of gene function by recombining a mutation of a gene on one of the *FRT*-carrying chromosomes (for instance, the analysis of the *chinmo* gene[6]). The applications of this technique are the same as with the TSG, although the TS-MARCM is

recommended in the analysis of the nervous system, where it has already been tested, as it allows membrane labeling.

G–TRACE

The G-TRACE technique allows memory expression of a gene. Cells will be labeled with one particular color if they expressed the gene at any time in their lineage history, whereas expression of the same gene in real time will result in labeling in both colors. This system is very useful to identify genes expressed at very early stages in development but also have later (different) functions. G-TRACE relies on the combination of the GAL4-*UAS* system and a Flp-out cassette (Fig. 3b and Table 3). A gene-specific GAL4 driver line controls *RFP* expression and reports current gene expression. The GAL4 also activates *FLP* expression, which leads to the excision of a stop cassette and inducing *GFP* expression under an ubiquitous promoter. *GFP* expression is permanent and inheritable, and reports the history of expression of this gene[7] (Fig. 4b). Therefore, G-TRACE provides both a spatial and a temporal readout of the expression of a gene of interest. This technique has been tested in different tissues, such as the brain, eye-antenna, lymph gland and wing. It can be broadly used as it only depends on the availability of a tissue or cell-specific GAL4 driver. Nevertheless, only a nuclear version of GFP is available, so neural morphology cannot be analyzed—an issue that can be easily overcome by replacing nuclear RFP or GFP with membrane-bound versions. Another limitation is that weak drivers might not trigger Flp-out efficiently, leading to unlabeled clones[7].

Multicolor systems: *Drosophila* Brainbow

A major challenge for *Drosophila* neurobiologists is to maximize resolution to dissect, neuron by neuron, the connectome of the fly brain. The *Drosophila* Brainbow (dBrainbow) and Flybow techniques recently developed by Hampel *et al.*[8] and Hadjieconomou *et al.*[9], respectively, use multiple colors to identify multiple individual neurons in a large cell clone and are based on the Brainbow technique developed in mice[10]. Whereas in the mouse this technique is restricted to very few promoters[10,47], researchers using *Drosophila* as a model can take advantage of the huge collection of GAL4 lines and extend the analysis to almost any cell type. These techniques allow studying relationships between different lineages or addressing questions of how a particular lineage contributes to a specific structure.

The dBrainbow method uses a construct that consists of a *UAS* sequence followed by a stop cassette and three cassettes encoding cytosolic fluorescent proteins of different colors flanked by incompatible *loxP* sites (Fig. 3c and Table 3). Heat shock–induced Cre recombination mediates excision between a single pair of matching *loxP* sites, allowing random expression of only one fluorescent protein in each cell. Owing to the irreversibility of the process, expression of the fluorescent protein is permanent in the progeny of the heat-shocked cells[8] (Fig. 4c). Adding a second copy of the *UAS-dBrainbow* transgene into the cell increases the available colors to six possible combinations of the three primary colors. This is a great system to study how different lineages interact to form a structure. For example, it allows labeling the progeny of two different neuroblasts in different colors, enabling the visualization of how these two populations of cells interconnect or spread away from each other. A disadvantage of this method is that it relies on the use of the Cre recombinase, which in flies displays toxicity[48] and poor inducibility[49]. These limitations are overcome by the Flybow system.

Multicolor systems: Flybow

Hadjieconomou and colleagues developed two Flybow versions based on inversions and excisions of a transgene coding for four different fluorescent proteins. Like dBrainbow, these techniques also provide spatiotemporal control of the system through the use of the

GAL4-*UAS* and in this case, a heat shock–controlled Flp[9] (Table 3). In the *Flybow 1.1* construct (*FB1.1*), a *UAS* sequence is followed by two cassettes flanked by *FRT* sites with different orientations. Each cassette contains the coding sequences of two inverted fluorescent proteins. GAL4-expressing cells induce the expression of the first fluorescent protein, GFP, by default. The heat-shock activation of Flp induces inversions between *FRT* sites in opposing orientation or excision when they are in the same orientation. These events generate stochastic expression of one of the fluorescent proteins. Color diversity can be increased by subsequent heat shocks (Fig. 4d). The use of this technique, as well as of dBrainbow, is recommended to study the development of complex tissues, such as the nervous system, where thousands of neurons are born from a few neuroblasts. Provided that the progeny of a neuroblast will be labeled in one color, one can visualize how groups of single color–labeled neurons establish connections with neurons expressing a different marker, which are the progeny of a different neuroblast. Thus, Flybow and dBrainbow facilitate the analysis of neural patterning.

Flybow uses membrane-bound fluorescent proteins instead of cytosolic proteins as dBrainbow does. Thus, Flybow might be better suited for experiments that require revealing fine neural morphologies such as when determining the exact type of neurons labeled by different driver lines. Flybow relies on a modified Flp, mFLP5, that targets the modified *FRT* site *mFRT7.1*. The mFLP5-*mFRT7.1* system exhibits low basal expression and high recombination efficiency and specificity. In addition, its features make possible the use of the Flybow system in combination with other Flp-*FRT*–based systems as the different recombinases do not cross-react. For instance, the *FB1.1* transgene can be used together with MARCM technology. By generating MARCM mutant clones in a *FB1.1* background, it is possible to visualize mutant neurons and perform high-resolution functional studies. Besides the nervous system, *FB1.1* has also been tested in the wing imaginal disc[9].

Flybow 2.0 was generated by the addition of an upstream stop cassette into the *Flybow 1.1* construct (Fig. 3c and Table 3). In this version, the expression of fluorescent proteins requires previous excision of this cassette by a canonical Flp, which facilitates sparse neuronal labeling and avoids unwanted GFP expression. Only cells with GAL4, Flp and mFLP5 activity will express the florescent proteins. This combination has already been tested in the fly visual system, where wild-type neurons labeled by using the *Flybow* transgene can be compared with MARCM mutant neurons that exhibit incorrect innervation patterns in the visual system[9].

Discussion

Starting with the implementation of the GAL4-*UAS* system in the early 1990s, *Drosophila* biologists have developed increasingly complex and controllable expression systems. The *Drosophila* community now has three independent binary expression systems at its disposal. These systems, in combination with split-molecule technology, repressible activation domains, Flp-out cassettes and other excision systems, and an ever increasing number of expression lines, theoretically allow for any gene to be expressed or manipulated in any cell type at any developmental stage. Once the community generates and characterize an exhaustive collection of swappable driver lines, a database of expression profiles will allow researchers to identify driver lines or combinations of lines and effector molecules that will allow an exquisite level of resolution for the manipulation of cell type(s) of interest.

Clonal analysis has also been improved since the development of the MARCM technique in the late 1990s with the new TSG and TS-MARCM methods that permit labeling the progeny of a cell in two different colors. In turn, the *Drosophila* Brainbow and Flybow systems label the lineage of each heat shocked neuroblast in one color, so one can analyze multiple cell

lineages, their interactions and the architecture of complex neural networks. Furthermore, the use of these systems is not mutually exclusive, but complementary. For instance, the *Flybow 1.1* construct can be used with canonical Flp for intersectional studies and it has also been successfully used with MARCM[9]. MARCM system has also been tested together with the QF-*QUAS* system[3]. The use of these techniques separately as well as in combination allows for more accurate analysis of the lineages giving rise to a specific structure and its detailed architecture. Brief reviews comparing these systems have been published in this journal[50,51]. Although the Flybow and *d*Brainbow have been tested mostly for studies in the nervous system, both can be used in any structure of the fly. Its use is ideal to analyze complex lineages where a single color system (that is, MARCM) falls short in helping to identify groups of cells among large populations. In turn, these multicolor systems are high resolution techniques that will provide further insights into the development of any tissue.

And what does the future hold? Future advances might focus on the implementation of more fluorescent proteins to increase the number of colors and facilitate the identification of cells within clones. An important drawback of the techniques described for clonal analysis consists of the difficulty to control the size of clones. Future improvements should allow generating single cell clones by specifically expressing Flp with cell-specific drivers or targeting activation of *FLP* under the control of a heat-shock promoter in single cells with a laser. In this approach a focused laser is used to raise the temperature of a single or select cell population causing activation of the heat-shock promoter driving *FLP* only in those cells targeted by the laser.

The stage is set for *Drosophila* biologists to decipher the logic of complex developmental programs and neural circuit formation. An unparalleled genetic tool-box of effectors molecules and expression systems that can be combined in a variety of manners to label, manipulate and mutate genes in selected populations of cells with high spatiotemporal resolution. Different expression systems together with multicolor-reporter transgenes now allow for a previously unachieved level of resolution for lineage and mutant analysis. Through the combined use of different driver lines and expression systems it should be possible to exclusively label and manipulate genes in any cell type of interest and study gene function in combination with morphological, physiological and behavioral analysis.

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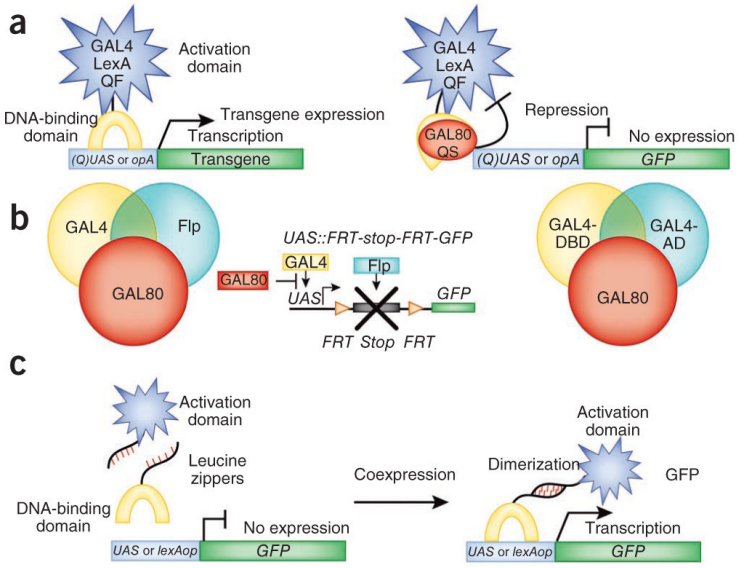

Figure 1.

Controlling expression patterns. (**a**) Two-component expression systems such as GAL4-*UAS*, LexA-*lexAop* or QF-*QUAS* consist of a transcriptional activator expressed in a specific pattern and a transgene under the control of a promoter that is largely silent in the absence of the transcriptional activator. These systems can be repressed by specific molecules such as GAL80 or QS. (**b**) In intersectional strategies for the restriction of transgene expression, GAL80 and Flp are used to restrict GAL4-driven expression. GAL80 and Flp are expressed using two different promoters that partially overlap with the expression of GAL4. *GFP* from the *UAS* :: *FRT-stop-FRT-GFP* construct is expressed only in cells that express both GAL4 and Flp, but not GAL80 (left and center). Split-GAL4 can be used with GAL80. Only cells expressing both hemi-drivers but not GAL80 show expression (right). (**c**) In split-molecule technology, activation domain and DNA-binding domain are fused to leucine-zipper motifs that reconstitute a functional transcriptional activator only in those cells that express both subdomains.

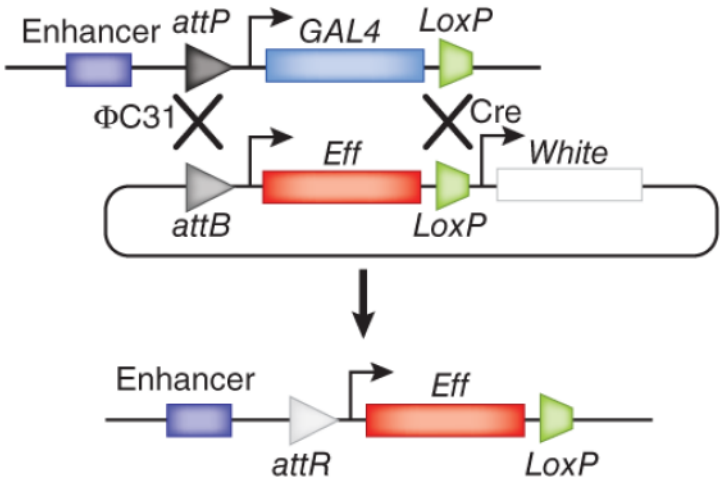

Figure 2.

Convertible enhancer trap strategy. The InSITE system allows GAL4 to be replaced by any effector sequence (*Eff*). The *mini-white* marker (*white*) is removed from the original enhancer trap using Cre recombinase. φC31 integrase allows recombination between the *attB* site on the donor *Eff* plasmid and the *attP* site of the original enhancer trap insertion, allowing replacement of *GAL4* by *Eff*. The Cre recombinase and φC31 integrase two-step process is simplified in the figure. Adapted from ref. 22.

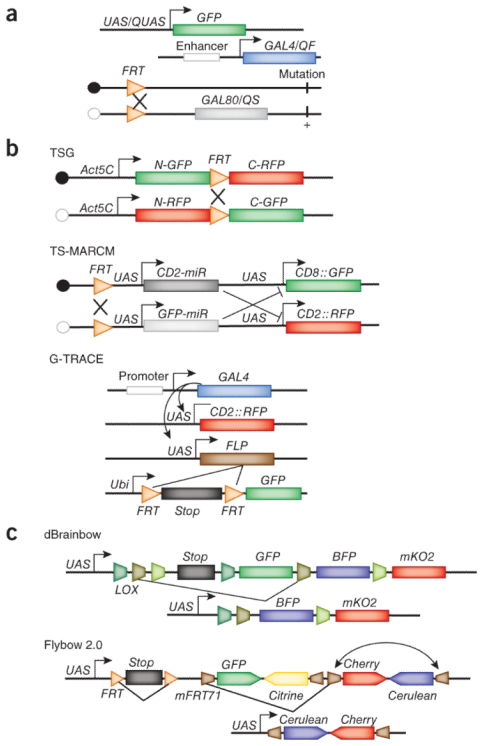

Figure 3.

Genetic system for clonal analysis. (**a**) MARCM and QMARCM. MARCM combines the Flp-*FRT* system with the suppressible ability of GAL80 over the GAL4-*UAS* binary system. The QF-*QUAS* system can similarly be used for MARCM with the transcriptional activator QF and its repressor QS. (**b**) Techniques for labeling cell clones with different colors. The TSG allows for two-color labeling through marker reconstitution of *N-GFP–C-GFP* and *N-RFP–C-RFP* domains after the recombination of *FRT* sites. Both transgenes are expressed under the actin 5 (*Act5C*) promoter. In TS-MARCM the expression of the membrane-bound markers (*CD8*::*GFP* or *CD2*::*RFP*) requires the release of the microRNA suppressors (microRNA to *CD2* (miR-CD2) or microRNA to *GFP* (miR-GFP)) through *FRT* site recombination. The G-TRACE reports real time expression (*CD2*::*RFP*) and stable inherited expression (*GFP*) of a gene of interest. Ubi, ubiquitous promoter. (**c**) Multicolor systems. In the dBrainbow technique Cre recombinase can generate multicolor labeling by randomly recombining matching *loxP* sites (represented by trapeze-shaped motifs of the same color). The Flybow method uses the flipase to induce inversions (arrow) and excisions, generating color diversity. Trapeze-shaped motifs represent *mFRT7.1* recognition sequences, and triangle-shaped boxes represent *FRT* sequences. The fluorescent proteins pointing to the right represent their correct orientation.

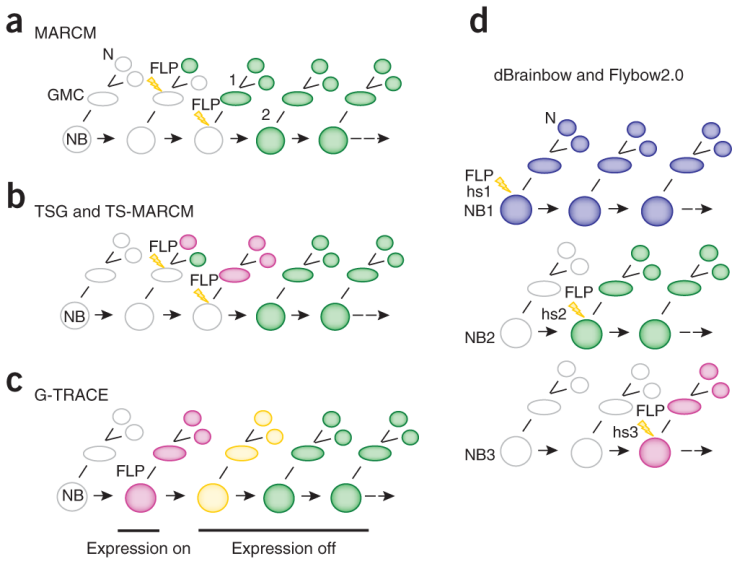

Figure 4.

Lineage models for clonal analysis systems. (**a**) The MARCM system can be used to generate single-cell clones when heat shock–induced recombination occurs on a ganglion mother cell (GMC) that divides into two neurons (N). Recombination in a neuroblast (NB) can generate sister-cell clones (1) or multicellular clones (2). (**b**) TSG and TS-MARCM can be used to generate sister-cell clones of two different colors when the recombination occurs in a GMC. NB clones can generate two cells with one of the markers (magenta in this example) and a multicellular clone with the other marker (green). (**c**) The G-TRACE allows for RFP labeling in cells expressing a gene in real time; GFP expression indicates the progeny of RFP expressing precursor cells. Cells in magenta will eventually express GFP and become yellow, but are shown in magenta to indicate GFP expression delay prior to Flp expression and excision of stop cassette. Green cells expressed RFP in the past but no longer do. (**d**) In dBrainbow and Flybow2.0 systems, the progeny of each cell clone (here a neuroblast, NB) is labeled in one color. The figure represents consecutive heat shocks (hs1–hs3) inducing recombination in different NBs, resulting in several lineages being labeled with distinct colors. The models represent NBs dividing asymmetrically.

Table 1

Expression systems

Expression system	Description	Recommended use
GAL4-*UAS*[1]	Binary expression system repressible by GAL80	Rescue or misexpression
LexA-*lexAop*[13]	Binary expression system repressible by GAL80	Combined with GAL4 for simultaneous gene manipulations and alone for high levels of gene over- or misexpression
QF-*QUAS*[3]	Binary expression system repressible by QS	Combined with GAL4 and/or LexA for intersectional gene expression or manipulations
Split GAL4 (ref.22) Split LexA[23]	Ternary expression system	Restrict gene expression through partially overlapping promoters; intersectional strategies to control gene expression
TARGET[18,19]	Uses GAL80^{ts} for temporal control of a GAL4-*UAS*–based expression system	Temporally controlled transgene expression
GeneSwitch[21] GAL4-ER[20]	Uses hormone-inducible GAL4 chimeras	Temporally controlled transgene expression
InSITE[22]	Uses multiple recombination systems to generate a swappable enhancer trap platform	Generating effector-swappable enhancer traps
Janelia Farm library[28]	Based on cloned enhancer elements	Targeting small populations of neurons for manipulation

Table 2

| Effectors: a basic *Drosophila* toolkit

Effector	Action	Target	Organism	Refs.
GAL4	Transcriptional activator	*UAS*	*Saccharomyces cerevisiae*	1,52–54
GAL80	Transcriptional repressor by direct binding to GAL4	GAL4	*S. cerevisiae*	53,54
LexA	Transcriptional activator	*lexAop*	*Escherichia coli*	55,56
Flp	Recombinase that excises DNA sequences flanked by two identical (homotypic) *FRT* sites	FRT	*S. cerevisiae*	25 – 27
Cre	Recombinase that excises DNA sequences flanked by two homotypic loxP sites	*loxP*	Bacteriophage P1	27,57
φC31	Integrase that mediates DNA integration in the genome. Unlike Cre and Flp, targets heterotypic *att* sites	*attB/attP*	*Streptomyces bacteriophage* φC31	27,58
QF	Transcriptional activator	*QUAS*	*N. crassa*	59 – 61
QS	Transcriptional repressor of QF by direct binding to QF	QF	*N. crassa*	61,62
Quinic acid	Controls the *Q* gene cluster by inhibiting QS	QS	Generic	61
VP16	Transactivation domain, insensitive to GAL80 repression	Multiple transcription factors	Herpes simplex virus	14,63,64

Table 3

| Clonal analysis systems

Clonal analysis system	Description	Recommended use
MARCM4	Labels and manipulates genes in specific cell clones	Cell labeling; lineage analysis; gene function
Q MARCM3	Generates cell clones in two colors by using two independent drivers or systems	Cell labeling; lineage analysis; gene function
TSG5	Labels the two daughter cells of a precursor in two different colors	Cell labeling; lineage analysis in nonneuronal tissues
TS-MARCM6	Labels the two daughter cells of a precursor in two different colors; unlike the TSG, has a membrane marker and allows the use of different drivers	Cell labeling; gene function; lineage analysis in neuronal and nonneuronal tissues
G-TRACE7	Provides cell clones with spatiotemporal information of the expression of a gene by using two different markers	Temporal analysis of gene expression
dBrainbow8	Generates multicolor labeling of multiple cell lineages under a specific driver	Cell labeling; interaction between lineages; neuronal network mapping
Flybow9	Generates multicolor labeling of multiple cell lineages under a specific driver	Cell labeling; interaction between lineages; neuronal network mapping; intersectional studies