Chapter 1
The Current State of the Neuroanatomy Toolkit in the Fruit Fly *Drosophila melanogaster*

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Abstract The fruit fly *Drosophila melanogaster* is a popular workhorse model organism that has tremendously contributed to our understanding of the nervous system across eukaryotic multicellular species. Through molecular, developmental, histochemical, anatomical, and physiological experimentation, studies that incorporate fruit flies have had immediate biomedical impact relevant to neurobiology and neuropathology. *D. melanogaster* is one of the most well-established eukaryotic multicellular model organisms, largely due to its sophisticated and expanding in vivo targeted neurogenetic manipulations. Here, we summarize the current status of techniques for precisely targeted spatiotemporal manipulation of the fly's nervous system, focused on the most recent developments within the field.

1.1 Introduction

The fruit fly *Drosophila melanogaster* is one of the very few model organisms with a large arsenal of genetic tools for highly sophisticated genetic manipulation (Venken and Bellen 2012, 2014; Venken et al. 2011a, 2016). Through this toolbox, fruit flies have significantly contributed to our understanding of developmental, biological, physiological, and behavioral aspects of the developing and adult

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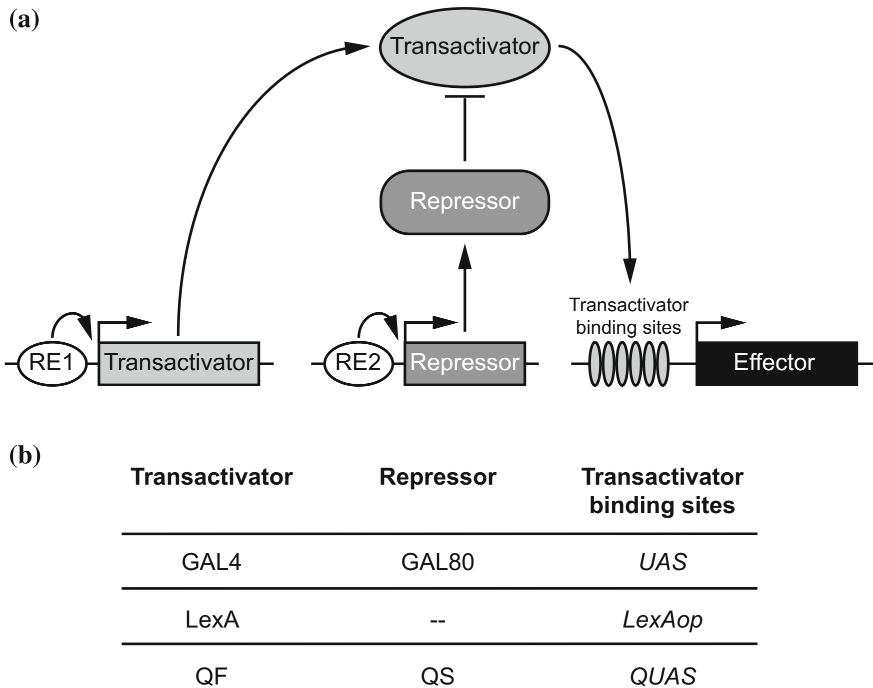
nervous system (Bellen et al. 2010), including basic neurobiological and behavioral functions such as vision (Silies et al. 2014), olfaction (Wilson 2013), taste (Freeman and Dahanukar 2015), circadian rhythm (Tataroglu and Emery 2014; Allada and Chung 2010), sleep (Donelson and Sanyal 2015), memory (Keene and Waddell 2007), pain (Leung et al. 2013; Tracey et al. 2003) and courtship (Dickson 2008), to name just a few. In addition, since the early 2000s, work in flies has contributed more and more to our understanding of the mechanisms and neuropathological characteristics associated with neurodevelopmental diseases that have their origins during development (Gatto and Broadie 2011), and neurodegenerative (McGurk et al. 2015), neurological (Shulman 2015), and neuropsychiatric disorders (van Alphen and van Swinderen 2013), which often occur much later in life. This toolbox comprises a large number of different molecular players that are used for a variety of purposes (Venken and Bellen 2012, 2014; Venken et al. 2011a, 2016; del Valle Rodríguez et al. 2012). The goal of this chapter is to provide a summary of the available genetic reagents that are used to spatiotemporally manipulate neurons.

1.2 Binary Activation Systems

Cellular manipulation in *D. melanogaster* is almost exclusively performed through binary activation systems (Fig. 1.1). A prototypical binary activation system has two parts: a transactivator and an effector. The transactivator is a heterologous transcription factor with a DNA-binding domain (DBD) and an activation domain (AD), typically expressed from a regulatory element, such as enhancer or promoter that will direct expression in a specific tissue or subset of cells (see Sect. 1.4). This artificial transcription factor can then drive the expression of any effector through binding at a synthetic promoter with specific multimerized binding sites (see Sect. 1.3). To further refine overexpression, the effector can be preceded or followed by additional RNA or protein regulatory elements that will tune expression levels in a negative or positive fashion, e.g., minimal promoters required for transcriptional initiation (Ni et al. 2009), translational enhancers (Pfeiffer et al. 2012), introns (Ni et al. 2008, 2009; Pfeiffer et al. 2010, 2012), RNA stabilizing elements (Pfeiffer et al. 2010, 2012), transcriptional termination signals (Pfeiffer et al. 2010, 2012; Brand and Perrimon 1993; Shearin et al. 2013), or protein-destabilizing domains (Nern et al. 2011). The different binary expression systems have incorporated the expression patterns of many 1000s of simple regulatory elements to efficiently and systematically drive expression of arbitrary effectors, e.g., fluorescent markers for cell labeling (see Sect. 1.3.1), or neuromodulators to influence neuronal physiological activity (see Chap. 7). Currently, there are three binary activation systems that are commonly used in *Drosophila*: the GAL4, LexA, and Q systems.



Transactivator	Repressor	Transactivator binding sites
GAL4	GAL80	*UAS*
LexA	--	*LexAop*
QF	QS	*QUAS*

Fig. 1.1 Binary transcriptional activation systems commonly used for neuroanatomical intersectional analysis in *Drosophila melanogaster* are GAL4, LexA, and QF. GAL4, LexA, and QF encode transactivators that can capture the expression patterns of regulatory elements (RE) either through random transposon integration or using cloned fragment fusions. The transactivator can activate expression of an effector gene construct that contains a multimerized copy of the transactivator's DNA binding site. The GAL4 and QF systems also have repressor proteins, i.e., GAL80 and QS, respectively, that target the activation domain of the transactivator protein, resulting in inhibited transactivation activity. The repressor's expression is regulated by a second regulatory element, i.e., same or different than the regulatory element driving the transactivator

1.2.1 The GAL4 System

The GAL4 system uses the heterologous GAL4 transcription factor, a regulator of galactose-induced genes in *Saccharomyces cerevisiae*, and an effector construct containing the GAL4 recognition site, the upstream activating sequence (*UAS*) (Giniger et al. 1985; Johnston and Hopper 1982; Laughon and Gesteland 1982). GAL4 was initially shown capable of inducing reporter gene expression outside of *S. cerevisiae*, including *Drosophila* (Fischer et al. 1988; Kakidani and Ptashne 1988). Following this demonstration, a two-part GAL4/*UAS Drosophila* toolkit was developed (Brand and Perrimon 1993). In contrast to the enhancer fusion approach that preceded it (Fischer et al. 1988; Kakidani and Ptashne 1988), the modular nature of the GAL4/*UAS*-system allowed construction of both driver lines (see

Sect. 1.4) and *UAS*-linked effector lines (see Sect. 1.3) that could be used in any combination by crossing a driver line with a desired expression pattern (i.e., determined by enhancer and/or promoter) to a fly carrying the desired effector construct. Since the introduction of the GAL4 system, collections of thousands of GAL4 lines and *UAS* effector lines have been generated (Venken et al. 2011a; Duffy 2002; Hayashi et al. 2002; Jenett et al. 2012; Jory et al. 2012; Li et al. 2014; Manning et al. 2012). We will discuss different types of GAL4 lines in detail below: some lines are enhancer traps or fusions (see Sect. 1.4.1), some are promoter traps or fusions (see Sect. 1.4.2), and some are protein traps (see Sect. 1.4.3). The value of these collections has been significantly enhanced through a number of large-scale imaging and characterization projects that have generated annotated and searchable databases of expression data throughout the nervous system as well as other tissues (Hayashi et al. 2002; Jenett et al. 2012; Jory et al. 2012; Li et al. 2014; Manning et al. 2012; Chiang et al. 2011; Peng et al. 2011).

In *S. cerevisiae*, GAL4-mediated expression is repressed by GAL80, which binds to the GAL4 AD and prevents transcriptional activation in the absence of galactose (Ma and Ptashne 1987a). Interestingly enough, GAL80 can also function as a negative regulator of GAL4 in a heterologous model system (e.g., *Drosophila*), a function that was first exploited in the context of the elegant MARCM (i.e., mosaic analysis with a repressible cell marker) system to positively mark clones in mosaic mitotic analysis (Lee and Luo 1999) (see Sect. 1.6). Subsequently, GAL80 has also become an important tool for intersectional refinement of enhancer trap expression patterns (see Sect. 1.5) (Pfeiffer et al. 2010; Suster et al. 2004).

While the GAL4 system provides spatial control of gene expression, for many experiments it is desirable to also have temporal control. For instance, many genes have dual roles in development as well as adult nervous system function, and phenotypes resulting from GAL4-mediated expression at both stages may obscure these dual roles of the gene. Variants of GAL4 have been developed that provide temporal control over gene expression. A hormone-inducible derivative of GAL4, i.e., the GAL4 DBD fused to the estrogen receptor domain, was shown to function in *Drosophila* oocytes (Han et al. 2000). Similarly, the more widely adopted GeneSwitch system utilizes a synthetic protein fusion consisting of a GAL4 DBD, progesterone receptor ligand binding domain and p65 activation domain, which can be induced in a dose-dependent manner with RU486 (mifepristone) (Nicholson et al. 2008; Osterwalder et al. 2001; Roman and Davis 2002; Roman et al. 2001).

Another mechanism that adds temporal control to the GAL4 system is the use of a temperature-sensitive mutation in GAL80 (Matsumoto et al. 1978). Shifting flies to the nonpermissive temperature abolishes repression of GAL4-mediated expression by GAL80^{ts} (McGuire et al. 2003). One limitation of these tools is their timescale of induction (and hence their temporal resolution), which ranges from 6 h to achieve steady-state expression and 36 h to return to baseline expression with GAL80^{ts}, and about 24 to 48 h to achieve maximal expression with GeneSwitch (McGuire et al. 2003). Finally, temperature itself strongly influences GAL4-mediated expression because most enhancer trap lines use promoter elements from the temperature-sensitive Hsp70 promoter (Brand and Perrimon 1993;

Mondal et al. 2007), although GAL4's transcriptional activity itself is temperature-independent (Mondal et al. 2007). Temperature shifts can cause unexpected physiological responses that influence many behavioral phenotypes (Kuo et al. 2012). Thus, it is important to control for this additional experimental variable in any experiments involving GAL80^{ts}.

1.2.2 The LexA System

In its native context in regulating the *Escherichia coli* SOS stress response, LexA functions as a transcriptional repressor (Walker 1984). However, when LexA is fused to a heterologous transcriptional AD, it can activate transcription from transgenes containing LexA operator (LexAop) sites in heterologous systems, including *D. melanogaster* (Lai and Lee 2006; Szuts and Bienz 2000). Development of the LexA system as a second binary expression system in *Drosophila* made orthogonal expression of multiple transgenes in the same animal possible. In addition, fusing LexA to the GAL4 or VP16 ADs generated GAL80-sensitive and -insensitive versions of LexA (Lai and Lee 2006), which have applications in intersectional targeting (see Sect. 1.5), and also enabled more sophisticated versions of the MARCM technology, e.g., dual-expression-control MARCM (Lai and Lee 2006) (see Sect. 1.6). Fusing estrogen and progesterone receptor domains to the LexA DBD provides spatiotemporal control by β-estradiol and RU486 respectively (Kuo et al. 2012).

1.2.3 The Q System

The Q system is based on a transcription factor, QF, from the *Neurospora crassa qa* gene cluster (Geever et al. 1989), which regulates quinic acid metabolism in its native context by binding to the so-called QUAS sites (Potter et al. 2010). Like the LexA system, the Q system provides an orthogonal system for labeling or manipulating specific populations of cells and also enables "coupled MARCM" experiments in which two-cell populations arising from a single-cell division can be independently labeled using the Q and GAL4 systems (see Sect. 1.6) (Potter et al. 2010).

Like GAL4, QF is targeted by a negative regulator, QS, which can repress QF-mediated expression (Huiet and Giles 1986). Repression by QS can also be disrupted in a dose-dependent manner by feeding flies quinic acid, providing a means to temporally control transgene expression (Potter et al. 2010; Potter and Luo 2011).

The initial QF construct exhibited some toxicity in *D. melanogaster*, precluding the establishment of pan-neuronal or pan-organismal driver lines. However,

subsequent protein engineering efforts have yielded nontoxic variants, QF2 and QF2w, as well as chimeric GAL4QF (i.e., a protein fusion between the GAL4 DBD and the QF AD) and LexAQF (i.e., a protein fusion between the LexA DBD and the QF AD) transactivators, which are QS suppressible and quinic acid inducible (Riabinina et al. 2015).

1.3 Neurogenetic Labeling

The three binary expression systems, GAL4, LexA, and Q, can be used to drive expression of genetically encoded reporters to label the entire cytoplasm or sub-compartments of neurons, i.e., cell compartments and organelles common to most cells (e.g., nucleus, mitochondria, endoplasmatic reticulum, and Golgi), or cellular compartments exclusive to neurons (e.g., synaptic vesicles, active zones, and dendrites) (Fig. 1.2). Fluorescent reporters can be used for live imaging or analysis of fixed specimens (i.e., directly or after immunohistochemistry using antibodies), and non-fluorescent reporter proteins can be used for immunohistochemistry. Alternatively, neuronal modulators or activity sensors can be targeted to a subset of neurons to affect or measure neuronal physiology respectively.

1.3.1 Fluorescent Protein Reporters

Currently, most existing fluorescent reporters are only GAL4 compatible. Expression of fluorescent reporters without an organelle—or compartment-targeting peptide label the entire cytoplasm and provide a full internal labeling of the host neuron (Pfeiffer et al. 2010; Halfon et al. 2002; Shearin et al. 2014; Yeh et al. 1995). While some earlier reporters inefficiently labeled the cytoplasm of entire neurons, codon optimization (Pfeiffer et al. 2010), or multimerization (Shearin et al. 2014) of the reporters has significantly improved labeling. On the other hand, fluorescent markers fused to membrane targeting motifs or membrane targeted domains solely label the cellular outline and their enrichment in membranes provides intricate detail about neuronal morphology (Pfeiffer et al. 2010; Lee and Luo 1999; Ritzenthaler et al. 2000; Ye et al. 2007; Yu et al. 2009). Protein fusions between synaptic vesicle proteins and reporters predominantly label synaptic vesicles and the presynaptic portion of the synaptic contact (Estes et al. 2000; Rolls et al. 2007; Zhang et al. 2002). A fluorescent protein fused to the active zone localized proteins *bruchpilot* (*brp*) (Wagh et al. 2006) or *cacophony* (*cac*) (Kawasaki et al. 2004) labels active zones. Dendrites are preferentially labeled by a synthetic fusion protein between a fluorescent reporter and the mouse protein ICAM5/Telencephalin (i.e., Denmark) (Nicolai et al. 2010) or an exon encoding a specific membrane targeting domain of Down syndrome cell adhesion molecule (Dscam) (i.e., Dscam[exon 17.1]) (Wang et al. 2004). A fluorescent protein fusion to the neurotransmitter

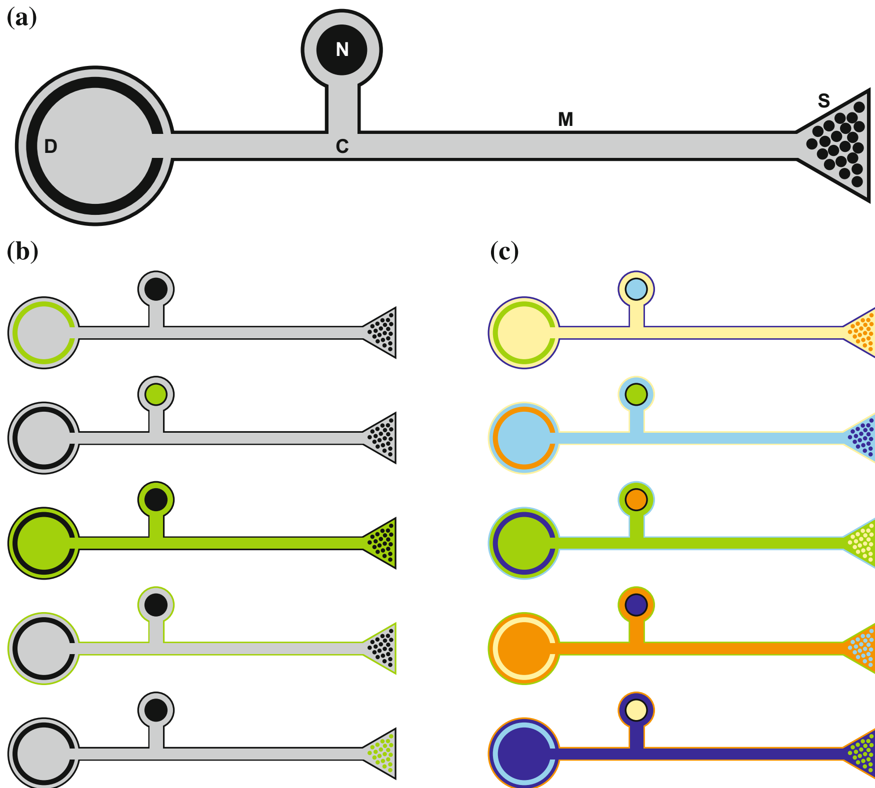
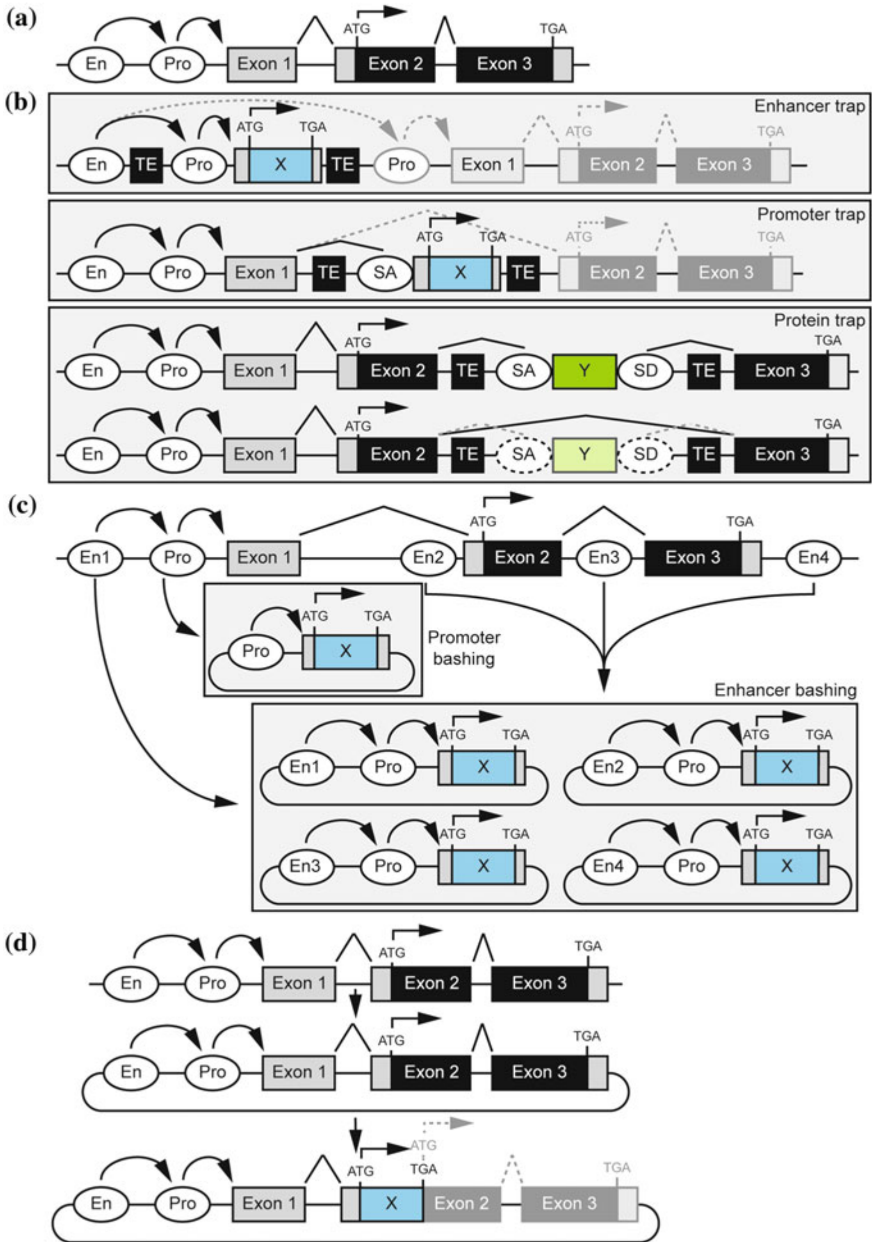


Fig. 1.2 Reporters useful for neuroanatomical analysis in *Drosophila melanogaster*. **a** Schematic of a typical *Drosophila melanogaster* neuron indicating five neuronal compartments most relevant to neuroanatomical analysis: dendrites (D), nucleus (N), cytoplasm (C), membrane (M), and synapse (S). **b** Classically, individual reporters are targeted to unique compartments and analyzed separately. **c** Future analysis may incorporate combinatorial reporters that could be activated through Brainbow strategies (see Sect. 1.6 and Fig. 1.12), i.e., each of the five Brainbow cassettes encodes five fluorescent proteins each labeling a separate neuronal compartment

receptor proteins resistant to dieldrin (Rdl) and nicotinic acetylcholine Receptor α7 (nAChRα7) can also be used to identify synapses (Leiss et al. 2009; Sanchez-Soriano et al. 2005). Protein fusions between fluorescent proteins and targeting elements specific for nuclei (Yasunaga et al. 2006), mitochondria (LaJeunesse et al. 2004), endoplasmatic reticulum (LaJeunesse et al. 2004), and Golgi (LaJeunesse et al. 2004) result in subcellular labeling enriched for the targeted organelle. Only recently fluorescent reporters as described above for the GAL4 system have also been generated for the LexA and Q systems, including several markers that label cytoplasm (Shearin et al. 2014; Yagi et al. 2010), membrane (Pfeiffer et al. 2010; Lai and Lee 2006; Potter et al. 2010; Diegelmann et al. 2008; Petersen and Stowers 2011), and synaptic vesicles (Shearin et al. 2013; Petersen and Stowers 2011).


(a)
En
Pro
Exon 1
ATG
Exon 2
TGA
Exon 3
(b)
Enhancer trap
En
TE
Pro
ATG
X
TGA
TE
Pro
Exon 1
ATG
Exon 2
Exon 3
TGA
Promoter trap
En
Pro
Exon 1
TE
SA
ATG
X
TGA
TE
Exon 2
Exon 3
Protein trap
En
Pro
Exon 1
ATG
Exon 2
TE
SA
Y
SD
TE
Exon 3
TGA
(c)
En1
Pro
Exon 1
En2
ATG
Exon 2
En3
Exon 3
TGA
En4
Promoter bashing
Enhancer bashing
X
(d)
En
Pro
Exon 1
ATG
Exon 2
Exon 3
TGA
X

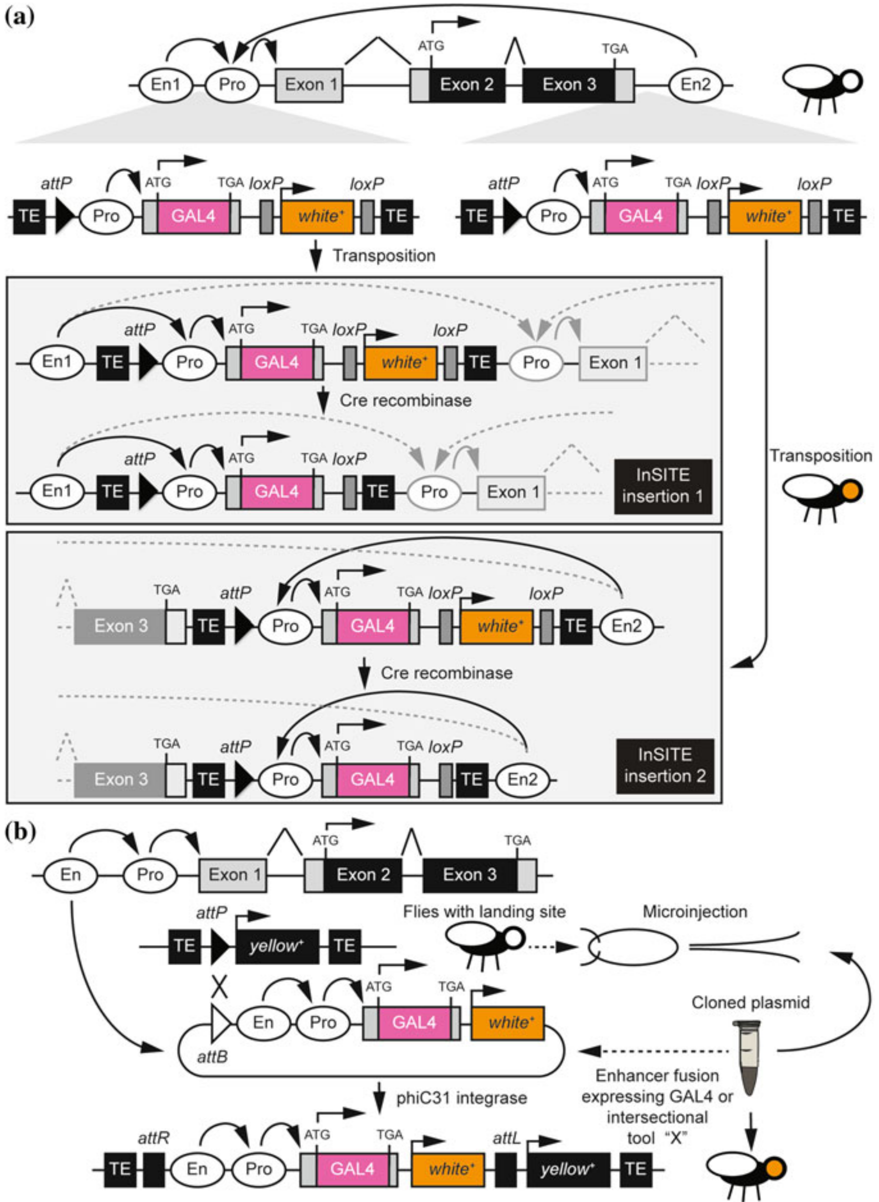
◂**Fig. 1.3** Overview of regulatory element trapping by transposons versus regulatory element cloning. **a** Diagram of a gene indicating enhancer (En), promoter (Pro), and exon/intron structure (i.e., 5′ and 3′ UTRs are indicated in *gray* while coding portions are indicated in *black*), and splicing patterns. **b** Diagrams of typical enhancer- (*top*), promoter- (*middle*), and protein-transposon trap (bottom) insertions containing genetically encoded cargo, i.e., intersectional tool "X" for enhancer- and promoter trap insertions, or protein tag "Y" for protein trap insertions. While theoretically genome regulation is thought to be absent downstream of the trap, leaky "read through" regulation may escape the trap (indicated by *gray dashed lines*, *grayly colored exons*, and *light green* "Y" encoded exon). **c** Diagram of enhancer and promoter bashing. Individual enhancers (En1, En2, En3, and En4) and promoters (Pro) are subcloned in appropriate plasmids, i.e., upstream of a binary activator for promoter bashing, or upstream of a minimal promoter and binary activator for enhancer bashing. While a promoter can be defined as a piece of DNA of arbitrary length upstream of the 5′ UTR that directly influences transcriptional initiation, enhancers can be located at different positions near or within the gene. **d** Diagram of the generation of a genomic clone encompassing all regulatory elements that can be upgraded to include an intersectional tool "X" by DNA cloning methods, e.g., recombineering (see Sect. 1.4.2 and Fig. 1.6b). While theoretically genome regulation is thought to be absent downstream of the TGA stop codon, leaky "read through" regulation may escape the event (indicated by *gray dashed lines* and *gray colored exons*). Enhancer (En), promoter (Pro), start codon (ATG), stop codon (TGA), intersectional tool (X), protein tag (Y), transposon end (TE), splice acceptor site (SA), splice donor site (SD)

1.3.2 Non-fluorescent Protein Reporters

Besides fluorescent markers some non-fluorescent reporters are useful as well. A fusion with horseradish peroxidase is useful for transmission electron microscopy (Larsen et al. 2003; Watts et al. 2004). Recently, a family of highly antigenic molecules was engineered combining the advantages of both fluorescent proteins (i.e., high solubility and stability, and well tolerated by cells) and peptide epitope tags (i.e., small size and readily available, well validated, and reliable primary antibodies) (Viswanathan et al. 2015). The GFP protein backbone was used as a scaffold for numerous copies (i.e., 10–15) of single peptide epitope tags. Each of these epitope tags can bind many primary antibodies significantly amplifying the signal. The resulting tags were dubbed 'spaghetti monster' fluorescent proteins. Spaghetti monsters were generated for several commonly used peptide tags, i.e., HA, Myc, V5, Flag, OLLAS, and strep II. Orthogonal spaghetti monsters were used to reveal stereotyped cell arrangements in the fly visual system through multicolor stochastic labeling (Nern et al. 2015) (see Sect. 1.6).

1.4 Regulating Binary Activators

The expression pattern of a binary transcriptional activator depends on the regulatory elements that drive its expression (Fig. 1.3a). Regulatory elements can be connected to binary transcriptional activators through random transposition of mobile elements with a "trap" that encodes a synthetic piece of DNA that captures genomic regulatory

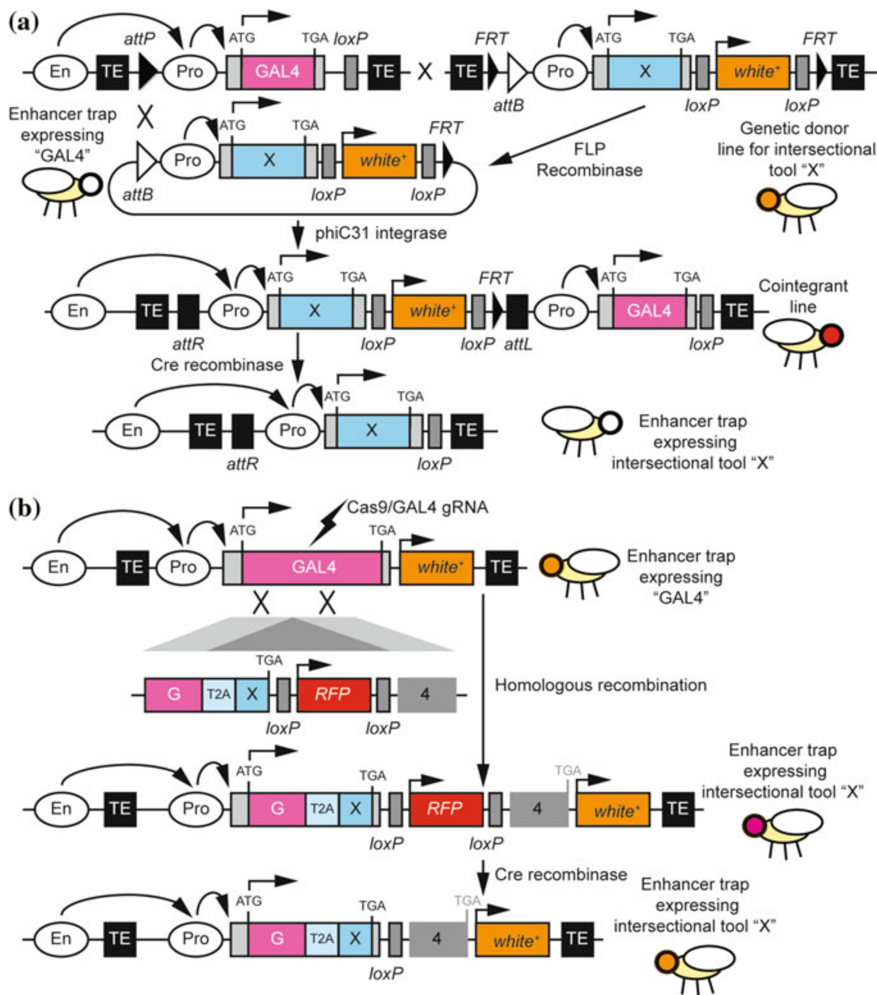

(a)
En1
Pro
Exon 1
ATG
Exon 2
Exon 3
TGA
En2
attP
TE
GAL4
loxP
white+
Transposition
Cre recombinase
InSITE insertion 1
InSITE insertion 2
(b)
En
yellow+
Flies with landing site
Microinjection
Cloned plasmid
attB
phiC31 integrase
Enhancer fusion expressing GAL4 or intersectional tool "X"
attR
attL

◀**Fig. 1.4** Enhancer analysis by enhancer element cloning and enhancer trapping by transposons. **a** Enhancer trapping illustrated by InSITE transposons. The same transposon can integrate at different locations in the genome. After isolation of individual insertion events, the transformation marker, flanked by *loxP* sites, can be removed by Cre recombinase. **b** In the pipeline, developed by Janelia Farm Research Campus, a cloned donor plasmid containing *attB* attachment site for phiC31-mediated site-specific integration, an enhancer, a minimal promoter, an intersectional genetic tool (e.g., GAL4 binary transactivator), and a transformation marker (the dominant color marker $white^{+}$) is microinjected into embryos, and integrates into an *attP* attachment-containing landing site. Using this system, enhancer expression patterns can be repurposed by cloning a different intersectional tool downstream of the enhancer and generating a new transgenic line by microinjection and phiC31-mediated integration. Transposon end (TE), enhancer (En), promoter or minimal promoter (Pro), intersectional tool (X), locus of crossover in P1 (*loxP*), attachment phage site (*attP*), attachment bacteria site (*attB*), attachment *left* site (*attL*), attachment *right* site (*attR*)

information surrounding the transposon insertion site (Fig. 1.3b). Traps come in different flavors: enhancer, promoter, or protein trap. An enhancer trap captures cumulative regulatory information from surrounding enhancers and silencers. Promoter and protein traps, on the other hand, capture all regulatory information of the host gene in which the transposon is integrated. Alternatively, a regulatory element and binary transcriptional activator are coupled together by bacterial cloning in a plasmid that is used for fly transgenesis. For historical reasons, as DNA elements regulating a specific gene or developmental process were often screened for by the tedious brute force process of random cloning, this method is called "bashing" (Fig. 1.3c). Finally, genomic DNA clones (Ejsmont et al. 2009; Venken et al. 2006, 2009), each potentially encompassing the majority if not all of a gene's entire regulatory repertoire can be used as starting material to dissect regulatory elements (Fig. 1.3d). To no one's surprise, each of these methods has advantages and disadvantages. Determining the expression behavior of enhancers requires empirical experimentation (Arnold et al. 2013; Kvon 2015), while the expression of genes can be deduced from large-scale RNA sequencing efforts (Graveley et al. 2011). The latter category is particularly useful to probe regulatory intersection between expression domains of previously characterized neuronal enhancers and genes that have important function during synaptic communication within the nervous system, e.g., neurotransmitters and neuropeptides (see Sect. 1.4.3).

1.4.1 Enhancer Trapping and Bashing

The concept of enhancer trapping was first demonstrated in *E. coli* by integrating transposons containing reporter genes near regulatory elements, upstream or downstream of the transposon insertion site, in order to study endogenous expression patterns (Casadaban and Cohen 1979). The development of *P* element-mediated transgenesis (Rubin and Spradling 1982) opened the door to enhancer trapping in *Drosophila* (O'Kane and Gehring 1987). The first *Drosophila* enhancer traps contained the *E. coli LacZ* gene, which enabled the visualization of expression patterns, but only after a colorimetric X-gal staining (O'Kane and



Gehring 1987). Replacing the LacZ gene with the GAL4 transcriptional activator provided limitless opportunities to express any reporter or modulator by binary activation regulated by any regulatory element (Brand and Perrimon 1993). Similar enhancer trap collections were made for GAL80 (Suster et al. 2004), GeneSwitch (Nicholson et al. 2008) and LexA (Miyazaki and Ito 2010), and expanded for GAL4 to accommodate the InSITE system (Gohl et al. 2011) (Fig. 1.4a).

To generate binary drivers with more restricted expression patterns, genomic DNA pieces encompassing putative enhancers are subcloned into transgenesis-competent plasmids upstream of a minimal promoter and GAL4 transcriptional activator (Fig. 1.4b). This method is generally known as enhancer bashing. The resulting plasmids are then integrated by *P* element-mediated transposition (Rubin and Spradling 1982), or at a specific docking sites in the fly genome

◂**Fig. 1.5** In vivo repurposing of enhancer traps by genetic crosses using InSITE and HACK methods. **a** The InSITE system allows the GAL4 transactivator in an enhancer trap transposon to be swapped for another intersectional genetic tool "X" using genetic crosses. An *attB*-containing genetic donor construct, flanked by *FRT* recombination sites, is liberated with FLP recombinase and is integrated by phiC31 integrase into the *attP* site of the enhancer trap construct. The cointegration event (i.e., donor construct integrated into acceptor enhancer trap construct), which is tracked by an eye color marker (*white*$^+$), is then treated with Cre recombinase in a second genetic cross to remove all genetic material located between *loxP* sites, i.e., GAL4 and the eye color marker, completing the swap of GAL4 to the new intersectional genetic tool "X". **b** Overview of the HACK method. An enhancer-driven GAL4 "acceptor" line, e.g., enhancer trap, can be converted to a new binary activator line through gene conversion after a Cas9/gRNA-mediated cut at GAL4. The "donor" element, located at a different genomic location has two targeting arms homologous to GAL4 flanking a T2A translational self-cleaving peptide fused to a novel binary activator, as well as the dominant eye color marker, red fluorescent protein (*RFP*) flanked by *loxP* sites, which can be removed by Cre recombinase in a second genetic cross. A similar strategy can be employed for transgenics generated by the JFRC pipeline. Transposon end (TE), enhancer (En), promoter or minimal promoter (Pro), intersectional tool (X), FLP recognition target (*FRT*), locus of crossover in P1 (*loxP*), attachment phage site (*attP*), attachment bacteria site (*attB*), attachment *left* site (*attL*), attachment *right* site (*attR*), 2A peptide of *Thosea asigna virus* (T2A), red fluorescent protein (RFP)

(Pfeiffer et al. 2010; Venken et al. 2006; Bischof et al. 2007; Groth et al. 2004; Knapp et al. 2015; Markstein et al. 2008), using the phiC31 integrase (Bischof et al. 2007; Groth et al. 2004), followed by extensive expression analysis (Jenett et al. 2012; Jory et al. 2012; Manning et al. 2012; Pfeiffer et al. 2008). Due to variable position effects that occur between different transposon insertion sites (Levis et al. 1985), site-specific integration is preferred; since transgenes with different regulatory elements can be integrated at the same docking site, position effects are mostly neutralized (Pfeiffer et al. 2008). Plasmids for enhancer bashing are available for fusions with GAL4 (Chiang et al. 2011; Pfeiffer et al. 2008; Apitz et al. 2004; Sharma et al. 2002).

At Janelia Farm Research Campus (JFRC), a collection of 7000 transgenic lines was generated and the expression patterns have been characterized in the adult brain and ventral nerve cord (Jenett et al. 2012), the embryonic central nervous system (Manning et al. 2012), and in larval imaginal discs (Jory et al. 2012). Since the JFRC collection is based upon cloned GAL4 enhancer fusions, repurposing an enhancer pattern using another binary system transactivator or intersectional tool can be accomplished by cloning the enhancer fragment upstream of the gene of interest and establishing a new transgenic line by microinjection (Pfeiffer et al. 2008, 2010). While creating a large number of lines by injection is labor-intensive, this system has the long-term advantage that once characterized, a large collection of enhancer trap lines does not need to be maintained in continuous culture (as is the case with other enhancer trap lines, since *Drosophila* cannot be readily cryopreserved), as any given driver line can be readily regenerated by microinjection when desired.

To simplify these labor-intensive strategies, a number of methods now unify all the tools for binary activation of gene expression under the same umbrella. These extensible genetic toolkits are all based on in vivo or in vitro exchange of the genes being driven by a captured regulatory element. One of these systems, Integrase

Swappable In vivo Targeting Element (InSITE) uses a two-step cassette exchange strategy with phiC31 integrase and Cre recombinase (Bischof et al. 2007) to convert a binary transactivator into another intersectional genetic tool (Fig. 1.5a) (Gohl et al. 2011). In the InSITE system, an enhancer trap line containing GAL4 and an appropriately positioned *attP* and *loxP* site serves as a target or "landing site" for phiC31-mediated integration of an *attB* and *loxP*-containing donor plasmid. The donor plasmid can be used to introduce other binary transactivators, hemidrivers, binary system repressors, or any other effector of interest. Once an integrant has been isolated, germline treatment with Cre recombinase can be used to remove GAL4 and to generate a cleanly swapped enhancer trap line. An independently developed method, G-MARET, is very similar to InSITE (Yagi et al. 2010).

One key design advantage of the InSITE system is that it can be carried out in vivo purely through genetic crosses, obviating the need to inject embryos with the plasmids necessary to generate the swaps (Gohl et al. 2011). To facilitate this process, chromosomally integrated *FRT*-flanked *attB* donor lines for commonly used intersectional tools have been established. Activating FLP recombinase liberates a circular episome from the chromosome analogous to an injected *attB* donor plasmid that can integrate into the *attP* site in an InSITE enhancer trap line (Fig. 1.5a). Because the recombinase and integrase reactions are very robust, generation of swaps is highly efficient either by injecting a donor plasmid or through genetic crosses only (Gohl et al. 2011).

A collection of more than 1000 InSITE GAL4 enhancer trap lines in an isogenic genetic background has been generated (Gohl et al. 2011; Silies et al. 2013). The chromosomal insertion sites of this collection have been mapped using a novel next-generation sequencing (NGS)-based strategy in which line identity was encoded in a small number of pools using digital error-correcting (Hamming) codes, and NGS libraries were prepared, enriched for *piggyBac* transposon ends using PCR, and sequenced (Gohl et al. 2014). Using this approach, the pattern of appearance of a transposon-adjacent sequence in the pools could then be used to determine the association between insertion site and line identity.

Most recently, a method was developed to convert any existing GAL4 line to a QF2 line using injections or genetic crosses, similar to InSITE. This method called Homology Assisted CRISPR Knock-in (HACK), utilizes the CRISPR/Cas9 system to induce double stranded breaks in a GAL4 transgene, followed by gene conversion at a QF2 donor transgene (Lin and Potter 2016) (Fig. 1.5b). While the method was demonstrated for conversion of GAL4 to QF2, it should be fairly straightforward to implement other binary activators and repressors in the pipeline.

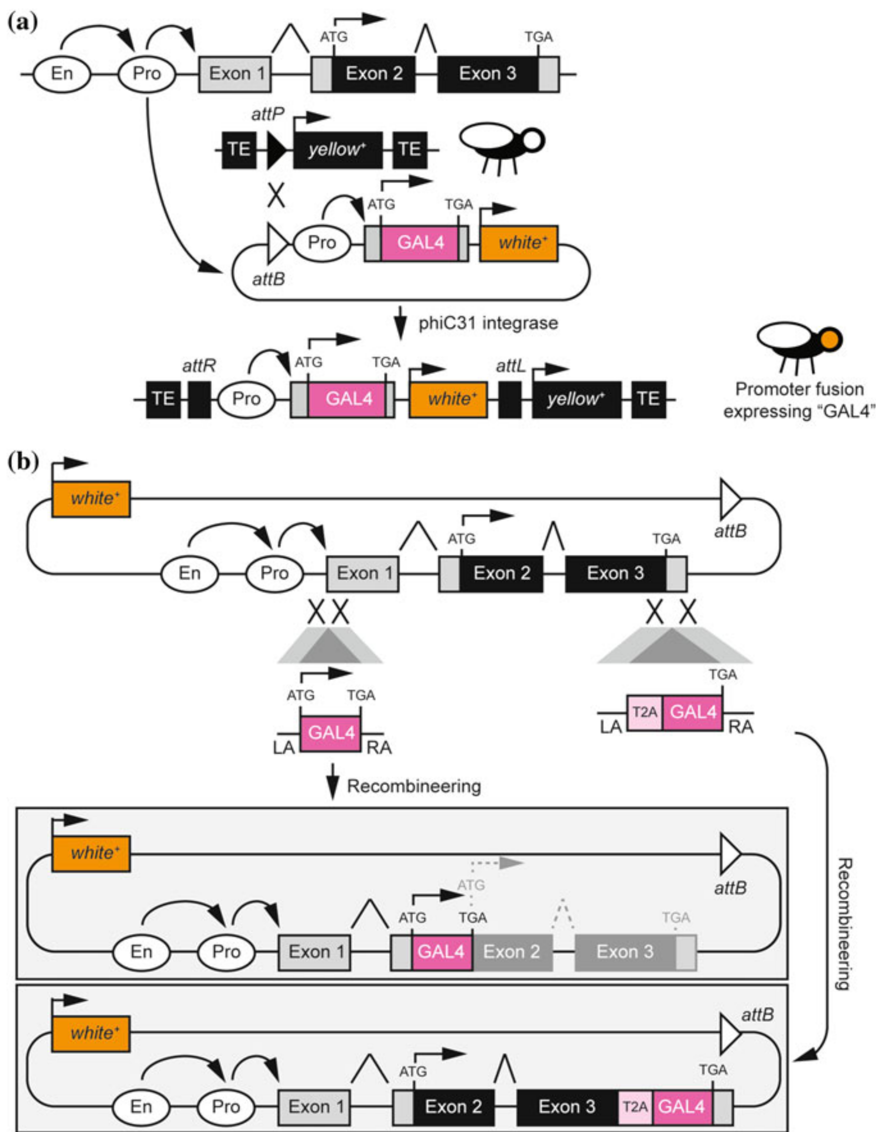
1.4.2 Promoter Bashing and Trapping

To generate binary drivers with expression patterns closely representing endogenous genes, genomic DNA pieces encompassing promoters are subcloned into transgenesis-competent plasmids upstream of the GAL4 transcriptional activator

(Fig. 1.6a). This method is generally known as promoter bashing. The resulting plasmids can then be integrated by transposition (Osterwalder et al. 2001; Roman et al. 2001), or at a specific docking site in the fly genome (Pfeiffer et al. 2010; Venken et al. 2006; Bischof et al. 2007; Groth et al. 2004; Knapp et al. 2015; Markstein et al. 2008), using the phiC31 integrase (Bischof et al. 2007; Groth et al. 2004). Again, site-specific integration is preferred over transposition since the latter results in variable position effects between different insertions (Levis et al. 1985). Plasmids for promoter bashing are available for fusions with GAL4 (Petersen and Stowers 2011; Pfeiffer et al. 2008), GeneSwitch (Osterwalder et al. 2001; Roman et al. 2001), LexA (Shearin et al. 2013; Petersen and Stowers 2011), and QF (Petersen and Stowers 2011). Such cloned promoters do not always accurately reflect endogenous expression of a gene, primarily because the cloned fragment may lack enhancer and/or repressor elements necessary for appropriate regulation (Gnerer et al. 2015).

A valuable alternative strategy is to use recombineering to integrate binary transcriptional activators in large genomic DNA clones that presumably cover the entire regulatory repertoire (Ejsmont et al. 2009; Venken et al. 2006, 2009; Sharan et al. 2009) (Fig. 1.6b). Binary transcriptional activators, such as GAL4 (Chan et al. 2011; Jin et al. 2012; Stowers 2011) and QF (Stowers 2011), can be amplified by PCR and readily introduced in the genomic locus through recombineering. Subsequently, recombineered plasmids are integrated in specific *attP* docking sites in the fly genome to neutralize genomic position effects (Pfeiffer et al. 2010; Venken et al. 2006; Bischof et al. 2007; Groth et al. 2004; Knapp et al. 2015; Markstein et al. 2008). Another approach to capturing and dissecting the entire regulatory region of a gene is through in situ enhancer bashing. This has been accomplished by introducing an *attP* landing site into a locus by gene conversion and using FRT mediated recombination to delete regulatory elements (Bieli et al. 2015a). Cloned rescue constructs containing full length, partial, or modified fragments of the deleted regulatory domain can be introduced to parse the functional elements of the regulatory domain (Bieli et al. 2015b).

To ensure full capture of all regulatory information acting on a gene, *Minos*-Mediated Integration Cassette (MiMIC) provides a trapping alternative for catching promoters (Venken et al. 2011b) (Fig. 1.7a). MiMIC is a *Minos*-based transposon with two inverted phiC31 *attP* sites flanking a marker that can be swapped with a replacement cassette using recombinase-mediated cassette exchange (RMCE) (Bateman et al. 2006). MiMIC insertions that are located in a 5′ UTR non-coding intron of a gene can be replaced with a splice acceptor site followed by a binary factor revealing the expression pattern of the gene. This was illustrated for GAL4 (Gnerer et al. 2015; Venken et al. 2011b), LexA (Gnerer et al. 2015), and QF (Venken et al. 2011b). This strategy is feasible for ~13% of MiMIC insertions (Venken et al. 2011b; Nagarkar-Jaiswal et al. 2015). Genes without a 5′ UTR non-coding intronic MiMIC insertion can easily be modified using CRISPR/Cas9-stimulated gene targeting (Gratz et al. 2014), and an ectopic targeting template accommodating promoter trapping (Fig. 1.7b).



1.4.3 Protein Trapping

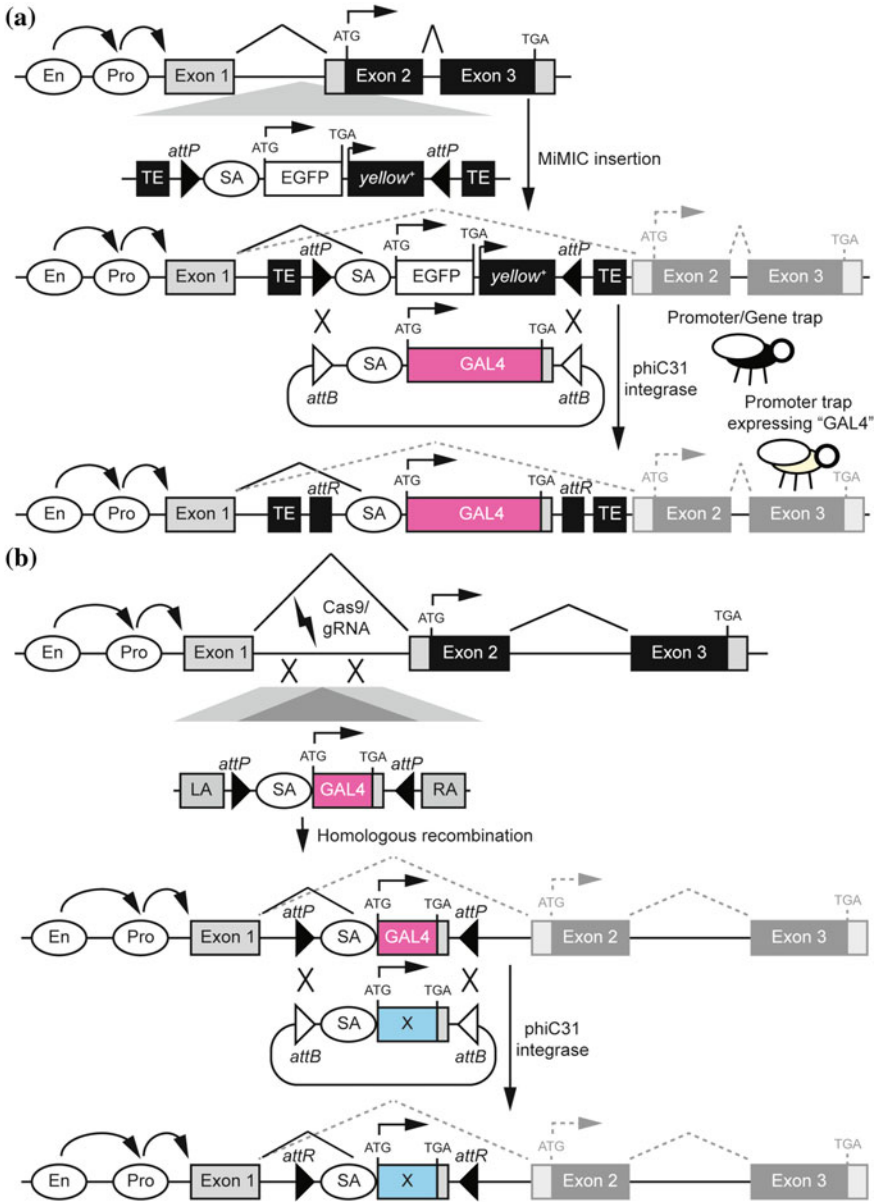
An alternative strategy to generate promoter traps is through protein trapping using the MiMIC system, introduced in the previous section. Under normal circumstances, a protein trap is made by converting a MiMIC transposon insertion in a coding intron into an artificial exon encoding a genetically encoded protein tag (e.g., superfolder GFP) to visualize endogenous protein localization. However, each

◂**Fig. 1.6** Promoter analysis by promoter bashing and recombineering in genomic DNA clones. **a** Similar to enhancer bashing (see Fig. 1.4b), promoter pieces can be cloned into a plasmid containing *attB* attachment site for phiC31-mediated site-specific integration, the GAL4 binary transactivator, and a transformation marker (the dominant color marker *white*$^{+}$). After microinjection, plasmids can integrate into an *attP* attachment-containing landing site. Using this system, promoter expression patterns can be repurposed by cloning a different intersectional tool (i.e., “X”) downstream of the promoter and generating a new transgenic line by microinjection and phiC31-mediated integration. **b** The integration of the GAL4 binary transactivator by recombineering into genomic DNA clones encompassing all regulatory elements. Starting clone and recombineered clone can be integrated in docking sites as described previously (see Figs. 1.4b and 1.6a). While theoretically translation is thought to be absent downstream of the recombineering event, leaky “read through” regulation may escape the event (indicated by *gray dashed lines* and *gray colored exons*). Alternatively, the inclusion of a T2A peptide strategy can generate a bicistronic transcript resulting in the expression of both the GAL4 binary transactivator and the host gene product (see also Figs. 1.5b and 1.8a, b). Using this system, promoter expression patterns can be repurposed by recombineering a different intersectional tool into the genomic clone and generating a new transgenic line by microinjection and phiC31-mediated integration. Enhancer (En), promoter (Pro), transposon end (TE), attachment phage (*attP*), attachment bacteria (*attB*), attachment *left* (*attL*), attachment *right* (*attR*), *left* homology arm for recombineering (LA), *right* homology arm for recombineering (RA), 2A peptide of *Thosea asigna virus* (T2A), splice acceptor site (SA), enhanced green fluorescent protein (EGFP)

of these intragenic intronic insertions can be converted into gene-specific binary factors, through the use of novel exchange cassettes containing a splice acceptor followed by a self-cleaving T2A peptide sequence fused to the coding sequence of the transcriptional activator followed by a 3′ UTR (Fig. 1.8a). This method was illustrated for GAL4 (Gnerer et al. 2015; Diao et al. 2015), LexA (Diao et al. 2015), QF2 (Diao et al. 2015), split GAL4 (Diao et al. 2015), and GAL80 (Gnerer et al. 2015; Diao et al. 2015). Similar to InSITE, this method also works through genetic crosses (Diao et al. 2015). This strategy is feasible for ~18% of all MiMIC insertions (Venken et al. 2011b; Nagarkar-Jaiswal et al. 2015). When a MiMIC insertion is not available in a gene, MiMIC-like elements compatible with phiC31-catalyzed RMCE can be integrated using CRISPR/Cas9 at any location in the fly genome (Diao et al. 2015) (Fig. 1.8b).

1.5 Refining Genetic Targeting by Intersectional Perturbations

Enhancer traps are rarely expressed in a single cell or cell type, or at a single stage in development. To further refine enhancer trap expression patterns, a number of intersectional genetic targeting approaches have been developed. These approaches effectively implement Boolean logic gates within cells, i.e., the integration of regulatory information coming from multiple expression patterns (Fig. 1.9a). Given their utility, modularity, and widespread adoption, binary systems form the basis of most intersectional methods. In addition, the FLP/*FRT* recombinase system provides another useful tool (Golic and Lindquist 1989), which can be used in


(a)
En
Pro
Exon 1
ATG
Exon 2
Exon 3
TGA
MiMIC insertion
TE
attP
SA
ATG
EGFP
TGA
yellow+
attP
TE
Promoter/Gene trap
attB
GAL4
phiC31
integrase
Promoter trap
expressing "GAL4"
attR
(b)
Cas9/
gRNA
LA
RA
Homologous recombination
X

◀**Fig. 1.7** Promoter analysis by promoter trapping using MiMIC transposons integrated in 5′ UTR non-coding introns. **a** Promoter trapping of a 5′ UTR non-coding intronic MiMIC transposon insertion by recombinase-mediated cassette exchange between two pairs of *attP* and *attB* sites. The exchanged cassette encodes a splice acceptor site followed by the GAL4 binary transactivator. Using this system, promoter expression patterns can be repurposed by cloning a different intersectional tool downstream of the splice acceptor and generating a new *transgenic line* by microinjection and phiC31-driven recombinase-mediated cassette exchange. **b** Genes that do not have a 5′ UTR non-coding intronic insertion can be trapped by CRISPR/Cas9 targeting of a promoter trap element, similar to what has been reported for protein traps within a MiMIC-style exchange element (see Fig. 1.8b). Subsequent recombinase-mediated cassette exchange catalyzed by phiC31 integrase can replace the GAL4 binary transactivator with a novel intersectional tool "X". In both cases, while theoretically genome regulation is thought to be absent downstream of the promoter trap, leaky "read through" regulation may escape the trap (indicated by *gray dashed lines* and *gray colored exons*). Enhancer (En), promoter (Pro), splice acceptor site (SA), enhanced green fluorescent protein (EGFP), transposon end (TE), attachment phage (*attP*), attachment bacteria (*attB*), attachment *left* (*attL*), *left* homology arm for homologous recombination (LA), *right* homology arm for homologous recombination (RA)

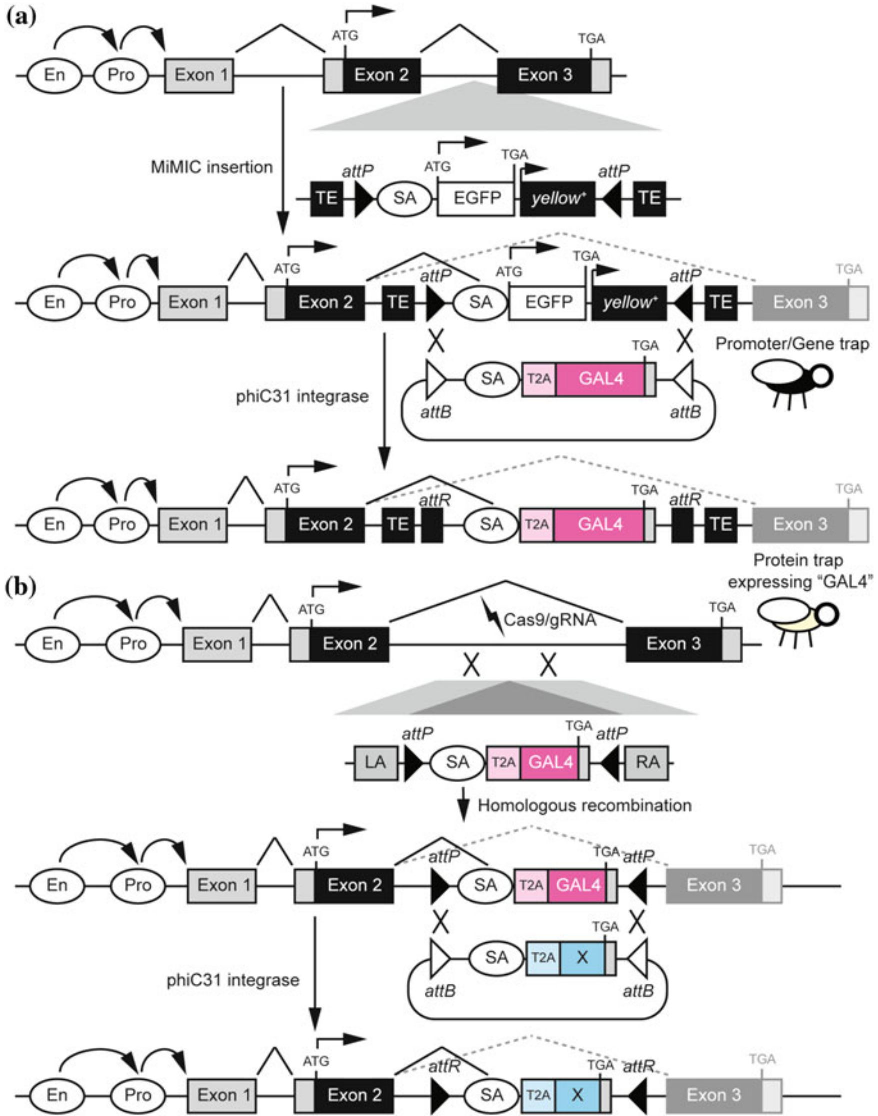
conjunction with binary expression systems for intersectional refinement of expression patterns (Bohm et al. 2010). While there are too many possible intersectional strategies to enumerate, with the tools currently available, essentially any desired basic logic gate can be implemented to refine overlapping expression patterns (Fig. 1.9b). Below we provide some examples and also highlight additional tools that enable specific intersectional operations.

1.5.1 OR Gates

OR gates, which combine the expression patterns of two separate transgenes, can be simply constructed by co-expressing two drivers from a single binary system, or by using two driver lines from different binary systems along with appropriate effector lines expressing the same gene. Since enhancer trap patterns are typically broader than desired, the use of a true OR gate is limited in practice as the goal is typically to refine rather than combine expression patterns. More commonly, two orthogonal driver lines will be used to drive expression independently in two distinct tissues, in order to, for instance, manipulate or monitor neighboring cell populations (Potter et al. 2010).

1.5.2 AND Gates

AND gates (Fig. 1.9c), and NOT gates (see Sect. 1.5.3) are the most useful operations in order to combinatorially refine expression patterns. There are multiple ways in which an AND gate (i.e., expressing an effector gene only in the cells that overlap between two expression patterns) can be constructed. One common way is to use two independent binary system drivers in conjunction with the FLP/*FRT*

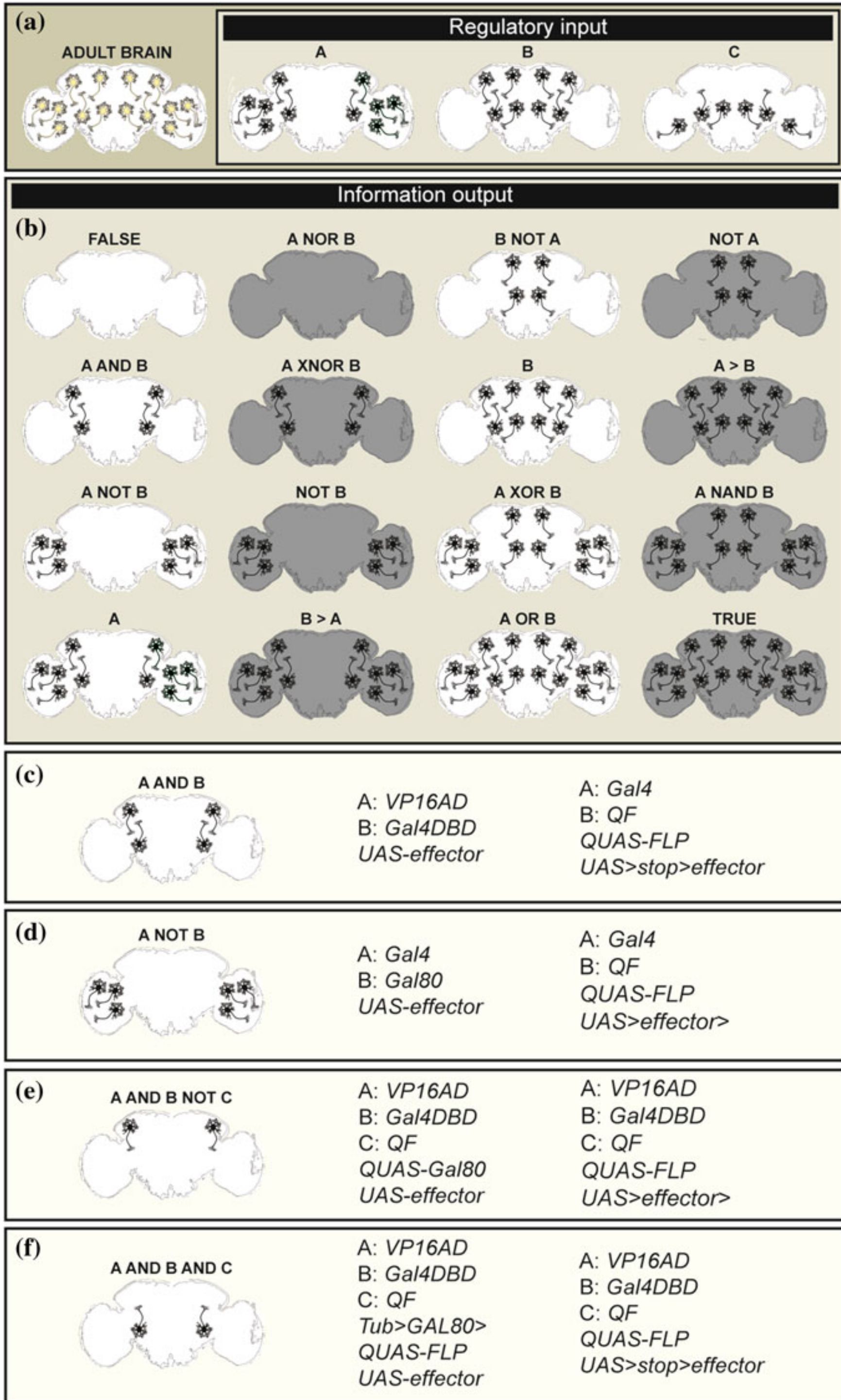


system. The FLP gene encodes a site-specific recombinase protein that catalyzes the recombination of a pair of *FRT* sites (Golic and Lindquist 1989). Depending on the relative orientation of the two *FRT* sites on a linear chromosome, recombination can result in either a deletion or an inversion of the intervening sequence. For use in intersectional targeting, two basic strategies, FLP-in (Fig. 1.10a) (Basler and Struhl 1994; Struhl and Basler 1993), and FLP-out (Fig. 1.10b) (Bohm et al. 2010), are used. In the FLP-in approach, an *FRT*-flanked "stop" cassette containing transcriptional or translational terminators is inserted between a ubiquitous or

◂**Fig. 1.8** Protein trapping by transposons and gene targeted protein traps. **a** Protein trapping of a coding intronic MiMIC transposon insertion by recombinase-mediated cassette exchange between two pairs of *attP* and *attB* sites. The exchanged cassette encodes a splice acceptor site followed by a T2A self-cleaving peptide fused to the GAL4 binary transactivator. *Note* that this is not a classical protein trap resulting in the incorporation of a protein tag in the middle of the host gene product (see Fig. 1.3b, *bottom*); this is rather a hybrid protein/gene trap resulting in C-terminal truncated gene product followed by intersectional tool "X". While theoretically genome regulation is thought to be absent downstream of the transposon trap, leaky "read through" regulation may escape the trap (indicated by *gray dashed lines* and *gray colored exons*). Using this system, gene expression patterns can be repurposed by cloning a different intersectional tool downstream of the splice acceptor site and generating a new transgenic line by microinjection and phiC31-driven recombinase-mediated cassette exchange. **b** Simplified overview of CRISPR/Cas9 targeting of a protein trap element in a coding intron of a target gene. The protein trap (embedded in a MiMIC-style exchange element cassette) encodes a splice acceptor site followed by a T2A self-cleaving peptide fused to the GAL4 binary transactivator. Subsequent recombinase-mediated cassette exchange catalyzed by phiC31 integrase can replace the GAL4 binary transactivator with a novel intersectional tool "X". While theoretically genome regulation is thought to be absent downstream of the Crispr/Cas9 t targeted trap, leaky "read through" regulation may escape the trap (indicated by *gray dashed lines* and *gray colored exons*). Enhancer (En), promoter (Pro), transposon end (TE), splice acceptor (SA), enhanced green fluorescent protein (EGFP), 2A peptide of *Thosea asigna virus* (T2A), intersectional tool (X), attachment phage site (*attP*), attachment bacteria site (*attB*), attachment *left* site (*attL*)

transactivator-inducible promoter and an effector gene of interest. Upon exposure to FLP recombinase, the stop cassette is removed, resulting in expression of the transgene in tissues where the promoter is active. In the FLP-out approach, the effector gene itself is flanked by *FRT* sites, resulting in deletion of the effector gene upon FLP expression. An AND gate can be created by using a FLP-in cassette expressed under the control of one binary transactivator and using a second transactivator to drive FLP expression (Fig. 1.10c).

Another elegant method of constructing an AND gate is to use a split hemidriver system (Fig. 1.10d). Since the AD of GAL4 is genetically separable from the DBD (Ma and Ptashne 1987b), it is possible to generate split-GAL4 proteins where the AD and DBD are each fused to heterodimerizing leucine zippers. When the two split-GAL4 proteins are expressed in the same cell, they are able to dimerize and reconstitute functional GAL4 activity (Pfeiffer et al. 2010; Luan et al. 2006). Since the LexA system uses a transactivator that is by design a chimeric fusion of a DBD and an AD, split-LexA hemidrivers can also be made (Ting et al. 2011). Notably, the establishment of *UAS*-LexA-DBD lines allows GAL4 lines to be intersected with the split-LexA system. The DBD and AD of QF are also separable and functional GAL4: QF and LexA:QF chimeras have been made (Riabinina et al. 2015). However, no split-QF hemidriver lines currently exist in *Drosophila*, though this system has been generated in the nematode worm *Caenorhabditis elegans* (Wei et al. 2012). The level of expression driven by hemidriver AND gates can also be controlled by using ADs of different strengths. In addition to the originally reported GAL4-AD hemidriver (which drives weak expression) and VP16AD hemidriver (which drives stronger expression) (Luan et al. 2006), a p65AD hemidriver has been generated which drives still stronger levels of effector expression (Pfeiffer et al. 2010).


(a)
ADULT BRAIN
Regulatory input
A
B
C
Information output
(b)
FALSE
A NOR B
B NOT A
NOT A
A AND B
A XNOR B
B
A > B
A NOT B
NOT B
A XOR B
A NAND B
A
B > A
A OR B
TRUE
(c)
A AND B
A: VP16AD
B: Gal4DBD
UAS-effector
A: Gal4
B: QF
QUAS-FLP
UAS>stop>effector
(d)
A NOT B
A: Gal4
B: Gal80
UAS-effector
A: Gal4
B: QF
QUAS-FLP
UAS>effector>
(e)
A AND B NOT C
A: VP16AD
B: Gal4DBD
C: QF
QUAS-Gal80
UAS-effector
A: VP16AD
B: Gal4DBD
C: QF
QUAS-FLP
UAS>effector>
(f)
A AND B AND C
A: VP16AD
B: Gal4DBD
C: QF
Tub>GAL80>
QUAS-FLP
UAS-effector
A: VP16AD
B: Gal4DBD
C: QF
QUAS-FLP
UAS>stop>effector

◂**Fig. 1.9** Logic gates for intersectional refinement of expression patterns in *Drosophila melanogaster*. **a** Schematic of the integration of regulatory information coming from three expression domains. Seven hypothetical neurons in each half brain are illustrated in the adult brain that can be activated by regulatory input, i.e., “A”, “B”, or “C” regulation. **b** Schematic diagrams of all sixteen logic gates, just coming from two regulatory inputs, i.e., “A” and “B”. *Dark* coloring indicates active regulation. (**c**, **d**) Most useful logic gates and representative transgenic implementations of intersectional logic gates. **c** A two-input AND gate, and two examples with requirements at the level of genetic components. **d** A two-input NOT gate, and two examples with requirements at the level of genetic components. **e**, **f** Examples of three-input combinatorial gating. **e** A three-input AND/NOT gate, and two examples with requirements at the level of genetic components. **f** A three-input AND gate, and two examples with requirements at the level of genetic components

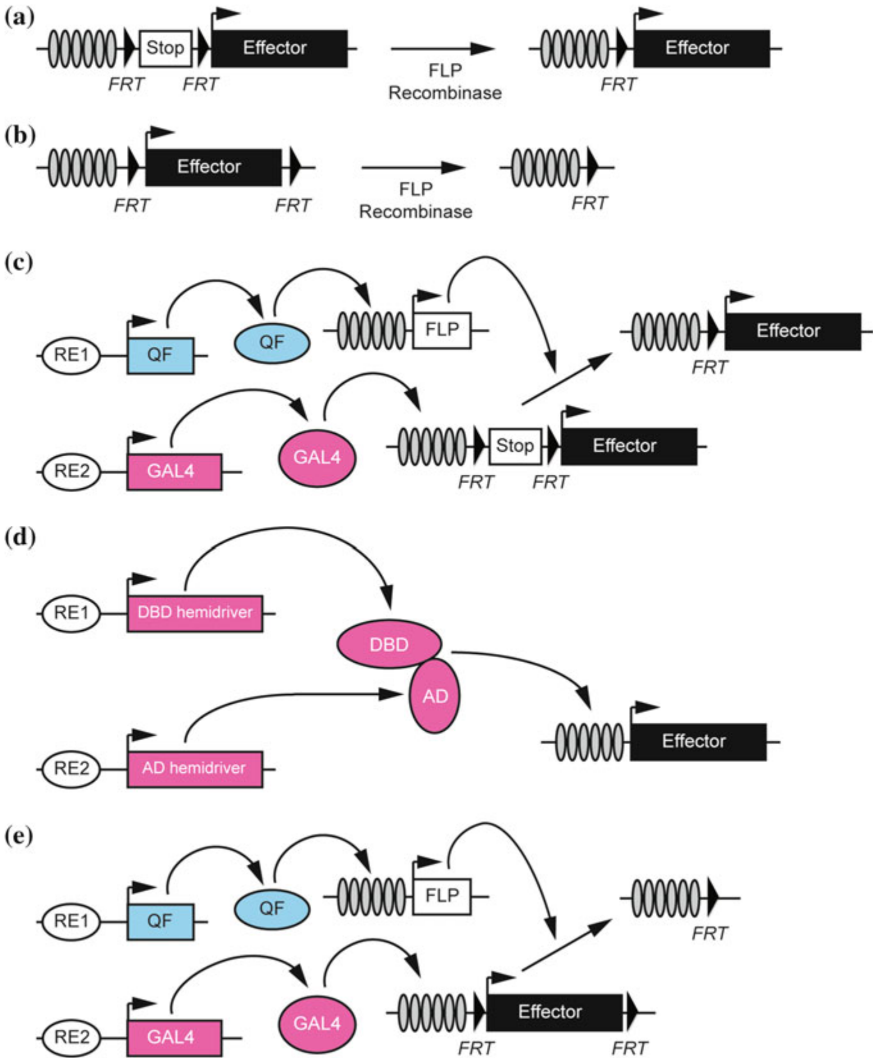
A specialized case of an AND gate is the GRASP (i.e., GFP reconstitution across synaptic partners) system (Feinberg et al. 2008; Gordon and Scott 2009), recently expanded toward multicoloring (Macpherson et al. 2015). In this system, the goal is not to refine expression of a binary system driven effector gene, but rather to report on synaptic interactions between adjacent cells. This is accomplished by expressing non-fluorescent split GFP proteins under the control of two orthogonal binary systems, GAL4 and LexA. When the two-cell populations targeted by these binary systems are in close proximity, which occurs at synapses, GFP function is reconstituted.

1.5.3 NOT Gates

The natural repressor proteins of GAL4 and QF (GAL80 and QS, respectively) provide a convenient means for generating NOT gates (i.e., subtracting one expression pattern from a second expression pattern) (Fig. 1.9d) (Lee and Luo 1999; Potter et al. 2010). NOT gates can also be implemented using FLP-out constructs, where the FLP-out construct is driven by one binary transactivator, and FLP is expressed under the control of another transactivator (Fig. 1.10e). An important consideration in using GAL80 or QS is that in order to get effective disruption of transactivator activity, the repressor protein must be expressed at a comparable level to that of the transactivator. Optimizing the transcriptional and translational regulatory elements associated with the effector or enhancer trap construct can help achieve the necessary high levels of repressor expression for a functional NOT gate (Pfeiffer et al. 2010, 2012).

1.5.4 Combinatorial AND/NOT Gating

While more esoteric logic gates can be devised, e.g., NAND, NOR, XOR, and XNOR gates (Fig. 1.9b), their practical utility is limited. A more useful



experimental application is the layering of multiple AND and/or NOT gates to dramatically refine an expression pattern. Examples of such layered logic gates are illustrated in Fig. 1.9e (i.e., A AND B NOT C) and Fig. 1.9f (i.e., A AND B AND C). The development of new binary activation systems and recombinases together with orthogonal recombination target sites will further broaden the combinatorial palette (Nern et al. 2011; Hadjieconomou et al. 2011).

◀**Fig. 1.10** Experimental intersectional paradigms commonly used in *Drosophila melanogaster*. **a** The FLP-in approach uses an effector transgene that is interrupted by an *FRT*-flanked stop cassette. In the presence of FLP recombinase, the stop cassette is excised, allowing expression of the effector gene. **b** In the FLP-out approach, the effector gene itself is flanked by *FRT* sites and is excised in the presence of FLP recombinase, preventing effector gene expression. **c** An AND gate based on FLP-in technology. The FLP-in is controlled by one binary transactivator (e.g., GAL4 driven by one regulatory input) while a second transactivator (e.g., QF driven by a second regulatory input) activates FLP expression. **d** An AND gate base on a split binary system. Split hemidrivers are constructed by fusing heterodimerizing leucine zippers to the modular DNA binding domain (DBD) or activation domain (AD) of transactivator proteins. Typically, each hemidriver half is expressed from regulatory elements (RE1 and RE2) and transactivator function is reconstituted only in cells that express both hemidriver halves. DBD hemidriver can be GAL4DBD or LexADBD, while AD hemidrivers can be GAL4AD, VP16AD, or p65AD. **e** A NOT gate based on implementing FLP-out technology. The FLP-out construct is driven by one binary transactivator (e.g., GAL4 driven by one regulatory input), while FLP is expressed by an orthogonal transactivator system (e.g., QF driven by a second regulatory input). Flp recognition target (*FRT*), regulatory element (RE), DNA binding domain (DBD), activation domain (AD)

1.6 Mitotic Analysis and Multicolor Stochastic Labeling Strategies in *D. melanogaster*

Comprehensive brain wiring maps are needed to understand how information flows in order to orchestrate behaviors. The ability to label isolated single neurons and to examine their entire projection patterns (dendritic and axonal processes) has been a critical anatomical limitation to study how neural circuits are organized.

Visual information is processed in the adult *Drosophila* optic lobe which contains ~60,000 neurons (Hofbauer and Campos-Ortega 1990), whose cell bodies are found in the outer brain surface and their projections cluster in internal neuropil structures. Classical studies using Golgi impregnations have allowed the study of the organization of the fly optic lobe and revealed the enormous cell diversity, with over 100 morphologically distinct cell types (Cajal and Sanchez 1915; Fischbach and Dittrich 1989; Strausfeld 1976). More recent studies (Gao et al. 2008; Morante and Desplan 2008) have made use of modern genetic tools to label individual neurons or groups with a Golgi staining-like resolution using stochastic recombination events with MARCM (Lee and Luo 1999) (Fig. 1.11), MARCM derivatives (Potter et al. 2010), or FLP-in techniques (Fig. 1.10a), especially Brainbow technologies (Fig. 1.12). Although it has been possible to collect large neural datasets with single-color MARCM labeling (Chiang et al. 2011; Costa et al. 2016) (Fig. 1.13a), a major limitation of these labeling studies has been the inability to resolve the morphology of individual cells when cells are in close proximity with one another (Fig. 1.13b). Thus, visualization of the neuronal morphology and the spatial arrangements to discriminate adjacent neurons and visualize cellular interactions within the same brain requires methods to distinguish the processes of multiple individual neurons in different colors (compare Fig. 1.13c, d), as discussed below (Fig. 1.12). While full electron microscopic reconstruction can be used to track and determine the connectivity of groups of neighboring neurons (Takemura

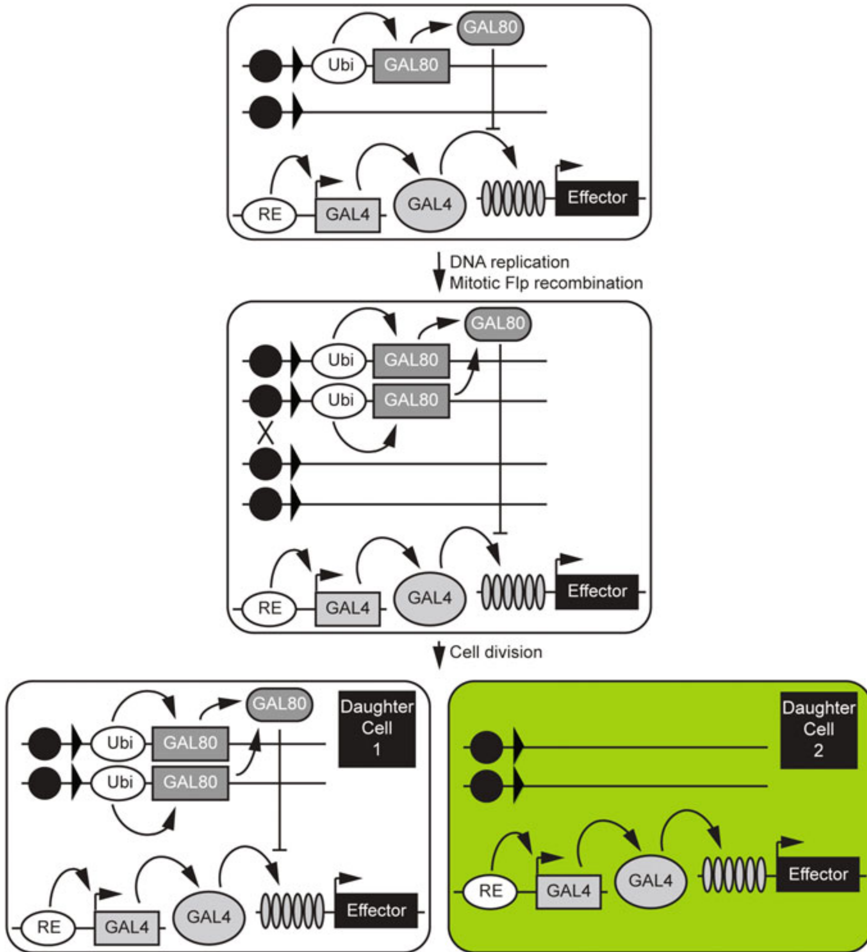


Fig. 1.11 Neuronal labeling strategies in *Drosophila melanogaster* using mitotic analysis. Schematic of typical mitotic analysis illustrated by mitotic analysis using a cell repressible marker (MARCM). Before cell division, all cells are unlabeled (i.e., the GAL80 repressor inhibits GAL4-mediated activation of effector). After cell division, one daughter cell remains unlabeled (i.e., twice the amount of the GAL80 repressor maintains inhibition of GAL4-mediated activation of effector), while the second cell becomes *labeled* or "*marked*" (i.e., lack of the GAL80 repressor ensures GAL4-mediated activation of effector). Ubiquitous promoter (Ubi), regulatory element (RE)

et al. 2013, 2015), such approaches are extremely labor-intensive and not practical for large-scale characterization of complex brain regions.

In this regard, the visualization of multiple individual neurons from defined cell populations has been greatly enriched by the development of methods for combinatorial multicolor stochastic labeling inspired by the mouse Brainbow technique

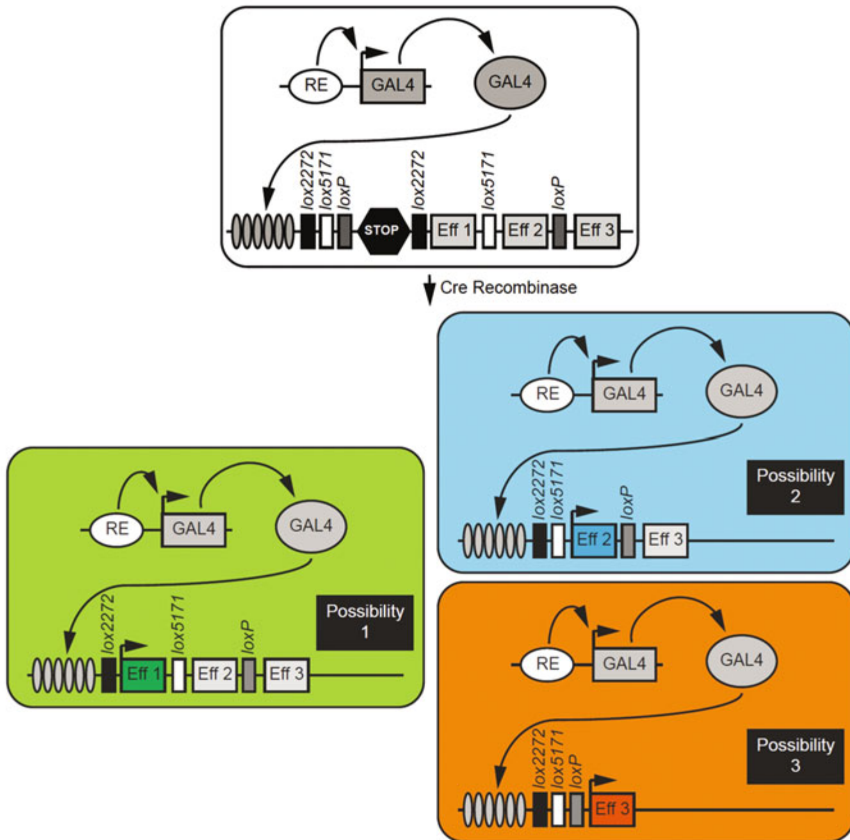


Fig. 1.12 Neuronal labeling strategies in *Drosophila melanogaster* using postmitotic single choice stochastic analysis. Schematic of typical postmitotic single choice stochastic analysis illustrated by dBrainbow. After Cre-mediated stochastic recombination, cells have activation of one effector (i.e., possibility 1), a second effector (i.e., possibility 2), or a third effector (i.e., possibility 3). Regulatory element (RE), locus of crossover in P1 (*loxP*), orthogonal *loxP* sites (*lox2272* and *lox5171*)

(Livet et al. 2007), that uses Cre/*LoxP*-mediated site-specific recombination to drive stochastic and combinatorial expression of multiple fluorescent proteins within a cell population. The use of multiple copies of this construct allowed 90 different distinguishable hues, enabling many individual neurons to be simultaneously identified (Livet et al. 2007).

Several adaptations of Brainbow are available in *Drosophila* (Hadjieconomou et al. 2011; Hampel et al. 2011) that combine the power to regulate transgene expression specifically in different neural populations using the GAL4/*UAS*-system (Brand and Perrimon 1993) with the label diversity provided by stochastic color choice. dBrainbow (Hampel et al. 2011) consists of a single *UAS*-reporter construct

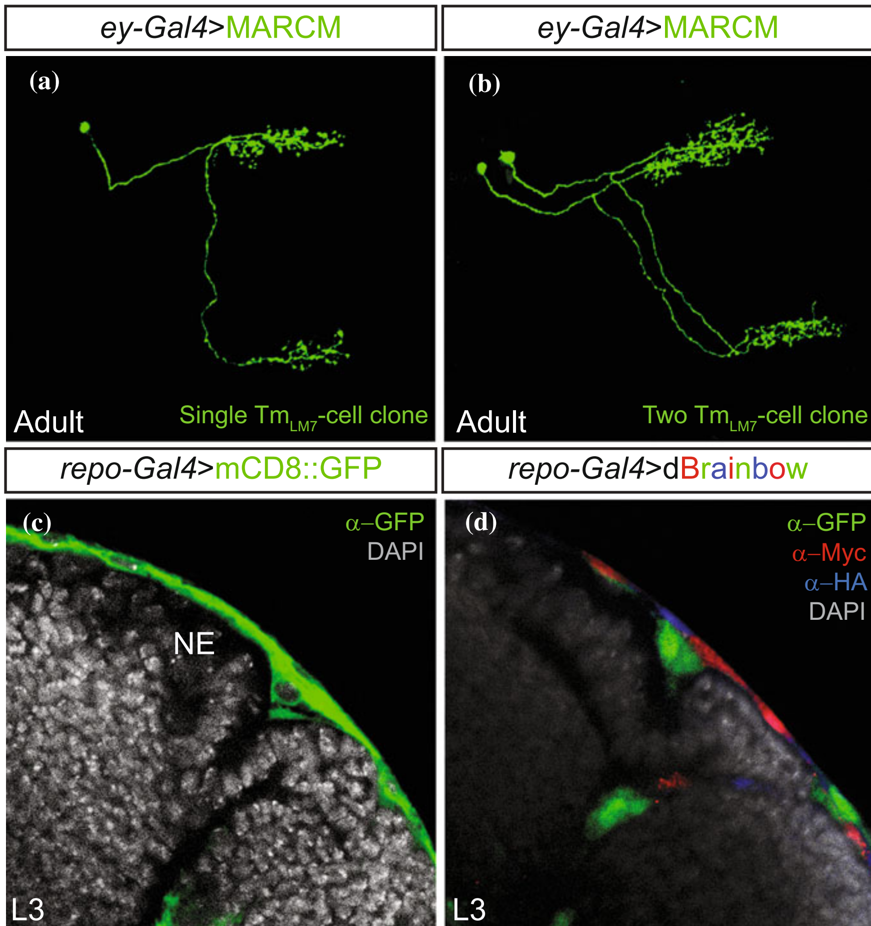


Fig. 1.13 Comparison between mitotic and postmitotic single choice stochastic neuronal labeling strategies in *Drosophila melanogaster*. **a** Single MARCM clones in adult TmLM7 neurons using *ey-GAL4*. **b** Two-cell MARCM clones in adult TmLM7 neurons using *ey-GAL4*. **c** Surface-associated glial cells are labeled by the pan-glial driver *repo-GAL4* driving *UAS*-mCD8::GFP (*green*) in a third instar larval optic lobe. DAPI counterstaining highlight all nuclei. *NE* neuroepithelial cells. **d** Surface-associated glial cells: expression of *UAS*-dBrainbow in all glia by *repo*-GAL4 reveals perineurial (*blue*), subperineurial (*red*) and optic-lobe-associated cortex (*green*) glia in a third instar larval optic-lobe. DAPI counterstaining highlight all nuclei. *NE* neuroepithelial cells

containing a transcriptional stop sequence followed by genes encoding three cytoplasmic fluorescent proteins, that are flanked by incompatible Cre recombinase recognition sites (*LoxP* sites) and thus allows three recombination outcomes. In the presence of a Cre recombinase, recombination between one of the identical pairs of lox sites will lead to the random and permanent selection of one of the three

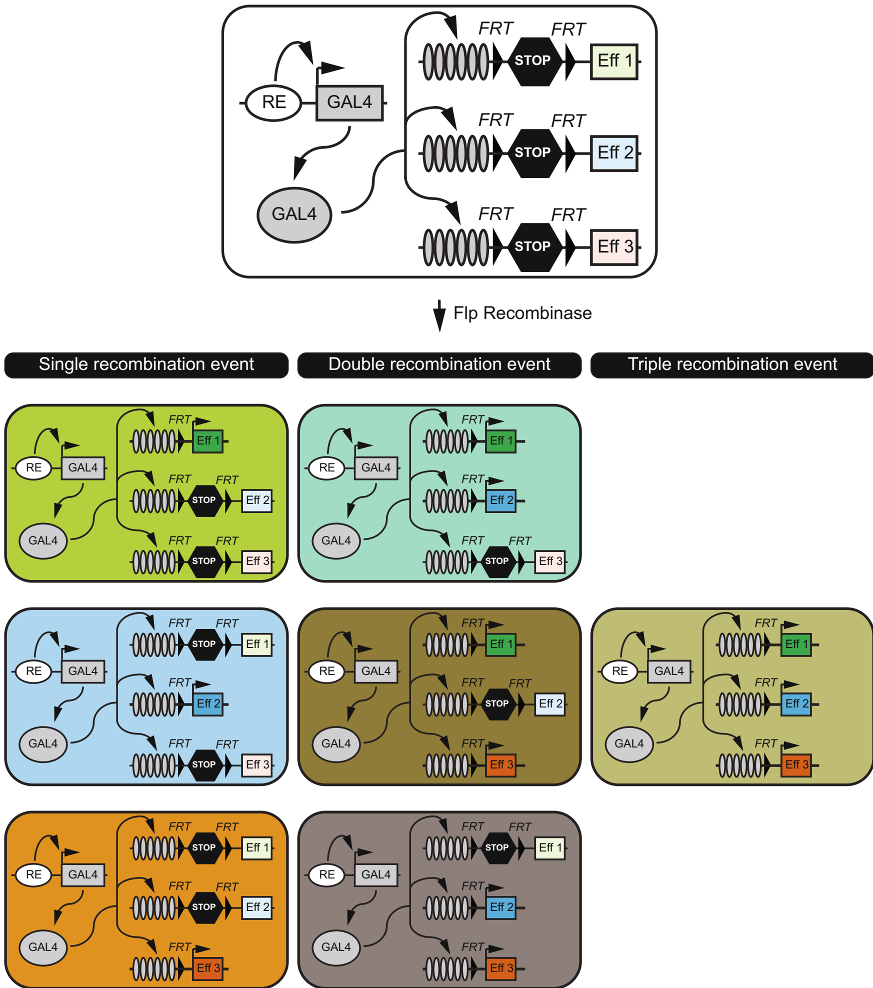


Fig. 1.14 Neuronal labeling strategies in *Drosophila melanogaster* using postmitotic multiple choice stochastic analysis. Schematic of multiple choice stochastic analysis illustrated by Multi-color FLP Out (MCFO) technology. After FLP-mediated stochastic removal of a transcriptional terminator present in each of three orthogonal labeling units (i.e., one unit controlling effector 1, a second unit controlling effector 2, and a third unit controlling effector 3), all combinatorial events are possible ($2^3 - 1$). All events indicating single recombination events (i.e., 3 events), two recombination events (i.e., 3 events), and three recombination events (i.e., 1 event) are shown. Regulatory element (RE), FLP recognition target (*FRT*), effector (Eff)

fluorescent proteins (Fig. 1.12). However, the use of Cre recombinase poses two problems in flies. First, it is potentially toxic when expressed at high levels (Heidmann and Lehner 2001). Second, there is a lack of efficient inducible Cre-lines (Siegal and Hartl 1996), resulting in labeling of clonal groups of cells

(Hampel et al. 2011). The LOLLIbow (live imaging optimized multicolor labeling by light-inducible Brainbow) method uses a photo-inducible form of Cre (split-Cre) to activate recombination in vivo after the illumination with a blue light (Boulina et al. 2013), allowing the acquisition of data at multiple times from the same sample. Thus, this method is ideal to analyze morphogenesis with single-cell resolution at embryonic, larval and pupal stages.

To bypass the limitations of Cre in flies, Flybow (Hadjieconomou et al. 2011) instead uses Flp recombinase to rearrange a single *UAS*-construct with two cassettes, each encoding two fluorescent proteins in opposing orientations flanked by *FRT* sites. Both Flybow and dBrainbow have been applied to study embryonic, larval, pupal, and adult nervous systems in fixed tissues, but also can be used for live imaging of endogenous proteins (Hadjieconomou et al. 2011; Hampel et al. 2011).

A novel strategy for multicolor stochastic labeling in the nervous system has been recently developed called Multicolor Flip-Out (MCFO) (Fig. 1.14) (Nern et al. 2015; Wolff et al. 2015). This method is a multicolor adaptation of the FLP-in technique (Basler and Struhl 1994) and employs a combination of stop cassette constructs with multiple copies of different epitope tags inserted into a myristoylated non-fluorescent GFP backbone (Pfeiffer et al. 2010; Viswanathan et al. 2015). These novel protein reporters called "spaghetti monster" fluorescent proteins (see Sect. 1.3.2) improve targeting the plasma membrane to identify fine neuronal processes. The heat shock inducible modified FLP/*FRT* system used in the Flybow and MCFO techniques allow dense or sparse multicolor stochastic labeling depending on the duration and timing of induction of the FLP recombinase expression (Nern et al. 2015; Hadjieconomou et al. 2011). Early heat shocks (when neuroblasts are dividing) favor labeling clonal groups of cells, while late heat shocks facilitate the identification of single postmitotic cells.

The cell labeling tools described so far are very useful for determining single-cell properties, by labeling a small percentage of cells within a tissue. However, multicolor labeling methods like TIE-DYE (three independent excisions dye) (Worley et al. 2013) and Raeppli (Kanca et al. 2014) have been designed to mark multiple cell lineages and allow whole-tissue labeling in fixed and live animals, respectively, aiming to distinguish the contribution of each of those lineages to the adult structure. Moreover, both systems allow simultaneous multicolor lineage analysis with overexpression or knockdown of *UAS*-constructs in a subset of the marked clones, allowing the comparison with control clones.

In summary, Brainbow-derived technologies offer an unprecedented opportunity to map cell diversity and arrangement of cells in developing and mature circuits, but also to dissect the mechanisms that contribute to organ morphogenesis.

1.7 Conclusions and Future Directions

Given the breadth of genetic tools available for precise, targeted control of gene expression, it is clear that the fruit fly, *D. melanogaster*, will continue to remain a major model system for the functional dissection of the nervous system. In the future, new genetic tools such as the CRISPR/Cas9 system (Gasiunas et al. 2012; Jinek et al. 2012), will enable increasingly precise manipulation of the nervous system. For instance, CRISPR can be used to introduce transgenic constructs at specific loci via homologous recombination, to repurpose binary system transgenic components (Lin and Potter 2016), to carry out high-throughput lineage tracing (McKenna et al. 2016), and in the future could potentially be used as another means of creating intersectional logic gates (for instance, by using sgRNAs targeting GAL4 or other binary system components to disrupt their expression in specific tissues).

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