Neuron
Primer

Genetic Manipulation of Genes and Cells in the Nervous System of the Fruit Fly

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DOI 10.1016/j.neuron.2011.09.021

Research in the fruit fly *Drosophila melanogaster* has led to insights in neural development, axon guidance, ion channel function, synaptic transmission, learning and memory, diurnal rhythmicity, and neural disease that have had broad implications for neuroscience. *Drosophila* is currently the eukaryotic model organism that permits the most sophisticated in vivo manipulations to address the function of neurons and neuronally expressed genes. Here, we summarize many of the techniques that help assess the role of specific neurons by labeling, removing, or altering their activity. We also survey genetic manipulations to identify and characterize neural genes by mutation, overexpression, and protein labeling. Here, we attempt to acquaint the reader with available options and contexts to apply these methods.

1. Introduction

The fruit fly *Drosophila melanogaster* is currently the model organism that allows the most sophisticated genetic manipulations of all higher eukaryotes. An arsenal of genetic tools permits the investigation of the complexity of the nervous system in unprecedented detail. *Drosophila* research has contributed to our understanding of nervous system development (Doe, 2008; Hartenstein et al., 2008), growth cone guidance and target recognition (Dickson, 2002), exocytosis and endocytosis at synapses (Bellen et al., 2010), synapse remodeling (Collins and DiAntonio, 2007), and the neural circuitry underlying behaviors such as courtship (Villella and Hall, 2008), diurnal rhythms and sleep (Crocker and Sehgal, 2010), aggression (Kravitz and Huber, 2003), and learning and memory (McGuire et al., 2005). Moreover, it is now obvious that *Drosophila* is a good model organism to study genes that are involved in human disease, especially neurodegenerative mechanisms associated with Alzheimer's disease, Parkinson's disease, polyglutamine and other triplet repeat expansion diseases, amyotrophic lateral sclerosis, and neurological disorders such as epilepsy, depression, and schizophrenia (Lu and Vogel, 2009; Lessing and Bonini, 2009; O'Kane, 2011). The toolkit is so extensive that it is becoming difficult to assess which tool is most appropriate for a particular application. The goal of this review is to provide a summary of the available genetic reagents and to frame the context in which to apply them.

Fly neurobiology encompasses many different fields of interest including the cell biology of neurons, development and degeneration of the nervous system, neural circuit architecture, and behavioral consequences of neural activity. Numerous neurons and genes are involved in these processes and essentially two strategies are now available: a neuron-centric and a gene-centric approach. The neuron-centric approach is based on techniques that label subsets of neurons. It permits removal of specific neurons, impairing neuronal function, or increasing neuronal activity, followed by assaying an output, for example a specific behavior. The ability to manipulate many different and specific neuronal populations is provided by thousands of individual stocks that express transcriptional activators such as GAL4, LexA, and QF in selective neuronal populations (Brand and Perrimon, 1993; Lai and Lee, 2006; Potter et al., 2010; Pfeiffer et al., 2010; Yagi et al., 2010).

The gene-centric approach is based on forward or reverse genetic methods. Forward genetic screens allow the unbiased identification of novel players. Reverse genetic approaches are designed to affect a gene of interest and include transposon mutagenesis, deletion mutagenesis, RNAi, and gene targeting. Both forward and reverse genetic approaches allow the assessment of phenotypes associated with these mutations to provide a better understanding of the role of genes and their corresponding proteins in the nervous system in vivo. Subsequently, gene products can be labeled with protein tags that permit protein visualization.

2: The *Drosophila* Nervous System

The fly brain is estimated to contain 90,000 neurons (K. Ito, personal communication), a million-fold fewer than the typical human brain (Meinertzhagen, 2010), but with a similar complexity of different neural cell types (Bullock, 1978). For example, the visual system of the fly contains at least 113 different classes of neurons based on Golgi stains (Fischbach and Dittrich, 1989), a number similar to vertebrate eyes, which contain about 100 different types of neurons and support cells (Dacey and Packer, 2003). Flies and mammals use the same neurotransmitters (GABA, glutamate, acetylcholine), share biogenic amines like dopamine and serotonin, and have numerous neuromodulatory peptides. Like mammals, flies have sodium channels that propagate action potentials, and the same families of potassium and calcium channels regulate membrane potential. In both systems, information passes between neurons at specialized contact

points called synapses, and these synapses have common protein architecture. Thus, insights about the nervous system obtained in *Drosophila* are often relevant for other species (Bellen et al., 2010).

There are some differences between fly and vertebrate nervous systems. In flies, the neuron to glia ratio is 10:1, while in vertebrates this ratio is 1:10. This difference may be due to the fact that in flies, glia wrap bundles or fascicles rather than individual neurons. Flies still contain many different types of glia (Hartenstein, 2011). Unlike vertebrate neurons, the cell bodies of *Drosophila* neurons are located in a cortical rind surrounding the brain neuropile composed of axons, dendrites, and synapses. Many fly neurons synapse with multiple postsynaptic targets, forming diads, triads, or tetrads (Takemura et al., 2008), and some fly neurites integrate both pre- and postsynaptic inputs. In general, fly neurons have relatively few synapses and in the visual system, they are typically in the range of 30–50 per neuron (Meinertzhagen and Sorra, 2001), whereas vertebrate neurons often have thousands of synapses. Unlike vertebrates, flies use glutamate as an excitatory neurotransmitter at the neuromuscular junction and acetylcholine in most sensory and central synapses.

The most significant features of neurons lie in the structural design by which they form a network to process sensory information and drive appropriate behavioral programs. Although electrophysiological correlates of behavior have been obtained in some invertebrate species (Marder and Rehm, 2005), structural information on synaptic networks is very difficult to obtain and much of the toolkit that has recently been developed aims at remedying this problem (Meinertzhagen et al., 2009). The best studied circuits in *Drosophila* are those that process olfactory and visual stimuli (Fischbach and Hiesinger, 2008; Imai et al., 2010; Borst et al., 2010). Our understanding of other peripheral sensory input circuits such as taste (Cobb et al., 2009), hearing and mechanotransduction (Kernan, 2007), and cold and heat (Garrity et al., 2010) is less well advanced. Similarly, the motor circuits governing escape behavior, larval crawling, and flight remain only partially defined (Crisp et al., 2008; Fotowat et al., 2009). Although neurons and circuits that regulate more complex behaviors such as learning and memory formation, arousal, ethanol responses, circadian rhythms, sleep, aggression, and courtship have been studied, many questions remain unanswered. The tools that are described here have been and will be valuable to further our understanding.

In summary, the fly nervous system contains a manageable number of neurons with a great diversity of neuronal types capable of producing complex behaviors. By analogy to screens for genes affecting the basic cellular processes of the nervous system in *Drosophila*, there is reason to suppose that investigation of the genes, neurons, and circuits underlying diverse fly behaviors will yield insights relevant across biological systems.

3: Genetic Access to Neuronal Populations

3a. Introduction

Several genetic techniques are available to label neurons in the fly brain. Regulatory elements that direct gene expression at a specific time and place can be placed upstream of a desired label or marker. However, the preferred methods employ binary expression systems where a fly stock expressing a transactivator or driver (e.g., GAL4) is crossed to a stock that bears a responder element (e.g., a *UAS-GFP* reporter or *UAS-Shibire^{ts1}* effector) to produce progeny in which a reporter gene is expressed at the desired time and place. The virtues of the binary expression systems include restricted expression of toxic proteins, amplification of expression levels, and, most importantly, the ability to express many different reporters and effectors in a specific cell type, or the same responder in many different cell types. This section will describe the different binary systems and the manner in which transactivator and responder elements can be manipulated to add spatial and temporal control. The methods for intersecting orthogonal binary expression systems are powerful, and techniques for clonally or stochastically marking subsets of neurons within a larger group are discussed.

3b. Binary Expression Systems

Binary systems consist of a transactivator that specifically binds to a DNA binding site resulting in the transcriptional activation of a downstream responder (Figure 1A). Repressors of the transactivator and compounds that activate or inactivate the transactivator or the repressor allow temporal or spatial control of gene expression.

GAL4 was the first binary system developed for use in *Drosophila*. The GAL4 transactivator binds *U*pstream *A*ctivating *S*equences (UAS) to initiate transcription of downstream responders (Fischer et al., 1988; Brand and Perrimon, 1993) (Figure 1B). GAL4 activity can be inhibited by the GAL80 repressor (Lee and Luo, 1999). The GAL4 system is extremely reliable and useful (Duffy, 2002) and recent improvements have increased expression levels and uniformity significantly (Pfeiffer et al., 2010).

The regulatory elements that dictate GAL4 expression simultaneously determine both temporal and spatial control. The spatial expression patterns can be restricted by several positive and negative intersectional techniques. The most widely used mechanism for achieving temporal control of GAL4 expression utilizes a temperature-sensitive GAL80 repressor (Figure 1B) (McGuire et al., 2003). An alternative strategy uses GAL4 variants that rely on various drugs for activation (Figure 1C) (Han et al., 2000; Osterwalder et al., 2001; Roman et al., 2001). While GAL4 activation in response to drugs is slow, this approach can be used to bypass GAL4 expression during development.

GAL4 expression levels and activity are increased at 28°C and reduced at 18°C, perhaps due to heat shock elements present in the promoter (Mondal et al., 2007). A temperature-sensitive (ts) version of GAL4 was developed to allow overexpression only at the permissive temperature (Mondal et al., 2007).

Efficacy of GAL4 was improved by codon optimization, messenger RNA stabilization, and substitution of higher-activity transcriptional activating domains (Pfeiffer et al., 2010). Extremely high levels of GAL4 can be toxic in some cells (Kramer and Staveley, 2003; Rezával et al., 2007; Pfeiffer et al., 2010), and optimal levels have been established. Expression levels of the responder were increased by varying the number of UAS sites and adding posttranscriptional regulatory elements; finally, a specific polyadenylation signal and the inclusion of an intron and posttranscriptional regulatory element enhanced GAL80 suppression of GAL4 significantly (Pfeiffer et al., 2010).

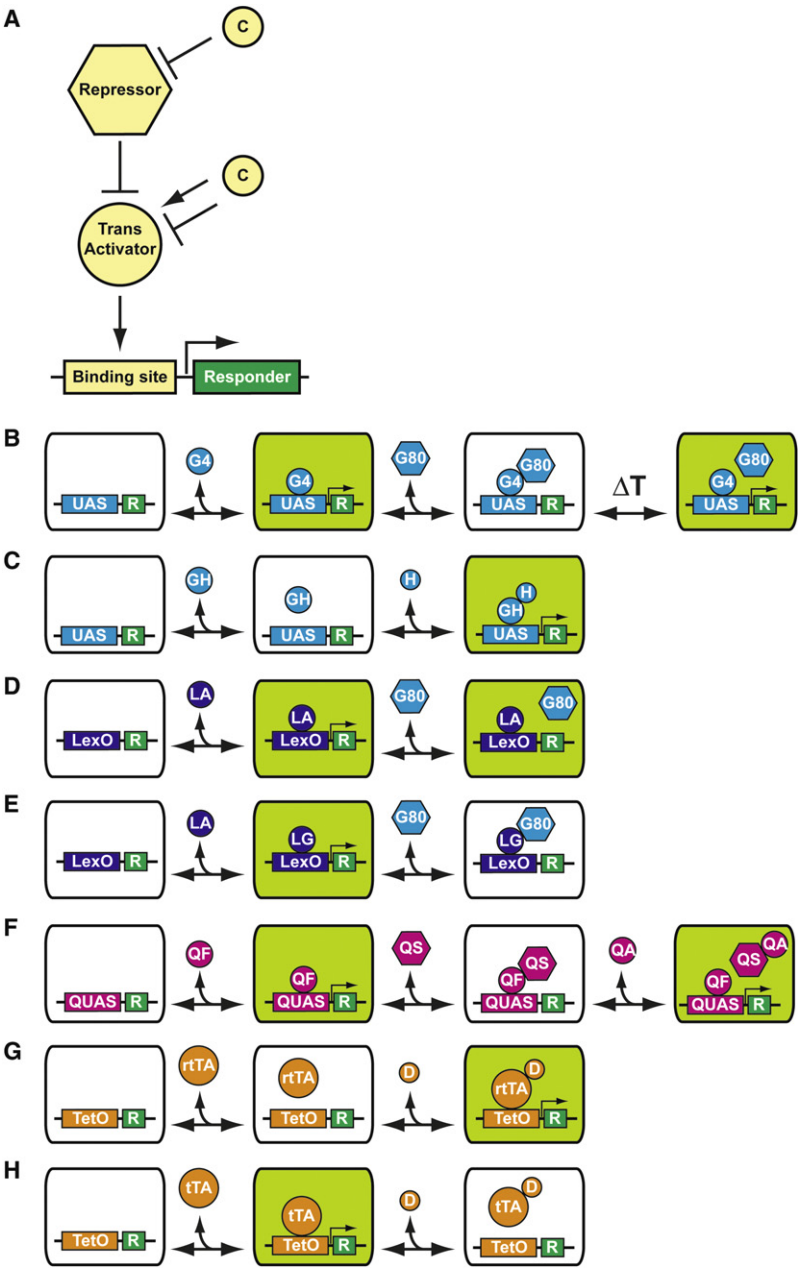

Figure 1. Binary Systems

(A) Overview of a binary system. The transactivator binds to a binding site to activate a responder. Expression of a repressor blocks the activity of the transactivator. Compounds (C) can modulate the activity of repressor and transactivator and permit temporal and spatial control.

(B) The GAL4 system. Cells in which GAL4 (G4) is expressed and able to activate transcription of the responder (R) are shown in green. A temperature shift to 30°C inhibits the temperature-sensitive version of GAL80 (G80) and permits GAL4 activity.

(C) The hormone (H) inducible GAL4 system with GAL4 DNA binding domain fused to a hormone binding domain (GH). This system is GAL80 insensitive.

(D) The LexA system with the LexA binding domain coupled to an activation domain different from GAL4 (LA). This system is GAL80 insensitive.

(E) The LexA system with the LexA binding domain coupled to the GAL4 activation domain (LG). This system is GAL80 sensitive.

(F) The QF system.

(G) The Tet-On system (rtTA) activated by doxycycline (D).

(H) The Tet-Off system (rTA) inactivated by doxycycline (D).

A different binary system is based on the LexA transactivator (Figures 1D and 1E). Fusion of the DNA binding domain of LexA to the transcription activation domain of the viral protein VP16 results in a potent GAL80-insensitive transactivator that can bind to LexA operator (LexOp) sites and drive expression of responder elements (Szüts and Bienz, 2000; Lai and Lee, 2006) (Figure 1D). VP16 can be replaced by a GAL4 transactivation domain that is GAL80 sensitive (Figure 1E). The LexA system has also been optimized by adding a nuclear localization signal and the p65 transcriptional activation domain (GAL80-insensitive) to LexA; lexAop operator sequences were modified to allow better inducible transcriptional activation levels and reduce leaky expression (Pfeiffer et al., 2010). Additionally, numerous GAL80-suppressible and GAL80-insensitive LexA activators were generated that exhibit lower toxicity at high expression levels than GAL4 (Yagi et al., 2010).

Another binary system, Q, was recently developed (Figure 1F) (Potter et al., 2010). The transactivator (QF) binds to *QF Upstream Activating Sequences* (QUAS), activating transcription of reporters. Interestingly, activity of the repressor (QS) is controllable by quinic acid (QA) and can be titrated by varying QA concentration, incorporating additional levels of regulation. Toxicity was reported for QF when expressed at high levels (Potter et al., 2010).

A final but less used binary system is based on the tetracycline system that includes the Tet-On (Figure 1G) (Bieschke et al., 1998; Stebbins et al., 2001) and the Tet-Off system (Figure 1H) (Bello et al., 1998; Stebbins et al., 2001; Stebbins and Yin, 2001). Both systems provide induction of a tetracycline operator sequence (TetO) driven reporter after adding (Tet-On) or removing (Tet-Off) tetracycline drugs. They are rarely used in *Drosophila*, but novel technologies that allow upgrading existing GAL4 drivers with other transactivators may revive their use (see below).

3c. Generation of Transactivators Expressed in Different Neural Populations

Regulatory elements are required to drive the expression of transactivators. Ideally, specific drivers for every neuronal population should be available. To create these drivers, the original *P* element enhancer detectors (''enhancer traps''), transposable elements that contain a minimal promoter upstream of the lacZ gene (O'Kane and Gehring, 1987), were modified by replacing the lacZ reporter with GAL4 (Brand and Perrimon, 1993). Upon

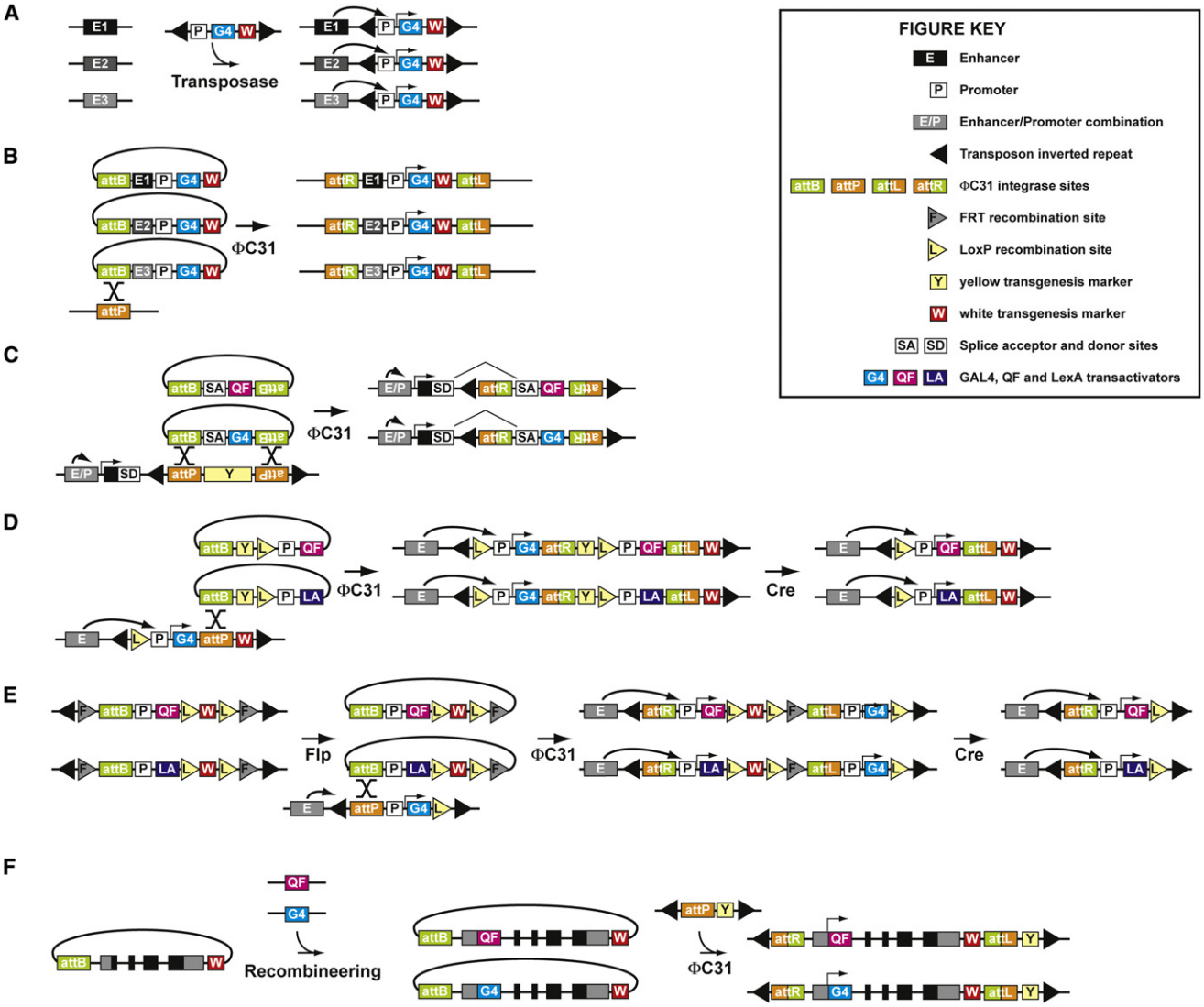

Figure 2. Generation of Transactivator Fly Lines

(A) Transposon enhancer trapping. A transposon containing a minimal promoter and GAL4 is mobilized into the genome through a transposase. Upon insertion in the genome it can be influenced by different enhancers.

(B) Plasmid transgenesis. Different enhancers are cloned in front of a minimal promoter and GAL4, and integrated into the same *attP* docking site with ΦC31integrase, allowing direct comparison of regulatory influences.

(C) The MiMIC system. A 5′UTR intronic MiMIC insertion can be converted into transactivator lines (GAL4 or QF) using a gene trap strategy and ΦC31-mediated RMCE.

(D) The G-MARET system. A previously generated GAL4 line that is under the influence of an enhancer can be converted into novel ones (QF or LexA) using ΦC31transgenesis. Unwanted sequences flanked by *LoxP* sites are removed with Cre recombinase.

(E) The InSITE system. A previously generated GAL4 line that is under the influence of an enhancer can be converted into novel ones (QF or LexA) using ΦC31transgenesis. Donor constructs contained within transposons can be mobilized in vivo with Flp recombinase. Unwanted sequences flanked by *LoxP* sites are removed with Cre recombinase.

(F) Recombineering. PCR cassettes containing binary factors (QF or GAL4) are recombined into a genomic DNA fragment and the resulting transgene is integrated using ΦC31transgenesis.

random transposition, genomic enhancers in the vicinity of the transposon control the expression of GAL4 (Figure 2A). The first binary analysis was pioneered by random *P* element mobilization (Brand and Perrimon, 1993). A large number of GAL4 lines (6,966) have been generated with this system (Hayashi et al., 2002). Similar enhancer trap collections were made for GAL80 (Suster et al., 2004), hormonally controlled GAL4 (Nicholson et al., 2008), and LexA (Miyazaki and Ito, 2010). A common theme of all these screens is that the obtained expression patterns are often relatively broad and include diverse neural types, limiting their usefulness for labeling specific neurons.

To generate drivers with more restricted patterns of expression, relatively small fragments of genomic DNA were subcloned into transgenesis-competent plasmids upstream of a promoter

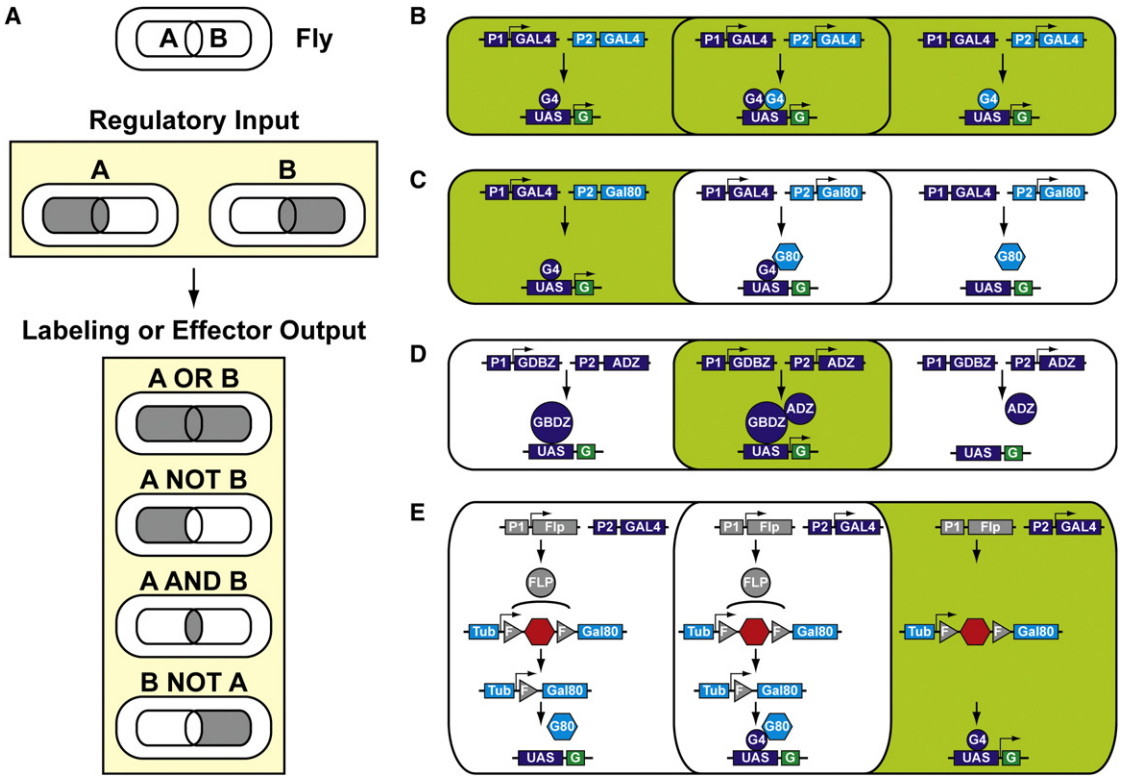

Figure 3. Intersectional Strategies to Refine Expression Patterns for Neuronal Labeling and Manipulation

(A) A few examples of hypothetical logic intersectional gates to illustrate potential responder outputs, based on just two regulatory inputs, within the entire fly. (B–E) Some examples of effector outputs that can be generated with available regulatory input tools. (B) Addition with two GAL4 lines. (C) Substraction with GAL80. (D) Intersection with split GAL4. (E) Substraction with Flp-In. Tub (constitutive tubulin promoter). Other illustration keys are the same as in Figure 2.

and GAL4 (Figure 2B). These plasmids were then integrated at a specific docking site in the fly genome using the ΦC31 integrase (Groth et al., 2004; Bischof et al., 2007) and tested for expression, resulting in thousands of GAL4 lines (Pfeiffer et al., 2008). Plasmids for enhancer- and promoter-bashing are available for fusions with GAL4, hormonally controlled GAL4 and LexA, or fluorescent proteins (Osterwalder et al., 2001; Sharma et al., 2002; Roman and Davis, 2002; Apitz et al., 2004; Barolo et al., 2004; Pfeiffer et al., 2008; Petersen and Stowers, 2011; Han et al., 2011a).

As the LexA and QF technologies have only recently been developed, there are relatively few drivers available (Lai and Lee, 2006; Diegelmann et al., 2008; Potter et al., 2010; Miyazaki and Ito, 2010). Obviously, enhancer trap screens or enhancer fusion lines could be created for LexA and QF (Pfeiffer et al., 2008). Alternatively, methods for replacing DNA in a place where an enhancer detector is already present have been developed. The original method is based on *P* element replacement or exchange, which relies on the tendency of a new *P* element to insert at the locus of one being excised (Gloor et al., 1991; Sepp and Auld, 1999). This can be used to swap GAL4 with a membrane marker within a specific neuronal population (Berdnik et al., 2006), for example. Another system is known as MiMIC (*mi*nos-*m*ediated *i*ntegration *c*assette) (Venken et al., 2011) (Figure 2C). MiMIC is a Minos-based tranposable element that contains two inverted *attP* sites that allow the replacement of DNA between both *attP* sites using RMCE (*r*ecombinase-*m*ediated *c*assette *e*xchange) (Bateman et al., 2006). MiMIC insertions that are in the first noncoding intron of a gene can be replaced with a splice acceptor site followed by a binary factor revealing the expression pattern of the gene (Venken et al., 2011). Alternatively, G-MARET (GAL4-based *m*osaic-inducible *a*nd *r*eporter-exchangeable *e*nhancer *t*rap) (Yagi et al., 2010) (Figure 2D) and InSITE (*in*tegrase *s*wappable *i*n vivo *t*argeting element (Gohl et al., 2011) (Figure 2E) allow replacement of a previously characterized GAL4 with other activators. Moreover, InSITE allows in vivo exchange by simple genetic crosses avoiding microinjection experiments (Gohl et al., 2011). A final method is the introduction of transactivators into genomic constructs by recombineering (Stowers, 2011) (Figure 2F).

3d. Intersectional Strategies

Binary drivers that label small neuronal populations are not available for many neuronal types (Pfeiffer et al., 2008). Sometimes the specific neuronal subpopulation cannot be labeled with one binary factor but two independent drivers share an expression domain in the neurons of interest. By combining different systems one can label specific neuronal subpopulations through intersectional strategies (Figure 3A). The simplest strategy is additive, where the expression pattern of two GAL4 drivers is combined (Figure 3B). Subtraction restricts the function of

Table 1. Binary Reporter Constructs for Visualization of Neurons

Regulation	Reporter	Location	Readout	References
GAL4				
5x/10xUAS	GFP	Cytoplasmic	Green	Yeh et al., 1995; Pfeiffer et al., 2010
5xUAS	2xGFP	Cytoplasmic	Green	Halfon et al., 2002
1x/3x/5x/10x/15x/20x/40xUAS	CD8-GFP	Membrane	Green	Lee and Luo, 1999; Pfeiffer et al., 2010
5xUAS/10x-UAS	myr-GFP	Membrane	Green	Ritzenthaler et al., 2000; Pfeiffer et al., 2010
5xUAS	CD8-DsRed	Membrane	Red	Ye et al., 2007
5xUAS	CD2-RFP	Membrane	Red	Yu et al., 2009b
5xUAS	mtdTomato-3xHA	Membrane	Red	Potter et al., 2010
5xUAS	HRP-CD2	Membrane	DAB/EM	Larsen et al., 2003; Watts et al., 2004
5xUAS	synaptotagmin-GFP	Synaptic vesicle	Green	Zhang et al., 2002
2xUAS	n-synaptobrevin-YFP	Synaptic vesicle	Yellow	Rolls et al., 2007
5xUAS	n-synaptobrevin-GFP	Synaptic vesicle	Green	Estes et al., 2000; Zhang et al., 2002
5xUAS	bruchpilot-GFP	Active zone	Green	Wagh et al., 2006
5XUAS	cacophony-GFP	Active zone	Green	Kawasaki et al., 2004
5xUAS	DenMark	Dendrites	Red	Nicolaï et al., 2010
5xUAS	Dscam[exon 17.1]-GFP	Dendrites	Green	Wang et al., 2004
5xUAS	Rdl-HA	Synapse	Antibody	Sánchez-Soriano et al., 2005
5xUAS	Dα7-GFP	Synapse	Green	Leiss et al., 2009
5xUAS	EYFP-Mito	Mitochondria	Yellow	LaJeunesse et al., 2004
5xUAS	EYFP-ER	Endoplasmatic reticulum	Yellow	LaJeunesse et al., 2004
5xUAS	EYFP-Golgi	Golgi complex	Yellow	LaJeunesse et al., 2004
5xUAS	GFP-NLS	Nucleus	Green	Yasunaga et al., 2006
Q				
5xQUAS	CD8-GFP	Membrane	Green	Potter et al., 2010
5xQUAS	tdTomato-3xHA	Membrane	Red	Potter et al., 2010
LexA				
8xLexO	CD2-GFP	Membrane	Green	Lai and Lee, 2006
8x/13x/16x/26xLexO	CD8-GFP	Membrane	Green	Pfeiffer et al., 2010
8xLexO	myr-mCherry	Membrane	Red	Diegelmann et al., 2008
8xLexO	mCherry-CAAX	Membrane	Red	Yagi et al., 2010

Examples of reporters used with the GAL4, QF and LexA binary systems for the visualization of neurons.

GAL4 to those cells that do not express its inhibitor, GAL80 (Lee and Luo, 1999) (Figure 3C). Another variation is an intersectional technique that relies on split binary systems, pioneered by the split-GAL4 system (Luan et al., 2006b) that was recently optimized (Pfeiffer et al., 2010) (Figure 3D). The GAL4 transcription factor is split into two hemidrivers, each of which is driven by separate regulatory elements. Where the expression domains overlap, both halves of GAL4 are expressed, heterodimerize via leucine zippers, and reconstitute a functional activator. A similar split strategy was recently developed for LexA (Ting et al., 2011). Another intersectional strategy combines GAL4 with Flp recombinase (Golic and Lindquist, 1989), each driven by separate regulatory elements. The expression of transactivator, responder, or repressor depends on recombinase activity removing an intervening stop cassette (Struhl and Basler, 1993). Alternatively, GAL80 can be activated by Flp-In so that only cells that express GAL4 and not Flp are capable of expressing a UAS-responder element (Bohm et al., 2010) (Figure 3E). Many combinations of the orthogonal binary expression systems and Flp recombinase can be envisioned (Potter et al., 2010; Bohm et al., 2010; Yagi et al., 2010; Potter and Luo, 2011). The development of new recombinases and alternative target sites further broadens the combinatorial palette (Nern et al., 2011; Hadjieconomou et al., 2011).

3e. Markers for Neuronal Labeling

The binary systems described in the previous section can be used to overexpress reporters to label neuronal subpopulations or subcompartments of these neurons (Table 1). Numerous fluorescent reporters are available. To label the entire cytoplasmic compartment, fluorescent proteins can be overexpressed (Yeh et al., 1995; Halfon et al., 2002; Pfeiffer et al., 2010). Fluorescent markers fused to membrane targeted domains label the cell outline (Lee and Luo, 1999; Ritzenthaler et al., 2000; Ye et al., 2007; Yu et al., 2009a; Pfeiffer et al., 2010). Fusions with synaptic vesicle proteins predominantly label the presynaptic compartment of synaptic contacts (Estes et al., 2000; Zhang et al., 2002; Rolls et al., 2007). Active zones can be labeled with bruchpilot-GFP (Wagh et al., 2006) or cacophony-GFP (Kawasaki

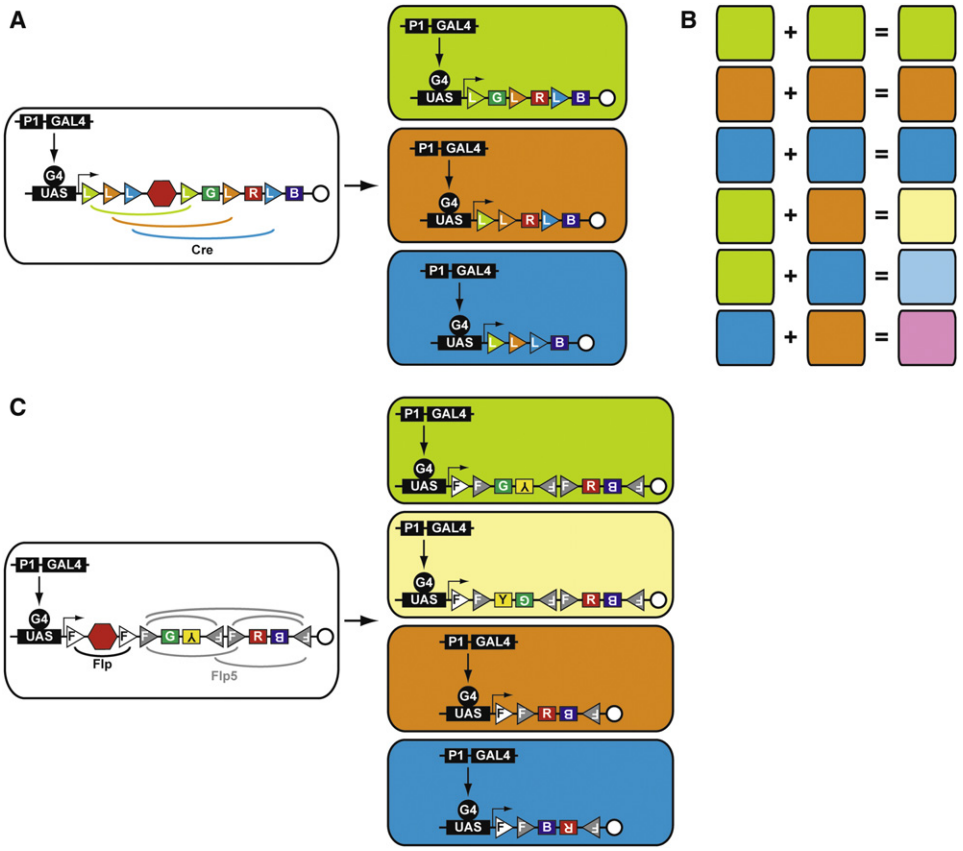

Figure 4. Multicolor Neuronal Labeling Techniques
(A) The dBrainbow system. Orthogonal *LoxP* sites are indicated in different colors. GFP (G), RFP (R), BFP (B).
(B) Color combinations when two copies of dBrainbow are used when various fragments of DNA are lost.
(C) The Flybow 2.0 system. By combining deletions and inversions of DNA, different markers can be expressed. Orthogonal *FRT* sites are indicated in different colors. YFP (Y). Other illustration keys are the same as in Figure 2.

et al., 2004). While there is no generic marker for postsynaptic sites, Denmark (Nicolaï et al., 2010) or Dscam[exon 17.1] (Wang et al., 2004) preferentially labels dendrites. Fusions to neurotransmitter receptor proteins such as UAS-Rdl-HA and UAS-Dα7-GFP can also be used to identify synapses (Sánchez-Soriano et al., 2005; Leiss et al., 2009). Markers that label subcellular organelles include fluorescent proteins fused to targeting elements specific for mitochondria, endoplasmatic reticulum, Golgi, and nucleus (LaJeunesse et al., 2004; Yasunaga et al., 2006). A fusion with horseradish peroxidase is useful for transmission electron microscopy (Larsen et al., 2003; Watts et al., 2004). Markers for LexA and Q are limited so far but include several membrane-localized reporters (Lai and Lee, 2006; Diegelmann et al., 2008; Pfeiffer et al., 2010; Yagi et al., 2010; Potter et al., 2010; Petersen and Stowers, 2011).

3f. Stochastic Labeling of Neurons

The neuronal labeling systems discussed above often reveal relatively broad expression domains that are reproducible for many but not all drivers (Pfeiffer et al., 2008). To characterize the morphology of individual neurons, stochastic labeling techniques were developed to label single neurons or small subpopulations. This allows determination of cellular morphology and tracing from pre-synaptic to post-synaptic neurites. These techniques are based on Flp recombinase and are referred to as Flp-On and MARCM (see below).

The Flp-On method is a stochastic labeling technique that can be used with any GAL4 driver (Gao et al., 2008b; Gordon and Scott, 2009; Bohm et al., 2010). A ubiquitously driven GAL80 flanked by *FRT* sites prevents GAL4 from activating a responder. A weak heat shock causes transient Flp expression from a hs-Flp transgene, removing GAL80 in a random subset of cells, resulting in GAL4 activation and labeling of some neurons within the GAL4 expression domain. Alternatively, a stop cassette between UAS and reporter is removed (Wang et al., 2003). The inclusion of additional constructs with other reporters can extend the number of neurons that can be individually labeled within a single specimen (G. Rubin, personal communication).

Two alternative multicolor labeling techniques based on the mouse Brainbow system (Livet et al., 2007) have recently been published (Figure 4). dBrainbow (Hampel et al., 2011) and Flybow (Hadjieconomou et al., 2011), like Brainbow, use recombinases to rearrange DNA cassettes expressing different fluorescent proteins, enabling each neuron within a GAL4 expression pattern to randomly select one of the available

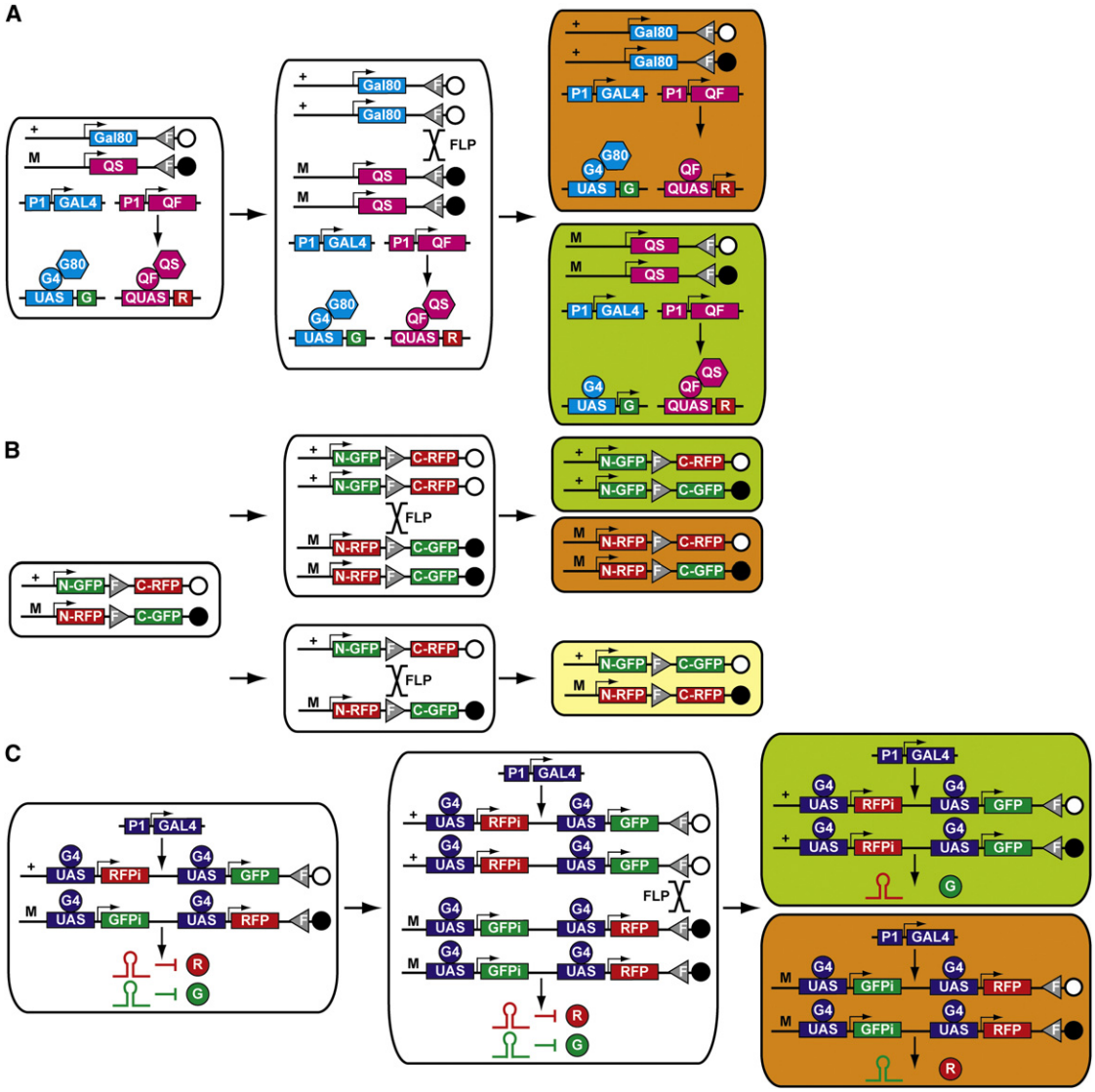

Figure 5. Stochastic Neuronal Labeling Techniques

(A) Schematic illustration of GAL4-MARCM, Q-MARCM and coupled MARCM. GFP (G), RFP (R), YFP (Y). This system allows the differential labeling of the two daughter cells of a single mitotic recombination event.

(B) The twin-spot generator. N-terminal portion of GFP and RFP (N-GFP and N-RFP), C-terminal portion of GFP and RFP (C-GFP and C-RFP). Wild-type chromosome (+) and mutant chromosome (M). The other illustration keys are the same as in Figure 2.

(C) The twin-spot MARCM system. RNAi against GFP (GFPi), RNAi against RFP (RFPi) also permits differential labeling of two daughter cells upon mitotic recombination.

fluorescent proteins for expression. dBrainbow uses Cre recombinase and orthogonal variants of its *loxP* DNA binding site while FlyBow uses Flp recombinase and *FRT* sites. A comparison of the two methods is presented in (Cachero and Jefferis, 2011).

3g. Generation of Genetic Mosaics

Key in the analysis of mutant phenotypes in specific tissues in *Drosophila* was the integration of *FRT* sites to permit efficient mitotic recombination. This permits the creation of two differently labeled daughter cells after division of the mother cell through chromosomal exchange, using the Flp recombinase. The *FRT* sites were positioned near centromeres permitting homozygosity of entire chromosomal arms, resulting in homozygous mutant cells in an otherwise heterozygous animal (Xu and Rubin, 1993). In conventional mitotic recombination the mutant neuron is typically not marked with a fluorescent marker since it is lost upon recombination. This was circumvented by incorporating the GAL80 repressor (Figure 5A) (Lee and Luo, 1999). This system is known as MARCM (*m*osaic *a*nalysis with a *r*epressible *c*ell *m*arker) (Lee and Luo, 1999). Upon mitotic recombination, GAL80 expression is lost in the mutant cells, resulting in GAL4 activation and transcriptional activation of the reporter. An example of how the intersection of lineage and GAL4 expression that MARCM provides has been used to map development and connectivity in the olfactory system has been described (Marin

Table 2. Binary Effector Contructs for Manipulation of Neural Activity

Name	Protein	Mechanism	References
Neuronal Cell Elimination			
UAS-DTA	WT version of diptheria toxin A	Cell death by protein synthesis inhibition	
UAS-DTI	Attenuated version of diphtheria toxin A	Cell death by protein synthesis inhibition	Han et al., 2000
UAS-FRT-stop-FRT-DTA	Inducible version of diphtheria toxin A	Cell death by protein synthesis inhibition	Lin et al., 1995
UAS-RCA	Ricin toxin	Cell death by protein synthesis inhibition	Moffat et al., 1992
UAS-RCA^{cs}	Cold sensitive version of ricin toxin	Cell death by protein synthesis inhibition	Allen et al., 2002
UAS-FRT-stop-FRT-RCA	Inducible version of ricin toxin	Cell death by protein synthesis inhibition	Hidalgo and Brand, 1997
UAS-grim	Proapoptotic gene *grim*	Triggers endogenous cell death pathway	Wing et al., 1998
UAS-hid	Proapoptotic gene *hid*	Triggers endogenous cell death pathway	Zhou et al., 1997
UAS-reaper	Proapoptotic gene *reaper*	Triggers endogenous cell death pathway	Zhou et al., 1997
UAS-reaperC	Strongest version of reaper	Triggers endogenous cell death pathway	Wing et al., 1998
UAS-hid; TubP-GAL80ts	Inducible version of hid	Triggers endogenous cell death pathway	McGuire et al., 2003
Inhibition of Neuronal Activity			
UAS-TNT or UAS-TeTxLc	Tetanus toxin	Blocks chemical synaptic transmission by cleaving nSyb	Sweeney et al., 1995
UAS-FRT-stop-FRT-TNT	Inducible version of tetanus toxin	Blocks chemical synaptic transmission by cleaving nSyb	Keller et al., 2002
UAS/QUAS-Shibire^{ts1}	Dominant-negative form of dynamin GTPase	Blocks synpatic transmission at ~30°C by interfering with vesicle recycling	Kitamoto, 2001; Potter et al., 2010
UAS/QUAS-FRT-stop-FRT-Shibire^{ts1}	Inducible version of Shibire^{ts1}	Blocks synpatic transmission at ~30°C by interfering with vesicle recycling	Stockinger et al., 2005; Potter et al., 2010
UAS-para^{RNAi}	RNAi against Para	Reduces expression of sodium channel required for action potentials	Zhong et al., 2010
UAS-Kir2.1-EGFP	Inward-rectifying K+ channel; PIP_2 dependent	Prevents membrane depolarization	Baines et al., 2001; Paradis et al., 2001
UAS-FRT-CD2-FRT-Kir2.1-EGFP	Inducible version of Kir2.1	Prevents membrane depolarization	Yang et al., 2009
UAS-dOrk-deltaC	Outward-rectifying K+ channel	Prevents membrane depolarization	Nitabach et al., 2002
UAS-EKO	Un-inactivatable Shaker K+ channel	Prevents membrane depolarization	White et al., 2001b
UAS-NpHR	Halorhodopsin	580 nm light activated chloride pump	Unpublished
Excitation of Neuronal Activity			
UAS-dnATPase (D369N)	Dominant-negative Na^+/K+ ATPase	Blocks membrane repolarization pump	Sun et al. 2001; Parisky et al., 2008
UAS-NaChBac	Bacterial sodium channel	Increases sodium conductance	Nitabach et al., 2006; Sheeba et al., 2008
UAS-TrpVR1	Vanilloid receptor activated by capsaicin	Increases cation conductance	Marella et al., 2006

Table 2. *Continued*

Name	Protein	Mechanism	References
Excitation of Neuronal Activity			
UAS-dTrpA1	Cation channel activated by warm temperatures	Permits cation conductance in response to temperature increase	Rosenzweig et al., 2005, 2008
UAS-FRT-stop-FRT-trpA1^{myc}	Inducible version of dTrpA1	Permits cation conductance in response to temperature increase	von Philipsborn et al., 2011
UAS-TrpM8	Cold-activated cation channel	Increases cation conductance in response to cold	Peabody et al., 2009
UAS-EAG-DN	Dominant-negative K+ channel	Decreases potassium currents required to repolarize neurons or lowers resting membrane potential	Broughton et al.,, 2004
UAS-Shaker-DN	Dominant-negative K+ channel	Decreases potassium currents required to repolarize neurons or lowers resting membrane potential	Mosca et al., 2005
UAS-Shaw-DN	Dominant-negative K+ channel	Decreases potassium currents required to repolarize neurons or lowers resting membrane potential	Hodge et al., 2005
UAS-P2X2	ATP-gated channel	Increases cation conductance in response to light uncaging of AMP	Lima and Miesenböck, 2005
UAS-ChR2	Channelrhodopsin	470 nm light activated cation channel	Schroll et al., 2006; Hwang et al., 2007

Examples of effectors used predominantly with the GAL4 binary system for the manipulation of neural activity.

et al., 2002). One can also reverse the MARCM strategy to address what is happening in labeled wild-type cells that are adjacent to unlabeled mutant cells by placing GAL80 onto the chromosome arm that carries the mutation of interest (Lee et al., 2000b).

A version of MARCM has also been developed for the Q system (Potter et al., 2010); MARCM and Q-MARCM can be combined to differentially label both progeny of a progenitor cell (Figure 5A). An alternative strategy for labeling both the wild-type and mutant daughters of a mother cell division are two constitutively expressed fluorescent proteins (EGFP and mRFP1) that are split in half, separated, and reconstituted upon a Flp-mediated event (Figure 5B) (Griffin et al., 2009), similar to the MADM technique in mice (Zong et al., 2005). In another approach two different membrane markers with two short hairpin RNAi suppressors against both membrane markers are incorporated (Figure 5C) (Yu et al., 2009a). The genetic wizardry to generate controlled mosaics is presented here in the context of labeling different neural populations but has also been utilized to map which neurons require particular gene function (see below).

4: Manipulation of Neuronal Activity

4a. Introduction

The brain of an adult fruit fly is capable of producing a wide range of coordinated behavioral sequences in response to current sensory stimuli and previous experiences. Just as in vertebrates, some areas of the brain are specialized for decoding particular sensory modalities or governing specific behavioral programs. This localization of function suggests a research strategy to identify the specific neurons necessary and sufficient to produce different behaviors. The genetic reagents to reproducibly target exogenous gene expression to specific cell populations (described above) can be used to drive the production of ion channels or toxins to manipulate neural activity and determine the effect on behavior (Brand and Perrimon, 1993; Lai and Lee, 2006; Luan and White, 2007; Pfeiffer et al., 2008, 2010; Olsen and Wilson, 2008; Simpson, 2009; White and Peabody, 2009; Potter et al., 2010; Yagi et al., 2010; Bellen et al., 2010) (Table 2). This section will discuss the options available for increasing or decreasing the activity of groups of neurons in order to identify those that are critical for a behavior.

The role of a gene in a particular process can be assayed by examining the measurable consequences—phenotypes—associated with its removal. An analogous experiment is to assay the role of a given neuron in a behavior by silencing or killing it. Lesion studies have been used to correlate behavioral function to areas of the vertebrate brain, and the genetic reagents available to Drosophilists allow this type of lesion study to be performed with cellular precision and reproducibility. Systematic elimination or silencing of groups of neurons will produce a map of brain regions and neurons critical for different behaviors that will pave the way for understanding how specific neurons encode and transform information.

4b. Causing Cell Death

One way to assess how a neuron or a group of neurons participate in a behavior or guidance decision is to eliminate their function and assay the phenotypic consequences. For example, GAL4 lines have been used to target expression of toxins or genes that initiate programmed cell death to particular cell populations in the embryonic nervous system to show that these cells serve as guideposts for axon guidance decisions of other neurons (Hidalgo et al., 1995; Lin et al., 1995; Hidalgo and Brand, 1997). Expression of bacterial toxins from Diphtheria and Ricin kills cells by disrupting protein synthesis (Kunes and Steller, 1991; Bellen et al., 1992; Moffat et al., 1992). Transgenes

expressing the most potent forms can be lethal, but attenuated and inducible versions exist (Bellen et al., 1992; Lin et al., 1995; Smith et al., 1996; Hidalgo and Brand, 1997; Han et al., 2000; Allen et al., 2002). Expression of the proapoptotic genes *grim*, *reaper*, or *hid* can trigger programmed cell death (Zhou et al., 1997); simultaneous expression of several apoptotic genes may be even more effective (Wing et al., 1998). Proapoptotic gene expression was used to determine the behavioral role of the cells releasing eclosion hormone (McNabb et al., 1997).

The efficacy of the cell killers varies in different neuronal types and developmental stages. Coexpression of a visible reporter such as UAS-GFP is prudent to confirm that the targeted cells have been destroyed. GAL4 lines often express throughout development and the UAS-toxin constructs described are constitutively active, meaning that they begin to kill cells as soon as they are expressed. If the GAL4 expression begins at the same time as the process under study, this is not a problem, but delaying the time of cell death may be desirable if an adult phenotype is under investigation. There are several options for adding temporal control to GAL4 expression that have already been discussed. In addition, a cold-sensitive version of the ricin protein makes cell death dependent on the temperature of the flies (Moffat et al., 1992).

4c. Blocking Synaptic Transmission by Interfering with Synaptic Vesicle Release

Killing a cell is an extreme manipulation that may have undesirable collateral consequences. Silencing a neuron, either by preventing the release of neurotransmitter or by blocking changes in membrane potential (see below) is a more precise way to determine its function. *Drosophila* neurons release neurotransmitters such as glutamate, GABA, and acetylcholine from synaptic vesicles in response to localized calcium influx through voltage-activated calcium channels. While synaptic vesicles share much of the release machinery with vesicles in other cell types, some proteins such as neural synaptobrevin (nSyb) are specific to neurons and enriched in synaptic vesicles. Disruption of these proteins can silence neurons by preventing the release of neurotransmitter-containing vesicles. Expression of the light chain of tetanus toxin (UAS-TNT or Tet or TeTxLc) cleaves nSyb and blocks vesicle release (Sweeney et al., 1995). UAS-TNT has been used to study the role of transmitter release in axon guidance and synapse formation (Tripodi et al., 2008) as well as to determine the role of many types of neurons in different behaviors (Kong et al., 2010b) although some neurons seem to be less susceptible to TNT (Thum et al., 2006). UAS-TNT was originally tested in glutamatergic motor neurons; the release machinery for biogenic amines, including serotonin, dopamine, octopamine, tyramine, and neuropeptides may differ. Some aminergic neurons do show phenotypes with UAS-TNT (Friggi-Grelin et al., 2003). UAS-TNT can also affect peptide release of the Eclosion Hormone releasing cells (McNabb and Truman, 2008), but it may not be fully effective on Pigment Dispersment Factor (Kaneko and Hall, 2000; Blanchardon et al., 2001; Umezaki et al., 2011) or Crustacean Cardiac Activating Peptide release (Luan et al., 2006a). A UAS-FRT-stop-FRT-TNT is available for intersectional experiments (Keller et al., 2002).

UAS-TNT has major virtues: it targets a neural-specific protein, and thus should only block vesicle release in neurons. Moreover, as it is a potent toxin, even low levels of expression are effective. Since UAS-TNT is constitutively active, chronic expression may lead to some circuit-level form of compensation for the silenced neurons, or cell damage within them.

A way to silence neurons acutely can bypass developmental roles, reduce pleiotropic effects, and minimize the opportunity for compensation. UAS-Shibire^{ts1}, a temperature-sensitive dominant-negative form of dynamin, a GTPase required for vesicle recycling, blocks chemical neurotransmission acutely (Kitamoto, 2001). Although UAS-Shibire^{ts1} affects vesicle recycling in many cell types, it may act most quickly in neurons where vesicle recycling is a rate-limiting step for neurotransmission. UAS-Shibire^{ts1} is effective in many different neuronal types, including photoreceptors and cholinergic neurons (Kitamoto, 2001), as well as peptidergic and aminergic neurons (Krashes et al., 2009; Alekseyenko et al., 2010). UAS-Shibire^{ts1} has been used to identify neurons involved in courtship, sleep, color vision, and taste discrimination (Kitamoto, 2002; Broughton et al., 2004; Pitman et al., 2006; Gao et al., 2008b; Masek and Scott, 2010). The acute temporal control afforded by UAS-Shibire^{ts1} allows investigation of neurons in adult behavior and even discrimination between neurons involved in learning and memory retrieval (Waddell et al., 2000; Dubnau et al., 2001; McGuire et al., 2001; Kasuya et al., 2009). A recombinase-inducible version, UAS-FRT-stop-FRT-Shibire^{ts1}, is suitable for further temporal control or use with intersectional methods (Stockinger et al., 2005) (see above). A QUAS version became recently available (Potter et al., 2010).

UAS-Shibire^{ts1} is now widely used to study the acute affects of neuronal silencing on cell morphology and animal behavior, but there are some cautionary notes. Since UAS-Shibire^{ts1} disrupts the recycling step, the vesicles must be released before it becomes effective, making UAS-Shibire^{ts1} a use-dependent blocker. The exact temperature threshold and mechanism of dominance are uncertain (Grant et al., 1998) although temperatures ranging from 29°C-34°C are used (see references in Table 2). The level of mutant dynamin required for blockade and the speed of inactivation may depend on neural type. The elevated temperature may affect normal performance of some behaviors. UAS-Shibire^{ts1} also causes build up of microtubules in some cells at permissive temperatures (Gonzalez-Bellido et al., 2009). Flies expressing constitutively dominant-negative and wild-type dynamin are available (Moline et al., 1999) and can be used as controls.

4d. Blocking Membrane Depolarization by Ion Channel Manipulation

Disruption of membrane depolarization is another way to silence neurons. Neurons open voltage-gated sodium channels (encoded by *para*) in response to membrane depolarization to propagate action potentials or graded changes. It is possible to reduce the number of sodium channels directly using UAS-Para RNAi (Zhong et al., 2010) or to block *para* conductance with tethered toxins (Wu et al., 2008), but the more common approach has been to increase potassium conductance, which lowers the resting membrane potential or acts as a shunting current to prevent depolarization. UAS-Kir2.1 encodes a mammalian inward rectifying K+ channel and its expression provides the most complete suppression of depolarization of the reagents

described here (Baines et al., 2001; Paradis et al., 2001). This channel requires PIP2 (Hardie et al., 2004), which suggests that levels of this cofactor may modulate Kir2.1's efficacy in some cells. A recombinase-inducible version allows temporally controlled expression (Yang et al., 2009). Another construct, electrical knockout, UAS-EKO, encodes a version of the Shaker voltage-gated K+ channel that cannot inactivate and opens at a voltage threshold closer to the resting potential; it only partially blocks the photoresponse but is an effective neuronal silencer in some cell types (White et al., 2001b). UAS-dOrk expresses a two-pore leak K+ channel and can suppress neuronal excitability (Nitabach et al., 2002). Several reviews compare these options and the consensus is that UAS-Kir2.1 is the strongest silencer (White et al., 2001a; Holmes et al., 2007). This kind of chronic manipulation of membrane potential may result in homeostatic compensation, so inclusion of Tub-GAL80^{ts} to increase temporal control of expression may be advisable. One nice feature of these three potassium channel effectors is that they are fused to GFP, which allows confirmation of location and level of their expression.

4e. Increasing Neural Activity Using Ion Channels

Neurons can be rendered more active by increasing sodium or calcium conductance or by reducing potassium conductance. The temperature activated cation channel UAS-dTrpA1 (Rosenzweig et al., 2005; Rosenzweig et al., 2008) has been a powerful reagent to acutely activate neural activity and has been used to identify neurons involved in sleep and courtship behavior (Parisky et al., 2008; von Philipsborn et al., 2011). An assessment of efficacy of continued expression of UAS-dTrpA1 shows that increased excitation can be maintained in some cells (Pulver et al., 2009). The acute activation in response to moderate temperature increase and the sustained depolarization have made UAS-dTrpA1 a favorite tool in many labs. UAS-TrpM8 encodes a cold-sensitive cation channel (Peabody et al., 2009); it can be used to confirm that neurons identified with UAS-dTrpA1 cause phenotypes in response to increased activity rather than the increase in temperature required to activate the channel. The chemical ligand capsaicin can activate mammalian TrpV1 channels expressed in flies and has been used to map gustatory inputs (Marella et al., 2006).

Finally, overexpression of a bacterial sodium channel, NaChBac, can increase neural excitability (Nitabach et al., 2006) but may have other effects in other cell types or over longer timescales (Sheeba et al., 2008).

Reduction of the potassium current can also increase neural activity. Dominant-negative versions of the tetrameric potassium channels Shaker, Eag, Shaw, and Shal have been made by truncation of the wild-type channels, usually after the N-terminal multimerization domain (Broughton et al., 2004; Hodge et al., 2005; Mosca et al., 2005; Ping et al., 2011). RNAi constructs against Shaw also increase neural activity (Hodge and Stanewsky, 2008). These reagents have been reviewed (Hodge, 2009). A drawback is that the dominant-negative ion channels are only effective in neurons that express the normal versions of these ion channels.

4f. Optogenetics in Flies

Optogenetics was pioneered by UAS-P2X2, a cation channel activated by caged ATP released by light. This channel has been used to identify neurons sufficient to induce jump-escape (Lima and Miesenböck, 2005), courtship song (Clyne and Miesenböck, 2008), and olfactory conditioning (Claridge-Chang et al., 2009). One drawback is that the caged ATP must be injected into the hemolymph and then activated by light exposure, limiting the kind of behavior that can be studied and reducing the number of flies that can be screened.

The advent of genetically encoded proteins that activate or silence neural activity in response to light has been an exciting development for the neuroscience field (Deisseroth, 2011; Peron and Svoboda, 2011; Toettcher et al., 2011). These reagents can be used to identify neurons relevant to particular behaviors—they are acute and the combination of genetic and optical targeting makes their spatial and temporal locus of action exquisitely controllable, but the magic of these reagents has yet to be tapped in flies. Because light delivery can be temporally controlled with the precision of neurons themselves, these tools allow us to input or disrupt information within neurons directly, and enable us to investigate what the neurons are actually doing when they are active in their networks.

Channelrhodopsin is a 470 nm light-activated cation channel (Boyden et al., 2005; Nagel et al., 2005). All-trans retinal is an essential cofactor and in flies, this must be supplied in larval and adult food. UAS-ChR2 has been used to study larval learning and pain, adult escape responses, proboscis extension, and CO2 avoidance (Schroll et al., 2006; Hwang et al., 2007; Suh et al., 2007; Zhang et al., 2007; Gordon and Scott, 2009; Zimmermann et al., 2009). ChR2 reagents in flies have been reviewed (Zhang et al., 2007) and the electrophysiological effects of ChR2 have been quantified at the larval neuromuscular junction (Pulver et al., 2009). Various ChR2 point mutations improve conductance, membrane targeting, and expression level (Kleinlogel et al., 2011). Efforts to shift the excitation spectrum to longer wavelengths (Zhang et al., 2008) may limit the effect of light-activation on behavior since flies do not see red light > 800 nm and improve light penetration through the cuticle. Red-shifting will also increase spectral separation from GCaMP and NpHR (described below). ChR2 has the potential to temporally mimic endogenous neural spiking activity, so its potential for interrogating the neural information code is enormous.

Halorhodopsin (NpHR), the 580 nm light-activated chloride pump, has been used in *Drosophila* (S. Pulver and L. Griffith, personal communication), but newer versions that contain enhanced membrane trafficking sequences may work even better (Gradinaru et al., 2008). The current light-gated silencers have low ion conductance, which means that they must be highly expressed to be effective. Arch, ArchT, and Mac, outward proton pumps driven by yellow/green or blue light, are in development in other systems (Chow et al., 2010; Han et al., 2011b) and may work well in flies.

Much of the current use of optogenetic reagents in flies has been done in the translucent embryonic and larval stages where light penetrates well. Adult brain tissue can be made more light accessible by partial removal of the cuticle, but this limits the range of behaviors that can be investigated and the number of flies that can be assayed. In addition, some behaviors may be affected by the light stimulus; this confound may be reduced by using reagents activated by red-shifted light which is out of

the flies' visual range. To use the optogenetic reagents to their fullest potential, we need more information about what kinds of activity patterns might normally be present in neurons.

4g. Alternatives, Caveats, and Opportunities

In general, we recommend using two methods that disrupt neural function in different ways to help confirm the importance of particular neurons and avoid artifacts due to the penetrance or peculiarities of a given effector. Showing that activating and blocking activity have reciprocal phenotypes also strengthens the implication that the neurons are critical decision points for the behavior. Using a very effective blocker helps when identifying subtle neuronal contributions to a behavior—one does not want to miss a phenotype because the effector expression level was below effective threshold. Finally, acute blockers are often more useful than constitutively acting ones.

The options for manipulating neural activity are varied and effective but there is always room for improvement. For example, an acutely inducible and reversible electrical blocker of neural activity would be a valuable addition to the arsenal of tools for manipulating neural activity. Some neurons may be able to release both a canonical neurotransmitter and a peptide; it would be advantageous to be able to selectively block each type of release. Finally, there is no blocker of electrical transmission through gap junctions that are encoded by innexin genes in *Drosophila*, making it more challenging to identify the roles that these connections play in adult brain function.

5: Imaging Neuronal Activity

5a. Introduction

Since the brain acts as an interconnected network, a particular class of neurons may contribute to many behaviors, and their role may be affected by the action of neighboring neurons. Genetic targeting methods can direct the expression of fluorescent reporters of neural activity so that relevant neurons can be observed in action to see how they respond to controlled sensory stimuli or during different behaviors. Recording neuronal activity aids in identification of neurons whose activity is correlated with sensory stimuli, and enables the study of how neurons encode and transform the input signals they receive. This section will discuss these reagents.

5b. Techniques

Optical techniques that use changes in fluorescence to measure neuronal activity are a powerful way to identify neurons that respond to particular sensory stimuli or whose activity correlates with specific behaviors. They are essential for neural circuit analysis, i.e., how activity in neurons encodes information.

When a neuron fires an action potential there is a large local increase in calcium concentration that can be detected by *g*enetically *e*ncoded *c*alcium *i*ndicators (GECIs) that can be targeted to neurons of interest. Most GECIs use a calcium binding peptide to trigger either circularization of a single split fluorophore (GCaMP) (Wang et al., 2003) or energy transfer (FRET) between two fluorophores (Cameleon, Camgaroo, and TN-XXL) (Fiala et al., 2002; Yu et al., 2003; Mank et al., 2008). Ratiometric imaging is advantageous in preparations that undergo movement because the baseline fluorescence serves as a reference and the change in wavelength shows the change in neural activity. The single-fluorophore sensor, UAS-GCaMP, has been significantly improved recently and has emerged as the reagent of choice (Tian et al., 2009). UAS-GCaMP3 is being used to monitor activity in intact behaving flies (Chiappe et al., 2010; Seelig et al., 2010). Optical signals from UAS-GCaMP and other GECIs have been compared to electrophysiological recordings to determine how the fluorescence change correlates with particular voltage changes (Jayaraman and Laurent, 2007; Hendel et al., 2008). How well this calibration generalizes to other types of neurons has not yet been determined. The absence of a change in fluorescence cannot yet be interpreted to mean that neurons show no activity, since graded potential changes or single action potentials are not reliably detectable. Calcium indicators based on red fluorescent proteins are in development and should allow simultaneous imaging of different neural populations.

It is also possible to image vesicle release with UAS-synaptopHluorin (Miesenböck et al., 1998; Ng et al., 2002), which undergoes an increase in fluorescence upon the pH change associated with vesicle fusion, or UAS-ANF-EMD, which is specifically released from dense-core peptidergic vesicles (Rao et al., 2001). Another alternative is UAS-Aequorin-GFP, a bioluminescent reporter that integrates activity over longer timescales (Martin et al., 2007). There are sensors for cAMP, glutamate and activated PKA that may also be useful reporters for specific types of activity (Shafer et al., 2008). UAS-CaMKII-UTR-GFP may detect increases in mRNA localization at more active synapses (Ashraf et al., 2006). Voltage sensors exist but are not in wide use (Siegel and Isacoff, 1997; Guerrero et al., 2002; Sjulson and Miesenböck, 2008; Akemann et al., 2010).

6: Visualization of Neural Circuit Connectivity

6a. Introduction

The neurons identified by the experimental strategies outlined above constitute pieces of a puzzle that must then be assembled into a connected whole. Linking these neuron parts into neural circuits requires determining the connectivity between them, the strength of these connections, and the excitatory, inhibitory, or modulatory nature of these connections. While this aspect of neural circuit mapping is the least well developed, an overview of the current tools is presented in this section.

6b. Techniques

The techniques described in the preceding sections are useful for identifying neurons whose activity causes or correlates with specific stimuli or behaviors. The next major challenge is determining how these neurons are connected into circuits. The GAL4 and LexA reagents (see above) can be used to target the expression of fluorescent proteins to these neurons to image their morphology by light microscopy. Confocal or two-photon imaging allows the entire three-dimensional trajectories of these neurons to be visualized and thus to determine areas of the brain they innervate. Neurons can be labeled with cytoplasmic, nuclear, or membrane-targeted reporters, and the polarity of neurons can be investigated using dendritically or synaptically localized fluorophores (Estes et al., 2000; Zhang et al., 2002; Wang et al., 2004; Nicolaï et al., 2010). This can give an indication of where in the brain a neuron receives information from and where it releases neurotransmitter to pass this information along.

If the expression pattern of a GAL4 line is sparse enough, individual neuronal trajectories can be followed directly. If the

original expression pattern is broad, single neurons within the pattern can be labeled by stochastically active reporter constructs (Wong et al., 2002; Busch and Tanimoto, 2010; Raghu and Borst, 2011) or in randomly selected different colors (Hampel et al., 2011; Hadjieconomou et al., 2011) to allow individual neurons to be followed in detail (see above). Various light level imaging projects based on lineage and single neuron clones are locating the major compartment level connections in the fly brain (Chiang et al., 2011) but moving from a "projectome," showing at a compartment level where neurons may go, to a "connectome," demonstrating which neurons actually form synaptic connections, remains a challenge for the future.

Three-dimensional confocal images of two different GAL4 expression patterns can be aligned to a common reference brain to evaluate the possibility that the neurons overlap or come into contact (Jenett et al., 2006; Jefferis et al., 2007; Peng et al., 2011). The computational and manual alignment algorithms are accurate to within ~5 μm, which is sufficient to determine whether two populations cannot possibly connect but not to conclusively demonstrate actual connectivity.

Reporter constructs that contain different fluorescent proteins, enzymes or proteins with epitopes recognized by antibodies are available. If the two distinct reporters are expressed under the control of different expression systems (GAL4, LexA, and Q) two neural populations can be imaged simultaneously and their potential overlap or proximity assessed (Lai and Lee, 2006; Gordon and Scott, 2009; Peng et al., 2011). If the reporters are subcellularly localized and the dendrites of one population are very close to synapses of the other, the hypothesis that these neurons are functionally connected is strengthened (von Philipsborn et al., 2011). It may also be possible to increase confidence by showing that candidate post-synaptic neurons express receptors for the neurotransmitter released by the presynaptic neurons.

A system called GRASP (GFP *r*econstitution *a*cross *s*ynaptic *p*artners) that detects cell-cell contact such as that which occurs at synapses has been developed in *C. elegans* and imported to *Drosophila* (Feinberg et al., 2008; Gordon and Scott, 2009). GRASP uses two transgenes, each encoding a complementary part of GFP, that are expressed in two populations of neurons that might be connected. If the membranes touch, the two halves of GFP bind and make a fluorescent (and antigenic) protein. This is a promising method for testing whether two neurons come into contact, but demonstrating that this contact happens at normally occurring synapses and that these synapses are functional requires additional experiments.

Photoactivatable GFP has been used to follow particular neural input pathways (Datta et al., 2008; Ruta et al., 2010). In this type of experiment, one group of neurons is labeled with a reporter, and then a dark or photoconvertible fluorescent protein is expressed in neurons that are potentially connected. The area near the first group is illuminated with the wavelength of light required to photoactive the protein expressed in the candidate partners. If these candidates are close enough to the light spot, the fluorescent protein gets activated and diffuses throughout these neurons, labeling them enough that they can be identified by their morphology. This approach may work best in convergent circuits with areas of dense innervations where a large fraction of the GFP can be photoconverted by a very local illumination. This method demonstrates that two groups of neurons are close enough to form synapses but does not demonstrate that they actually do so.

Future development of methodology to demonstrate connectivity and explore the weights of particular synaptic connections is warranted. Trans-neuronal tracers based on lectins and neurotrophic viruses have been used to propose connectivity in vertebrate systems (Horowitz et al., 1999; Wickersham et al., 2007), but none have yet been successfully adapted for use in flies. Electron microscopy can show that synapses exist between two neurons and identification of the neurons in question is possible by completely reconstructing their trajectories or by labeling them with a genetically encoded enzyme (such as horseradish peroxidase) that produce an electron-dense reaction product. The optogenetic methods for activating neurons and the genetically encoded calcium indicators of neuronal activity can be combined with electrophysiological recordings to test functional connectivity and synaptic strength. One of the biggest hurdles remaining for deciphering neural circuits in *Drosophila* is demonstrating functional connectivity.

7: Forward Genetic Approaches to Isolate Novel Neuronal Genes

7a. Introduction

Mutations in genes expressed and required in the nervous system can be generated by reverse genetics (see below) or forward genetics. Forward genetic approaches are focused on phenotypic driven identification of mutations in genes involved in a certain biological process (St Johnston, 2002); for example, axon guidance, synaptic transmission, or behavior. Here, we will discuss and compare different strategies and mutagens and the advantages and caveats of various forward screening methodologies.

7b. Transposon Mutagenesis

Forward genetic screens based on transposon mutagenesis to identify new loci affecting neuronal features have so far been based on *P* elements (St Johnston, 2002) and *piggyBac* (Schuldiner et al., 2008). Two main strategies can be envisaged: one based on using existing collections, and one based on creating and screening a novel collection of transposon insertions. The most productive strategy is to screen existing collections of transposon insertions (Bellen et al., 2004, 2011; Matthews et al., 2005). The main advantage of these collections is that the identified phenotypes are often associated with the transposon insertion, there is generally a single insertion, and the insertion site is molecularly mapped or easily mapable. However, there are also drawbacks: access to these large collections is problematic, not all the phenotypes observed are associated with the insertions itself due to second-site hits (Liebl et al., 2006), and the screens typically cover many fewer genes than an EMS screen (see below). Indeed, many insertion stocks only carry one mutation, and because of insertion preference it is often impossible to reach saturation of the genome with a single transposons (Bellen et al., 2011). Finally, most insertional mutations are hypomorphic. However, the latter caveat is also a real advantage that has been exploited for quantitative and/or behavioral traits (Anholt and Mackay, 2004).

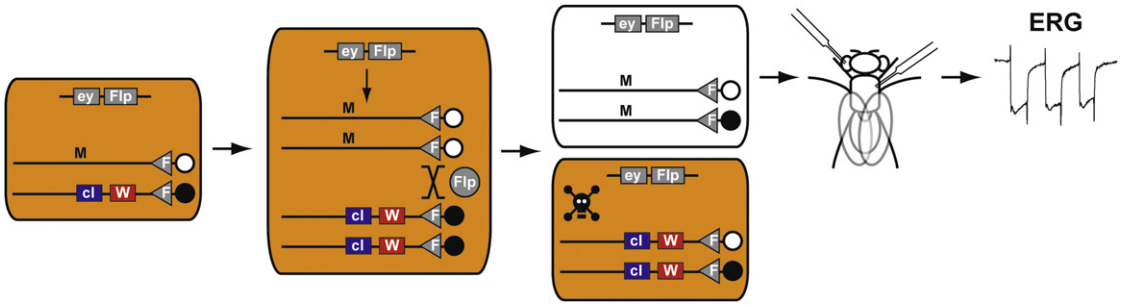

Figure 6. Forward Genetic Screens to Identify Novel Neuronal Genes
A mosaic Flp/*FRT* screen. Mutations generated by EMS on an *FRT* chromosome are crossed to another *FRT* chromosome containing a recessive cell lethal (*cl*) and dominant *white+* eye marker. The mutant chromosome (M) is made homozygous by Flp recombinase driven by a regulatory element expressed in the eye (*ey*) during cell division. Homozygous WT tissue dies, resulting in mostly homozygous mutant tissue that is white and that can be investigated with a phenotypic assay such as an electroretinogram (ERG). Other illustration keys are the same as in Figure 2.

The second approach is to create a collection of tranposons and screen for interesting phenotypes. This has mostly been done with *P* elements (Rørth, 1996; Bourbon et al., 2002; Peter et al., 2002; Oh et al., 2003) and *piggyBac* (Hacker et al., 2003; Horn et al., 2003; Mathieu et al., 2007; Schuldiner et al., 2008) and can be combined with mosaic analysis in an *FRT* background, i.e., flies that contain centromeric *FRT* sites on 2L, 2R, 3L and 3R (Mathieu et al., 2007; Schuldiner et al., 2008). These screens have been quite productive but are labor intensive.

Transposons have been useful in identifying numerous new genes that affect behavior, including loci required for olfaction (Kulkarni et al., 2002; Rollmann et al., 2005), aggression (Edwards et al., 2009), sleep (Cirelli et al., 2005; Koh et al., 2008), and ethanol induced behavior (LaFerriere et al., 2008; Corl et al., 2009; Kong et al., 2010a; King et al., 2011).

7c. Ethylmethane Sulfonate Mutagenesis

Forward chemical mutagenesis screens based on ethylmethane sulfonate (EMS) (Alderson, 1965) have led to isolation of pioneering genes that laid the foundation of our understanding of many neurobiological processes, such as neuronal identity (Doe, 2008), neuronal specification (Hartenstein et al., 2008), growth cone guidance (Seeger et al., 1993), visual perception and retinal neurodegeneration (Benzer, 1967; Pak et al., 1970), synaptic transmission (Suzuki et al., 1971), diurnal rhythmicity (Konopka and Benzer, 1971), learning and memory (Dudai et al., 1976), and sleep (Cirelli, 2003).

EMS is the most widely used chemical mutagen in *Drosophila*. A detailed protocol for EMS mutagenesis has been described (Bökel, 2008). If designed properly, EMS screens are typically saturating in nature, which is not the case for any of the other screening strategies. The power of any genetic screen typically depends on the ease and speed of the phenotypic assay, which is almost invariably the rate-limiting factor. The key feature of any well-designed EMS screen is that many thousands of flies can be screened quickly in a primary screen; this also permits lowering the dose of EMS to 10–12 mM (one mutation per 120–150 amino acids) rather than the typical 25 mM (Lewis and Bacher, 1968), which significantly reduces the number of nucleotide changes per chromosome, produces healthier stocks, and, most importantly, allows faster and more reliable mapping. Unlike most other mutagens, the molecular lesions caused by EMS are essentially random, ensuring that most genes of interest will be targeted and that multiple lesions will be found in each gene. Whole-genome sequencing now allow us to reliably and efficiently map EMS induced lesions at very reasonable costs (Blumenstiel et al., 2009; Wang et al., 2010) (H.J.B., unpublished data).

7d. Forward Genetic Screening in Specific Tissues

The identification of novel genes that affect specific biological processes in a specific tissue are based on creating mosaic animals (Xu and Rubin, 1993). Flp-mediated mitotic recombination screens result in the generation of homozygous mutant tissue in an otherwise heterozygous animal, limiting the effect of a possible detrimental or lethal mutant phenotype at an earlier developmental stage. Advantageously, such screens can often be designed as F1 screens where single progeny of mutagenized flies can be directly screened, mutations isolated, and balanced to generate stable stocks if the screen does not jeopardize viability and fertility of the heterozygous animals that carry clones. These screens are most conveniently performed with EMS.

Forward genetic Flp/*FRT* screens are based on creating clones in specific cells, tissue or organs using specific Flp drivers (Figure 6). Flp expression results in homozygous mutant tissue associated with a phenotypic outcome that can be scored easily. The most widely used driver is an eye specific driver, *ey*-Flp (Newsome et al., 2000), or *ey*-GAL4; UAS-Flp (Stowers and Schwarz, 1999). To obtain clones that are large enough it is important to use a driver that is expressed early in development. Moreover, clone size can be enhanced with the use of homologous chromosomes that carry a recessive cell lethal mutation, or a *Minute*. The large clones in the eye have allowed screening for morphological defects of eye cells (Newsome et al., 2000), simple behavioral paradigms such as phototaxis (Verstreken et al., 2003), electrophysiological function using electroretinograms (Ohyama et al., 2007), or bristle abnormalities on the head cuticle (Tien et al., 2008). These screens can be also combined with different MARCM strategies (see above).

7e. Mapping the Lesion that Causes the Mutation

Forward genetic screens generally require a strategy to genetically and/or molecularly map the mutation. In the case of transposons, the insertion site is often known or can be easily mapped (Hui et al., 1998; Bellen et al., 2011).

Mutation mapping becomes more challenging for EMS mutagenesis. However, mapping is greatly facilitated on autosomes since 95% of the fly genome is covered by deficiencies (Cook et al., 2010a). Alternatively, mapping can be performed using meiotic recombination and single-nucleotide polymorphisms (SNPs) (Chen et al., 2008).

Perhaps the easiest mapping method accessible to all *Drosophila* researchers are defined *P* element insertions. For autosomal mutations mapping to about 1 cM is easy, cheap, and fast if they display a robust visible or lethal phenotype (Zhai et al., 2003). Thousands of *P* element or other transposon insertions with dominant markers are available.

Deficiency or meiotic mapping is not easy for lethal mutations and male sterile mutations on the *X* chromosome, since males only carry one *X* chromosome. These, as well as viable mutations, can now be mapped via duplication mapping since duplication stocks covering more than 95% of the *X* chromosome are now available (Venken et al., 2010; Cook et al., 2010b).

The most rapid and cost-effective way to identify EMS induced lesions is to first obtain a rough mapping position in a 50–300 kb (0.5–1 cM) interval using transposon, deficiency, or duplication mapping. This is now followed by whole-genome sequencing (Blumenstiel et al., 2009). Note that even low EMS levels induce many SNPs along a chromosome and that without rough mapping it is very difficult to assign a lesion to a phenotype. Finally, it is important to rescue the phenotype of the identified mutations with a genomic rescue clone. Injection-ready clones from genomic libraries covering more than 95% of the fly genome are available (Venken et al., 2009; Ejsmont et al., 2009). Moreover, these genomic rescue constructs can be modified by recombineering to introduce tags for protein labeling or conditional inactivation (Venken et al., 2008, 2009; Ejsmont et al., 2009).

8: Reverse Genetic Approaches to Mutate Specific Neuronal Genes

8a. Introduction

Reverse genetics is driven by interest in a particular gene and requires technologies that allow selective disruption of a gene (Adams and Sekelsky, 2002; Venken and Bellen, 2005). Broadly speaking, five strategies are available to reduce gene activity: transposon excision, altering transposons inserted in the gene, RNA interference (RNAi), and gene targeting through either homologous recombination or zinc finger nucleases.

8b. Transposon Mutagenesis and Manipulations

The most commonly used transposable elements that have been introduced into the fly field are the *P* element, *piggyBac* and *Minos* (Venken and Bellen, 2007). The goal of the Gene Disruption Project (GDP) is try to obtain at least one transposon insertion in every fly gene to allow their manipulation. The GDP has generated and/or sequenced over 150,000 insertions and more than 15,000 transposon insertions have been deposited in the Bloomington *Drosophila* Stock Center. Currently about 65% of all annotated *Drosophila* genes carry insertions (Bellen et al., 2011).

P elements mobilize efficiently and can excise imprecisely to generate deletions. They exhibit a strong insertional bias for promoters and origin of replications binding sites (Bellen et al., 2011). *piggyBac* transposons do not show a strong insertional bias, but mobilize less efficiently than *P* elements, and only excise precisely (Thibault et al., 2004; Witsell et al., 2009). *Minos* elements have very little insertional bias, transpose stably, and efficiently (Metaxakis et al., 2005; Bellen et al., 2011; Venken et al., 2011), and excise imprecisely (Metaxakis et al., 2005; Witsell et al., 2009). The percentage and overall size of imprecise excisions for *P* elements and *Minos* can be increased when performed in a *mus309* mutant background (Witsell et al., 2009).

8c. Deletion mutagenesis

Deletions are true null alleles of genes. Deletions can be generated through X-ray mutagenesis, imprecise excision of a *P* element or *Minos* (see above), excision of sequence between any two *P* element transposons located at different positions of the same chromosome (Cooley et al., 1990; Parks et al., 2004; Paré et al., 2009), the deletion-generator strategy (Huet et al., 2002; Mohr and Gelbart, 2002; Myrick et al., 2009), or Flp-mediated recombination between two *FRT* sites each located in a transposon located at different positions of the same chromosome (Ryder et al., 2004, 2007; Parks et al., 2004; Cook et al., 2010a). *FRT* deletions now cover 98% of the chromosomes (Cook et al., 2010b). These stocks are the most widely used stocks in the fly community as they permit mapping of mutations through complementation tests.

8d. RNA Interference

RNAi is the simplest way to affect gene function quantitatively. First performed through embryonic microinjections (Kennerdell and Carthew, 1998), RNAi was demonstrated in vivo using the GAL4/*UAS* system (Fortier and Belote, 2000; Lam and Thummel, 2000; Kennerdell and Carthew, 2000). Four genome-wide RNAi libraries aimed at targeting all fly genes have been or are being generated. The first library, encompassing 22,270 lines covering 88% of all the predicted protein-coding genes, was generated in a *P* element (Dietzl et al., 2007). The addition of the Dicer-2 enzyme improved knockdown levels for RNAi transgenes that generally resulted in a hypomorphic phenotype (Dietzl et al., 2007), although introduction of Dicer-2 may lead to toxicity. In the neurobiology field, this library has been used to screen for Notch signaling components during external sensory organ development (Mummery-Widmer et al., 2009), heat nociception (Neely et al., 2010), and stem cell renewal (Neumüller et al., 2011). Particularly elegant uses of RNAi lead to the identification of Sex Peptide receptor and the neurons that respond to it (Yapici et al., 2008; Häsemeyer et al., 2009). A second library consisting of 11,496 lines covering 6,047 genes was generated in a *P* element as well and was used to identify novel components involved in the circadian clock network (Matsumoto et al., 2007) (R. Ueda, personal communication). Unfortunately, since the integration site of *P* elements cannot be controlled, position effects result in variable knockdown. This problem was partially circumvented by using the site-specific ΦC31 integrase system (Groth et al., 2004; Bischof et al., 2007). These libraries are still being constructed (Dietzl et al., 2007; Ni et al., 2009). Another advantage of the ΦC31 system is that RNAi parameters can directly be compared to each other and therefore be optimized (Ni et al., 2008; Ni et al., 2009). These studies also illustrated that short hairpin RNAs (shRNA) modeled on an endogenous microRNA are an effective alternative for classical dsRNA mediated RNAi in the generation of genome-wide RNAi

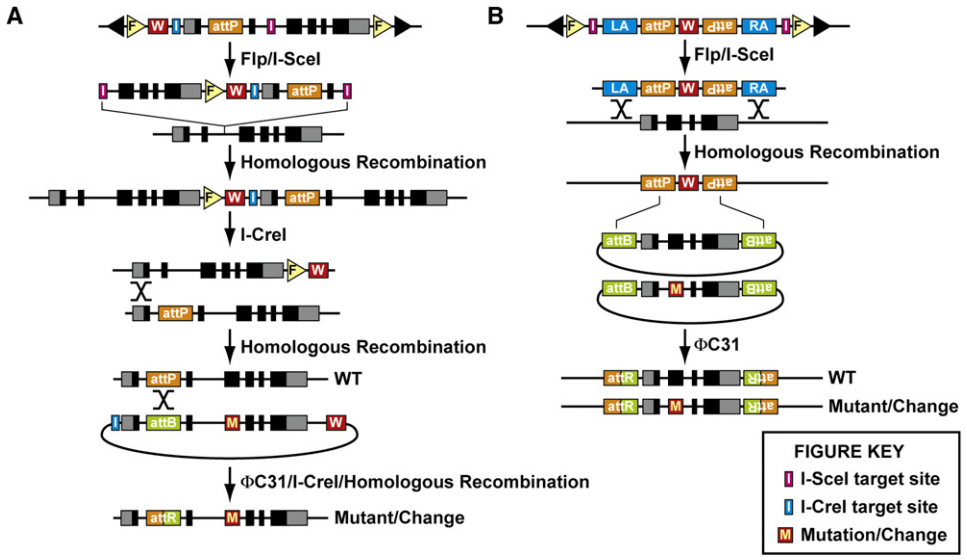

Figure 7. Reverse Genetic Techniques to Manipulate Neuronal Genes
(A) Ends-in gene targeting illustrated by SIRT.
(B) Ends-out gene targeting illustrated by RMCE/IMAGO. Other illustration keys are the same as in Figure 2. See text for details.

libraries (Ni et al., 2011). shRNA-mediated RNAi can be directed toward alternative exons and allowed studying the function of alternative splice variants (Shi et al., 2007; Yu et al., 2009b).

RNAi experiments can result in unwanted phenotypes due to off-target knockdown. RNAi rescue strategies provide a solution to this problem: one exploits genome-wide libraries of a related species, *Drosophila pseudoobscura* (Kondo et al., 2009; Ejsmont et al., 2009; Langer et al., 2010), since genes and their regulatory regions of *Drosophila pseudoobscura* are similar enough to rescue genes of *Drosophila melanogaster*, but divergent enough to resist the RNAi machinery. Another strategy uses GAL4 to express a UAS rescue construct with altered codon usage that resists the RNAi degradation (Schulz et al., 2009).

In summary, advantages of RNAi experiments are that they can be performed in a tissue-specific fashion using the GAL4-UAS system. Disadvantages are that off-target effects are not uncommon and knockdowns are almost always incomplete.

It is difficult to compare the efficiency of different screening strategies. An RNAi screen to identify novel players in the Notch pathway (Mummery-Widmer et al., 2009) did not identify any of the genes that have been isolated using Flp/*FRT* screens with EMS mutagenesis (Jafar-Nejad et al., 2005; Acar et al., 2008; Tien et al., 2008) with one exception (Rajan et al., 2009).

8e. Gene Targeting and Homologous Recombination

Homologous recombination or gene targeting can be used to generate modifications or mutations in specific genes in their normal chromosomal context. Gene targeting in *Drosophila* is performed using one of two methods: ends-in gene targeting and ends-out gene targeting (Wesolowska and Rong, 2010). The result of ends-in gene targeting is a local duplication at the targeting site, due to the integration of the entire targeting vector (Rong and Golic, 2000, 2001). This duplication can be resolved during a second round of homologous recombination catalyzed by the meganuclease *I-Cre*I (Rong et al., 2002), resulting in precisely engineered alleles of several genes required in the nervous system that include point mutations, deletions, gene swaps, protein tags, GAL4 insertion, or splice form reduction (Demir and Dickson, 2005; Stockinger et al., 2005; Brankatschk and Dickson, 2006; Hattori et al., 2007; Hattori et al., 2009; Spitzweck et al., 2010).

Ends-in targeting experiments are generally time consuming. Therefore, *s*ite-specific *i*ntegrase mediated *r*epeated *t*argeting (SIRT) was developed to facilitate downstream modifications (Gao et al., 2008a) (Figure 7A). SIRT combines homologous recombination and the ΦC31-mediated site-specific transgenesis system (Groth et al., 2004; Bischof et al., 2007). During ends-in targeting SIRT introduces an *attP* site for ΦC31. Subsequently, this *attP* site allows limitless genome modifications including point mutations and deletions through transgenesis of modified genomic fragments contained within an *attB* plasmid (Gao et al., 2008a). Drawbacks of SIRT are that remnants (*att* sites) are left behind in the genome.

Ends-out gene targeting is generally used to replace parts of the genome, resulting in the generation of deletions and truncations (Gong and Golic, 2003) and is now the preferred method (O'Keefe et al., 2007; Huang et al., 2008). The advantage of ends-out targeting is that alleles are created during a single gene targeting event, possibly followed by efficient removal of unwanted sequences flanked by *LoxP* sites through Cre recombinase (Siegal and Hartl, 1996). One drawback of ends-out targeting is that it always leaves remnants behind in the genome. Fortunately, these remnants can be engineered in the targeting construct as desired, such as peptide tags (Yamamoto-Hino et al., 2010) or a GAL4 transcriptional activator (Manoli et al., 2005; Sokol et al., 2008). Two additions have been incorporated to facilitate ends-out gene targeting. A first addition is a negative selection marker based on an apoptotic gene to eliminate all nonhomologous targeting events (Huang et al., 2008). A second

addition is geared toward subsequent rounds of manipulations based on integrases. One is ΦC31-mediated RMCE (Choi et al., 2009; Weng et al., 2009), also known as the *i*ntegrase-*m*ediated *a*pproach for *g*ene knock*o*ut (IMAGO) method (Choi et al., 2009) (Figure 7B). A second one is based on regular ΦC31-mediated transgenesis followed by Cre reduction known as genomic engineering (Huang et al., 2009) or *in s*itu *i*ntegration for *r*epeated *t*argeting (InSIRT) (Iampietro et al., 2010). Genome engineering has recently included the Bxb1 integrase for subsequent genome manipulations (Huang et al., 2011). These manipulations allowed creation of knockin alleles (Choi et al., 2009; Huang et al., 2011), a conditional knockout allele (Choi et al., 2009), small deletions (Huang et al., 2009; Iampietro et al., 2010), point mutations (Huang et al., 2009), or insertion of protein tags and other DNA elements (Huang et al., 2009). However, both applications still leave small remnants (*att* and/or *LoxP* sites) in the genome.

Although gene targeting alleles are obtained at a target locus, nontargeted background or second-site lethal mutations do arise (O'Keefe et al., 2007; Roy and Hart, 2010). This can be circumvented through repeated backcrossing to the parental strain from which mutants were derived (O'Keefe et al., 2007; Roy and Hart, 2010).

An alternative and emerging method of gene targeting is mediated by zinc finger nucleases. Zinc finger nucleases are chimeric proteins generally consisting of three zinc finger domains, each recognizing a nucleotide triplet, fused to a Fok1 nuclease domain. They function as a dimer. Zinc finger nucleases have been used to generate mutations by nonhomologous end joining (Bibikova et al., 2002; Beumer et al., 2006) or homologous recombination with an ectopic template as a substrate (Bibikova et al., 2003; Beumer et al., 2006). Creating mutations via zinc finger nucleases seems attractive, especially since an embryo injection protocol has been established (Beumer et al., 2008). However, the method is not widespread and not all loci can be targeted.

9: Genetic Labeling of Neuronal Proteins

9a. Introduction

Critical information about genes and the proteins they encode is their cellular and subcellular distribution. These data are typically determined by in situ hybridization experiments and immunohistochemical stainings using antibodies raised against the protein encoded by the gene. However, several powerful genetic methods are now available to visualize protein expression patterns through tagging of genomic rescue constructs, gene targeting, and protein trapping.

9b. Tagging of Genomic Rescue Constructs

Generally, genomic rescue constructs are obtained by traditional cloning into plasmids that are compatible with *P* element transgenesis (Rubin and Spradling, 1982; Spradling and Rubin, 1982; Le et al., 2007) or ΦC31-mediated site-specific integration (Groth et al., 2004; Bischof et al., 2007). A valuable alternative to generate tagged genomic rescue constructs emerged recently through recombineering (Sharan et al., 2009). Recombineering can be performed with different recombination templates such as PCR products that encompass protein tags or oligonucleotides that encompass specific mutations (Sharan et al., 2009). Recombineering was first introduced into the *Drosophila* field as a versatile transgenic platform named P[acman] (P/ΦC31 artificial chromosome for manipulation) (Venken et al., 2006). Recombineering can be used to retrieve small to large DNA fragments containing genes from existing genome-wide BAC libraries for *Drosophila melanogaster* via gap-repair in *Escherichia coli*. The ΦC31 integrase integrates the *attB* containing constructs into defined *attP* containing docking sites (Groth et al., 2004; Venken et al., 2006; Bischof et al., 2007; Markstein et al., 2008). Subsequent recombineering steps can then be performed to introduce changes that include point mutations and deletions in *Escherichia coli* for structure/function analysis (Pepple et al., 2008; Leonardi et al., 2011). Alternatively, different tags for visualization of protein expression, subcellular protein localization, or acute protein inactivation using FIASH-FALi (Venken et al., 2008; Kasprowicz et al., 2008) can be introduced in the genomic locus by recombineering and subsequently integrated in specific *attP* docking sites in the fly genome (Figure 8A).

The P[acman] methodology was adapted to create two genomic DNA libraries in a modified P[acman] vector with an average insert size of 21 kb and 83 kb, respectively (Venken et al., 2009). An additional library with an average insert size of 36 kb was generated within a fosmid backbone (Ejsmont et al., 2009). These libraries allow rescue of mutations, as well as structure/function analysis and protein tagging of more than 95% of all annotated genes of the fly genome. These tagged clones should be very useful to determine the expression patterns of numerous uncharacterized genes and their corresponding proteins in the nervous system. Moreover, recombineering can be extrapolated toward high-throughput efforts to tag hundreds of genes within a small time frame (Poser et al., 2008).

9c. Endogenous Protein Tagging by Gene Targeting and Protein Trapping

An alternative approach to tag genes/proteins is to tag the endogenous locus. This can be done via gene targeting (see above) or protein trapping. Protein trapping is based on inserting artificial exons in genes. The methodology is simple but depends on the presence of a transposon in the gene of interest. The insertion of artificial exons or protein traps in *Drosophila* is typically based on transposons (Figure 8B). As such, the endogenous protein becomes labeled with the tag that was engineered in the artificial exon. Protein trapping was pioneered with the green fluorescent protein that was incorporated in a *P* element screen, resulting in some protein trap insertions (Morin et al., 2001; Clyne et al., 2003; Rolls et al., 2007). A similar *piggyBac* based strategy resulted in more lines (Besse et al., 2007). Subsequently, genome-wide efforts with *P* element and *piggyBac* transposons were performed, resulting in several hundred tagged lines (Kelso et al., 2004; Buszczak et al., 2007; Quiñones-Coello et al., 2007). Finally, a large scale protein trapping effort based on a hybrid *piggyBac*/*P* element was used to establish expression patterns for 535 protein trap lines inserted into genes expressed in the *Drosophila* brain (Knowles-Barley et al., 2010).

The MiMIC system that has been described previously (Figure 2C) allows for a more versatile protein-trapping approach. Any MiMIC that is inserted in an intron between two coding exons of any gene can be used to incorporate any tag using RMCE (Figure 8C). The insertion of a tag in the middle of proteins is quite efficient and does not seem to disrupt protein function much. In addition, plasmids containing seven different tags in

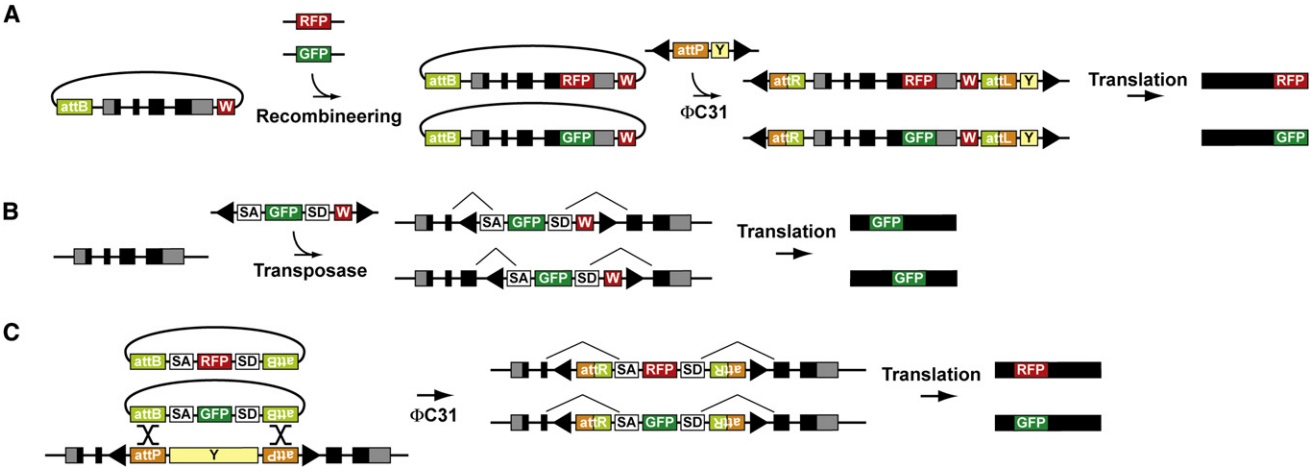

Figure 8. Genetic Techniques for Neuronal Protein Labeling

(A) Recombineering-mediated protein tagging illustrated for C-terminal tagging. PCR fragments encompassing protein tags (GFP or RFP) are recombined into a genomic rescue fragment. The resulting transgenes are integrated using ΦC31transgenesis and used to obtain the expression pattern of the host gene.

(B) Transposon protein trapping. A transposon encoding an artificial exon encompassing splice acceptor, GFP and splice donor site, can integrate into coding introns and reveal the expression pattern of the host gene.

(C) MiMIC protein trapping. Coding intronic insertions of MiMIC can be converted to protein traps by ΦC31-mediated RMCE using plasmids encoding artificial exons encompassing a splice acceptor, GFP or RFP, and a splice donor site. The resulting swap events reveal the expression pattern of the host gene. Illustration keys are the same as in Figure 2. See text for details. For a description of endogenous gene tagging by gene targeting, please see Figure 7.

all reading frames are available permitting the tagging of hundreds of genes. This is particularly useful for large genes which contain many introns (Venken et al., 2011).

10: En Route to the Courtship Circuit

10a. Introduction

The approaches and reagents described above can be used to address many experimental questions. This last section provides an example of how these diverse tools have been creatively used to identify many of the components of a neural circuit driving fly courtship behavior.

10b. The Courtship Circuit

The identification of neurons involved in male courtship behavior in *Drosophila* provides a beautiful case study for how the tools reviewed here have been used to gain fundamental insights. Mutations that affect nearly all aspects of male courtship behavior without altering the non-nervous system physical aspects of gender were discovered and mapped to a male-specific isoform of the fruitless gene (Ryner et al., 1996; Ito et al., 1996). Specific mutations were engineered into the *fruitless* gene to test the hypothesis that male-specific splicing was responsible for male courtship behavior (Demir and Dickson, 2005). The expression pattern of the male-specific isoform of the Fruitless transcription factor was determined by antibody staining to include approximately 2,000 neurons of many different neuronal types in the central nervous system (Lee et al., 2000a). These neurons can be manipulated by GAL4 lines that mimic the FruM expression pattern: several enhancer traps inserted near FruM capture parts of its expression (Dornan et al., 2005; Kimura et al., 2005) and specific GAL4 knockin alleles have been generated (Manoli et al., 2005; Stockinger et al., 2005). Analysis of these expression patterns revealed subtle gender differences in which Fru controls cell number in some lineages (Kimura et al., 2005). Differences in extent of arborization and potential connectivity differences became more apparent when subsets of the expression pattern were examined with MARCM and Flp-Out techniques that allowed the visualization of individual lineages and neurons within the FruM expression pattern (Cachero et al., 2010; Yu et al., 2010). The areas of gender dimorphism suggest the location of neurons involved in male-specific behaviors.

Silencing all FruM neurons with UAS-Shibire^{ts1} strongly reduces courtship behavior (Demir and Dickson, 2005; Manoli et al., 2005; Stockinger et al., 2005) and their activation with *UAS-P2X2* is sufficient to trigger courtship song (Clyne and Miesenböck, 2008). The function of particular classes of Fruitless neurons has been investigated by using GAL4 lines that intersect with the FruM expression pattern and reagents that disrupt FruM specifically or alter neural activity. The FruM-positive median bundle neurons were shown to be critical for correct behavioral sequence generation using RNAi against FruM (Manoli and Baker, 2004). A screen using the intersection between FruM and GAL4 neuronal expression facilitated identification of additional brain regions involved in male courtship (Meissner et al., 2011). Statistical analysis of courtship behavior when randomly generated subsets of the FruM expression pattern were feminized or inhibited suggested brain regions key for courtship initiation (Kimura et al., 2008). Expression of the neural activator UAS-dTrpA1 with intersectional techniques and random generation of FlpOut clones identified neurons likely to be sufficient to evoke specific aspects of *Drosophila* courtship song production (Kohatsu et al., 2011; von Philipsborn et al., 2011).

Male courtship behavior is influenced by a range of sensory inputs (Krstic et al., 2009; Koganezawa et al., 2010), especially the olfactory system (Billeter et al., 2009). Photoactivatable GFP has been used to trace connectivity from the olfactory receptors that detect female flies through the antennal lobe to

second order projections (Datta et al., 2008; Ruta et al., 2010). Anatomical analysis suggests a compartment-level convergence of FruM neurons (Yu et al., 2010) and expression of dendritic and synaptic reporters in candidate partners suggests connectivity (von Philipsborn et al., 2011). New understandings of the neural basis for courtship behavior have been reviewed (Manoli et al., 2006; Dickson, 2008; Benton, 2011).

The courtship circuit has several advantages: a single gene (or isoform) expressed in many neural components, sexually dimorphic anatomy and behavior, some known sensory inputs, and corroborative historical data from gynandromorphs and feminization screens (Hall, 1979; Ferveur et al., 1995; Broughton et al., 2004), but the astute use of genetic tools to manipulate neurons has led to our current understanding of the neural circuit driving male courtship behavior. Recent work has demonstrated functional imaging of FruM neurons during a facsimile of courtship behavior (Kohatsu et al., 2011), which will allow interrogation of how neurons within the circuit respond to specific sensory stimuli and how their activity correlates with behavioral output. The same experimental setup could be used to deliver specific activity patterns with light-stimulated channelrhodopsin to determine how these neurons affect behavioral outcomes. Although there is still much work to be done to connect the identified components of a courtship circuit and find the missing links, the potential to understand how a neural circuit actually works to drive this complex behavior is unmatched. Similar smart use of the powerful tools described here should enable mapping of circuits driving a range of different behaviors. This will permit circuit comparisons, identification of neurons that participate in several circuits, and the investigation of the way decisions between behavioral programs are made.

11. Conclusions

To understand how the nervous system of an animal controls a particular behavior, one needs to identify the neurons involved, determine how their activity influences the behavior, and explore how they connect to other participating neurons. The abundance of tools for spatially and temporally targeting gene expression to specific neurons, manipulating or observing their activity, and assaying behavioral consequences makes *Drosophila* a premier system for exploring the principles guiding the development and function of neural circuits. The ability to make specific mutations in neural genes and label particular proteins to determine their cellular and subcellular locations within neurons makes *Drosophila* a formidable model for the study of the cell biology of neurons as well. Lastly, the canonical strength of *Drosophila*—high-throughput forward genetic screens to identify novel genes as levers into understanding critical neural processes—has only been enhanced by modern tissue-specific mosaic targeting and recent advances in DNA sequencing that speed up the laborious mutant mapping steps. We hope that this overview of the research tools available and the examples of how they have been used inspire their application to new questions.

ACKNOWLEDGMENTS

We apologize to those whose work we did not cite because of our focus and space limitations. We would like to thank Stephanie Albin, Bruce Baker, Juan Botas, Herman Dierick, Vivek Jayaraman, Jon-Michael Knapp, Claire McKeller, Gerry Rubin, Andrew Seeds, and Alex Vaughan for comments on the manuscript. We appreciate personal communications with Ryu Ueda, Kei Ito, Gerry Rubin, Stefan Pulver, and Leslie Griffith. Our research was supported by the U.S. National Institutes of Health grants T32 GM007526 (K.J.T.V.), R01 GM067858 (H.J.B.), and RC4 GM096355 (H.J.B.) and the Howard Hughes Medical Institute (J.H.S. and H.J.B.).

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