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Targeted manipulation of neuronal activity in behaving adult flies

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ABSTRACT

The ability to control the activity of specific neurons in freely behaving animals provides an effective way to probe the contributions of neural circuits to behavior. Wide interest in studying principles of neural circuit function using the fruit fly *Drosophila melanogaster* has fueled the construction of an extensive transgenic toolkit for performing such neural manipulations. Here we describe approaches for using these tools to manipulate the activity of specific neurons and assess how those manipulations impact the behavior of flies. We also describe methods for examining connectivity among multiple neurons that together form a neural circuit controlling a specific behavior. This work provides a resource for researchers interested in examining how neurons and neural circuits contribute to the rich repertoire of behaviors performed by flies.

1. INTRODUCTION

The study of behavior often requires watching an animal move its body to accomplish different tasks. This is because the performance of any behavior requires movement of some part of the body. For example, a fruit fly moves a wing to sing a courtship song, a fish moves its tail fin to swim away from a predator, or a human moves his or her fingers to type the letters that make up a book chapter. To study behavior, one must ultimately confront the problem of how to observe the movements being performed and how to quantify them (Anderson and Perona 2014; Egnor and Branson 2016). Additionally, these very movements may cause technical challenges when researchers wish to manipulate the activity of specific neurons while simultaneously assessing the effect of the manipulation on a behavior.

The field of neuroscience has recently seen a number of innovations that better equip researchers to study neural circuit function in freely moving animals. The first are tools for expressing any protein coding sequence of interest in behaviorally relevant neurons (Venken et al. 2011b; Huang and Zeng 2013). Many of these expression systems are integrated into the genome or, in the case of some vertebrates, introduced into specific brain regions using viruses. The second are tools and techniques developed for the "remote control" of neuronal activity. For example, light-gated ion channels enable the manipulation of the activity of specific neurons using light (Boyden et al. 2005; Bernstein et al. 2012). Third, methods have been developed for the recording, classification, and quantification of behavior. For example, machine vision-based tracking of animal movement has greatly improved the consistency and throughput of behavioral analyses (Anderson and Perona 2014; Egnor and Branson 2016). Finally, additional tools have been developed to examine the functional relationships among different neurons that each contribute to a given behavior. This includes assessment of the functional connectivity between neurons using genetically encoded indicators of neural activity (Broussard et al. 2014). This suite of innovations now empower researchers to make substantial progress in probing the functions of neural circuits across a range of different species (White 2016).

These tools can be combined in the fruit fly (*Drosophila melanogaster*) to greatly simplify dissection of the behavioral contributions of specific neurons. This is in part because of the ease with which flies containing multiple transgenes can be generated to enable the manipulation of specific neurons, often at single cell resolution. With such exquisite specificity, the range of different tools for visualizing and manipulating behaviorally relevant neurons can be brought to bear on questions of how neural circuits control behavior. Another advantage of flies is their amenability to large-scale screens for identifying previously unknown behaviorally relevant neurons. Such screens offer the prospect of uncovering different types of neurons that together constitute the neural circuit mediating a particular behavior. Collectively, these different tools and approaches can be used to study the rich set of innate behaviors performed by flies such as walking, flight, grooming, feeding, mating, fighting, and escape. Moreover, behavioral and circuit-based studies in flies have provided new insights into basic topics in neuroscience such as learning and memory, sensory-motor integration, neuromodulation, sleep, behavioral choice, behavioral sequencing, motor control, and sensory systems (Huston and Jayaraman 2011; Yoshihara and Ito 2012; Kaun et al. 2012; Perry and Barron

2013; Pavlou and Goodwin 2013; Wilson 2013; Tataroglu and Emery 2014; Borst 2014; Borgmann and Büschges 2015; Owald et al. 2015; Hoopfer 2016; Masek and Keene 2016; McKellar 2016). Given that many of the tools used to study neural circuits have only recently become available, we anticipate that the coming years will experience a rapid growth in our understanding of how neural circuits within the fruit fly nervous system are organized to produce particular behaviors.

The aim of this manuscript is to provide a theoretical and practical resource for both beginning and experienced researchers who are interested in studying the roles of neurons and neural circuits in fruit flies. We describe how to identify and manipulate the activity of behaviorally relevant neurons in freely behaving flies. This includes information about expression systems, reagents for manipulating neural activity, behavioral hardware, rearing flies for neural manipulation experiments, and assessing the behavioral impact of neural manipulations. Further, we describe methods for examining how different identified neurons are organized into neural circuits to collectively control behavior. We include discussions about the advantages and disadvantages of different reagents and approaches so that the reader can make informed decisions about the experimental approaches that best suit their needs. To illustrate different techniques, we refer to experiments where researchers study neurons whose activation elicits specific behaviors. However, the approaches discussed here can be applied to the study many other aspects of nervous system function.

2. BINARY EXPRESSION SYSTEMS

Critical for probing the neural basis of behavior in flies are binary expression systems, such as GAL4/UAS, that allow for visualization and manipulation of behaviorally relevant neurons (Venken et al. 2011b; del Valle Rodríguez et al. 2012). GAL4 is a yeast-derived transcription factor that binds to its *upstream activating sequence* (UAS) to drive transcription of any coding sequence of interest placed under the control of UAS. Binary expression systems are designed to take advantage of the enhancer activity of *Drosophila* genomic regulatory elements that control when and where genes will be expressed in the body (Brand and Perrimon 1993; Duffy 2002). Genomic enhancers are used to direct expression of GAL4 in different subsets of neurons in *enhancer trap* or *enhancer fusion* transgenic flies. Enhancer traps arise when a transposable element (for example P element or Minos) containing a minimal transcriptional promoter upstream of the GAL4 coding sequence is randomly inserted into different locations in the genome. The expression pattern of GAL4 then is then directed by the minimal promoter in conjunction with genomic regulatory elements that are local to the transposable element's insertion site (O'Kane and Gehring 1987; Brand and Perrimon 1993). Enhancer traps are historically the most common method for driving expression in neural subsets.

Enhancer fusions contain genomic regulatory elements that are fused with a minimal transcriptional promoter and GAL4, and then inserted into the genome. Most enhancer fusion transgenic fly lines were made with the enhancer fusion inserted into a defined genomic location that contains the *attP* target sequence for *phiC31* mediated site-specific integration (Groth et al. 2004; Pfeiffer et al. 2008; Jenett et al. 2012) (Barry Dickson, personal communication). Many transgenic lines have been generated, each containing a stably inserted *attP* sequence at a different genomic location to provide different possible sites for integration (Groth et al. 2004; Venken et al. 2006; Bischof et al. 2007; Markstein et al. 2008; Ni et al. 2009; Knapp et al. 2015). Ideally the *attP* site is transcriptionally neutral such that only the specific genomic regulatory elements in the enhancer fusion direct the expression pattern of GAL4. Insertion of the enhancer fusion into an *attP* site offers the advantage that it avoids unwanted behavioral consequences of random insertions into different genomic locations. This is in contrast to the transposable element method used for enhancer traps that can introduce mutations (Spradling et al. 1999). Importantly, enhancer fusion transgenic fly lines can easily be remade because the sequences of the genomic fragments and *attP* insertion sites are known (Pfeiffer et al. 2008). In contrast, enhancer trap-based lines cannot be easily remade in the case the fly stock is lost.

Thousands of enhancer trap and enhancer fusion GAL4 transgenic fly lines have been generated, whose collective neural expression patterns cover most, if not all of neurons in the nervous system (Yoshihara and Ito 2000; Gohl et al. 2011; Jenett et al. 2012) (Barry Dickson, personal communication). Some express GAL4 in specific neuronal types such as dopaminergic neurons, whereas others express in an assortment of neurons. The expression patterns of many GAL4 lines are publicly accessible (e.g., http://flweb.janelia.org/cgi-bin/flew.cgi, http://stockcenter.vdrc.at/control/main) and stocks for several large collections are available at the Bloomington *Drosophila* Stock Center and Vienna *Drosophila* Resource Center (http://flystocks.bio.indiana.edu/Browse/gal4/gal4_main.htm, http://stockcenter.vdrc.at/control/main). Additional binary expression systems have been developed for use in Drosophila such as LexA/LexAop and QF/QUAS (Lai and Lee 2006; Potter et al. 2010; Pfeiffer et al. 2010; Riabinina et al. 2015). The advantage of having multiple binary expression systems is that they can be used for independent expression of different coding sequences in the same fly.

GAL4 and other binary expression systems can be used to express any coding sequence of interest in subsets

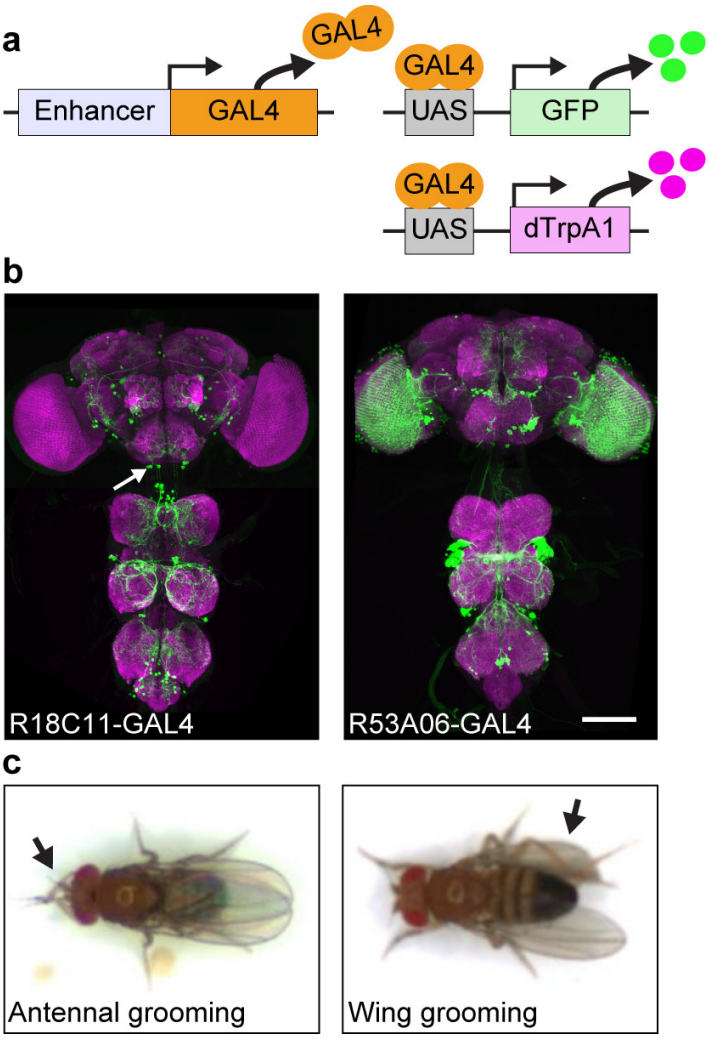

Figure 1. GAL4-mediated expression for visualizing neurons and manipulating their activity. (**a**) The GAL4/UAS system: GAL4 transcription factor binds to the UAS sequence to direct transcription of a gene of interest (*UAS-GFP* and *UAS-dTrpA1* shown). Genomic regulatory elements that function as transcriptional enhancers direct the expression of GAL4 to particular cells in the nervous system. (**b**) Two different example enhancer GAL4 fusion expression patterns visualized by expression of GFP in the brain and ventral nervous system. Samples were co-stained with anti-GFP (green) and an antibody that marks synapses, anti-Bruchpilot (magenta), for visualizing the neuropile. Scale bar, 100 µm. Images are published in the following references (Seeds et al. 2014; Hampel et al. 2015). (**c**) Thermogenetic activation of neurons within each pattern using dTrpA1 can elicit grooming of the head (left, R18C11-GAL4) or wing (right, R53A06-GAL4). Flies were imaged while on a temperature-controlled peltier plate. Arrows point to the legs performing the grooming movements.

of neurons in the *Drosophila* CNS (central nervous system) (**Figure 1a**). This is accomplished by crossing GAL4 lines to flies containing a transgene with UAS fused upstream of the coding sequence. For example, the expression pattern of a GAL4 line is visualized using a UAS-fused gene for a fluorescent protein such as *green fluorescent protein* (*UAS-GFP*, **Figure 1b**). A variety of fluorescent proteins of different colors have been identified and developed for visualizing neurons. Fluorescent proteins can also be fused to proteins that are targeted to different parts of a neuron such as the membrane, nucleus, or synapses. Membrane targeting is useful for visualizing the morphology of an entire neuron, whereas synaptic targeting marks its inputs and outputs. Reagents for visualizing neurons have been previously reviewed in (Venken et al. 2011b; Sivanantharajah and Zhang 2015). In addition to visualizing subsets of neurons, GAL4 can be used to express ion channels for manipulating their activity. This enables experiments to probe the role(s) of specific neurons in behavior (**Figure 1c**, discussed in section 3). Moreover, the activity of neurons can be visualized during behavior or in response to sensory stimuli by expressing genetically encoded indicators of neural activity (discussed in section 8).

3. THERMO- AND OPTOGENETIC NEURAL ACTIVATION

The activity of neurons can be manipulated using a range of different neural activators or inhibitors (Venken et al. 2011b; Inagaki et al. 2014; Klapoetke et al. 2014). We focus our discussion on neural activators that can be induced acutely using temperature or light. These cation channels have the advantage that they can be kept inactive throughout development of the nervous system and then acutely induced in the adult. *Thermogenetic* activation relies on expression cation channels of the *transient receptor potential* (TRP) family whose conductances change significantly in the presence of warmth or cold (dTrpA1 and TRPM8 respectively) (Hamada et al. 2008; Peabody et al. 2009). The type of *optogenetic* activation described in this chapter relies on channelrhodopsins that are induced by particular wavelengths of light, such as blue light-induced Channelrhodopsin-2 (ChR2) and red light-induced CsChrimson or ReaChR (Boyden et al. 2005; Lin et al. 2013b; Inagaki et al. 2014; Klapoetke et al. 2014; Dawydow et al. 2014). The conductance changes of these channels in response to temperature or light allow for the “remote” activation of neurons with tight temporal control in intact and freely behaving flies.

A critical consideration in designing neural activation experiments is the temporal dynamics of the induced channel activities and how these activities impact the firing behavior of neurons. Some experiments call for a long duration of neural activation over the course of many seconds or minutes. Thermogenetic activation via dTrpA1 can drive neuron spiking and affect behavior over long time courses when exposed to constant warmth (Parisky et al. 2008; Pulver et al. 2009; Bernstein et al. 2012; Seeds et al. 2014; Hoopfer et al. 2015). However,

some dTrpA1-activated neurons may show spike frequency adaptation. For example, the continual activation of Gr5a gustatory receptor neurons causes a decay in their spiking within a few seconds, and they are no longer able to elicit a proboscis extension reflex (Inagaki et al. 2014). However, brief thermogenetic activation of Gr5a neurons using an infrared laser can elicit this behavior (Keene and Masek 2012). These results indicate that longer-duration thermogenetic activation may cause adaptation in some types of neurons but not in others. Tonic exposure of channelrhodopsins to light has also been shown to cause spike frequency adaptation, which can be circumvented using pulsed rather than constant photostimulation (Pulver et al. 2009; Inagaki et al. 2014). However, one form of ChR2 called ChR2-XXL has been developed that has a slower time course of channel closure, thus enabling longer-duration neural activation (Dawydow et al. 2014). In the case where experiments call for more precise and shorter-duration neural activation, channelrhodopsins offer temporal precision of neural activation in the millisecond time scale, as opposed to hundreds of milliseconds for thermogenetic channels (Boyden et al. 2005; Bath et al. 2014).

It is important to keep in mind that the temperature or light changes required for thermo- and optogenetic activation can cause unwanted secondary effects on behavior. For example, higher temperatures may increase grooming behavior or reduce mating (Seeds et al. 2014; Vaughan et al. 2014). Similarly, optogenetic activation can introduce confounding behavioral artifacts. For example, flies can see the light pulses, which can elicit startle responses, potentially disrupting ongoing behaviors (Klapoetke et al. 2014). In the case where vision is not required for the behavior, these light-induced behavioral artifacts have been avoided by using blind flies with a homozygous allele of the *norpA* gene (de Vries and Clandinin 2013). Exposing flies to the smallest necessary temperature change or lowest light power necessary for neural activation can minimize these secondary behavioral effects.

Red light-activated channelrhodopsins offer significant advantages over those that are blue light-activated. As mentioned above, flies can see the blue light used to activate ChR2 (Yamaguchi et al. 2010), which may cause confounding behavioral responses to the light. Blue light also penetrates poorly through the fly cuticle, making it difficult to deliver enough light to activate ChR2-expressing neurons in the CNS (Inagaki et al. 2014). Of note, ChR2-XXL is more sensitive to lower blue-light levels than ChR2, which enables blue-light activation of neurons in the CNS (Dawydow et al. 2014). In contrast, CsChrimson and ReaChR are activated by red light that readily penetrates the cuticle and effectively activates neurons in the CNS (Inagaki et al. 2014; Klapoetke et al. 2014). Red light is also less visible to flies, and therefore causes fewer behavioral effects than blue light (Inagaki et al. 2014; Klapoetke et al. 2014). In this respect, CsChrimson has an advantage over ReaChR, in that its peak wavelength sensitivity is further red shifted by about 45 nm (Klapoetke et al. 2014). This longer wavelength is less visible to flies, further reducing the behavioral artifacts associated with optogenetic activation.

4. REARING FLIES FOR THERMO- AND OPTOGENETIC BEHAVIORAL EXPERIMENTS

Specific conditions should be met when rearing flies for use in thermo- or optogenetic experiments. For thermogenetic experiments, flies need to be reared at temperatures that do not activate dTrpA1 or TRPM8 to avoid ectopic neural activation during development (< 25 °C for dTrpA1, > 18 °C for TRPM8) (Hamada et al. 2008; Peabody et al. 2009). Channelrhodopsin-expressing flies should be kept in dark containers and/or their vials wrapped in aluminum foil to prevent neurons from being activated by ambient room light. Flies also need to be fed the channelrhodopsin cofactor, all-*trans*-retinal, because they do not produce appreciable endogenous levels. Only flies expressing the ChR2 mutant ChR2-XXL do not require all-*trans*-retinal food supplementation (Dawydow et al. 2014), possibly because its high affinity for all-*trans*-retinal enables it to access low endogenous concentrations. Flies expressing other channelrhodopsins are typically reared on food supplemented with all-*trans*-retinal at concentrations ranging from 0.1 to 0.5 mM (de Vries and Clandinin 2013; Inagaki et al. 2014; Klapoetke et al. 2014; Reyn et al. 2014; Hoopfer et al. 2015; Ohyama et al. 2015). Flies have been reared on regular food and transferred to all-*trans*-retinal-supplemented food a few days before they were used for experiments. However, we find that flies expressing channelrhodopsin in some neural types that were reared without all-*trans*-retinal supplemented food can show motor defects or lethality (Hampel, Seeds, and Hibbard, unpublished observations). This indicates that feeding flies all-*trans*-retinal throughout development protects some neurons from potentially detrimental effects of channelrhodopsin overexpression.

While temperature and light exposure are specific to the inducers of neural activity, more general factors must be considered when rearing flies for behavioral experiments. First, the housing conditions in which flies are reared can affect their behavior. For example, flies reared in isolation are more aggressive than if they are group housed (Hoffmann 1990; Ueda and Kidokoro 2002; Wang et al. 2008). Housing also determines whether flies have mated, which affects many aspects of social behavior. For example, males with previous mating experience modify their courtship behavior to increase their chance of future mating success (Saleem et al. 2014). Mated females show increased rejection towards males that court them (Connolly and Cook 1973). Therefore,

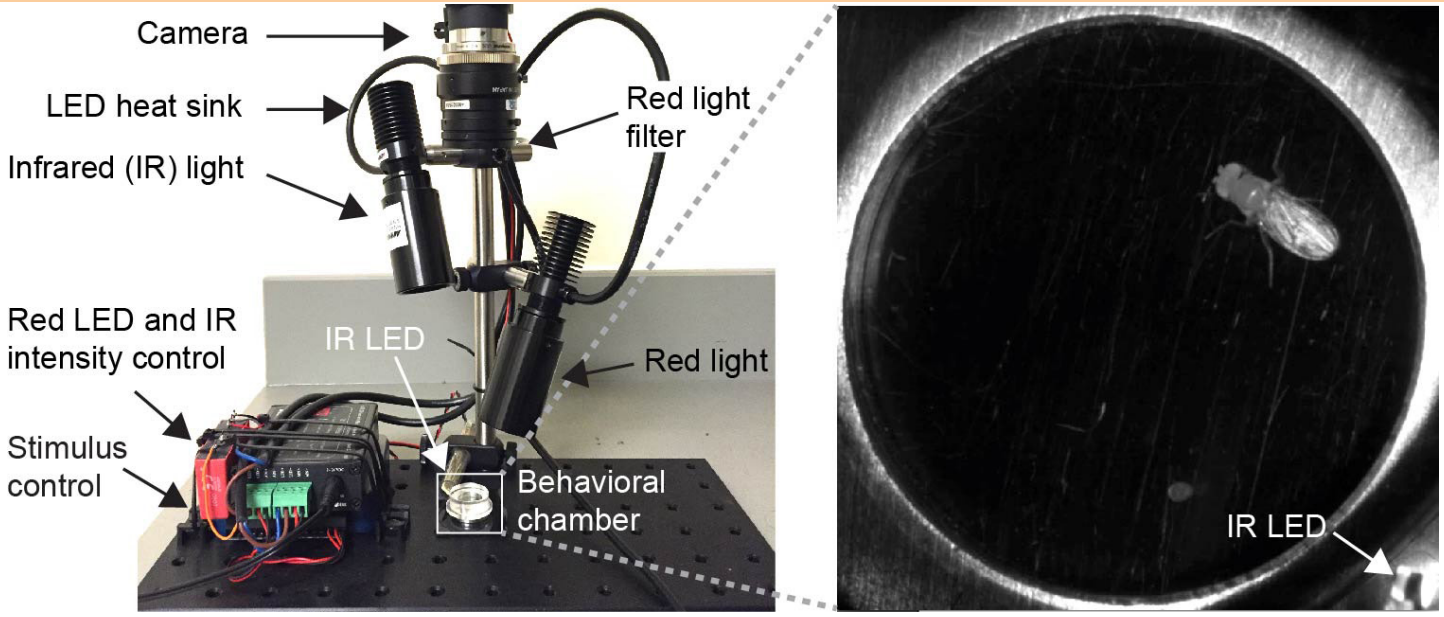

Figure 2. Basic optogenetic apparatus (left). Freely behaving fly in an IR illuminated chamber (right). The IR LED indicates that red light illumination is in progress for optogenetic activation.

it is important to consider whether flies are reared in isolation or in groups when designing experiments. Second, the time of day can affect behaviors such as locomotion, eclosion, feeding, and mating (Allada and Chung 2010). To ensure consistency of behavioral experiments, many groups use circadian-entrained flies that are all tested at the same time of day (Vinayak et al. 2013). Third, hunger influences fly behaviors such as food searching (Root et al. 2011; Gruber et al. 2013), innate avoidance (Bräcker et al. 2013), learning (Krashes et al. 2009), and locomotion (Knoppien et al. 2000; Lee and Park 2004; Albin et al. 2015). Furthermore, the food recipe and number of flies reared on a particular volume of food can affect behavior (Guo et al. 1996). Fourth, flies experience age-dependent changes in behavior such as their propensity to mate or the degradation of their locomotor activity (Grotewiel et al. 2005). Therefore, many experiments use flies that are all the same age. Fifth, flies are often anesthetized using CO2; however, its use in preparing flies for behavioral experiments can have dramatic and long-lasting effects on many different behaviors (Nicolas and Sillans 1989; Seiger and Kink 1993). Long recovery times (i.e., 24 hours) have been recommended to mitigate the effects of CO2 on behavior (Greenspan 2004); however, other experiments indicate that this may not be long enough (Barron 2000). Cold anesthesia can be used in place of CO2, as it is reported to cause less severe behavioral side effects (Barron 2000). One way to circumvent the behavioral effects of anesthesia is to transfer flies using an aspirator (Zaninovich et al. 2013). Sixth, genetic background can affect behavior. For example, different sub-strains of Canton Special (CS), a wild-type strain that is frequently used as a control for behavioral experiments, show remarkably different behavior in the same experimental paradigm (Colomb and Brembs 2014). This shows the importance of controlling for genetic background in behavioral experiments. Backcrosses into a common genetic background will ensure consistency between control and experimental flies.

5. HARDWARE FOR THERMO- AND OPTOGENETIC BEHAVIORAL EXPERIMENTS

Different systems have been designed for thermo- or optogenetic manipulation of neural activity in freely behaving flies. These systems have several common features: 1) hardware for delivering thermo- or optogenetic activation to the flies, 2) chambers that are permissive to the behavior being studied, 3) hardware for recording the behavior, 4) and a design that enables practical experimental implementation (e.g., getting flies in and out, cleaning, etc.). Below we discuss features of behavioral systems that have been designed to address these different issues.

An advantage of thermogenetic systems is that they can be relatively simple to build at low cost. The different systems used for warming or cooling flies include temperature-controlled rooms or chambers, water baths, heating blocks or pads, or peltier plates (Marella et al. 2012; Mann et al. 2013; Flood et al. 2013; Seeds et al. 2014; Sun et al. 2014; Hampel et al. 2015). Chambers that house the flies have been designed with features that facilitate rapid warming such as mesh floors, or heat-conducting floors that are in direct contact with a heating element (Seeds et al. 2014; Harris et al. 2015). Alternatively, the flies and/or chambers can be pre-warmed prior to an experiment (Keleman et al. 2012; Burke et al. 2012; Seeds et al. 2014; Asahina et al. 2014). These simple systems expose the fly's

entire body to temperature induction that typically occurs over a time frame of seconds to minutes. More spatially specific and rapid induction can be achieved by using infrared lasers that produce heat when focused on a particular body part and thereby activate neurons within the targeted area. For example, proboscis extension can be elicited when an infrared laser focused on the mouthparts of an immobilized fly induces dTrpA1 in sugar-sensing gustatory receptor neurons (Keene and Masek 2012). Taking this approach one step further, a computer vision-targeted infrared laser has been devised to precisely warm specific body parts on a freely walking fly, such as the antennae, head, or thorax, to locally activate neurons in different parts of the nervous system (e.g., antennal sensory neurons, brain, or ventral nervous system) (Bath et al. 2014).

Optogenetic systems illuminate flies with particular wavelengths of light. This requires a light source (laser- or LED-based) to illuminate flies from above or below and a means of controlling the intensity and timing of light exposure. Different examples and designs are available for building relatively simple and inexpensive optogenetic systems for whole-fly illumination (Pulver et al. 2011; de Vries and Clandinin 2013; Inagaki et al. 2014; Klapoetke et al. 2014). **Figure 2** shows an example optogenetics system with components commonly found in other systems. Systems have also been developed that direct laser pulses to specific body parts of a moving fly (Wu et al. 2014). Other systems restrict light to particular regions of a chamber allowing flies to "choose" whether the neurons are activated (or not) by moving into (or out of) the illuminated region, or coupling neural activation with particular localized features of a larger chamber, such as odors (Suh et al. 2007; Lin et al. 2013a; Aso et al. 2014b; Klapoetke et al. 2014; Lin et al. 2015).

Flies must be contained for observation in a behavioral chamber for experiments where they need to be freely behaving. It is important to consider that behavior can be greatly affected by different chamber features such as the size, accessibility of the walls and ceiling to the flies, and environment conditions (e.g., presence of food, other flies, etc.). For example, a male fly will spend less time courting a female in a large chamber than in a small chamber wherein the male is always in proximity to the female (Ewing and Ewing 1984; Zawistowski and Richmond 1987; Griffith and Ejima 2009). Chambers with low ceilings restrict flies to the floor for studying non-flight behaviors such as locomotion or grooming (Seeds et al. 2014; Triphan et al. 2016). Of note, chamber heights that are too low restrict the performance of behaviors that involve a raised posture, such as copulation or aggression (Hotta and Benzer 1976; Simon and Dickinson 2010). A systematic study of chamber conditions has revealed that a 3.5 mm chamber height is permissive to most non-flight behaviors performed by *Drosophila melanogaster* (Simon and Dickinson 2010). A chamber with a low ceiling, narrow walls, and a "dead end" has been designed that prevents flies from turning around, thus forcing them to walk backwards (Bidaye et al. 2014). In contrast, chambers can be designed with high ceilings to permit flight (Reynolds and Frye 2007; Straw et al. 2011; Ardekani et al. 2013; van Breugel and Dickinson 2014).

Environmental conditions within chambers also affect the behaviors of flies. For example, a fly may vary its behavior if there are other flies in the chamber, and this may be influenced by whether the flies are male or female. Chambers containing male-female combinations promote courtship, whereas male-male combinations can promote aggression. Furthermore, flies are more likely to show aggressive behavior when female or a food source is present, and the amount of food within the chamber can influence the probability and nature of aggressive behavior (Hoffmann 1987; Chen et al. 2002; Lim et al. 2014). Flies can also leave pheromones or other chemicals behind in chambers, which can affect the social behaviors of new inhabitants (Suh et al. 2004; Wang and Anderson 2010; Lin et al. 2015). Thus, cleaning chambers between experiments will reduce residual pheromones that could influence behavioral outcomes in future experiments (Zawistowski and Richmond 1987). We have also found that static electricity within chambers can cause increased grooming, which may disrupt the performance of a particular behavior of interest. For example, a static electric field might pull the antennae out of place, a condition that elicits antennal grooming (Hampel et al. 2015). Treatment of behavioral chambers with antistatic agents (e.g., UltraSpray, United SCP) resolves this problem. These examples demonstrate the importance of considering environmental conditions in designing chambers for behavioral experiments.

Although the behaviors of flies can be measured without directly observing their movements (Mendes et al. 2013; Itskov et al. 2014; Seeds et al. 2014; Albin et al. 2015; Egnor and Branson 2016), here we focus on video recording and annotating the movements of flies. Chambers should not only allow flies to perform the behavior of interest, but their design must also facilitate the recording of fly movements such that specific features of the movements can be annotated. In particular, the height and width of chambers are important factors to consider with respect to the camera set up. Chamber height determines the vertical space in which flies can move, and different heights require different methods to ensure that flies are visible at all times. For chambers with high ceilings, methods have been developed for tracking flies in three-dimensional space through the use of multiple cameras and computational reconstruction of flight trajectories (Straw et al. 2011; Ardekani et al. 2013; van Breugel and Dickinson 2014). For non-flight behaviors, one camera can be used in conjunction with a low ceiling to restrict flies to the focal length of the camera lens. The chamber width and camera

resolution should be empirically determined to ensure that the recorded video captures features of the fly that are important for observing the behavior being studied. For example, measurements of walking trajectory do not require as high an image resolution or frame rate as observation of the legs performing grooming movements. Thus, chamber size should be large enough for viewing the behavior, but also small enough to enable recording of high-quality video. In designing the behavioral chambers, one has to ensure that flies can be well viewed for behavioral annotation. For example, flies tend to cluster at the periphery of chambers with vertical walls, and will often climb onto the walls and ceilings (Simon and Dickinson 2010). This means that fly bodies will be recorded in multiple orientations (e.g., dorsal and ventral), which can complicate behavioral annotation. Flies can be effectively restricted to the floor of the chamber by treating the walls and ceilings with slippery transparent coatings (e.g., Sigmacote (Sigma-Aldrich), Rain X (SOPUS products), or SurfaSil (Thermo Fisher Scientific)) and designing chambers to have walls that slope at an angle to the floor (Simon and Dickinson 2010; Goda et al. 2014; Hoopfer et al. 2015).

Proper illumination of flies within the chamber is critical for obtaining high-quality video recordings. Special consideration should be given to the type of illumination used for optogenetic experiments because visible light may activate neurons expressing channelrhodopsins. Therefore, many optogenetic recording setups visualize the flies using infrared illumination (Inagaki et al. 2014; Hoopfer et al. 2015; Hampel et al. 2015). Bear in mind that flies are unable to see infrared light and are therefore effectively blind under these conditions, which is a problem for studying any behavior that relies on vision. Furthermore, filters on the camera can prevent the bouts of photostimulation from interfering with visualization of the fly in the video (**Figure 2**). In contrast, recordings of thermogenetic experiments can be made using visible or infrared light. The illumination source can be positioned above or below the behavior chamber. For set ups in which the camera is mounted above the behavior chamber, illumination from above allows for clear visualization of the fly body (Seeds et al. 2014; Hampel et al. 2015), whereas illumination from below produces a high-contrast silhouette (Branson et al. 2009; Simon and Dickinson 2010).

6. ANNOTATING BEHAVIORS ELICITED BY NEURAL MANIPULATIONS

One effective strategy for identifying behaviorally relevant neurons and determining how they control behavior is to use thermo- or optogenetics to manipulate the activity of neurons within the expression patterns of different GAL4 lines (e.g., enhancer-GAL4/*UAS-dTrpA1*). Such experiments require that behavioral performance is readily recognizable so that it can be effectively annotated and quantified. To illustrate techniques and approaches for quantifying the behaviors of freely moving flies in response to neural manipulations, we focus on experiments with neurons whose activation elicits specific behaviors. Although we focus on neural activation, similar experimental approaches that block neural activity have also revealed how specific movements are elicited (Gordon and Scott 2009; Mann et al. 2013). Furthermore, the approaches described here can be applied to the study of other neural circuit functions and behaviors.

Behavior can be quantified through manual annotation of video recordings or by machine vision-based behavioral tracking (Egnor and Branson 2016). Manual annotation involves watching videos of an experiment and annotating behavior “by eye” according to criteria that the researcher uses to define what a particular behavior is. For example, walking behavior could be defined as when a fly moves more than one body length with no pause in leg movements (Seeds et al. 2014). This manual approach can be upgraded with the aid of software that enables marking of behavioral events within the time course of the video such as VCode (Hagedorn et al. 2008), JWatcher (Blumstein and Daniel 2007), or BORIS (Friard and Gamba 2016). Although manual annotation is an effective way to study behavior, it has the drawbacks of being labor-intensive and highly dependent on the judgment of the researcher. This subjective aspect has the potential for inconsistency in how the same video might be annotated by two different researchers, or even by the same researcher at different times. Therefore, machine vision-based behavioral tracking algorithms have been developed to improve annotation consistency, throughput rate, and quantitative analysis of behavior (Anderson and Perona 2014; Egnor and Branson 2016). Such algorithms track multiple statistics of the trajectories of flies and/or their body parts through time (e.g., translational speed, angular speed, or distance to another fly), which can be used to define classifiers for particular behaviors (Dankert et al. 2009; Branson et al. 2009; Robie et al. 2010; Straw et al. 2011; Tsai and Huang 2012; Donelson et al. 2012; Schusterreiter and Grossmann 2013; Ardekani et al. 2013; Bidaye et al. 2014; Dell et al. 2014; Berman et al. 2014). These statistics can also be fed into supervised machine-learning algorithms, such as the *Janelia Automatic Animal Behavior Annotator* (JAABA), where the researcher can train new behavior classifiers by manually annotating a small amount of video based on their own intuition about the behavior (Branson et al. 2009; Kabra et al. 2013). Other methods have been developed that do not presuppose human-assigned behaviors but instead assign behavioral events based on statistically-defined structure of the tracked movements (Berman et al. 2014). These different approaches can provide detailed descriptions of behavior

through time, allowing for the extraction of quantitative statistics such as the behavioral duration, frequency, and probability of transitioning between different behaviors.

One major advantage of flies is their amenability to high-throughput screens of hundreds or thousands of GAL4 lines to identify those that direct expression in behaviorally relevant neurons. Because neural activation can elicit strong behavioral phenotypes that are easy to distinguish from controls, visual observation has been an effective way to screen many GAL4 lines quickly. For example, all flies of a particular GAL4 line will perform the same grooming behavior when the targeted neurons are activated (Flood et al. 2013; Seeds et al. 2014; Hampel et al. 2015). Flood et al. carried out a screen of 835 GAL4 lines to catalog different behaviors that can be independently elicited with thermogenetic neural activation, including grooming, flight, feeding, and egg laying (Flood et al. 2013). This work provides a great example of the different behaviors that can be easily observed with neural activation. Other screens have focused on identifying GAL4 lines that elicit specific behaviors such as grooming, courtship song, feeding, or walking (Philipsborn et al. 2011; Seeds et al. 2014; Bidaye et al. 2014; Hampel et al. 2015; Albin et al. 2015). Tracking algorithms have been used to screen for GAL4 lines that express in neurons involved in aggression and climbing (Asahina et al. 2014; Hoopfer et al. 2015; Triphan et al. 2016), and are anticipated to be increasingly used for identifying lines involved in other behaviors. These different screens have proven to be effective for identifying behaviorally relevant neurons. Furthermore, the unbiased screening of different GAL4 lines has led to the identification of neurons that would not have been anticipated a priori to underlie particular behaviors.

Once GAL4 lines are identified, different experimental approaches can be employed for more detailed studies of how activation of neurons within the GAL4 pattern elicits behavior. Below are examples of approaches that have been taken. Experiments have been designed that take advantage of the precise temporal control of neural activators to assess the dynamics of neural activation on behavior. This has revealed how neurons can elicit persistent behaviors or cause alterations in behavioral states (Inagaki et al. 2014; Bath et al. 2014; Hoopfer et al. 2015; Hampel et al. 2015). Different odorant or food conditions have been used to study their contributions to feeding, attraction, avoidance, social behaviors, and learning (Gao et al. 2013; Aso et al. 2014b; Lim et al. 2014; Ramdya et al. 2014; Albin et al. 2015). Wild type, mutant, and flies with neural manipulations have been used to test different cues of conspecific flies that can affect social behaviors (Wang et al. 2011; Hoopfer et al. 2015). “Fly robots” have been used to decipher how different visual and tactile cues affect social behaviors (Pan et al. 2012; Agrawal et al. 2014; Ramdya et al. 2014; Clowney et al. 2015). Flies have been coated in dust to elicit competing grooming behaviors and study the mechanisms of behavioral choice (Seeds et al. 2014). Notably, many of these different behavioral experiments were done after using approaches described below to refine GAL4 expression patterns to just the behaviorally relevant neurons.

7. INTERSECTIONAL TECHNIQUES FOR IDENTIFYING BEHAVIORALLY RELEVANT NEURONS

In the previous section we described how GAL4 lines have been identified that express in neurons whose activation is sufficient to elicit specific behaviors. However, since GAL4 lines often express in a large population of neurons that includes both the neurons that are able to elicit a behavior of interest and other neurons that are not involved in the behavior, it becomes a challenge to attribute the behavioral effect of the neural manipulation to any particular neurons. But this is exactly what is necessary to understand how the nervous system is organized to produce behavior. How can one effectively subdivide a population of neurons in a GAL4 pattern to isolate just those that elicit the behavior? The wealth of transgenic tools in *Drosophila* offers different solutions to this challenge.

There are numerous examples of two different enhancer trap or enhancer fusion lines that drive expression in a shared neuron that is involved in a behavior of interest and in unshared neurons that that are not involved in the behavior (example shown in **Figure 3a**). Positive intersectional techniques can be employed to reproducibly target expression only in these shared neurons between two enhancer patterns (**Figure 3b**). In contrast, negative intersectional techniques involve suppressing expression at this intersection, effectively reducing the number of neurons within a particular GAL4 expression pattern (Suster et al. 2004; Sivanantharajah and Zhang 2015). We focus on positive intersectional techniques here, as they are the most widely used for reproducibly targeting and manipulating specific neurons. We also discuss the advantages and disadvantages one should consider when deciding on a particular method.

The two main strategies for producing positive intersections are *split GAL4* (spGAL4) and recombinase-based. spGAL4 takes advantage of the fact that GAL4 has two modular domains that are both necessary to drive transcription, a *DNA binding domain* (DBD) and a transcriptional *activation domain* (AD). These two domains can be co-expressed as separate proteins fused to leucine zipper motifs, and the zippers mediate heterodimer formation to reconstitute the transcriptional activity of GAL4 (Luan et al. 2006; Pfeiffer et al. 2010). The trick is that each spGAL4 domain can be expressed under the control of a different enhancer, and only when these two enhancers express in the same neurons will the two domains

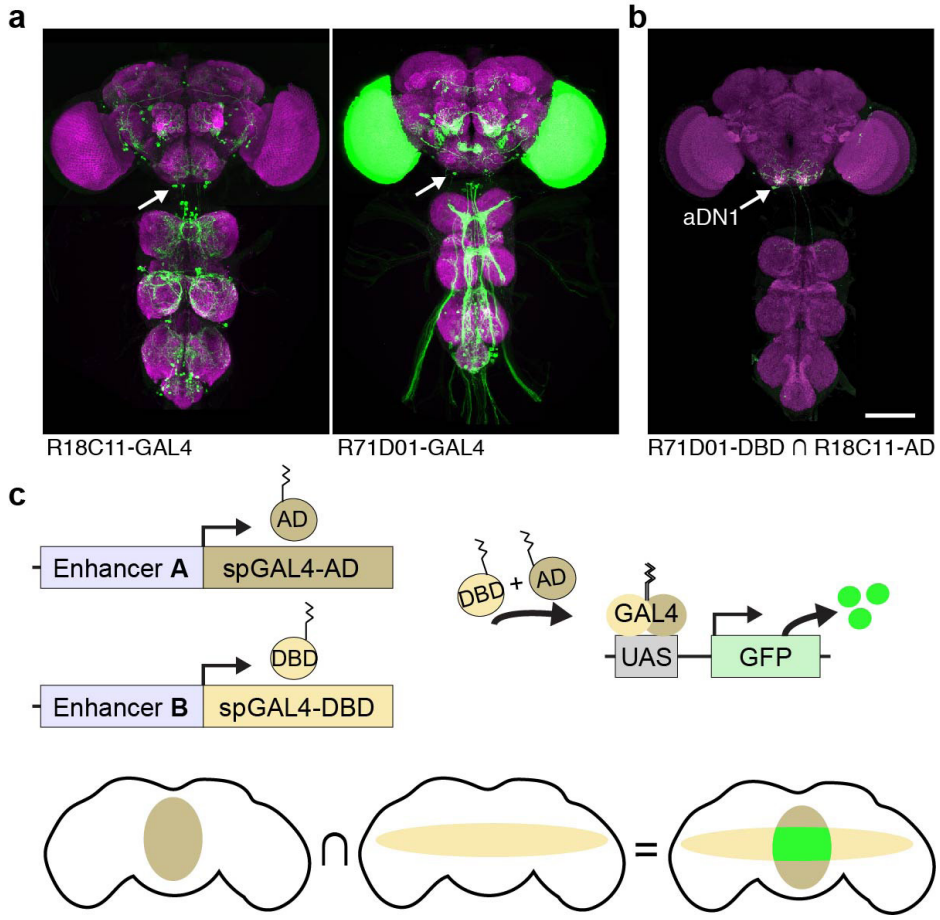

Figure 3. spGAL4 intersectional strategy for identifying behaviorally relevant neurons from broader expression patterns. (**a**) CNS expression patterns of two different GAL4 lines that elicit antennal grooming with neural activation expressing GFP (left and middle). White arrows point to the only common neurons between the two patterns that elicit antennal grooming (Hampel et al. 2015). Scale bar, 100 µm. (**b**) GFP expression pattern of a positive intersection between the two enhancer-driven patterns shown in a (spGAL4 intersectional method used). The intersection targets a single neuron (aDN1, white arrows) whose activation elicits antennal grooming. (**c**) spGAL4 system: enhancer elements drive expression of the GAL4 activation domain (spGAL4-AD) and the GAL4 DNA binding domain (spGAL4-DBD) in different patterns. The neurons labeled in both expression patterns will express both domains, which together reconstitute GAL4 through leucine zippers. Reconstituted GAL4 binds to the UAS sequence and drives expression of a downstream coding sequence, such as GFP as shown in **a**.

heterodimerize to reconstitute active GAL4 (**Figure 3c**). One advantage of spGAL4 is that it drives highly reproducible expression of UAS-controlled transgenes in the intersected neurons, which contrasts with recombinase-based methods (described below). spGAL4 requires only three transgenes: 1) an enhancer-spGAL4-AD, 2) an enhancer-spGAL4-DBD, and 3) a coding sequence of interest expressed under UAS control. A split LexA (spLexA) system has also been developed that takes advantage of the modularity of LexA in the same way as the spGAL4 system (Ting et al. 2011).

A second strategy for producing a positive intersection uses *flippase* (FLP), a recombinase that catalyzes the removal of sequences between two *FLP recognition target sequences* (FRTs). There are several ways in which this activity has been harnessed to enable intersected neurons to express a UAS-controlled transgene. One method is to remove a FRT-flanked stop sequence from a UAS-controlled transgene so that a protein of interest is expressed in cells that are positive for both GAL4 and FLP (e.g., *UAS-FRT-Stop-FRT-GFP*, **Figure 4a**) (Stockinger et al. 2005; Philipsborn et al. 2011; Rezával et al. 2012; Alekseyenko et al. 2013). This method requires only three transgenes: 1) an enhancer-FLP line, 2) an enhancer-GAL4 line, and 3) a coding sequence of interest expressed under UAS-FRT-Stop-FRT control. A collection of enhancer trap FLP lines have been produced that can be used for such positive intersections (Bohm et al. 2010). Alternatively, enhancer-LexA lines can be used to drive expression of FLP (*LexAop-FLP*) in place of an enhancer-FLP line (Shang et al. 2008). Another method for performing FLP-based intersections relies on the strong repression of GAL4 activity by its natural regulator in yeast, GAL80. When GAL80 is ubiquitously expressed in all cells of the fly, it represses GAL4 activity and blocks activation of UAS-controlled transgenes (Lee and Luo 1999). GAL4 activity can

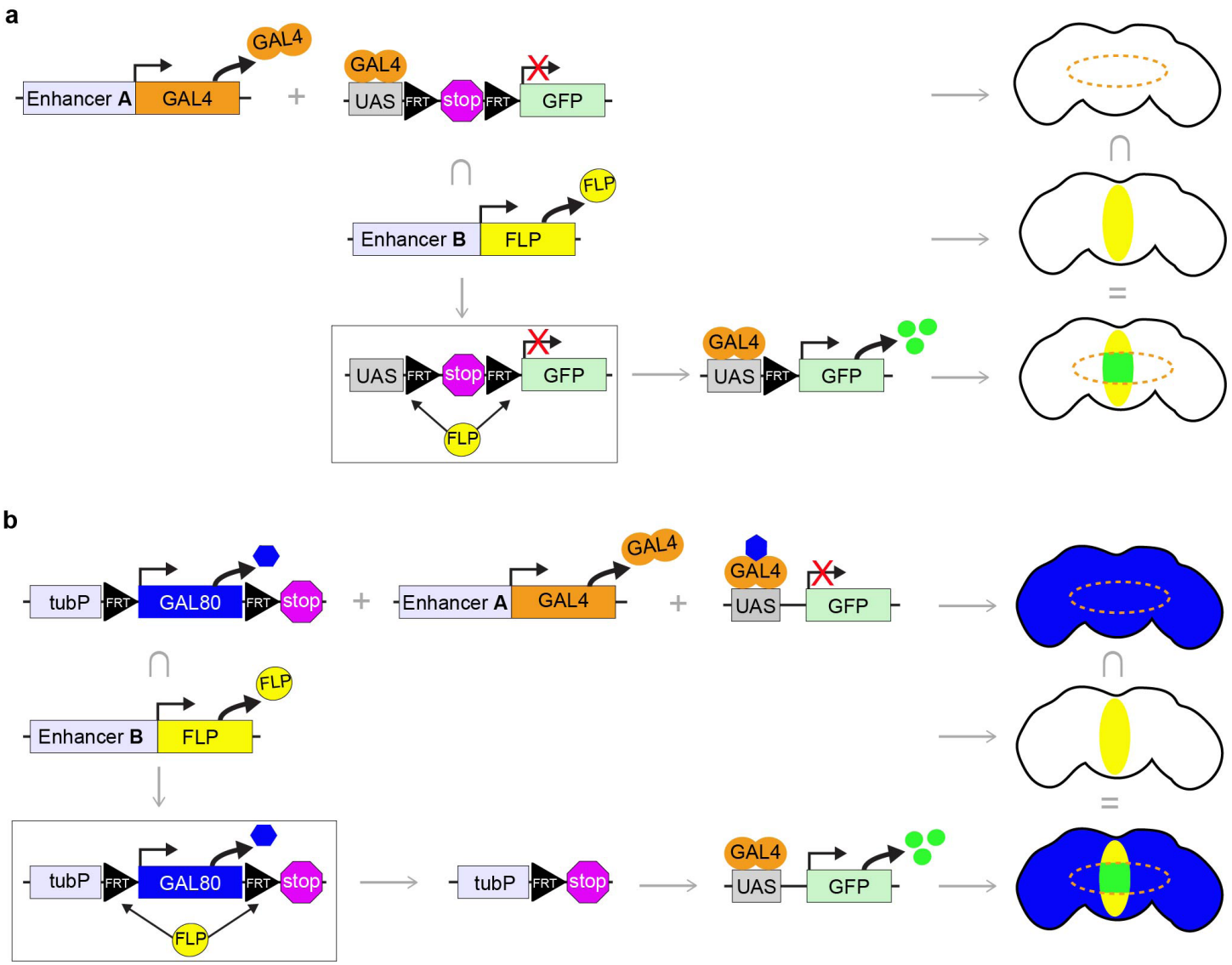

Figure 4. FLP-based intersectional strategies for refining broader expression patterns. (**a**) A flip-in strategy requires three transgenes. Regulatory enhancer elements drive the expression of GAL4 and FLP in partially overlapping cells. The UAS-fused gene of interest (GFP shown) is expressed in the GAL4 pattern, but only translated in FLP expressing cells that can remove a FRT-flanked stop codon. Figure inspired by (Sivanantharajah and Zhang 2015). (**b**) The GAL80 flip-out method requires four transgenes. Regulatory enhancer elements drive the expression of GAL4 and FLP in partially overlapping patterns. Expression of the repressor GAL80, which inhibits GAL4 activity by binding to its transcriptional activation domain, is driven pan-neurally by the tubulin regulatory sequences (tubP). Only cells that express FLP eliminate GAL80 repression to GAL4 through recombination of FRT sites that flank the GAL80 gene. Therefore, only in cells that co-express GAL4 and FLP will transcribe the gene of interest (GFP shown).

be restored in cells in which GAL80 is removed, and this can be achieved when the GAL80 coding sequence is flanked by FRTs and the cells express FLP (**Figure 4b**) (Gordon and Scott 2009; Bohm et al. 2010). Thus, expressing GAL4 and FLP in two partially overlapping enhancer patterns removes GAL80 and allows GAL4 to activate a UAS-controlled transgene in the intersectional cells (Shang et al. 2008; Bohm et al. 2010; Pool et al. 2014). Notably, this method requires four transgenes: 1) a ubiquitously expressed, FRT-flanked GAL80 cassette, 2) an enhancer-FLP line, 3) an enhancer-GAL4 line, and 4) a coding sequence of interest expressed under UAS control.

The FLP-based methods described above require the use of a FLP line with a known pattern of expression (or a collection of enhancer-FLP lines that can be screened), but FLP can also be used in the absence of such lines. An early approach that is labor-intensive but can be essential in some circumstances is to express FLP under heat-shock control in the context of a ubiquitously expressed FRT-flanked GAL80 transgene, to again allow GAL4 activity in cells in which the GAL80 is removed. The key to this approach is to use a regimen of FLP heat-shock induction that results in a low frequency of GAL80 removal from cell to cell so that specific neurons with GAL4 activity are

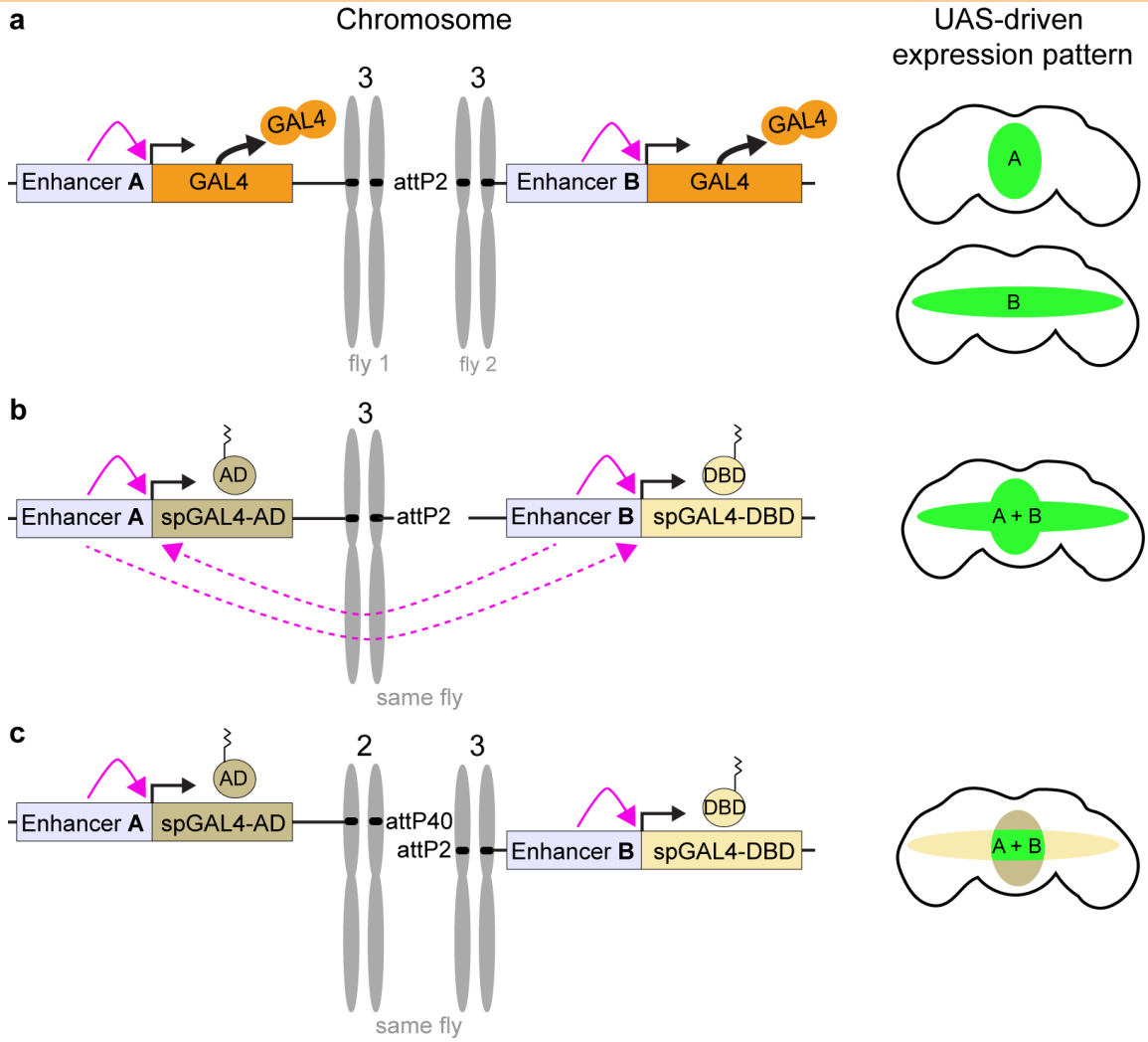

Figure 5. The importance of using appropriate genomic landing sites to avoid transvection. (**a**) Two different enhancer-GAL4 transgenes (Enhancer A-GAL4; Enhancer B-GAL4) inserted in the same genomic landing site (*attP2*) in two different flies drive expression of a gene of interest in independent patterns (green). (**b**) Two different enhancer-transgenes (Enhancer A-spGAL4-AD; Enhancer B-spGAL4-DBD) inserted in the same genomic landing site (*attP2*) within the same fly can each influence the activation of either transgene's minimal promoter in either *cis* (magenta arrows), or *trans* (magenta dashed arrows), or in *cis* and *trans* combined. One possible outcome of the latter scenario is shown: Enhancers A and B activate transcription of both spGAL4-AD and -DBD to reconstitute GAL4 in both enhancer-driven expression patterns (magenta arrows and dashed arrows). See the following reference for more detailed discussion of possible outcomes of transvection (Mellert and Truman 2012). (**c**) Two different enhancer transgenes (Enhancer A-spGAL4-AD; Enhancer B-spGAL4-DBD) inserted in distinct genomic landing sites (attP40 and attP2) in the same fly drive transcription of the spGAL4-AD and spGAL4-DBD in the corresponding pattern of each respective enhancer. This leads to reconstitution of GAL4 only in cells that are shared between both enhancer expression patterns.

stochastic from one fly to the next (Gordon and Scott 2009; Marella et al. 2012; Mann et al. 2013). These flies must also have transgenes to manipulate neuronal activity (e.g., *UAS-dTrpA1*) and to report the activity of GAL4 in the manipulated neurons (e.g., *UAS-GFP*). Each fly is then tested for whether a neural manipulation affects the behavior of interest, and is subsequently dissected so that its CNS can be imaged to identify which neurons had GAL4 activity. By correlating the behavioral output of a large number of flies with the anatomical location of the neurons that were manipulated, behaviorally relevant neurons from a broader GAL4 expression pattern are identified based on whether they show positive expression and affect the behavior of interest.

The intersectional method employed may depend on the type of GAL4 line that was used to manipulate the neurons of interest (e.g., enhancer trap or enhancer fusion). FLP-based methods have the advantage of being highly versatile in this respect because they can be implemented with any GAL4 line (Shang et al. 2008; Sivanantharajah and Zhang 2015). An alternate way to implement positive intersections using any GAL4 line of interest is to drive

expression of the LexA-DBD under UAS control (*UAS-LexA-DBD*) (Ting et al. 2011). Because spLexA was constructed with leucine zippers and works with the same activation domains that are used for spGAL4, it can be used in conjunction with available AD fly lines that have been constructed for use with spGAL4 (Luan et al. 2006; Ting et al. 2011).

In contrast to the FLP- and spLexA-based methods described above, spGAL4 requires the construction of new fly lines that express spGAL4 halves in the same neurons as the original GAL4 lines of interest. In the case where the GAL4 line is an enhancer fusion, the genomic enhancer is fused to either spGAL4-AD or spGAL4-DBD (Pfeiffer et al. 2008; Pfeiffer et al. 2010). The enhancer-spGAL4 fusion construct is then inserted into an *attP* site in the genome using *phiC31* integrase. Such constructs should generally be inserted into the same *attP* site used for the original GAL4 line, because this increases the likelihood of recapitulating that expression pattern (Pfeiffer et al. 2010). Unfortunately, inserting both spGAL4 halves into the same *attP* site can lead to transvection across paired chromosomes, causing each of the spGAL4 halves to be partially or fully expressed in the enhancer pattern of the opposite half (Mellert and Truman 2012). This can undermine the intersectional approach by causing the reconstituted spGAL4 to have a broader expression pattern than anticipated (**Figure 5a,b**). Because of this and other potential problems conferred by transvection, it is advisable to only use one transgene per *attP* site in a particular fly genotype (Mellert and Truman 2012). One *attP* combination that has been successfully used for spGAL4-AD and DBD insertions is *attP2* and *attP40* (Aso et al. 2014a; Hampel et al. 2015) (**Figure 5c**); however, the quality of this site pair can vary depending on the enhancer fusion used. Importantly, enhancer fusions inserted at *attP* locations other than where they were originally characterized can yield different expression patterns because of genomic positional effects (Pfeiffer et al. 2010). Therefore, if insertion of a spGAL4-half at one *attP* site does not recapitulate the expected expression pattern, the spGAL4-half may need to be inserted into a different site. A number of different *attP* integration sites have been generated to facilitate this (Groth et al. 2004; Venken et al. 2006; Bischof et al. 2007; Markstein et al. 2008; Ni et al. 2009; Knapp et al. 2015).

spGAL4 halves can be swapped with GAL4 in enhancer trap lines as well. One particular enhancer trap collection called the *integrase swappable in vivo targeting element* (InSITE) system has been designed specifically for such a purpose. InSITE lines are constructed such that GAL4 can be replaced with other genes such as spGAL4 halves via recombinase-mediated cassette exchange, thus enabling its expression in the same pattern (Gohl et al. 2011). This system is advantageous because spGAL4 flies can be generated genetically through simple fly crosses, in contrast to the enhancer fusions that require the generation of new transgenic flies through embryo injections. The disadvantage of InSITE is that spGAL4 replacements can only be done using InSITE enhancer trap lines. Another approach has been developed for use with a collection of *Minos-Mediated Integration Cassette* (MiMIC) transposons, where GAL4, spGAL4, or any other coding sequence can be expressed in the pattern of a native gene of interest (Venken et al. 2011a; Diao et al. 2015). Finally, an exciting new technique called *homology assisted CRISPR Knock-in* (HACK) offers the possibility of replacing GAL4 from any existing enhancer trap or enhancer fusion line with a coding sequence of interest by performing two simple crosses (Lin and Potter 2016). Although we are not aware of a case where HACK has been used to replace GAL4 with spGAL4 halves, we anticipate that this technique will become a method of choice for performing such swaps.

Positive intersections can be implemented in a couple of different ways depending the circumstance of the particular experiment. In the case where two identified enhancer patterns are suspected to share a common behaviorally relevant neuron, an intersection can be performed with these two patterns (e.g., enhancer-1-spGAL4-DBD and enhancer-2-spGAL4-AD). In a different scenario, a single enhancer pattern is identified that contains a neuron of interest, but there is no known second enhancer pattern that could be used to produce a positive intersection. In this case, the enhancer-spGAL4 of interest (e.g., enhancer-1-spGAL4-DBD) can be screened against a library of spGAL4-ADs to identify combinations that target the neuron(s) of interest (Luan et al. 2006; Luan et al. 2012). In the case where a FLP-based method is being used, a GAL4 line that expresses in a neuron of interest is screened against a FLP enhancer trap library (Bohm et al. 2010; Rezával et al. 2012; Pool et al. 2014). Although it is feasible that intersectional approaches can refine expression patterns to a single neuron (**Figure 3a,b**), in many cases the neurons of interest are not the only ones targeted by a particular spGAL4 or FLP/FRT pattern. This complicates the interpretation as to which neuron in the pattern is responsible for the phenotype. Therefore, it is optimal to identify multiple intersectional combinations that target the same neuron of interest but differ in their “contaminating” neurons. This strengthens an argument that the behavioral phenotypes caused by manipulating neural activity are due to the particular shared neuron (Tuthill et al. 2013; Hampel et al. 2015).

8. ASSESSING FUNCTIONAL CONNECTIVITY AMONG NEURONS

The approaches described above can reveal individual neurons that elicit stereotyped behaviors when activated. In some cases, different neurons have been identified that elicit the same behavior. For example, at least four different

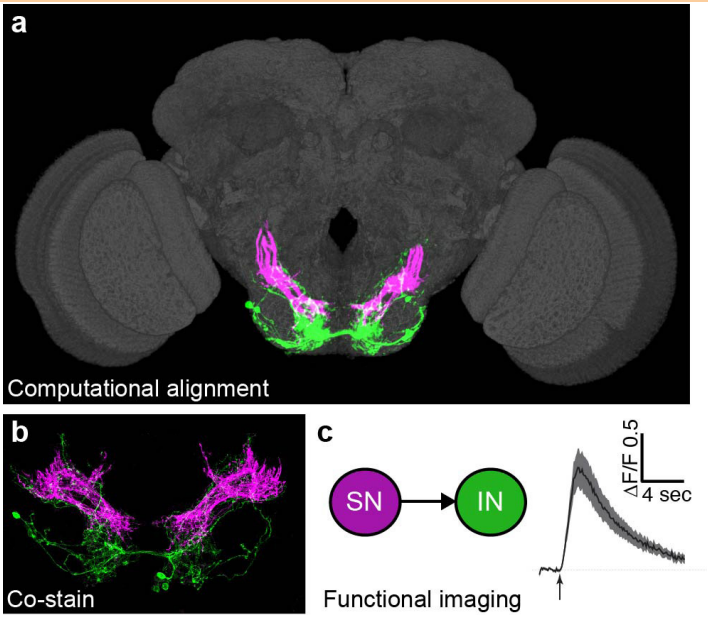

Figure 6. Approaches to assess neural proximity. (**a**) Computationally aligned neurons (green and magenta) from two individual brains to a standard brain (CTMK and FluoRender software). (**b**) Antibody co-stain using GFP and RFP (green and magenta respectively) that expressed in two neurons within the same brain (same neurons as shown in a). (**c**) Schematic for testing connectivity between neurons. Antennal mechanosensory neurons (SN) expressing CsChrimson are activated with red light and simultaneously changes of fluorescence are measured in putative downstream interneurons (IN) expressing GCaMP using two photon calcium imaging (response trace on right). Some of this data is published in the following reference (Hampel et al. 2015).

neuronal types can elicit grooming of the antennae with neural activation (Hampel et al. 2015). This raises the question of whether they may be part of the same functionally connected circuit. Below we describe methods for piecing together how such neurons are organized and function as circuits. This involves analyzing the proximity between neurons and determining their functional connectivity. In other cases, individual neurons have been isolated, but the identities of other connected neurons remain unclear. We discuss techniques that can be employed to identify such missing components of behavioral circuits. This section is intended to provide a brief overview of different circuit-mapping techniques with references for further reading on particular topics of interest.

One way to assess whether different neurons interact is to use light microscopy data to determine whether their neurites are in close proximity. Ideally this is achieved by examining the neurons in the same brain. Such neuroanatomical mapping is difficult because it is technically challenging to independently visualize more than two neurons in the same fly (discussed more below). Computational methods can be employed to align confocal Z-stack images of different neurons to visualize their gross anatomical relationships. That is, different neurons are registered to a "standard" brain and then displayed within the same 3-dimensional space. One widely used free software for registering brain confocal stacks is the *computational morphometry toolKit* (CMTK) (https://www.nitrc.org/projects/cmtk/), and others are available such as BrainAligner (Peng et al. 2011). One problem that arises is that the 3-dimensional renderings of neurons become increasingly messy as more are added, especially when each confocal stack contains multiple neurons. Therefore, additional tools such as the free software FluoRender enable the display of selected neurons from these alignments for the clearest possible visualization of the putative circuit (Wan et al. 2012). Another such free software package is Volocity (by PerkinElmer), and commercially available programs include Amira (by FEI) and Imaris (by Bitplane). These different tools can be used to examine the spatial relationships between neurons to gain a first approximation about whether their neurites are in close enough proximity to form synaptic connections (**Figure 6a**). Additionally, computational alignment provides a means to display many neurons in the same brain for figures or movies to visualize multi-neuron structures (Aso et al. 2014a; Hampel et al. 2015). Furthermore, the computational alignment of individual neurons to a standard brain affords the possibility to use a program called NBLAST to perform a number of different useful searches (Costa et al. 2016). For example, NBLAST can identify neurons having the same or overlapping morphology as a particular neuron of interest, or to identify GAL4 lines that express in a particular neuron.

The proximity between two neurons can be examined in the same brain using different binary expression systems. For example, one neuron can express GFP using GAL4/UAS (GAL4 or a spGAL4 combination), whereas another neuron can express a different colored fluorescent protein using another binary system such as the LexA/LexAop or QF/QUAS (**Figure 6b**). It is possible that three binary expression systems could be employed to independently express in three different neurons, however it would be challenging to get the minimum of six required transgenes into the same fly. Generating fly lines that have multiple transgenes inserted as an array into the same genomic location would be one way to do such an experiment (Knapp et al. 2015). Another technique that provides information about the proximity between two neurons is called *GFP reconstitution across synaptic partners* (GRASP) (Feinberg et al. 2008; Gordon and Scott 2009). This technique can be used to determine if two neurons had membrane contacts during development and/or in the developed brain.

The ability to independently target expression in two neurons whose neurites are in close proximity enables testing whether activation of the putative upstream neuron excites or suppresses activity of the downstream neuron.

The activity of neurons can be assessed through the use of different genetically encoded indicators of neural activity (Grienberger and Konnerth 2012; Masuyama et al. 2012; Broussard et al. 2014; Fosque et al. 2015; Gao et al. 2015; Dana et al. 2016). For example, *genetically encoded calcium indicators* (GECIs) increase their fluorescence as calcium levels rise in active neurons. One example of how to assess functional connectivity is to use LexA/LexAop to express the neural activator CsChrimson in a putative upstream neuron while GAL4/UAS is used to express the GECI GCaMP6 in the putative downstream neuron (Chen et al. 2013; Klapoetke et al. 2014). The upstream neuron is then optogenetically activated while the downstream neuron expressing GCaMP6 is monitored for changes in fluorescence (**Figure 6c**). These experiments can be done with the nervous system kept in the body or using a dissected brain (Kallman et al. 2015; Zhou et al. 2015; Clowney et al. 2015; Hampel et al. 2015; Cohn et al. 2015; Shirangi et al. 2016). The functional connectivity between neurons that is demonstrated by measuring GECI responses cannot indicate whether the connections are direct, and it is always possible that intermediate neurons are involved. To distinguish between the possibilities of monosynaptic versus polysynaptic connectivity, as well as to characterize the physiological properties of the functional connection, electrophysiology experiments are required (Gruntman and Turner 2013; Kohl et al. 2013; Fişek and Wilson 2014; Tuthill and Wilson 2016).

Whereas activation of specific neurons enables testing whether they are sufficient to elicit a particular behavior, blocking the activity of these neurons enables testing whether they are necessary for performance of the behavior. Different reagents that are available for inhibiting neurons are discussed in the following references (Venken et al. 2011b; Sivanantharajah and Zhang 2015). Taking this approach one step further, two binary expression systems can be used to independently manipulate different neurons and test necessity and sufficiency for behavior with respect to their functional connectivity. For example, sensory neurons that elicit antennal grooming can be activated while simultaneously blocking synaptic transmission of putative downstream interneurons that express tetanus toxin light chain (Hampel et al. 2015). The inhibition of some interneurons reduced or abolished antennal grooming, showing that those neurons are normally necessary to elicit the full antennal grooming response to sensory neuron activation. Assessing the relative roles of different neurons in this way can reveal how they are organized into a neural circuit that underlies a particular behavior.

Individual neurons involved in a particular behavior are often identified without knowing the identities of their postsynaptic partners. One strategy to identify such downstream circuitry uses enhanced versions of *photoactivatable GFP* (PA-GFP), which photoconvert from a low-fluorescence to a high-fluorescence form under two-photon illumination (Patterson and Lippincott-Schwartz 2002; Ruta et al. 2010). A small region of axonal arborizations of the identified neuron is subjected to two-photon illumination with the expectation that the locally associated dendrites of its postsynaptic partner, which expresses PA-GFP, is labeled by photoconverted PA-GFP that also diffuses throughout the neuronal processes and cell body (Datta et al. 2008; Ruta et al. 2010; Fişek and Wilson 2014; Clowney et al. 2015). This approach has been used to identify second, third, and fourth order neurons in a pheromone circuit (Ruta et al. 2010), demonstrating the impressive utility of PA-GFP-based approaches for circuit mapping. Furthermore, functional connectivity among the neurons can be assessed as the putative upstream neurons are activated while testing for responses in the PA-GFP-identified downstream neurons (Ruta et al. 2010; Fişek and Wilson 2014; Clowney et al. 2015).

9. COMPLEMENTARY APPROACHES

We close by highlighting approaches for interrogating neural circuit function that complement those discussed above. First, are large-scale efforts to identify and target individual neurons in specific brain regions rather than identifying neurons using the above described behavioral screening methods. For example, spGAL4 combinations have been identified that express in the different neurons innervating the fly mushroom body (Aso et al. 2014a) and visual system (Tuthill et al. 2013; Nern et al. 2015). The activity of each neuron type was then systematically manipulated to assess their contributions to different behaviors (Tuthill et al. 2013; Aso et al. 2014b). Such approaches facilitate the systematic dissection of neurons that make up specific brain regions, and the assessment of their contributions to behavior. Second, serial section *electron microscopy* (EM) images of brain volumes are increasingly being reconstructed, which can reveal neural circuit connectivity to near completion (Saalfeld et al. 2009; Takemura et al. 2013; Ohyama et al. 2015; Berck et al. 2016; Schneider-Mizell et al. 2016). Importantly, this structural information can be used to pose hypotheses about circuit function that can then be tested in conjunction with genetic approaches described above for targeting and manipulating different circuit components (Ohyama et al. 2015). The complementary use of genetic tools and EM microscopy has great potential for studies of circuit connectivity and function. Finally, different approaches have been developed for recording the activity of specific neurons in behaving flies. This includes the use of GECIs or whole-cell patch clamp recordings as flies are either tethered or freely moving in a chamber (Maimon et al. 2010; Seelig et al. 2010; Haberkern and Jayaraman 2016; Grover et al. 2016). In this chapter we have described a set of tools and techniques that collectively enable the

interrogation of neural circuit function at different levels of inquiry, from individual neurons, to neural circuits, to behavior.

10. ACKNOWLEDGEMENTS

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