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REVIEW

Recent Advances in the Genetic Dissection of Neural Circuits in *Drosophila*

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Abstract Nervous systems endow animals with cognition and behavior. To understand how nervous systems control behavior, neural circuits mediating distinct functions need to be identified and characterized. With superior genetic manipulability, *Drosophila* is a model organism at the leading edge of neural circuit analysis. We briefly introduce the state-of-the-art genetic tools that permit precise labeling of neurons and their interconnectivity and investigating what is happening in the brain of a behaving animal and manipulating neurons to determine how behaviors are affected. Brain-wide wiring diagrams, created by light and electron microscopy, bring neural circuit analysis to a new level and scale. Studies enabled by these tools advances our understanding of the nervous system in relation to cognition and behavior.

Keywords *Drosophila* · Neural circuit · Neuroanatomy · Neurogenetics · Systems neuroscience

The nervous system endows an animal with cognition and behavior. To understand how cognitive processes and behavior are coordinated and performed, neural circuits mediating distinct functions need to be identified and characterized. With a nervous system of intermediate complexity, a rich behavioral repertoire, and genetic tractability, *Drosophila* is a model organism at the leading edge of neural circuit analysis. In this review, we give a brief introduction to the state-of-the-art genetic tools commonly used by Drosophilists to precisely target discrete neuronal populations, to reveal neuronal morphology and connectivity, and to monitor and manipulate neuronal activity. We discuss what brain wide-wiring diagrams created by light and electron microscopy might bring to neural circuit analysis. Modeling and evolutionary comparison of neural circuits could aid the understanding of neural circuits, in addition to the genetic dissection of the molecular and neuronal architectures for cognition and behavior.

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Genetic Targeting of Neuron Populations

Genetic dissection of neural circuits in *Drosophila* involves delineating neuronal subtypes, neuronal interconnectivity and signaling, as well as functions in cognition and behavior. Unlike conventional dissection based solely on anatomical knowledge, genetic dissection relies on genetic tools that allow the expression of effector genes in subsets of neurons, thus affording the targeted labeling and/or manipulation of identified neurons. In flies, these tools are mostly based on binary expression systems. Recently, the binary expression systems have been further improved to allow more precise labeling of neurons, and easier repurposing of drivers.

Genetically Accessing Neurons via Binary Expression Systems

Using the transposable *P* element to introduce transgenes into *Drosophila* has revolutionized the genetic study of

Drosophila [1]. The *P* element-mediated transformation with the yeast transcription activator GAL4 revealed that the expression of the foreign gene *GAL4* can be controlled by local genomic elements, such as enhancers [2]. Introducing both GAL4 and its binding sequence *UAS* (*Upstream Activating Sequence*) into flies led to the invention of the most used binary expression system, GAL4/*UAS* [3], in which the transgene following *UAS* was expressed only in the cells expressing GAL4, thus allowing targeted expression of effector genes simply by crossing a GAL4 line with a *UAS* line (Fig. 1A).

GAL4 lines were previously constructed with the *GAL4* coding sequence following a minimal promotor (enhancer trap) [4–6] or a defined promoter (enhancer fusion) [3] integrated into the genome with the *P* element at random loci. Expression of the enhancer trap GAL4 depends on the local cis-regulatory sequences such as enhancers. Recently, the community has been increasingly drawn to newer collections of driver lines such as the Janelia FlyLight [7, 8] and Vienna Tiles (VT) lines [9] that are generated with a modular design: optimized vectors, defined cis-regulatory sequences, and characterized genomic loci for PhiC31-mediated targeted integration. The expression patterns of most FlyLight and VT lines are visualized and searchable, which makes it easier to find useful drivers expressing in brain regions of interest and greatly facilitates the dissection of neural circuits.

Binary expression systems other than GAL4/*UAS* are also frequently used to allow independent expression of different transgenes in different neuron populations. The LexA/*LexAop* system introduces both bacterial transcription repressor LexA's DNA binding domain fusing the viral transcription factor VP16 acidic activation domain (VP16AD) or the GAL4 trans-activating domain (GAD) or human p65 activation domains (p65AD) and LexA binding sequence *LexAop* into flies [8, 10]. The QF/*QUAS* system borrows the transcriptional activator QF and its binding sequence *QUAS* from *Neurospora crassa* [11, 12].

Spatiotemporally Refining Genetic Targeting

There are about 100, 000 neurons in the fly nervous system. Only in rare cases do drivers label sparse or even a single pair of neurons. Genetic strategies have been developed to narrow down the expression of drivers and make it possible to study brain functions at single-cell resolution.

One strategy takes advantage a repressor of the transcription activator. GAL80 binds to the GAD and inhibits GAL4 action on *UAS*, thus independently targeting GAL4 and GAL80 allows expression of the *UAS*-transgene in neurons expressing GAL4 but not GAL80, achieving a narrowed set-difference expression pattern [13] (Fig. 1B). *elav-GAL80* [14] represses the expression of GAL4 pan-neuronally and is often used to ascertain that GAL4-driven

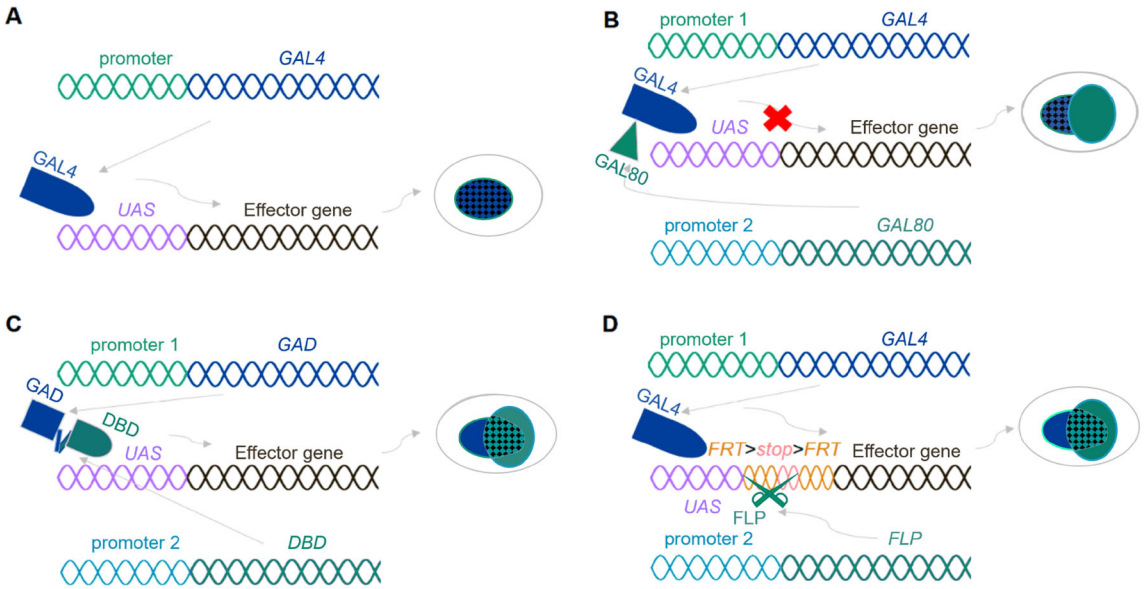

Fig. 1 Genetic targeting of transgene expression in *Drosophila*. **A** GAL4/*UAS* binary expression system. Expression of the transgene (indicated by dotted region) following the *UAS* is dictated by GAL4, whose expression is under the control of a regulatory sequence such as a promoter. **B** GAL4/*UAS*/GAL80 allow a set-difference expression of transgene. The *UAS*-transgene is expressed in cells expressing GAL4 controlled by promoter 1 but not GAL80 (which binds to GAL4 and inhibits its action on *UAS*) controlled by promoter 2. **C** split-GAL4 allows an intersectional expression of transgene. The transgene following the *UAS* is expressed in cells expressing both split-GAL4 parts, the GAD and DBD, which are under the control of promoter 1 and promoter 2, respectively. **D** FLP/*FRT* adds another layer of control over a binary expression system such as GAL4/*UAS*. The *UAS*-transgene is expressed in cells expressing both GAL4 and FLP, which stochastically excise the *FRT-stop* cassette to allow the effector gene to be expressed.

expression of the effector, in neural tissue indeed, is responsible for the observed effect; *tsh-GAL80* [15] can mask the expression of GAL4 in most ventral nerve cord (VNC) neurons, and is useful for differentiating neurons in the brain from those in the VNC in some cases. The temperature-sensitive and ubiquitously expressed GAL80 (*tubP-GAL80^{ts}*) is used to achieve temporal control over transgene expression by transferring the restrictive 18°C to permissive 30°C to relieve GAL8 repression, *e.g.*, to have transgene expressed only during the adult stage to circumvent developmental effects [3, 16]. In the QF/QS/*QUAS* tertiary expression system, suppression of QF by its repressor QS can be relieved by feeding quinic acid to flies, allowing spatiotemporal control of the transgene expression [11].

Another strategy takes advantage of reconstitution of a functional transcription factor from its split parts. Split-GAL4 fuses the complementary DNA-binding domain (DBD) and transcription-activation domain GAD to each of a pair of heterodimerizing leucine zippers (Zip$^-$ and Zip$^+$), to allow them to reconstitute functional GAL4. Transgenes under *UAS* control are expressed only in cells expressing both DBD and GAD (and thus reconstituted GAL4). Targeting the DBD and GAD independently produces intersectional expression of two drivers [17] (Fig. 1C). The orthogonal modular design of constructs for FlyLight facilitates the generation of split-GAL4 lines with the same regulatory sequences and thus mostly reproducible expression patterns of GAL4 lines. Recently, as more split-GAL4 lines have been characterized [18], systematic dissection of neuronal subtypes in various complex brain areas such as the lamina [19], lobula complex [20], mushroom bodies output neurons [21], and the sexually dimorphic P1 neurons [22] has become feasible. A split-LexA system that independently targets the DNA-binding LexA and trans-activating VP16AD has also been developed to allow intersectional expression [23].

Combining split-GAL4 and GAL80 can further narrow down the expression of transgenes. However, while GAL80 represses GAD-mediated transcription, it does not effectively inhibit split-GAL4 that uses p65AD or VP16AD as transcriptional activators. Killer Zipper (KZip$^+$), a dominant-negative repressor of split-GAL4 that disrupts the formation of the split-GAL4 heterodimer, has been introduced to allow targeted expression in neurons expressing both parts of split-GAL4 but not GAL80 [24].

Stochastic Labeling by Site-Specific Recombination

Recombination-mediated excision of functional cassettes in the transgene adds a separate layer of control over the binary expression systems. The yeast recombinase Flippase (FLP) catalyzes mitotic recombination between the *FRT* (*flippase recognition target*) sequences on homologous chromosomal loci. FLP/*FRT* is introduced into *Drosophila* [25, 26] for the induction of stochastic labeling by inserting FLP-excisable *FRT* cassettes into effector constructs such as the *UAS*-transgene and targeted recombination. Directed flanking *FRT*s can generate "flip out" excision of the flanked cassette while oppositely-directed flanking *FRT*s can generate inversion of the flanked cassette. An *FRT*-flanked cassette with a transcriptional terminator (*FRT-stop*) inserted after *UAS* and just before the transgene is often used to "flip in" the transgene in neurons with FLP, which excises the *FRT-stop* cassette, and narrows down expression of the transgene in neurons intersectionally defined by both independently-targeted GAL4 and FLP [27] (Fig. 1D). For example, FLP under control of the brain-restricted *Otd* promoter *Otd-FLP* has been used in combination either with *tubP > GAL80 >* (> indicates *FRT* site) or with *UAS > stop >* genes allows effector transgenes to be expressed in the brain [28–30]. Heat-shock-inducible FLP (*hs-FLP*) allows control over the timing and volume of recombination events which eventually results in varying degrees of labeling. Other site-specific recombination systems have also been introduced into *Drosophila*, such as Cre/*LoxP* from bacteriophage [31], KD/*KDRT* (*KD-recombinase Target Recognition*) from *Kluyveromyces drosophilarum*, R/*RSRT* from *Zygosaccharomyces rouxii*, and B3/*B3RT* (*B3 recombinase Target Recognition*) from *Zygosaccharomyces bisporus* [32].

MARCM (Mosaic Analysis with a Repressible Cell Marker) uses hs-FLP/*FRT* and the ubiquitously-expressed GAL80 to induce mosaic cells with transgene expression in targeted tissue without transgene expression [13]. MARCM affords sparse expression of reporters or effectors in a targeted neuron population, and allows study of the morphology and development as well as the behavioral functions of neural circuits at single-cell resolution [33].

FINGR (Flippase-induced Intersectional GAL80/GAL4 Repression) combines the *FRT-stop* with GAL80 to allow *FRT*-dependent "flip-in" of GAL80 to repress GAL4 expression with a collection of enhancer-trap Flippases [34] and allows more restricted targeting in neurons with GAL4 but without FLP expression.

Repurposing GAL4 to GAL80, Split-GAL4, and QF2

Tasks such as revealing the relationship between two neuronal populations entail simultaneous targeting of each by independent binary systems. As most available drivers are GAL4 lines, other binary drivers such as LexA and QF that reproduce the GAL4 expression are needed. Therefore, easy methods to repurpose GAL4 lines to other drivers are of great interest to the community. Previous attempts such

as InSITE (Integrase Swappable *in vivo* Targeting Element) build platforms to allow *GAL4* be replaced by *LexA*, *QF*, or *GAL80* by crosses. However, the system can only replace specially-constructed IT-GAL4s but cannot replace existing GAL4s [35]. Recently, the HACK (Homology Assisted CRISPR Knock-in) method was invented to replace *GAL4* in the genome with split-*GAL4*, *GAL80* [36], and *QF2* [37] by harnessing gene conversion that uses the clustered regularly-interspaced palindromic repeats (CRISPR)/Cas9 system to induce double-strand DNA breaks in the *GAL4* sequence and then homology-directed repair to unidirectionally transfer a donor homologous sequence. The FlyLight project allows relatively convenient generation of LexA or QF2 or split-GAL4 lines with largely reproducible expression as GAL4 lines by using the same regulatory sequences [38]. However, the HACK method can convert GAL4 lines only by crosses without plasmid construct and injection, thus is particularly useful to repurpose enhancer trap GAL4 lines whose expression patterns are difficult to reproduce since regulatory sequences are difficult to define.

Revealing Neuronal Morphology by the Expression Pattern of Reporters

Visualizing Neuronal Morphology with Fluorescent Proteins

The expression patterns of drivers can be visualized by light microscopy with the targeted expression of reporters such as fluorescent proteins. Besides the most commonly used green fluorescent protein (GFP) [39], fluorescent proteins with different spectral properties such as red fluorescent proteins (such as tdTomato [40], mCherry [41], and dsRed [42, 43]) and GFP-derived yellow fluorescent proteins (such as Venus[44] and Citrine[45]), and cyan fluorescent proteins (such as Cerulean[46] and mTurquoise[47]) are also frequently used. To reveal different cellular components or compartments, GFP has been engineered by fusing it to various subcellularly localized molecules or by post-translational modification. GFP fused with mCD8 [13] or myristoylated GFP [8] is frequently used to mark cell membranes. GFP tagged with the synaptic vesicle-associated protein Synaptotagmin 1 (Syt::GFP) allow labeling of presynaptic compartments [48]. A hybrid protein of mCherry and the mouse protein Icam5, which is specifically and highly enriched in somatodendritic compartments (DenMark), is used to mark postsynaptic sites [49]. GFP fused with the nuclear localization signal from the large T antigen of Simian virus 40 allows nuclei to be labeled [50] and is useful to discern cell identity and count cell numbers. RedStinger, DsRed(T4) tagged with a nuclear localization signal, is also used to mark nuclei [43] (Table 1).

Revealing the Composition of Neural Circuits with Stochastic Multi-color Labeling

Plexuses of neurons in neural circuits are often intractable for one fluorescent reporter to discern its neuronal diversity and arrangement. Stochastic multicolor labeling can visualize multiple neurons in distinct colors in the same preparation, and thus allows the tracing of neuronal lineages and projections to reveal how neural circuits are arranged. Brainbow has been developed in the mouse to label neurons with different colors by stochastic expression of one of multiple fluorescent proteins based on Cre/*Lox* recombination [51]. Brainbow has been introduced into *Drosophila* using two approaches: Flybow uses a modified inducible FLP/*FRT* system to drive the inversion and excision of *FRT*-flanked cassettes of different membrane-tethered fluorescent proteins to allow stochastic expression of one of four epitopes [52] (Fig. 2A); dBrainbow uses Cre and three mutually-exclusive *Lox* sites to drive the stochastic expression of one of three epitope-tagged proteins, and double copies of dBrainbow can generate six-color labeling [53] (Fig. 2B). MCFO (MultiColor Flip Out), allows multicolor stochastic labeling by controlling the expression of multiple distinct epitope-tagged proteins with recombinase-mediated excision of transcription-terminating cassettes [54] (Fig. 2C). This has been used to reveal the shapes and arrangements of cells in the visual system [20] and central complex [55], among other brain regions.

Monitoring Neuronal Activity by a Genetically-Encoded Ca^{2+} Indicator or Voltage Indicator

The functioning of neural circuits is reflected in dynamic and patterned neuronal activity. Ca^{2+} and voltage dynamics are common indicators of neuronal activity and can be monitored by light microscopy imaging with genetically-encoded Ca^{2+} indicators (GECIs) and voltage indicators (GEVIs), respectively. Targeted expression of GECIs or GEVIs allows the monitoring of neuronal activity in labeled neurons or neuronal compartments (Table 1).

Monitoring Neuronal Activity with Genetically-Encoded Ca^{2+} Indicators

Intracellular Ca^{2+} is a widely-used indicator for neuronal activity. Various genetically-encoded indicators have been developed, based on fluorescent proteins and Ca^{2+}-binding proteins such as Calmodulin (CaM), allowing activity to be monitored in a targeted neuronal population.

Table 1 Common effectors / methods for labeling, monitoring or manipulating neurons.

Effectors/methods	Description
Fluorescent reporters	
UAS-GFP	mCD8::GFP [8] and myr::GFP [39] to mark cell membranes
UAS-RFP	tdTomato [40], mCherry [41], dsRed [42, 43]
UAS-YFP	Venus [44] and Citrine [45]
UAS-CFP	Cerulean [46] and mTurquoise [47]
Subcellular marker	
UAS-DenMark	Mouse Icam5-tagged mCherry to mark the postsynaptic sites [49]
UAS-syt::GFP	Syt1-fused GFP to label presynaptic compartments [48]
UAS-GFP::nls	Nucleus-localized GFP [50]
UAS-RedStinger	Nucleus-localized DsRed(T4) [43]
UAS-C3PA-GFP	Intracellular diffusion of focused photoactivated PA-GFP to reveal neuronal processes [92, 93]
Genetically-encoded Ca^{2+} indicators	
*UAS-IVS-GCaMP6**	Most-used GFP-based GECIs [56] GCaMP6s, GCaMP6f [58]
*UAS-RCaMP**	mRuby-based GECIs jRCaMP1a, b [64]
*UAS-R-GECO**	mApple-based GECIs jRGECO1a, 1b [64]
*UAS-twitch-2C**	FRET-based ratiometric fluorescent Ca^{2+} reporter [60, 61]
UAS-CaMPARI	Ratiometric reporter reflects Ca^{2+} activity under photoconverting UV [67]
CaLexA	Transcriptional reporter for sustained Ca^{2+} activity
TRIC	Transcriptional reporter for sustained Ca^{2+} activity
Genetically-encoded voltage indicators	
UAS-ArcLight	GEVI with improved signal and signal-to-noise ratio [80]
*UASp-Asap2f**	GEVI with improved sensitivity [81]
Neuronal manipulation	
UAS-rpr	Pro-apoptotic molecule Reaper[84] to ablate cells
UAS-hid	Pro-apoptotic molecule Hid [85] to ablate cells
*UAS-TNT**	Tetanus toxin light chain to suppress synaptic vesicle fusion and block neurotransmission [87]
*UAS-shibire^{ts}**	Temperature-sensitive dominant-negative of Dynamin to disrupt synaptic vesicle exo/endocytosis and block neurotransmission [88]
UAS-NachBac	Bacterial Na^+ channel to increase neuronal excitability [89]
*UAS-Kir2.1**	K^+ inwardly-rectifying channel to silence neuronal firing [90]
UAS-Ork1.Δ-C	Open rectifier K^+ channel to silence neuronal firing [91]
UAS-TrpA1	Thermosensitive cation channel to activate neurons [94]
*UAS-ChR2**	Blue light-sensitive Channelrhodopsin to activate neurons [95]
*UAS-ReaChR**	Red light-sensitive Channelrhodopsin to activate neurons [96]
*UAS-CsChrimson**	Red light-sensitive Channelrhodopsin to activate neurons [97]
UAS-NpHR.YFP	Yellow light-sensitive Halorhodopsin to suppress neurons [98–100]
UAS-GtACR1	Cyan (GtACR1) or blue (GtACR2) light-sensitive
UAS-GtACR2	Channelrhodopsin to silence neurons [101]
*UAS-P2X2.Y**	ATP-gated non-specific cation channel to activate neurons [102]

**LexAop* lines also available.

Monitoring Transient Ca^{2+} in Neurons

Transient Ca^{2+} can be monitored using GCaMP, a single molecule GFP-based Ca^{2+} probe with the CaM and its receptor, the M13 fragment from myosin light chain kinase, attached to the C and N termini of a circularly-permutated EGFP (cpEGFP) [56]. Ca^{2+}-binding to CaM induces a conformational change of CaM-M13 and thus the cpEGFP, resulting in a change in fluorescence intensity. It is widely used to image neuronal responses [57]. The latest

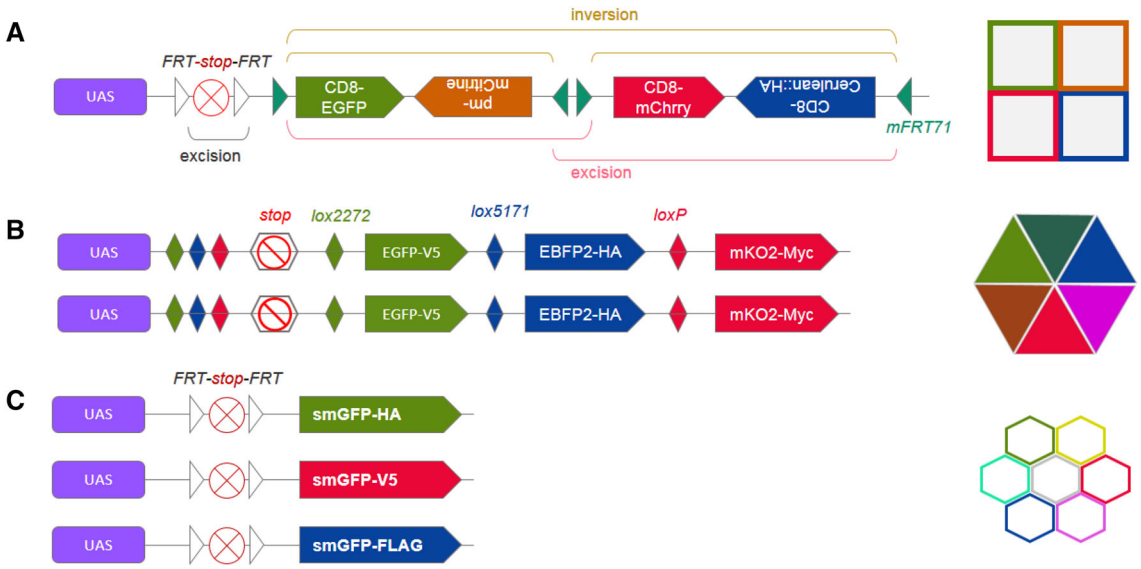

Fig. 2 Stochastic multicolor labeling of cells. **A** Flybow uses *hs-FLP* (enzyme for *FRT*), *hs-mFlp5* (enzyme for *mFRT71*) to generate *FRT*-mediated inversion and excision to allow expression of one of four fluorescent proteins in subsets of targeted neurons. **B** dBrainbow uses hs-Cre to induce excision of the stop cassettes to allow one of the three fluorescent protein to be stochastically expressed in targeted neurons. Two copies of the constructs generate six-color labeling. **C** MCFO uses hs-FLP to stochastically excise *FRT*-flanked interruption cassettes and allow the combinatorial expression of three markers.

version, GCaMP6, can even detect single action potentials in the soma [58]. Subcellularly-targeted GCaMP allows the compartmentalized imaging of Ca^{2+}. Syt::GCaMP, which attaches GCaMP6 to the C terminus of Syt1, then locates to and detects Ca^{2+} influx specifically in presynaptic terminals [59].

Transient Ca^{2+} can also be monitored using Twitch-2C, a ratiometric fluorescent Ca^{2+} reporter that utilizes 'Twitch' sensors based on the C-terminal domain of *Opsanus* troponin C and fluorescence resonance energy transfer (FRET) [60]. A conformational change mediated by Ca^{2+} binding alters the FRET between two fluorophores (mTurqoise2 and cpCitrine174), resulting in a change in the ratio of cyan to yellow fluorescence, which can be monitored by ratiometric imaging [61].

Red versions of GECIs, such as R-GECO (based on mApple) [62] and RCaMPs (based on mRuby) [63], of which the recent versions jRCaMP1a, b or jRGECO1a [64] have sensitivity comparable to GCaMP6, provide an additional channel of imaging to allow dual-color imaging of separate neuronal populations when used together with GCaMP to simultaneously monitor two populations of neurons [65].

GECIs can be used for neuron imaging in acutely dissected live nervous system or head fixed animal. Flyception allows more temporarily precise real-time imaging in freely-moving flies through an imaging window with the head cuticle removed in a specially-designed arena [66].

Capturing Neuronal Ca^{2+} Activity in a Light-Defined Time-Window

CaMPARI (calcium-modulated photoactivatable ratiometric integrator) takes advantage of the photoconvertible green fluorescent protein (FP) that irreversibly converts to a red fluorescent species upon illumination with violet light. The construct attaches a circular permutation of a photoconvertible FP to CaM and M13 peptide to allow irreversible conversion from green to red fluorescence with an increase in Ca^{2+} and the simultaneous presence of UV light (405 nm). It is used to label the increased activity of targeted neurons during behavior by simultaneous UV photoconversion [67]. CaMPARI has recently been used to identify clock neuron classes that respond to changes in temperature [68], and verify the gustatory response of interneurons involved in bitter processing in affixed adult flies [69], and to monitor the response of dopamine neurons to odor [70] and octopaminergic neurons to behavioral interruption training in freely-behaving larvae [71].

Transcriptional Reporter for Ca^{2+} level

CaLexA (Ca^{2+}-dependent nuclear import of LexA) takes advantage of a Ca^{2+}-responsive transcription factor, nuclear factor of activated T cells, and fuses it to VP16AD and mutant LexA that lacks a nuclear localization signal. Sustained neuronal activation promotes nuclear transport of the LexA chimera and drives transgene expression under the control of *LexAop* [72].

TRIC (transcriptional reporter of intracellular Ca^{2+}) combines split GAL4 parts with CaM and its targeting peptide CaMKII to reconstitute functional GAL4, contingent upon the Ca^{2+} level. By fusing GAD to CaM and DBD to CaMKII, Ca^{2+}-binding facilitates the association of CaM with CaMKII and thus the reconstitution and nuclear transport of functional GAL4. Thus, the expression of reporter genes under GAL4/*UAS* control reflects a sustained neuronal activation [73].

CaLexA and TRIC are both useful for reporting chronic neuronal activation [74, 75]. A real-time CaLexA-LUC assay combining *UAS-CaLexA* with *LexAop-LUC* has revealed the activity of circadian neurons in intact freely-moving flies [76]. A similar Tric-LUC has also been developed [77].

Monitoring Neuronal Activity with Genetically-Encoded Voltage Indicators

Electrophysiology is the standard method to monitor the electrical dynamics of a neuron. Whole-cell patch-clamp recording has been increasingly used to monitor central neurons and map neural circuits [78, 79]. The electrophysiological responses of neurons can also be monitored by light microscope imaging with GEVIs, which allow the optical detection of voltage transients at the subcellular level in targeted neurons. ArcLight, combining superecliptic pHlourin and the S1–S4 voltage-sensor domain of the *Ciona intestinalis* voltage-sensitive phosphatase, can report both subthreshold events and action potentials in intact neurons with an improved signal and signal-to-noise ratio [80]. ASAP is another GEVI with improved sensitivity and allows two-photon imaging of graded potentials in flies [81]. ASAP2f [82], an improvement on ASAP, produces larger fluorescence changes and sustains stable responses during continuous imaging. With superior sensitivity and kinetics, the potential of GEVIs for optical electrophysiology is mostly hindered by that the temporal scanning speed does not match the dynamics of neurons [83].

Neuronal Manipulation

Neurons can be manipulated to interrogate their functions. Ablating neurons genetically by pro-apoptotic molecules such as Reaper [84] or Hid [85], or making neurons dysfunctional by blocking transmission or repressing electrical pulses can reveal the necessity of neurons for behaviors. Activating neurons can uncover behavior-initiating/inducing neurons, like those that control the feeding motor program [86].

Neuronal transmission can be blocked using tetanus toxin light chain (TNT), which suppresses synaptic vesicle fusion and thus neurotransmission [87], or Shibire, a dominant-negative form of Dynamin, an essential component of the synaptic vesicle exo/endocytosis machinery [88]. Neuronal excitability can be promoted by the bacterial depolarization-activated Na^+ channel NaChBac [89] or silenced by the inwardly-rectifying K^+ channel Kir2.1 [90] or by open rectifier K^+ channel dOrk-ΔC [91]. Targeted expression of specific effectors allows neuronal activity to be conditionally modulated by light, temperature, or chemicals (Table 1).

Optogenetics Controls Neuronal Activity with Light

Optogenetics tools use light-sensitive ion channels to achieve manipulation of neuronal activity with high temporal resolution. Neurons can be activated by blue light with Channelrhodopsin-2 (ChR2), a blue light (470 nm)-sensitive nonselective cation channel derived from the green alga *Chlamydomonas reinhardtii* [95], However, blue light has a visual effect on flies and does not penetrate the cuticle well. The side-effects can be circumvented by using red light. ReaChR (Red-activatable Channelrhodopsin) is derived from *ChR1* in *Chlamydomonas reinhardtii* with a red-shifted spectral sensitivity that allows neuronal activation by red light (590 nm–630 nm) [96]. CsChrimson, derived from red light (590 nm)-responsive ChR1 in the red alga *Chlamydomonas noctigama*, has been introduced into *Drosophila* to activate neurons with red light [97].

The Cl^- channel Halorhodopsin from the archaebacterium *Natronomonas pharaonis* (NpHR) is able to suppress neuronal firing upon activation by yellow light (570 nm) [98–100]. The theta anion Channelrhodopsins from algal *Guillardia* (GtACRs) are have been introduced into flies for optogenetic silencing of neurons by cyan (GtACR1, at 515 nm) or blue light (GtACR2, at 470 nm) [101].

Thermogenetics Controls Neuronal Activity with Temperature

Neuronal function can be modulated by temperature in the poikilotherm *Drosophila* with temperature-sensitive molecules. Neuronal transmission can be temporally blocked by temperature-sensitive $Shibire^{ts1}$ at temperatures above 29°C [88]. Neurons can be activated by the thermosensitive cation channel TrpA1 if shifted from the restrictive 22°C or below to above 25°C [94].

Chemogenetics Controls Neuronal Activity with Chemicals

The ATP (adenosine 5'-triphosphate)-gated non-specific cation channel receptor P2X2 was used in flies as the first demonstration of controlling neuronal activity by light [102]. It was initially used in flies for light activation of neurons by injecting the flies with encased ATP which can be uncaged by light. *Drosophila* lacks endogenous ATP-gated channels and thus the released ATP can specifically activate neurons expressing P2X2.

Revealing Trans-synaptic Neuronal Connections

Neurons function in circuits. Understanding neuronal function entails identifying the neuronal projections, connecting neurons, and synaptic contacts.

Tracing Neuronal Processes

Neuronal projection of sparsely labeled neurons can be easily visualized by fluorescent reporter. However, neuronal projection within a neural plexus is difficult to discern, which can be relieved by the PA-GFP (photoactivatable GFP), a GFP variant that greatly increases its fluorescence with 488 nm excitation light after intense 413 nm radiation. With targeted expression of PA-GFP in neurons [103], light treatment at specific loci harboring part of the neuronal processes can convert the PA-GFP to its high-fluorescence form and diffusion of the converted GFP allows the projection pattern of a subset of targeted neurons to be highlighted [92, 93]. Although it is invasive to achieve precise light treatment, PA-GFP allows higher spatial specificity than that permitted by genetic targeting, and can reveal connections to discrete brain regions using a broadly-expressed PA-GFP [104].

Trans-neuronal Tracing

To reveal the circuitry in which neurons perform functions, it is necessary to map the downstream and upstream neurons. The relationship between two populations of neurons can be grossly addressed by simultaneously expressing different markers in each to see if overlap or contiguity exists in their expression patterns. To systematically map trans-synaptic connected neurons, new strategies have been developed to label the downstream neurons of target neurons.

trans-Tango allows anterograde trans-synaptic tracing, based on the Tango assay in which a synthetic signaling pathway consisting of two fusion proteins converts the activation of a cell-surface receptor such as G protein-coupled receptor to reporter expression via site-specific proteolysis. With trans-Tango, ligands presented at presynaptic sites in targeted neurons (labeled by *UAS-myrGFP*) trans-synaptically activate receptors and release membrane-tethered QF to drive expression of the *QUAS*-controlled reporter in postsynaptic neurons via ubiquitously-expressed Tango that uses the QF/*QUAS* to drive a marker (*QUAS-mtdTomato*) in the receptor cells [105] (Fig. 3a). This has been used to trace the APDN-TuBusup-EB circuit that regulates sleep-wake arousal [65, 106], and the connections revealed by trans-Tango can be functionally verified with *UAS*-driven P2X2 and *QUAS*-driven GCaMP [65].

TRACT (TRAnsneuronal Control of Transcription) can also label anterograde trans-synaptic neurons based on ligand-induced intramembrane proteolysis. *Drosophila* n-Synaptobrevin (n-Syb)-fused artificial ligands in LexA-labeled neurons are presented on the presynaptic membrane, and the trans-synaptic interaction with an artificial receptor induces intramembrane proteolysis in post-synaptic neurons to liberate a membrane-bound GAL4 variant, and ultimately drives the expression of a *UAS*-controlled reporter [107] (Fig. 3B). In principle, combined use of TRACT and trans-Tango allows simultaneous labeling of the primary (*UAS*-driven reporter) and secondary (*QUAS*-riven reporter) postsynaptic neurons of a LexA driver.

Revealing Potential Synaptic Contacts Between Neuronal Populations

As synapses are beyond the resolution of the ordinary light microscope, apparent contacts of neurons are not necessarily synaptic connections, so genetic methods have been developed to aid in marking putative synaptic connections.

GRASP (GFP Reconstitution Across Synaptic Partners) makes use of the trans-cellular complementation of split GFP parts (spGFP1-10 and spGFP11) to detect the membrane proximity of two cells. The spGFPs, neither of which is fluorescent alone, are separately expressed in the outer membranes of two populations of cells. If two adjacent cells are in enough proximity, the spGFP could reconstitute into a functional fluorescent GFP, revealing potential synaptic contacts (Fig. 4A). The initial version of GRASP used CD4 to present spGFPs to all the outer membranes of cells [108]. To improve the specificity of synaptic labeling, one of the two spGFPs was anchored to synaptic molecules to target synaptic sites. spGFP1-10 was fused to the transmembrane synapse protein Neurexin for better synaptic targeting [109]. Fusing one of the spGFPs to the C terminus of n-Syb (syb:GRASP) allows directional and activity-dependent GRASP labeling, since the presynaptic vesicle membrane n-Syb can only present the spGFP to the synaptic cleft after vesicle fusion to the cell

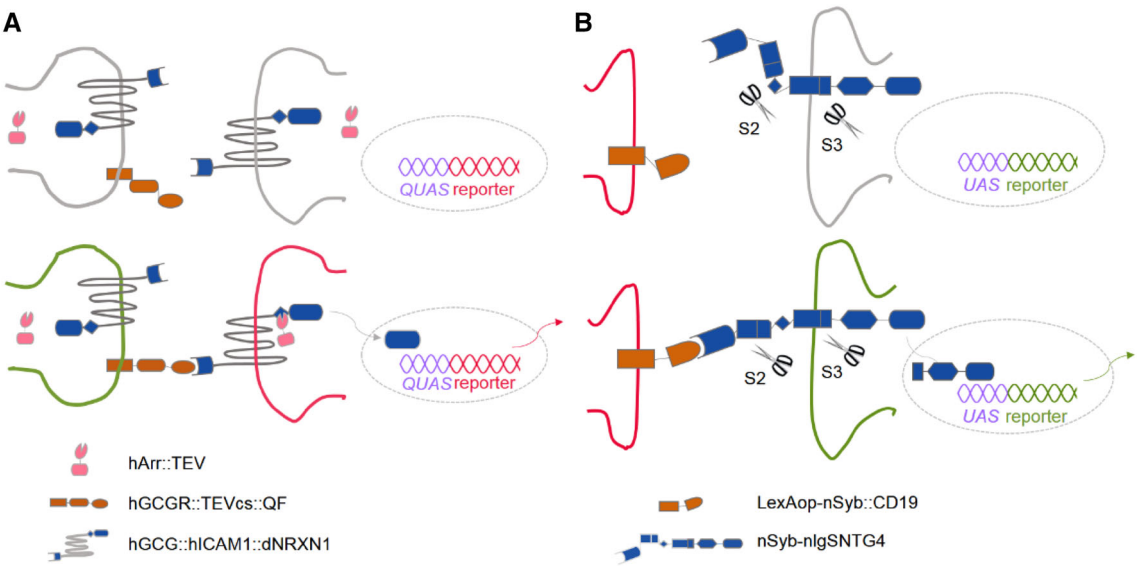

Fig. 3 Methods for anterograde trans-synaptic tracing. **A** Trans-Tango allows anterograde tracing based on pan-neuronally-expressed Tango of human glucagon (hGCG) and presynaptically targeted expression of the glucagon. The trans-Tango is composed of two chimera proteins: pan-neuronally expressed hGCGR::TEVcs::QF tethers the transcription activator QF to the membrane via linking it to the human glucagon receptor (hGCGR) with a linker containing the cleaving site for N1a protease from the tobacco etch virus (TEVcs); hArr::TEV allows recruitment of TEV to activated hGCGR via human β-arrestin2, and one *QUAS* reporter. Overall, GAL4/*UAS* targeted expression of hGCG::hICAM1::dNRXN1 presents hGCG at presynaptic sites via the synaptic protein Neurexin1 (dNRXN1) and extracellular domain of human cell adhesion molecule (ICAM1), and activates hGCGR in postsynaptic neurons and recruits hArr::TEV to cleave QF off the membrane to translocate into the nucleus and initiates reporter expression under *QUAS* control. **B** TRACT allows anterograde tracing of neuronal circuitry by ligand-induced intramembrane proteolysis. TRACT is composed of CD19 presynaptically localized with nSyb in neurons targeted by LexA/*LexAop*, and the pan-neuronally-expressed fusion protein nlgSNTG4 that tethers transcription factor GAL4 and CD19 antibody ID3 to the synaptic sites of cell membranes via the intracellular domain of neuroligin (NLGN), the Notch regulatory region (NRR) and transmembrane domain of Notch. The presynaptic ligand CD19 binding to postsynaptic receptors ID3 partially unfolds the NRR to allow for cleavage of the S2 site by endogenous metalloproteases, and further allows cleavage (S3) by γ-secretase-mediated intramembrane proteolysis of the intracellular domain containing GAL4 which then translocates to the nucleus to activate the transcription of reporter genes such as GFP under *UAS* control.

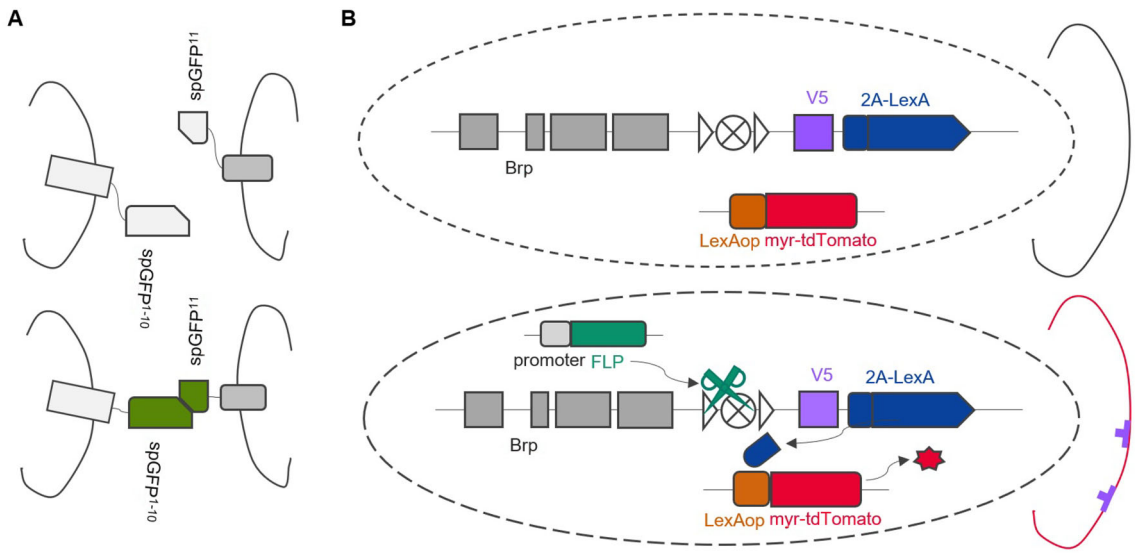

Fig. 4 Synaptic labeling. **A** GRASP detects membrane proximity permitting synaptic contacts between two neurons by the fluorescence of a reconstituted functional GFP from complementary spGFPs expressed in each of the neurons. **B** STaR allows Brp-fused tags with V5 expressed in FLP-mediated stochastically-labeled neurons (with myr-tdTomato) to mark the active zone, mimicking the endogenous pattern of Brp in a cell-type-specific fashion.

membrane [110]. Recently, t-GRASP (targeted GFP Reconstitution Across Synaptic Partners) tested different pre-synaptic and post-synaptic/dendritic proteins for targeting both spGFPs, and further improved the synaptic specificity [111].

STaR (Synaptic Tagging with Recombination) allows inducible tagging of synaptic proteins to visualize synapses in the processes of sparsely-identified neurons, to aid in identifying synapses for specific neurons using ordinary fluorescent light microscope. It is hardly possible to discern dense synaptic puncta in neuronal processes of small size using antibodies against synaptic proteins, which are usually broadly expressed. STaR uses bacterial artificial chromosomes to harbor an engineered genomic region of synaptic protein with a tag sequence separated by a recombination recognition sequence flanked by a stop sequence that can be excised by recombinase, so the induced expression of tagged synaptic proteins display a level and spatiotemporal pattern similar to that of the endogenous synaptic protein. STaR of Brp, a component protein of presynaptic active zones, with a V5 tag, allows FLP-mediated stochastic expression of the V5-tagged Brp to label active zones [112] in defined neurons that are labeled with the *LexAop*-driven transgene with a co-translating 2A-LexA cassette [113] downstream of the V5 tag (Fig. 4B). Using GFP instead of V5 also allows labeling of active zones of specific neurons in live animals, and can be used to monitor structural synaptic plasticity [114–116]. STaR also allows co-labeling of presynaptic protein and postsynaptic receptors, thus revealing the contact sites of synaptic partners [112].

Functional Neuronal Interconnectivity

Synaptic connections between neurons, as indicated by membrane proximity via GRASP, or reconstructed from electron microscopic volumes are not necessarily functional. Functional connections between neurons are assayed by manipulating one neuron and simultaneously monitoring the other, and it is expected that proper activation of the upstream neurons would induce a delayed but timed response in downstream neurons. The most precise method to date uses paired electrophysiological recording electrodes from two neurons to record one neuron's response to stimulation of the other. Other methods can also do the trick, e.g., one type of neuron can be activated optogenetically by light with CsChrimson [102, 117, 118] or ReaChR [119], or chemogenetically by applying ATP to the acutely dissected brain with P2X2 [76, 115, 120]; meanwhile, activity of the other type can be monitored by electrophysiological recording [121] or imaging with GCaMP. Such assays could also reveal indirect functional connections or population responses.

The Connectome Approach to Neural Circuits

Brain-Wide Circuit Reconstructed by Light Microscopy

The whole-brain wiring diagram, reconstructed by aligning tens of thousands of stochastically-labeled single neurons into a standard brain, provides a blueprint to evaluate the organization of the brain and to infer how different brain regions are interconnected [122]. This single-cell resolution brain reconstruction also aids in interrogating neuron interconnectivity and producing hypotheses of information flow [123, 124]. NBLAST allows identified neurons be fitted to a standard brain and gives hints about their identity and connections [125] as well as similarity [126] based on morphology.

Harnessing machine learning for behavioral classification and big data computing, brain/behavior correlation has been mapped by large-scale analysis of the expression patterns of neurons and the corresponding behaviors induced by their activation in *Drosophila* larvae [127] and adults [128]. Such approaches produce a behavior reference atlas for neuronal subsets, providing hints on brain regions that might be implicated specific behaviors.

Brain-Wide Circuit Reconstructed by Electron Microscopy

Electron microscopy (EM) affords synaptic resolution circuit reconstruction [129], and is the gold standard for circuit mapping. With rapid improvements in serial-section EM and data acquisition/processing pipeline, EM volumes and reconstructions of more and more parts of the brain have been obtained. The connectome of the fly visual circuitry [130, 131], the EM volume of the entire nervous system of the *Drosophila* larva [132], the complete wiring diagram of the *Drosophila* larval antennal lobe [133], mushroom bodies [134], adult olfactory receptor neurons, and projection neurons [135] have been completed. Recently, an EM volume of the whole adult fly brain was obtained, and reconstruction of the mushroom bodies based on the dataset revealed new synaptic partners [136]. More neural circuits and interconnectivity are expected to be reconstructed from this dataset, culminating with a brain-wide connectome, all neurons and their synaptic connections in *Drosophila* brain. Despite the challenges ahead in processing and annotating the daunting volume of EM data, a post-connectome era is heralded for neural circuit studies in *Drosophila*.

EM reconstruction can comprehensively map neuronal types and connectivity, reveal interesting but previously unidentified neurons and connections, shedding light on

novel circuit motifs, produce hypotheses at the circuit level that can be cross-validated with results obtained by light microscopy, and provide a basic reference for *Drosophila* neural circuit studies. Considering that the operation of neural circuits can be dynamically reconfigured by neuromodulators [137], combining EM results with the precise genetic manipulation of neural circuits and revealing behavioral assays could help elucidate the operations and functions of neural circuits.

Concluding Remarks and Perspectives

Even as humble as a fruit fly, it must respond to external visual, chemical, mechanosensory stimuli, find food and mates, choose sites for oviposition, modify its behavior with past experiences. How does the tiny fly achieve the feats with circa 1000, 1000 neurons? Here we have briefly reviewed the common genetic tools currently used in *Drosophila* to solve such mysteries. Brain functions are executed in neural circuits. The sophisticated array of genetic tools available makes flies one of the best organisms for neural circuit cracking. With precise monitoring and manipulation of the nervous system possible at single-cell resolution, it's possible to establish causal relationship between neurons and behavior and gain mechanistic understanding of the brain in relation to cognition and behaviors.

Furthermore, it's instructive to make sense of neural circuits and behavior from modeling and evolutionary perspectives.

Theoretical modeling provides explainable systems with functionally-defined components. The predictive power of modeling guides functional studies of circuits and behavior. The Hassenstein–Reichardt elementary motion detector (EMD) has been proposed as a model for motion vision [138], and it has guided efforts to define the visual circuit implementing the EMD [130, 139]. Recent studies revealed a neural circuit for heading orientation, elegantly fitted by the ring attractor model [120, 140].

Insect brains share a common layout, but also differ [141]. How the sameness and differences in brain and behavior arise in evolutionarily close species provides a window for studying the conservation and variation of neural circuits. With the annotated genomes of non-model organisms and CRISPR/Cas9 for convenient genome editing, neuroethologists can draw more on neurogenetic tools. Transferring *Drosophila* circuit study techniques to non-model organism [142] would reveal the evolution of neural circuits [143], in adaption to particular needs in related species [144].

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