

# Chapter 2

## Genetically Encoded Markers for *Drosophila* Neuroanatomy

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### Abstract

The description of the anatomy of neural circuits provides a framework for predictions about their functions. During the last 2 decades, the explosion of genetically encoded tools for manipulating and visualizing the neural circuits in the fruit fly allowed important advances in correlating neural circuits and behavior. In this chapter, we review the properties of the main genetically encoded markers that are used to study *Drosophila* neuroanatomy, including data on toxicity when available.

**Key words:** Genetically encoded markers, Binary systems, Neuroanatomy, Subcellular localization

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### 1. Introduction

The brain of *Drosophila* is organized into structural and functional units, or compartments, that include neurons deriving from a single neuronal lineage (reviewed in (1)). In order to limit the space for reasonable predictions about their function, an accurate and comprehensive description of the connectivity between and within compartments is required. Ideally, this would include information about the directionality of the information flow between neurons.

Development of versatile and genetically encoded tools to study fruit fly neuroanatomy was originally based on P element technology and the GAL4/UAS binary system (2). The use of green and red fluorescent proteins, GFP and RFP (isolated respectively from the jellyfish *Aequorea* and from the reef coral *Discosoma*) (3), and the subsequent engineering of brighter and more photostable variants characterized by a palette of different spectra (4, 5) further enriched the toolbox. Recent innovations including the development of alternative binary systems, LexA/LexAOp (6) and

the latest—and therefore still less characterized QF/QUAS (7) as well as mosaic approaches taking advantage of several recombinase variants allowed the development of even more highly versatile and extremely sophisticated tools (8, 9).

In this introductory chapter, we would like to provide an overview of the principal features of genetically encoded markers available to visualize *Drosophila* neurons, including data on toxicity when available. All these markers are available as UAS constructs and transgenic stocks listed in FlyBase. In addition, some of them also exist as LexA-responsive (LexAOp) reporter lines (6). It is likely that all markers will be available for all expression systems in the near future. Tables displaying FlyBase references for the cited markers and company reference numbers for commercial antibodies can be found at the end of the chapter.

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## 2. Labeling the Entire Neuron

Genetically encoded neuronal reporters can be distinguished based on their subcellular localization—nuclear, cytoplasmic, or associated to the membrane, to the cytoskeleton, or to specific organelles. Differences in labeling properties between reporter molecules also depend on their structural properties. In particular, their molecular weight and whether they are active or not as monomers influence their diffusion in thin cellular processes. Importantly, markers also differ regarding their innocuity or toxicity to neurons. For instance, the expression of most cytoskeleton-bound reporters was shown to perturb neuronal morphology and even induce lethality under some circumstances (10).

### 2.1. Cytoplasmic markers

The most common cytoplasmic markers consist of  $\beta$ -galactosidase ( $\beta$ -Gal), an *Escherichia coli* enzyme—encoded by the gene *lacZ* (11)—and GFP (3). The two proteins differ in size: GFP is small (27 kDa) and acts mostly as a monomer, while  $\beta$ -Gal acts as a large 464kDa tetramer (12, 13). As a consequence, GFP diffuses better in neurites as compared to  $\beta$ -Gal. In addition, in contrast to  $\beta$ -Gal, whose expression is visualized through immunostaining or by providing a chromogenic substrate (X-Gal), GFP emits fluorescence in a spontaneous manner, allowing for live imaging studies. Low levels of GFP expression can also be detected using specific monoclonal or polyclonal commercially available antibodies.

Both UAS-GFP (3, 14, 15) and UAS-lacZ (16) reporter lines were generated more than a decade ago and are generally considered to be innocuous for neurons. However, a recent publication indicates that, in long-term assays, GFP expression could, unlike  $\beta$ -gal, affect fly health and result in a decrease in longevity and in locomotor activity (17).

## 2.2. Nuclear Markers

Though nuclear markers cannot provide information about circuitry itself, they can be useful in a quantitative approach—counting the number of cells belonging to a certain population, or for developmental studies. They consist of the fusion of fluorescent as well as nonfluorescent reporters together with a nuclear localization signal (*nls* sequence). Most commonly used nuclear UAS lines include UAS-lacZ.NZ (18), UAS-GFP<sup>nls</sup> (19) and UAS-RedStinger (consisting of the fast-maturing RFP variant DsRed.T4 associated to a *nls* sequence) (20). In addition, the chromosome-associated fluorescent markers H2Av-GFP and H2Av-RFP produced by the fusion of the fluorescent markers with the Histone H2A variant protein can also be used to visualize all nuclei (21, 22).

## 2.3. Membrane-Bound Markers

The most widely used membrane-associated reporter is the product of the fusion of the mammalian lymphocyte marker CD8 and GFP (23). Another lymphocyte membrane-bound protein, CD2, is also used and visualized thanks to a commercially available monoclonal antibody (24). Expression of one or both constructs does not seem to provoke any deleterious effects in neurons. This may be due to the fact that both CD8 and CD2 have no homologues in the fly and therefore are less likely to interfere with endogenous cellular processes (23–25). Though cytoplasmic and membrane-bound GFP do label the entire neuron, the two reporters should not be viewed as equivalent (25). Indeed, thin and thick processes differ in their cytoplasm versus membrane ratio. Therefore, membrane-bound reporters should be preferred when focus is directed towards thin fibers, whereas cytoplasmic markers better label thick processes and larger structures such as varicosities (23, 25).

CD2-HRP, a fusion between CD2 and horseradish peroxidase (HRP), constitutes another membrane-bound marker designed for electron microscopy usage. Indeed, HRP catalyses the formation of an electron-dense product from diaminobenzidine (DAB) that can be visualized by EM (26). The use of this tool was reported for ultrastructural studies in pupal and adult brains (27–29).

## 2.4. Cytoskeleton-Bound Markers

In order to target reporter genes all along the neurites, several cytoskeleton-bound reporter genes were generated by the fusion of lacZ or GFP together with tau or kinesin proteins. Also, the tau protein itself can be used as a probe and labeled using an antibody (30). However, both kinesin and tau fusion constructs proved to be toxic to neurons and to induce lethality in combination with several GAL4 lines (30). In addition, tau-GFP, tau-lacZ reporters as well as a human form of tau alone were shown to induce morphological defects in axonal arborization (10).

## 2.5. Secretory Pathway and Mitochondrial Markers

Genetically encoded markers for the secretory pathway, Endoplasmic Reticulum (ER) and Golgi Apparatus (GA) include the ER marker Lys-GFP-KDEL (31) and the GA markers ManII-GFP

(32) and GalT-GFP (31). Outposts of both ER and GA were detected in *Drosophila* neurites though their precise subcellular localization varies depending on neuronal type. For example, the ER marker GFP-KDEL, as well as the GA marker ManII-GFP, were observed in the entire axons when expressed in developing photoreceptor neurons (33). In contrast, in larval md neurons, ManII-GFP is specifically localized in the soma and at dendrites branching points (32). Therefore, it seems that the presence of secretory outposts is actually not restricted either to dendrites or axons but rather depend on particular developmental or physiological states of the neurites.

A genetically encoded mitochondrial marker, mito-GFP, was also established and reported to label all neuronal mitochondria when expressed in *Drosophila* neurons (34, 35).

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### 3. Presynaptic Markers

#### 3.1. Syt 1-GFP

Synaptotagmin 1 (Syt 1; also simply named Syt) is the founding member of a large family of vesicular  $\text{Ca}^{2+}$  sensor proteins that regulate membrane traffic in neurons and other cell types (reviewed in (36)). Among the seven *Drosophila* synaptotagmins, only two, Syt 1 and Syt 4, are present at most if not all synapses (37, 38). In particular, Syt1 is associated to synaptic vesicles in probably all *Drosophila* presynaptic terminals (37). Accordingly, Syt1::GFP became a commonly used presynaptic marker (39) and a valuable tool for mapping neuronal circuits (see for example (40, 41)). Concerning toxicity of the fusion protein for the neurons, the only published data reveal that, in contrast to N-Syb-GFP (see below), expression of Syt1-GFP in a subset of CNS neurons throughout development (using the Gal4 driver G4) is fully viable (39). However, controls for more subtle defects, in particular on synaptic morphology, were not reported.

#### 3.2. N-Syb-GFP

In *Drosophila*, two Synaptobrevins were isolated, a ubiquitous form, Syb, and a neuronal form N-Syb (42–44). N-Syb is associated to the membrane of synaptic vesicles and is required for evoked neurotransmitter release (45).

Similar to Syt1-GFP, the fusion construct N-Syb-GFP is often used as a reporter of the presynaptic compartment of *Drosophila* neurons, in particular in studies aiming at describing neuronal circuits (see for instance (46–48)). A first fusion construct of N-Syb with the GFP variant S65T was published (49, 50), followed by a similar fusion to the next generation GFP variant eGFP (39). More recently, UAS lines bearing a N-Syb::RFP fusion (monomeric DsRed) have also been established (51). The only information about the toxicity of N-Syb::eGFP fusion protein comes from

Zhang et al. (39) and indicates that expression of the construct in a large subset of neurons throughout development induces lethality at the pupal stage.

### 3.3. *Brp-GFP*

The *Drosophila* coiled-coil domain protein Bruchpilot (BRP) was first identified as the epitope recognized by the presynaptic active zone specific monoclonal antibody NC82 (52, 53). BRP is present at the electron dense T-bars where it is crucial for the structure of the active zone and the release of neurotransmitter, in particular by interacting directly with  $\text{Ca}^{2+}$  channels (reviewed in (54)). A UAS-BRP-GFP reporter was constructed by fusing GFP to the N terminus of BRP, and its subcellular localization was shown to match endogenous BRP expression (52). A UAS-BRP-mRFP line was also recently generated (55). However, no data is available concerning the innocuity/toxicity of these two transgenes.

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## 4. Somatodendritic and Postsynaptic Markers

### 4.1. *DenMark*

The recently published DenMark is a fluorescent protein resulting from the fusion of the red fluorescent protein mCherry (5) with the mouse ICAM5/telencephalin (41). In the mammalian telencephalon, ICAM5 is specifically expressed in dendrites (56). Thanks to ICAM5, expression of DenMark is highly enriched at membranes of the somatodendritic compartment and is also detected at postsynaptic sites. We showed that DenMark expression does not affect neuronal physiology or dendritic morphology. Similar to CD8-GFP, this could be explained by the fact that ICAM5 has no invertebrate homologue and is therefore less likely to interfere with endogenous processes. Importantly, we found that in immature neurons, DenMark expression is not polarized and is detected in axonal as well as in somatodendritic compartments. Since the same was observed for another somatodendritic marker, DsCam 17.1—GFP (see below), we assume that this finding may reflect the developmental dynamic of neuronal polarization rather than a lack of specificity of the marker. Finally, an antibody against the mCherry progenitor DsRed (Clontech #) also labels DenMark and can be used to compensate for low levels of expression with some GAL4 driver lines (41).

### 4.2. *Dscam 17.1-GFP*

The Dscam 17.1 protein corresponds to one of the putative 38016 membrane-bound cell adhesion molecules potentially encoded by the *Dscam 1* (*Drosophila* Down syndrome cell adhesion molecule) locus. In *Drosophila*, the multiple roles of Dscams in the formation of neural circuits are achieved through homophilic or heterophilic interactions (57). Subcellular localization of the Dscam proteins is controlled by alternative splicing of the transmembrane domain

encoded by exon 17. Isoforms containing the alternative exon 17.2 are localized to the axons, while the exon 17.1 targets the proteins at the somatodendritic compartment (58). Accordingly, Dscam 17.1-GFP fusion proteins present a somatodendritic localization and have already been adopted as a tool for mapping neuronal circuits (1, 40, 59, 60). As mentioned above, similar to DenMark, Dscam17.1-GFP is detected in axons of immature neurons (41). However, unlike DenMark, Dscam17.1-GFP is toxic at high dose—overexpression using the panneuronal elav-Gal4 driver is fully lethal (41). In addition, given the multiple functions of Dscams in the development of neuronal circuits (57), further investigations concerning the effects of Dscam17.1-GFP expression on fine dendritic morphology would be useful.

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## 5. Cautionary Considerations

Genetically encoded markers used in combination with the GAL/UAS constitute powerful and versatile tools for the *Drosophila* neuroanatomist. However, like any tool, they have their limitations and pitfalls. For instance, as already discussed, they can be toxic for the neurons, provoking either cell death or more problematically, affecting fine morphology. As already discussed, markers deriving from exogenous proteins seem to be less prone to affect neuronal development and physiology, perhaps because they are less likely to interfere with too many endogenous cellular functions. In contrast, markers derived from endogenous important cell components, like cytoskeleton-bound probes or Dscam-GFP—are highly toxic (10, 30, 41). In this context, it is important to keep in mind that control experiments indicating that markers do not affect the morphology of neurons are still lacking for most of them.

Another important feature of certain genetically encoded markers is the specificity of their subcellular localization. As described above, many markers were designed by fusing a fluorescent (in most of the cases GFP) or a nonfluorescent marker (usually  $\beta$ -gal) together with full-length proteins, protein domains, or signal peptides controlling their subcellular localization. However, depending on the amount of fusion protein expressed in neurons, the cellular machinery governing protein trafficking and/or localization can become saturated resulting in the loss of the specificity of the fusion protein localization. Obviously, saturating concentration of the marker will depend on the nature of the marker itself, the “strength” of the GAL4 driver, the number of copies of UAS and GAL4 driver constructs and—due to the thermosensitivity of the GAL/UAS system (61)—the temperature at which flies are kept. For instance, significant reduction in subcellular

specificity of the somatodendritic marker DenMark was observed in flies homozygous both for the mushroom body driver 201Y-GAL4 and UAS-DenMark constructs (41).

Similarly, antibodies recognizing markers such as GFP or DsRed are very useful for detecting low expression levels, especially in single or sparse neurons. However, they can also draw misleading interpretation when used with specific subcellular fusion constructs. Indeed, specific subcellular localization often consists of an enrichment of a marker at a specific compartment rather than its complete absence from the “nonlabeled” compartment. Thus, immunolabeling can lead to a saturation of the signal and thereby mask the difference between enriched and nonenriched structures.

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## 6. What’s Missing?

Despite the wealth of tools already available, one can think about several extra ones that could enrich the toolbox and potentially lead to qualitative improvements.

First, concerning the directionality of neuronal information flow, available tools encompass presynaptic and somatodendritic markers. However, we still lack a specific marker for postsynaptic densities. Second, a tool to visualize synapses was recently developed in *Caenorhabditis elegans* (62). The GRASP (GFP Reconstitution Across Synaptic Partners) method is based on the expression of two complementary fragments of GFP at the membrane of two distinct cells. When the two fragments come in contact—for instance when both cells form a synapse—the GFP is reconstituted and emits fluorescence. GRASP was then rapidly implemented in the fruit fly by taking advantage of the two binary systems, GAL/UAS and LexA/LexAOp (63). However, since both GFP fragments are expressed at membranes, GFP reconstitution occurs not only at synapses but also at any contact point between the cells. Therefore, a synapse-specific GRASP system, possibly based on the fusion of both GFP fragments with proteins respectively enriched at pre- and postsynaptic sites, still needs to be developed. Third, a limitation inherent to the GRASP system is linked to the stability of the GFP. Indeed, once reconstituted, the fluorescent protein will remain attached between the pre- and postsynaptic sites preventing its internalization. Besides possible toxic consequences, this will “fix” the synapse at the stage where the GFP was reconstituted. In contrast, a transsynaptic marker which, unlike GRASP, could be internalized and recycled would in principle allow monitoring plasticity of synaptic contacts between cells in living animals.

## 7. Products and Tools

### 7.1. Constructs

We provide here the Flybase references for constructs corresponding to the genetically encoded markers described in this chapter. We restricted the list to the constructs implemented in the major binary system, GAL4/UAS. However, new LexA-responsive LexAOp reporter lines are regularly generated and rendered available in stock centers.

|                | Construct               | # Flybase   |
|----------------|-------------------------|-------------|
| Cytoplasmic    | UAS-GFP                 | FBtp0001403 |
|                | UAS-LacZ                | FBtp0000355 |
| Nuclear        | UAS-GFP <sup>nls</sup>  | FBtp0001204 |
|                | UAS-RedStinger          | FBtp0018199 |
|                | UAS-LacZ <sup>nls</sup> | FBtp0001611 |
| Membrane-bound | UAS-CD8::GFP            | FBtp0002652 |
|                | UAS-CD2                 | FBtp0000378 |
|                | UAS-CD2::HRP            | FBtp0019068 |
| Organites      | UAS-GFP::KDEL           | FBtp0021899 |
|                | UAS-ManII::GFP          | FBtp0039245 |
|                | UAS-GAIT::GFP           | FBtp0041224 |
|                | UAS-mito::GFP           | FBtp0041285 |
| Presynaptic    | UAS-sytI::GFP           | FBtp0016185 |
|                | UAS-Nsyb::GFP           | FBtp0013062 |
|                | UAS-BRP::GFP            | FBtp0022731 |
| Postsynaptic   | UAS-DenMark             | FBtp0056788 |
|                | UAS-DsCam17.1::GFP      | FBtp0021447 |

### 7.2. Antibodies

The following list provides references for the antibodies mentioned in the text.

|                 | Company                    | Host species |
|-----------------|----------------------------|--------------|
| Anti-β gal      | Cappel                     | Rabbit       |
| Anti-GFP        | mAB 3E6 Invitrogen #A11120 | Mouse        |
|                 | Invitrogen #A11122         | Rabbit       |
| Anti-CD2        | Serotec clone OX-34        | Rat          |
| Anti-dsRed      | Clontech #632496           | Rabbit       |
| Anti-bruchpilot | DHSB NC82                  | Mouse        |
| Anti-synapsin   | DHSB 3C11                  | Mouse        |
| Anti-Ncadherin  | DHSB DN-EX#8               | Rat          |

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