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Dual-labeling method for electron microscopy to characterize synaptic connectivity using genetically encoded fluorescent reporters in *Drosophila*

Nobuaki K. Tanaka^{a,1}, Louis Dye^a, and Mark Stopfer^{a,*}

^aNational Institutes of Health; National Institute of Child Health and Human Development, 35 Lincoln Drive, msc 3715, Bethesda, MD 20892, USA

Abstract

Light and electron microscopy (LM and EM) both offer important advantages for characterizing neuronal circuitry in intact brains: LM can reveal the general patterns neurons trace between brain areas, and EM can confirm synaptic connections between identified neurons within a small area. In a few species, genetic labeling with fluorescent proteins has been used with LM to visualize many kinds of neurons and to analyze their morphologies and projection patterns. However, combining these large-scale patterns with the fine detail available in EM analysis has been a technical challenge. To analyze the synaptic connectivity of neurons expressing fluorescent markers with EM, we developed a dual-labeling method for use with pre-embedded brains. In *Drosophila* expressing genetic labels and also injected with markers we visualized synaptic connections among two populations of neurons in the AL, one of which has been shown to mediate a specific function, odor evoked neural oscillation.

Keywords

olfactory circuit; olfaction; synapse; GFP; DAB; antennal lobe

1. Introduction

In recent years analysis of neural circuitry has benefited from the introduction of genetic expression systems that can cause neurons to express a variety of proteins (Luo et al., 2008). Fluorescent proteins can reveal the morphology and projection patterns of neurons under light microscopy, and help guide electrodes to record from specific neurons. Other genetic tools can allow imaging neural activity with calcium reporters, and controlling neural activity with effector genes. These genetic approaches have all helped advance a functional understanding of neuronal circuits in model animals such as *Drosophila*. However, understanding the functions of neurons and neuronal circuitry requires understanding their synaptic connections, which are generally not visible under the light microscope. There remains a need for a practical method for combining standard methods for viewing connectivity among brain areas under LM and methods for identifying synaptic connections and synaptic details under EM.

*Correspondence to Dr. Mark Stopfer National Institutes of Health, NICHD, 35 Lincoln Drive, msc 3715, Bethesda, MD 20892, USA
Phone: +1-301-451-4534 Fax: +1-301-402-4777 stopferm@mail.nih.gov.

nktanaka@cp.kyoto-u.ac.jp (N. K. Tanaka), stopferm@mail.nih.gov (M. Stopfer).

¹Present address: Career-Path Promotion Unit for Young Life Scientists, Kyoto University, Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan

In *Drosophila*, the GAL4 enhancer-trap system can be used to label various kinds of neurons by causing them to express fluorescent proteins. This approach has been helpful for studying the organization of olfactory system (Vosshall et al., 2000; Jefferis et al., 2001; Wong et al., 2002; Marin et al., 2002; Tanaka et al., 2004; Couto et al., 2005; Fishilevich and Vosshall, 2005; Shang et al., 2007; Tanaka et al., 2008). We sought to extend this approach by developing a new dual labeling method for pre-embedded *Drosophila* brains that could be used to identify synaptic connections between particular types of neurons under EM.

We applied this new approach to analyze olfactory circuitry. Olfactory responses begin in olfactory receptor neurons (ORNs), each of which sends an axon to a glomerulus specified by the ORN's receptor (Vosshall et al., 2000; Ressler et al., 1994). In these glomeruli, ORNs form synapses onto the dendrites of local neurons (LNs in insects, juxtaglomerular cells in vertebrates) and projection neurons (PNs in insects, mitral and tufted cells in the vertebrate) (Kay & Stopfer, 2006). In *Drosophila*, two major populations of GABAergic local neurons (LN1 and LN2) have been identified in the antennal lobe (AL), the insect's primary olfactory center, analogous to the olfactory bulb of vertebrates (Okada et al., 2009; Tanaka et al., 2009). These two populations of LNs were originally identified and are most readily distinguished by their GAL4 enhancer traps: LN1 by NP1227, and LN2 by NP2426. Both types of LN innervate most, but not always all glomeruli, but each type has a different arborization pattern within each glomerulus. The LN1 cells arborize in parts of the glomerulus that specifically do not overlap with the terminal areas of ORNs, while LN2 cells arborize throughout the entire glomerulus (Tanaka et al., 2009). This anatomical distinction suggested these two populations of LNs might have different synaptic connectivity and thus serve distinct functions in olfactory processing. Indeed, we recently reported that synaptic transmission from LN2, but not LN1 cells is required for the generation of odor evoked neural oscillations (Tanaka et al., 2009). Earlier reports have established that LNs form output synapses onto ORNs and PNs in cockroach (Distler et al., 1997a,b). In *Drosophila*, LNs have been shown to inhibit the output of ORNs and thus help control the gain of olfactory responses (Olsen et al., 2008). It seems clear that LNs serve important circuit functions, but determining the synaptic connectivity of specific types of LNs has been difficult.

Previously, neurons in the *Drosophila* AL made to express green fluorescent protein (GFP) and then immunolabeled with a post-embedding method have been identified in EM images (Shang et al., 2007). However, this approach typically damaged membrane structures, making it difficult to observe and characterize synapses. Thus, we began by working to establish a procedure that included a detergent step to allow antibody conjugated with electron-dense nanogold to penetrate the cell membrane to label the GFP-positive cells while maintaining the integrity of the membrane structure. We injected dextran conjugated with tetramethylrhodamine and biotin into neurons of interest and stained them with 3,3'-diaminobenzidine tetrahydrochloride (DAB) precipitation. Further, we tested a pre-embedding method in which brains were labeled before they were cut into ultrathin sections. This approach offered several advantages: it readily allowed us to evaluate the success of immunolabeling before cutting ultrathin sections; by including most labeling steps in the wholemount preparation, it helped us avoid the nonuniform distortions that can sometime occur from one ultrathin section to the next during postembedding steps; and thus, it permitted reconstruction from serial sections. Using this new method, we tested whether LN2 cells make synapses onto ORNs.

2. Materials and methods

2.1. *Drosophila* rearing

We used female flies expressing GFP in LN2 (genotype: *y w NP2426-GAL4/w; UAS-GFP/+*) (Tanaka et al., 2009). Flies were reared in a box whose temperature was maintained at 23°C and humidity at more than 50% and were fed Jazz-mix (Fisher Scientific).

2.2. Dual labeling method for electron microscopy

Flies were anesthetized by placing them in vials on ice for less than a minute, and were then restrained with wax and epoxy in a plastic chamber. The tip of the antenna was opened with forceps and injected with dextran conjugated with tetramethylrhodamine and biotin (D-7162, Molecular Probes). This compound began to dissolve in *Drosophila* saline (in mM): NaCl 103, KCl 3, TES 5, trehalose 10, glucose 10, sucrose 7, NaHCO₃ 26, NaH₂PO₄ 1, CaCl₂ 1.5, MgCl₂ 4, adjusted to 280 mOsm with sucrose and pH 7.25 with HCl; Tanaka et al., 2009) as it was applied to the olfactory sensory organ. Fresh saline was then applied over the top of the fly body, and a window was opened on the top of the head. After removing the fat and air sacs above the brain, the perineural sheathes around the AL were gently removed with forceps. Within 35 min after the dye injection the brain was dissected out and fixed in 0.5% glutaraldehyde, 4% paraformaldehyde made in phosphate buffered saline (PBS, pH 7.4) at room temperature for 1 hr. Brains were then washed twice in PBS for 5 min and in 0.1% Triton-X/PBS for 5 min, and were then incubated at 4°C overnight without shaking in rabbit anti-GFP antibody (A11122, Invitrogen, diluted at 1:100) in 8% goat serum/0.1% Triton made in PBS. Brains were then incubated overnight without shaking in Alexa Fluor 568-conjugated streptavidin (S-11226, Molecular Probes, 0.5 mg/l) and goat anti-rabbit IgG conjugated with nanogold (#2004, Nanoprobes, diluted at 1:50) in 8% goat serum/0.1% Triton made in PBS at 4°C. To find a treatment to allow antibody conjugated with nanogold to penetrate through the cell membrane, we used goat anti-rabbit IgG conjugated with nanogold and Alexa Fluor 594 (FluoroNanogold-anti-rabbit Fab'-Alexa Fluor 594, #7304, Nanoprobes, diluted at 1:50) and then determined whether the GFP signal overlapped with that of Alexa Fluor 594. Brains were then washed in 8% goat serum/0.1% Triton made in PBS, then in PBS, and were then post-fixed with 2% glutaraldehyde/PBS for 30 min at room temperature. After another washing in PBS and then in distilled water, brains were silver-enhanced (2012 HQ silver, Nanoprobes) for 7 min in a dark room at room temperature. Brains were then rinsed vigorously in distilled water for more than 5 min, and then in PBS, and were then incubated in an avidin-biotin-peroxidase complex (ABC kit, Vectastain) for 1 hr. After washing the brains with PBS, the brains were incubated with 0.067% 3,3'-diaminobenzidine tetrahydrochloride (DAB; D5905, Sigma) in 0.1 M Tris-HCl (pH 7.4) for 30 min and were reacted with 0.03% H₂O₂ in 0.067% DAB in 0.1 M Tris-HCl (pH 7.4) for about 8 min, which precipitated electron-dense material. The brains were then washed in Sorenson's buffer and post-fixed with 0.2% osmium tetroxide/Sorenson's buffer for 1 hr. Then brains were again washed in Sorenson's buffer, then rinsed in 0.1 M acetate buffer, and then dehydrated in ethanol. The brains were infiltrated with increasing concentrations of epon resin (EMbed 812, Electron Microscopy Sciences) by decreasing the concentrations of ethanol. The epon resin was polymerized in an oven set at 60°C overnight. Ultrathin sections at 70 nm thickness were then cut with a Reichert Ultracut E ultramicrotome, mounted on LUXFilm TEM films (#12812-CU, Ted Pella, Inc.), and post-stained with 2% uranyl acetate (Polysciences, Inc.) in 50% methanol and Reynolds lead citrate. Images were obtained with a Jeol 1010 transmission electron microscope operating at 80 kV.

3. Results

Because the LN2 cells are most readily distinguished from LN1 cells by their different GAL4 enhancer-trap lines, we began by using genetic tools to label these neurons, and then used immunogold labeling to render the genetic markers electron dense. We prepared flies in which GFP is expressed specifically in LN2 cells (GAL4 enhancer-trap strain NP2426 crossed with UAS-GFP) (Tanaka et al., 2009). However, we then suffered grievously using a common antibody labeling procedure for paraformaldehyde fixation: after we fixed the brains with glutaraldehyde, although GFP fluorescence remained evident, we were unable to tag the GFP with antibodies, even when using conventional detergent concentrations (Triton-X or saponin: 0.2%, or mixture of 0.1% saponin and Triton-X). One potential reason was that the perineural sheath or cell membrane, once cross-linked by glutaraldehyde, would not allow the antibody to pass through. Thus, we varied the concentration of glutaraldehyde (0.05% to 2%) while using a high concentration of Triton-X (0.2%). But, again, although GFP fluorescence was evident, the antibody did not label the GFP. We next tried dissecting away the perineural sheath of the AL with forceps (Tanaka et al., 2009). This finally enabled immunolabeling of GFP (Fig. 1). We found that, even when the cortex around the AL was desheathed, but the sheath of the AL was left intact, antibody could successfully label the GFP expressed within the AL. These results show that the barrier to antibody labeling was the fixed perineural sheath, and not the fixed cell membrane, and not the inability of the antibody to recognize fixed antigen.

However, we found this method often damaged membrane structures to an extent that prevented us from characterizing synapses with EM. By testing a wide range of concentrations of both of fixative and detergent, we developed a recipe to successfully balance the requirements of labeling GFP with antibody and keeping the membrane structure sufficiently intact: 0.5% glutaraldehyde and 4% paraformaldehyde for prefixation and 0.1% Triton-X for detergent. To render the immunostaining products detectable under EM we used a standard method by which a secondary antibody conjugated with nanogold was enhanced with silver (http://www.nanoprobe.com/tech_help/TechSE.html).

To visualize the presynaptic side of the overlap between ORNs and LN2s we next developed a method for staining a second group of neurons with DAB in addition to the first group of neurons with immunolabeled GFP. Here, we injected Dextran conjugated with biotin and tetramethylrhodamine into the antenna to label the ORNs housed there. Biotin can form an avidin complex with peroxidase, which then precipitates DAB. In combining immunolabeling and DAB staining, we found the most important procedural factor was the order: silver enhancement must precede DAB staining (otherwise, the DAB signal was degraded).

Our new dual procedure of labeling ORNs with DAB and LN2 cells with silver-enhanced nanogold allowed us to visualize LN2 cells presynaptic to ORNs (Fig. 2A). Additionally, we identified synapses from LN2 cells onto the neighboring neurites of LN2 cells (Fig. 2B). In some of these cases an LN2 cell could form output synapses onto not only an LN2 but also onto a neuron clearly not labeled by either nanogold or DAB (which may be an LN1, a PN, or a centrifugal neuron). Because LN2 cells are GABAergic (Okada et al., 2009; Tanaka et al., 2009), the synapses we observed would inhibit ORNs and other LN2s. Because DAB staining sometimes obscured the structure of presynaptic densities, we could not conclude whether ORNs made synapses onto LN2 cells.

4. Discussion

Fast inhibition provided by LN2 cells causes populations of PNs to synchronize in *Drosophila*, leading to odor-elicited neural oscillations (Tanaka et al., 2009). A well-accepted model for the mechanism underlying odor-evoked oscillatory synchronization includes synapses from LNs to PNs and from PNs to LNs, but does not include synapses from LNs to ORNs (Bazhenov et al., 2001). Our demonstration of such synapses suggests that LN2 cells may not only help control the gain of olfactory responses by inhibiting the ORNs (Olsen et al., 2008), but may also, by regulating the output of ORNs, coordinate the precise timing of spikes in the AL.

A traditional way to confirm synaptic connections between a specific pair of neurons is to use electrophysiological techniques, such as causing one neuron to spike and testing whether the other is promptly excited or inhibited. However, in many situations this approach is inefficient and impractical. Another way is to label the two neurons with materials of different electron density and then visualize synapses with an EM, a method that often requires injecting dye or directing labeled antibodies against a neurotransmitter. However, application of this method is limited, because multiple types of neurons can share the same neurotransmitter, and because individual cells labeled by bulk dye injection can be difficult to trace.

Our genetic approach offers several advantages. Using transgenic lines it is possible to label various kinds of neurons specifically and repeatedly. It is possible to identify a neuron's type without tracing it under EM. The synaptic architectures of neurons can be easily studied within complex contexts such as the projection patterns at the light microscopic level, electrophysiological properties, and responses to sensory stimuli.

Simple variations to our dual-labeling method can extend its utility. For example, use of another transcriptional system (Lai and Lee, 2006) can induce the expression of a genetically encoded marker such as membrane-bound horseradish peroxidase (Watts et al., 2004), permitting the labeling of specific populations of neurons without dye injection. A strategy of combining multiple transcriptional systems will help reveal and explain neural circuitry within the brains of animals amenable to genetic manipulation.

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References

- Bazhenov M, Stopfer M, Rabinovich M, Huerta R, Abarbanel HD, Sejnowski TJ, Laurent G. Model of transient oscillatory synchronization in the locust antennal lobe. *Neuron*. 2001; 30:553–67. [PubMed: 11395014]
- Couto A, Dickson BJ. Molecular, anatomical, and functional organization of the *Drosophila* olfactory system. *Curr Biol*. 2005; 15:1535–47. [PubMed: 16139208]
- Distler PG, Boeckh J. Synaptic connections between identified neuron types in the antennal lobe glomeruli of the cockroach, *Periplaneta americana*: I. Uniglomerular projection neurons. *J Comp Neurol*. 1997a; 378:307–19. [PubMed: 9034893]

- Distler PG, Boeckh J. Synaptic connections between identified neuron types in the antennal lobe glomeruli of the cockroach, *Periplaneta americana*: II. Local multiglomerular interneurons. *J Comp Neurol*. 1997b; 383:529–40. [PubMed: 9208997]
- Fishilevich E, Vosshall LB. Genetic and functional subdivision of the *Drosophila* antennal lobe. *Curr Biol*. 2005; 15:1548–53. [PubMed: 16139209]
- Jefferis GSXE, Marin EC, Stocker RF, Luo L. Target neuron prespecification in the olfactory map of *Drosophila*. *Nature*. 2001; 414:204–8. [PubMed: 11719930]
- Kay LM, Stopfer M. Information processing in the olfactory systems of insects and vertebrates. *Semin Cell Dev Biol*. 2006; 17:433–42. [PubMed: 16766212]
- Lai SL, Lee T. Genetic mosaic with dual binary transcriptional systems in *Drosophila*. *Nat Neurosci*. 2006; 9:703–9. [PubMed: 16582903]
- Luo L, Callaway EM, Svoboda K. Genetic dissection of neural circuits. *Neuron*. 2008; 57:634–60. [PubMed: 18341986]
- Marin EC, Jefferis GS, Komiyama T, Zhu H, Luo L. Representation of the glomerular olfactory map in the *Drosophila* brain. *Cell*. 2002; 109:243–55. [PubMed: 12007410]
- Okada R, Awasaki T, Ito K. Gamma-aminobutyric acid (GABA)-mediated neural connections in the *Drosophila* antennal lobe. *J Comp Neurol*. 2009; 514:74–91. [PubMed: 19260068]
- Olsen SR, Wilson RI. Lateral presynaptic inhibition mediates gain control in an olfactory circuit. *Nature*. 2008; 452:956–60. [PubMed: 18344978]
- Ressler KJ, Sullivan SL, Buck LB. Information coding in the olfactory system: evidence for a stereotyped and highly organized epitope map in the olfactory bulb. *Cell*. 1994; 79:1245–55. [PubMed: 7528109]
- Shang Y, Claridge-Chang A, Sjulson L, Pypaert M, Miesenböck G. Excitatory local circuits and their implications for olfactory processing in the fly antennal lobe. *Cell*. 2007; 128:601–12. [PubMed: 17289577]
- Tanaka NK, Awasaki T, Shimada T, Ito K. Integration of chemosensory pathways in the *Drosophila* second-order olfactory centers. *Curr Biol*. 2004; 14:449–57. [PubMed: 15043809]
- Tanaka NK, Tanimoto H, Ito K. Neuronal assemblies of the *Drosophila* mushroom body. *J Comp Neurol*. 2008; 508:711–55. [PubMed: 18395827]
- Tanaka NK, Ito K, Stopfer M. Odor-evoked neural oscillations in *Drosophila* are mediated by widely branching interneurons. *J Neurosci*. 2009; 29:8595–603. [PubMed: 19571150]
- Vosshall LB, Wong AM, Axel R. An olfactory sensory map in the fly brain. *Cell*. 2000; 102:147–59. [PubMed: 10943836]
- Watts RJ, Schuldiner O, Perrino J, Larsen C, Luo L. Glia engulf degenerating axons during developmental axon pruning. *Curr Biol*. 2004; 14:678–84. [PubMed: 15084282]
- Wong AM, Wang JW, Axel R. Spatial representation of the glomerular map in the *Drosophila* protocerebrum. *Cell*. 2002; 109:229–41. [PubMed: 12007409]

Fig 1.
Desheathing allowed antibody to penetrate antennal lobe tissue. The top of the right antennal lobe (AL) was desheathed, resulting in successful anti-GFP antibody labeling (magenta). Green: GFP expressed in the LN2 cells in the AL. Scale bar = 50 μ m.

Fig. 2.

Double labeling revealed synaptic connections among ORNs and LN2s. **(a)** An ORN is stained with DAB, and LN2 cells are labeled with silver-enhanced gold. Asterisks indicate the synapse of an LN2 onto an ORN **(a)** and the presynaptic site of an LN2 onto an LN2 and an unlabeled neuron (?) **(b)**. Scale bar = 500 nm.