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Abstract. Knowledge of neuronal wiring and morphogenesis in *Drosophila* is essential to understand brain function and dysfunction. The immunoenzyme method based on horseradish peroxidase/diaminobenzidine (HRP/DAB) provides high-contrast images to resolve details underlying neuronal architecture. However, the poor staining penetration and a lack of corresponding three-dimensional imaging methodology limit its application. Herein, we modified the HRP/DAB method to stain neuronal circuits in the whole brain of *Drosophila*. Furthermore, we found that imaging with the micro-optical sectioning tomography system provided a fast and automatic method that could dissect cell-specific neuroanatomical architecture at a submicron voxel resolution. [DOI: 10.1117/1.JBO.19.9.090506]

Keywords: micro-optical sectioning tomography; horseradish peroxidase staining; three-dimensional reconstruction; neuroimaging.

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1 Introduction

As a model system in neurobiological studies, *Drosophila* has the advantage of utilizing simple brain circuits for diverse behaviors. Moreover, with the vast array of genomics, proteomics, powerful genetic tools, and large collection of available mutants, *Drosophila* has become an immensely popular experimental animal model.[1] Thus, understanding the brain-wide neuroanatomical architecture of *Drosophila* is essential for formulating hypotheses of neural information flow and deciphering neural mechanisms of brain function and dysfunction.[2]

Currently, two techniques are commonly used for neuroanatomy of the *Drosophila* brain: (1) staining with metal impregnation by Golgi methods and (2) labeling neurons with fluorescent reporters by genetic methods.[3] The Golgi method randomly stains a small population of neurons; early studies have provided an indispensable first step in visualizing the cellular composition of the fly brain. However, using this method, our ability to bridge cell morphology to functional properties is hampered due to its lack of specificity and reproducibility. In contrast, using genetic approaches to target genetic reporters, such as fluorescent protein, tells us the distribution and morphology of specific types of neurons.

Two alternatives exist for detecting expressions of the reporter genes: immunofluorescence (IF) and immunoenzyme staining. Although widely used, IF still suffers from drawbacks, including photobleaching, photofading, and autofluorescence. In contrast, the immunoenzyme staining technique provides colored (absorption-based, opposite to fluorescence), permanent precipitates. Through immunoenzyme staining based on horseradish peroxidase/diaminobenzidine (HRP/DAB), Kimura et al. have observed sexual dimorphism of neurons for investigating the function of the fruitless gene.[4] However, DAB penetrates poorly in thick tissue specimens, giving inconsistent results in deeper-lying cells.[5,6] Meanwhile, the colored reaction product of HRP/DAB cannot be axially resolved by bright-field microscopy and, subsequently, this leads to the lack of three-dimensional (3-D) information to distinguish adjacent neurons or neurites. These two issues restrict the application of HRP/DAB staining to the detection of neurons located in the surface layer.

Here, we set out to develop an immunoenzyme staining method to visualize the 3-D structure of the entire *Drosophila* neural network. In order to accomplish this, we modified the immunoenzymatic neuron labeling technique to attain a uniform staining effect over the whole fly brain. By means of the micro-optical sectioning tomography (MOST) system,[7,8] which combines histological ultrathin sectioning and bright-field line-scan imaging, we accomplished invariable axial resolution and unlimited imaging depth. Thus, by combining the improved HRP/DAB staining methodology and the MOST technique, we have developed a fast, automatic, and high spatial resolution method to image specific neurons in the *Drosophila* brain.

2 Materials and Methods

2.1 *Sample Preparation*

Drosophila melanogaster were grown at 25°C. The flies used were wild-type Canton-S, TPH-GAL4, and UAS-mCD8::GFP. The antibodies used were rabbit antiGFP (1:250, Invitrogen, Carlsbad, California), ChemMate Envision/HRP Kit (DAKO, Glostrup, Denmark), and Alexa 488 goat anti-rabbit (1:500, Invitrogen).

Fly brains were dissected on ice and fixed overnight in 4% paraformaldehyde at 4°C. For immunoenzyme staining, brains were transferred to 0.3% hydrogen peroxide (H_2O_2) in methanol for 10 min to quench endogenous peroxidase activity and treated with citrate antigen retrieval solution (pH 6.0) for 15 min at 95°C after washout of H_2O_2. After blocked 30 min with 5% normal goat serum, brains were incubated for 48 h at 4°C in primary antibodies and then for 24 h at 4°C with secondary antibodies. Next, brains were developed for 30 to 50 min in 0.4 mg/mL DAB (Sigma, St Louis, Missouri) containing 0.005% H_2O_2 and sequentially dehydrated in 50, 70, 85, 95, and 100% alcohol, 100% alcohol-acetone (1:1), and 100% acetone (3×). After dehydration, brains were sequentially infiltrated in 50, 75, and 100% (3×) Spurr resin (SPI, West Chester, Pennsylvania) for 30 min each, followed by fresh 100% Spurr resin overnight.

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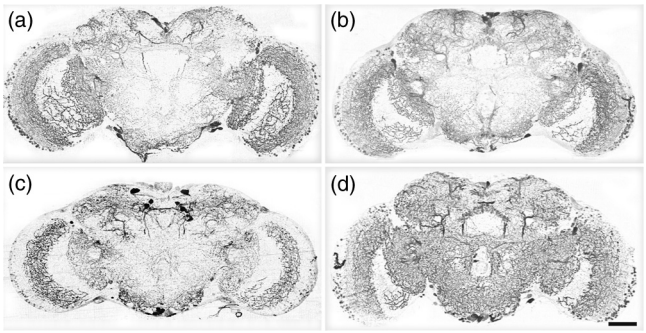

Fig. 1 Projection images (thickness = 35 μm) of fly brains stained under different immunoenzyme staining conditions. The incubation conditions of the second antibody were 4 h at room temperature (a), 12 h at 4°C (b), and 24 h at 4°C [(c) and (d)], respectively. The developing times in the diaminobenzidine (DAB) solution were 15 min [(a) and (c)] and 30 min [(b) and (d)]. Scale bar = 50 μm.

Finally, brains were embedded in 100% Spurr solution and polymerized for 36 h at 60°C. For immunofluorescent staining, fly brains were absolved from H_2O_2 and citrate treatment, and finally mounted in the glycerolbased Vectashield.

2.2 *Data Acquisition and Image Processing*

Using the MOST system, embedded fly brains were ultrathin sectioned (1 μm thickness), simultaneously imaged by a 40× water-immersion objective, and recorded by a line-scan charge-coupled device (voxel size = $0.35 \times 0.35 \times 1.0$ μm^3). As a reference, immunofluorescent specimens were imaged on a Zeiss LSM 780 confocal microscope with 1 μm z-steps using a 40× water-immersion lens (voxel size = $0.21 \times 0.21 \times 1.0$ μm^3).

Raw MOST images were preprocessed as previously described,[7] interpolated to $0.35 \times 0.35 \times 0.35$ μm^3 with Lanczos interpolation, and volume-rendered in three dimensions using Amira (Visage Software, San Diego, California).

3 Results

To attain uniform staining through the whole fly brain, we modified the immunoenzyme staining protocol. We found that incubation conditions of the HRP-conjugated secondary antibody and the DAB solution were the most important factors determining staining penetration. The maximal projection of a stack of coronal sections (thickness: 35 μm) in the center part of the fly brain was used to assess stain penetration (Fig. 1). As shown in Fig. 1(a), the shorter time the brains were incubated in the secondary antibody and the DAB solution, the less the brain was stained. Prolonging the incubation time of either step promoted stain penetration; however, the signal in the center of the brains remained weak [Figs. 1(b) and 1(c)]. We found that the best condition to obtain uniform brain-wide staining was to incubate for 24 h in secondary antibodies and 30 min in DAB solution [Fig. 1(d)]. In addition, for smoothing the high background caused by prolonging the reaction time, a 10-min immersion in 0.3% H_2O_2 was used to quench endogenous peroxidase.

To verify the invariable axial resolution and unlimited imaging depth of the MOST system, we compared immunoenzyme images from the MOST system [Figs. 2(a) to 2(c)] and IF images from the confocal microscope [Figs. 2(d) to 2(f)]. To distinguish between methods, a local brain region at the same location was zoomed-in. As can be seen from Figs. 2(b) and 2(e), the resolution in the x–y plane is similar for both types of imaging. The difference, however, could be observed when the same local brain region was rotated along the y axis [Figs. 2(c) and 2(f)]; the neural fibers acquired by the MOST system were continuous and uniform in three dimensions [Fig. 2(c)], while they became spread out and fuzzy along the z axis in the confocal system [Fig. 2(f)]. These results confirmed that the MOST system ensured consistent axial resolution and image quality in depth, and also verified the stronger signaling and contrast of the modified immunoenzyme staining method.

Finally, we acquired 3-D datasets of serotonin-specific neural circuits driven by the TPH-Gal4 transgenic line in the whole fly brain at a submicron voxel resolution. Taking advantages of the fast imaging speed and automated data collection of the MOST system, the average imaging time of one whole fly brain was ~10 min, much faster than that of traditional confocal microscopy. Meanwhile, no additional registration was needed because of the accurate spatial positioning of the obtained images.

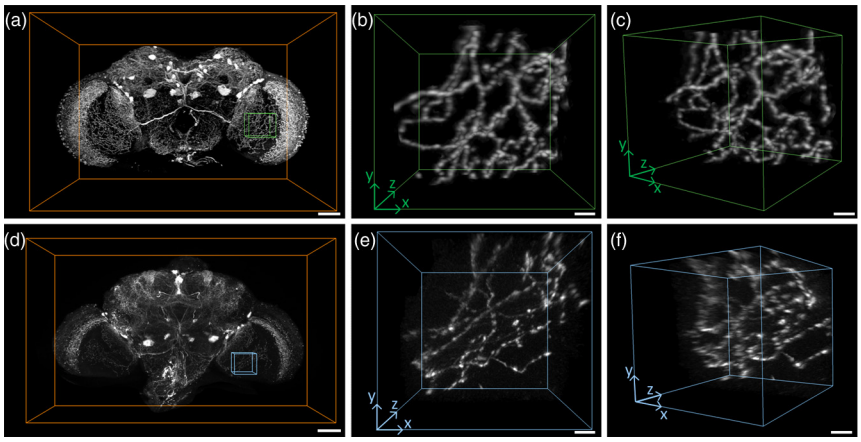

Fig. 2 Images reconstructed from a stack of coronal sections (thickness: 56 μm) acquired by the micro-optical sectioning tomography system (a) and confocal microscopy (d). (b) and (e) Enlarged views of local brain regions (volume size: $50 \times 45 \times 55$ μm^3) marked in (a) and (d). (c) and (f) The same local brain region in (b) and (e) rotated 30 deg around the y axis. Scale bar = 50 μm [(a) and (d)] and 5 μm [(b), (c), (e), and (f)].

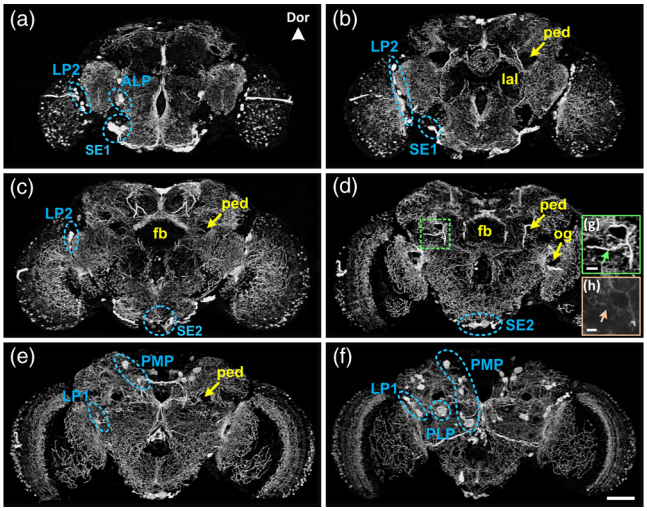

Fig. 3 [(a) to (f)] Coronal projection images of one immunostained *Drosophila* brain {thickness: 21 μm [(a) to (d)] and 35 μm [(e) and (f)]}. (g) Enlarged views of the region marked in (d). (h) Region corresponding to (g) imaged by confocal microscopy. Arrows mark neural processes. The nomenclature for naming the cell clusters is according to the previous studies.[9–11] LP1, cells between the lobula and the protocerebrum, posterior lateral protocerebrum; LP2, cells between the medulla/central neuropil; ALP, anterior cell body rind, lateral to midline; PLP, posterior lateral protocerebrum; PMP, posterior cell body rind, medial to the calyx, running dorso-ventral; SE1: anterior subesophageal neurons; SE2, posterior to SE1; Dor, dorsal; fb, fan-shaped body; lal, lateral accessory lobes; ped, mushroom body pedunculus; og, optic glomerulus. Scale bar = 50 μm [(a) to (f)] and 10 μm[(g) and (h)].

To show the brain-wide distribution of the serotonin-specific neural processes, a series of coronal projection images of the entire fly brain are presented in Fig. 3. As shown, serotonin-containing neurons are widely distributed in most brain areas, but are comparatively sparse in the fan-shaped body, mushroom body pedunculus, lateral accessory lobes, and optic glomeruluses. According to the location of the cell bodies, serotonin neurons driven by the TPH-Gal4 transgenic line were classified into several distinct clusters: anterior lateral protocerebrum (ALP), lateral protocerebrum (LP), subesophageal (SE), posterior lateral protocerebrum (PLP), and posterior medial protocerebrum (PMP). A large portion of the serotonergic neurons could be observed in our results and their distributions are consistent with previous studies.[9–11] Furthermore, our results provide some unique neuronal structure. As shown in an enlarged view of local brain region, the neural process (marked by an arrow) can be resolved clearly in Fig. 3(g) rather than being faintly visible as in Fig. 3(h) (corresponding immunofluorescent results). To our knowledge, this is the first 3-D immunoenzyme-stained *Drosophila* brain dataset to offer a high-contrast, comprehensive outlook on the fly brain and could, thus, be a valuable comparison tool for more specific, targeted studies.

4 Summary

In summary, by combining the improved immunoenzyme staining method and MOST technology, we demonstrated fast, automatic, and high spatial resolution imaging of specific neurons in the *Drosophila* brain. By optimizing the experimental conditions of HRP-conjugated secondary antibodies and DAB developing, the penetration of the immunoenzyme stain could be extended to the whole fly brain. Furthermore, using thin-section imaging via the MOST system, we were able to achieve a 3-D volume rendering of the HRP/DAB-stained fly brain. Compared with conventional histological methods, automation of data collection in the present study greatly improved the imaging speed of anatomical studies, avoided the burdensome manual operation, and ensured the coherence of the acquisition conditions such that the datasets were more standardized. High axial resolution and better image uniformity would provide great conveniences for subsequent image analyses. Furthermore, in combination with advanced genetic tools, such as the mosaic analysis with a repressible cell marker (MARCM) system and flippase (FLP)-out, this method offers the potential to provide a finer atlas of the *Drosophila* nervous system.

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