

RapiClear[®]

Maximize Signal-to-Noise Ratio

SunJin Lab

Optical Clearing Innovation

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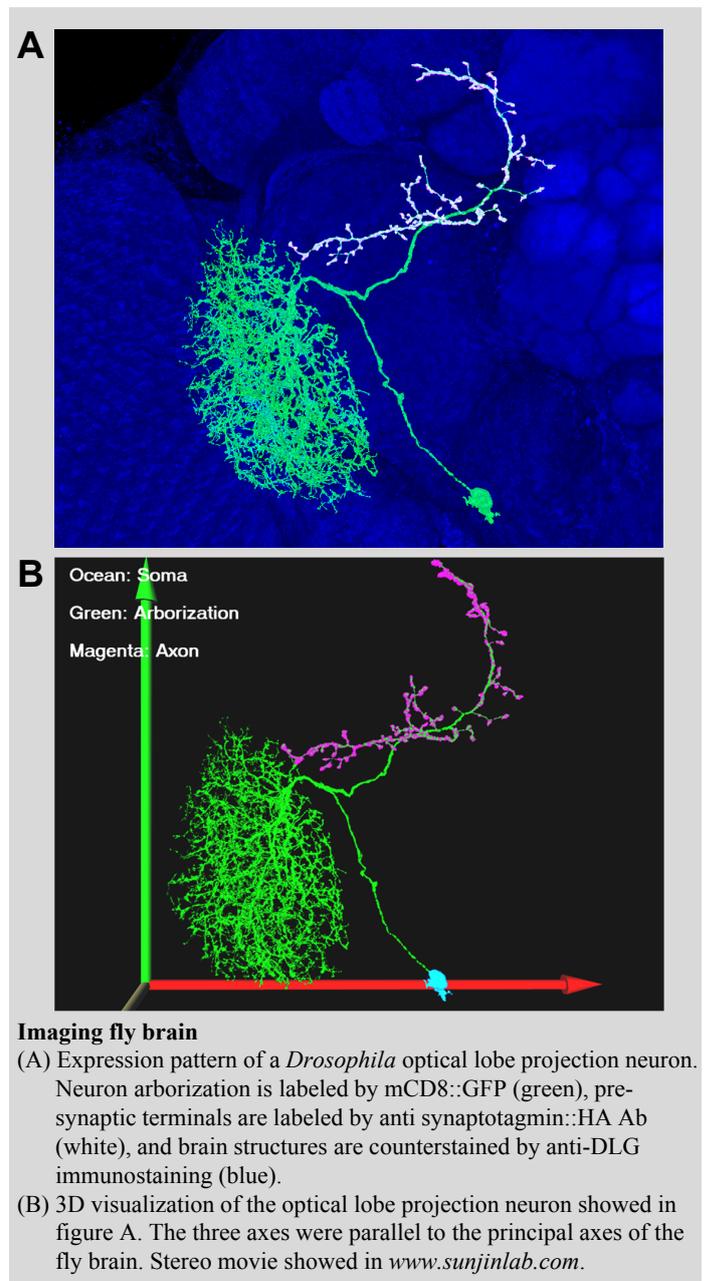
Protocol for *Drosophila* Brain Immunostaining

Preparing any type of chemical please read the MSDS book to determine what types of safety clothing and equipment you should wear

MATERIALS

REAGENTS and EQUIPMENTS

- 1X PBS (100 mM Na₂HPO₄/NaH₂PO₄, pH 7.2)
- Triton[™] X-100 (Sigma-Aldrich, cat. no. T8787)
- Sodium azide (Sigma-Aldrich, cat. no. S2002)
- Paraformaldehyde (Electron Microscopy Sciences, #15713-S)
- Normal goat serum (Lampire Biological Laboratories, #S2-0609)
- Primary antibody: for example, the 4F3 mouse anti-discs large (DLG) antibody (1:50) is used to label general neuropils in *Drosophila* brain or rabbit anti-HA antibody (Abcam; 1:2000).
- Secondary antibodies and streptavidin conjugated florescence dye: these may include Alexa Fluor-546 anti-mouse and biotinylated goat anti-rabbit (1:250) and Alexa Fluor 635 streptavidin (1:500).
- RapiClear 1.47 (SunJin Lab Co. #RC147001/RC147002)**
- Vacuum oven and pump (Risen Inc. RUD-30L)
- 24-well immunostaining plate
- Microwave oven (2,450 MHz, 1100 Watts). The microwave energy was measured by heating one liter of water, to be 34.98±1.60 kcal, at room temperature.
- Orbital shaker
- 1.5-ml microcentrifuge tubes
- Two pairs of sharp forceps (Dumont, no. 55)
- Loop (~0.5 mm diameter)
- Dissection dishes
- Kimwipes
- Coverslips
- Slides
- Clear nail polish
- iSpacer (SunJin Lab Co., #IS006 or IS007)**
- Confocal microscope equipped with an argon-krypton laser (458, 488, or 514 nm) and two HeNe lasers (543 and 633 nm).
- 40X water-immersion objective lens (N.A. value ≥ 1.2) or 63X water-immersion objective lens (N.A. value ≥ 1.4).



STOCKS

- 20% PBST Add 20 ml Triton-X 100 to 80 ml PBS and store at 4°C.
- 1% sodium azide Add 0.5 g sodium azide to 50 ml PBS. Store at 20°C. **!CAUTION Toxic.**
- Fixation solution Add 10 ml 16% w/v paraformaldehyde to 30 ml 0.25% (vol/vol) PBST in a 50 ml tube. Must be prepared fresh and placed at 4°C. **!CAUTION Toxic.**
- Washing Buffer Add 30 g NaCl and 5 ml 20% PBST to 995 ml PBS. Store this nonhazardous buffer at 4°C.
- Blocking buffer 10% (vol/vol) NGS containing 0.5 ml 20% PBST, 0.5 ml NGS and 0.1 ml 1% sodium azide in 3.9 ml PBS. Store this solution for only a short period (overnight at the very most) at 4°C.
- Dilution Buffer Add 62.5 ul 20% PBST, 50 ul NGS and 100 ul 1% sodium azide to 4,787.5 ul PBS. Store this solution for only a short period (overnight at the very most) at 4°C.
- Primary antibody 1:50 mouse anti-DLG monoclonal antibody and 1:2000 rabbit anti-HA polyclonal antibody in dilution buffer.
- Secondary antibody 1:250 biotinylated goat anti-rabbit and 1:200 Alexa-546 anti-mouse in dilution buffer.
- Florescence dye 1:500 Alexa Fluor 635 streptavidin in dilution buffer.

PROCEDURES

DISSECTION

- Anaesthetize adult flies on ice.
- Place flies onto a dissection dish and immerse them in PBS.
- Remove the head cuticle from the brain and clean the brain with gentle forceps manipulation under a dissecting microscope.
- Collect the dissected brains using a loop without touching the brain and place them in a 24-well plate with 100 ml PBS in each well on ice.

FIXATION

- The brain is placed in 4% paraformaldehyde in PBS on ice and rapidly fixed with microwave irradiation for 90 s on a rotation plate, three times. **!CAUTION** Seal the well with a color tape to avoid dehydration.
- Keep the brain in blocking buffer at room temperature for 2 hours.
- Expel the air trapped in the tracheal system by keeping the

sample in blocking buffer within a vacuum chamber (depressurize to -70 cmHg and keep it for 10 min), 5 cycles.

- Keep the brain in the blocking buffer in 4°C overnight.

IMMUNOHISTOCHEMISTRY

- Brain samples are washed with washing buffer for 30 min at room temperature, 3 times.
- Incubate with anti-DLG as well as anti-HA antibodies (120 µl/well) on an orbital shaker at 4°C for 2 days.
- Wash with washing buffer for >2 hrs at room temperature, 3 times.
- Keep the samples in washing buffer on an orbital shaker at 4°C for overnight.
- Incubate the samples with biotinylated anti-rabbit and Alexa-546 anti-mouse (120 µl/well) on an orbital shaker at 4°C for 2 days.
- Wash with washing buffer for >2 hrs at room temperature, 3 times.
- Incubate with Alexa Fluor 635 streptavidin on an orbital shaker at 4°C for overnight.
- Wash with washing buffer for >2 hrs at room temperature, 3 times.
- Mount the brains in a drop of *RapiClear*TM 1.47 under a coverslip separated by a iSpacer, so the brain shape is not flattened.

IMAGING

- Each image stack contains 120~160 optical sections with 0.32 x 0.32 x 1.0 µm³ voxel size taken under a 40X or 63X objective lens. Following settings were used: scanning speed 7, resolution 1,024 X 1,024 voxels, zoom 0.7, optical slice thickness 1 µm, 50% overlap between two slices and averaged 4 times each scanning line.
- Two channels were simultaneously scanned: GFP-labeled neurons were excited using a 488 nm ArKr laser and Alexa Fluor 635-labeled pre-synaptic terminals were excited using a 633 nm HeNe laser. Alexa Fluor 546-labeled neuropilar structures were excited using a 543 nm HeNe laser

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