Protocol

Visualizing Axons in the *Drosophila* Central Nervous System Using Immunohistochemistry and Immunofluorescence

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INTRODUCTION

How axons in the developing nervous system navigate to their correct targets is a fundamental question in neuroscience. Studies of axon guidance in the embryonic central nervous system (CNS) of *Drosophila melanogaster* have proved instrumental to the identification of molecules and mechanisms that regulate wiring. The relative simplicity of the *Drosophila* embryonic CNS, the advantages of genetic approaches, and the ability to analyze the pathfinding decisions of well-defined axon groups or even individual axons have all contributed to our understanding of the fundamental mechanisms of axon guidance and target selection. In this protocol, we describe methods for using fluorescent antibody staining as well as horseradish peroxidase (HRP) immunohistochemistry to reveal axon-projection patterns and identify specific guidance molecules. In the presence of 3,3′-diaminobenzidine tetrahydrochloride (DAB), HRP forms a brown, stable precipitate that allows visualization of axon populations under white light. This can be quite advantageous when teaching basic embryonic anatomy and dissection techniques, because unlike fluorescent antibody staining, HRP can be visualized in a lighted room. HRP detection does not allow precise colocalization of signals, nor can multiple colors be visualized simultaneously. These objectives are easily achieved with fluorescent antibody staining.

RELATED INFORMATION

Images generated by two-color fluorescent staining with markers used to assess midline crossing, commissure choice, lateral position, and motor axon guidance are shown in Figure 1. Procedures for the visualization of surface pools of receptors and the surface distribution of secreted cues are presented in **Live Dissection and Surface Labeling of Proteins in *Drosophila* Embryos** (Bashaw 2010).

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <**!**>, and recipes for reagents marked with <**R**>.

Reagents

The antibodies listed below are those used to stain the embryos visualized in Figure 1. However, many other antibodies are available for the detection of subsets of neurons and glia in the embryonic CNS. Secondary antibodies conjugated with HRP are required for immunohistochemistry.

Antibodies, primary (diluted to final concentrations in blocking solution)

Anti-BP102 (1:50 dilution; mouse monoclonal; labels most CNS axons) (DSHB)

Anti-FasII (1:50 dilution; mouse monoclonal; labels subsets of interneurons and all motor neurons) (DSHB)

Anti-GFP (green fluorescent protein; 1:500 dilution; rabbit polyclonal) (Molecular Probes/Invitrogen)

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Antibodies, secondary (diluted to final concentrations in blocking solution)
Cy3-conjugated goat anti-mouse (1:1000 dilution) (Jackson ImmunoResearch)
Alexa488-conjugated goat anti-rabbit (1:500 dilution) (Molecular Probes/Invitrogen)
<!>Bleach (Clorox)
Blocking solution (PTX containing 5% normal goat serum)
<!>DAB (3,3′-diaminobenzidine tetrahydrochloride) (Sigma-Aldrich D5905; supplied as 10-mg tablets)
Drosophila embryos
<!>Formaldehyde (histological grade; 37%, w/v)
Glycerol (70% in PBS)
<!>Heptane
<!>Hydrogen peroxide (3%, v/v)
<R>Juice plates
<!>Methanol
Normal goat serum (GIBCO 16210-064)
Heat the goat serum for 30 min at 65°C. Divide into 5-mL aliquots and store at -20°C.
<R>PBS (10X stock; pH 7.0)
Dilute to 1X prior to use.
<R>PTX
Tween 20

Equipment

Microcentrifuge tubes (1.5-mL)
Micropipettor (P1000) and tips
Nytex mesh
Pasteur pipettes
Rotating platform (e.g., Nutator mixer)
Scintillation vials (glass)
Squirt bottle
Vortex mixer

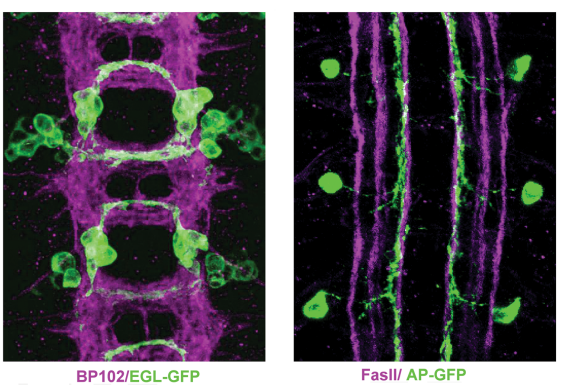

FIGURE 1. Visualization of subsets of neurons in fly embryos. Stage 16 *Drosophila* embryos expressing UASTauMycGFP under the control of either Eagle-Gal4 (*left*) or Apterous-Gal4 (*right*) are stained with anti-GFP (green) and MAb BP102 (magenta; *left*) or anti-FasII (magenta; *right*). EglGal4 specifically labels two clusters of commissural neurons. One cluster, the EG neurons, consists of ~10-12 cells that project their axons across the midline in the anterior commissure. The second cluster, the EW neurons, consists of three to four cells that project their axons in the posterior commissure. The BP102 and Egl markers can both be used to assess midline crossing, and Egl is also a good marker for examining commissure choice. ApGal4 labels three ipsilateral interneurons per hemisegment and FasII labels several bundles of longitudinal interneurons as well as motor neurons. Both Ap and FasII can be used to evaluate lateral positioning and test for abnormal midline crossing; FasII is also an excellent marker to look at motor axon guidance. Anterior is up in both panels. (For color figure, see doi: 10.1101/pdb.prot5503 online at www.cshprotocols.org.)

METHOD

Collection and Fixation of Embryos

1. Collect *Drosophila* embryos (for 20-24 h at 25°C) on small apple (or grape) juice plates.
2. Dechorionate the embryos by immersing them in 50% bleach for 3-5 min. Squirt the bleach directly onto the plate.
3. Strain the embryos through Nytex mesh and rinse with plenty of water.
4. Transfer the embryos to glass scintillation vials and fix with 10 mL of 3.7% formaldehyde (diluted in either water or PBS) and 10 mL of heptane by rocking on a rotating platform for 15 min.
   The embryos should float at the interface.
5. Remove the lower formaldehyde phase with a Pasteur pipette. Take care to remove as much fixative as possible.
6. Add 10 mL of methanol. Shake and vortex for 1 min.
   Many embryos should sink.
   See Troubleshooting.
7. Remove the methanol/heptane and, using a P1000 pipette, transfer the embryos to a microcentrifuge tube.
8. Rinse the embryos two to three times with methanol. Store at –20°C or proceed to staining.
   See Troubleshooting.

Basic Antibody Staining

In all steps (except rinses), mix the reagents by rocking on a rotating platform.

9. Rinse the embryos twice in PTX.
   Some antibodies (e.g., anti-Robo) do not perform well when incubated in Triton X-100; try Tween 20 instead.
   See Troubleshooting.
10. Wash once in PTX for 5 min.
11. Add 250 µL of blocking solution. Rock for 5-10 min.
12. Add the primary antibody. Use dilutions of between 1:25 and 1:100 for monoclonals and between 1:500 and 1:2000 for polyclonals. Incubate overnight at 4°C.
13. Rinse three times in PTX.
14. Wash twice in PTX for 5 min each.
15. Add the secondary antibody. Use dilutions of 1:250 for HRP conjugates and between 1:500 and 1:2000 for fluorescent conjugates.
16. Incubate for ≥30 min at room temperature.
17. Wash as in Steps 13 and 14.

HRP Immunohistochemistry

For fluorescent staining, omit Steps 18-21 and proceed with clearing.

18. Incubate in 300 µL of DAB (0.3 mg/mL in PBS containing 0.1% Tween 20).
19. Add 5 µL of 3% hydrogen peroxide.
20. Develop for 2-30 min.
    See Troubleshooting.
21. Stop the reaction by rinsing several times in PTX.
22. Rinse once in PBS.

23. Clear in 70% glycerol (in PBS) for at least 2 h.
Embryos can be stored forever in glycerol at 4°C. Do not freeze!

TROUBLESHOOTING

Problem: When the methanol is added, the solution turns milky.
[Steps 6 and 8]
Solution: In Step 5, it is important to remove as much of the lower fixative phase as possible. If too much fixative is left behind, the solution will turn milky when the methanol is added, obscuring the view of the embryos. This can be remedied by adding more methanol.

Problem: The embryos clump together when PTX solution is added.
[Step 9]
Solution: Unfortunately, if this happens, the embryos must be discarded and a fresh collection obtained. This occurs when residual heptane is left on the embryos. It is important to rinse the embryos adequately with several changes of methanol after fixation.

Problem: A weak signal is observed despite a long developing time.
[Step 20]
Solution: The most common reason for a weak signal in HRP-staining experiments is inferior hydrogen peroxide, which has been stored for too long or exposed to light. Use a new bottle.

REFERENCES

Bashaw GJ. 2010. Live dissection and surface labeling of proteins in *Drosophila* embryos. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot5504.

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