Protocol

A Solid-Phase Immunostaining Protocol for High-Resolution Imaging of Delicate Structures in the Drosophila Larval Central Nervous System (CNS)

Elizabeth A. Daubert and Barry G. Condron

Department of Biology, University of Virginia, Charlottesville, Virginia 22903-4328, USA

1Corresponding author (Condron@virginia.edu)

INTRODUCTION

This protocol describes a method for mounting and immunostaining Drosophila larval tissue in preparation for high-resolution fluorescent imaging of fine structures in the central nervous system (CNS). Affixing the tissue directly to the coverslip and then moving the coverslip between wash solutions provides a simple solid-phase method of immunostaining that assists in preserving fine structures. This method also easily allows for manipulations and/or viewing of the live sample prior to fixation if desired. Finally, putting the tissue in direct contact with the coverslip places fine structures immediately adjacent to the objective lens. We also briefly describe a method to create three-dimensional (3D) models of confocal Z-stacks in order to better characterize fine structures by measuring their volume and obtaining 3D Cartesian coordinates in space.

RELATED INFORMATION

Here we specifically describe the imaging of serotonergic varicosities within the neuropil of the larval CNS (Sykes and Condron 2005), but this protocol could be expanded to a variety of tissues. Representative images obtained using this protocol are shown in Figure 1.

MATERIALS

Reagents

Figure 1. Representative images. (A) Z-projection of third instar larval ventral nerve cord stained for serotonin. Abdominal segments A4-A8 are shown at 100X magnification. Note the delicate neuropil staining as well as cell bodies. (B) The A7 segment has been cropped and rebuilt in 3D using Improvision Volocity rendering software. The A7 segment is rotated away from the viewer as compared to A. (C) The cell body has been cropped away from the left hemisegment to reveal the varicosities to be analyzed. (D) The varicosities counted and measured by the rendering software are highlighted to compare to C.
\textit{Drosophila} larvae, mostly clean of fly food/yeast paste

- 90% glycerol mount
- 4% paraformaldehyde in PBS for immunostaining
- PBS (pH 7.2)

Prepare 10X PBS without \(\text{CaCl}_2\) or \(\text{MgCl}_2\). Filter through a 0.22-\(\mu\)M filter, and dilute 1:10 with \(\text{H}_2\text{O}\) to make working 1X PBS solution.

PBST for immunostaining

Primary antibody

\textit{Multiple antibodies may be used for double and triple labeling.}

- 1% PS insect medium

Secondary antibody (fluorescent; e.g., AlexaFluor)

\textbf{Equipment}

- 3D rendering software (Improvision Volocity, http://www.improvision.com)
- Coverslips, 18 mm\(^2\) (No. 1 for sample attachment, No. 2 for live imaging setup)
- Dissecting microscope with 20X-30X magnification
- Dissecting tools (Fine Science Tools No. 5 forceps and/or small tungsten needles)
- Fingernail polish
- Fluorescent confocal microscope with 100X oil lens (with camera)
- Glass microscope slides
- Hypodermic needle
- Image editing software (e.g., Adobe Photoshop)
- Kimwipes
- Petri dishes (35 x 10 mm)
- Petroleum jelly (e.g., Vaseline)
- Pipettes and pipette tips (200 \(\mu\)l)
- Shaker/rocker for all washes and incubations (e.g., Reliable Scientific 12-inch x 16-inch shaker set to \(\sim\)25 rpm)

\textbf{METHOD}

1. Under a dissecting microscope, dissect the CNS from the larva in at least 3 ml of 1% PS insect medium at room temperature. Immediately transfer the CNS to 3 ml of clean medium. If
manipulations and/or live viewing are desired, continue with Step 2. If the sample is to be fixed immediately, proceed immediately to Step 7.

*It is crucial that the CNS be immediately transferred to clean medium upon dissection. Substances released from dissected larvae (yeast, digestion enzymes) will diminish the tissue quality if allowed to remain with dissected tissue.*

**Manipulations/Live Viewing of Sample**

2. Pipette the CNS tissue to a clean, dry No. 1 coverslip in ~10 µl of medium. Orient the tissue so that the desired viewing region is adjacent to the coverslip.

3. Prepare a glass slide by putting two No. 2 coverslips on it (attached with petroleum jelly), leaving space between them to form a small well (see Fig. 2).

4. Invert the coverslip with the tissue samples, and place the inverted coverslip on the glass slide so that the tissue is positioned in the well (see Fig. 2).

5. Flush the well with insect medium (alone or containing pharmacological treatments) as follows:
   - i. Place the slide on an incline, and apply liquid to the top of the well with a pipette.
   - ii. Place a Kimwipe at the bottom of the well to draw liquid through the well via capillary action. *Medium should be flushed through often (every 30 min) if the sample is to remain in this state for an extended time before fixation (e.g., time-lapse imaging, drug treatments).*

6. When fixation is desired, flush the well with the 4% paraformaldehyde solution, and fix for 1 hour at room temperature. Carefully remove the coverslip with forceps, and proceed to Step 10.

**Immediate Fixing of Sample**

7. Place a coverslip in a 35 x 10-mm Petri dish with 2 ml of 1% PS insect medium.

8. Using a pipette, move the dissected CNS tissue to the coverslip. Using forceps or a small needle, gently press the tissue to the coverslip, taking care not to crush the sample. The tissue should be
oriented so that the most delicate structures to be imaged are adjacent to the glass of the coverslip. Provided the coverslip has not been previously exposed to protein, the CNS should easily stick to it. Jostling the dish should reveal that the tissue is adhered to the coverslip.

9. Add 3-4 ml of 4% paraformaldehyde solution to the sample, and incubate for an hour at room temperature. After fixation, the protocol can be interrupted and tissue can be stored in 1X PBS at 4°C overnight if necessary.

**Incubation of Samples with Antibodies**

10. Quickly rinse the samples twice in 3 ml of 1X PBS. Use a pair of forceps to rapidly move the coverslip (with attached tissue) between dishes of wash solutions. Briefly drain the coverslip between washes. Move the coverslip between rinses and washes quickly to avoid drying out. All rinses and washes can be performed in 35 x 10-mm Petri dishes with 3-4 ml volumes.

11. Rinse the samples twice in 3 ml of PBST.

12. Place the samples in 2 ml of PBST containing an appropriate primary antibody dilution, and incubate them with gentle agitation as follows:
   i. For second instar and younger specimens, incubate overnight at 4°C.
   
   ii. For third instar specimens, incubate for two nights at 4°C or overnight at room temperature to help ensure antibody penetration.

13. Wash the samples extensively in 3 ml of PBST at room temperature with gentle agitation as follows:
   i. After incubation at 4°C in Step 12, the samples can be washed three times for 10 minutes each.
   
   ii. After incubation at room temperature in Step 12, wash the samples six times over 2 hours to obtain maximum reduction of background signal.

14. Place the samples in 2 ml of PBST containing an appropriate fluorescent secondary antibody dilution (e.g., AlexaFluor secondary at 1:1000). Incubate the samples for 2-8 hours at room temperature or overnight at 4°C.

15. Wash the samples three times in 3 ml of PBST over a total of 45 minutes at room temperature.

16. Rinse the samples twice in 3 ml of 1X PBS.

17. Place the samples back into 3 ml of 4% paraformaldehyde (PFA) solution for 10 minutes at room temperature. This locks the antibodies onto the sample.

18. While the samples are in PFA, draw a square ring of petroleum jelly onto a slide using a hypodermic needle.

19. Using the end of a 200-µl pipette tip, dab a large drop of 90% glycerol mount solution onto the slide within the petroleum jelly ring.

20. Take the samples out of PFA, and rinse them in 3 ml of 1X PBS.
21. Rinse the samples in 3 ml of H₂O.

22. Remove the coverslip from the H₂O using forceps, and gently dab excess liquid from the edges using a Kimwipe.

23. Flip the coverslip so that the samples are facing down, and place it gently onto the petroleum jelly ring on the slide.

24. Seal the coverslip to the slide using fingernail polish.

25. Place the sealed slide overnight at -20°C before imaging. *Leaving the mounted sample here overnight allows the glycerol and DABCO in the mount solution to exchange with H₂O in order to change the refractive index of the tissue for imaging.*

**Imaging and Visualizing Varicosities**

26. Set the confocal microscope with 100X magnification to record a stack from the start of neuropil staining to just beneath cell body staining, being sure to image the neuropil first. Image with 1 x 1 binning and 0.1-µm-thick sections. *Exposure times may vary from 150 to 1000 msec, depending on immunofluorescent intensity.*

27. Export images to image editing software (e.g., Adobe Photoshop). Adjust the levels to reduce background and enhance contrast (autolevel) for each image of the stack, and adjust the bit depth to 8 bits. *Bit adjustment will be necessary to allow the 3D rendering software to process the large amount of data.*

28. Import the leveled stack into 3D rendering software (Improvision Volocity) and create a Z-projection. Crop the image to the region of interest. *The cropping step could also be performed with the image editing software in Step 27.*

29. If desired, crop off the cell bodies, leaving only the varicosities. *Isolating the varicosities is necessary if the program is to count and/or measure varicosities subsequently.*

30. Set Volocity to count objects falling within a volume range of 0.2-8 µm³ and to separate touching objects and reduce noise.

31. Use Volocity to measure the objects counted to obtain varicosity volumes, Cartesian coordinates, and so on.

**TROUBLESHOOTING**

**Problem:** Samples fall off coverslip.

**[Step 8]**

**Solution:** Samples will fall off simply because they were not stuck down properly to start. There should be no protein on the coverslip or else the tissue will not stick to it before fixation. Make sure to move the tissue to the coverslip in clean media (i.e., free of any matter from the dissection) to avoid this problem.
Problem: Poor signal.

[Step 12]

Solution: Increasing the wash times, especially after primary antibody incubation, often increases the signal relative to the noise. However, depending on the antibodies used, type of tissue used, and other factors, the antibody concentrations, antibody incubation times/temperatures, and wash times may all require optimization.

Problem: Sample fades quickly during imaging.

[Step 25]

Solution: Sufficient clearing time in 90% glycerol mount is necessary to obtain the best possible images. Samples must remain in the glycerol mount solution overnight at -20°C. Thicker or denser tissues may require longer time periods. If samples have received sufficient exposure to the glycerol mount solution and fading remains a problem, remake the 90% glycerol mount solution with new DABCO.

DISCUSSION

Affixing the nervous tissue directly to a coverslip in this protocol ensures that the neuropil will be as close as possible to the objective lens, which will help reduce photobleaching of delicate staining during imaging. Attachment of tissue to the coverslip also reduces immunostaining time as the samples can be quickly drained between each wash. Fixing the CNS directly to the coverslip is also useful when working with tissue that tends to be lost by sticking to the walls of microcentrifuge tubes, such as embryonic CNS. However, owing to the large wash volumes necessary, antibody volumes must be adjusted and rare or expensive antibodies may be used excessively.

REFERENCES


Caution

General warning

This material contains hazardous components. Please see recipe for full details.

Recipe

1% PS insect medium

Schneider’s insect medium (Sigma)

Penicillin-streptomycin solution (5000 units/ml penicillin, 5 mg/ml streptomycin) (P4458; Sigma)

Add 10 ml of penicillin-streptomycin solution to 1 liter of Schneider’s insect medium. Store the solution at
4°C-8°C.

Recipe

4% paraformaldehyde in PBS for immunostaining

- 100 ml 1X PBS
- 4.0 g paraformaldehyde (laboratory grade)

Place the PBS and paraformaldehyde in a bottle together, and put the bottle in a water bath set to 70°C-75°C. Swirl the solution occasionally until all of the paraformaldehyde is dissolved (may take a couple of hours). Cool the solution to room temperature, and filter it through a 0.22-µm filter to remove any particulate matter. Store it at 4°C-8°C.

Recipe

90% glycerol mount

- 18 ml glycerol
- 2 ml 1X PBS
- 0.5 g DABCO

Stir at room temperature until DABCO goes into solution. Store at 4°C-8°C.

Recipe

PBS

- 137 mM NaCl
- 2.7 mM KCl
- 10 mM Na₂HPO₄
- 2 mM KH₂PO₄

To prepare 1 liter of PBS(Phosphate-buffered Saline), dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄, and 0.24 g of KH₂PO₄ in 800 ml of distilled H₂O. Adjust the pH to 7.4 (or 7.2 if required) with HCl. Add H₂O to 1 liter. Dispense the solution into aliquots and sterilize them by autoclaving for 20 minutes at 15 psi (1.05 kg/cm²) on liquid cycle or by filter sterilization. Store the buffer at room temperature. If necessary, PBS may be supplemented with 1 mM CaCl₂ and 0.5 mM MgCl₂. Can be made as a 10x stock.
Recipe

PBST for immunostaining

1 g BSA

5 ml 20% Triton X-100

1 liter 1X PBS

Stir the solution at room temperature to dissolve the BSA. Filter it through a 0.22-µm filter, and store at 4°C-8°C.

Copyright © 2007 by Cold Spring Harbor Laboratory Press. Online ISSN: 1559-6095 Terms of Service All rights reserved. Anyone using the procedures outlined in these protocols does so at their own risk. Cold Spring Harbor Laboratory makes no representations or warranties with respect to the material set forth in these protocols and has no liability in connection with their use. All materials used in these protocols, but not limited to those highlighted with the Warning icon, may be considered hazardous and should be used with caution. For a full listing of cautions, click here.

All rights reserved. No part of these pages, either text or images, may be used for any reason other than personal use. Reproduction, modification, storage in a retrieval system or retransmission, in any form or by any means-electronic, mechanical, or otherwise-for reasons other than personal use is strictly prohibited without prior written permission.