Protocol

Immunohistochemistry and Morphometric Analysis of *Drosophila* Larval Body Wall Neuromuscular Junction Preparations

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The *Drosophila* neuromuscular junction (NMJ) is an excellent model for studying vertebrate glutamatergic synapses. Researchers have uncovered fundamental mechanisms at the fly NMJ that are conserved in higher-order organisms. To gain molecular and structural insight into these and other structures, immunolabeling is invaluable. In this protocol, we describe how to use immunolabeling to visualize embryonic/larval presynaptic and postsynaptic structures at the NMJ. We also include details about amplification of weak immunohistochemistry signals and how to use these signals to quantify synaptic growth via bouton counting. Boutons are bead-like structures at motor axon terminals that house synapses, and the number of boutons reflects the size of the NMJ. We also describe how to identify the different bouton types.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Dissected larvae/embryos from Protocol: ***Drosophila* Late Embryonic through Late Larval Stage Body Wall Dissection: Dissection Tools and Techniques** (Ashley and Carrillo 2024)

Normal goat serum (5%) in 0.1% PBST <R>

Phosphate-buffered saline (PBS) <R> (1×)

Prepare without calcium and magnesium.

Phosphate-buffered Triton (PBST; 0.1%) <R>

Primary and secondary antibodies for tissue immunolabeling

Goat anti-horseradish peroxidase (HRP)-TRITC (Jackson ImmunoResearch 123-025-021)

This antibody cross-reacts with several neuronal cell surface molecules (Jan and Jan 1982), such that even brief incubations can easily label all neurons.

Goat anti-mouse Alexa 647 (Thermo Fisher A32728)

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From the *Drosophila* Neurobiology collection, edited by Bing Zhang, Ellie Heckscher, Alex C. Keene, and Scott Waddell.

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Goat anti-rabbit Alexa 488 (Thermo Fisher A32731)
Mouse anti-Alexa 647 (ICL M647-65A-400)
Mouse anti-NC82 (Developmental Studies Hybridoma Bank [DSHB] NC82)
Mouse anti-Synapsin (DSHB, SynORF1)
Rabbit anti-Discs large (Dlg) (Koh et al. 1999)
Vectashield (Vector Laboratories)

Equipment

Cover glass (22-mm × 22-mm; #1.5)
Fluorescence microscope with 40× or 63× objective
Forceps (Dumont #3)
Glass slides (25-mm × 75-mm)
Kimwipes (Kimtech)
Microcentrifuge tubes (0.5-mL)
Nail polish (clear; Ted Pella 114-7)
Nutator (TCS Scientific, or other supplier) or orbital shaker (Fisher 10320808 [or equivalent])
Tally counter (or other device to keep a running count)

METHOD

At the end of Protocol: **Drosophila *Late Embryonic through Late Larval Stage Body Wall Dissection: Dissection Tools and Techniques*** *(Ashley and Carrillo 2024), late-embryonic and first-instar preparations will be in nine-well depression plates, and later larval preparations will be in 0.5-mL tubes. Perform all larval incubations in 0.5-mL tubes on a nutator, and all late-embryonic and first-instar incubations in a nine-well depression plate on an orbital shaker (50–60 rpm). Perform all steps at room temperature unless indicated otherwise. All buffer exchanges should be done carefully to avoid damaging tissue (a glass pipette or P1000 tip pushed into a P200 tip will allow for exchange without drawing the third-instar preparations into the pipette).*

A protocol for general immunolabeling is found in Steps 1–8. An alternative protocol for immunolabeling with signal amplification that involves tertiary antibodies is found in Steps 9–24. Steps 25–28 describe how to score bouton number and growth.

Immunolabeling of Tissue

1. Remove the PBST from the microcentrifuge tubes containing dissected larvae from Protocol: ***Drosophila* Late Embryonic through Late Larval Stage Body Wall Dissection: Dissection Tools and Techniques** (Ashley and Carrillo 2024). Replace PBST with 300 µL of 5% normal goat serum in 0.1% PBST and block for 1 h at room temperature.
2. Combine primary antibodies in 5% normal goat serum in 0.1% PBST. In the case of Figure 1, the following dilutions were used:
   - A dilution of 1:40,000 rabbit anti-DLG was used. However, this is not a commercially available antibody. For the same labeling, a 1:100 dilution of mouse anti-DLG (DSHB) would be used.
   - A dilution of 1:100 mouse anti-Synapsin (DSHB) was used.
3. Remove the 5% normal goat serum in 0.1% PBST and replace it with 300 µL of primary antibody mix. Incubate the samples overnight at 4°C.
4. Remove the primary antibody mix and replace it with 300 µL of PBST. Incubate for 10 min at room temperature. Repeat this wash twice with fresh PBST.
5. Combine the secondary antibodies into a secondary antibody mix. In our example, we used the following dilutions into 300 µL of 0.1% PBST:
   - 1:500 goat anti-rabbit Alexa 488
   - 1:50 goat anti-HRP-TRITC

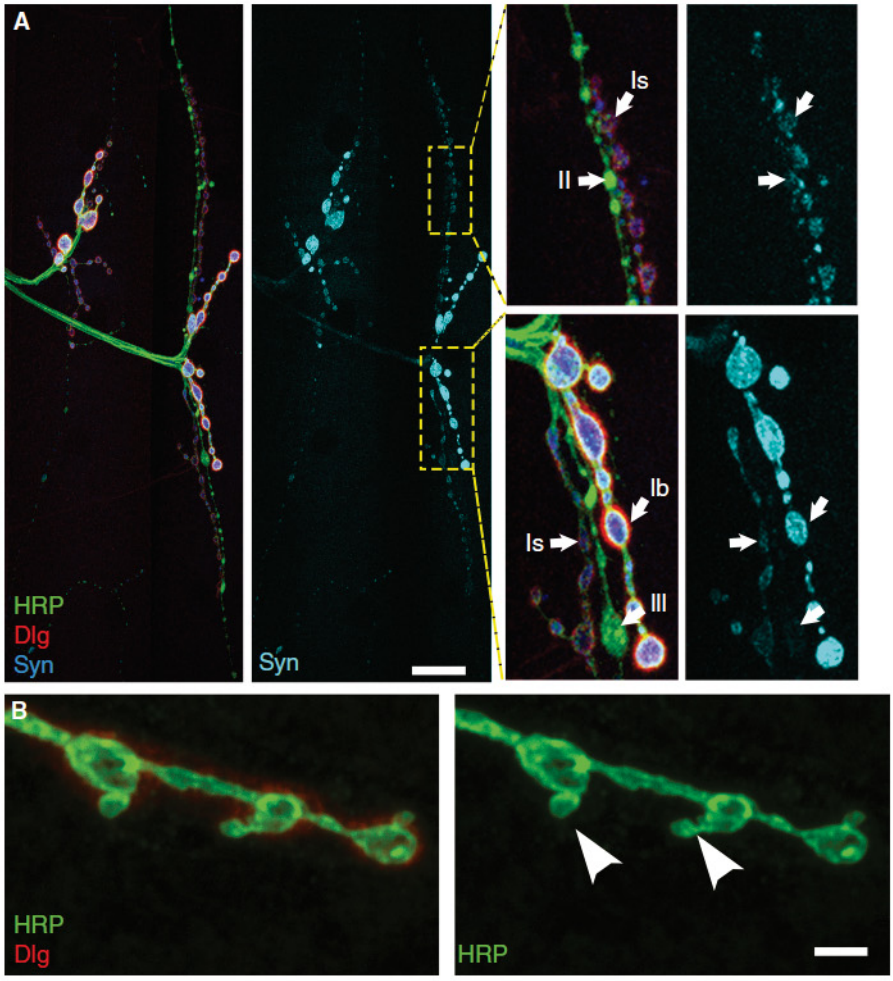

FIGURE 1. Bouton identification. (*A*) Wild-type muscle 12 and muscle 13 neuromuscular junctions (NMJs) labeled with anti-horseradish peroxidase (HRP, green), anti-Dlg (red), and anti-Synapsin (Syn, blue) antibodies. For single-channel panels, anti-Synapsin was changed to teal for easier visualization. Dashed yellow box panels highlight the morphology of the various types of boutons. Note that Synapsin is enriched in Is and Ib boutons and a small amount in type II, but not in type III, boutons. (*B*) An Ib NMJ arbor with satellite boutons (arrowheads). Scale bars: *A*, 20 µm; *A* [*inset*],*B*, 5 µm.

- 1:500 goat anti-mouse Alexa 647

  Anti-HRP is not a secondary antibody, but it does not require overnight incubation to yield clear results and is thus included here.

6. Replace PBST with 300 µL of secondary antibody mix and incubate for 2 h at room temperature.
7. Replace the secondary antibody mix with 300 µL of fresh PBST and wash for 10 min at room temperature. Repeat this wash twice with fresh PBST.
8. Prepare a clean slide for each genotype. Add 40 µL of Vectashield to each slide. Carefully remove samples from the 0.5-mL tube using #3 forceps, blot gently on a Kimwipe, and place into the drop of Vectashield. Move samples in the Vectashield until they are all oriented such that the cuticle is facing the slide, and the inside of the larval preparation is facing upward. Carefully lower the coverslip onto the Vectashield. Seal the coverslip to the slide using clear nail polish.

Proceed to Step 25.

Signal Amplification Using Tertiary Antibodies

This technique (adapted from [Ashley et al. 2018]) is ideal for enhancing signal from antibodies with low background, but it requires high exposure/gain to obtain clear images. The antibody enhancement works through addition of two additional steps to bolster the signal of the initial primary antibody (Fig. 2A). In this case, we chose to amplify the active zone marker BRP, as this protein has a clear signal with very low background. This is ideal, as this type of enhancement may also increase the background signal.

9. Remove the PBST from the microcentrifuge tubes containing dissected larvae from Protocol: ***Drosophila* Late Embryonic through Late Larval Stage Body Wall Dissection: Dissection Tools and Techniques** (Ashley and Carrillo 2024). Replace PBST with 300 µL of 5% normal goat serum in 0.1% PBST and block for 1 h at room temperature.
10. Prepare the primary antibody mix in 5% normal goat serum in 0.1% PBST. For example, dilute the anti-BRP antibody 1:50 into 5% normal goat serum in 0.1% PBST.

    In this case, we used the anti-BRP (DSHB NC82) antibody as an example, as it has a very punctate signal.
11. Remove the 5% normal goat serum in 0.1% PBST from the samples and replace with 300 µL of primary antibody mix.
12. Incubate samples overnight at 4°C.
13. Replace the primary antibody mix with 300 µL of 0.1% PBST and wash the sample for 10 min at room temperature on a nutator. Repeat the wash step two additional times.
14. Dilute goat anti-mouse Alexa 647 secondary antibody 1:500 in 0.1% PBST.

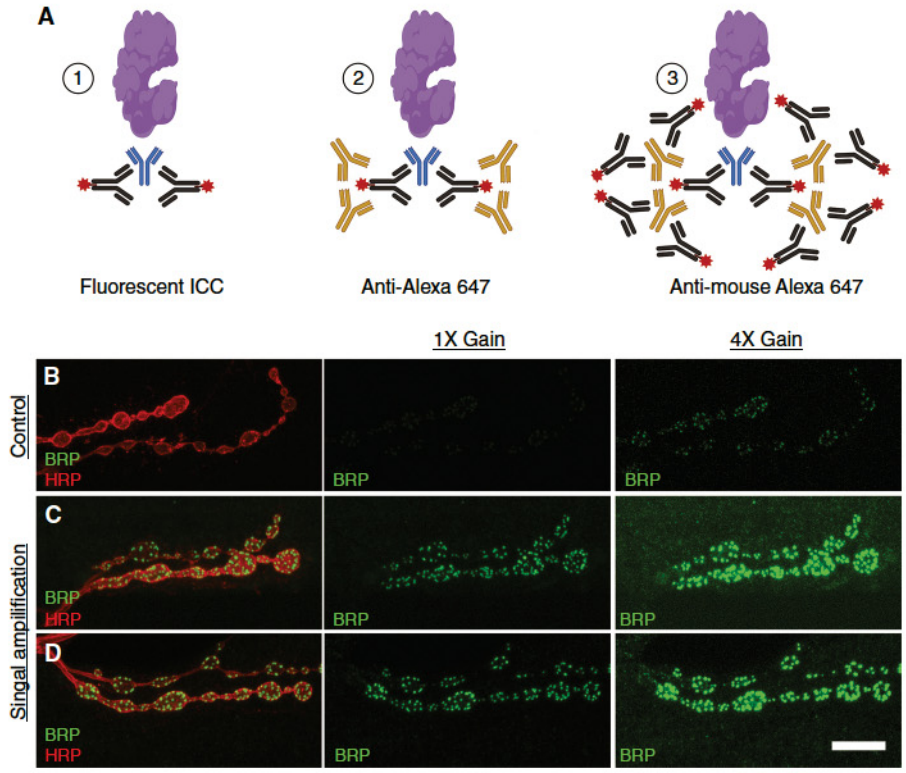

FIGURE 2. Antibody signal enhancement. (*A*) Cartoon representation of standard fluorescent immunocytochemistry (ICC) with an Alexa 647-conjugated secondary antibody (panel *1*), application of mouse anti-Alexa 647 antibody (panel *2*), and application of anti-mouse-Alexa 647 (panel *3*) to drastically enhance the immunofluorescent signal (made with Biorender.com). (*B*) An NMJ labeled with anti-HRP (red) and anti-Bruchpilot (BRP, green) with standard immunochemistry (same as panel *1* in *A*). (*C*,*D*) Two examples of NMJs labeled with the same primary and secondary antibodies as in *B*, but with additional amplification antibodies applied. The 4× gain was applied to all BRP signals to demonstrate that the anti-BRP signal in *B* is present, albeit far dimmer. Scale bar, 10 µm.

15. Replace the PBST in the tube with 300 µL of secondary antibody mix and incubate samples for 2 h at room temperature.
16. Replace secondary antibody mix with 300 µL of 0.1% PBST and wash for 10 min at room temperature. Repeat the wash step two additional times.
17. Prepare the tertiary antibody mix by diluting mouse anti-Alexa 647 antibody 1:800 in 0.1% PBST.

    This is the key step in the amplification, as this antibody recognizes the fluorophore Alexa 647 with very little background signal.
18. Remove the PBST and replace with 300 µL of tertiary antibody mix. Incubate for 2 h at room temperature.
19. Replace the tertiary antibody mix with 300 µL of 0.1% PBST and wash for 10 min at room temperature. Repeat the wash step two additional times.
20. Prepare the quaternary antibody mix in 0.1% PBST. We used the following dilutions:

    Here, we included the fiducial marker anti-HRP to label neuronal membranes. If other, nonamplified antibodies were included in the primary antibody mix, this step is where the secondary antibodies for those would be included.

    - 1:500 goat anti-mouse Alexa 647
    - 1:50 goat anti-HRP-TRITC
21. Remove the PBST and replace with 300 µL of quaternary antibody mix. Incubate for 2 h at room temperature.
22. Replace quaternary antibody mix with 300 µL of 0.1% PBST and wash for 10 min at room temperature. Repeat the wash step two additional times.
23. Prepare a clean slide for each genotype. Add 40 µL of Vectashield to each slide. Carefully remove the samples from the 0.5-mL tube, blot gently on a Kimwipe, and place into the drop of Vectashield. Move samples in the Vectashield until they are all oriented such that the cuticle is facing the slide, and the inside of the larval preparation is facing upward. Carefully lower the coverslip onto the Vectashield. Seal the coverslip to the slide using clear nail polish.
24. Move samples to the confocal microscope for standard imaging. The amplified antibody signal will be significantly stronger than the original nonamplified signal (Fig. 2B).

    In this case, we left out the tertiary antibody from one set of samples but processed otherwise the same. As can be seen, a 4× increase in gain is required to observe the original conditions at levels similar to those of the enhanced preparations.

Morphometric Analysis (Scoring Bouton Number and Growth)

25. Using a fluorescence microscope, under an at least 40× objective, bring the NMJs of interest into focus and identify structures:
    - Anti-HRP: labels neuronal membranes (Jan and Jan 1982)
    - Anti-Dlg: labels the muscle postsynaptic area around type I boutons

      Note that anti-Dlg signal is brighter around Ib boutons than around Is boutons (Guan et al. 1996).
    - Anti-Synapsin: labels both type I boutons and faintly labels type II boutons
26. Using HRP and Dlg intensity, determine which boutons are Is and Ib (Fig. 1A).

    Due to differences in cell surface proteins on the presynaptic membrane, HRP intensity appears much stronger in type Ib boutons than in Is boutons, making it easier to distinguish between the two. Similarly, the ultrastructure of the postsynaptic membrane is more complex around type Ib boutons than around Is boutons; hence, the increased anti-Dlg staining around type Ib boutons.
27. Count the number of each type of bouton with the aid of the tally counter to avoid losing count.

    Synapsin will allow for easier scoring of Is and Ib boutons, as the boutons are far more clearly defined (Fig. 1A, inset).

28. If satellite boutons are present (Fig. 1B, arrowheads), score these separately.
Satellites are small buds present on the surface of the bouton, separated by a short stalk.

RECIPES

Normal Goat Serum (5%) in 0.1% PBST

Reagent	Vendor	Amount
Goat serum	Fisher 7332500100ML	10 mL
Phosphate-buffered		190 mL
Triton		
(PBST; 0.1%) <R>		

Combine and mix gently. Store for up to 1 mo at 4°C.

Phosphate-Buffered Saline (PBS)

Reagent	Amount to add (for 1× solution)	Final concentration (1×)	Amount to add (for 10× stock)	Final concentration (10×)
NaCl	8 g	137 mM	80 g	1.37 M
KCl	0.2 g	2.7 mM	2 g	27 mM
Na_2HPO_4	1.44 g	10 mM	14.4 g	100 mM
KH_2PO_4	0.24 g	1.8 mM	2.4 g	18 mM
If necessary, PBS may be supplemented with the following:				
$CaCl_2 \cdot 2H_2O$	0.133 g	1 mM	1.33 g	10 mM
$MgCl_2 \cdot 6H_2O$	0.10 g	0.5 mM	1.0 g	5 mM

PBS can be made as a 1× solution or as a 10× stock. To prepare 1 L of either 1× or 10× PBS, dissolve the reagents listed above in 800 mL of H_2O. Adjust the pH to 7.4 (or 7.2, if required) with HCl, and then add H_2O to 1 L. Dispense the solution into aliquots and sterilize them by autoclaving for 20 min at 15 psi (1.05 kg/cm^2) on liquid cycle or by filter sterilization. Store PBS at room temperature.

Phosphate-Buffered Triton (PBST; 0.1%)

1 mL of 10% (v/v) Triton X-100
100 mL of phosphate-buffered saline (PBS) <R> (1×)
Prepare PBS without calcium and magnesium. Combine PBS and Triton and mix gently.

Store for up to 1 mo at 4°C.

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