Protocol

Using the Proximity Ligation Assay to Visualize Colocalization of Proteins at the *Drosophila* Larval Neuromuscular Junction

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In the nearly 50 years since the neuromuscular junction (NMJ) was first established as a model synapse, its molecular composition has been extensively characterized. Early work relied on fluorescent signals to determine whether proteins localized to the pre- and postsynaptic regions. As more synaptic molecules were identified, determining the localization of these proteins relative to each other became important. Conventional microscopy lacks the resolving power to assess whether two proteins are within an appropriate distance to bind directly or be part of a larger complex. Super-resolution and immunoelectron microscopies can improve spatial resolution, but these techniques can be difficult to execute and troubleshoot, and access to these instruments is limiting. However, another approach, proximity labeling, overcomes many of these limitations by using a DNA secondary label that can only be amplified if the two proteins of interest are within 40 nm of each other, which is ~5× greater than the resolving power of conventional microscopy. In this protocol, we describe the use of the proximity ligation assay, which combines immunohistochemistry with DNA amplification, to reveal protein colocalization in the *Drosophila* NMJ.

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

Fixed larval tissue in a 0.5-mL microcentrifuge tube (see Step 1)

In this case, we used Mhc-CD8-GFP-Sh flies (which have a muscle-specific promoter driving a GFP containing the C terminus of the Shaker potassium channel, causing it to localize to the postsynaptic region) (Zito et al. 1999) and w1118 flies (wild-type control; Bloomington Drosophila *Stock Center 5905).*

Duolink anti-mouse PLUS (Duolink kit, Sigma-Aldrich DUO92102)
Duolink anti-rabbit MINUS (Duolink kit, Sigma-Aldrich DUO92102)
Duolink buffer kit (Duolink kit, Sigma-Aldrich DUO92102)
Duolink In Situ Detection Reagents Orange (Duolink kit, Sigma-Aldrich DUO92102)

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Goat anti-horseradish peroxidase (HRP) Alexa 647 (Jackson ImmunoResearch 123-605-021)

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

Normal goat serum (5%) in 0.1% PBST <R>
Phosphate-buffered saline (PBS) <R> (1×)
Phosphate-buffered Triton (PBST; 0.1%) <R>
Primary antibodies raised in two different species (e.g., rabbit and mouse)

In our example, we used mouse anti-DLC (Developmental Studies Hybridoma Bank 4F3) and rabbit anti-GFP (Thermo Fisher A11122).

Vectashield (Vector Laboratories)

Equipment

Centrifuge tubes (0.5-mL)
Confocal microscope
Glass coverslips (25-mm × 25-mm; #1.5)
Glass slides (75-mm × 25-mm)
Incubator at 37°C
Kimwipes (Kimtech)
Nail polish (clear, Ted Pella 114-7)
Nutator (TCS Scientific, or other supplier)
Refrigerator or 4°C cold room

METHOD

Proximity ligation assay (PLA) was developed by Fredriksson et al. (2002) and adapted to the Drosophila *neuromuscular junction (NMJ) by Wang et al. (2015). For a schematic visualization of the technique, see Figure 1.*

1. Collect fixed samples (in this case, body wall muscle preparations) in a 0.5-mL centrifuge tube (see Protocol: ***Drosophila* Late Embryonic through Late Larval Stage Body Wall Dissection: Dissection Tools and Techniques** [Ashley and Carrillo 2024a] and Protocol: **Immunohistochemistry and Morphometric Analysis of *Drosophila* Larval Body Wall Neuromuscular Junction Preparations** [Ashley and Carrillo 2024b] for more details). At room temperature, remove the fixative with a pipette, being careful to not draw up the samples, and replace the fixative with 300 µL of PBS to wash. Gently agitate the samples on a nutator for 10 min at room temperature.
2. Remove the PBS and replace with 300 µL of 0.1% PBST to permeabilize the tissue. Incubate on a nutator for 10 min at room temperature. Repeat the 0.1% PBST wash two additional times for 10 min each at room temperature.
3. Remove the PBST and apply 300 µL of 5% normal goat serum in 0.1% PBST for 1 h at room temperature with agitation on a nutator.
4. Dilute the primary antibodies into 300 µL of 5% normal goat serum in 0.1% PBST.

   In our example, we used 1:100 mouse anti-DLC and 1:000 rabbit anti-GFP.
5. Remove the 5% normal goat serum in 0.1% PBST and replace with the primary antibody solution. Incubate the samples overnight at 4°C on a nutator.
6. The next day, remove the primary antibody solution and replace with 300 µL of 0.1% PBST. Incubate for 10 min at room temperature on a nutator. Repeat the 0.1% PBST wash two additional times for 10 min per wash.

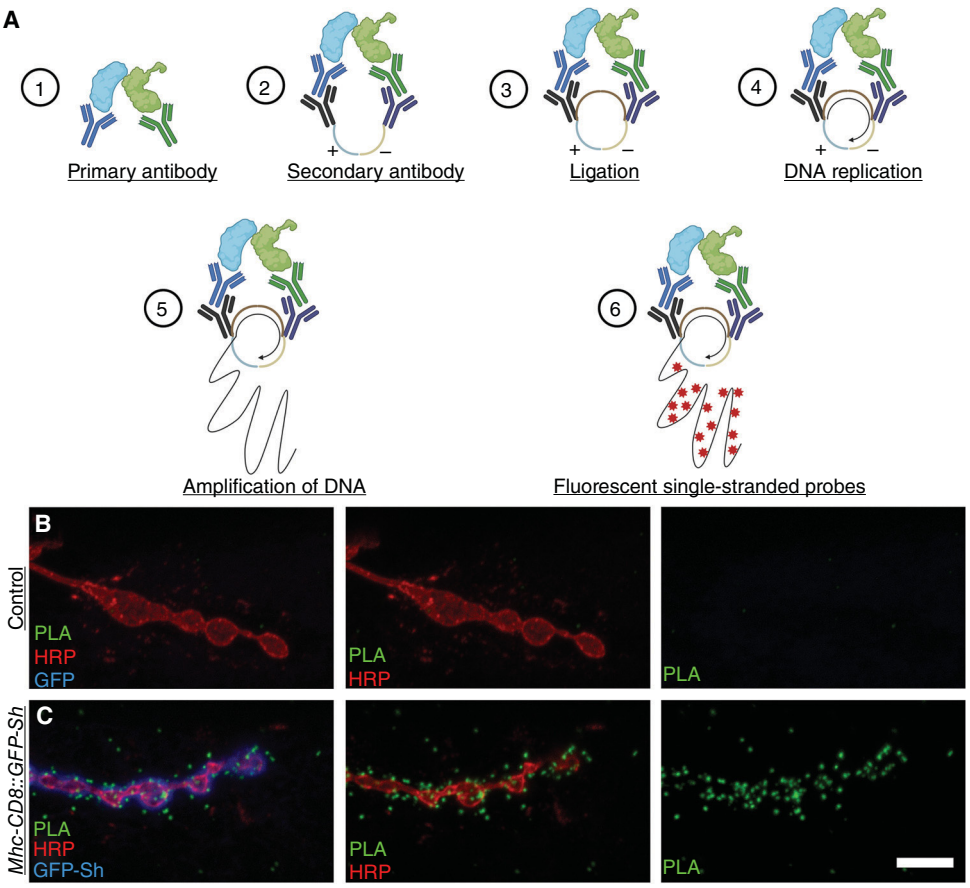

FIGURE 1. Proximity ligation assay (PLA). (*A*) Cartoon representing key steps in the PLA: two proteins are labeled with primary antibodies (panel *1*); secondary antibodies, each carrying a complementary DNA fragment (labeled plus and minus strands), bind to the primary antibodies (panel *2*); a third fragment of DNA is added, and, if the proteins are within 40 nm of each other, the three DNA fragments are joined together with a ligase (panel *3*); a polymerase reaction begins (panel *4*); DNA replication generates a long DNA strand (panel *5*); and the resulting single-stranded DNA is labeled with single-stranded probes (panel *6*) (made with Biorender.com). (*B*,*C*) Neuromuscular junctions labeled with PLA (green) and antibodies against green fluorescent protein (GFP, blue) and horseradish peroxidase (HRP, red). (*B*) Control animal has HRP but no GFP antigen. (*C*) An animal expressing GFP with a Shaker (Sh) tail, which has been shown to bind Dlg (Zito et al. 1999). Scale bar, *B*,*C*, 5 µm.

7. Dilute the following antibodies 1:50 in 300 µL of the Duolink antibody diluent buffer, all in the same tube: anti-mouse PLUS and anti-rabbit MINUS PLA antibodies and anti-HRP Alexa 647 antibody.

   The directions in the PLA kit suggest 1:5 to 1:25 dilutions of secondary antibodies in their buffer, but we found an improved signal-to-noise ratio using a 1:50 dilution. In our example, we also added a 1:100 dilution of anti-HRP Alexa 647 to label neurons.

8. Remove the last PBST wash from the samples and add the secondary antibody solution. Incubate the samples with secondary antibodies for 2 h at room temperature on a nutator.
9. Remove antibodies and replace with 300 µL of the Duolink Buffer A. Incubate for 5 min at room temperature. Repeat the Buffer A wash one additional time for 5 min at room temperature.
10. Prepare the ligation mix containing 80 µL of H_2O, 20 µL of 5× ligation buffer (Duolink kit), and 1.25 µL of ligase (Duolink kit). Remove Buffer A from the samples and replace it with the ligation mix. Incubate the samples for 1 h at 37°C.
11. Remove the ligation mix and wash samples with 300 µL of Buffer A twice for 2 min each at room temperature on a nutator.

12. Prepare the amplification mix containing 80 µL of H_2O, 20 µL of 5× amplification buffer (Duolink kit), and 1.25 µL of polymerase (Duolink kit). Tap gently to mix. Remove Buffer A from the samples and replace with the amplification mix.
13. Incubate the samples for 2 h in an incubator at 37°C.
14. Wash the samples with 300 µL of Duolink Buffer B twice for 10 min each at room temperature on a nutator.
15. Prepare 300 µL per tube of diluted Duolink Buffer B 1:100 in H_2O.
16. Wash the samples for 1 min at room temperature in 300 µL of the 1:100 dilution of Buffer B on a nutator.
17. Prepare a clean slide for each genotype. Add 40 µL of Vectashield to each slide. Carefully remove 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.
18. Move the slides to the confocal microscope and check the regions of interest. In this case, we counterstained with anti-HRP, which will label all neurons. Through careful imaging, we were able to see several clear puncta that were present in the experimental samples but not in the controls, highlighting colocalization (Fig. 1).

DISCUSSION

As described above, PLA enables detection of proteins that are within 40 nm of each other, representing a fivefold increase over optical resolution. The processing of PLA samples requires additional steps compared to standard immunofluorescence techniques, but the PLA kits include all the necessary reagents, and, when combined with the appropriate secondary antibodies, various fluorescent combinations resolve protein–protein colocalization. Prior studies used standard fluorescence colocalization and yeast two-hybrid to reveal that the scaffolding protein Dlg and the Shaker potassium channel form a complex (Tejedor et al. 1997). We used this protein complex as an example for our PLA assay (Fig. 1).

RECIPES

Normal Goat Serum (5%) in 0.1% PBST

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

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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