Protocol

Labeling of Cell Surface Proteins at the *Drosophila* Larval Neuromuscular Junction Using Binding Partner Peptides

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Determining the precise localization of interacting proteins provides fundamental insight into their putative function. Classically, immunolabeling of endogenous proteins or generating tagged versions of proteins has been used to localize interacting proteins. However, in many cases, the interacting partner of a protein of interest is unknown. For cell surface proteins, it is possible to determine the localization of interacting proteins if one of the binding partners is known. This approach is based on generating purified, recombinant, tagged extracellular domains (ECDs) of a protein of interest, and incubating tissue to allow the recombinant protein to bind to its interacting partner(s). In this protocol, we detail the cloning of secreted, tagged ECDs from cell surface proteins, transfection of cloned plasmids into S2 cells, collection of secreted domains, concentration of the cell culture medium to enrich for the ECDs, and labeling of tissue with these ECDs.

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

Complementary DNA (can be ordered from *Drosophila* Genomics Resource Center [DGRC] for each gene, or RT-PCR-amplified from RNA extracted from tissue)

Copper sulfate (100 mM)

Filter-sterilize using a 0.22-µm filter flask and store at room temperature.

Drosophila larvae or embryos dissected as described in Protocol: ***Drosophila* Late Embryonic through Late Larval Stage Body Wall Dissection: Dissection Tools and Techniques** (Ashley and Carrillo 2024)

Effectene (QIAGEN) or dimethyldioctadecyl-ammonium bromide (DDAB) (Han 1996)

Escherichia coli DH5-α

Ice

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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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Normal goat serum (5%) in 0.1% PBST <R>
Paraformaldehyde (4%, w/v) (20% [w/v] solution [Electron Microscopy Sciences 15713] diluted with 1× PBS)
Phosphate-buffered saline (PBS) <R> (1×)

Prepare without calcium and magnesium.

Phosphate-buffered Triton (PBST; 0.1%) <R>
pECIA14 plasmid (Addgene 47051)
pENTR/D-TOPO cloning kit (Thermo Fisher)
LR clonase (Thermo Fisher 11791020)
Primary antibody: rabbit anti-alkaline phosphatase (Abcam ab118856)

This antibody is no longer available. Abcam recommends several replacements on their website.

Protease inhibitors (Sigma-Aldrich P8849)
S2 cell growth medium <R>
S2 cells
Secondary antibodies
Goat anti-horseradish peroxidase-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-rabbit Alexa 488 (Thermo Fisher A32731)
Sodium azide (1 M)
Taq enzyme and appropriate PCR reagents, including primers to the region of interest (see Step 2)
Vectashield (Vector Laboratories)

Equipment

Amicon Ultra centrifugal filters (Millipore UFC810008)

The specific Amicon tube will depend on the molecular weight of the protein of interest; therefore, choose the appropriate molecular weight cutoff.

Centrifuge tubes (50-mL)
Confocal microscope
Forceps (Dumont #2 and #3)
Kimwipes (Kimtech)
Maxiprep kit
Microcentrifuge tubes (0.5- and 1.5-mL)
Nail polish (clear, Ted Pella 114-7)
Nutator (TCS Scientific, or other supplier)
Parafilm
PCR machine
Petri dish (145-mm × 20-mm)
Refrigerated centrifuge (Beckman Coulter Allegra X-15R or equivalent refrigerated centrifuge at 4°C)
T-75 tissue culture flasks

METHOD

This protocol was adapted from Fox and Zinn (2005). Perform all steps at room temperature unless otherwise indicated.

Figure 1A is a cartoon of the procedure, in which a tagged extracellular domain fragment is incubated with the unfixed tissue, where it binds to its partner and is fixed in place, and the tag is recognized by specific primary and secondary antibodies.

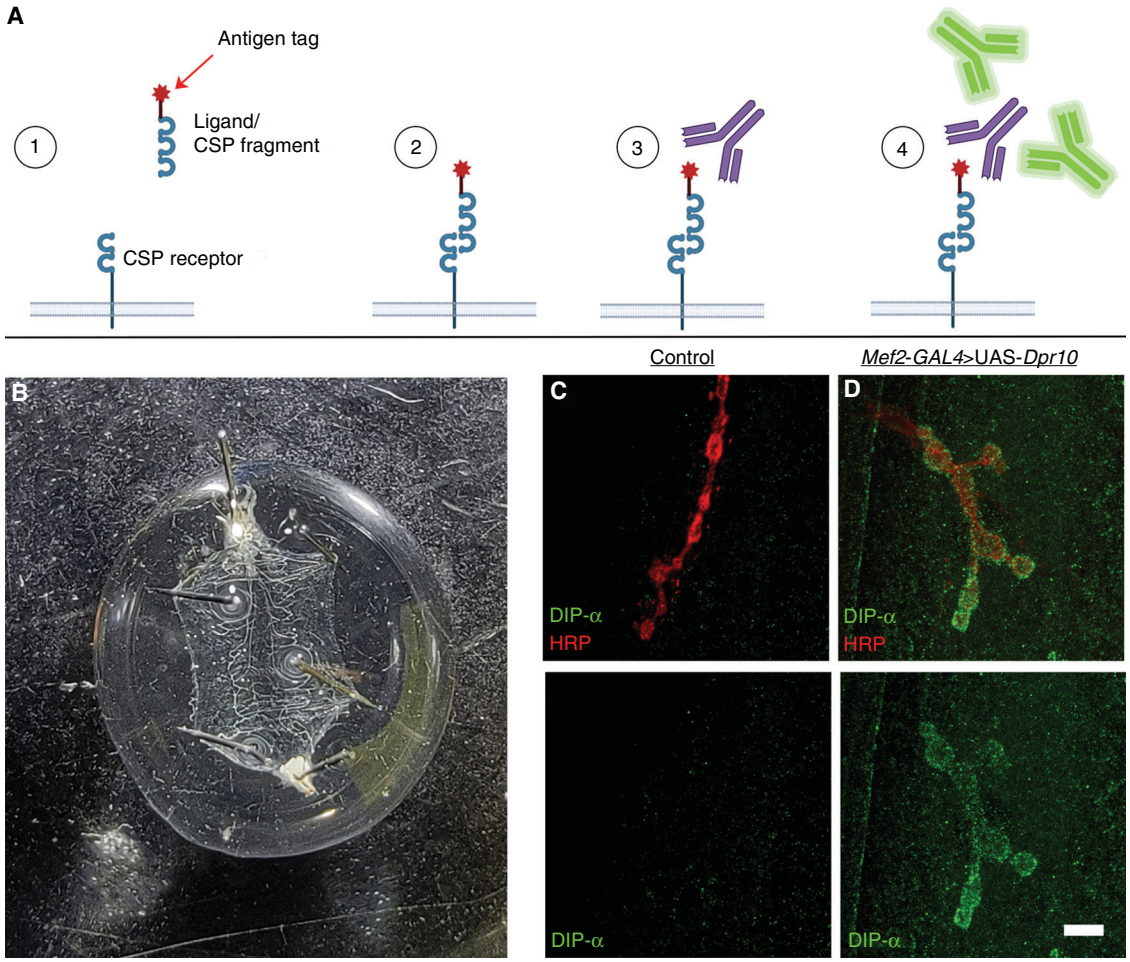

FIGURE 1. Cell surface protein (CSP) labeling. (*A*) Cartoon representing the process of incubating a sample with a tagged version of either the ligand or a soluble version of a receptor-binding partner (panel *1*), the two proteins interacting (panel *2*), using primary antibodies specific to the exogenous protein (panel *3*), and labeling with secondary antibodies to identify the protein complex (panel *4*) (made with Biorender.com). (*B*) A dissected third-instar larva with a small volume of solution with the protein of interest covering the surface. (*C*,*D*) Third-instar neuromuscular junctions (NMJs) incubated with soluble Dpr-interacting protein-α (DIP-α). (*D*) Note that, upon muscle overexpression of Dpr10, a DIP-α-binding partner, the DIP-α signal (green) is significantly increased, suggesting that Dpr10 and DIP-α interact. The NMJ is labeled with anti-horseradish peroxidase (HRP) antibody (red). Scale bar: *C*,*D*, 10 µm.

ECD Fragment Design and Production

1. Identify the protein of interest to test for binding to its receptor/ligand.

   In this case, we chose the extracellular domain of the Dpr-interacting protein-α (DIP-α), to examine where its binding partners localize.

2. Amplify the sequence of the gene of interest from source complementary DNA and clone it into the pECIA14 (C-terminal 6XHis, FLAG, and alkaline phosphatase) plasmid (Özkan et al. 2013).

   i. Use Taq enzyme and standard PCR techniques to amplify the extracellular region of the protein of interest.

   ii. Combine the PCR product with the pENTR/D-TOPO vector according to the manufacturer's instructions.

   iii. Using the LR clonase, combine the entry vector with the pECIA14 destination vector according to the manufacturer's instructions.

iv. Transform the mixture into standard DH5-α *Escherichia coli* bacteria and grow the bacteria via standard techniques.

Due to the nature of the cloning, nearly all colonies should be positive. Verify colonies with sequencing.

v. Purify the plasmid using a maxiprep kit of choice.

3. Grow S2 cells (Echalier et al. 2018) in S2 cell growth medium at 25°C in T-75 flasks. Split 1:4 (cells:medium) every 3–5 d.

Actively growing cells transfect with higher efficiency. We typically split 1:1, 24 h prior to transfection.

4. Transfect S2 cells using a commercially available product such as Effectene or DDAB (Han 1996). For the volumes needed here, transfect 40 mL of cells at a density of 1×10^6/mL in a 50-mL centrifuge tube with 20 µg of plasmid, and then split the cells between two T-75 flasks for growth at 25°C.

5. Eighteen hours after transfection, induce expression by adding copper sulfate to the medium at a final concentration of 1 mM. Continue to grow cells undisturbed at 25°C.

6. Harvest growth medium 3 d after induction as follows: As the cells are semiadherent, resuspend the cells from the bottom of the flask through gentle pipetting and transfer to a conical tube. Centrifuge the tube at 800*g* for 10 min at room temperature. Carefully collect the supernatant and discard the pellet.

As these are soluble extracellular fragments, the protein will be secreted into the medium; so here we are saving the medium and discarding the cells and debris.

7. To stabilize the supernatant, add protease inhibitors to a final dilution of 1:100 and sodium azide to a final concentration of 3 mM before storage at 4°C.

The supernatant is stable for several months at 4°C.

8. Collect 4 mL of supernatant and load it into an Amicon tube to concentrate the protein fragments of interest. Centrifuge the Amicon tube at 1500*g* for 30 min at 4°C or until the volume is reduced to 500 µL. Transfer the concentrated solution to a 1.5-mL centrifuge tube and keep on ice until ready to use.

Dissection and Incubation with ECD Fragments

9. Dissect larvae or embryos (see Protocol: ***Drosophila* Late Embryonic through Late Larval Stage Body Wall Dissection: Dissection Tools and Techniques** [Ashley and Carrillo 2024]) with a density of only two per dissection dish. Do not fix the tissue.

In our example, we dissected wild-type flies and animals that were overexpressing the DIP-α binding partner, Defective proboscis extension 10 (Dpr10), in the muscles. Our previous work has shown that this drastically increases the available binding partners around the synapse, and thus the amount of DIP-α peptide that accumulates there (Ashley et al. 2019).

10. Remove most of the PBS from the dissecting dish with a Kimwipe.

The Sylgard surrounding each preparation should be dry.

11. In the Sylgard dish, carefully cover each dissected animal with 75 µL of concentrated protein fragment from Step 8 (Fig. 1A,B).

This step essentially places a bubble of concentrated protein over the preparation. Do not move the chamber excessively, as this will break the bubble.

12. To minimize evaporation, place the Sylgard dish into a humidified chamber made from a 145-mm Petri dish containing a wet Kimwipe to maintain humidity.

13. Incubate in the humidified chamber for 1.5 h at room temperature.

14. Remove the Sylgard dish from the humidified chamber, remove the solution over the preparations, and wash with 1 mL of PBS for 10 min at room temperature. Remove PBS and repeat the wash two additional times. Finally, remove the PBS and replace with 1 mL of 4% paraformaldehyde. Incubate for 30 min at room temperature.

15. Remove the fixative and replace with 1 mL of PBS. Using #3 forceps, carefully move the samples to a 0.5-mL tube and wash three times, 10 min each, with 500 µL of 0.1% PBST.
16. Remove the PBST and replace with 5% normal goat serum in 0.1% PBST for 1 h at room temperature.
17. Dilute rabbit anti-alkaline phosphatase 1:500 in 5% normal goat serum in 0.1% PBST to a final volume of 300 µL.

    If other primary antibodies are to be used to better define regions of interest, they should be included in this mix.
18. Incubate the sample in the antibody solution overnight at 4°C on a nutator.
19. Remove the primary antibody mix and wash three times, 10 min each, with 500 µL of 0.1% PBST at room temperature on a nutator.
20. Prepare the secondary antibody mix. Into 0.1% PBST, dilute goat anti-rabbit Alexa 488 1:500 and goat anti-HRP-TRITC 1:50 in a total volume of 300 µL.
21. Remove the PBST from the sample. Incubate the sample in secondary antibody mix for 2 h at room temperature on a nutator.
22. Remove the secondary antibody mix and wash three times, 10 min each, with 0.1% PBST on a nutator.
23. 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 #2 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.
24. When the nail polish is dry, move to the confocal microscope for imaging. Image both control and experimental animals at the same settings (Fig. 1C,D).

    See Troubleshooting.

TROUBLESHOOTING

Problem (Step 24): There is too much background noise in the peptide channel.
Solution: This problem has multiple potential solutions. Consider the following:

- Peptide concentration can vary greatly based on transfection level and construct. Try 50% and 25% dilutions of the original peptide.
- Anti-alkaline phosphatase antibody may have a nonspecific reaction with cytoplasmic proteins; do not permeabilize the preparation (work with PBS instead of PBST). After incubation with the primary antibody, wash three times, 10 min each, with PBS, and then fix for 10 min with 4% paraformaldehyde (this will cross-link the anti-alkaline phosphatase antibody to the surface). Wash three times, 10 min each, with PBST, and then proceed to immunolabel with the remaining primary antibodies.

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.

S2 Cell Growth Medium

Reagent	Vendor	Quantity
Schneider's medium	Sigma-Aldrich S0146	500 mL
100× penicillin–streptomycin	Sigma-Aldrich P4333	5 mL
Fetal bovine serum	Sigma-Aldrich F4135	50 mL

Combine all reagents and filter-sterilize using a 0.22-μm filter flask. Store for up to 3 mo at 4°C.

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