Protocol

Drosophila Late Embryonic through Late Larval Stage Body Wall Dissection: Dissection Tools and Techniques

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One of the challenges of studying synaptic structure and function is accessibility. Some of the earliest readily identifiable and accessible synapses were from the frog and various arthropods. To address questions regarding mechanisms that underlie synaptic development and function, genetically tractable systems were required, and researchers turned to the *Drosophila melanogaster* embryonic/larval neuromuscular preparation. *Drosophila* embryos are transparent and can be labeled with antibodies or probes and imaged in whole-mount preparation for structural analysis. Embryos can also be dissected to visualize the entire body wall musculature as well as finer details including live protein trafficking and protein–protein interactions. Whereas younger dissected embryos can be mounted directly onto charged slides, more mature embryos and larvae develop a cuticle that impedes this adherence, so different techniques must be applied. In this protocol, we detail how to manufacture dissection tools and collect embryos, and discuss the individual steps of dissecting late-stage embryos, early first-instar larvae, and late-stage third-instar larvae.

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

Drosophila flies (to produce embryos and larvae)

Fixative

We describe the use of 4% (w/v) paraformaldehyde (20% [w/v] solution [Electron Microscopy Sciences 15713] diluted with 1× PBS). Alternatives include Bouin's fixative (Electron Microscopy Sciences 15990), 100% methanol, or 100% acetone.

Grape agar plates <R>

Prepare an appropriate number of plates according to the recipe. Prepare additional grape agar according to the recipe but pour ~20 mL into the base of a 90-mm Petri dish to cut slabs for dechorionation (see Step 26). Pour the agar mixture into the dish, and cover with the lid when stored at 4°C.

NaOH (1 M)

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

Prepare without calcium and magnesium.

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

Sylgard plates <R>

Yeast paste

Combine active dry yeast with an approximately equal volume of H_2O in a 50-mL tube and mix thoroughly with a laboratory spatula. Prepare ~25% of the tube volume and store at 4°C with the cap loosened. Yeast will expand violently if not careful. The consistency should resemble stiff peanut butter; if too liquid, add more yeast; if too dry, add more H_2O.

Equipment

Banana plug receptacle and male banana plug (optional; see Steps 7–9)

Bottle (6-oz, polypropylene; Genesee 32-130)

Brass pin vise (80-mm; Ted Pella 13560)

Clay (any modeling clay)

Depression plate, nine-well (Corning 7220-85) (optional; see Step 55)

Dissecting microscope (at least 10× zoom; e.g., Leica M125 C with a 1× objective or Zeiss Discovery V8 with a 1.6× objective)

Higher magnification (10×) is needed to resolve embryo structures.

Electrical tape or heat shrink wrap (see Step 1)

Electrical wires (18 AWG, red and black, ~50-cm)

Forceps (Dumont #2, #3, and #5)

Glass slide (plain; Fisher 12-544-1)

Graphite rod (molded, 0.5-in outer diameter; total length of ~2 cm)

Incubator

Laboratory tape (Fisher 159015R)

Metal file or sandpaper (see Step 2)

Micro-alligator clip (smooth-jawed)

Microcentrifuge tubes (0.5-mL)

Micromanipulator (manual, mounted to a tilting base on a steel baseplate; WPI M3301-M3-R) (optional; see Step 6)

Needle (18-gauge or smaller)

Nutator (TCS Scientific, or other supplier) or orbital shaker (Fisher 10320808 [or equivalent])

Petri dishes and lids (90-mm × 15-mm and 35-mm × 10-mm)

For 35-mm × 10-mm Petri dishes, use Falcon 351008 because some other brands do not tightly fit the opening of the bottles.

Plastic rod (~15-cm long; 6- to 8-mm diameter) (optional; see Step 8)

Plate (black, plastic; AmScope PP-95)

Power supply (variable, 100-W, AC)

We use a Leitz 050-262 microscope lamp supply but there are many similar power supplies available online.

Probe (standard, wooden-handled; Fisher 13-820-024)

Razor blade

Scissors (strong, sharp; Westcott Titanium)

Soldering iron and solder

Steel pins (100-µm; Fine Science Tools 26002-10; use scissors to cut down to 4- to 6-mm)

Tape (double-sided; e.g., Scotch)

Transfer pipette (1-mL)

Tungsten wire (100-µm)

Vannas spring scissors (fine, 3-mm blade [Fine Science Tools 15000-08] and very fine, 2-mm blade [Fine Science Tools 15000-03])

METHOD

Steps 1–19 describe how to generate tungsten tools for dissection of embryos or first-instar flies. Steps 20–25 describe embryo collection. Steps 26–38 describe special steps for late-embryo and first-instar dissection. Finally, general larval dissection and fixation are discussed in Steps 39–56.

Generating the Ideal Sharpened Tungsten Tools

This technique is adapted from Collins (2012). Both tungsten pins to be used in dissection and an embryo dissection tool are generated in this section. If dissecting third-instar larvae, these tools are not necessary.

Setup of the Tungsten Wire-Sharpening Rig

This technique uses NaOH and exposed live wires; therefore, please handle with extreme caution. Trace amounts of hydrogen gas are produced as the tungsten is sharpened; therefore, use in a well-ventilated area.

1. Solder an alligator clip to the end of a red electrical wire and cover the exposed wire with either electrical tape or heat shrink wrap.
2. Using a metal file or sandpaper, carve a notch around the circumference of a graphite rod, ~1.5 cm from one end. Wrap the exposed end of a black electrical wire around this notch, twist it back on itself, and then solder the wire to itself. This is the ground wire.
3. With the power supply unplugged, attach the red and black wires to the positive and negative terminals, respectively, on the power supply.
4. Add 1 M NaOH to a Petri dish to fill it ~1 cm and place the graphite ground into the solution. Make sure not to touch the exposed wire around the carbon rod to the solution.
5. Using sharp Westcott scissors, cut several 8-mm lengths of tungsten wire to make pins. Work in multiples of six, as each dissection requires six pins. Always make extras, as these are easily damaged or lost. To make the embryo dissection tool, cut 10-mm pieces of tungsten wire.

(Optional) Micromanipulator Control of Electrode Sharpening

This procedure allows for easy removal of the alligator clip and tungsten wire, which facilitates visualization of the sharpened wire under a microscope (Fig. 1A, inset).

6. Mount a manual micromanipulator such that it is almost perpendicular to the NaOH bath (Fig. 1A).
7. Solder a banana plug receptacle to the red wire, instead of the alligator clip.
8. Mount the receptacle to a plastic rod and tighten the rod into the micromanipulator.
9. Solder the alligator clip to the male banana plug.

Proceed to Step 10 for pin sharpening instructions and Step 16 to generate the embryo dissection tool.

Tungsten Pin Sharpening Technique

10. Load the cut 8-mm piece of tungsten wire into the alligator clip with ~5–6 mm exposed beyond the jaws.
11. Set the power supply to 10 V and, using either the manual manipulator or by hand, submerge the wire in NaOH until it almost touches the jaws. Turn on the power supply for 20 sec and then turn it off.

This will erode the shaft of the wire and create a shoulder (Fig. 1B).

12. Once the shoulder on the wire has formed, turn on the power supply and dip the wire in and out of the bath 15 times, ~1–2 mm deep and ~1 sec per dip.

This sharpens the remaining material (Fig. 1C).

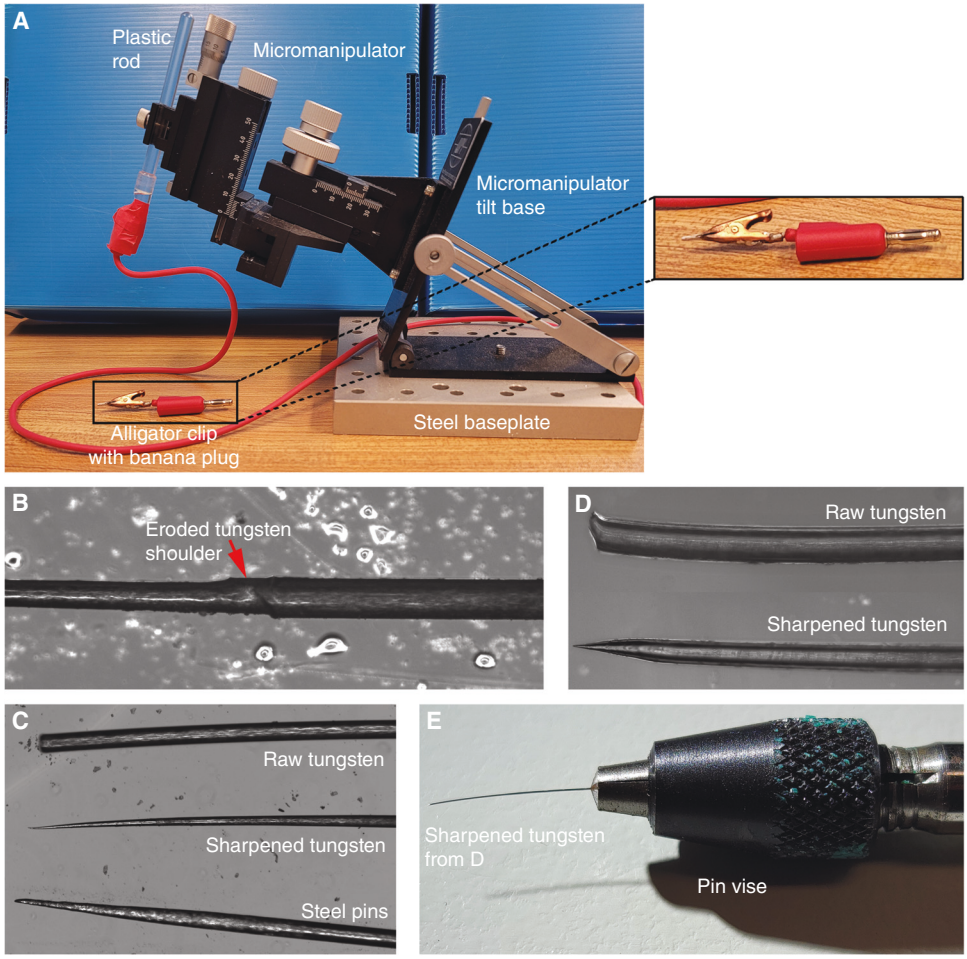

FIGURE 1. Tungsten wire-sharpening setup for embryo/larval dissection. (*A*) Custom tungsten-sharpening setup with a micromanipulator mounted to a tilting base. (*B*) High-magnification view of the tungsten wire after the shoulder has been eroded to thin the wire before further sharpening. (*C*) Comparison of raw tungsten wire (*top*), eroded and sharpened tungsten first-instar-dissecting pin (*middle*), and a steel third-instar-dissecting pin (*bottom*). The sharpened tungsten pin is needed for late-embryo/first-instar dissection because of the small size of the animal. (*D*) Comparison of raw tungsten wire (*top*) and the sharpened wire for embryo dissection (*bottom*). (*E*) Sharpened tungsten wire for embryo dissection mounted into a brass pin vise.

13. Test the pin by gently inserting it into a Sylgard plate.

    These pins must go ~1 mm into the Sylgard. If they are inserted too far, the tips will start to bend and will be unusable.

14. Repeat Steps 10–14 for the remaining pins.

15. (Optional): Using the gradations on the micromanipulator, precisely set the depth for initial thinning of the wire, such that all pins will have the same shape.

Generating the Sharpened Tungsten Embryo Tool

16. Load the cut 10-mm piece of tungsten wire into the alligator clip with ~5–6 mm exposed beyond the jaws.

17. Using either the manual manipulator or by hand, with the power supply set to 10 V, dip the tip of the tungsten wire (~0.5- to 1-mm) in and out of the NaOH bath 15–20 times.

    As lateral strength is required for these tools, these wires do not need to be thinned initially as described in Step 11.

18. Check the wire tip under a dissecting microscope.

    The tip should be so sharp that it may be difficult to clearly observe under a dissecting microscope (Fig. 1D). Note the shorter taper than seen in Figure 1C.

19. Repeat Steps 16–18 until several sharpened wires are produced. Clamp one into the pin vise to create an embryo-dissecting tool (Fig. 1E).

Embryo Collection

20. Prepare the embryo collection chamber by using a needle to poke holes all around a 6-oz polypropylene bottle to provide adequate ventilation.

    Caution: The needle is very sharp.

21. Place a small amount of yeast paste (~5-mm diameter circle) in the center of a 35-mm × 10-mm grape agar plate.

22. Add at least 100 flies (60/40 female/male ratio) to the polypropylene bottle from Step 20 and quickly cover the bottle with the grape plate, yeast side toward the bottle. Secure with laboratory tape.

    The plate will fit snugly into the mouth of the bottle.

23. Place the chamber in a 25°C incubator and leave overnight to allow flies to acclimate to their new environment.

    Additional humidity is not needed.

24. When ready for embryo collections, every 1–2 h, change the plate by tapping the chamber against a bench with the plate side up so that the flies fall to the bottom of the bottle, and then replace the plate with a fresh grape plate and yeast.

    Embryos will be deposited along the surface of the plate and within the yeast paste. Changing the plate every 1–2 h helps to yield well-synchronized embryos.

25. Incubate embryos for the desired amount of time in an incubator at 25°C.

    Late-stage embryos will develop at ~22–24 h postfertilization at 25°C (Lee et al. 2009). The first-instar stage is from 24 to 48 h postfertilization, the second instar is from 48 to 72 h postfertilization, and the third instar is from 72 to 120 h postfertilization at 25°C. The final period of third-instar development, known as wandering third-instar, is ~8 h long and is characterized by the larvae ceasing foraging and climbing the sides of the vial to pupariate. Unless otherwise noted, all third-instar anatomical comparisons are performed at this final stage (Hales et al. 2015).

    To dissect late-stage embryos, proceed to Step 26.

    To dissect larvae, proceed to Step 39.

Late-Embryo Dissection Technique

Due to the small size of these animals, special techniques must be used.

26. Using a razor blade, cut a small slab of agar, ~5-mm × 10-mm, from a 90-mm grape agar plate and move it to an unlabeled slide (Fig. 2A).

27. Cut a 20-mm length of double-sided tape, and place adjacent to agar slab on the slide (Fig. 2A).

28. Using a small paint brush, transfer the embryos from the surface of the grape plate to the double-sided tape.

29. Using the tip of the wooden-handled probe, gently roll the embryos on the double-sided tape, and the embryos will pop free of their chorion membrane (Fig. 2B). Transfer the dechorionated embryos to the agar slab.

    Freshly dechorionated embryos will easily stick to the probe, allowing for transfer to the agar slab.

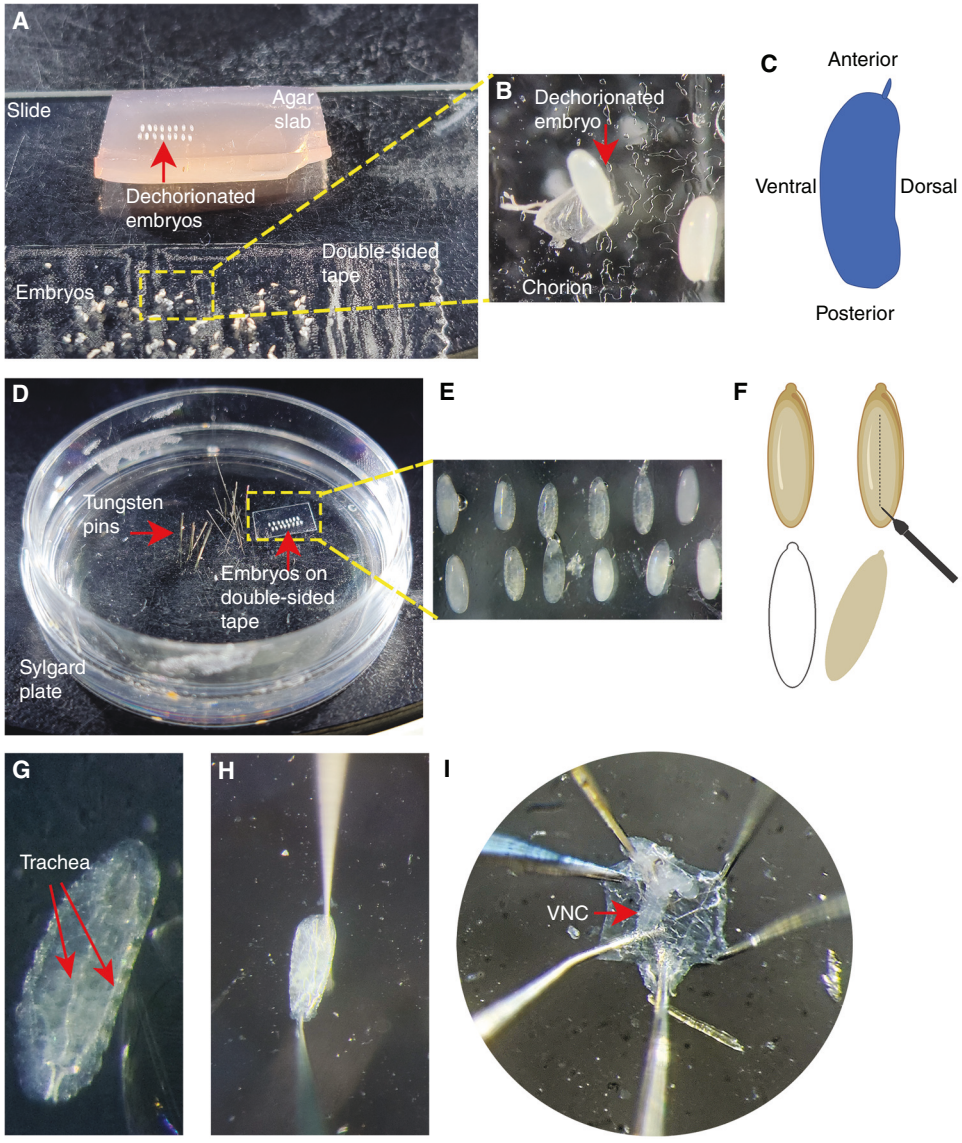

FIGURE 2. Stage 17 embryo dissection. (*A*) Slide with an agar slab and double-sided tape. Embryos have been placed on double-sided tape and dechorionated using a wooden-handled probe. Dechorionated embryos have been transferred to the agar slab and aligned properly. (*B*) High-magnification image of an embryo being removed from the chorion membrane. (*C*) Cartoon representation of a dechorionated embryo; note that the anterior is marked by the presence of a micropyle protrusion. (*D*) Sylgard dissection plate with embryos on a rectangle of tape. (*E*) High-magnification view of the embryos on double-sided tape. (*F*) Cartoon demonstrating the process of removing the embryo from the vitellin membrane (made with Biorender.com). (*G*) A stage 17 embryo removed from the vitelline membrane; note the presence of tracheae (arrows). (*H*) A stage 17 embryo pinned at the head and tail with sharpened tungsten pins. (*I*) A fully dissected stage 17 embryo; note the intact ventral nerve cord (VNC) (arrow).

30. Align embryos on the agar slab anterior side up and ventral toward the user using the #3 forceps (Fig. 2A,C).

    The embryos are quite fragile, so use caution in handling. It is easier to use just the tip of the forceps to push the embryo into position.

31. Prepare an ~5-mm × 10-mm piece of double-sided tape by lightly sticking the tape down on a glass surface and cutting to size with a razor blade.

32. Using the #2 forceps, move the tape to the short edge of a plain glass slide.

33. Holding the slide over the aligned embryos, gently touch the tape down to the agar block.

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The embryos should now be adhered to the double-sided tape.

34. Using #3 forceps, gently lift the tape and embryos from the slide and move them to a Sylgard plate. Press down firmly to stick the tape onto the Sylgard plate (Fig. 2D,E).
35. Add ~750 µL of 1× PBS to fill the plate about three-quarters.
36. Place the Sylgard plate under a dissecting microscope to clearly visualize the samples.
37. Using the pin vise tungsten/embryo dissection tool, lightly pierce the vitelline membrane close to the posterior end. While keeping the tip of the embryo dissection tool just under the surface of the vitelline membrane (clear membrane over the embryo), angle the tip upward and push the tool gently toward the anterior edge of the embryo, moving along the midline.

    This will easily slice the vitelline membrane, and if the embryos are mid–late stage 17, they will easily dislodge because the cuticle has developed (Fig. 2F,G).
38. Lightly pierce the embryo to move it from the vitelline membrane to the Sylgard plate. Continue to Step 43 below and follow the notes for younger animals.

Standard Larval Dissection and Fixation for Immunolabeling

As written, this procedure is for third-instar larvae. Modifications for dissection of earlier-stage larvae or late embryos (called "younger animals" below) are indicated as notes.

39. Prepare a Sylgard dish such that there are six groups of six 100-µm steel pins arranged in the center of the dish.

    This allows for six dissections per plate, so one can adjust accordingly (Fig. 3A).

    For younger animals, use 100-µm tungsten pins (Fig. 1C).
40. Wrap the dish in a small ring of clay on the black side of the AmScope plastic plate to hold it immobile while dissecting and moving pins (Fig. 3B,C).

    The plate also allows for easier movement, as it provides a larger surface area to turn and manipulate the dish.
41. Fill the dissecting dish with PBS, until it is about three-quarters full (~1 mL of PBS).
42. Using a paint brush, pick up either a larva or younger animal to dissect and rinse in a separate dish of PBS. Using the paintbrush or #3 forceps, carefully align the larva with the anterior toward the center of the dish and dorsal side up.

    The trachea run on either side of the dorsal surface, so align the animal such that these are both on the top of the preparation (Fig. 2G).
43. Using the #2 forceps to grasp the pins, pin the head just behind the mouth hooks.

    Use #3 forceps for younger animals.

    For all animals, make sure to angle the pins away from the body wall to ensure unobstructed visibility (Fig. 2H,I).
44. Next, pin the posterior end of the animal between the trachea and just anterior of the posterior spiracles (Fig. 3D).
45. Turn the dissecting dish such that the larva is perpendicular to the user by gently rotating the plastic base plate.
46. Using 3-mm spring scissors, cut across the posterior dorsal side, just above the tail pin, and try to sever both trachea (Fig. 3D).

    For younger animals, use 2-mm blade scissors instead of 3-mm blade scissors. These scissors have much finer blades.
47. Lifting the lower scissor blade, cut anterior until just before the scissors touch the pin (make sure not to puncture the ventral body wall).

    Some find it easier to rotate the dish 90° counterclockwise before making this cut.

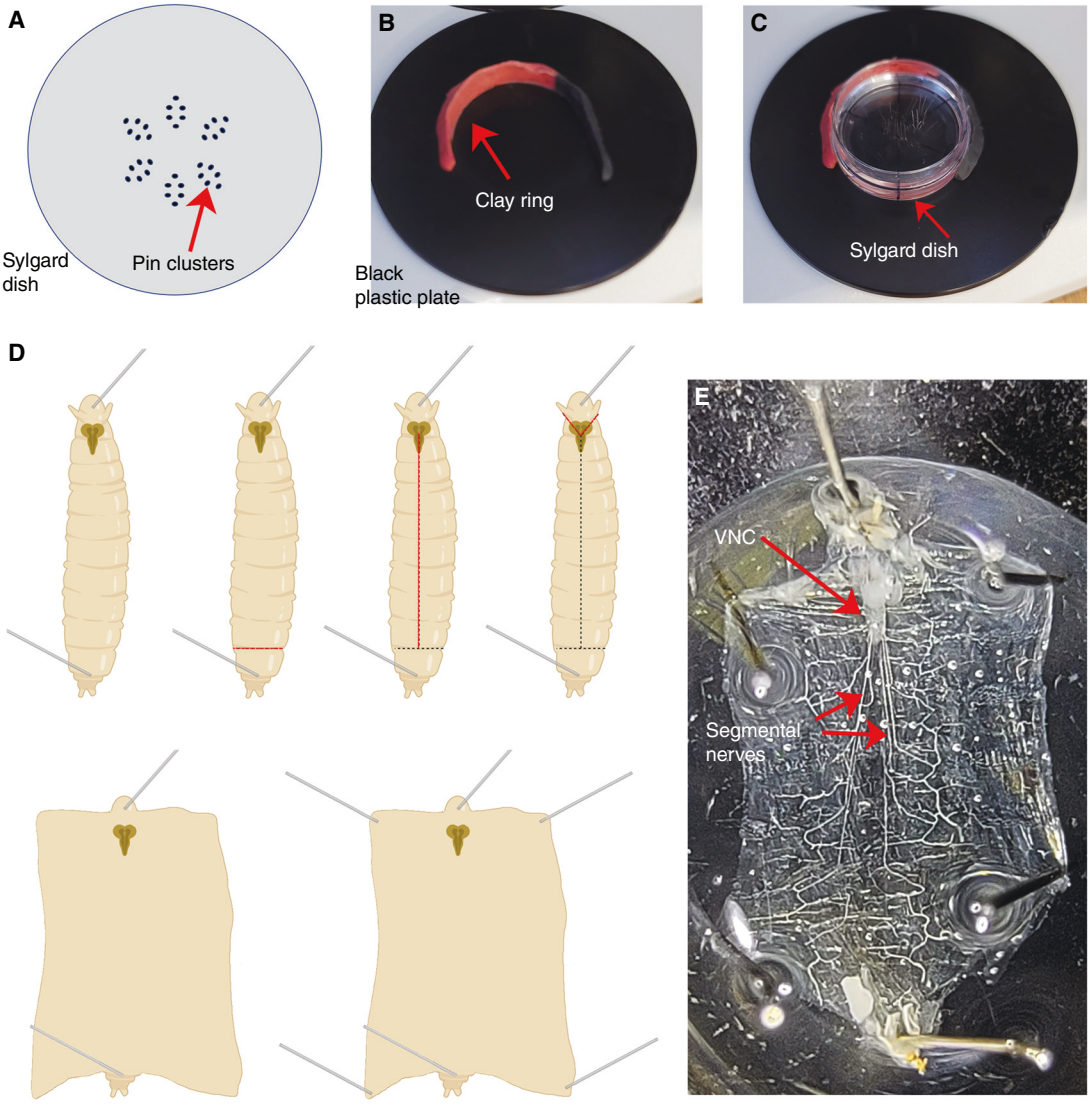

FIGURE 3. Larval dissection. (*A*) Cartoon of a Sylgard dish with steel pins clustered in the center. (*B*,*C*) Clay ring on a black plastic plate to hold the Sylgard dish in place and allow both hands to be used for dissecting. To rotate the dish, the black plastic plate is simply turned with unused fingers. (*D*) Schematic of larval dissection steps; red lines denote the next incision (made with Biorender.com). (*E*) A dissected third-instar larva pinned under saline. Arrows point to the VNC and segmental nerves.

48. Carefully cut a "Y"-shaped incision near the anterior end by turning the blade (or the dish) first 45° to the left and then 45° to the right of center.
49. Use the #3 forceps to remove the gut and fat bodies, but be careful to not dislodge the brain.

    The easiest technique is to use the forceps to grab one of the tracheae toward the posterior end and lift and pull forward.

    Use #5 forceps for younger animals.
50. Carefully pin the larva open using the remaining four pins from that cluster.

    Once again, make sure to angle the pins away from the body wall to ensure unobstructed visibility (Fig. 2I).
51. Once fully pinned down, use the 1-mL transfer pipette to rinse the preparation with PBS and clear away any additional material (Fig. 3E).
52. Repeat with the remaining five larvae/embryos.

To perform binding partner analysis, proceed to Protocol: ***Labeling of Cell Surface Proteins at the*** **Drosophila** ***Larval Neuromuscular Junction Using Binding Partner Peptides*** *(Ashley and Carrillo 2024a).*

53. Rinse once with 1 mL of PBS and fix samples. Remove PBS and replace with 1 mL of 4% paraformaldehyde. Incubate for 30 min at room temperature in 4% paraformaldehyde.

    Other fixatives could be used here, including Bouin's fixative, methanol, or acetone.

54. Remove the fixative and replace with 1 mL of PBS, and wash preparations for 10 min at room temperature without agitation.

55. After the wash, carefully remove pins using forceps (#2 for larval preparations, #3 for younger animals) and, grasping the edge of the preparation with the forceps, carefully transfer the preparation to a 0.5-mL microcentrifuge tube containing 300 µL of PBS.

    Use #3 forceps to remove the pins from younger animals.

    Perform immunolabeling in a nine-well depression plate containing 300 µL of PBS for younger animals.

56. Remove PBS and replace with 300 µL of PBST. Wash preparations three times with 300 µL of PBST for 10 min (per wash) on a nutator at room temperature.

    Proceed immediately to Protocol: ***Immunohistochemistry and Morphometric Analysis of*** **Drosophila** ***Larval Body Wall Neuromuscular Junction Preparations*** *(Ashley and Carrillo 2024b).*

RECIPES

Grape Agar Plates

Reagent	Quantity
Agar	12 g
H_2O	300 mL
Grape juice (Welch's or equivalent)	100 mL
Sucrose	5.35 g

1. Combine H_2O and agar together in a microwave-safe flask, and heat until just boiling. Use caution not to boil over.
2. Stop the microwave until the bubbles subside.
3. Restart the microwave and heat until bubbles rise, watching carefully to stop before boiling over.
4. Again, stop the microwave until bubbles subside.
5. Repeat Steps 3 and 4 until the bubbles become large and the agar is dissolved.
6. Meanwhile, heat the grape juice and sugar together until the sugar is dissolved.
7. Combine the two solutions and pour into the lids of 35-mm Falcon Petri plates (see Protocol: *Drosophila* Late Embryonic through Late Larval Stage Body Wall Dissection: Dissection Tools and Techniques [Ashley and Carrillo 2024 Cold Spring Harb Protoc doi:10.1101/pdb.prot108499]).

   Pour such that there is a convex meniscus over the edge of the lid. This fills about 50 35-mm Petri dish lids.

8. After cooling, store in a lidded container at 4°C until ready to use. These are stable up to 2 wk, but discard if agar begins to dry and pull away from the sides.

   Warm to room temperature before use in the egg-laying chamber.

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.

Sylgard Plates

1. Using the Sylgard 184 Silicone Elastomer Kit (Dow Corning), prepare a 10:1 mixture of elastomer base and curing agent by weight.

   Elastomer base is very viscous, so we typically weigh this first and calculate the amount of curing agent required. For example, for 11 g of elastomer, 1.1 g of curing agent is needed.

2. Using a metal spatula, stir together the two components until the mixture appears homogenous (i.e., no swirls of unincorporated curing agent). Mix gently, as vigorous mixing will incorporate lots of air into the mix, making the plates less clear. If a vacuum chamber is available, a 30-min incubation in a vacuum chamber can drastically reduce excess bubbles.
3. Pour 5 mm of Sylgard into the base of each 35-mm × 10-mm Petri dish.

   Twenty grams of elastomer makes about four plates.

4. Cure Sylgard for 2–3 d at room temperature, or for ~30 min in a 65°C incubator.

   Higher curing temperature will thin the mixture and allow more air to escape, resulting in Sylgard plates that are very clear. Plates are stable at room temperature; however, it is recommended to cover between uses, as the smooth surface can easily collect dust.

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