Protocol

Dissection of *Drosophila* Wandering Larval Brains for Patch-Clamping Neurons

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An enormous amount of neuroscientific knowledge has been gained from studying the larval stage of *Drosophila*. From an electrophysiological point of view, the larval neuromuscular junction has played an important role in this quest for knowledge, as it presents practical advantages such as accessibility and a stereotypic pattern. The physiological properties of larval central neurons have been less explored, with information regarding mainly a few identified motoneurons available to date. This protocol describes a quick and easy dissection of the brain of wandering third-instar *Drosophila* larvae to produce an ex vivo preparation in which central neurons can be patch-clamped. Immobilizing fresh and tiny tissues, such as larval brains, to perform successful patch-clamp recordings is a crucial step; here we explain in detail how this can be achieved using cyanoacrylate glue.

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 wandering third-instar larvae of the desired genotype (such as *RN2-Gal4 [eve-Gal4] > UAS-GPF/RFP* for labeling of identified motoneurons)

Larval external saline <R>

This is different from the classical HL saline (Feng et al. 2004). The solution can be prepared in advance if sucrose and calcium chloride are omitted (add before use).

Vetbond 3M Tissue Adhesive (*N*-butyl cyanoacrylate)

Equipment

Dissecting microscope (e.g., Leica S6E)
Glue pipettes (any electrode pipette)
Microcentrifuge tubes (0.5-mL)
Microscope slides (75-mm × 25-mm)

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Paintbrush (fine)
Pasteur pipettes
Pipette tips (1-mL)
Plastic tubing (fine, flexible; e.g., Tygon T3601-13)
Scissors (see Step 8)
Sharpened forceps <R> (two; see Steps 3 and 4)

The fine forceps must be in perfect shape for dissections to be completed quickly and with minimal effort. Unfortunately, no matter how careful you are with your favorite pair of forceps, they will bend at some point. Subtle damage to forceps is fixable by sharpening, and it is always better to invest some time sharpening your bent forceps than to spend a whole day attempting to dissect with defective tools. Sharpening forceps is an art; becoming good at it requires practice, but is worthwhile, as the forceps that you sharpen may even be better than a new pair.

Sylgard-coated coverslips and Petri dishes <R>

Before the day when you will patch-clamp, prepare many Sylgard-covered coverslips; you can prepare many at once so that you have to do this only once in a while. Sylgard-covered coverslips can be used only once; therefore, during a day of patch clamping, many coverslips will be used.

See Discussion.

METHOD

1. Fill a Sylgard-coated plastic dish halfway with larval external saline, and, using a fine paintbrush, place a wandering third-instar larva in the saline (Fig. 1A).
2. Using two pairs of fine forceps, split the larva about two-thirds down its length.

   See Troubleshooting.
3. Take the head portion (the longer one) and use two forceps to tear the cuticle from the open posterior end toward the anterior, tearing the cuticle on the dorsal side (between the large parallel tracheas) all the way up to the mouth hooks. Remove the cuticle.

   Try to leave the mouth hooks in place, as they provide a good "handle" for manipulating the central nervous system (CNS) later (see Step 5).

   See Troubleshooting.
4. Locate the CNS, confirm that it is undamaged, and remove all additional tissue that interferes with access to the ventral nerve cord (VNC) such as the gut, imaginal discs, and salivary glands (Fig. 1B).
5. Add a drop of larval external saline to a Sylgard-coated coverslip. Transfer the clean brain (handling it by the attached mouth hooks) onto the Sylgard-coated coverslip with a

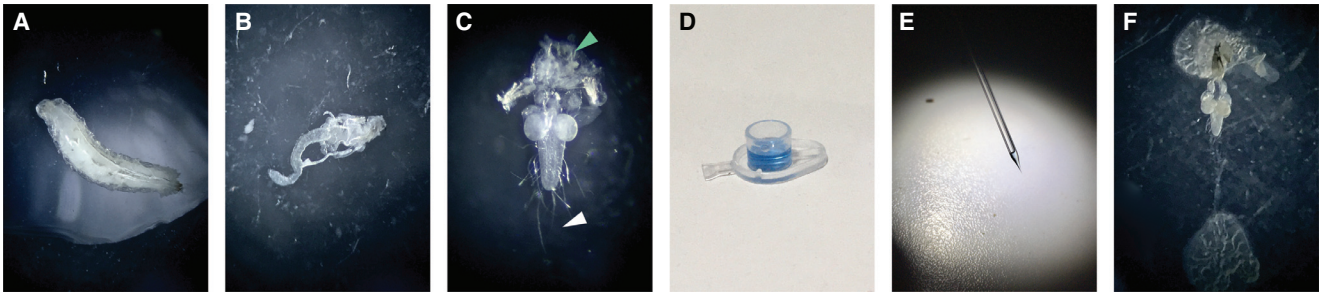

FIGURE 1. Step-by-step preparation of a wandering larval brain for whole-cell patch-clamp electrophysiology. (*A*) Wandering third-instar larva placed in a Sylgard-coated plastic dish filled halfway with larval external saline. (*B*) Larval brain surrounded by additional tissue after the larva was split about two-thirds down its length and its cuticle was removed. (*C*) Cleaned brain with mouth hooks (green arrowhead) and nerves (white arrowhead) attached. (*D*) Glue container made from a lid cut from a 0.5-mL microcentrifuge tube. (*E*) Tip of glass pipette filled with glue. (*F*) Larval brain glued to a Sylgard-coated coverslip.

drop of larval external saline. Place the brain with its ventral side down in the middle of the saline drop.

The tissue will naturally attach to the fresh Sylgard on the coverslip.

6. Comb the nerves down onto the Sylgard using forceps (Fig. 1C).

   The fresh (unused in this context) Sylgard that coats the coverslips is mildly sticky; therefore, once placed, the brain will attach to it. However, this attachment to the Sylgard is quite loose; therefore, the brain could be repositioned if necessary. To do this, pull the brain using the mouth hooks and replace it in the correct orientation on another part of the Sylgard-covered coverslip, as the area where it originally adhered will lose its stickiness.

7. Prepare a "gluer" by attaching a 1-mL tip to 30–40 cm of Tygon tubing.
8. Prepare a glue container by cutting a lid from a 0.5-mL microcentrifuge tube with scissors (Fig. 1D) and adding cyanoacrylate glue to the lid.
9. Use cyanoacrylate glue to immobilize the brain.

   i. Place any electrode pipette at the end of a "gluer," break its tip by gently rubbing it on the bottom of the glue container, and apply negative pressure (from the blue tip of the gluer) to aspirate some cyanoacrylate glue onto the tip (Fig. 1E).

   ii. Use positive pressure to blow the glue slowly between the brain and mouth hooks.

   See Troubleshooting.

   iii. Glue down the nerves (Fig. 1F).

   Cyanoacrylate glue will harden as soon as it contacts the solution. To avoid glue clogging the pipette tip, apply some positive pressure (gently) as you insert the pipette into the saline, but be careful not to release the glue all over your preparation while doing so.

   See Discussion.

10. After completing the dissection, orient the preparation under the dissecting microscope.

    For the larval preparation, the brain lobes should be on top of the field of view, with the VNC going down (Fig. 1F).

11. Transfer the coverslip with the glued brain preparation to the electrophysiology setup, keeping in mind its orientation.

    Proceed immediately to Protocol: ***Patch-Clamping* Drosophila *Brain Neurons*** *(Fernandez-Chiappe and Muraro 2022a).*

TROUBLESHOOTING

Problem (Steps 2 and 3): The CNS is damaged during the dissection.

Solution: There are two crucial moments when the CNS may be damaged: while dividing the larva in two (Step 2) or when removing the cuticle (Step 3).

- If the ending of the VNC is missing, the damage occurred while splitting the larva. Unfortunately, if the VNC is damaged, it cannot be used for patch clamping. Clean the dish and start a new dissection, but this time tear the larva at a more posterior place (which makes cuticle removal more laborious and time-consuming but results in an undamaged VNC).
- The other step in which the CNS may be damaged is the removal of the cuticle (Step 3). Always tear the cuticle on the dorsal side to avoid the brain, which is located ventrally.

Problem (Step 9.ii): The glue at the tip of the glue pipette hardens before it can be blown out.

Solution: If clogging still occurs, the pipette may be saved by breaking the tip a little further to remove the clogged portion. To do this, insert the pipette through the Sylgard down to the coverslip and rub the tip on the glass at the bottom to break the clogged part. When pulling the pipette of the Sylgard, the liquid glue will flow out of the pipette again, allowing gluing of the brain. Be careful

with this technique, however, because if the tip is broken too much, all the glue will flow out at once, damaging the preparation by overgluing. If this happens, the brain should be discarded and a new dissection should be started from the beginning.

DISCUSSION

Electrophysiological recordings require the tissue to be immobilized by some means. To achieve this, harps (tiny pieces of metal in the shape of a harp that hold down tissue such as a mouse brain slice) can be used, but these are not convenient for small fly tissues such as larval or adult brains. Pinning down the tissue is another means of immobilization, but this causes extra damage to the tissue. Also, pins may obstruct access when approaching the tissue with the electrode or the protease pipette.

Both of our dissection protocols (this one and Protocol: **Dissection of *Drosophila* Adult Brains for Patch-Clamping Neurons** [Fernandez-Chiappe and Muraro 2022b]) propose gluing down the tissue instead of holding it down by other means. This is a quick and easy way to immobilize the tissue, but an adequate substrate onto which the tissue can be effectively glued down is necessary. Sylgard is a silicone product that perfectly serves this purpose. Once prepared following the manufacturer's instructions (using the Sylgard 184 Silicone Elastomer Kit [Dow Corning]), Sylgard can be poured into small Petri dishes (plastic, 35-mm in diameter) to create a perfect arena for dissections; these can be washed and recycled over and over. Sylgard can be also poured onto small coverslips on which the desired tissue can be glued down.

Gluing brains to coverslips is a challenging part of this procedure. To master gluing down a brain, you must first master gluing. Take time to accustom yourself to how strongly you need to rub the tip of the pipette to cause a good amount of glue to flow in when you aspirate through the gluer (as shown in Fig. 1E). Next, take a Sylgard-covered coverslip and place a drop of larval external saline (without larvae) on it. Under the dissection microscope, practice blowing glue using positive pressure onto the Sylgard surface, aiming to produce small drops of glue in a row. If you feel adventurous, attempt to write your name in glue on the Sylgard surface. If you manage this, you are ready to glue some brains! Patch pipettes need to be freshly polished (see Steps 8–13 in Protocol: **Preparation of Pipettes and Pipette-Filling Devices for Patch-Clamping *Drosophila* Neurons** [Fernandez-Chiappe and Muraro 2022c]), so unused patch pipettes from a previous session or the halves of pipettes left over from setting up the appropriate puller settings (see Steps 20–28 in Protocol: **Preparation of Pipettes and Pipette-Filling Devices for Patch-Clamping *Drosophila* Neurons** [Fernandez-Chiappe and Muraro 2022c]) could be used for gluing.

RECIPES

Larval External Saline

Reagent	Final concentration (1×)
NaCl	135 mM
KCl	5 mM
$MgCl_2 \cdot 6H_2O$	4 mM
$CaCl_2 \cdot 2H_2O$	2 mM
TES	5 mM
Sucrose	36 mM

In advance of the experiment, prepare a 10× stock of this solution as follows.

1. Combine the reagents above without calcium chloride or sucrose.
2. Adjust the pH to 7.15 with 10 M NaOH.

   This solution can be stored for many months at 4°C.
3. Before use, dilute to 1× and add calcium chloride and sucrose as necessary for the final concentrations shown.

Sharpened Forceps

MATERIALS

Equipment

Dissecting microscope
Forceps (fine; e.g., Dumont #55, Fine Science Tools 11255-20)
Sharpening stone (e.g., Dan's Black Arkansas)

METHOD

1. Set the sharpening stone under a dissecting microscope, and, with the pair of forceps pressed closed, start rubbing one side and then the other to sharpen the tip, with the aim of making the ends meet again. Alternate sharpening a little on one side followed by a little on the other side; also change the angle between the forceps and the sharpening stone.

   A drop of H_2O on the sharpening stone may help to achieve better sharpening.

2. After a few minutes (depending on the material of the forceps and the extent of damage, it may take less or more time), wet soft tissue paper and wipe the forceps' tip clean from the handle toward the tip to remove the shredded metal.
3. Observe the result of the sharpening under the dissecting microscope. Test the forceps by attempting to pick up a small item such a dissection pin.
4. Continue sharpening (Step 1) if the tip is not yet good enough.

Sylgard-Coated Coverslips and Petri Dishes

MATERIALS

Equipment

Coverslips (small, square, 18 × 18-mm or round, 10-mm in diameter)
Falcon tubes (50-mL)
Incubator at ~50°C
Petri dishes (plastic, 35-mm and 100-mm in diameter)
Sylgard 184 Silicone Elastomer Kit (Dow Corning)
Syringes (1-mL)

METHOD

Sylgard-Coated Coverslips

1. Spread coverslips in many large Petri dishes (diameter of 100 mm or more) so that they are not touching each other, but the whole dish is covered.
2. Prepare the Sylgard following the manufacturer's instructions (basically by mixing the two components at a 10:1 ratio in a 50-mL Falcon tube) and mix well.
3. Fill a 1-mL syringe with the Sylgard mixture. Release a single drop on top of each coverslip.

   One drop is enough; do not add more or it will go over the coverslips, joining them all together and creating an unusable art installation.

4. Place Petri dishes flat in an incubator for a few hours or overnight at ~50°C until the Sylgard is hard.

   Sylgard will remain liquid if stored at –20°C for some time (months); keep leftover material at –20°C for future preparation of Sylgard-covered coverslips.

Sylgard-Coated Petri Dishes

5. Prepare the Sylgard following the manufacturer's instructions (basically by mixing the two components at a 10:1 ratio in a 50-mL Falcon tube) and mix well.
6. Pour Sylgard into a 35-mm Petri dish to cover its surface completely with a thick layer (approximately half its height).
7. Place the Petri dish flat in an incubator overnight at ~50°C until the Sylgard is hard.

REFERENCES

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