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Protocol

Preparation of Pipettes and Pipette-Filling Devices for Patch-Clamping *Drosophila* Neurons

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An essential requirement of every laboratory procedure is to have all materials ready when they are needed, so that the experimental flow is not disrupted. This is particularly true for patch clamping; therefore, effort must be devoted in advance to produce materials such as patch pipettes. This can be a fiddly business; hence, this protocol provides step-by-step advice on how to pull and polish patch-clamp pipettes. It also includes a brief description on how to prepare homemade filling devices to deliver saline efficiently and inexpensively into the pipettes. The protocol ends with guidelines on how to change the filament of a Sutter horizontal puller, a dreaded yet necessary activity that should be learned by anyone who wishes to become an expert patch clamper.

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

Blu Tack, wax, or plasticine (see Step 5)
Internal saline solution for the appropriate stage (select one):
- Adult internal saline <R>
- Larval internal saline <R>

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

Equipment

Binder with filament-changing sheets
Bunsen burner
Eppendorf tube (2-mL)
Forceps
Glass capillaries (thick- or thin-walled, borosilicate; e.g., Sutter Instrument BF150-86-7.5 or BF150-110-7.5)

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Thin-walled capillaries are used for producing pipettes to deliver the protease for local disruption of the superficial glia. With the glia removed, neuronal somas are exposed. Thick-walled capillaries are used to produce the actual patch pipettes.

Gloves (nonpowdered)
Microforge (e.g., Narishige MF2-LS2) or Fire Polish Spacer block (see Steps 8–13)
Micropipette puller (e.g., Sutter Instrument P-97 or P-1000)
Pasteur pipettes
Petri dishes (≥10-cm, ideally square; see Step 5)
Pipette tips (200-µL)
Puller filaments (e.g., Sutter Instrument FB255B)
Scissors
Screwdriver
Syringe (1-mL)

METHOD

This protocol describes how to pull patch and protease pipettes (Steps 1–7), how to polish patch pipettes after they are pulled (Steps 8–13), how to produce pipette-filling devices (Steps 14–19), and how to change the filament on a Sutter horizontal puller (Steps 20–28).

Pulling Pipettes

1. Program a Sutter puller (P-97 or P-1000).
   - For patch pipettes, program four cycles (loops).

     This is necessary for patch pipettes to be the correct shape and size. Do not attempt to do fewer or more cycles; otherwise, the shape of the pipette and thickness of the tip wall will not be satisfactory.

   - For protease pipettes, program four or five cycles.

     Four or five cycles should be adequate, as long as the tip size is appropriate.

   Bear in mind that the puller settings will depend on the type and size of the filament that you have installed on the puller and on the type and size of capillary glass that you use. The settings in Tables 1 and 2 could serve as a guide if you have an FB255B box filament installed and you are using BF150-86-7.5 glass for patch pipettes and BF150-110-7.5 glass for protease pipettes. Although it may be useful as a starting point, be aware that puller program settings will need to be modified for each puller every time the filament is changed (see Steps 20–28), with time as the filament wears off and even on a daily basis, depending on ambient conditions.

   The programs on a Sutter puller (P-97 or P-1000) consist of several cycles of precisely heating a filament to melt the glass in the middle of the capillary and pulling from both sides until the glass breaks, producing two pipettes. Many variables can be programmed for each cycle, such as heat (the amount of current that goes through the filament), pull (the hard pull, which should not be used for patch electrodes; this is only for sharp electrodes), velocity (which relates to how much the puller bars separate), and time (how long the puffing air cools down the glass) or delay (a different way of cooling the glass by puffing air with a certain delay).

TABLE 1. Patch-clamp pipette program settings on a P-1000 Sutter Instrument puller equipped with a FB255B box filament and using thick-walled BF150-86-7.5 glass capillaries

Loop	Heat	Pull	Velocity	Delay
1	535	-	21	1
2	535	-	21	1
3	535	-	21	1
4	570	-	21	1

Ramp test value = 566 on BF150-86-7.5 glass. All numbers are in arbitrary units.

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TABLE 2. Protease pipette program settings on a P-1000 Sutter Instrument puller equipped with a FB255B box filament and using thin-walled BF150-110-7.5 glass capillaries

Loop	Heat	Pull	Velocity	Delay
1	570	-	20	1
2	570	-	20	1
3	570	-	20	1
4	570	-	20	1
5	570	-	20	1

Ramp test value = 566 on BF150-86-7.5 glass (beware, although thin-walled glass capillaries are used for protease pipettes, the values given here relate to a ramp of 566 on thick-walled glass).

2. Use the Sutter puller to melt the glass in the middle of the capillary and pull from both sides until the glass breaks, producing two pipettes.
3. Determine the appropriate settings by trial and error.
   i. Test the pipettes under the microscope to observe the taper and tip (patch and protease pipettes).
   ii. Fill the pipettes with internal saline (adult or larval as appropriate) and test the tip size by measuring their resistance (patch pipettes only).

      For protease pipettes, the tip size should be in the range of 10–20 µm. For patch pipettes, the tip size should be in the range of 1–3 µm, and, when tested with a 5-mV seal test, should be in the range of 6–7 MΩ (to achieve 9–12 MΩ after polishing, see Steps 8–13).

   Table 3 provides advice on how to change the programs settings if the shape or size of the pipettes is not what is desired. As with any experiment, do not modify many parameters at the same time. Start by changing a few points for heat. Changing only one parameter and only on the last cycle may sometimes solve the problem (or not!).

   Further and extensive advice on how to produce patch-clamp pipettes can be found in Sutter's Pipette Cookbook.

4. Produce pulled pipettes as needed.

   For patch pipettes, perform Steps 8 and 9 or Steps 10–13 before storage.

5. To store pulled pipettes, add a strip of Blu Tack (or wax or plasticine) along the middle of a large Petri dish (square ones are ideal).
6. Stick the pipettes on the strip of Blu Tack, with their larger end facing down and the tip facing upward so that it does not break.
7. Keep the lid of the Petri dish on to prevent the pipettes from becoming dusty.

Polishing Pipettes

Patch pipettes can be polished with a microforge (Steps 8–9) or an adapter for the Sutter puller (Steps 10–13). Polish more patch pipettes than the ones you plan to use so that you have spares in case you break some or in case some are overpolished and therefore not usable. Patch pipettes need to be pulled and fire-polished freshly on the same day of use; leave unused polished patch pipettes for gluing the following day.

TABLE 3. Advice provided by Sutter Instrument on how to alter puller program settings to achieve desired changes in the size and shape of micropipettes

Parameter	Increase	Decrease
Heat	Smaller tips	Larger tips
	Longer taper	Shorter taper
	Higher resistance	Lower resistance
Pull	Smaller tips	Larger tips
	Longer taper	Shorter taper
Velocity	Smaller tips	Larger tips
Time/delay	Shorter taper	Longer taper

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Polishing with the Microforge

8. Press the pedal of a microforge, such as the MF2-LS2 by Narishige, to generate heat through a filament to polish the tip of the pipette.

   Fire polishing patch pipettes makes the glass tip smooth, and this helps the electrode attach to the cell. A microforge is a piece of equipment consisting of a microscope that magnifies the tip of the pipette and a pedal.

9. Observe the tip of the pipette retract to the desired point as it is being polished using the microforge's grid.

   Basically, with a microforge, you can visually follow the whole polishing process.

Polishing with an Adapter for the Sutter Puller

10. Design a single loop program on the puller to set conditions.
11. Use an adapter for the Sutter puller (a "Fire Polish Spacer block," sold separately, but it is not expensive) to hold a patch pipette in position close to the puller filament.
12. Polish the tip of the pipette.
13. Experiment with the heat level until the resistance of the polished pipettes is in the correct range.

    Fire polishing in this way is blind; the process cannot be visually followed. However, the effect of the polishing will be apparent as an increase in the average resistance of the polished pipettes compared to that of unpolished ones when tested at the setup. Although more time is needed to set the conditions to achieve good fire polishing in this way, this is a quick and effective polishing method once the settings are in place.

Producing Homemade Pipette-Filling Devices

14. Light a small Bunsen burner with the smallest flame possible.

    Commercially available flexible needles can be purchased to fill pipettes with solutions; however, these tend to be expensive. A homemade equivalent can be produced by using 200-µL tips and a Bunsen burner.

15. Grasp a 200-µL tip by its tip and hold it above the heat with the flame around the middle, rotating it until the heat makes the largest part fall under its own weight. At this point, quickly remove the tip from the flame. Pull slowly from both ends to stretch it.
16. Place the tip aside to cool down.
17. Use scissors to cut on the stretched part. Discard the tip end.
18. Cut the base of the tip so that it fits on a 1-mL syringe.

    You have produced a homemade pipette-filling device, similar to a MicroFil.

19. Check that the stretching has not sealed the plastic. To do this, push air through it with the syringe; if it is sealed, do not discard it immediately, cut the tip a little more and push air through it again.

    It may take a few attempts to do it correctly; so be prepared to produce a lot of melted tip art on the first day when you try this procedure. Once you understand how to do it, you may wish to produce many pipette-filling devices for future use. Beware, not all 200-µL tips behave the same way under the flame: Depending on the brand, some will be useful to produce a homemade pipette-filling device and some will not; it is just a matter of trial and error. We have found that GBO (Greiner Bio-One) 10–200-µL tips work well.

Changing the Filament on a Sutter Horizontal Puller

20. Following the manufacturer's instructions and using a screwdriver, loosen the screws that press the metallic plates that hold the filament in place. Remove the old filament.
21. Clean the metallic plates with tissue to remove the soot that was deposited there when the filament exploded.

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22. Wearing nonpowdered gloves, position a new filament in place, manipulating it with forceps.

    Never touch a filament with your bare hands, as it may become greasy. Wear nonpowdered gloves in case you need to nudge it into place.

23. Using the screwdriver, tighten the screws but only slightly, so that they hold position but you can still move the filament to its final place. Load a capillary glass into the puller to aid the centering process.

    Be careful when moving the glass toward the filament, as it may touch it if the filament is too off-center.

24. Position the filament so that the glass goes through the middle of the box or trough.

    Depending on the filament type that you are using, puller filaments come in two shapes, box and trough; either can be used. Centering the filament may require you to move around the puller, examining it from different angles.

25. Ensure that the filament is on top of the air jet (which cools the filament after each heating step).
26. Tighten the screws completely. Check again that the filament has not moved during the final tightening.
27. Produce two pipettes by pulling a capillary. Check that they are approximately of the same shape and tip size.

    If they are not, it is likely that the filament is off-centered and needs to be repositioned.

28. Determine the settings needed for the programs to produce the pipettes you need.
    i. Conduct a ramp test on the new filament. Record the results on a filament-changing sheet stored in a binder saved only for this purpose.
    ii. Compare the ramp value of the new filament to the ramp value of the previous filament (the one that exploded).

        The closer these values are to each other, the higher the chances are that the programs will work sooner.

    iii. Adapt the programs by taking into account the difference in the ramp values and modifying them accordingly.

        For example, if the new ramp value is five heat points higher than the previous one, increase the heat by five points in the pulling programs.

    iv. Examine the taper and tip of the pipettes under the microscope (patch and protease pipettes), fill the pipettes with internal saline (larval or adult as appropriate), and test the tip size by measuring their resistance (patch pipettes only).
    v. Keep changing the program settings, taking into account the advice in Steps 1–7, to produce the perfect patch and protease pipettes.
    vi. Record the settings of these programs in your binder for future reference.

        Changing the puller filament is easy and requires a few minutes; determining the settings needed may require more time.

    While changing the filament and program settings, you may produce many pipettes that are not usable. Save the ones that you have not tested with solutions (the other half of bad pipettes), as these can be used for gluing preparations.

DISCUSSION

To be successful at patch clamping, it is crucial to use the appropriate pipettes; hence, effort must be devoted to this task before a patching session. In the accompanying protocols, we describe how to obtain whole-cell patch-clamp recordings from fly neurons (see Protocol: **Dissection of *Drosophila* Wandering Larval Brains for Patch-Clamping Neurons** [Fernandez-Chiappe and Muraro 2022a], Protocol: **Dissection of *Drosophila* Adult Brains for Patch-Clamping Neurons** [Fernandez-Chiappe and Muraro 2022b], and Protocol: **Patch-Clamping *Drosophila* Brain Neurons** [Fernandez-Chiappe and Muraro 2022c]). Three types of pipettes are necessary to follow these protocols: glue pipettes, protease pipettes, and patch pipettes. Glue pipettes can be any pipette because, for the gluing step, the tip of the pipette

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will be broken slightly to fill it with cyanoacrylate glue; therefore, these are the easiest to obtain. Patch-clamp pipettes for fly neurons are not substantially different from patch-clamp pipettes for mammalian neurons; therefore, plenty of information exists about how to produce them (Sutter's Pipette Cookbook). On the contrary, protease pipettes are not used in many experimental procedures and, because the tip must be a precise size, which is larger than that of patch pipettes, these pipettes are the most difficult to produce.

After producing many pipettes, the puller filament will eventually need to be replaced. Horizontal pullers such as the P-97 or P-1000 puller from Sutter Instrument are equipped with a thin and fragile platinum filament (e.g., FB255B, Sutter Instrument) that heats up precisely to the required temperature and quickly cools down to allow manufacture of the pipettes necessary for protease application and for patch-clamping neurons. Unfortunately, this type of filament does not last forever (several months to a year, depending on usage and luck); therefore, you will need to change it eventually. Always keep some spare puller filaments (e.g., FB255B, Sutter Instrument) at hand. The puller filament may explode, emitting a loud noise and stopping the working of the puller (the filament does not heat up when the pull button is pressed). But, even before it explodes, the filament may start producing uneven and unreliable pipettes. If this is the case and, if after visual inspection of the filament, it appears defective (having lost its shine, thinning and subtly wrinkling in some parts), it may be better to exchange it with a new filament instead of modifying the programs to obtain better pipettes. Although adapting the programs as the filament ages may work for a while, at some point the time it takes to adjust programs and the time and materials lost while producing unusable pipettes will be the equivalent of the time spent changing the puller filament.

Another reason to change the puller filament is accidental damage caused by the operator. This is not uncommon because the filament is very fragile and even a gentle impact with a capillary will cause it to lose its shape. When using the puller, always be careful to avoid any contact with the filament. If something has touched the filament, even if there is no visible change to it, you will need to run a ramp test following instructions from the puller manufacturer (see Steps 28.i–28.ii) and readjust the programs or change the filament if the damage is extensive.

RECIPES

Adult Internal Saline

Reagent	Final concentration
Potassium gluconate	102 mM
NaCl	17 mM
$CaCl_2{\cdot}2H_2O$	0.085 mM
EGTA	0.94 mM
HEPES	8.5 mM

Adjust pH to 7.2 with 5 M KOH. To avoid swelling or shrinkage of cells, be conscientious when weighing the components, and confirm the final concentration of osmolytes with an osmometer (if available). Aliquot 1.9 mL in a 2-mL Eppendorf tube and store for up to many months at −20°C.

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Larval Internal Saline

Reagent	Final concentration
$MgCl_2.6H_2O$	2 mM
EGTA	2 mM
KCl	5 mM
HEPES	20 mM
KCH_3SO_3	140 mM

Prepare the KCH_3SO_3 by combining 0.78 g of KOH and 0.91 mL of methanesulfonic acid per 100 mL of internal saline. Add the other reagents to the KCH_3SO_3, adjusting the pH of the solution to 7.4 with 10 M KOH. To avoid swelling or shrinkage of cells, be conscientious when weighing the components, and confirm the final concentration of osmolytes with an osmometer (if available). Aliquot and store at −20°C (keeps for many months).

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