

Video Article

Primary Neuronal Cultures from the Brains of Late Stage Drosophila Pupae

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Abstract

In this video, we demonstrate the preparation of primary neuronal cultures from the brains of late stage Drosophila pupae. The procedure begins with the removal of brains from animals at 70-78 hrs after puparium formation. The isolated brains are shown after brief incubation in papain followed by several washes in serum-free growth medium. The process of mechanical dissociation of each brain in a 5 ul drop of media on a coverslip is illustrated. The axons and dendrites of the post-mitotic neurons are sheered off near the soma during dissociation but the neurons begin to regenerate processes within a few hours of plating. Images show live cultures at 2 days. Neurons continue to elaborate processes during the first week in culture. Specific neuronal populations can be identified in culture using GAL4 lines to drive tissue specific expression of fluorescent markers such as GFP or RFP. Whole cell recordings have demonstrated the cultured neurons form functional, spontaneously active cholinergic and GABAergic synapses. A short video segment illustrates calcium dynamics in the cultured neurons using Fura-2 as a calcium indicator dye to monitor spontaneous calcium transients and nicotine evoked calcium responses in a dish of cultured neurons. These pupal brain cultures are a useful model system in which genetic and pharmacological tools can be used to identify intrinsic and extrinsic factors that influence formation and function of central synapses.

Video Link

The video component of this article can be found at <http://www.jove.com/video/200/>

Protocol

Preparations before day of culturing:

1. Make sterile dissecting solution.
2. Make sterile DMEM and keep in 10 ml aliquots at 4°C for 2 weeks.
3. Make sterile DDM2 supplements and freeze in 50 or 100 µl aliquots for 1 month.
4. Make ConA/laminin.
5. Coat coverslips.

Optional: Make sterile CNBM and store frozen for up to 4 months.

On day of culturing

I. Prepare Enzyme Solution (ES) in laminar flow hood

1. Put 5 ml of dissecting solution (DS) in a 15 ml centrifuge tube.
2. Weigh 0.8 mg of L-Cysteine in a 0.6 ml eppendorf and add 150 µl of DS. Pipette up and down until crystals are gone.
3. Add 150 ml of DS/Cysteine to the 5 ml DS and mix well.
4. Add 50 U (units) of Papain.
5. Add 7 ml of 0.1 N Na OH and mix well. Solution will clear within 30 min. when activated.
6. Filter enzyme solution with 0.2 mm syringe filter into a sterile 1.5 ml screw cap microfuge tube

Papain: Want 50 U in 5 ml DS. Each batch is slightly different, so must calculate how much:

$$37.3 \text{ mg/ml and } 28.3 \text{ U/mg}$$

$$37.3 \times 28.3 = 1055.59 \text{ U/ml (1000 ml)}$$

50 U = 47.4 ml

II. Make DDM2 media

On day of culturing: add the following supplements to make DDM2

To 10 ml of DMEM add the supplements, just before culturing:

1. 100µl Transferrin
2. 100µl Putrescine
3. 100µl Selenium
4. 100µl Progesterone
5. 50µl Insulin
6. 10µl 20-Hydroxyecdysone

Note: Make enough DDM2 for all cultures planned (each brain plated on a single coverslip in an individual 35 mm Petri dish, 1.5 ml of media/culture)

Step III-VI can be at a done nonsterile lab bench and should be completed in 45 minutes or less.

III. Pupae Collection

1. Select 10-15 pupae from sides of vial under a dissecting microscope.
2. Place pupae in the lid of a dry 35 mm Petri dish.

Note: We generally use brains from pupae between 55-78 hours after puparium formation (APF). It is slightly harder to dissect out the brain from the younger pupae since the head capsules tend to collapse, while the overall level of neurite outgrowth is slightly lower from the oldest pupae. In the Canton-S wildtype strain, these stages are recognized by pigmented eyes that are light brown to slightly reddish, with little pigment elsewhere.

IV. Decapitation under dissecting microscope

1. Place two drops of sterile dissecting solution in lid of Petri dish, next to pupae.
2. Gently hold a single pupa with fine forceps in left hand and use a 27 gauge syringe needle attached to a 1 cc syringe in the right hand to remove the cuticle at the anterior end of pupa.
3. Squeeze pupa slightly with forceps and as head emerges use 27 gauge needle to sever neck.
4. With tip of the needle or the forceps, transfer head to drop of dissecting solution.
5. Repeat until you have 10-15 heads in a single drop.

V. Removal of the brain from head under dissecting microscope

1. In each hand hold a 1 cc syringe with a 27 gauge needle.
2. Use these to position the fly head in the drop of dissecting solution so the proboscis is facing you and the dorsal surface of the head is north.
3. Insert the left needle into the proboscis to pin the head down and use the right needle to make a slit in the right eye that goes from the ventral to the dorsal surface.
4. Insert the right needle near the left in the region of the proboscis and then use the left needle to gently depress the cuticle over the left eye, pushing the brain toward the slit in the right eye. The brain should emerge from the slit on the right, relatively intact, often with both optic lobes still attached and sometimes the pigmented eye tissue as well.
5. Push the brain onto the back of the needle and transfer to a drop of sterile dissecting saline in a new sterile, 35 mm Petri dish.
6. Collect 10-15 brains for each genotype.

VI. Removal of optic lobes and enzyme treatment

1. In each hand hold a 1 cc syringe with a 27 gauge needle and use these to gather all brains in one small area of the drop of dissecting solution
2. Use syringes as cutting tools to remove the optic lobes from each brain so the tissue remaining for culture is the central brain region.
3. Use a 20 µl pipetman, set at 5 µl, to transfer all the brains of a single genotype to an eppendorf tube containing 1 ml of sterile papain solution.
4. Brains incubated in papain for 10-15 minutes on a rotator set at 60 rpm.
5. Centrifuge at 3000 rpm for 3 minutes.

Inside sterile laminar flow hood – all steps where tissue is exposed to air should be done in laminar flow hood for the remaining steps.

VII. Washing

1. Remove enzyme solution and replace with sterile dissecting solution.
2. Centrifuge at 3000 rpm for 3 minutes.

3. Wash with sterile dissecting solution a total of 3 times.
4. Remove dissecting saline and replace with DDM2.
5. Centrifuge and wash a total of 2 times with DDM2.
6. Transfer brains and media to a sterile 35 mm Petri dish.

VIII. Trituration and Plating under dissecting microscope

1. Place 5 μ l of DDM2 in the center of a coverslip.
2. Transfer 1 brain to this drop of DDM2 with a yellow tip.
3. Under the microscope, use 27 gauge needles to dice up brain into small pieces (should take <1 minute).
4. Under visual guidance, break the tip of a glass pipet that has been pulled to a fine point so that the larger pieces can be gently sucked into the tip of the pipet and expelled back into the medium. We use mouth suction tubes that come standard with 100 μ l hematocrit capillary glass.

Note: Take care not to get bubbles in the media and you will need to empirically determine the best size pipet to use. Good ones allow you to dissociate the brain with some individual cells and still some large clumps, in about 30 seconds/brain. When you look at these at higher power, approximately 10-20% of the neurons should still have some processes remaining and there will still be some large clumps. If you dissociate too much, then all the cells will be round and they will have sustained so much damage they will not survive. This step takes practice and must be done quickly since there is a large surface-to-volume ratio and the osmolality and pH of the drop of DDM2 can change quickly. Tissue needs to be placed in CO₂ incubator as quickly as possible.

5. Write on the petri dish lid: "Genotype, date and time".
6. Put the 35 mm petri dish in a 100 mm petri dish (with wet kimwipe) and let settle in the CO₂ incubator for 30 min.
7. Flood the 35 mm dish with 1.5 ml of DDM2.
8. Keep cultures in the incubator at 5% CO₂ incubator (23°C).

Note: If you don't have access to a cooling CO₂ incubator, use a standard mammalian tissue culture incubator and either put it in a cool room and heat to 23°C, or put in the lab, turn off temp, and use ice in a tray at bottom of incubator to regulate temp around ambient.

IX. Feeding Cells

1. On Day 1 (next day), add 0.5 ml of CNBM/B27 (Conditioned Media) to make 3:1 DDM2:CNBM/B27.
2. On Day 5 replace 1 ml of media for 3:1 DDM2:CNBM/B27.
3. Keep feeding cells with 3:1 DDM2:CNBM/B27 every 4-5 days.

Note: Cultures can be grown without the non-neuronal conditioned media but the neurons appear a bit more fragile and are more difficult for use in electrophysiology experiments.

Discussion

Neurons harvested from the brains of embryonic/postnatal rodents can be grown in primary cell culture where they extend neurites and form functional synaptic connections. Methods for preparation of these cultures are well established and studies in rodent neuronal cultures have played a critical role in identifying genes and environmental factors involved in regulation of synapse formation and function (Banker and Goslin, 1991). While insect neurons from a variety of species can also be grown in culture, the only insect neurons shown to form functional synaptic connections in culture are from *Drosophila* (Rohrbough et al, 2003). Neuroblasts harvested mid-gastrula stage *Drosophila* embryos grown in defined media give rise to neurons that are electrically excitable and form functional, interneuronal synaptic connections (O'Dowd, 1995; Lee and O'Dowd, 1999). To identify factors that regulate synaptic form and function in neurons known to be involved in mediating adult behaviors we developed the technique illustrated in this video for harvesting and culturing neurons from brains of late stage *Drosophila* (Su and O'Dowd, 2003). One of the key features of this procedure was to use papain, instead of collagenase or other harsh enzymes traditionally used in preparation of insect neuronal cultures. Papain results in dissociated neurons retaining short axonal processes that survive, regenerate neuritic processes, and form functional interneuronal synaptic connections. Whole recordings in identified cell populations, including mushroom body Kenyon cells, show that fast excitatory synaptic transmission in the cultures is mediated by nAChRs while inhibition is mediated GABA receptors (Su and O'Dowd, 2003). Our recent electron microscopic analysis demonstrates that the neurons in culture form synapses with structural features that are similar to both chemical and electrical synapses in the adult brain (Oh et al., 2007). As illustrated in the video, the cultures are also amenable to Fura-2 calcium imaging studies and we have used these to investigate the cellular mechanisms underlying spontaneous and evoked changes in intracellular calcium levels in identified cell populations including Kenyon cells and cholinergic neurons (Jiang et al., 2005; Campusano et al., 2007). With the extensive genetic tool kit available in *Drosophila* this culture system provides a very useful tool for identifying intrinsic and extrinsic regulators of central synapse structure, function, and plasticity.

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References

1. Banker, G. Goslin, K. (Editors) *Culturing Nerve Cells*. Cambridge: MIT Press. (1991)
2. Rohrbough, J., O'Dowd, D.K., Baines, R.A., and Broadie, K. (2003) Cellular bases of behavioral plasticity: Establishing and Modifying synaptic circuits in the *Drosophila* Genetic System. *J. Neurobiol.* 54: 254-271.
3. O'Dowd, D.K. (1995) Voltage-gated currents and firing properties of embryonic *Drosophila* neurons grown in a chemically defined medium. *J. Neurobiol.* 27: 113-126. (1995)
4. Lee, D., and O'Dowd, D.K. Fast excitatory synaptic transmission mediated by nAChR in cultured *Drosophila* neurons. *J. Neurosci.* 19: 5311-5321 (1999)
5. Su, H and O'Dowd, D.K. Fast synaptic currents in *Drosophila* mushroom body Kenyon cells are mediated by alpha-bungarotoxin-sensitive nAChRs and picrotoxin-sensitive GABA receptors. *J. Neurosci.* 23: 9246-9253. (2003)
6. Jiang, S.A., Campusano, J.M., Su, H., and O'Dowd, D.K. *Drosophila* mushroom body Kenyon cells generate spontaneous calcium transients mediated by PLTX-sensitive calcium channels. *J. Neurophysiol.* 94: 491-500. (2005)
7. Campusano, J.M., Su, H., Jiang, S.A., Sicaeros, B., and O'Dowd, D.K. nAChR-mediated calcium responses and plasticity in *Drosophila* Kenyon cells. *Develop Neurobiol.* 67: 1520-1532. (2007)
8. Oh, H-W, Campusano, J.M., Hilgenberg, L.G.W., Sun, X., Smith, M.A. and O'Dowd, D.K. Ultrastructural analysis of chemical synapses and gap junctions between *Drosophila* brain neurons in culture. *Develop Neurobiol.*, in press. (2007)