

Video Article

Preparation of Dissociated Mouse Cortical Neuron Cultures

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URL: <https://www.jove.com/video/562>

DOI: [doi:10.3791/562](https://doi.org/10.3791/562)

Keywords: Neuroscience, Issue 10, cellular, molecular, neurobiology, neuron, calcium/sodium imaging, primary cultures, mouse

Date Published: 12/19/2007

Citation: Hilgenberg, L.G., Smith, M.A. Preparation of Dissociated Mouse Cortical Neuron Cultures. *J. Vis. Exp.* (10), e562, doi:10.3791/562 (2007).

Abstract

This video will guide you through the process for generating cortical neuronal cultures from late embryo and early postnatal mouse brain. These cultures can be used for a variety of applications including immunocytochemistry, biochemistry, electrophysiology, calcium and sodium imaging, protein and/or RNA isolation. These cultures also provide a platform to study the neuronal development of transgenic animals that carry a late embryonic or postnatal lethal gene mutation. The procedure is relatively straight forward, requires some experience in tissue culture technique and should not take longer than two to three hours if you are properly prepared. Careful separation of the cortical rind from the thalamo-cortical fiber tract will reduce the number of unwanted non-neuronal cells. To increase yields of neuronal cells triturate the pieces of the cortical tissue gently after the enzyme incubation step. This is imperative as it prevents unnecessary injury to cells and premature neuronal cell death. Since these cultures are maintained in the absence of glia feeder cells, they also offer an added advantage of growing cultures enriched in neurons.

Video Link

The video component of this article can be found at <https://www.jove.com/video/562/>

Protocol

Preparations before day of culturing:

- Prepare sterile dissecting solution (DS).
- Prepare NBM/B27 (Neurobasal Medium with B27 supplements).
- Autoclave ddH₂O and sterilize glass coverslips, if needed.
- Coat tissue culture dishes or glass coverslips with poly-D-lysine.

Poly-D-Lysine Coating:

Prepare the day before culturing under sterile conditions.

- Thaw aliquot of 10X PDL and place on ice.
- Add 9 ml sterile ultra-filtered water to 1 ml of PDL and mix well (1X).
- Coat surfaces with 1X PDL overnight at room temperature as follows:
 - glass coverslips (Bellco Glass, Inc. Cat.# 1943-00012): 75 µl/coverslip
 - OR
 - 24 well culture plate: 300 µl/well
 - OR
 - 35mm culture dish: 1ml/dish
- Rinse 5X with sterile water (use sterile Pasteur pipettes to aspirate liquid).
- Remove water until surface is completely dry.

Culturing procedure (done in laminar flow hood under sterile conditions)

1. Cut out block of agar and glue it onto the support block of the microtome Vibraslicer (Campden Instruments Ltd.) using super glue.
2. When using late embryonic stage (E17-18) mouse fetuses, euthanise dam, remove uterus and free individual fetuses from embryonic sack. Place fetuses into sterile Petri dish and continue as outlined below.
3. Decapitate mouse fetus or pup (follow guidelines approved by your Institutional Animal Care and Use Committee).
4. Remove skin and skull and place brain onto Whatman filter paper disk in a 60mm petri dish filled with cold DS.
5. Cut off the cerebellum using a sterile razor blade.
6. Pick up brain using a spatula and drain excess fluid on filter paper, then transfer brain to Vibraslicer support block and glue brain in place (caudal side up and ventral part facing agar).
7. Fill chamber with cold DS. Set speed selector to 8-9.

8. Cut 200-400 μm sections, beginning from olfactory bulb. Once the blade of the Vibraslicer enters the cortex, begin cutting 600 μm coronal sections. An E17-18 mouse brain typically yields 3-4 usable slices, while a P0 mouse brain yields 4-5 usable slices.
9. Transfer brain slices to 35 mm petri dish labeled DS 2 using reverse end of un-plugged 10 ml glass Pasteur pipet.
10. Dissect out cortex and remove meninges using glass needles pulled from capillary tubes.
11. Cut cortical rinds into small pieces, 0.5-1 mm in length.
12. Filter ES through 0.2 μm filter attached to a 5 cc syringe into a 35 mm petri dish.
13. Transfer the cortical tissue pieces to petri dish containing filtered ES.
14. Incubate at 37°C for 30 min.

In the meantime:

1. Clean up hood, Vibraslicer and dissecting tools.
2. Add 5 ml of warm NBM/B27 into a 60 mm petri dish.

After 30 min. incubation:

1. Transfer tissue to 10 ml DS. Let settle for 1 min.
2. Transfer tissue to first Hi tube swirls and let settle for 2-3 min.
3. Transfer to second Hi. Repeat as above.
4. Transfer to first Li. Repeat as above.
5. Transfer to second Li. Repeat as above.
6. Transfer to third Li. Repeat as above.
7. Transfer tissue to the 60 mm dish with media.

Under the dissection microscope:

1. Clean debris from tissue and triturate each piece by gently passing through a pulled glass pipet (use decreasing bore sizes) to loosen up the tissue.
2. Transfer cell suspension to 15 ml conical tube containing the rest of NBM+B27 (Make a total of 13 ml for a 24 well plate or 11 ml for 5 - 35 mm dishes. Mix gently and wait for large pieces of tissue to settle).
3. Transfer cell suspension (0.5 ml/well for a 24 well plate or 2 ml / 35 mm culture dish).
4. When using glass coverslips, add 80 μl cell suspension per coverslip and allow 1 hr in tissue culture incubator for cells to adhere before flooding the chamber with more NBM/B27 media.
5. Maintain cultures at 37°C and 5% CO_2 .

Next Day

24 well plate: Feed each well with 0.5 ml non-neuronal cell conditioned NBM/B27 medium (cNBM/B27; Protocol for preparation of cNBM/B27 medium appears below)

35mm dishes: Replace 0.5 ml with cNBM/B27 medium.

Maintain culture by replacing 0.5ml of medium with fresh cNBM/B27 every 2-3 days.

Although the culture media does not promote glia cell proliferation, cultures can be treated with 5 μM FDU (5-Fluoro-2'-deoxyuridine, Sigma F0503) at day 3-5 in culture to further reduce the number of glial cells if needed.

SET-UP for Dissection and Culture

Sterilize dissecting tools in 70% EtOH or autoclave them:

- Scissors (med. and small) Spatulas (med. and small)
- Tweezers (med. and small) Razor blade
- Blade for vibraslicer Buffer bath
- Small metal wedge for blade Support for brain

Other materials:

- Petri dishes (60 mm)
- Petri dishes (35 mm)
- Filter paper
- Glass pipet 10 ml
- Sterile disposable pipets, 5,10 and 25 ml
- Syringe filter, 0.2 μm
- Syringe 5 cc
- Centrifuge tubes, 15 ml
- Sterile Pasteur glass pipets
- PDL coated culture dishes or glass coverslips

Label six 15 ml centrifuge tubes and follow preparation:

Tube #1: Enzyme solution:

Add 50 U of papain (Worthington LS 03126) to 5 ml DS containing:

- 100 µl of L-cystein (0.8mg)
- 7 µl 0.1N NaOH
- 50 µl APV (5mM)

Leave solution out at room temperature to clear. Note that enzyme solution will appear 'cloudy' at first and needs to clear before use.

Tube #2 & #3: Hi Enzyme Inhibitor:

3 ml DS + 300 µl BSA/Ti + 30 µl APV (5mM).

Mix gently to avoid bubbles, then divide into two 1.5ml aliquots.

Tube #4 - #6: Low Enzyme Inhibitor:

8 ml DS + 80 µl BSA/Ti + 80 µl APV (5mM).

Mix gently to avoid bubbles, divide into three 2.6 ml aliquots.

Place tubes numbered #2 through #6 on ice. Leave tube #1 (Enzyme Solution) at room temperature.

SOLUTIONS AND ALIQUOTS

- Solution A (Buffered Saline) Sigma Formula wt 500 ml [conc.]
- Sodium Chloride NaCl S-9625 58.45 80.0g 137mM
- Potassium Chloride KCl P-4504 74.56 4.0g 5.4mM
- Sodium Phosphate Dibasic anhydrous Na_2HPO_4 S-0876 142.0 0.24g 0.17mM
- Potassium Phosphate Monobasic anhydrous KH_2PO_4 P-5379 136.09 0.3g 0.22mM

Weigh out all ingredients and mix until dissolve with 400 ml ultra filtered water. Bring final volume to 500 ml. Place in a clean bottle and autoclave. Store at 4°C and label "DS Solution A".

- Solution B (Hepes) Sigma Formula wt 250 ml [conc.]
- Hepes Hepes H-3375 283.3 20.97g 9.9mM

Add ultra-filtered water up to 200 ml. Mix until dissolved and bring final volume to 250 ml. Place in a clean bottle and autoclave. Store at 4°C and label "DS Solution B".

- Working Solution 500 ml [conc.]
- Ultra filtered water 400 ml
- Stock solution A 25 ml
- Stock Solution B 14 ml
- D (+)-Glucose Sigma G-8270 3.0 g 33.3mM
- Sucrose Sigma S-0389 7.5 g 43.8 mM

Adjust pH to 7.4 with 1N NaOH. Bring final volume to 500 ml with ultra filtered water. Decant into a clean glass bottle and autoclave. Store at 4°C. Label "Dissecting Solution".

Poly-D-Lysine Preparation:

1. Prepare 10x stock solution of poly-D-lysine (PDL; Sigma P-7280) in sterile H_2O at 1mg/ml.
2. Make 1.0 ml aliquots. This solution can be stored at -20°C for up to 3 months.

MEDIA

1. Add 10ml of B-27 Supplement (Gibco 17504-044) to 500 ml NBM bottle (Neurobasal Medium, Gibco 21103-049).
2. Make 40 ml aliquots and store at 4°C.

APV

1. Add 10 ml sterile H_2O to vial of 10 mg APV (2-Amino-5-phosphonopentanoic acid, Sigma A-5282) and mix thoroughly.
2. Prepare 180 µl aliquots and store at -20°C.

BSA/Ti

1. Dissolve 1 g BSA (Bovine Albumin. Sigma A-7030) and 1 g Trypsin Inhibitor (Sigma T-9253) in 10 ml DS.
2. Adjust pH to 7.4 with 1N NaOH.

3. Sterilize by filtering through 0.2 μ m syringe filter.
4. Divide into 400 μ l aliquots and store at -20°C.

L-CYSTEINE

In an Eppendorf tube dissolve 0.6 mg L-Cysteine (Sigma C-7755) in 200 μ l DS.

AGAR (4%)

1. Dissolve 4 g of Bacto Agar (Difco 0140-01) in 100 ml sterile water. Keep at 4°C until needed for culture.
2. Microwave Agar to melt and fill a 35 mm petri dish. Let sit to cool down and polymerize.

PAPAIN (Worthington LS 03126)

NON-NEURONAL CULTURES IN NUNC CULTURE BOTTLES

COATING CULTURE BOTTLE (4 Nunc bottles)

1. Add 9 mL sterile water to each of 3 tubes of 10X PDL to make 1X PDL.
2. Transfer 30 ml 1X PDL in the first bottle.
3. Move slightly until all the growth surfaces are covered. Let sit for 1 min.
4. Transfer 1x PDL to second bottle. Let sit for 1 min.
5. Repeat with the third and forth bottles.
6. Rinse all the four bottles 2X with 150 ml sterile H₂O.

PREPARE ENZYME SOLUTION (ES):

1. In a 0.6 ml centrifuge tube weight out 0.8 mg L-Cysteine (Sigma C7755), add 150ml DS and vortex until crystals are dissolved.
2. Transfer 5 ml DS to a 15 ml conical tube, then add 150ml L-Cysteine.
3. Add 50 units Papain. (Worthington LS 03126)
4. Add 7ml 0.1N NaOH.

PREPARE MEM (GIBCO # 11090-081)

1. Remove 65 ml from the 500 ml MEM bottle. Save 10 ml to prepare Glucose.
2. Add 5 ml Pen/Strep (Gibco #15070-063).
3. Add 10 ml 1M Glucose (Sigma G-8270) (1.8 g/ 10 ml MEM)
4. Add 50 ml Fetal Bovine Serum (Gibco # 16140-071) or Bovine Calf Serum (Omega BC-04).
5. Mix well, aliquot and store at 4°C.

NEUROBASAL MEDIUM/B-27 SUPPLEMENT (NBM/B27)

NBM, 500 ml (Gibco #21103-049); B-27 (50X), 10 ml (Gibco #17504-044)

1. Add the entire content of B-27 vial to the NBM bottle and mix.
2. Aliquot and store at 4°C.

DISSECTION OF BRAIN TISSUE

1. Dry dissecting tools from 70% EtOH before dissection.
2. Transfer 10 ml DS in each of 3-15 ml tube.
3. Select mouse pups (P0 – P3), you will need three brains for 4 bottles.
4. Add cold DS to 35 mm petri dish.
5. Decapitate mouse pup and dissect out brains.
6. Place brains into the petri dish containing cold DS.
7. Chop tissue into pieces, small enough to pass through a glass pipette.

ENZYME DISSOCIATION

1. Remove as much DS as possible from the dish with a Pasteur pipette.
2. Filter ES through a 0.2 μ m filter and 5cc syringe.
3. Place ES in the dish with tissue.
4. Incubate tissue at 37°C incubator (CO₂ free) for 30 min.

WASHING TISSUE

1. Transfer tissue with glass pipette to the first DS tube, making sure to get as little ES as possible.
2. Centrifuge for 30 sec at 1500 rpm.
3. Transfer tissue and repeat procedure two more times.

TRITURATION

1. Place 38 ml of MEM/FBS into a 50ml centrifuge tube

2. Add 3 ml of MEM/FBS to a 35 mm dish and transfer tissue to this petri dish.
3. Dissociate the tissue by gently triturating it through a 1000 μ l eppendorf pipet tip.
4. Transfer cell suspension to the tube containing 38ml of MEM/FBS and mix well.

PLATING

1. Stand the culture bottles upright to get equal amounts of the cell suspension and media in each bottle.
2. Put 90 ml of MEM/FBS in each culture bottle.
3. Add 10 ml of cell suspension in each bottle.
4. Label: Non-Neuronal, initials and date. Incubate at 37°C and 5% CO₂.

FEEDING

1. On Day 3 or 4, feed cells with warm MEM/FBS (decant all the media and replace it with 100 ml of MEM/FBS). Cultures may take 7-10 days to become confluent.
2. On Day 7-10 change the media for NBM/B27.
3. Next day, collect the media. Filter with 0.22 μ m
4. Make aliquots of 40 ml and feed culture with MEM/FBS.
5. Next 2 or 3 days, change media for NBM/B27
6. Next day, collect and repeat the procedure.
7. Keep collecting media for approx. 2 weeks.

Disclosures

The authors have nothing to disclose.