Preparation of Dissociated Mouse Cortical Neuron Cultures

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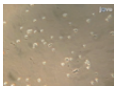
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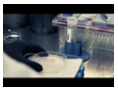
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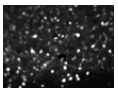
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Abstract

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

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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.

Protocol

Preparations before day of culturing:

- Prepare sterile dissecting solution (DS).
- Prepare NBM/B27 (Neurobasal Mediom with B27 supplements).
- Autoclave ddH_2O and sterilize glass coversilps, 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 600µm 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

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Keywords

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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 5µM 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:

- 100µl 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 µm syringe filter.
4. Divide into 400 µl aliquots and store at -20°C.

L-CYSTEINE

In an Eppendorf tube dissolve 0.6 mg L-Cysteine (Sigma C-7755) in 200 µl 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 H_2O.

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 µm filter and 5cc syringe.
3. Place ES in the dish with tissue.
4. Incubate tissue at 37°C incubator (CO_2 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 1000µl 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% CO_2.

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 0.22µm
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.

Materials

Name	Company	Catalog Number	Comments

5-Fluoro-2'-deoxyuridine	Sigma-Aldrich	F0503	
Sodium Chloride	Sigma-Aldrich	S9625	
Vibraslicer	Campden Instruments		
Potassim Chloride	Sigma-Aldrich	P4504	
Sodium Phosphate Dibasic	Sigma-Aldrich	S0876	
Potassium Phosphate Monobasic	Sigma-Aldrich	P5379	
Hepes	Sigma-Aldrich	H3375	
D (+)-Glucose	Sigma-Aldrich	G8270	
Sucrose	Sigma-Aldrich	S0389	
Poly-D-Lysine	Sigma-Aldrich	P7280	
B27 Supplement	Invitrogen	17504-044	
Neurobasal Media (NBM)	Invitrogen	21103-049	
2-Amino-5-phosphonopentanoic acid	Sigma-Aldrich	A5282	
Bovine Albumin	Sigma-Aldrich	A7030	
Trypsin Inhibitor	Sigma-Aldrich	T9253	
Sodium Hydroxide, 1N solution	Fisher Scientific	SS266-1	
L-Cysteine	Sigma-Aldrich	C7755	
Bacto Agar	Difco Laboratories	0140-01	
Papain	Worthington Biochemical	LS 03126	
Sterile 0.2 &#65533;m Syringe Filter	Fisher Scientific	DDA02025S0	
Glass Coverslips, No1, Ã˜12mm	Bellco Glass	1943-00012	
Minimum Essential Media (MEM)	Invitrogen	11090-081	with Earle's saltswithout L-glutamine, needed for growing non-neuronal cultures
Penicillin/Streptomycin	Invitrogen	15070-063	for non-neuronal culture
Fetal Bovine Serum	Invitrogen	16140-071	needed for non-neuronal cultures
Nunclon "Triple Flask" Tissue Culture Bottle	Fisher Scientific	12-565-25	Use for non-neuronal cultures. these flasks are very convenient when producing "conditioned Neurobasal Media/B27", but any

			other tissue culture flasks or dishes can be used instead.
Glass bottom "Imaging dishes"	MatTek Corp.	P35G-1.5-10-C	Glass bottomed, Ã˜35mm culture dishes ideal for Calcium or Sodium Imaging when using an inverted imaging setup. But expensive!

Ask the Author

15 Comments

WOW - this really really interesting.

MORE of this please !!!!

1 (/video/562/preparation-of-dissociated-mouse-cortical-neuron-

Can this protocol be applied to earlier embryonic stages, such as E11.5 ~ E13.5?

2 (/video/562/preparation-of-dissociated-mouse-cortical-neuron-

That's a tough one. I have not done any dissociated cultures from brains that early in development. What I can say is that at E15 - E16 the cerebral cortex is relatively thin and although the cortical layers are clearly visible, it is more difficult to dissect. So the short answer is I don't know, but I don't see why it should not work.

Cutting the brains on a vibra slicer will be difficult. The tissue is very soft and the ventricles will most likely not support the cortex as the blade tries to cut through the brain. It might be easier to separate the cerebral cortex from the rest of the brain and then dice it into smaller pieces. I imagine the initial yield of dissociated cells to be a lot less than at a later stage but the cortex at early stages of development is mitotically active and the number of neurons may increase in the cultures. At E11.5 - E13.5 the tissue is also less developed (fewer neurites) so the dissociation should be easier and less disruptive.
I would give it a try and my advise is to experiment with the incubation times used for the enzyme digestion, lower the papain concentration or both. More recently I have adopted new way of triturating the tissue after the enzyme digestion and washing procedure. Instead of pulled glass capillaries I now use sterile 200µl Eppendorf pipette tips. This is much more gentle on the cells and causes less debris. Good luck and let me know if it worked.

2.1 (/video/562/preparation-of-dissociated-mouse-cortical-neuron-

Protocol states that use 75ml of 1xPDL / coverslip.

I think it's a typographical error. it should have been 0.75ml/coverslip.Please confirm.

3 (/video/562/preparation-of-dissociated-mouse-cortical-neuron-

Thank you for letting us know. We are looking into this immediately.

3.1 (/video/562/preparation-of-dissociated-mouse-cortical-neuron-

I think your protocol is too complex and not aggreable for doing more than one brain at a time. I do 9-10 pre-natal brains in the same time you do one and my culture is fairly pure and works wonderfully for our experiments. Obviously, your culture would be very pure, but is it worth the time and effort?

4 (/video/562/preparation-of-dissociated-mouse-cortical-neuron-

Thank you for your comment. If you need to process that many brains (9-10) in parallel then applying this protocol will be problematic. For one (at least for the instrument we are using) the platform for the vibra slicer has only space for 5 brains in a row (six if you squeeze them in). Also, the time it takes to separate the cortical rinds from the rest of the brain and all subsequent steps will take that much longer and will invariably affect the number of viable neurons.

Your question "is it worth the time and effort?" is probably best answered by the operator and will depend on the intended use.

4.1 (/video/562/preparation-of-dissociated-mouse-cortical-neuron-

I�m studying this in the University and this video is very helpful thanks!!

5 (/video/562/preparation-of-dissociated-mouse-cortical-neuron-

What is the dissection solution made of? is there a protocol for it? thanks

6 (/video/562/preparation-of-dissociated-mouse-cortical-neuron-

I am attempting to isolate proteins from cortical neurons that have been growing between 3 and 21 days. How do you suggest detaching the cells from the dishes? Is it necessary to use trypsin/EDTA or can I just scrape the neurons off? If trypsin is necessary, how much do you recommend using? I am using poly-lysine coated 10cm dishes.

7 (/video/562/preparation-of-dissociated-mouse-cortical-neuron-

Beth, I messed up. Please see my comment below. Depending on the size of the culture dishes and cell density you can calculate the surface area of your culture dishes estimate the expected protein yield. We have used 10cm dishes, but to get a decent neuronal cell density these dishes require a lot of brain tissue (one entire P0 mouse brain/100mm dish, which will contain approx. 1 - 1.5mg of total protein).

7.1 (/video/562/preparation-of-dissociated-mouse-cortical-neuron-

thanks for the video, it was very helpful. How long do you culture the neuron before you do electro physiological experiments.

8 (/video/562/preparation-of-dissociated-mouse-cortical-neuron-

reply to #7: We routinely scrape off the cells in Tris buffer or lysis buffer on ice. Using trypsin might affect membrane proteins you may wish to investigate later on. You might want to calculate your buffer volume depending on how many cells you have on the culture dishes (also varies with culture age). A high density culture plated on a 35mm culture dish typically yields 200-300 micro gram of total protein. The buffer volume will depend on whether you would like to prepare total, cytosolic and or a membrane enriched fraction that contains a reasonable amount of protein so you can load the desired amount onto a lane on your gel (assuming you want to do western blots). A good starting volume of buffer should be ≈ 150 to 200 micro liter.

8.1 (/video/562/preparation-of-dissociated-mouse-cortical-neuron-

reply to #8: That depends pretty much on your experiments or question you pursue. Excitatory input develops very early in cultured cortical neurons (first week in culture), while inhibitory input develops slower (10-12 days in culture). In this respect you might want to look up a paper by Li Z. et al. published in J. Neurobiol. (1999).

8.2 (/video/562/preparation-of-dissociated-mouse-cortical-neuron-

Why not add Glumax or Gutamine into your Neurobasal/B27 medium? My cortical culture looks nice in the first week and then died after DIV7. I used P0 mice, Neurobasal A with B27 and Glumax.

Thanks

9 (/video/562/preparation-of-dissociated-mouse-cortical-neuron-

Were the cultures maintained with glia conditioned media? Maintaining neuronal cultures with NBM/B27 alone will not always yield "healthy" cells, especially if you would like to keep them longer. We have used Glumax only once and the cultures did not look anything like the sister cultures maintained with glia conditioned NBM/B27. If you don't have conditioned media around you might want to try adding 1-2% serum to your NBM/B27. However, keep in mind that serum containing media will increase glia proliferation in your cultures (you can use anti-mitotic agents like FDU or AraC three days after plating).

9.1 (/video/562/preparation-of-dissociated-mouse-cortical-neuron-

Your culture looks very nice. Thanks for the protocol.
I assumed that video features the high density culture with that yields 200-300 micro gram of total protein as you explained in one of your comments. Could you please clarify what approximate density of cells per cm^2 shown on the video?

10 (/video/562/preparation-of-dissociated-mouse-cortical-neuron-

is there any possibility to see this video without subscription? i am try to culture adult neurons, and any hint on a clean preparation will be very precious

11 (/video/562/preparation-of-dissociated-mouse-cortical-neuron-

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You can reach me at ward.parry@jove.com

11.1 (/video/562/preparation-of-dissociated-mouse-cortical-neuron-

I Have a question. Why glutamine is not included in NBM which is required for neuronal growth. Similarly glutamine is not included in astrocyte media as well?

12 (/video/562/preparation-of-dissociated-mouse-cortical-neuron-

I have a question regarding the myelination status of your cultures:
Do you have oligodendrocytes in your culture, if yes, how many percent do you assume? Do they contribute to the myelination of your axons? Thanks, EA

13 (/video/562/preparation-of-dissociated-mouse-cortical-neuron-

Hi. Very helpful especially for a newbie in primary neuronal culture prep. Just two areas to clarify. In the preparation of the APV, you store in aliquotes of 180uL. However, this solution is used in the preparation of Enzyme solution, where I would assume is a typo where 50mL APV was used. Can you please confirm if it is 50uL and also, why store in 180uL aliquotes. Is APV stable to freeze thaw?THANKS

14 (/video/562/preparation-of-dissociated-mouse-cortical-neuron-

Also, I cannot find the protocol for preparing condition non-neuronal NBM/B27.

15 (/video/562/preparation-of-dissociated-mouse-cortical-neuron-

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