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Mouse Primary Astrocyte (mPA) isolation and culture

Forked from Mouse Primary Astrocyte (mPA) isolation and culture

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We use this protocol and it's working

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Disclaimer

This protocols was adapted from the the work of Boxun Li in the Gersbach lab at Duke University.

Abstract

This protocols describes methods for isolating and culturing primary astrocytes from mice.

Materials

Cytosine arabinoside (AraC) (1000X)
Dissolve 4.86 mg AraC (Sigma, C1768) in 2 ml distilled water to make 1,000x stock solution of 10 mM and filter the solution with 0.22 µm filters. Store at -20 °C and protect from light.
Hydrocortisone (1000X)
Stock concentration: 10mM in DMSO. Dissolve 0.108g of Hydrocortisone (sigma H-0888) in 30ml of DMSO. Filter with 0.22 µm filters and save at -20.
Lo Ovomucoid (10X)
To 150ml of DPBS (Thermo Fisher Scientific, 14287-080), add 3 g BSA (Sigma A8806). Mix well. Add 3 g Trypsin inhibitor (Worthington, LS003086) and mix to dissolve. Adjust pH to 7.4; requires the addition of approximately 1ml of 1N NaOH. When completely dissolved, bring to 200 ml DPBS and filter through 0.22 µm filter. Make 1.0 ml aliquots and store at -20˚C.

200 ml DPBS
3g BSA
3g trypsin inhibitor

Hi Ovomucoid (6X)
To 150 ml DPBS (Thermo Fisher Scientific, 14287-080), add 6 g BSA (Sigma A8806). Add 6 g Trypsin inhibitor (Worthington, LS003083) and mix to dissolve. Adjust pH to 7.4; requires the addition of at least 1.5ml of 1N NaOH. When completely dissolved, filter through 0.22 µm filter. Make 1.0 ml aliquots and store at -20˚C.
Insulin (0.5 mg/ml; 100X)
To 20 ml sterile water add 10 mg insulin (Sigma, I-6634), 100ul 1.0 N HCl. Mix well. Filter through 0.22µm filter. Store at 4˚C for 4 to 6 weeks.
NAC (5 mg/ml; 1000X)
Dissolve 50 mg N-acetyl cysteine (Sigma A8199) in 10 ml Neurobasal (Gibco, 21103-049) (will be yellowish). Filter through 0.22 µm filter and make 500 µl aliquots; store at -20˚C.
Poly D-Lysine Water (PDL) (100X)
Add 5 ml ddH2O in a 5 mg bottle of PDL (Sigma P6407). Filter through 0.22µm filter. Make 100 µl aliquots; store at -20˚C.
DNase stock solution - Required concentration for the primary cell isolation: 12500Unit/mL
Use DNaseI (Worthington, LS002007, 100 mg). EBSS (Gibco, 14155-063-500ml; Free of CaCl2, MgCl2 and PhenolRed). Calculate the amount of 1X EBSS to dissolve DNase1 to get 12500Unit/ml. Once dissolved, Filter through 0.22µm filter. Make 200ul Aliquot. store -20˚C.
Papain - Worthington Biochemicals, LK003176
DPBS (Dulbecco's Phosphate Buffered Saline) – (Gibco, 14287-080)
DMEM (Gibco, 11960-044)
HI-FBS (Gibco, 10437-028)
Penicillin-Streptomycin (Pen/strep) (10,000 U/mL) (Gibco, 15140148)
L-Glutamine (GIBCO 25030)
Sodium Pyruvate (GIBCO 11360)
Trypsin (0,05% Trypsin-EDTA) (Gibco, 25300-054)

Normal Goat Serum (NGS) - (ThermoFisher 01-6201)

Astrocyte Growth Media (AGM)

1 This recipe makes 500ml of filtered astrocyte growth media. Store in tissue culture fridge after use.

2 Wash filter with a bit of DMEM and vacuum off.

3 To the filter holder, add the following media components:

Component	Volume	Location in lab
DMEM (#11960)	450ml	TC fridge
HI*-FBS	50ml	TC freezer
Penstrep (100x)	5ml	TC freezer
L-Glutamine (100x)	5ml	TC freezer (use a fresh aliquot)
Sodium Pyruvate (100x)	5ml	TC fridge
Insulin	5ml	TC fridge
N-Acetyl Cysteine	500µl	TC freezer – labelled NAC
Hydrocortisone	500µl	TC freezer – labelled H

*Heat Inactivated

4 Filter vacuum and store in tissue culture fridge.

Astrocyte Culture

5 PREP DAY = DIV0 (Days in vitro 0)
Take prep DPBS (Cat #14287-080) from the cold room (Don't need to add Phenol red for only astrocytes).

6 Preparation of Culture Flasks for Astrocytes

6.1 SPRAY DOWN EVERYTHING IN THE TISSUE CULTURE HOOD. Take out fresh flask of DPBS from cold room and add phenol red (1:1000).

6.2 Dilute 100µl PDL (poly-D-lysine--see Materials) aliquots stored in -20°C in 10ml water. Add 10ml to each flask.

6.3 Coat 75cm² culture flasks with 10 µg/ml PDL for at least 30 mins at room temperature.

6.4 Wash 3 times with sterile ddH2O, then add 15ml AGM to each flask. Let sit in incubator with lid slightly twisted off during prep to let the CO2 levels and temperature equilibrate.

7 Preparation of Solutions for Astrocytes for 9 Pups

8 Prepare the following solutions:

	A	B	C	D
	Tube Label	**DPBS**	**# of Aliquots + Additive**	**Notes**
	Lo OVO	9mL	1 1mL aliquot	Add 100µL DNase before use
	Hi OVO	6mL	1 1mL aliquot	

9 Warm 90mL AGM (for 1 litter) in the water bath.

10 For each litter – prepare 2 universal tubes with papain resuspended in PBS. Each vial gets 20ml PBS, 2 vials of papain, and 800µl DNaseI (add DNaseI right before adding tissue). Place in the water bath to warm. You will finish preparing the papain near the end of the dissection.

11 Papain = gentler form of trypsin. To prepare, add 10mL of prewarmed DPBS to papain vial (stored at -20C). Dissolve and return to universal tube. Remember to add 800µl DNase (stored at -20C) per universal tube just before adding the tissue.

Mouse Brain Dissection

12 Prepare dissection surface by cleaning with ethanol, placing an absorbent pad on the benchtop, and preparing tools. Will need small curved spring scissors, larger scissors for decapitation, and forceps.

12.1 Use tools ONLY in tissue culture room. Tools should also be rinsed with water – NEVER use detergent.

13 Prepare 4 60mm dishes, adding 6ml of DPBS into each dish. Each dish would contain 3 brains, and one dish is used for dissection.

14 Decapitate pups and de-skin the heads. Remove skull using small scissors to trim around but above the ears up to the midline. Skull should peel off.

15 Remove the brains and place into a 60mm dish with DPBS.

16 Cut off the cerebella. Remove basal ganglia by trimming axons and scooping out the tissue. Remove meninges from both surfaces of the brain using forceps. Remove hippocampus from both hemispheres. This will leave isolated cortex.

17 Transfer cortices to a fresh 60mm dish with DPBS.

18 Chop the cortices with sterile razorblade such that each tissue chunk is less than $1mm^3$.

Tissue Digestion

19 Resuspend the papain. And add the DNase.

20 Cut off the tip of sterile plastic pipette to make a larger opening.

21 Using the plastic pipette, suck up the chopped tissue and DPBS. Tap the tip of the pipette onto the papain solution in 20 ml universal tubes so tissue settles towards bottom of tip, then add to tube, minimizing amount of liquid transferred. Swirl gently to get a 'tornado effect' before letting the brain tissue settle. Add half of the tissue to each of the tubes (ideal would correspond to 5 brains / tube for mouse).

22 Incubate at 33°C for 45 minutes, swirling the tissue at 15-minute intervals.

22.1 Never above 34°C – papain is temperature sensitive. Do NOT swirl at the 45min time-point.

23 Just before the end of the papain incubation, add DNase to the Lo Ovo (400µl).

24 Aspirate off the supernatant with a fresh vacuum pipet. Be sure to suck off the bubbles first and take care not to aspirate tissue. Try to remove as much media as possible.

25 Add 2 ml Lo-Ovo gently by dribbling down the side of the tube. Triturate using a 1ml pipette about 10 passes, starting out slowly and then speeding up. Let the tissue settle about 90 seconds. Check that there is a suspension, otherwise, 1ml of Lo-Ovo can be added to help in resuspension. Add the remainder of Lo-Ovo to dissociated cells and move to a 50ml conical tube. You can combine tubes at this point.

25.1 Ovo breaks up tissue into single cells and inactivates papain. Aspirate 1ml and release gently in a circular manner around the vial.

26 Centrifuge the cells at 1100rpm for 11 minutes at room temperature.

26.1 This pellet is often fragile, can go up to 1400rpm for spin.

27 Aspirate off the supernatant with vacuum.

27.1 Very delicate and important step affecting yield. Do not aspirate all solution, ~1ml of media can be left to avoid disturbing pellet when aspirating supernatant.

28 Resuspend the cells in 2 ml Hi-Ovo. Triturate using a 1ml pipette about 10 passes. Add the remainder of Hi-Ovo.

28.1 Cells do not thrive well in Hi-Ovo so be sure to finish step 9 under 2 minutes.

29 Centrifuge the cells at 1100rpm for 11 minutes at room temperature.

30 Aspirate off the supernatant with vacuum. Here the protocol splits in two for neurons and astrocytes.

Astrocyte Prep

31 Resuspend the cells in 2-3ml warmed AGM. Bring up to 10ml. Filter the cell mixture through 100µm strainer 1 mL at a time into a 50ml Falcon tube. Remember to Ethanol/flame sterilize forceps. It also helps to wet the filter by stabbing it into the tube with a P1000 pipet with 1ml of AGM.

32 Centrifuge the filtered cells at 900rpm for 9 minutes at room temperature.

33 Aspirate off the supernatant and resuspend the cells in 2 mL AGM.

34 Bring up the volume to 10ml to count cells. Can pool cells from multiple universal tubes if necessary.

35 Count the cells. Make sure to zoom in to check. Plate cells at very high density: 30 million cells per 75cm² culture flask as minimum. Better if can be between 40-45 million. Add the required amount to the prepped flasks.

36 Store in incubator with cap slightly unscrewed.

37 Roughly 3 pups will yield ~40 million cells, plate in a T75 flask.

Astrocyte Shake-off (DIV3)

38 Warm up AGM and DPBS in water bath.

39 Check astrocytes for normal morphology under microscope.

40 Wash in 10ml DPBS at least 3 times.

41 Add 20ml of DPBS and shake hard 8 times on each hand (amount of shaking needs to be determined on an individual basis).

42 Aspirate the DPBS and dislodged cells.

43 Add another 20ml of DPBS. Check under microscope to see if cell debris and other cells have been dislodged. There should be patches of 'holes' for which the astrocytes can grow into. If insufficient, shake more/harder.

44 Add 15ml of AGM to the flask.

45 Store in incubator.

Feed Astrocytes with AraC (DIV5)

46 Add AraC (cytosine arabinoside) to AGM at 1:1000. Warm in incubator. [AraC stock is 10mM].

47 Replace half of the media with the AraC media (final concentration of AraC = 1:2000).

Passage astrocytes (DIV 7-8)

48 Optional: if astrocytes are not used on DIV7, replace half the media with AGM + AraC (1:2000).

49 Warm up Neuronal Maturation Medium (for co- culture with neurons or astrocyte mono-culture control) and Trypsin.

50 Remove old media in flask.

51 Rinse flask with DPBS 3 times.

52 Add 10mL pre-warmed trypsin to each flask. Incubate for 5 mins in 37C incubator, lying flat (not stacked) to have even heat.

53 Vacuum trypsin after the 5 mins incubation without dislodging the cells.

54 Tap flask to loosen astrocytes and rinse off with 10mL DPBS twice (total volume of ~20mL per flask).

55 Centrifuge at 600 xg for 5 mins to pellet astrocytes.

56 Resuspend astrocytes in 2mL for every flask at the beginning of today. Use P1000 pipette to make sure dissociation into single cells.

57 Count astrocytes in Trypan Blue (1:1) on a hemocytometer. Note: counting with automated cell counter might be challenging due to the irregular shape and processes of astrocytes.

58 Dilute astrocytes to 500k/mL in Neuronal Maturation Medium. Start co-culture with neurons at a 1 astrocyte:5 neurons ratio with D0 (in suspension) or D1 (attached) neurons that are plated on a PDL-coated tissue-culture vessel. Or start astrocyte mono-culture as a control at the same number (needs to be diluted in Neuronal Maturation Medium).
o Note: Neuronal differentiation protocol and Neuronal Maturation Medium are detailed in: https://www.protocols.io/view/excitatory-neuron-differentiation-from-inducible-n-ewov1kqxpgr2/v1

Acknowledgements

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