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Glia-Free Cortical Neuron Culture

In 1 collection

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ABSTRACT

Protocol for feeding neuronal cultures with astrocyte-conditioned media and staining them for synapses.

MATERIALS

STOCK SOLUTIONS:

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 sterilize the solution by filtration.
Store at -20 °C and protect from light.

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 approx. 1ml of 1N NaOH.
When completely dissolved bring to 200 ml DPBS, filter through 0.22 µm filter.
Make 1.0 ml aliquots and store at -20˚C.

Hi Ovomucoid (6X)
To 150 mls 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 -GP filter.
Make 1.0 ml aliquots and store at -20˚C.

4% BSA (20X)
Dissolve 8 g BSA (Sigma A4161) in 150 ml DPBS (Thermo Fisher Scientific, 14287-080).
Dissolve at 37˚C.Adjust pH to 7.4 (with approx. 1ml 1N NaOH).
Filter 0.22 µm filters.
Make 1.0 ml aliquots; store at -20˚C.

Borate buffer:
Add 4.64 g Boric acid to 500 ml ddH20. Adjust pH to 8.4 with NaOH. Filter and store at 4C.

Poly D-Lysine Borate (PDL) (100X)
Add 5 ml borate buffer in a 5 mg bottle of PDL (Sigma P6407).
Filter through 0.22µm filter.
Make 100 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.

BDNF – (Peprotech, 450-02). The master stock should be prepared at 1mg/ml in 0.2%BSA DPBS and stored at -80˚C as 100ul aliquots.
Preparation of 1000x Working Stocks
Thaw on ice one master stock aliquot (100ul) of BDNF. Cool down on ice 2ml of filtered 0.2% BSA (Sigma A-8806) in DPBS solution. Add BDNF master stock aliquot to the BSA solution. Mix well but gently to avoiding foaming. Aliquot in 0.5ml tubes 20ul each, concentration 50ug/ml (1000X). Flash freeze the aliquots in liquid nitrogen and store at -80C. Working concentration: 50ng/ml, final concentration on neurons 25ng/ml**.**

CNTF – (Peprotech, 450-13). Master stock should be prepared at 1mg/ml in 0.2%BSA DPBS and is stored at -80˚C as 50ul aliquots.
Preparation of 1000x Working Stocks
Thaw on ice one master stock aliquot (100ul) of CNTF. Cool down on ice 2.5ml of filtered 0.2% BSA (Sigma A-8806) in DPBS solution. Add CNTF master stock aliquot to the BSA solution. Mix well but gently to avoiding foaming. Aliquot in 0.5ml tubes 20ul each, concentration 20ug/ml (1000X). Flash freeze the aliquots in liquid

nitrogen and store at -80C. Working concentration: 20ng/ml, final concentration on neurons 10ng/ml**.**

Forskolin (5 mM; 1000X)
Add 12ml DMSO to 50 mg of Forskolin (Sigma, F6886).
Make 20 ul aliquots; store -20˚C.

500 mM Tris HCl, pH 9.5 (10X)
Dissolve 12.1 g Trizma base into 200 ml dH_2O
Ph to 9.5 with HCl.

DNase stock solution -Required concentration for the prep: 12500Unit/mL 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.
BSL1 (Baneiraea Simplicifolia Lectin 1) - (Vector Laboratories L-1100) - Resuspend BSL1 in PBS 1X to a final concentration of 2mg/ml. Once dissolved, Filter through 0.22µm filter. Make 25ul Aliquot. store -20˚C.

Laminin (Culturex 3400-010-01) mixture
Insulin – (Sigma I1882) - 10mg of insulin in 20ml of TC water. Vortex to make sure that the powder dissolves well before filtering.
Syringe Filter 0.22 µm – Millipore SLGPR33RB

Nitex filter - filter 3-20/14 from Tetko, Inc. Cut into 3-inch squares, wrap in small packets of foil, and autoclave.

Papain - Worthington Biochemicals, LK003176

DPBS (Dulbecco's Phosphate Buffered Saline) – (Gibco, 14287-080)

Penicillin-Streptomycin (10,000 U/mL) (Gibco, 15140148)
L-Glutamine(GIBCO 25030)

Sodium Pyruvate (GIBCO 11360)
Neurobasal media (Gibco, 21103-049)
B27 (50x) (Gibco, 17504-044)

Coverslips (Assistant Deutsche Spiegelglas micro sheet, 92100100030, 12mm)

AffiniPure Goat anti-Rat IgG + IgM (H+L) (Jackson Immunoresearch, 112005044)

AffiniPure Goat anti-Mouse IgG + IgM (H+L) (Jackson Immunoresearch,

115005044)

Mouse antineural cell adhesion molecule L1 Hybridoma (Developmental Studies Hybridoma Bank, ASCS4)

Lectin (Sigma, I1882)

Trypsin (0,05% Trypsin-EDTA) (Gibco, 25300-054)

Prepare Neuronal Growth Media (NGM)

1 This recipe makes 20ml of neuronal media. Make fresh per use.

To a 50ml tube, add the following media components:

Reagent	Volume
Neurobasal	19ml
Pen/strep (100x)	200µl
Glutamine (100x)	200µl
Sodium Pyruvate (100x)	200µl
B27 (50x)	400µl

2 Sterile filter these components through a syringe filter. Place media in the incubator with thecap unscrewed. Allow the media to warm and equilibrate for at least 45 minutes. (Media will have an orange color and bubbles)

3 Add growth factors right before the time to use the media.

BDNF	20µl
CNTF	20µl
Forskalin	20µl

Preparation of Coverslips (day before prep)

4

Note

Note: Make sure coverslips have been washed shaking for at least 3 weeks with 70% ethanol before use.

5 Flame sterilize the forceps after spraying with ethanol.

6 Wash coverslips with ddH2O in a large petri dish (150 x 15 mm).

7 Set coverslips in a large petri dish (150 x 15 mm). Coat with 100ul of poly-D-lysine (PDL) solution (100ul PDL (100X) in 10ml of borate buffer).

8 Let the coverslips sit in PDL-B for at least 30 mins in the hood RT.

9 Make laminin mixture: 10 µl aliquot of mouse laminin solution + 5 ml Neurobasal media.

10 Add 100µl of this laminin mixture to each coverslip. Place the petri dish in a 37°C incubator overnight

Preparation of Panning Dishes (day before prep)

11 Use 10cm petri dishes for positive panning and 15cm petri dishes for negative panning.

12 For negative plates:
To 2 15cm Petri dishes add 30 ml 50mM Tris-HCL pH9.5 + 72 µl of anti-rat IgG + IgM to one plate and 30 ml Tris-HCL pH9.5 + 72 µl of anti-mouse IgG + IgM to the other. Incubate at 4C overnight.

13 For positive plates:
To 2 10cm plates add 12 ml Tris-HCL pH9.5 + 54 µl anti-mouse IgG and IgM. Incubate at 4°C.

Cortical Neuron Isolation (Prep Day)

14 Firstly, take theL1 antibody out to thaw at RT

15 Prepare the following solutions (enough for 9 pups)

Tube Label	DPBS	# of Aliquots + Additive	Notes
Lo OVO	36ml	2 2ml aliquot	Add 400µl DNase before use
Hi OVO	20ml	2 2ml aliquot	
BSA Buffer	76ml	2 2ml aliquot	Divide among 2 50ml Falcon Tubes
Panning (X2 tubes)	45ml	500µl insulin + 5ml BSA buffer	Insulin, do not use if >6 weeks' old

Preparation of negative panning dishes

16 Create 2 Lectin negative panning dishes: 2x 15cm Petri dishes with 30ml prep DPBS. Add 25µl Unconjugated Griffonia Simplicifolia Lectin I. Shake plate gently in a '+' manner to ensure even distribution. Incubate at RT for at least 1 hour outside the hood.

17 Wash the mouse and rat negative panning dishes 3x with DPBS. Add 12ml BSA blocking buffer to

each. Incubate for at least 1 hr at RT outside the hood.

18 Wash the Positive panning dish 3x with 10ml DPBS carefully. Add 5ml of mouse-anti L1 mixture. Incubate at RT until just before use outside the hood.

19 After the lectin has incubated for 1 hr (usually during papain digestion), wash the Lectin negative panning dishes with DPBS three times. Add 12ml of BSA buffer. Let it at RT outside the hood. This step is usually done in between the Low Ovo centrifuge step or High Ovo centrifuge step

Note

This step is usually done in between the Low Ovo centrifuge step or High Ovo centrifuge step

Rat brain dissection

20 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.

21 Prepare 3 60mm dishes adding 5ml of DPBS to collect 6 cortices each, and one 10cm dish with 12ml of DPBS for the dissection.

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

23 Remove the brains and place them into the 10cm dish with DPBS.

24 Using the forces remove meninges and dissect the basal ganglia, hippocampus and the cerebella. This will leave isolated cortex.

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

26 Chop the cortices with scissors to increase the surface for enzymatic digestion.

Tissue digestion

27 Resuspend the papain. And add the DNase.

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

29 Using the plastic pipette, suck up the chopped tissue and DPBS. Tap the tip of the pipet onto the papain solution in 20 ml universal tubes without squeezing the pipet or adding liquid. Swirl gently to get a 'tornado effect' before letting the brain tissue settle

30 Incubate at 32oC for 45 minutes, swirling the tissue at 15-minute intervals.

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

32 Once the 45-minute incubation is completed, aspirate the DPBS from the universal tubes. The tissue will stay at the bottom of the tube.

33 Add 2 ml Lo-Ovo gently by dribbling down the side of the tube. Triturate using a 1ml pipette about 10 passes up and down. Check that there is a single cell suspension, otherwise, 1ml of Lo-Ovo can be added to help in resuspension. Add the remainder of Lo-Ovo to dissociated cells.

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

35 Aspirate off the supernatant with a vacuum without disturbing the pellet.

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

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

38 Aspirate off the supernatant with a vacuum.

39 Resuspend the pelleted cells in 3ml panning buffer in each tube using a P1000 pipette, up and down 10 times. Add another 7mL to bring to 10mL.

40 Filter the cells through a Nitrex mesh (use sterile forceps to make a conical shape into a 50ml tube). Bring the volume of the filtrate to 15mL with more panning buffer. To prepare the mesh in a cone shape, place the mesh at the mouth of the tube and make a conical shape with the help of the forceps and pipette through which the tissue suspension will filter through. Use panning buffer to wet the mesh.

Panning steps

41 Remove the DBPS/BSA from the first Lectin plate into a waste beaker. Add the filtered cells to this first Lectin plate. Let incubate at RT for 10 min.

42 At the end of the incubation, shake the plate forcefully on the benchtop but keep the solution inside the plate and away from the lid. Shake such that it performs one full rotation.

43 Remove the DBPS/BSA from the second Lectin plate. Dump the cell/buffer mixture into this second Lectin plate. Collect the last bit of loose cells with a P1000. Let incubate at RT for 10 min.

44 At the end of the incubation, shake the plate forcefully but keep the solution inside the plate and away from the lid. Shake such that it performs one full rotation.

45 Remove the BSA from the first negative panning plate. Dump the cell/buffer mixture onto this plate. Collect the last bit of loose cells with a P1000. Let incubate at RT for 15 min.

46 At the end of the incubation, shake the plate forcefully but keep the solution inside the plate and away from the lid. Shake such that it performs one full rotation.

47 Remove the BSA from the second negative panning plate. Dump the cell/buffer mixture onto this plate. Collect the last bit of loose cells with a P1000. Let incubate at RT for 15 min. (Just before the end of the incubation, prepare the positive panning dishes by pouring off the L1 primary solution and wash the plate 3x gently with DPBS).

48 At the end of the incubation, shake the plate forcefully but keep the solution inside the plate and away from the lid. Shake such that it performs one full rotation.

49 Remove the DPBS from the positive panning plates and add the cells from the second negative panning dish.

50 Incubate for 45min at RT.

Prepare 24 well plate with coverslips

51

Note

It is best to prepare your plates with coverslips during the above panning steps

52 Add 500µl warmed and equilibrated NGM to each well of the 24 well plate.

53 Pour neurobasal media onto the coverslip dish (without aspirating the existing neurobasal media on coverslips) just enough to dislodge the coverslips.

54 Pick up with sterile forceps and put the right side up (with the PDL/lamining coating) into each well.

Neuronal collection from positive panning

55 Using a 5ml serological pipette set to slow, gently remove the media (and loose unstuck cells). Wash at least 5 times with 5ml of Panning buffer by tilting the dish slightly towards self so that DPBS pools at the marked triangle. Gently place the dish back so the buffer covers the entire dish. Keep the 5th wash on the plate and check under a microscope until it looks clean from debris.

56 Remove the last wash of the panning buffer and add 5ml of panning buffer to collect the neurons. Use a P1000 pipette to carefully pipet up and down all over the surface of a dish. Collect the neurons in a 50ml tube. Add another 5ml of panning buffer, and check under the microscope to make sure all the neurons were collected. Place the cells in a 50ml conical tube and centrifuge at 1100rpm for 11 min at RT.

57 Resuspend the neuronal pellet in 2ml of warmed and equilibrated NGM.

58 Count the cells using a hemocytometer or an automated cell counter.

59 Plate the cells onto their coverslips at a density of 70,000/well. Store in the incubator at 10% CO2