JUN 26, 2023

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.261gedp57v47/v1

Protocol Citation: Justin T Savage, Juan Ramirez, Dolores Irala, Cagla Eroglu 2023. Astrocyte Isolation and ACM Production. **protocols.io** https://dx.doi.org/10.17504/protocols.io.261gedp57v47/v1

Protocol status: Working

Created: Jun 09, 2023

Last Modified: Jun 26, 2023

PROTOCOL integer ID: 83130

Astrocyte Isolation and ACM Production

In 1 collection

Justin T Savage[1], Juan Ramirez[1], Dolores Irala[2], Cagla Eroglu[1,2,3]

[1]Department of Neurobiology, Duke University Medical Center, Durham, NC 27710;
[2]Department of Cell Biology, Duke University Medical Center, Durham, NC 27710;
[3]Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC 27710

Eroglu_Lab

Justin T Savage

ABSTRACT

Protocol for isolating rat cortical astrocytes and producing astrocyte conditioned media for synaptogenesis assays.

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.

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 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.
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)

DMEM – (Gibco, 11960044)

FBS – (Gibco, 10437028)

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

Sodium Pyruvate (GIBCO 11360)

Trypsin (0,05% Trypsin-EDTA) (Gibco, 25300-054)
Vivaspin tube (Sartorius, Cat. VS2012. MWCO 5000; 20 mL tubes)
Low protein binding tubes (Eppendorf 022431081)

Minimal media (Neurobasal medium minus phenol red, 100 U/mL Pen/Strep, 2 mM L-Glutamine, 1 mM Na Pyruvate

Cortical Astrocyte Isolation (Prep Day)

1 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

2 Prepare Astrocyte Growth Media (AGM)
This recipe makes 500ml of filtered astrocyte growth media. Store at 4C.
Combine and filter the following reagents:

- DMEM 450ml
- FBS 50ml
- Penstrep (100x) - 5ml
- Glutamate (100x) - 5ml
- Sodium Pyruvate (100x) - 5ml
- Insulin - 5ml
- N-Acetyl Cysteine - 500µl
- Hydrocortisone - 500µl

Preparation of culture flasks

3 Dilute 100µl of PDL in 10ml water.

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

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

Rat brain dissection

6 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.

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

8 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.

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

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

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

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

Tissue digestion

13 Resuspend the papain. And add the DNase.

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

15 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

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

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

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

19 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.

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

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

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

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

24 Aspirate off the supernatant with a vacuum.

25 Resuspend the cells in 2-3ml warmed AGM. Bring up to 10ml. Filter the cell mixture through 30□m Nitex membrane 1 mL at a time into a 50ml Falcon tube. Remember to Ethanol/flame sterilize forceps.

26 Centrifuge the filtered cells at 1100rpm for 10 minutes at room temperature.

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

28 Bring up the volume to 10ml to count the cells.

29 Plate cells at very high density: 15 million cells per 75cm2 culture flask as a minimum. Better if can be between 17-20 millon. Add the required amount to the prepped flasks.

30 Store in incubator with cap slightly unscrewed.

DIV 3 Astrocyte Shake-off

31 Warm-up AGM and DPBS in a water bath

32 Check astrocytes for normal morphology under a microscope

33 Wash the cells with 10ml DPBS at least 3 times

34 Add 15ml of DPBS and shake the flasks at least 10 times (this allows for other cell types to detach from the coating).

35 Aspirate the DPBS and dislodged cells

36 Add another 15ml of DPBS. Check under a microscope to see if cell debris and other cells have been dislodged. There should be patches of 'holes' into which the astrocytes can grow into. If insufficient, shake more/harder.

37 Add 15ml of AGM

38 Store in incubator

DIV 5 Feed Astrocytes with AraC

39 Warm-up AGM in a water bath and add AraC (1:10000)

40 Check astrocytes for normal morphology under a microscope

41 Remove old AGM and replace with fresh AGM+AraC (15ml/flask)

42 Store in the incubator

DIV 7 Astrocyte Passage

43

Note

This protocol is for 1 flask of astrocytes. Scale up accordingly.

44 Warm-up trypsin, AGM and DPBS in a water bath

45 Dilute 100µl of PDL in 10ml water and coat a 10 cm culture dish with 10ml of 10 µg/ml PDL for at least 30 mins at room temperature.

46 Wash 3 times with sterile ddH2O, then add 10ml AGM to the plate. Let sit in the incubator during the astrocyte passaging to let the CO2 levels and temperature equilibrate.

47 Remove old AGM media in the flask

48 Rinse the flask with PBS without magnesium and calcium 3 times

49 Add 10 ml pre-warmed trypsin to each flask. Incubate for 5 minutes at 37˚C in the incubator, lying flat (not stacked) to have even heat.

50 Tap flask to loosen astrocytes and collect with 10ml pipette all the astrocytes that are now are dislodged.

51 Centrifuge at 1.1 x 1000RPM for 10mins to pellet the astrocytes

52 Vacuum off the supernatant

53 Resuspend the pellet in 2ml of AGM. Pipette up and swirl to dislodge the pellet. Dispense and swirl gently until a cell suspension is achieved.

54 Plate the astrocytes in the 10cm dish at 3 million as a minimum. Better if can be between 4-5 million.

55 Store in the incubator.

Astrocyte Conditioning Medium

56 Allow freshly plated cells to grow up to 4 days in AGM.

57 Wash plates with DPBS 2~3 times.

58 Add 10 mL serum-free conditioning media (see materials), and incubate without disturbing (4~5 days).

59 Prepare a centrifugal concentrator by washing it with distilled water by centrifuge ~8 mins @ 4000g @ 4C.

60 Pour off H2O from centrifugal concentrator

61 Collect ACM into 50 mL tubes, and centrifuge (1100g, 5 mins) to pellet cell debris.

62 Carefully transfer ACM from 50 mL tubes to centrifugal concentrator w/o disturbing pellet.

63 Spin centrifugal concentrator for ~60 mins @ 4000 g 4C (or down to around 1 mL, 20X).

64 Prepare sterile low protein binding tubes for aliquots.

65 Make ~100ul aliquots for experiments plus one extra aliquot for measuring protein concentration, freeze with liquid N2, and keep at -80 until use.