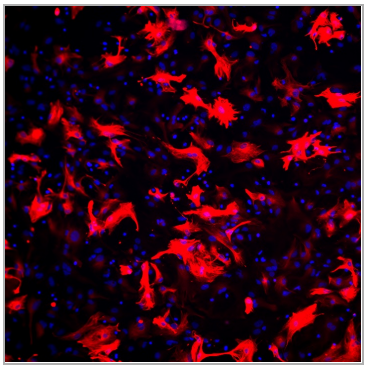

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We use this protocol and it's working

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Isolation and Culture of Mouse Cortical Astrocytes

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ABSTRACT

We provide a detailed protocol to isolate and culture primary astrocytes from the cortex of postnatal day 1 or 2 (P1/P2) mice.

GUIDELINES

This protocol is designed to generate primary cultures of astrocytes from P1/P2 mice cortex. The protocol is written based on the dissection of brain tissues from 2 pups, but it can be scaled up as needed.

The protocol is structured in five sections:
1. Tissue Dissection
2. Culture plating and maintenance
3. Subculturing
4. Microglia shaking (optional)
5. FBS inactivation

The length of the protocol is ~19 days

Please Note: Breeding and euthanasia of all animal work should be performed in accordance with an institutionally approved animal care and use protocol.

MATERIALS

REAGENTS

HBSS-G (HBSS/0.6% glucose) with P/S

Hanks' balanced salt solution (1X HBSS) Ca2+ and Mg2+-free *(Invitrogen #14170)*
Dissolve 3gr glucose *(Sigma #G7528-1KG)* in 500mL 1X HBSS
Add 1% P/S (5000 units) *(Invitrogen #15140122)*
Stericup Filter Units - pore size: 0.22 um *(Millipore, S2GPU05RE)* – use 500mL filter inside tissue culture hood with aspiration
The HBSS can be stored in the fridge (4C) for several weeks.

Poly-D, L-lysine hydrobromide (PDLL) *(Sigma #P9011)*
Dissolve 5mg in 500ml PBS (0.01mg/ml)
Filter and store at 4C
Stericup Filter Units - pore size: 0.22 um *(Millipore, S2GPU05RE)* – use 500mL filter inside tissue culture hood with aspiration

Trypsin, .25% (1X) *(Fisher #25200-056)*
final concentration: 0.125% Trypsin
Dilute 1:1 with HBSS-G+P/S before use

Deoxyribonuclease I (DNAse I) from bovine pancreas *(Sigma #D5025, 15KU)*
STOCK 10X 1mg/ml in HBSS. Aliquot and store @ -20C. Use at a final concentration of 0.1mg/ml.
Thaw **DNAse** on ice. DNAse is very temperature sensitive, so make sure to keep it cold until you use it, and do not vortex

Filtered 8% BSA *(Sigma, A9647-50G)* in PBS
4g of BSA dissolved in 50mL PBS
filter and store at 4C
Stericup Filter Units - pore size: 0.22 um *(Millipore, S2GPU05RE)* – use 500mL filter inside tissue culture hood with aspiration

Astrocyte culture media
DMEM-F12 *(GIBCO, 11320-033)*
10% heat-inactivated fetal bovine serum: see protocol *(Omega FB-11 or Millipore TMS-0135B)*
1% Penicillin/Streptomycin *(Invitrogen #15140122)*
Store @ 4C. Warm up @ 37C

Trypan Blue: *(Life technologies #1394110)*

EQUIPMENT:
Cell counter: *(Biorad, model:TC 20 automated cell counter)*
Counting slides: *(Biorad #145-0011)*
10cm Culture Dish: (*Genclone #25-202)*
T75 flask filtered cap: *(Fisher #12565349)*
Centrifuge: *(Eppendorf, Centrifuge 5810R)*
15mL tube: *(Olympus, 29-103)*

BEFORE START INSTRUCTIONS

Coating tissue culture plates

1. Coat desired number of T75 flasks with Poly-D-Lysine . Keep in the tissue culture incubator at 37C for 1 hour
2. Rinse plates two times with sterile H2O.
3. Leave the plates drying in the TC incubator while isolating astrocytes.

Prepare reagents (details in materials)

1. Astrocyte media
2. HBSS-G + P/S
3. 0.25% Trypsin in HBSS-G + P/S

(Optional) Filter reagents with 0.22um filters

Tissue dissection

Before starting:

- Spray down the dissection bench with 70% isopropanol.
- Prepare dissection tools
- Prepare an ice bucket for chilling dissection plates.
- Place on ice 15ml tubes with 14ml HBSS-G for cortices isolated from 2 pups
- Pre-warm astrocyte media and 1:1 0.25% Trypsin/ HBSS-G + P/S and warm HBSS-G + P/S at 37°C in a water bath. Use 5ml of Trypsin/HBSSG for each 15ml tube and 25ml of media for 2 pups.
- Thaw 10X DNAse 1 (1mg/ml) on ice (can remove from -20C on step 6). Dilute 1:10 in astrocyte culture medium before use (0.1mg/ml final concentration). Use 1ml/pup

Tissue Dissection

2h

1 Use a 10 cm Petri dish with ice-cold HBSS-G+P/S to dissect the brain from the skull. Insert the tip of tweezers in to the eyes to keep the head fixed.

2 Using a microscope, cut up from the cranial floor to the nose. Open skull like a book. Lift the cortices with tweezers

3 Dissect the cortex by removing hindbrain and olfactory bulbs, and trim away meninges. Use tweezers to lift the meninges without puncturing the cortex tissue.

4 Transfer the cortex to the 15ml tube containing 14ml of HBSS-G +P/S on ice. 2 heads per 15mL tube

5 Use a clean 10cm Petri dish with ice-cold HBSS-G+P/S for each pup to reduce contaminations.

Culture Plating and Maintenance

2h

6 Move inside the biosafety cabinet. Spray 15 mL tubes with alcohol. Aspirate the HBSS-G + P/S, leaving as little of the dissection medium as possible without disturbing the tissue or exposing it to air.

7 Add 5ml/tube of warm 1:1 digestion medium (HBSS-G+P/S)/(0.25% trypsin). Incubate 5 minutes at 37C. Swirl the tube every 2 minutes. Now, is a good time to defrost the DNase I.

Note: previously, 20 minutes caused over digestion, do not incubate for long

8 While tissues are incubated with the digestion medium, remove poly-D-lysine from the T75 flasks, rinse twice with sterile H2O2, and remove any excess water.

9 After 5 minutes of digestion, remove the tubes from the water bath, and spray them with 70% isopropanol before moving them into the hood.

10 Let the tissue pieces settle to the bottom of the tube for 1 minutes. Aspirate HBSS-G+P/S/trypsin, leaving as little of the dissection medium as possible, but without disturbing the tissue or exposing it to air.

11 Add 5mL of warm HBSS-G +P/S to each tube to rinse the tissue and remove any excess of trypsin. Let pieces settle and then aspirate the HBSS-G +P/S.

12 Dilute 1:10 DNase in the culture medium. Add 1 ml of astrocyte media/DNaseI to each 15 mL tube containing the tissue.

13 Triturate tissue by pipetting up and down using a 1ml tip (7 to 15 times). **Avoid** introducing bubbles. Let any chunk of non-dissociated tissue sink at the bottom of the tube for 1-2 minutes.

14 Filter the dissociated tissues from multiple tubes through the same 40um mesh pre-rinsed with 1-2 ml of culture medium. Collect the filtered dissociated tissues in a 50 ml conical tube, and at the end, rinse the mesh with 1-2ml of culture medium.

15 Overlay the cell suspension on an 8% BSA cushion in a 50 ml tube. Tilting the 50mL tube at a slight 45° angle will help keep two separate layers.

16 Spin cells for 6 minutes at 1200rpm at room temperature.

17 Discard the supernatant containing the dead cells, 8% BSA, and media. Be careful to not aspirate or disturb the cell pellet.

18 Resuspend the cell pellet with 1mL of warm culture medium for each pup dissected. (ex if 8 pups were dissected, the total volume should be 8mL)
If a blood ring is present in the cell pellet when resuspending the cell pellet, avoid carrying over the blood.

19 Count the cells. Add 10uL of resuspended cells + 90uL of culture medium to dilute your cells 1:10. Mix 10uL of cell dilution with 10uL of Trypan Blue and place 10uL on each slide. The average number of cells must be multiplied by 10 because of the dilution factor.

20 Plate the cells in **half** the final volume of your culture dish. For example, plate 9.75 million cells per T75 flask or 130,000 cells per cm^2. Incubate at 37C with 5% CO2 for 5-10 minutes, then tap the edge of the plates to release loose debris. Then, aspirate media.

21 Add the full volume (10mL for 10cm plate) of fresh warm astrocyte media. Incubate at 37 °C in with 5% CO2.

22 Change **half** of the culture medium the day after plating and every three days until the culture reaches 80% confluency. Note: astrocyte morphology will begin to appear around DIV 3.

Subculturing

30m

23 Monitor astrocytes and split culture as it becomes ~80% confluency.
Note: Before splitting the culture, following the shaking protocol described below is recommended for a higher yield of astrocytes.

24 Wash cells 2x with 5mL of PBS. Add 1mL trypsin and pipette it around the culture dish. If astrocytes are not detaching, incubate plate at 37C and check on it every 2-3 minutes under the microscope

25 After cells are detached, add 5mL of warm astrocyte media to neutralize trypsin. Collect cells in a 15mL tube and spin at 1200rpm at RT for 6 min

26 Aspirate supernatant but do not disturb cell pellet. Resuspend the cellular pellet in 5 mL fresh warm astrocyte medium.
OPTIONAL= freeze as astrocytes passage 1

27 Count the cells and plate 27,400 cells/cm^2. Bring the total volume of each 10cm plate up to 10mL. Plates should be pre-coated with Poly-D-Lysine.

28 Incubate at 37 °C in with 5% CO2 incubator. Change the medium every 3 days.

29 10-14 days after the 1st passage, astrocytes are plated with a density of 38,680 cells/cm^2 in the desired vessel (e.g. 6-well plate, chamber slides, etc). After 24-48hr cells can be used for treatments and downstream experiments. We collect on DIV 19 and do not keep them longer than DIV 23.
Optional: freeze astrocytes at passage 2

Microglia shaking (optional)

1h 30m

30 **Optional:** After 7- 8 days in vitro, at the time of the first passage, there may be overlaying microglia on the astrocyte monolayer or are floating in the culture media.

31 To detach microglia from the astrocyte layer, shake the T75 flask at 250rpm for 1 hour on an orbital shaker. Check every 20 minutes to make sure astrocytes don't detach. Shake vigorously by hand for 1-2 mins. Discard the supernatant containing microglia, or if you wish to culture and examine microglia, spin it down and plate it for culture.
Note: If microglia layer is not visible; this section is skipped

32 Rinse the remaining confluent astrocyte layer twice with PBS, continue with splitting the culture (step 24)
OPTIONAL= freeze astrocytes and label as passage 1

Heat Inactivation Protocol for FBS

1h

33 Thaw FBS. If the serum was thawed in a refrigerator, allow it to reach room temperature before placing it in the water bath.

34 Set water-bath temperature to maintain the product at 56± 2°C.

35 Control bottles should be equivalent to the product bottle. Controls bottles are volumed to the

same level as the product being heat inactivated and fitted with a thermometer suitable for monitoring 56°C. For example, if inactivating 50mL of FBS, then the control bottle should have 50mL of water.

36 The thermometer should not touch the sides or bottom of the control bottle.

37 Place the bottles in the water-bath.

38 Check the temperature of the control bottle frequently as the temperature approaches 56± 2°C.

39 When the temperature of the control reaches 56± 2°C start the timer for 30 minutes. If a shaking water bath is not available, ensure bottles are swirled every 10 minutes during the entire process. Swirl bottle thoroughly every 10 minutes or if applicable turn on the oscillating shaker unit.

40 After 30 minutes turn off the oscillating shaker and remove the bottles from the water bath. Cool to room temperature and either aliquot or return to the freezer.