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Protocol status: Working
We use this protocol and it's working

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Generating regionally specified astrocytes

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ABSTRACT

This protocol outlines the differentiation of stem cells into regionally specific ventral midbrain and cortical astrocytes.
The protocol is a 4–5-month procedure and requires media changes 3 times a week.

GUIDELINES

Seeding density reference and expansion

- 3 spheres are plated into a 48 well plate > 24 well plate >12 well plate > 6-well plate > T-25 > T75 > T175
- P0 is when you first seed them into 2D format
- At P10 VMDA astrocytes > MAX Passage
- At P12 Cortex astrocytes > MAX Passage
- At P10 Spinal cord astrocytes > MAX Passage

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Keywords: ASAPCRN, astrocytes, stem cells, regional astrocytes, diseased astrocytes

MATERIALS

Material input

- PPMI stem cells
- D13 VMDA neurons
- D13 cortical neurons

Key materials

- Advanced DMEM/F-12 **Thermo Fisher Catalog #12634010**
- Glutamax (100x) **Gibco - Thermo Fischer Catalog #35050-061**
- Gibco™ N-2 Supplement (100X) **Thermo Fisher Scientific Catalog #17502048**
- Antibiotic-Antimycotic 9100x0 [Anti-Anti] **Thermo Fisher Scientific Catalog #15240062**
- B-27™ Plus Supplement (50X) **Thermo Fisher Catalog #A3582801**
- Basic FGF (FGF2), Human **Gold Biotechnology Catalog #1140-02**
- EGF, Epidermal Growth Factor, human **Bio Basic Inc. Catalog #RC216-15.SIZE.100ug**
- PDS Kit Papain Vial **Worthington Biochemical Corporation Catalog #LK003176**
- DNase I, RNase free **Thermo Fisher Scientific Catalog #EN0525**

SAFETY WARNINGS

For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet)

Preparation

1 Prepare astrocyte base media by combining;

- 484.5 mL Advanced DMEM/F12
- 5 mL GlutaMAX
- 5 mL N2
- 5 mL ANTI-ANTI
- 500 µL B-27+VITA

Sphere formation

2 1d 0h 15m

1. Disassociate D13 VMDA diff or D19 Cortex diff using Accutase for 00:03:00 to 00:06:00
2. Collect cells as small clumps and inhibit using respective diff media + Ri 1:1000
3. Spin down cells at 1300 rpm for 00:03:00
4. Aspirate media and resuspend in respective diff media (with factors required for diff) + Ri 1:1000.
5. Seed 20 000 cells/ well in a 96-well round bottom plate
6. Spin plate at 1300 rpm for 00:03:00
7. After 24:00:00, change the media with Astro base + EGF (20ug/ml stock; 20 ng/ml final 1:1000 dilution) and hLIF (10ug/ml stock; 20ng/ml final 1:500 dilution)

Maintenance

3

- When spheres have been in hLIF and EGF for 4 weeks change the media to EGF (20ug/ml stock; 20 ng/ml final 1:1000 dilution) + FGF2 (100ug/ml stock; 20ng/ml final 1:5000 dilution)
- EGF and FGF2 is maintained for a minimum of 2 months.

Neurosphere slicing

4 1d 0h 5m

1. When spheres become 0.5cm or larger it is time to chop them to axonotomise the neurons

2. Aspirate media and slice spheres using an autoclaved blade or two needles. Slice spheres in one direction then rotate plate 90° and slice again. Continue to rotate until you've reached 360°
3. Add 5 mL of PBS and move sliced spheres into a 15ml Falcon tube
4. Let sphere settle and aspirate PBS
5. Resuspend in 0.5 mL DNase (1mL in large amount of starting material) and incubate at 37 °C for 00:05:00. Shake tube every few minutes to distribute DNase through fragments
6. To DNase/cell mixture add 9 mL Astro base supplemented w/FGF2 and EGF and re-plate in a new low attachment 10cm plates
7. After 24:00:00, media change spheres with 10 mL Astro base supplemented w/FGF2 and EGF to wash out DNase

Plating down using Papain

53m

5

53m

1. When spheres have been in EGF+FGF2 for 2 months and have been sliced a minimum of 3 times they can be plated down in 2D format
2. Coat plates using 1:80 MG
3. Use the Worthington kit manual to reconstitute the Papain powder and make up all other reagents
4. Transfer spheres to a 15ml falcon tube and wash with PBS-/-. Aspirate PBS -/- once spheres have settled
5. Add Papain DNase mixture to the spheres (Usually double the amount of Papain to volume of spheres) and incubate at 37 °C for 00:15:00 to 00:30:00. Perform triturates at every 5-minute interval
   **NOTE: If your spheres are healthy and quite large you might need to manually pull them apart before adding Papain like what we do for cutting
6. At the end of 15 minutes, triturate again and let large clumps settle to the bottom and take off all supernatant (this contains a lot of single cells)
7. Add supernatant to new tube and inhibit with base media
8. Add new Papain, and repeat step 3-5 until clumps are disassociated (you will not be able to dissociate the whole thing so some clumps will be left, no longer than 45 minutes).
9. Spin cells that were inhibited at 1000 rpm for 00:04:00
10. Aspirate supernatant and resuspend in 300 µL ovomucoid inhibitor +DNase (see Worthington manual)
11. In a separate tube, add 1 mL of inhibitor albumin. Slowly transfer the cell solution onto the top of the protein gradient and leave to settle. **Note: the Worthington manual says to spin down, this is not necessary
12. Transfer the top layer of cells to a fresh tube
13. Centrifuge at 1000 rpm for 00:04:00
14. Resuspend in EGF + FGF2 **(DO NOT ADD Ri)**

Freezing astrocytes

6 To freeze astrocytes at the precursor stage, perform prep as normal however resuspend in Astrocyte base media + 10% DMSO

M

2w

7 To mature the astrocytes, maintain in CNTF 1:1000 + base media for 2 weeks