Protocol

Generation of inflammation-responsive astrocytes from glial progenitors derived from human pluripotent stem cells

Astrocytes are an essential component of the central nervous system for neuronal support and response to injury and disease. Here, we present a protocol to generate glial progenitor cells (GPCs) from human-induced pluripotent stem cells (iPSCs) that can be further differentiated into inflammation-responsive astrocytes. This two-step protocol has the advantage of reducing the time of astrocyte differentiation since GPCs can be frozen and stored.

Renata Santos, Arianna Mei, Maria C. Marchetto
renata.santos@inserm.fr (R.S.)
mcmarchetto@ucsd.edu (M.C.M.)

Highlights
Generation of glial progenitors from iPSC that can be stored frozen
Rapid differentiation of functional astrocytes from glial progenitors
Generation of astrocytes responsive to pro-inflammatory cytokines
Protocol

Generation of inflammation-responsive astrocytes from glial progenitors derived from human pluripotent stem cells

Renata Santos,1,2,4,5,* Arianna Mei,1 and Maria C. Marchetto1,3,4,*

1Laboratory of Genetics, The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, CA 92037, USA
2Université Paris Cité, INSERM U1266, Institute of Psychiatry and Neuroscience of Paris, Laboratory of Dynamics of Neuronal Structure in Health and Disease, 102 rue de la Santé, 75014 Paris, France
3Department of Anthropology, University of California San Diego, 9500 Gilman Drive, La Jolla, CA 92093, USA
4Technical contact
5Lead contact
*Correspondence: renata.santos@inserm.fr (R.S.), mcmarchetto@ucsd.edu (M.C.M.)
https://doi.org/10.1016/j.xpro.2022.101261

SUMMARY

Astrocytes are an essential component of the central nervous system for neuronal support and response to injury and disease. Here, we present a protocol to generate glial progenitor cells (GPCs) from human-induced pluripotent stem cells (iPSCs) that can be further differentiated into inflammation-responsive astrocytes. This two-step protocol has the advantage of reducing the time of astrocyte differentiation since GPCs can be frozen and stored.

For complete details on the use and execution of this protocol, please refer to Santos et al. (2017) and Vadodaria et al. (2021).

BEFORE YOU BEGIN

General considerations and preparations

Note: Prepare all stock solutions listed below and make the necessary working aliquots (Table 1); media using recipes below, but only when needed. The mTeSR1-, ScienCell astrocyte- and DMEM/F-12-based media are stable for up to 2 weeks at 4°C (protected from light).

The iPSC must be thawed in advance and passaged once before starting the differentiation procedure (7–14 days). Proliferation rate of iPSCs and GPCs depends on the cell line used; therefore, timing may need to be adapted for each experiment.

△ CRITICAL: All procedures are performed in a class II type A2 biosafety cabinet with standard aseptic technique. Cell culture and all incubations are performed in a humidified incubator at 37°C with 5% CO₂.

△ CRITICAL: Plan to test the cultures regularly for Mycoplasma contamination.

Alternatives: We have also had success using this protocol with human embryonic stem cells (Santos et al., 2017).

Note: Human iPSCs were obtained and used according to legal and ethical guidelines.
Matrigel coating of culture plates

**Timing:** 1 h using Matrigel aliquots

1. Thaw Matrigel on ice or at 4 °C overnight, aliquot under sterile conditions into 1.5 mL tubes of 0.5 mg, 1 mg and 2 mg and store in the freezer at −80 °C.

   **CRITICAL:** Matrigel should be kept cold until plating to avoid polymerization, which occurs at temperatures above 10 °C. We recommend the use of pre-cooled pipets, tips, and tubes when aliquoting and handling Matrigel.

   **CRITICAL:** Each lot of Matrigel comes with a different concentration; take this into account when preparing aliquots.

2. Thaw Matrigel aliquots on ice. Use 0.5 mg Matrigel per 6-well plate or 8 μg/cm².

3. Dilute 1 mg Matrigel in 24 mL ice-cold DMEM/F-12.
   a. Aliquot 24 mL of cold DMEM/F-12 into 50 mL conical tubes and place on ice.
   b. Take 1 mL of media with a cold 5 mL pipette, mix with the Matrigel by pipetting up and down, add to the rest of the medium and mix well.

4. Add 2 mL of diluted Matrigel per well of a 6-well plate (or 35 μL/cm²).

5. Incubate for at least 30 min in the incubator at 37 °C and wash once with warm DMEM/F-12 prior to use.

   **Pause point:** Matrigel-coated dishes can be stored for up to 1 week at 37 °C or 2 weeks in the refrigerator sealed in parafilm or Saran wrap to prevent drying. Dried coated dishes should be discarded. Before use, incubate the plates for 30 min at 37 °C if stored at 4 °C.

Poly-L-ornithine (PLO)-laminin coating of culture plates

**Timing:** 1–2 days

---

Table 1. Storage and working concentrations of reagents used to culture and differentiate iPSCs

| Reagent              | Stock concentration | Working concentration | Dilution factor | Storage          |
|----------------------|---------------------|-----------------------|-----------------|-----------------|
| Accutase             | 1 x; prepare 5 or 10 mL aliquots | 1 x                  | N/A^a           | −20 °C          |
| Accumax              | 1 x; prepare 5 or 10 mL aliquots | 1 x                  | N/A^a           | −20 °C          |
| B-27 without vitamin A | 50 x; directly aliquoted | 1 x                  | 1:50            | −20 °C          |
| BSA                  | 0.1% in PBS         | 0.1%                  | 1:1             | −20 °C          |
| Collagenase IV       | 10 mg/mL in DMEM/F-12 | 1 mg/mL              | 1:10            | −20 °C          |
| DMSO                 | 100%                | 10%                   | 1:10            | Room temperature |
| EGF                  | 20 μg/mL^b          | 20 ng/mL              | 1:1,000         | −20 °C          |
| FBS                  | 100%                | 10%                   | 1:10            | −20 °C          |
| FGF2                 | 20 μg/mL^b          | 20 ng/mL              | 1:1,000         | −20 °C          |
| Laminin              | 1 mg/mL             | 5 μg/mL for plate coating in PBS | 1:200 | −20 °C |
| Laminin              | 1 mg/mL             | 1 μg/mL for culture media | 1:1,000 | −20 °C |
| Matrigel             | 0.5 mg/ 1 mg/2 mg   | 8 μg/cm²              | N/A^a           | −80 °C          |
| N-2                  | 100 x; directly aliquoted | 1 x                  | 1:100           | −20 °C          |
| Noggin               | 500 μg/mL^b         | 500 ng/mL             | 1:1,000         | −20 °C          |
| PBS                  | 10 x                | 1 x                   | 1:10            | Room temperature |
| PDGF-AA              | 100 μg/mL^b         | 10 ng/mL              | 1:10,000        | −80 °C          |
| Penicillin-streptomyccin | 100 x              | 1 x                   | 1:100           | −20 °C          |
| PLO                  | 10 mg/mL in H2O     | 10 μg/mL for plastic  | 1:100 for plastic | −20 °C |
|                     |                     | 50 μg/mL for glass    | 1:200 for glass | −20 °C |
| Y-27632              | 10 mM in PBS        | 10 μM                 | 1:1,000         | −20 °C          |

^aNot applicable.

^bIn 0.1% BSA in PBS.
6. Thaw PLO aliquots and prepare a solution in water at 10 μg/mL for plastic plates or 50 μg/mL for glass coverslips.
7. Pipette enough volume of PLO solution to cover the surface of each well or dish, swirl.
8. Incubate overnight at room temperature.
9. Wash thoroughly 3× with water to remove all PLO residues, which are toxic to the cells.
10. Thaw laminin aliquots and prepare a solution in PBS at 5 μg/mL, the same for plastic plates or glass coverslips.
11. Pipette enough volume of laminin solution to cover the surface of each well or dish, swirl.
12. Incubate overnight at room temperature or for 2 h at 37°C. No need to wash before use.

**Pause point:** PLO-laminin-coated dishes can be stored for up to 3–4 months frozen at –20°C sealed in parafilm or Saran wrap with enough solution to prevent drying. Dried coated dishes should be discarded. Before use, thaw and incubate the plates for 30 min at 37°C.

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Chemicals, peptides, and recombinant proteins | | |
| Accutase | STEMCELL | AT-104 |
| Accumax | STEMCELL | 07921 |
| Astrocyte media (AM medium) | ScienCell | 1801 |
| B-27 supplement (50X), minus vitamin A | Thermo Fisher Scientific | 12587010 |
| BSA (0.1%) | Sigma-Aldrich/Merck | A8806 |
| Collagenase IV (Gibco) | Thermo Fisher Scientific | 17104-019 |
| DMEM/F-12 for wash (but also the one below can be used for washing) | Thermo Fisher Scientific (Gibco) | 21331020 |
| DMEM/F-12 GlutaMAX supplement | Thermo Fisher Scientific (Gibco) | 10565018 |
| DMSO | Sigma-Aldrich/Merck | D2690 |
| EGF (human) | HumanZyme | HZ-7012 |
| Fetal bovine serum-TET Free (FBS) | Omega Scientific | FB-15 |
| FGF2 (human) | Joint Protein Central | Human FGF2 |
| Laminin | Invitrogen | 23017-015 |
| Matrigel | Corning | 354234 |
| mTeSR1 | STEMCELL | 85850 |
| N-2 supplement (100X) | Thermo Fisher Scientific | 17502048 |
| Noggin (human) | Proteintech | HZ-1118 |
| PBS, pH7.4 | Thermo Fisher Scientific | 10010023 |
| PDGF-AA (human) | PeproTech | 100-13A |
| Penicillin/Streptomycin | ScienCell | 0503 |
| Poly-L-Ornithine (PLO) | Sigma-Aldrich | P3655 |
| Rock Inhibitor, Y-27632 | AdooQ | A21448 |
| Critical commercial assays | | |
| Papain Dissociation System | Worthington | LK003150 |
| Experimental models: Cell lines | | |
| Human iPSCs | Laboratory of genetics – Gage; Salk Institute | N/A* |
| Human GPCs | Laboratory of genetics – Gage; Salk Institute | N/A* |
| Other | | |
| 6-well tissue culture plates | Corning | 3516 |
| Ultra-low-attachment 6-well plates | Corning | 3471 |
| Cell lifter | Corning | 3008 |
| Cryovials | Thermo Scientific | 375418 |
| CO2 resistant shaker | Thermo Scientific | 88881101B |

*Not applicable.
MATERIALS AND EQUIPMENT

Work with sterile plates, dishes, pipet tips, pipettes, microcentrifuge tubes and centrifuge tubes. Prepare reagents and media under sterile and endotoxin-free conditions. Working media can be sterilized by filtration using 0.22 μm prior to use. We recommend avoiding the use of antibiotics in culture and differentiation of iPSCs.

For embryoid bodies culture, low-attachment plates are needed, as well as an orbital shaker inside the 37°C 5% CO₂ incubator.

Note: Reagents can be stored in the conditions above for up to 1 year, unless not recommended by the manufacturer.

△ CRITICAL: To avoid frequent freeze-thaw cycles of frozen aliquots adapt the volume to the average volume used in one week. Once thawed, keep at 4°C.

| AM medium                         | Working concentration | Volume  |
|-----------------------------------|-----------------------|---------|
| Astrocyte media                   | –                     | 500 mL  |
| AGS (astrocyte growth supplement) | 100×                  | 5 mL    |
| FBS 2%                            | 100%                  | 10 mL   |

Maximum 2 weeks at 4°C protected from light.

| DMEM/F-12 glutamax N-2 B-27 medium | Working concentration | Volume  |
|------------------------------------|-----------------------|---------|
| DMEM/F-12 Glutamax                 | –                     | 500 mL  |
| N-2                                | 1:100                 | 5 mL    |
| B-27                               | 1:50                  | 10 mL   |

Maximum 2 weeks at 4°C protected from light.

| EBs patterning medium             | Working concentration | Volume  |
|-----------------------------------|-----------------------|---------|
| Astrocyte media                   | –                     | 100 mL  |
| AGS                               | 1:100                 | 1 mL    |
| FBS 2%                            | 1:100                 | 2 mL    |
| Noggin 500 ng/mL                  | 1:1,000               | 100 μL  |
| PDGF-AA 10 ng/mL (first 2 weeks only) | 1:10,000               | 10 μL   |

Maximum 1 week at 4°C protected from light.

| GPCs expansion medium             | Working concentration | Volume  |
|-----------------------------------|-----------------------|---------|
| Astrocyte media                   | –                     | 100 mL  |
| AGS                               | 1:100                 | 1 mL    |
| FBS 2%                            | 1:100                 | 2 mL    |
| FGF2 20 ng/mL                     | 1:1,000               | 100 μL  |
| EGF 20 ng/mL                      | 1:1,000               | 100 μL  |

Maximum 1 week at 4°C protected from light.

| Astrocyte induction medium        | Working concentration | Volume  |
|-----------------------------------|-----------------------|---------|
| DMEM/F-12 Glutamax                | –                     | 100 mL  |
| N-2                               | 1:100                 | 1 mL    |
| B-27                              | 1:50                  | 2 mL    |
| FBS 10%                           | 1:100                 | 10 mL   |

Maximum 1 week at 4°C protected from light.
**STEP-BY-STEP METHOD DETAILS**

**Preparing iPSCs for GPCs differentiation**

- **Timing:** 2–4 weeks

This step is necessary for increasing cell viability during thawing and expansion to produce colonies of human iPSCs for GPCs differentiation. Human iPSCs are cultured under feeder-free conditions and passaged at least once before starting GPCs differentiation to ensure cells are in an active growth phase before starting differentiation.

1. **Thaw iPSC lines.**
   - a. Prepare Matrigel-coated plates as described before and warm DMEM/F-12 and mTeSR1 media.
   - b. Prepare 15 mL centrifuge tubes containing 12 mL DMEM/F-12 (1 tube per vial with $1 \times 10^6$ cells to be thawed) for cell wash.
   - c. Prepare mTeSR1 ROCK inhibitor medium by adding $10 \mu$M Y-27632 (1:1,000 dilution of stock solution); 2 mL/well of a 6-well plate are needed.
   - d. Collect vials to be thawed on dry ice from cryostorage. Immediately transfer vials to a 37°C water bath.
   - e. Remove vial from water bath before thawing is complete, when approximately half of the content is still frozen.
   - f. Gently add 500 $\mu$L of warm DMEM/F-12 using a 5 mL pipet, pipet up and down and when completely thawed, transfer to a centrifuge tube containing 12 mL DMEM/F-12 medium.
   - g. Centrifuge at 200 g, 5 min at room temperature.
   - h. Gently aspirate supernatant leaving approximately 100 $\mu$L of media in the bottom of the tube, gently flick with finger to resuspend cell pellet.
   - i. Aspirate Matrigel solution from 6-well plates. No washing is required before plating the cells.
   - j. Gently add 2 mL of mTeSR1 ROCK inhibitor medium to resuspended cells and transfer entire volume to 1 well of 6-well plate.
   - k. Place plate in the incubator; gently shake the plate side to side and back and forth for a homogeneous distribution of the cells.
   - l. The next day, replace media with 2 mL of fresh mTeSR1 to remove ROCK inhibitor Y-27632. Colonies should appear within one week.

**CRITICAL:** Do not thaw completely the cells in the water bath to avoid cell death.

**CRITICAL:** Complete removal of DMSO present in storage media by washing is very important due to its toxicity.

**CRITICAL:** For media changes, always pipet carefully close to the wall of the wells to avoid detaching the cells.

**Note:** The use of ROCK inhibitor Y-27632 increases viability.

**Note:** Matrigel can be replaced by other extracellular matrices; we used Cultrex (R&D Systems, 3433-005-01) with similar results. mTeSR plus (Stem Cell, cat#100-0276) can replace mTeSR1 medium for growth and maintenance of iPSCs.

**Troubleshooting:** If experiencing low viability thawing cells to 12-well is recommended.

2. **Maintenance of iPSC cultures.**
a. Change mTeSR1 medium completely every day; this provides growth factors that reduce spontaneous differentiation.

**Note:** You can double feed once a week.

### 3. Passage of iPSC colonies.

a. When colonies cover 80%–90% of the plate they are ready to be passaged.
b. Prepare Matrigel-coated plates as described before and warm DMEM/F-12 and mTeSR1 media and PBS.
c. Prepare collagenase IV medium by adding 1 mg/mL collagenase IV to DMEM/F-12 (1:10 dilution of stock solution); 1 mL/well of a 6-well plate is needed.
d. Mark on the plate differentiated cells for removal using a microscope.
e. Wash 1× with 3 mL warm PBS.
f. Add 1 mL of collagenase IV medium per well of a 6-well plate and incubate 15 min in the 37°C incubator; at this point the edges of the colonies start curling up and are ready to be detached.
g. Wash 2× with 3 mL of warm DMEM/F-12 medium.
h. Remove the differentiated cells with a plastic tip.
i. Wash 1× with 3 mL of warm DMEM/F-12 medium.
j. Add 1 mL mTeSR1 medium per well and gently scrap the cells using a cell lifter; stop once the cells are detached from the plate to keep small colonies and avoid dissociation into single cells.
k. Resuspend in sufficient mTeSR1 medium (2 mL/well) to replate at 1:4–6 on 6-well Matrigel-coated plates.
l. Place plate in the incubator; gently shake the plate side to side and back and forth for a homogeneous distribution of the cells.

⚠️ **CRITICAL:** Cells should remain in small colonies when passaged; single iPS cells have lower viability.

**Alternatives:** ReLeSR reagent (STEMCELL, cat. #05872) and gentle dissociation can be used to passage iPSCs.

### GPCs differentiation and culture

**Timing:** 5–6 weeks

This step describes differentiation of iPSCs into GPCs using embryoid bodies (EBs) patterning, as well as expansion of GPC lines and cryopreservation.

### 4. GPCs generation from iPSCs.

a. Grow iPSCs in mTeSR1 medium on 3 wells of 6-well Matrigel-coated plates until 80% confluent.
b. Warm DMEM/F-12 for wash.
c. Prepare collagenase IV medium by adding 1 mg/mL collagenase IV to DMEM/F12 (1:10 dilution of stock solution); 1 mL/well of a 6-well plate is needed.
d. Prepare 15 mL centrifuge tubes containing warm 9 mL mTeSR1 ROCK inhibitor medium by adding 10 μM Y-27632 (1:1,000 dilution of stock solution); 1 tube per cell line is needed.
e. Mark on the plate differentiated cells for removal using a microscope.
f. Wash 1× with warm PBS.
g. Add 1 mL of collagenase IV medium and incubate 15 min in the 37°C incubator; at this point the edges of the colonies start curling up and are ready to be detached.
h. Wash 2× with 3 mL of warm DMEM/F-12 medium.
i. Remove the differentiated cells with a plastic tip.
j. Wash 1× with 3 mL of warm DMEM/F-12 medium.

k. Add 1 mL mTeSR1 medium/well, scrap the cells using a cell lifter and resuspend the aggregates of cells from the 3 wells in 9 mL mTeSR1 ROCK inhibitor medium (equivalent to 1×10⁶ cells/mL).

l. Transfer to 3 wells of a low attachment 6-well plate and incubate with gentle shaking (90 rpm) in the 37°C incubator.

m. Day 1: A homogeneous suspension of spherical EBs (ca. 200 μm) has formed. To collect the EBs, transfer the media carefully to 15 mL conical tubes and centrifuge at 200 x g for 1–2 min at room temperature, gently aspirate supernatant leaving approximately 200 μL of media in the bottom of the tube, gently flick with finger to resuspend cell pellet add 3 mL Sci- enCell Astrocyte media with AGS plus 2% FBS (AM medium) and supplemented with Noggin 500 ng/mL (1:1,000 dilution of stock solution) plus PDGF-AA 10 ng/mL (1:10,000 dilution of stock solution) resuspend by pipetting up and down gently 2–3 times with a 5 mL pipette and transfer to the same well. Keep agitation at 90 rpm.

n. Days 3, 5, 7, 9, 11, 13: perform complete media change using 3 mL/well of the same media composition. EBs will increase in size and become translucent (Figure 1). Keep agitation at 90 rpm.

Note: As EBs grow (4–5 times initial size in 3 weeks) media can be changed by simply using gravity in inclined plates under the flow hood.

o. Days 15, 17, 19: switch to AM medium with just PDGF-AA 10 ng/mL (1:10,000 dilution of stock solution). Keep agitation.

p. On day 21 the EBs are homogeneous, spherical, translucent, ca. 900 μm in size, and are ready for dissociation.

i. Prepare 6-wells PLO-laminin coated plates.

Figure 1. Differentiation of iPSCs into astrocytes through a glial progenitor intermediate step
(A) Time line.
(B) Representative bright field images through the different steps of differentiation (iPSC, EB, GPC and astrocyte).
(C) Representative images of immunostainings characteristic of the cell lines generated using this protocol. GPC lines expressing A2B5, NF1A and vimentin and 4-week old astrocytes expressing S100β, CD44, GFAP and vimentin.
For each cell line, prepare deoxyribonuclease (DNase)/papain solution as indicated in the Worthington kit instructions (Papain dissociation system, https://www.worthington-biochem.com/PDS/cat.html):

1 vial of DNase resuspended with 0.5 mL of Earle’s balanced salt solution; add 0.25 mL to papain solution.

1 vial of papain resuspended with 5 mL of Earle’s balanced salt solution.

Collect EBs from the 3 wells to a 15 mL conical tube and centrifuge at 200 g for 1 min at room temperature or let them settle at the bottom of the tube.

Resuspend by flicking and wash 1x with 5 mL of warm PBS.

Resuspend EBs with 5.25 mL of ribonuclease/papain solution and transfer to a well of a 6-well plate.

Incubate for 1 h in the 37°C 5% CO₂ incubator and pipette vigorously up and down with a 1 mL pipette every 15 min.

Check under a microscope to confirm that the suspension is composed of single cells.

Count the cells.

Plate the cells 200,000–300,000 cells/mL (45,000–60,000 cells/cm²) in AM medium supplemented with FGF2 20 ng/mL (1:1,000 dilution of stock solution), EGF 20 ng/mL (1:1,000 dilution of stock solution), laminin 1 µg/mL (1:1,000 dilution of stock solution) and ROCK inhibitor Y-27632 10 µM (1:1,000 dilution of stock solution) in PLO-laminin coated plates (use 2 mL/well for 6-well plates).

The next day, replace medium with 2 mL of fresh AM supplemented with FGF2 20 ng/mL, EGF 20 ng/mL (expansion medium) to remove ROCK inhibitor Y-27632.

CRITICAL: Initial cell density is important for differentiation and expansion of GPC lines.

Expansion, passage and preparation of frozen master stock of GPCs lines.

Perform complete medium change every other day using 2–3 mL/well of expansion medium (AM supplemented with FGF2 20 ng/mL, EGF 20 ng/mL, laminin 1 µg/mL).

When cells cover over 80% of the plate can be passaged using Accutase.

Prepare 15 mL centrifuge tubes containing warm 12 mL DMEM/F-12 (1 tube per well of a 6-well plate) for cell wash. Warm Accutase solution.

Wash 1x with warm PBS.

Add 1 mL of warm Accutase per well of a 6-well plate and incubate 5–10 min in the 37°C incubator.

Collect the cells by pipetting up and down and transfer to a centrifuge tube containing 12 mL DMEM/F-12 medium for washing.

Centrifuge at 200 g, 5 min at room temperature.

Resuspend in sufficient expansion medium to replate at a 1:6–8 ratio (10,000–20,000 cells/cm²) on 6-well PLO-laminin coated plates.

For cryopreservation, dissociate with Accutase as for passaging (steps b–g). After centrifugation, remove supernatant and add drop by drop AM medium with 10% DMSO (1 mL per well of a 6-well plate will result in 1 x 10⁶ cells per vial).

Transfer to a cryovial and freeze at −80°C in an appropriate freezing container for 12–24 h, then store in a liquid nitrogen tank.

CRITICAL: GPCs can be grown sparse (5,000 cells/cm²) as they are highly proliferative.

Note: Freezing down 5–10 vials containing 1 x 10⁶ cells at P1 or P2 will allow sufficient vials to perform several studies with the same batch of GPCs. This will increase reproducibility of the results and protect from cell line loss due to contamination or other difficulties.

Note: GPCs can be expanded and maintained for at least 10 passages with no decline in proliferation and ability to differentiate into astrocytes.
6. Thaw GPC lines.
   a. Prepare 15 mL centrifuge tubes containing warm 12 mL DMEM/F-12 (1 tube per well of a 6-well plate) for cell wash. Prepare 6-wells PLO-laminin coated plates.
   b. Cells from one frozen vial are usually plated on a full 6-well plate at a density of 15,000 cells/cm².
   c. Thaw the cells quickly in a water bath at 37°C and transfer to a 15 mL centrifuge tube containing warm 12 mL DMEM/F-12 for washing.
   d. Centrifuge at 200 g for 5 min at room temperature.
   e. Gently aspirate supernatant leaving approximately 100 μL of media in the bottom of the tube, gently flick with finger to resuspend cell pellet.
   f. Add 12 mL of expansion medium (AM supplemented with FGF2 20 ng/mL, EGF 20 ng/mL, laminin 1 μg/mL).
   g. Plate on 2 mL/well in a 6-well PLO-laminin coated plate.

   **Note:** The recovery rate of GPCs is estimated to between 60% and 80%, depending on the cell lines.

   △ CRITICAL: Complete removal of DMSO present in storage media by washing is very important due to its toxicity.

**Astrocyte differentiation**

**Timing:** >4 weeks

This step describes differentiation of GPCs into astrocytes.

7. Day 1: plate GPCs at 15,000 cells/cm² in expansion medium (AM supplemented with FGF2 20 ng/mL, EGF 20 ng/mL, laminin 1 μg/mL) in 6-well plates.

   **Note:** Differentiation can be started from passaged GPCs from passage 1–9 or from GPCs plated from frozen vials with equal efficiency.

8. Day 2: start differentiation by changing the expansion AM medium to DMEM/F-12 Glutamax supplemented with N-2, B-27 and 10% FBS (induction media).

9. Day 3–14: change media completely every other day.

10. Day 15: split cells to non-coated plates using Accutase.
    a. Prepare 15 mL centrifuge tubes containing warm 12 mL DMEM/F-12 (1 tube per well of a 6-well plate) for cell wash. Warm Accutase solution (or Accumax).
    b. Wash 1× with warm PBS.
    c. Add 1 mL of warm Accutase (or Accumax) and incubate 5–10 min in the 37°C incubator.
    d. Collect the cells by gently pipetting up and down and transfer to a centrifuge tube containing 12 mL DMEM/F-12 medium for washing.
    e. Centrifuge at 200 x g, 5 min at room temperature.
    f. Resuspend in sufficient induction medium to replate at a 1:6 ratio (20,000–25,000 cells/cm²) on 6-well non-coated plates.

11. After day 15: Perform complete medium change every other day using 2–3 mL/well of induction medium (DMEM/F-12 Glutamax supplemented with N-2, B-27 and 10% FBS).

   △ CRITICAL: If astrocytes get stickier and difficult to dissociate, replace Accutase by Accumax.

   △ CRITICAL: Plating cells onto non-coated plates at day 15 of differentiation purifies the cultures from progenitors and other types of cells while keeping the more mature astrocytes.
**Note:** After splitting at day 15 astrocytes continue to proliferate. FBS can be reduced to 5% or 2% to reduce proliferation rate without affecting maturation.

**Note:** Use of ROCK inhibitor Y-27632 10 μM for splitting astrocytes at day 15 improves viability but it is not required.

**Pause point:** Astrocytes can be stored frozen from day 7 up to 4 weeks post differentiation in induction medium plus 10% DMSO.

**EXPECTED OUTCOMES**

This protocol has been used successfully with more than 25 iPSC lines from which 15 were published (Figueiredo et al., 2021; Santos et al., 2017; Vadodaria et al., 2021).

The GPCs should be >90% pure as detected by expression of nestin, A2B25 and NFIA in immunofluorescence experiments (Santos et al., 2017; Vadodaria et al., 2021). The astrocytes should be >90% pure with expression of vimentin, CD44, S100β and GFAP (Santos et al., 2017; Vadodaria et al., 2021) (Figure 1).

The astrocytes should respond rapidly to inflammatory stimuli, such as IL-1β and TNF-α. Secretion of pro-inflammatory cytokines, such as IL-6 and IL-8, can be detected after 5 h in the media or intracellularly by flow cytometry assay (Santos et al., 2017; Vadodaria et al., 2021).

**LIMITATIONS**

Differentiation efficiencies and cell yields can vary between cell lines and experiments.

This protocol uses 10% FBS for astrocyte differentiation, which is not compatible with all types of experiments. For example, when performing co-cultures with neurons change to 1% FBS. FBS can also be completely replaced by 10% Knockout Serum Replacement (Thermo Fisher Scientific, cat. # 10828010) as reported (Heard et al., 2021).

**TROUBLESHOOTING**

**Problem 1**

GPCs show neuronal contamination.

**Potential solution**

It is likely that agitation during the EB patterning phase is not sufficient, use 90 rpm. Agitation is absolutely necessary for patterning of embryoid bodies and avoids neuronal contamination.

**Problem 2**

Low viability after splitting GPCs or astrocytes.

**Potential solution**

This can be due to long incubations with Accutase or papain enzymes, omitting the enzyme inhibition step, vigorous pipetting or use of cold reagents. ROCK inhibitor Y-27632 10 μM can be added to increase survival after splitting GPCs and astrocytes.

**Problem 3**

Hyperproliferation during astrocyte differentiation.

**Potential solution**

Astrocytes proliferate rapidly during the first 2 weeks of differentiation before splitting to non-coated plates. If proliferation is too high there is a risk of detachment of the cells, in this case, the
cells can be split at any time between day 7 and day 15. After 2 weeks proliferation is reduced as astrocytes mature.

**Problem 4**
Differentiating astrocytes detach from the plate.

**Potential solution**
This may happen if cell density is too high. To reduce risk of detachment in long cultures, astrocytes can be split before day 15 post-differentiation or plated onto POL-laminin coated plates in step 10.

**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Renata Santos (renata.santos@inserm.fr).

**Materials availability**
This study did not generate new unique reagents.

**Data and code availability**
This study did not generate any unique datasets or code.

**ACKNOWLEDGMENTS**
We are grateful for the funding provided to MCM by the Larry L. Hillblom Foundation and UCSD Department of Anthropology Startup funds. We would like to thank the Salk Institute Core Facilities: Next Generation Sequencing (NGS) Core Facility with funding from NIHNCI CCSG: P30 014195, the Chapman Foundation and the Helmsley Charitable Trust; The Stem Cell Core Facility, The Razavi Newman Integrative Genomics and Bioinformatics Core Facility with funding from NIH-NCI CCSG: P30 014195; and Flow Cytometry Core Facility with funding from NIHNCI CCSG: P30 014195. We would like to thank Fred H. Gage and the members of the Gage laboratory for their support.

**AUTHOR CONTRIBUTIONS**
R.S., A.M., and M.C.M. conceived the project and developed the methodology. R.S. and A.M. performed experiments. R.S. wrote the manuscript. A.M. and M.C.M. edited the manuscript.

**DECLARATION OF INTERESTS**
The authors declare no competing interests.

**REFERENCES**
Figueiredo, T., Mendes, A.P.D., Moreira, D.P., Goulart, E., Oliveira, D., Kobayashi, G.S., Stern, S., Kok, F., Marchetto, M.C., Santos, R., et al. (2021). Inositol monophosphatase 1 (IMPA1) mutation in intellectual disability patients impairs neurogenesis but not gliogenesis. Mol. Psychiatry 26, 3558–3571.

Heard, K.J., Shokhirev, M.N., Becronics, C., Fredlender, C., Zahid, N., Le, A.T., Ji, Y., Skime, M., Nelson, T., Hall-Flavin, D., et al. (2021). Chronic cortisol differentially impacts stem cell-derived astrocytes from major depressive disorder patients. Transl. Psychiatry 11, 608.

Santos, R., Vadodaria, K.C., Jaeger, B.N., Mei, A., Lefcochlos-Fogelquist, S., Mendes, A.P.D., Erikson, G., Shokhirev, M., Randolph-Moore, L., Fredlender, C., et al. (2017). Differentiation of inflammation-responsive astrocytes from glial progenitors generated from human induced pluripotent stem cells. Stem Cell Rep. 8, 1757–1769.

Vadodaria, K.C., Mendes, A.P.D., Mei, A., Racha, V., Erikson, G., Shokhirev, M.N., Oefner, R., Heard, K.J., McCarthy, M.J., Eyler, L., et al. (2021). Altered neuronal support and inflammatory response in bipolar disorder patient-derived astrocytes. Stem Cell Rep. 16, 825–835.