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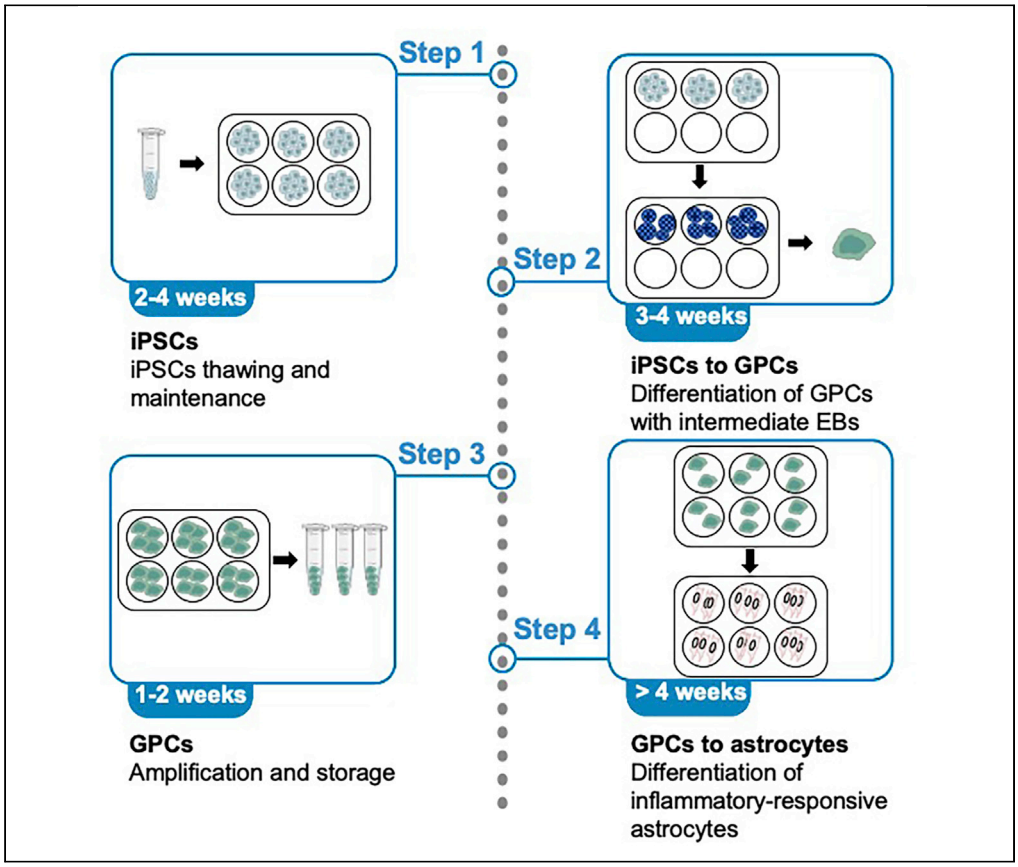

Astrocytes are an essential component of the central nervous system for neuronal support and response to injury and disease. Here, we present a protocol to generate glial progenitor cells (GPCs) from human-induced pluripotent stem cells (iPSCs) that can be further differentiated into inflammation-responsive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

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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

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Protocol

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

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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% CO_2.

⚠ 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.

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

Reagent	Stock concentration	Working concentration	Dilution factor	Storage
Accutase	1×; prepare 5 or 10 mL aliquots	1×	N/A[a]	−20°C
Accumax	1×; prepare 5 or 10 mL aliquots	1×	N/A[a]	−20°C
B-27 without vitamin A	50×; directly aliquoted	1×	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/cm^2	N/A[a]	−80°C
N-2	100×; directly aliquoted	1×	1:100	−20°C
Noggin	500 μg/mL[b]	500 ng/mL	1:1,000	−20°C
PBS	10×	1×	1:10	Room temperature
PDGF-AA	100 μg/mL[b]	10 ng/mL	1:10,000	−80°C
Penicillin-streptomycin	100×	1×	1:100	−20°C
PLO	10 mg/mL in H_2O	10 μg/mL for plastic 50 μg/mL for glass	1:100 for plastic 1 :200 for glass	−20°C
Y-27632	10 mM in PBS	10 μM	1:1,000	−20°C

[a]Not applicable.
[b]in 0.1% BSA in PBS.

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/cm^2.
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/cm^2).
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

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 (50×), 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	D2650
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 (100×)	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[a]
Human GPCs	Laboratory of genetics – Gage; Salk Institute	N/A[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
CO_2 resistant shaker	Thermo Scientific	88881101B

[a]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% CO_2 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 1×10^6 cells to be thawed) for cell wash.
   c. Prepare mTeSR1 ROCK inhibitor medium by adding 10 μM 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 μL 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 μL 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.
   m. 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.

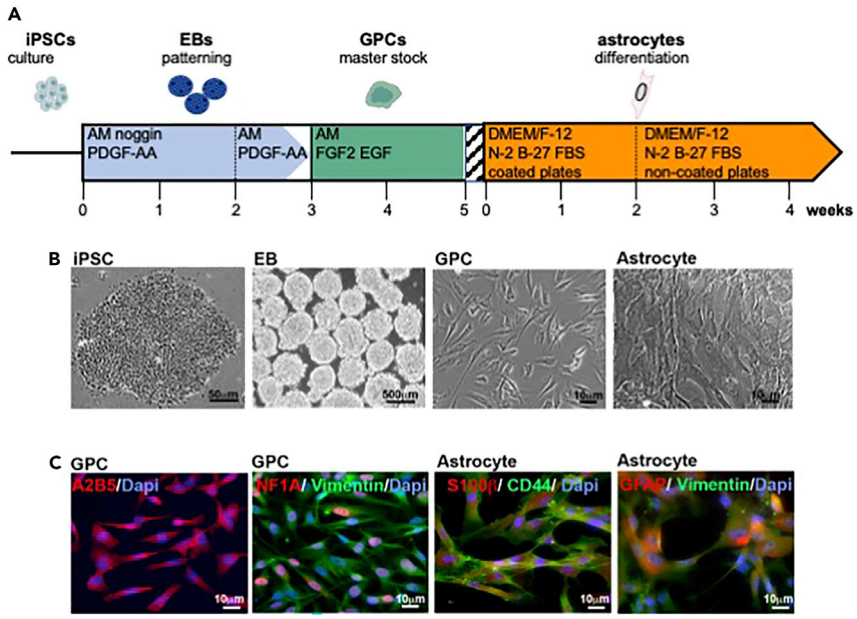

Figure 1. Differentiation of iPSCs into astrocytes through a glial progenitor intermediate step

(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 S100ß, CD44, GFAP and vimentin.

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 × 10^6 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 ScienCell 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.

ii. 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.

iii. 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.

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

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

vi. Incubate for 1 h in the 37°C 5% CO_2 incubator and pipette vigorously up and down with a 1 mL pipette every 15 min.

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

viii. Count the cells.

q. Plate the cells 200,000–300,000 cells/mL (45,000–60,000 cells/cm^2) 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).

r. 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.

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

a. 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).

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

c. 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.

d. Wash 1× with warm PBS.

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

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

g. Centrifuge at 200 g, 5 min at room temperature.

h. Resuspend in sufficient expansion medium to replate at a 1:6–8 ratio (10,000–20,000 cells/cm^2) on 6-well PLO-laminin coated plates.

i. 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 × 10^6 cells per vial).

j. 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/cm^2) as they are highly proliferative.

Note: Freezing down 5–10 vials containing 1 × 10^6 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/cm^2.
   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/cm^2 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/cm^2) 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, S100ß and GFAP (Santos et al., 2017; Vadodaria et al., 2021) (Figure 1).

The astrocytes should respond rapidly to inflammatory stimuli, such as IL-1ß 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 preforming 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 splitted 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.

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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.

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