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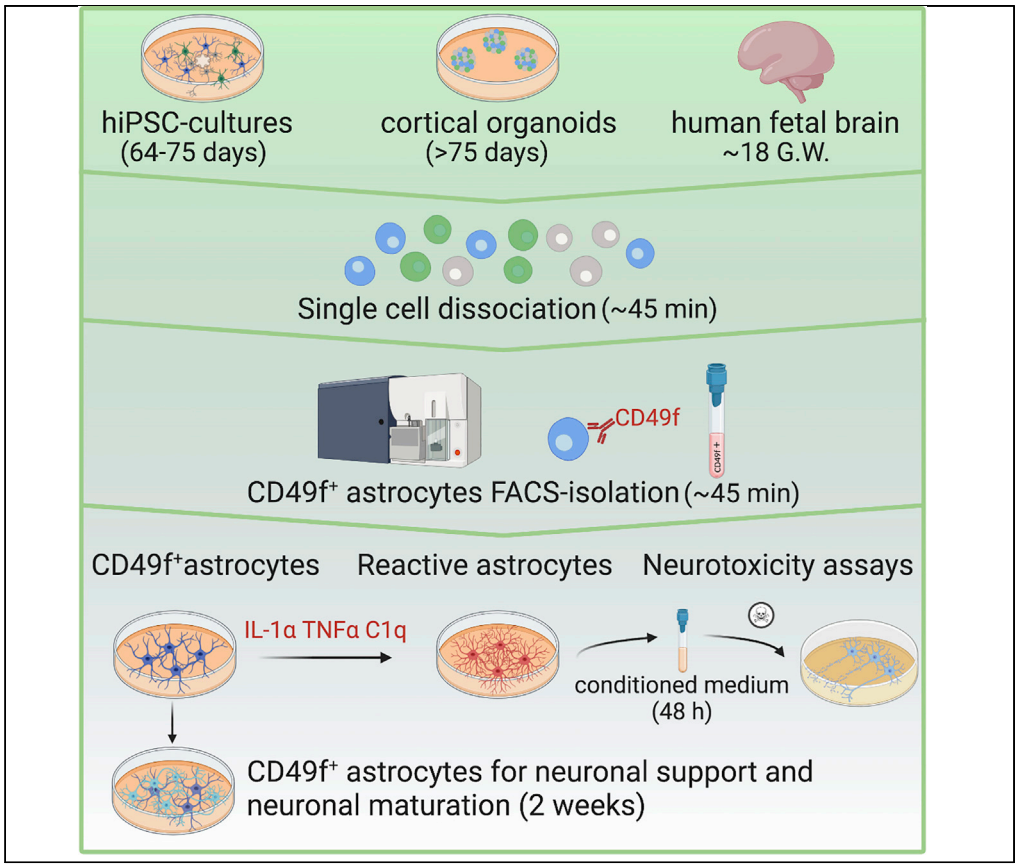
Protocol

Isolation of Human CD49f^{+} Astrocytes and *In Vitro* iPSC-Based Neurotoxicity Assays



Given the critical roles of astrocytes in neuroinflammation and neurological diseases, models for studying human astrocyte biology are in increasing demand. Here, we present a protocol to isolate human astrocytes from induced pluripotent stem cell (iPSC)-based cultures, neural organoids, and primary tissue, using the surface marker CD49f. Moreover, we provide protocols for *in vitro* co-cultures of human iPSC-derived neurons and astrocytes, as well as for neurotoxicity assays that expose neurons to conditioned media from reactive astrocytes.

Lilianne Barbar, Tomasz Rusielewicz, Matthew Zimmer, Kriti Kalpana, Valentina Fossati

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HIGHLIGHTS

A protocol for isolation of human CD49f^{+} astrocytes

Adaptations for isolation from iPSC cultures, 3D organoids, and fetal brain

Adaptable for different endpoints (e.g., co-cultures, functional studies)

An *in vitro* neurotoxicity assay exposing iPSC neurons to astrocyte-conditioned medium

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Isolation of Human CD49f^{+} Astrocytes and *In Vitro* iPSC-Based Neurotoxicity Assays

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SUMMARY

Given the critical roles of astrocytes in neuroinflammation and neurological diseases, models for studying human astrocyte biology are in increasing demand. Here, we present a protocol to isolate human astrocytes from induced pluripotent stem cell (iPSC)-based cultures, neural organoids, and primary tissue, using the surface marker CD49f. Moreover, we provide protocols for *in vitro* co-cultures of human iPSC-derived neurons and astrocytes, as well as for neurotoxicity assays that expose neurons to conditioned media from reactive astrocytes.

For complete details on the use and execution of this protocol, please refer to Barbar et al. (2020).

BEFORE YOU BEGIN

Prepare Fluorescent Activated Cell Sorting (FACS) Buffer

Timing: 15 min

Note: You can store FACS buffer at 4°C for up to 6 months.

1. Prepare FACS buffer.

Reagent	Final Concentration	Volume or Mass
DPBS	-	460 mL
BSA Fraction V (7.5%)	0.5%	33 mL
EDTA	2 mM	2 mL
Glucose	20 mM	1.8 g
Pen/Strep	1%	5 mL

2. Vacuum filter in tissue culture hood and keep sterile.

Prepare 1× Propidium Iodide (PI) Solution

Timing: 15 min

Note: Prepare it fresh for every sort.

Note: Work under sterile conditions.

Note: PI solution should be protected from light.

3. Transfer 5 mL of FACS buffer to a new tube.
4. Add 1 μL of 1 mg/mL stock solution of propidium iodide.
5. Invert or pipette to mix.
6. Keep on ice until use.

Geltrex Coating for iPSC Maintenance

Timing: 1 h

Note: Work under sterile conditions.

7. Coat plates with 1:100 Geltrex in DMEM/F-12.
8. Incubate at 37°C and 5% CO_2 for 1 h.

Note: Plates can be kept at 37°C and 5% CO_2 for up to 24 h.

9. Aspirate Geltrex from plates using a vacuum aspirator directly before plating cells.

Polyornithine/Laminin (PO/lam) Coating for iPSC-Astrocyte Cultures

Timing: 2 days

Note: Work under sterile conditions.

10. Coat plates with 0.1 mg/mL poly-L-ornithine (PO) in sterile ddH_2O.
11. Incubate at 37°C and 5% CO_2 for 8–12 h.

Note: Incubation can be done for as little as 1 h if needed.

12. Aspirate PO using a vacuum aspirator.
13. Wash wells with sterile ddH_2O.
14. Aspirate sterile ddH_2O using vacuum.
15. Repeat steps 13 and 14 two more times, for a total of three washes.

⚠ CRITICAL: Polyornithine can be toxic to plated cells if not washed properly.

16. Coat plates with 10 μg/mL laminin in DMEM/F-12.
17. Incubate at 37°C and 5% CO_2 for 8–12 h.

Note: Incubation can be done for as little as 4 h if needed.

18. Aspirate laminin from plates using vacuum, directly before plating cells.

Pause Point: It is possible to store PO/lam-coated plates by aspirating the laminin, letting dry, and then parafilming to seal the lid. Plates can be stored at 4°C for up to a month; however, we recommend preparing the coating fresh for each experiment.

PEI/lam (Polyethylenimine/Laminin) Coating for iPSC-Neuronal Cultures

Timing: 2 days

19. Dilute polyethylenimine (PEI) to 0.1% in 0.1 M borate buffer at pH 8.4 and sterile-filter, using Rapid-Flow™ Sterile Single Use Vacuum Filter Units.

Note: PEI solution can be stored at 22°C–25°C for 1 month.

20. Coat plates with 0.1% PEI solution.
21. Incubate at 37°C and 5% CO_2 for 8–12 h.

Note: Incubation can be done for as little as 1 h if needed.

22. Aspirate PEI using a vacuum aspirator.
23. Wash wells with sterile ddH_2O.
24. Aspirate sterile ddH_2O using a vacuum aspirator.
25. Repeat steps 23 and 24 four more times, for a total of five water washes.

⚠ CRITICAL: PEI is toxic to plated cells if not washed properly.

26. Coat plates with 10 μg/mL laminin in DMEM/F-12.
27. Incubate at 37°C and 5% CO_2 8–12 h.

Note: Incubation can be done for as little as 4 h if needed.

28. Aspirate laminin from plates using a vacuum aspirator directly before plating cells.

Pause Point: It is possible to store PEI/lam-coated plates by aspirating the laminin, letting dry, and then parafilming to seal the lid. Plates can be stored at 4°C for up to a month; however, we recommend preparing the coating fresh for each experiment.

Note: polyethylenimine (PEI) dendrimer is used to coat tissue culture lab-ware in place of Polyornithine as we have found PEI to show superior attachment properties of neurons over the course of experiments lasting more than two weeks.

Prepare Reagents for Papain Digestion

Timing: 15 min

29. Reconstitute the papain by adding 5 mL of Earle's medium (part of papain kit) to the papain bottle.
30. Reconstitute the DNase (part of papain kit) with 500 μL of Earle's medium.
31. Mix the reconstituted DNase with the papain.

⚠ CRITICAL: Mix gently since DNase is sensitive and prone to denaturation.

32. Add 32 mL of Earle's medium to the ovomucoid inhibitor (part of papain kit) bottle to reconstitute it. Mix well to dissolve the contents completely.

Note: Keep inhibitor on ice once reconstituted. The inhibitor solution after reconstitution is stable at 4°C and can be stored and re-used.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-GABA (1:500)	Sigma-Aldrich	A2052; RRID: AB_477652
Anti-MAP2 antibody (1:1,000)	Abcam	ab5392; RRID: AB_2138153
Anti-VGLUT1 (1:250)	Synaptic System	135316; RRID: AB_2619822
Anti-Vimentin antibody (1:25)	Abcam	ab8978; RRID: AB_306907
Monoclonal Anti-Glial Fibrillary Acidic Protein (GFAP) antibody produced in mouse (1:1,000)	Sigma-Aldrich	G3893; RRID: AB_477010
PE Rat Anti-Human CD49f (1:50)	BD Biosciences	555736; RRID: AB_396079
Chemicals, Peptides, and Recombinant Proteins		
2-mercaptoethanol	ThermoFisher	21985023
3,3′,5-Triiodo-L-thyronine	Sigma-Aldrich	T2877
Accutase	Life Technologies	A11105-01
AraC (Cytosine β-D-arabinofuranoside)	Sigma-Aldrich	C1768-100 mg
Ascorbic Acid	Sigma-Aldrich	A4403
B-27 Supplement with Vitamin A	ThermoFisher	17504044
B27 Supplement without Vitamin A	ThermoFisher	12587010
B27 Supplement, Minus Antioxidants	ThermoFisher	10889038
Biotin	Sigma-Aldrich	4639
Boric Acid-Borate buffer pH 8.4	Poly Scientific	S1605
BrainPhys™Neuronal Medium	StemCell Technologies	05790
BSA Fraction V solution (7.5%)	ThermoFisher	15260037
Complement Component C1q Native Protein	MyBioSource	MBS143105
DAPT	Tocris	2634
Distilled deionized Water (ddH_2O)	Fisher Scientific	15-230-204
DMEM/F-12	ThermoFisher	11320033
DMEM/F-12 GlutaMAX supplement	ThermoFisher	10565042
DPBS	ThermoFisher	14190250
EDTA	ThermoFisher	15575020
GDNF	R&D Systems	212-GD-MTO
Geltrex	ThermoFisher	A1413301
Glucose	Sigma	G6152
HEPES	Sigma-Aldrich	H4034
Insulin Solution, Human	Sigma-Aldrich	19278
Interleukin-1α from rat	Sigma-Aldrich	I3901
MEM Non-essential amino acids (NEAA) solution (100×)	ThermoFisher	11140050
mTeSR1 medium	StemCell Technologies	85850
N2 supplement	Life Technologies	15070063
N6,2′-O-Dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt (cAMP)	Sigma-Aldrich	D0260

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Natural mouse laminin	ThermoFisher	23017015
Neurobasal™ Medium	ThermoFisher	21103049
Neurotrophin 3 (NT3)	EMD Millipore	GF031
PD0325901	Reprocell	04-0006
Penicillin-Streptomycin	ThermoFisher	15070063
Polyethylenimine	Sigma-Aldrich	408727
Poly-L-ornithine hydrobromide	Sigma-Aldrich	P3655
Propidium Iodide	ThermoFisher	P3566
Recombinant Human BDNF	R&D Systems	248-BDB-MTO
Recombinant Human HGF Protein	R&D Systems	294-HG-025
Recombinant Human IGF-I/IGF-1 Protein, CF	R&D Systems	291-G1-200
Recombinant Human PDGF-AA Protein, CF	R&D Systems	221-AA-050
Recombinant Human TNF-alpha Protein	R&D Systems	210-TA-020
Stemolecule LDN-193189	Stemgent	04-0074
Stemolecule SB431542	Stemgent	04-0010
SU5402	Sigma-Aldrich	SML0443
T3	Sigma-Aldrich	T2877
XAV939	Tocris	3478
Y27632 (ROCK inhibitor)	Stemgent	04-0012-H-10
Critical Commercial Assays		
IncuCyte® Caspase-3/7 Green Apoptosis Assay Reagent	Sartorius	4440
IncuCyte® NucLight Rapid Red Reagent for nuclear labeling	Sartorius	4717
Papain Dissociation System	Worthington	LK003153
Biological Samples		
Fetal human brain tissue	Dr. Jean M. Hébert's laboratory	N/A
Experimental Models: Cell Lines		
Human Induced Pluripotent Stem Cell Lines[a]	The NYSCF Repository	nyscf.org/repository
Software and Algorithms		
FlowJo v9	BD Biosciences	RRID: SCR_008520; https://www.flowjo.com/
ImageJ	National Institutes of Health	RRID: SCR_003070
IncuCyte® Analysis Software	Sartorius	https://www.essenbioscience.com/en/products/software/incucyte-base-software/
Prism Software	GraphPad	RRID: SCR_002798 https://www.graphpad.com/scientific-software/prism/
Other		
BD FACSAria Cell Sorter	Beckman	N/A
CellCarrier-96 Ultra Microplates, tissue culture treated, black, 96-well with lid	PerkinElmer	6055300
Disposable scalpel	Fisher Scientific	NC9999403

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Falcon™ Cell Strainers, 40 μm	Fisher Scientific	08-771-1
Falcon™ Cell Strainers, 70 μm	Fisher Scientific	08-771-2
Incucyte® S3 Live-Cell Analysis System	Sartorius	N/A
Nalgene™ Rapid-Flow™ Sterile Single Use Vacuum Filter Units	ThermoFisher	569-0020
Thermo Scientific™ CO_2 Resistant Shakers	Fisher Scientific	88-881-101
Thermo Scientific™ Nunc™ Cell-Culture Treated Multidishes, 24-well plates	Fisher Scientific	12-565-163
Thermo Scientific™ Nunc™ Cell-Culture Treated Multidishes, 6-well plates	Fisher Scientific	14-832-11
Wide Orifice Pipet Tips, Aerosol Filter Wide Orifice Pipettor	VWR	89049-168

[a]The protocol can be applied to any embryonic or iPSC line; the data presented are based on iPSC lines generated with the fully automated NYSCF Global Stem cell Array® (Paull et al., 2015) and available through the NYSCF repository (nyscf.org/repository).

MATERIALS AND EQUIPMENT

Alternatives: the list of reagents described in the Key Resources Table are the ones tested and available in our laboratory. Equivalent chemicals, plasticware, media, antibodies from different vendors may be suitable alternatives and can be tested by the users.

Alternatives: The Phycoerythrin (PE) rat anti-human CD49f antibody from BD Biosciences can be replaced by any other commercially available antibody against the CD49f antigen. Also, PE-conjugation can be replaced by alternative fluorochromes.

Note: All media can be prepared and stored at 4°C for up to 2 weeks.

PDGF Medium

Reagent	Final Concentration	Volume (μL or mL)
DMEM/F-12, GlutaMAX	n/a	474 mL
PenStrep (100×)	1×	5 mL
2-Mercaptoethanol (1,000×)	1×	500 μL
MEM Non-Essential Amino Acids (NEAA) Solution (100×)	1×	5 mL
N2 supplement (100×)	1×	5 mL
B27 Supplement without Vitamin A (50×)	1×	10 mL
Insulin solution, human (10 mg/mL)	25 μg/mL	1.25 mL
PDGF-AA (0.1 mg/mL)	10 ng/mL	50 μL
IGF-1 (0.2 mg/mL)	10 ng/mL	25 μL
HGF (0.1 mg/mL)	5 ng/mL	25 μL
NT3 (0.1 mg/mL)	10 ng/mL	50 μL
T3 (600 μg/mL)	60 ng/mL	50 μL
Biotin (1 mg/mL)	100 ng/mL	50 μL
cAMP (50 mM)	1 μM	10 μL
Total	N/A	500 mL

Glial Medium

Reagent	Final Concentration	Volume (µL or mL)
DMEM/F-12, GlutaMAX	N/A	94 mL
PenStrep (100×)	1×	1 mL
2-Mercaptoethanol (1,000×)	1×	100 µL
MEM non-essential amino acids (NEAA) solution (100×)	1×	1 mL
N2 supplement (100×)	1×	1 mL
B27 supplement without Vitamin A (50×)	1×	2 mL
Insulin solution, human (10 mg/mL)	25 µg/mL	250 µL
T3 (600 ug/mL)	60 ng/mL	10 µL
Biotin (1 mg/mL)	100 ng/mL	10 µL
cAMP (50 mM)	1 µM	2 µL
HEPES (1 M)	10 mM	1 mL
Total	N/A	100 mL

Neuronal Induction d0-d9 Medium

Reagent	Final Concentration	Volume (µL or mL)
DMEM/F-12, GlutaMAX	N/A	120.5 mL
Neurobasal	N/A	120.5 mL
GlutaMAX (100×)	1×	1.20 mL
N2 supplement (100×)	1×	2.5 mL
B27 supplement without Vitamin A (50×)	1×	5 mL
SB431542 (20 mM)	20 µM	250 µL
LDN193189 (500 µM)	100 nM	50 µL
XAV939 (10 mM)	1 µM	25 µL
Total	N/A	250 mL

Neuronal Induction d10-d14 Medium

Reagent	Final Concentration	Volume (µL or mL)
DMEM/F-12, GlutaMAX	N/A	60.5 mL
Neurobasal	N/A	60.5 mL
GlutaMAX (100×)	1×	605 µL
N2 Supplement (100×)	1×	1.25 mL
B27 Supplement without Vitamin A (50×)	1×	2.5 mL
XAV939 (10 mM)	1 µM	12.5 µL
Total	N/A	125 mL

Neuronal Basal Medium

Reagent	Final Concentration	Volume (µL or mL)
BrainPhys	N/A	485 mL
B27 supplement with Vitamin A (50×)	1×	10 mL
PenStrep (100×)	1×	5 mL
Total	N/A	500 mL

Neuronal Maturation Medium

Reagent	Final Concentration	Volume (μL or mL)
Neuronal Basal Media	N/A	200 mL
BDNF (40 μg/mL)	40 ng/mL	200 μL
GDNF (40 μg/mL)	40 ng/mL	200 μL
Laminin (1.2 mg/mL)	1 μg/mL	167 μL
cAMP (500 mM)	250 μM	100 μL
Ascorbic Acid (200 mM)	200 μM	200 μL
PD0325901 (20 mM)	10 μM	100 μL
SU5402 (20 mM)	10 μM	100 μL
DAPT (20 mM)	10 μM	100 μL
ROCK Inhibitor (Y27632; 10 mM)[a]	10 μM	200 μL
Total	N/A	200 mL

[a]ROCK inhibitor is added only on d15 and omitted on the following days

Neuronal Maintenance Media

Reagent	Final Concentration	Volume (μL or mL)
Neuronal Basal Media	N/A	200 mL
BDNF (40 μg/mL)	40 ng/mL	200 μL
GDNF (40 μg/mL)	40 ng/mL	200 μL
Laminin (1.2 mg/mL)	1 μg/mL	167 μL
cAMP (500 mM)	250 μM	100 μL
Ascorbic Acid (200 mM)	200 μM	200 μL
Total	N/A	200 mL

STEP-BY-STEP METHOD DETAILS

Below, we provide distinct protocols for achieving single-cell suspension from neural cultures of hiPSC-derived cells (Astrocyte Purification 1a), 3D cortical organoids (Astrocyte purification 1b), and human fetal brains (Astrocyte Purification 1c). We then introduce protocols for astrocyte-neuron co-cultures, astrocyte-conditioned media collection, and neurotoxicity assays.

Astrocyte Purification 1a: Dissociation of hiPSC-Derived Mixed Cell Cultures in a Monolayer

Timing: 45 min

This protocol is optimized to dissociate iPSC-astrocyte cultures generated with our previously described differentiation protocol (Douvaras and Fossati, 2015; Barbar et al., 2020). We recommend digesting a full 6-well plate, with an expected yield of 300,000–600,000 CD49f$^+$ astrocytes per plate, depending on the cell line and the batch of differentiation. Astrocytes can be isolated as early as day 64.

Note: All steps should be performed under sterile conditions. After the sort, the CD49f$^-$ fraction can also be plated, which will be enriched for neurons and oligodendrocyte lineage cells.

1. Pre-warm Accutase at 37°C in a water bath.
2. Gently remove supernatant from the cells using a 10 mL pipette.

⚠ CRITICAL: Cells should never (or minimally) be exposed to air; therefore, we recommend leaving a thin layer of liquid.

⚠ CRITICAL: Do not touch the cell layer with the pipette, or the cells could detach.

3. Add 1 mL of pre-warmed Accutase per well of a 6-well plate.
4. Incubate for 25–30 min at 37°C and 5% CO_2 in incubator.

Note: This extended incubation time is necessary because the cell layer is overly confluent at this stage, and it does not adversely affect significantly cell viability. Papain treatment can also be used as an alternative. Accutase can be directly added to the well, without performing a PBS wash.

5. Return the plate to tissue culture hood.
6. Add 1 mL of DMEM/F-12 per well on top of Accutase (1:1 dilution).
7. Triturate 8–10 times per well using a P1000 pipette set to 1,000 μL.
8. Pass the cell suspension containing Accutase and DMEM through a 70 μm cell strainer into 50 mL conical tube pre-filled with DMEM/F-12, such that final Accutase dilution is >7×.

Note: Cells from the same iPSC line, but from different wells, are combined into one tube.

9. Transfer the cell solution from a 50 mL conical tube to 15 mL conical tubes for spinning.
10. Centrifuge at 300 × *g* for 4 min at 22°C–25°C.
11. Continue to "Astrocyte Purification 2: Preparing Cells for FACS" (step 34).

Astrocyte Purification 1b: Dissociation of hiPSC-Derived 3D Organoids

Timing: 2 h

In this section, hiPSC-derived organoids are dissociated into single cells for FACS using an enzymatic digestion kit. Organoids were generated using a previously established protocol that generates cortical neurons, oligodendrocyte lineage cells, and astrocytes; astrocytes arise no earlier than d75 (Madhavan et al., 2018).

Note: These organoids are patterned toward cortical development, whereas the previous monolayer protocol(Douvaras and Fossati, 2015) uses ventralizing and caudalizing agents (SA and RA respectively) to generate spinal cord astrocytes.

Note: This protocol is based on instructions provided with the Worthington Papain Dissociation kit (http://www.worthington-biochem.com/pds/default.html) and optimizations from a previous protocol (Velasco et al., 2019a, 2019b).

12. Gently transfer 3–4 organoids to an ultra-low attachment 6-well plate using a wide-bore 1,000 μL pipette tip. Aspirate any excess media, leaving just enough to not let the organoids dry.
13. Using a clean disposable scalpel, cut the organoids into small pieces (<0.5 mm).
14. Immediately add 1 mL of prepared Papain + Dnase solution to the organoids. Pipette 3 times.
15. Transfer the plate to an orbital shaker set at a speed of 70 rpm inside an incubator at 37°C and 5% CO_2. Incubate for 30 min.
16. Using a P1000, gently dissociate the organoids further to break them apart into even smaller pieces. Triturate 10 times very gently.
17. Return the plate to the orbital shaker and incubate for another 10 min.
18. During this time, prepare the inhibitor solution by adding 2 mL of Earle's medium and 1.2 mL of reconstituted Inhibitor solution to a 15 mL conical tube (prepare one tube per organoid sample).
19. Using a P1000, gently break apart the digested organoid sample further by pipetting up and down 10 times.

20. Transfer the cell suspension to the 15 mL conical tube with the prepared inhibitor solution, close the tube tightly, and mix the contents by inverting it a few times.
21. Centrifuge the cell suspension at 300 × *g* for 4 min at 22°C–25°C.
22. Continue to "Astrocyte Purification 2: Preparing Cells for FACS" (step 34).

Astrocyte Purification 1c: Dissociation of Fetal Human Brain Tissue

Timing: 2 h

In this section, fetal human brain tissue is dissociated into single cells in preparation for FACS, using the CD49f antibody.

Note: This protocol is based on instructions provided with the Worthington Papain Dissociation kit and optimizations from a previous protocol (Velasco et al., 2019a, Velasco et al., 2019b).

23. Gently transfer fetal brain chunks (a total of around 0.5 cm^2) to a Petri dish, using sterile forceps or a wide-bore 1,000 µL pipette tip. Aspirate any excess media, leaving just enough to not let the brain tissue dry.
24. Using a sterile disposable scalpel, cut the brain chunks into smaller pieces (<1 mm), and immediately add 2.5 mL of prepared Papain + Dnase solution to the fetal brain tissue. Pipette 3 times.
25. Transfer the dish to an orbital shaker set at a speed of 70 rpm inside an incubator at 37°C and 5% CO_2. Incubate for 30 min.
26. Using a P1000, gently dissociate the tissue further to break it apart into even smaller pieces. Triturate 10 times very gently.
27. Return the dish to the orbital shaker and incubate for another 10 min. During this time, prepare the inhibitor solution by adding 5 mL of Earle's medium and 3 mL of reconstituted Inhibitor solution to a 15 mL conical tube.
28. Using a P1000, gently break apart the digested fetal brain sample further by pipetting up and down 10 times.
29. Transfer the cell suspension to the 15 mL conical tube with the prepared inhibitor solution, close the tube tightly, and mix the contents by inverting it a few times.
30. Centrifuge the cell suspension at 300 × *g* for 4 min at 22°C–25°C.
31. Resuspend in 1 mL sterile ddH_2O for 30 s for red blood cell lysis, then immediately add 10 mL of PBS and mix.
32. Centrifuge the suspension at 300 × *g* for 4 min at 22°C–25°C.
33. Continue to "Astrocyte Purification 2: Preparing Cells for FACS" (step 34).

Note: Other methods of red blood cell lysis may be used.

Note: CD31 can be used in combination with CD49f to exclude $CD31^+/CD49f^+$ endothelial cells and isolate $CD31^-/CD49f^+$ astrocytes from primary samples.

Astrocyte Purification 2: Preparing Cells for FACS

Timing: 40 min

In this step, cells are stained for PE-conjugated CD49f and appropriate control samples are prepared, prior to FACS.

34. Retrieve the tubes from the centrifuge and aspirate supernatant, making sure not to disturb the pellet and leaving a minimal amount of liquid.
35. Using a standard 20 µL pipette, transfer 2 µL of pellet to a 15 mL conical tube containing 200 µL FACS buffer (for unstained control), and 2 µL of pellet to a 15 mL conical tube containing 200 µL

FACS buffer with 1× Propidium Iodide PI solution (for PI-only control) and place control tubes on ice.

36. Resuspend remaining pellet (sample) in 200 μL FACS buffer with 1:50 PE Rat Anti-Human CD49f antibody and mix by gently pipetting up and down.

Note: 200 μL of FACS buffer with 1:50 PE Rat Anti-Human CD49f is the volume recommended for cells derived from one full 6-well plate.

37. Incubate for 20 min on ice.
38. Add 2 mL of FACS buffer per sample of CD49f-stained cells and spin at 300 × *g* for 5 min at 22°C–25°C.
39. Aspirate supernatant.
40. Transfer 2 μL of CD49f-PE-stained cell pellet to a 15 mL conical tube containing 200 μL FACS buffer (for CD49f-only control) and place control tube on ice.
41. Resuspend remaining pellet (total sample) in 200 μL FACS buffer with 1× PI solution and place on ice.

Astrocyte Purification 3: Fluorescence-Activated Cell Sorting

Timing: 30–45 min per 6-well plate

This section covers sorting the astrocytes using a cell sorter, such as BD FACS-Aria, but any alternative sorter can be used. A 100-μm nozzle with a sheath pressure of 23 psi is recommended for cultured cells, and a 130-μm nozzle for fetal samples.

Depending on the quality of the dissociation and the viability and stickiness of the samples, a 130-μm nozzle with a sheath pressure of 10 psi may minimize clogging.

Gating strategy and representative plots for hiPSC-derived mixed cell cultures in a monolayer, 3D organoids, and fetal brain are shown in Figure 1.

Note: Keep samples under sterile conditions at all times if you intend to re-plate cells after sort.

Note: It is preferable to filter the cell suspension through a 40–45 μm filter directly before sorting each sample to avoid clogs.

42. Load cells on cell sorter.
43. Gate a Region of Interest (ROI) which excludes debris and dead cells on an FSC-A versus SSC-A dot plot.
44. Gate singlets first by an FSC-A versus FSC-H plot followed by an SSC-A versus SSC-H plot.
45. To set the Live gate, gate Propidium Iodide (PI)-negative cells using the PI-only control on an FSC-A versus PI plot.
46. Finally, gate $CD49f^{+}$ and $CD49f^{-}$ cells using the PI-only control.
47. Sort cells into a 15 mL tube containing 4 mL of PDGF media.

Note: A gentle, manual resuspension of sample is preferred to vortexing prior to sorting.

Note: Keep the tubes containing the sorted cells on ice until you are able to perform the plating (Astrocyte Purification 4: Plating Cells Post Sort) step (48).

Astrocyte Purification 4: Plating Cells Post Sort

Representative images of $CD49f^{+}$ sorted astrocytes are shown in Figure 2.

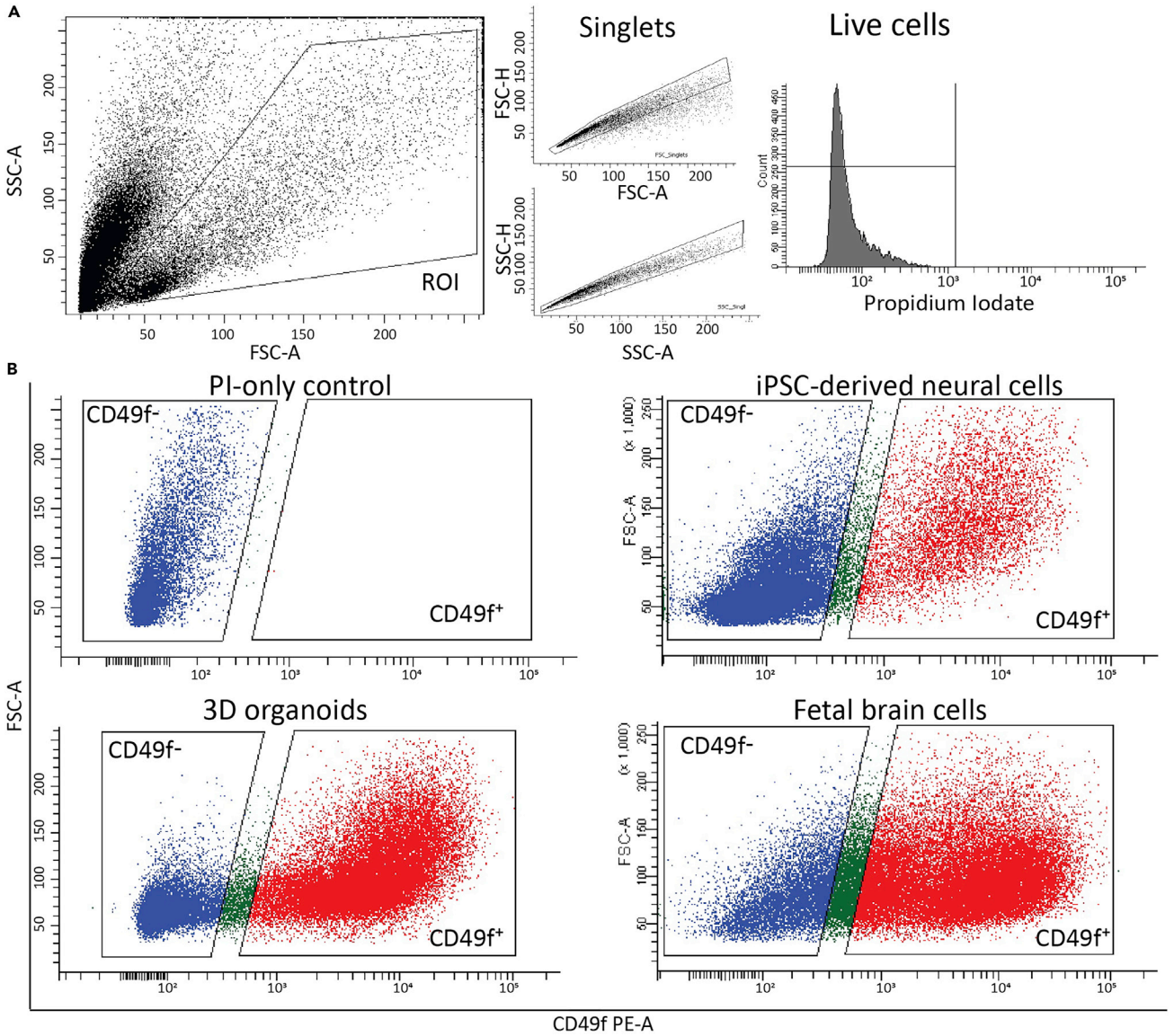

Figure 1. CD49f FACS Purification

(A) Gating strategy for selecting live cells, by excluding debris, doublets, and PI-positive, dead cells.

(B) Representative FACS plots for unstained control and for samples derived from iPSC differentiation in monolayer (iPSC-derived neural cells), 3D organoids, and fetal brain cells.

Timing: 15 min

48. Spin cells at 300 × *g* for 5 min at 22°C–25°C.
49. Aspirate supernatant.
50. Resuspend cells in PDGF medium at desired concentration for plating.
51. Aspirate laminin from PO/lam-coated plates.
52. Plate cells in PDGF medium into well(s), at a density of ~15,000–75,000 cells per cm^2 (based on the counts from the sort), depending on desired confluence.

Note: Lower confluence (~15,000–30,000 cells per cm^2) could be best for lower density imaging or imaging-based phagocytosis assays, while higher confluence (~50,000–

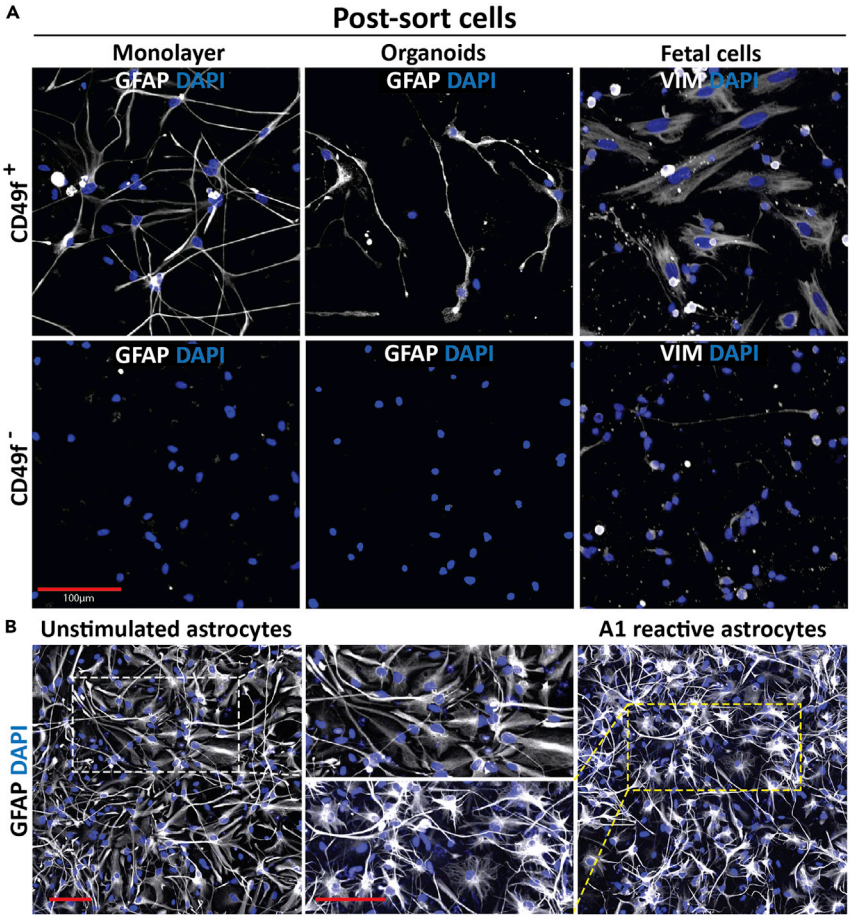

Figure 2. Cultures of CD49f$^+$ Astrocytes

(A) Representative immunofluorescent images of post-sorted cells from the monolayer protocol, the 3D organoid protocol and from fetal brain cells. Note the enrichment of GFAP$^+$ astrocytes in the CD49f$^+$ fraction compared to the CD49f$^-$ fraction.

(B) Human iPSC-derived CD49f$^+$ astrocytes can be stimulated *in vitro* with TNFα, IL-1α, C1q to become A1 reactive astrocytes that show a drastic change in their morphology 24 h after stimulation, as highlighted in the cropped images. Scale bars, 100 µm.

75,000 cells per cm^2) is recommended for collecting supernatant for colorimetric or fluorometric assays such as the glutamate uptake assay. Cells can also be initially plated at low density and allowed to expand to the optimal confluence for the specific downstream analysis planned.

Note: Adding ROCK Inhibitor at this stage does not appear to improve cell viability.

53. Place plate(s) in incubator and shake up-down and right-left, ensuring that cells are evenly distributed across each well.
54. The following day, check that cells are viable and that they attached to the plate. Gently, and without disturbing the cells, pipette out half the media per well and add the same volume of glial media.
55. Repeat step (54) every other day to feed cells until performing desired assay.

Note: CD49f$^+$ astrocytes are proliferating and will expand over the following days. We do not recommend passaging at this stage, as the viability is reduced.

Note: CD49f$^+$ astrocytes can be frozen in serum-free freezing media such as Synth-a-Freeze, but the viability is only ~50%, and a thorough characterization of freeze-thawed cells has not been performed.

Proceed to "Astrocytes Conditioned Media Collection" and "Neurotoxicity Assay."

Neuronal Differentiation

Timing: 30+ days

CD49f$^+$ astrocytes can be co-cultured with hiPSC-derived neurons to support their maturation. Alternatively, neurons can be generated to assess toxicity of A1 reactive astrocyte-conditioned medium. Here we provide a protocol to generate cortical neurons (Figure 3A), based on modifications from previously published protocols (Maroof et al., 2013; Qi et al., 2017). This protocol generates mixed subtypes of cortical neurons (Figure 3B) and can be adapted to smaller or larger scales by using alternative plate formats and adjusting the number of initial iPSCs to be seeded and the volume per well.

Note: We typically maintain iPSC cultures with mTeSR1 medium, on Geltrex coating, but alternative pluripotent media/matrices can be used to maintain iPSC culture in pluripotency.

56. Plate 100,000 hiPSCs/well in a 12-well plate previously coated with 0.15 mg/mL Geltrex (1:100 of stock) for 1 h at 37°C and add 1 mL of hiPSC maintenance medium (e.g., mTeSR1) with 10 μM ROCK inhibitor.

⚠ CRITICAL: Adding ROCK inhibitor is critical for improving iPSC viability.

57. The following day (d0), gently aspirate medium and immediately add 2 mL/well of neuronal induction d0-d9 medium.

Note: Colonies are ready for induction when they are well defined and 40% confluent, and this normally occurs 24 h after plating at the density described above in 56.

58. Repeat step (57) every day until d10.
59. On d10, aspirate medium using a vacuum aspirator and immediately add 2 mL/well of neuronal induction d10-d14 medium.
60. Repeat step (59) every day until d15.

61. On d15, dissociate cells to a single-cell suspension.
    a. Aspirate supernatant from the cells.

    ⚠ CRITICAL: When aspirating media, it is important to minimize cell exposure to air and therefore it is recommended to leave a thin layer of medium.

    b. Add 500 μL of Accutase per well of a 12-well plate.
    c. Incubate for 10–15 min at 37°C in incubator.
    d. After the incubation step, return the plate to tissue culture hood and gently triturate 8–10 times per well using a P1000 pipette set to 500 μL.
    e. Transfer Accutase cell solution into 15 mL conical tube pre-filled with DMEM/F-12 such that the final Accutase dilution is >7×.
    f. Spin at 300 × *g* for 5 min.

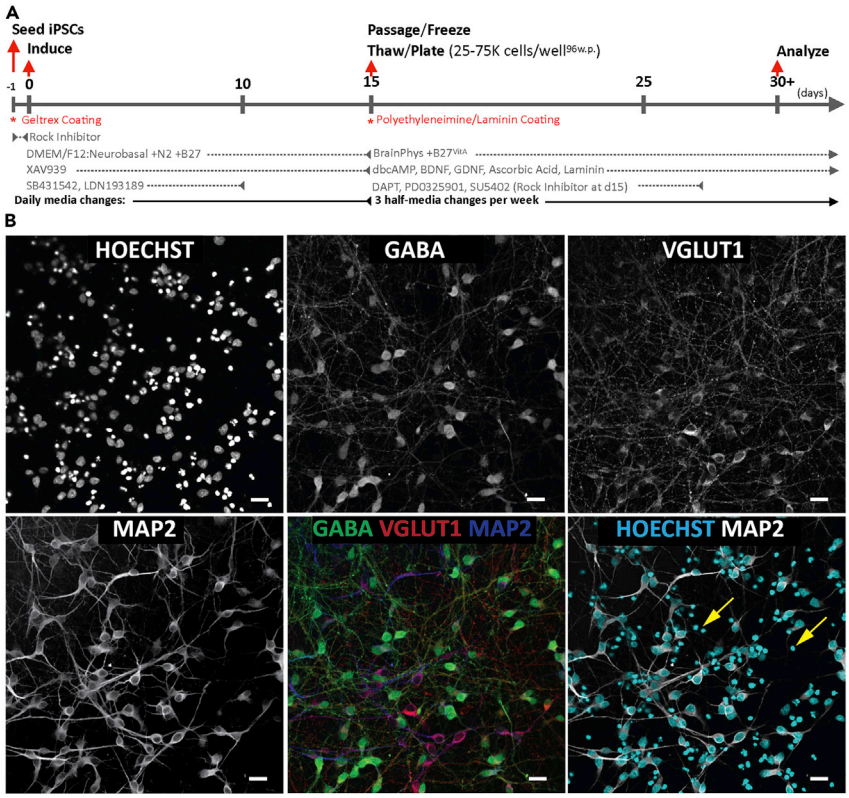

Figure 3. iPSC Differentiation to Cortical Neurons

(A) Schematic of the differentiation protocol, depicting major steps and differentiating agents.

(B) Immunofluorescent characterization of iPSC-derived neurons at the end of the differentiation protocol, showing that the culture is a mix of GABAergic neurons ($GABA^+$) and glutamatergic neurons ($VGLUT1^+$). Merge of MAP2 dendritic marker, VGLUT1, and GABA staining is also shown. MAP2 and Hoechst merge highlights the presence of dead cells, that stick to the coated plate and cannot be easily removed but are clearly identified as pyknotic nuclei (yellow arrows). Scale bar, 20 μm.

Pause Point: Cells can be frozen, by resuspending the pellet in Synth-a-Freeze or any alternative freezing solution recommended for iPSCs or neurons. Pooling every two wells of a 12-well plate into one cryovial is recommended. This will yield ~5–8 million live cells per vial at thaw (expected viability using Synth-a-Freeze is around 50%).

Note: From each well of a 12-well plate, 5–8 million live cells are generally obtained when passaging.

g. For plating cells, resuspend cells in neuronal basal medium with 10 μM ROCK inhibitor and mix by gently pipetting up and down 6 times.

⚠ CRITICAL: Adding ROCK inhibitor is critical for improving viability of neural progenitors.

h. Aspirate laminin from PEI/lam-coated plates.

i. Plate cells in neuronal medium with 10 μM ROCK inhibitor into well(s), at a density of 1.56 x $10^5/cm^2$.

Note: we recommend the 96-well plate (96wp) format for most downstream assays, particularly for neurotoxicity assay. Plate 50,000 cells/well of 96wp, in a total volume of 200 μL per well.

j. Place plate(s) in incubator and shake up-down and right-left, ensuring that cells are evenly distributed across each well.

62. The next day, check that cells have attached and gently remove all media using a pipette. Minimize the time cells are exposed to air. Quickly add neuronal maturation medium (200 µL per well for a 96wp format), making sure not to disturb the cells, by touching the walls instead of the bottom of the well with the pipette tip.
63. Feed cells every other day by gently pipetting out half of the culture media per well and carefully adding back the same volume of neuronal maturation medium.
64. On d28, pipette out half the culture media per well and add back the same volume of neuronal maintenance medium.
65. Feed cells every other day from this point on as described in (64).

Note: iPSC-derived neurons can be used for co-culture experiments (66) or for neurotoxicity experiments (78).

Note: Past d28, neurons can be kept in culture, feeding every other day.

⚠ CRITICAL: Pipette in and out very gently when changing media to prevent cells from detaching.

Astrocyte-Neuron Co-culture

Timing: 20 days

This section describes astrocyte-neuron co-cultures to study the effect of astrocytes on neurons in terms of neuronal maturation and/or morphology.

66. On d33 of the neuronal differentiation, gently pipette out half of the culture medium per well, as described previously.
67. After performing the CD49f$^+$ astrocyte sort as described above, resuspend CD49f$^+$ astrocytes in PDGF medium and plate them on top of the neurons at a density of 47 k/cm^2, such that half the volume consists of neuronal maintenance medium, and the other half of PDGF medium.
68. The following day, pipette out half the culture medium per well and add back the same volume of neuronal medium, with or without TNFα (60 ng/mL), IL-1α (6 ng/mL), and C1q (800 ng/mL), such that the final cytokine concentration in each well is 30 ng/mL for TNFα, 3 ng/mL for IL-1α, and 400 ng/mL for C1q.
69. Feed cells twice per week until d53:
    a. Pipette out half of the culture medium per well.
    b. Add back the same volume of neuronal medium, with or without TNFα (30 ng/mL), IL-1α (3 ng/mL), and C1q (400 ng/mL).
70. Perform your desired endpoint experiments (e.g., immunofluorescence, electrophysiology, etc.).

Note: If planning electrophysiology experiments, plate neurons on d15 on PEI/lam-coated acid-washed coverslips.

Note: Some cell death usually occurs during the differentiation, and dead cells tend to stick to the coated plates. They will be visible when imaging the plates as pyknotic nuclei (DAPI or Hoechst stained, see Figure 3B). We do not recommend washing them out, as this could result in lifting of live cells as well.

Astrocyte-Conditioned Medium Collection

Timing: 45 min

This section continues from the section "Astrocyte Purification 4: Plating Cells Post Sort" and describes how to collect astrocyte-conditioned medium from unstimulated (A0) and A1-stimulated reactive astrocytes. Conditioned media can be used subsequently for the Neurotoxicity assay (78).

71. Directly after sorting, plate $CD49f^+$-sorted astrocytes onto PO/lam-coated 24-well plates at 200,000/well, in PDGF medium (500 µL/well).
72. The following day remove half of the medium per well (250 µL) and add the same volume of BrainPhys medium with 1× PenStrep and 1× B27 supplement with Vitamin A per well.
73. Feed cells every other day as described in (72).
74. At day 8 post-plating, pipette out all the media per well and immediately gently add 600 µL per well of BrainPhys with 1× PenStrep and 1× B27 supplement minus antioxidants, with or without TNFα (30 ng/mL), IL-1α (3 ng/mL), and C1q (400 ng/mL).
75. Return plate to incubator and incubate for 48 h.
76. Return plate to tissue culture hood and transfer supernatant (conditioned media) from each well to a different Eppendorf tube using a P1000 pipette set to 500 µL.
77. Proceed with the following step right away. We recommend using fresh conditioned media as opposed to frozen or stored conditioned media.

Neurotoxicity Assay

Timing: 45 min

In this step, we describe the neurotoxicity assays, performed by exposing neurons to astrocyte-conditioned medium and assessing apoptotic cells via caspase staining over 3 days (Figure 4).

78. Prepare fresh "IncuCyte medium":
    a. Prepare BrainPhys, 1× PenStrep (1:100 dilution), and 1× B27 supplement minus antioxidants (1:100 dilution).

    Note: Total volume prepared depends on the amount of conditioned media collected (250 µL of IncuCyte medium needed for each 500 µL of conditioned media collected).

    b. Add 15 µM IncuCyte Caspase-3/7 Green Apoptosis Assay Reagent (1:333 dilution) and IncuCyte NucLight Rapid Red Reagent (1:667 dilution) for nuclear labeling to fresh media prepared in 78a.
    c. Pipette up and down 8–10 times to mix.

79. Add 250 µL of IncuCyte medium to each 500 µL of conditioned media collected (final dilution of IncuCyte Caspase-3/7 Green Apoptosis Assay Reagent and IncuCyte NucLight Rapid Red Reagent will be 1:1,000 and 1:2,000, respectively).
80. Mix by pipetting up and down.
81. Bring 96wp of d66 neurons grown as described above to culture hood.

    Note: This could also be performed on mature neuronal cultures at a different day of the differentiation.

82. Gently remove all media from each well using a pipette and immediately gently add 120 µL of media per well.
83. Place plate in the IncuCyte live cell imager (in an incubator at 37°C and 5% CO_2), and image every 6 h for 72 h in the phase, green, and red channels.

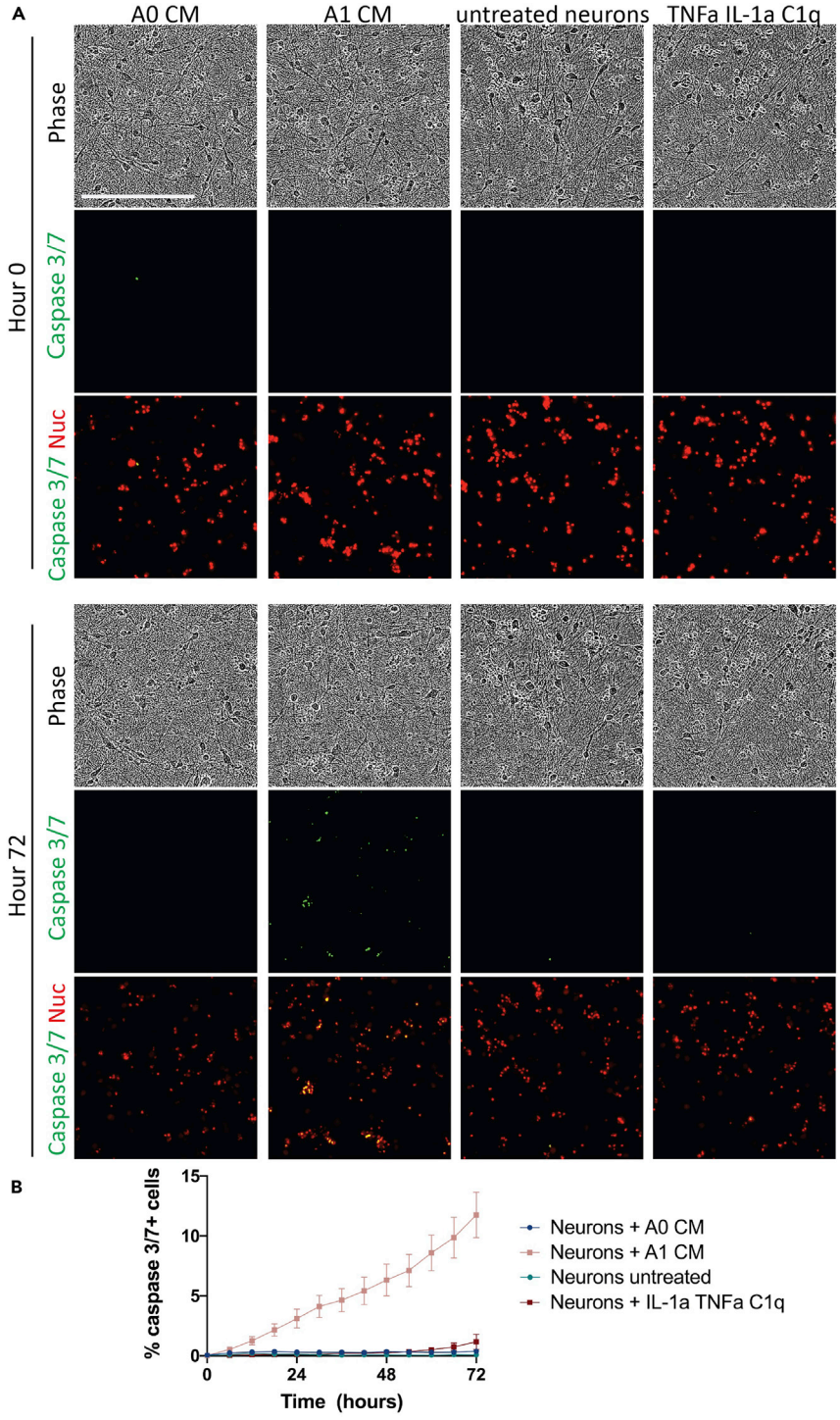

Figure 4. Neurotoxicity Assay

(A) Representative fields of neurons undergoing live imaging analysis using IncuCyte, at the beginning (0 h) and after 72 h. Phase images highlight neuronal morphology, green channel shows caspase $3/7^{+}$ apoptotic cells and red channel shows all nuclei. Note the presence of yellow apoptotic nuclei primarily in neurons exposed to A1 astrocyte-conditioned medium. Scale bar, 200 μm.

Figure 4. *Continued*
(B) Quantification of caspase 3/7$^+$ cells shows significant increase in apoptosis when neurons are exposed to A1 astrocyte-conditioned medium, compared to A0 astrocyte-conditioned medium, control medium (neurons untreated) and IL-1α, TNFα, C1q. This quantification is based on the data presented in Figure 7 (Astrocytes.3) of the original study (Barbar et al., 2020).

Note: The IncuCyte system is ideal for automated acquisition and analysis of live cells over time, however, apoptosis assays can be performed with any other microscope with temperature and CO_2 control or any other traditional system available in the lab.

Optional: In parallel on the same plate, image wells without the IncuCyte Caspase-3/7 Green Apoptosis Assay Reagent and the IncuCyte NucLight Rapid Red Reagent to correct to background autofluorescence.

EXPECTED OUTCOMES

The frequencies of CD49f$^+$ cells isolated from iPSC cultures, cortical organoids, and human fetal brain are summarized in Table 1. When performing the iPSC differentiation in monolayer, we generally obtain ~300,000–600,000 CD49f$^+$ astrocytes from a 6-well plate. The detailed differentiation protocol and the characterization of CD49f$^+$ astrocytes were described previously (Douvaras and Fossati, 2015; Barbar et al., 2020). Astrocyte isolation from monolayer cultures can be performed as early as day 64 of differentiation. iPSC lines from different genetic backgrounds may have different yields of CD49f$^+$ astrocytes.

As we previously reported (Barbar et al., 2020), over 80% of the CD49f$^+$ astrocytes generated using the monolayer protocol are GFAP$^+$ and AQP4$^+$, while about 30% of the CD49f$^+$ astrocytes isolated from 3D organoids (Madhavan et al., 2018) are GFAP$^+$, and over 70% are AQP4$^+$. The astrocytic identity of CD49f$^+$ cells was investigated through bulk RNA sequencing, single-cell RNA sequencing, and immunofluorescence. Bulk RNA sequencing of astrocytes isolated from hiPSC-derived mixed cell cultures in a monolayer showed that CD49f$^+$ cells clustered closely with fetal astrocytes (Barbar et al., 2020). However, single-cell RNA sequencing revealed that CD49f$^+$ cells consist of a mix of astrocytes at different stages of maturity, including mature astrocytes, as previously defined (Sloan et al., 2017).

Additionally, immunofluorescent analysis showed that sorting for CD49f enriches for cells that express several canonical astrocyte markers (Barbar et al., 2020).

Isolated CD49f$^+$ astrocytes can be immediately processed for downstream analyses, including but not limited to, single-cell and bulk RNA sequencing; alternatively, they can be plated down and grown for performing functional assays including glutamate uptake, phagocytosis of synaptosomes or myelin debris, cytokine release, calcium signaling, ATP stimulation, and for collecting conditioned media. Protocols for all these assays can be found in our original publication (Barbar et al., 2020). Sorted astrocytes can also be co-cultured with other CNS cells (e.g., microglia, neurons, oligodendrocytes).

Astrocytes co-cultured with neurons significantly improve neuronal maturation (e.g., increased spontaneous excitatory post-synaptic currents) (Barbar et al., 2020). Astrocytes can be stimulated to become reactive (A1-like) after exposure to TNFα, IL-1α, and C1q. We previously showed by transcriptome and western blot analyses that 24 h of stimulation are sufficient to induce the reactive phenotype. However, we recommend collecting the conditioned medium from reactive astrocytes after 48 h to allow accumulation of toxic factors in the supernatant.

Astrocytes treated with TNFα, IL-1α, and C1q (A1-like) have a significantly decreased ability to promote neuronal maturation. Moreover, conditioned medium from reactive A1-like astrocytes

significantly increases apoptosis of human iPSC-derived neurons (Barbar et al., 2020), through a toxic mechanism that is still not completely understood (Liddelow et al., 2017).

LIMITATIONS

The CD49f purification is based on specific differentiation protocols developed by our group. For monolayer differentiation we recommend (Douvaras and Fossati, 2015) and for 3D organoid differentiation we recommend (Madhavan et al., 2018). The yield of CD49f$^+$ astrocytes may vary when using alternative differentiation protocols. All iPSC lines used in this study were from healthy donors, and we cannot anticipate the yield of CD49f$^+$ astrocytes from patients with diseases affecting astrocytes. The isolation of CD49f$^+$ astrocytes from fetal brains is based on gestational week 18 (without abnormalities) and the yield may vary at different stages, or in the presence of pathologies.

iPSC-derived neurons described here and in our original study were generated using the protocol above. It is possible that the effects of astrocytes on promoting neuronal maturation and/or neurotoxicity may change when using alternative protocols for neuronal differentiation.

TROUBLESHOOTING

Problem 1

Sorted cells contain contaminant cells; i.e., cells not showing astrocyte morphology and negative for GFAP, AQP4, CD49f (steps 43–47 and step 54).

Potential Solution

This is likely due to a poor sorting efficiency. Make sure that sorting was performed properly, and that the sorter did not have any clogging or flow instability. Double check sorting gates and adjust them to make them more stringent. Contaminant cells could be progenitors, neurons, or oligodendrocyte lineage cells. Cultures can be expanded for a few days and sort can be repeated to purify CD49f+ astrocytes.

Problem 2

Astrocytes yield is low (step 54).

Potential Solution

Astrocyte yield may vary from line to line (e.g., different genetic background, different passage number), and can be affected by batch-to-batch variability in the reagents and cells. Check that reagents and growth factors have been properly reconstituted and stored. When working with a new line a small-scale experiment can be performed to determine the efficiency for that specific line, and the scale of future experiments can be adjusted accordingly.

Problem 3

CD49f$^+$ astrocytes expand their processes the day after plating, but then retract them and appear dead (step 54).

Potential Solution

This is likely because the plating density was too low, so we recommend plating cells at a higher density. Ensure counts are accurate before plating, as FACS counts can be overestimated. An additional count with a hemocytometer or a cell counter can be performed before plating for confirmation. Also, make sure that coating was performed as suggested and there are no issues with the matrices used (e.g., expiration, poor storage at wrong temperature, poor gelification).

Problem 4

CD49f$^+$ astrocytes begin to form clustered webs instead of a flat homogeneous monolayer (step 55).

Table 1. Frequencies of Live Cells and CD49f$^+$ Sorted Cells from FACS, Following Digestion from hiPSC-Derived Mixed Cultures, Organoid, and Fetal Brain

Sample	% Live Cells (Mean)	Standard Deviation	% CD49f+ (Mean)	Standard Deviation	n
hiPSC mixed cultures	89.5	8.3	40	10.4	65
Organoids	98.5	1.3	14.65	2.9	2
Fetal	93.1	-	62.9	-	1

The percentage of live cells was calculated from total ungated events and expressed as mean value. The percentage of CD49f$^+$ cells was calculated from the live-gated population and expressed as mean value. Standard deviation and number of samples (n) are also provided. Organoids were digested for FACS at day117 and day 169.

Potential Solution

This is likely because the cells have grown too dense. Avoid keeping the sorted astrocytes in culture for longer than one week after they have reached 100% confluency.

Problem 5

Neurons begin to cluster and lift after they are growing in neuronal maintenance media (step 65).

Potential Solution

This is likely due to residual proliferating progenitors. Using the recommended protocol, the majority of cells become post-mitotic neurons; however, ~2% of the culture will include proliferating progenitors, which may eventually overtake the culture. AraC can be added to the medium at a concentration of 2 μM between days 30 and 34 to eliminate mitotic cells (prior to astrocyte co-culture or conditioned media treatment).

Problem 6

All imaged neurons are dying when imaging for the neurotoxicity assay, including neurons not treated with conditioned medium (step 83).

Potential Solution

This is likely due to phototoxicity. Image less frequently and/or reduce exposure time, particularly in the 488 nm channel.

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Valentina Fossati (vfossati@nyscf.org).

Materials Availability

iPSC lines from healthy controls and from various diseases can be requested through the NYSCF repository at nyscf.org/repository.

Data and Code Availability

There are no datasets and codes related to this protocol.

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AUTHOR CONTRIBUTIONS

Conceptualization, V.F.; Investigation, L.B., M.Z., T.R., and K.K.; Writing – Original Draft, V.F. and L.B.; Writing – Review & Editing, all authors; Funding acquisition, V.F.

DECLARATION OF INTERESTS

NYSCF. Patent pending.

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