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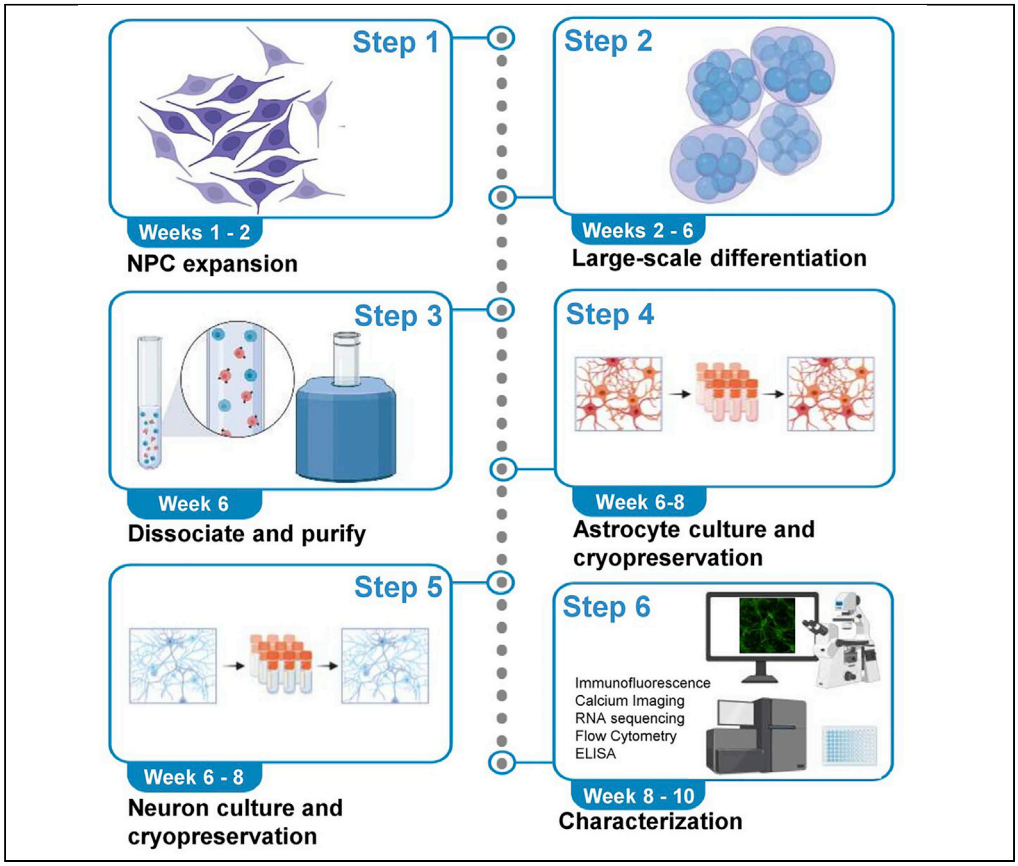
A microcarrier-based protocol for scalable generation and purification of human induced pluripotent stem cell-derived neurons and astrocytes



Here, we describe a protocol for a microcarrier (MC)-based, large-scale generation and cryopreservation of human-induced pluripotent stem cell (hiPSC)-derived neurons and astrocytes. We also detail steps to isolate these populations with a high degree of purity. Finally, we describe how to cryopreserve these cell types while maintaining high levels of viability and preserving cellular function post-thaw.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Jacob Knittel, Gayathri Srinivasan, Carlye Frisch, Nicholas Brookhouser, Sreedevi Raman, Albert Essuman, David A. Brafman

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Highlights

A protocol for large-scale production of hiPSC-derived neurons and astrocytes

Detailed protocol for purification and cryopreservation of neurons and astrocytes

In-depth description for biochemical and functional cell characterization

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A microcarrier-based protocol for scalable generation and purification of human induced pluripotent stem cell-derived neurons and astrocytes

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SUMMARY

Here, we describe a protocol for a microcarrier (MC)-based, large-scale generation and cryopreservation of human-induced pluripotent stem cell (hiPSC)-derived neurons and astrocytes. We also detail steps to isolate these populations with a high degree of purity. Finally, we describe how to cryopreserve these cell types while maintaining high levels of viability and preserving cellular function post-thaw.

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

BEFORE YOU BEGIN

Employing this protocol requires the culture of healthy hiPSCs that (i) exhibit characteristic hiPSC morphology, (ii) express pluripotency markers such as OCT4, NANOG, and SOX2, (iii) retain the ability to differentiate *in vitro* into populations representative of the three main germ layers, and (iv) display a normal complement of 46 chromosomes. HiPSCs used to develop this protocol have been cultured in Essential (E8) medium (ThermoFisher) on Cultrex® BME-coated plates and routinely passaged with Accutase (ThermoFisher). However, we anticipate that this protocol is compatible with other culture substrates (e.g., Matrigel™, recombinant vitronectin) and medium (e.g., mTeSR™ Plus, mTeSR1™) for the feeder-independent culture of hiPSCs as described by others (Chen et al., 2011; Ludwig et al., 2006). In addition, this protocol has been used with a broad spectrum of hiPSC lines derived using a variety of reprogramming methods from various somatic cell patient sources (Brookhouser et al., 2021). As such, we envision that this protocol will be compatible with any hiPSC line of choice. Finally, this protocol describes the thawing and expansion of hiPSCs in a 6-well plate format. To that end, users will need to adjust the protocol if other plate formats are employed.

All preparation of reagents and cell culture should be performed in a sterile laminar flow hood with appropriate aseptic technique.

Coat plates with Cultrex® BME

Timing: 30 min

Table 1. Stock and working concentration of key reagents

Reagent	Solvent	Stock concentration	Working concentration	Storage conditions	Aliquot size
Cultrex® Basement Membrane Extract (BME)	Cold DMEM	8–12 mg/mL	~64–96 μg/mL	−80°C for 1 year	0.2 mL per 1.5 mL Eppendorf tube
Y-27632 (ROCKi)	Nuclease free water	5mM	5μM	−20°C for 1 year	100 μL per 1.5 mL Eppendorf tube
Noggin	PBS	200 μg/mL	50 ng/mL	−20°C for 3 months	50 μL per 1.5 mL Eppendorf tube
Dorsomorphin	DMSO	12.5 mM	0.5 μM	−20°C for 1 month	20 μL per 1.5 mL Eppendorf tube
EGF	Nuclease free water	100 μg/mL	30 ng/mL	−20°C for 6 months	50 μL per 1.5 mL Eppendorf tube
FGF	Nuclease free water	100 μg/mL	30 ng/mL	−20°C for 3 months	50 μL per 1.5 mL Eppendorf tube
BDNF	PBS w/0.1% BSA	100 μg/mL	20 ng/mL	−20°C for 3 months	50 μL per 1.5 mL Eppendorf tube
GDNF	PBS w/0.1% BSA	100 μg/mL	20 ng/mL	−20°C for 3 months	50 μL per 1.5 mL Eppendorf tube
D-(+)-Glucose	Milli-Q water	100 mM	22.5 mM	4°C for 1 year	400 mL in 500mL glass bottle
Vitronectin-derived peptide (VDP)	Nuclease free water	5mM	5μM	−80°C for 6 months	200 μL per 1.5 mL Eppendorf tube
DNase I	Nuclease free water	20,000 U/mL	125 U/mL (refer to datasheet for activity)	−20°C for 1 year	100 μL per 1.5 mL Eppendorf tube
L-Cysteine	Milli-Q water	200 mM	1 mM	Prepare fresh	
Ovomucoid Inhibitor Solution	PBS	50 mg/mL	15–30 mg/mL	Prepare fresh/use right away to prepare low/high ovomucoid inhibitor aliquots	
Calcein AM	DMSO	5mM	50 nM	−20°C, protected from light for up to 7 days	50 μL per 1.5 mL Eppendorf tube
Fluo-4 AM	DMSO	2.5 mM	1 μM	−20°C, protected from light for up to 7 days	10 μL per 1.5 mL Eppendorf tube
Pluronic™ F-127	Milli-Q water	10% v/v	0.02% v/v	RT until expiration date	N/A
Poly-L-Ornithine (PLO)	Nuclease free water	100 μg/mL	4 μg/mL	−20°C for 6 months	5 mL per 15 mL conical tube
Laminin (Ln)	PBS	500 μg/mL	4 μg/mL	−20°C for 6 months	500 μL per 1.5 mL Eppendorf tube

All hiPSCs will be cultured on Cultrex® BME in feeder-independent culture systems. This step describes the procedure to coat tissue culture plates with Cultrex® BME. Stock vials of Cultrex® BME should be prepared in cold DMEM as described in Table 1.

1. Thaw one aliquot of Cultrex® BME on ice 12–24 h at 4°C.
2. Make a working solution of Cultrex® BME by diluting one aliquot in 25 mL cold DMEM/F-12 in a 50 mL conical tube.

   Note: The working solution of Cultrex® BME should be maintained on ice and can be stored up to 2 weeks at 4°C.

3. Add enough volume (approximately 1.5 mL per well) to cover the entire surface of the number of needed wells in a 6-well plate. Gently move the plate in several quick, short, back-and-forth, and side-to-side motions to evenly coat the plate.
4. Incubate at 37°C for 30 min.

   Note: Coated plates can be kept in an incubator at 37°C for 2 days prior to use.

Thawing hiPSCs

Timing: 30 min

This step describes thawing of hiPSCs onto Cultrex® BME-coated plates. All media should be warmed to 20°C–25°C prior to use. Prior to thawing hiPSCs all stock and working concentration of relevant reagents should be made as described in Table 1.

5. Quickly thaw a vial of hiPSCs (~3 × 10^6 cells/vial) in a bead bath and add the cell suspension to 4 mL DMEM in a 15 mL conical flask.
6. Centrifuge cells at 200 × g for 5 min at RT.
7. Aspirate the supernatant without disturbing the cell pellet.
8. Resuspend cell pellet in 5 mL E8 supplemented with 5 μM ROCKi.

⚠ CRITICAL: It is important to add ROCKi when thawing and passaging cells to aid in cell survival.

Note: We recommend that for ease-of-use and to allow for consistent cell counts that users employ single cell passaging with StemPro™ Accutase™. As such, to aid with single cell survival we recommend the use of ROCKi (Watanabe et al., 2007). However, given the documented issues with prolonged single cell passaging as it relates to genomic instability in hiPSCs, the user can employ colony passaging using EDTA (or similar reagents; (Beers et al., 2012)) until precise cell counts are required for the neural induction of hiPSCs.

9. Count the cells using a hemocytometer.
10. To another 15 mL conical, add the appropriate amount of cell suspension to plate the cells at 4 × 10^4 cells/cm^2 Add appropriate amount of E8 supplemented with 5 μM ROCKi to bring the total volume in the conical to 3 mL/well.

Note: Upon thawing a new hiPSC line, it is recommended to plate the cells in multiple wells at various densities ranging to 3–6 × 10^4 cells/cm^2 to establish the optimal plating density for each line and user.

11. Aspirate the Cultrex® BME from the 6-well plate.
12. Add the 3 mL of cell suspension to the well.
13. Place the plate in a 37°C, 5% CO_2 incubator.
14. Gently move the plate in several quick, short, back-and-forth, and side-to-side motions to evenly disperse the cells across the plate.
15. Change the medium daily with fresh E8 without ROCKi.

Passaging hiPSCs

Timing: 30 min

This step describes how to passage cells. HiPSCs should be passaged when they reach 70–80% confluence in approximately 3–4 days (Figure 1A, left panels).

16. Prior to passaging, coat the number of desired wells with Cultrex® BME as described in steps 1–4.
17. Aspirate the medium from the well.
18. Add 1 mL of Accutase per well of a 6-well plate.
19. Incubate the plate with Accutase at 37°C for 5 min.
20. Wash the surface with 1 mL of DMEM to further dissociate the cells from the plate (blast cells with P1000 where necessary)
21. Transfer cell suspension to a 15 mL conical tube.
22. Centrifuge at 200 × g for 5 min at RT.

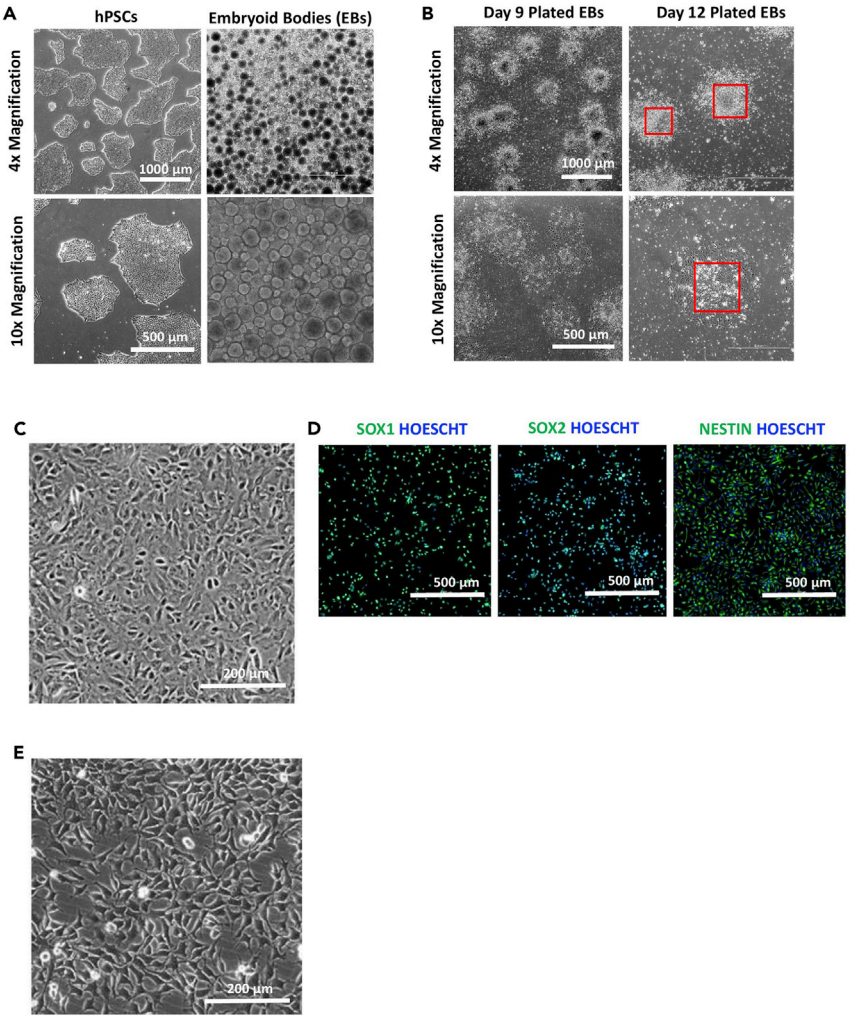

Figure 1. Representative images of cells at different times during the protocol

(A) Brightfield images of hiPSCs in monolayer (left panels) and suspension EB (right panels) culture. Scale bar = 1000 µm (upper panels), 500 µm (lower panels).

(B) EBs 9 (left panels) and 12 days (right panels) after differentiating. Rosette like structures begin to emerge on day 3 after replating. Scale bar = 1000 µm (upper panels), 500 µm (lower panels). Red box indicates the portion of the rosette that should be isolated using the rosette selection reagent.

(C) Representative image of morphology of hNPCs.

(D) Immunofluorescence showing expression of SOX1, SOX2, and NESTIN in hNPC cultures at passage 6. Scale bar = 500 µm.

(E) Representative image of morphology and confluence of hNPC post-thaw.

Note: Cells can be frozen at this step.

a. Resuspend cell pellet in Freezing Medium (90% FBS and 10% DMSO) at a density of 2×10^6 cells/mL.
b. Transfer 1 mL of the cell suspension to each cryovial.
c. Place cryovials in a Mr. Frosty™ and transfer to −80°C.
d. Transfer cells to liquid nitrogen storage the following day.
e. Cells can be stored in liquid nitrogen for up to 3 years.

23. Aspirate the supernatant without disturbing the cell pellet.

24. Resuspend cell pellet in 5 mL E8 supplemented with 5 μM ROCKi.
25. Count the cells using a hemocytometer.
26. Plate cells in 3 mL of E8 supplemented with 5 μM ROCKi per well at an appropriate density (see step 10).

Note: HiPSCs should be expanded for a minimum of 2 passages post-thaw prior to initiating neural differentiation.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Neun, clone A60, 1:500	EMD Millipore	Cat#: MAB377 RRID:AB_2298772
Anti-S100B antibody, 1:500	Sigma	Cat# S2532 RRID:AB_477499
Biotin-CD44	BioLegend	Cat#: 103004 RRID:AB_312955
Donkey anti-Mouse IgG (H+L) Alexa Fluor 488, 1:200	Thermo Fisher Scientific	Cat#: A21202 RRID:AB_141607
Donkey anti-Mouse IgG (H+L) Alexa Fluor 647, 1:200	Thermo Fisher Scientific	Cat#: A31571 RRID:AB_162542
Polyclonal Rabbit Anti-MAP2 antibody, 1:500	MilliporeSigma	Cat#: AB5622 RRID: AB_91939
Monoclonal Mouse Anti-TUJ1 antibody, 1:1000	Fitzgerald	Cat#: 10R-T136A RRID:AB_1289248
Rat anti-CTIP2 antibody, 1:300	Abcam	Cat# ab18465, RRID:AB_2064130
Rabbit anti-TBR1, 1:200	Abcam	Cat# ab31940, RRID:AB_2200219
Mouse anti-SATB2 antibody, 1:50	Abcam	Cat# ab51502, RRID:AB_882455
Rabbit anti-FOXG1 antibody, 1:500	Abcam	Cat# ab18259, RRID:AB_732415
Donkey anti-Rabbit IgG (H+L) Alexa Fluor 647, 1:200	Thermo Fisher Scientific	Cat# A-31573, RRID:AB_2536183
Goat anti-Rat IgG (H+L) Alexa Fluor 488, 1:200	Thermo Fisher Scientific	Cat# A-11006, RRID:AB_141373
Mouse anti-PSD95 antibody, 1:300	Abcam	Cat# ab2723, RRID:AB_303248
Sheep anti-VGLUT1 antibody, 1:50	Abcam	Cat# ab79774, RRID:AB_1604186
Donkey anti-Sheep IgG (H+L) Alexa Fluor 647, 1:200	Thermo Fisher Scientific	Cat# A-21448, RRID:AB_2535865
PE Mouse Anti-Human CD44	BD Biosciences	Cat#: 550989 RRID: AB_394000
PE Mouse IgG1, κ Isotype Control	BD Biosciences	Cat#: 555749 RRID: AB_396091
Chemicals, peptides, and recombinant proteins		
Accutase	Life Technologies	Cat#: 12604013
Astrocyte Medium	ScienCell	Cat#: 1801
B-27 Supplement (50×)	Life Technologies	Cat#: 17504001
BDNF (100 μg/mL)	STEMCELL Technologies	Cat#: 78005.1
Bovine Serum Albumin	Sigma-Aldrich	Cat #: A9418
BrainPhys™ Neuronal Medium and SM1 Kit	STEMCELL Technologies	Cat#: 5792
Calcein AM (50 μg)	Thermo Fisher Scientific	Cat#: C3100MP
CryoStor CS10 Cell Freezing Medium	STEMCELL Technologies	Cat#: 7930
Cultrex Reduced Growth Factor Basement Membrane Extract, PathClear (8–12 mg/mL)	R&D Systems	Cat#: 343301001
D-(+)-Glucose	VWR	Cat#: 97061-164
DI water	N/A	N/A
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich	Cat#: D2650-100ML
DMEM F-12 (1×)	Life Technologies	Cat#: 11330057
DMEM, high glucose (1×)	Thermo Fisher Scientific	Cat#: 11965118
DNase I 100 mg	Sigma-Aldrich	Cat#: 11284932001
Dorsomorphin (12.5 μM)	R&D Systems	Cat#: 3093
Dulbecco's phosphate-buffered saline (DPBS)	Life Technologies	Cat#: 14190-250
EBSS Solution A 10×	VWR	Cat#: AAJ67697-K2
EBSS Solution B 10×	VWR	Cat#: AAJ67697-K2
EGF (100 μg/mL)	STEMCELL Technologies	Cat#: 78006.2
Essential 8 Medium (E8) (1×)	Thermo Fisher Scientific	Cat#: A1517001
FACS Buffer (Stain Buffer [FBS])	BD Biosciences	Cat#: 554656

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Fetal Bovine Serum (FBS) (1×)	Thermo Fisher Scientific	Cat#: 26140079
FGF (100 μg/mL)	STEMCELL Technologies	Cat#: 78003.2
Fixation Buffer	BD Biosciences	Cat#: 554655
Fluo-4, AM, cell permeant 10 × 50 μg	Thermo Fisher Scientific	Cat#: F14201
GDNF (100 μg/mL)	STEMCELL Technologies	Cat#: 78058.1
GlutaMAX (100×)	Life Technologies	Cat#: 35050-061
Hoechst 33342 Solution (20 mM)	Thermo Fisher Scientific	Cat#: 62249
Hydrochloric acid (HCl) 1N	Sigma-Aldrich	Cat#: H9892-100ML
Laminin (Ln) (1mg/mL)	Life Technologies	Cat#: 23017-015
L-Cysteine hydrochloride monohydrate	VWR	Cat#: 97061-202
N2 Supplement (100×)	Life Technologies	Cat#: 17502001
Noggin (200 μg/mL)	R&D Systems	Cat#: 6057-NG/CF
Papain suspension 50 mM	Worthington Biochemical Corporation	Cat#: LS003126
Penicillin Streptomycin (100×)	Life Technologies	Cat#: 15140-122
Phosflow Perm Buffer III	BD Biosciences	Cat#: 558050
Poly-L-Ornithine (PLO) MW 30,000–70,000 (50mg)	Sigma-Aldrich	Cat#: P3655-50MG
Propidium Iodide (1.5 mM)	Thermo Fisher Scientific	Cat#: P1304MP
Protease Inhibitor Mini Tablet	Thermo Fisher Scientific	Cat#: A32955
ROCKi, 5 mM (Y-27632 dihydrochloride) (1mg/mL)	R&D Systems	Cat#: 1254
Sodium bicarbonate 7.5% Solution	VWR	Cat#: 45000-704
Sodium hydroxide	Sigma-Aldrich	Cat#: S8045-500G
STEMdiff Neural Rosette Select Reagent	STEMCELL Technologies	Cat#: 5832
Triton X-100	Sigma-Aldrich	Cat#: T8787
Trypsin Inhibitor (ovomucoid inhibitor) (2 gm)	Worthington Biochemical Corporation	Cat#: LS003086
Tyrode's solution HEPES-Buffered	VWR	Cat#: AAJ67607-AP
Vitronectin Derived Peptide (VDP) (100 mg)	AnaSpec	Cat#: SQ-ASPE-76797: CGKKQRFRHRNRKG
Critical commercial assays		
Human APO E (AD2) ELISA 96 assays	Thermo Fisher Scientifc	Cat#: EHAPOE
Pierce™ Detergent Compatible Bradford Assay Kit	Thermo Fisher Scientific	Cat#: 23246
Experimental models: Cell lines		
NRCM-1 hiPSC	NINDS Cell Repository	Cat#: ND50028
UCSD239i-APP2-1	WiCell Repository	Cat#: WB67856
Software and algorithms		
Fiji	ImageJ	https://imagej.net/software/fiji/
Other		
0.2μm syringe filters	VWR	Cat#: 28145-501
1.5 mL Eppendorf tubes	VWR	Cat#: 20901-551
10cm Tissue Culture Dishes, PS	VWR	Cat#: 25382-166
10mL pipettes	VWR	Cat#: 89130-898
15 mL conical	VWR	Cat#: 89174-468
24-Well Plate, 1.5 Coverslip Thickness, 13 mm Glass Diameter	MatTek	Cat#: P24G-1.5-13-F
25mL pipettes	VWR	Cat#: 89130-900
500 mL filter flask	VWR	Cat#: 10040-436
50mL centrifuge tube filter (0.2μm)	VWR	Cat#: 89220-710
50mL conical	VWR	Cat#: 490001-624
5mL pipettes	VWR	Cat#: 89130-896
5mL polypropylene tubes	VWR	Cat#: 89497-784
5mL syringes	VWR	Cat#: BD309646
6-well Multiwell Plate, PS, cell-repellent	VWR	Cat#: 30618-022
Cell strainer (40μm)	VWR	Cat#: 21008-949
CELLSTAR® 6-Well Plate Tissue Culture Treated, Sterile, Flat Bottom, Chimney Style, with Lid.	VWR	Cat#: 82050-842
Centrifuge	VWR	Cat#: BKB06314
Corning enhanced attachment microcarriers (MCs)	VWR	Cat#: 30617-554

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Cryovials	VWR	Cat#: 10018-738
Disposable spatulas	VWR	Cat#: 80081-188
Freezing container, Nalgene™ Mr. Frosty	MilliporeSigma	Cat#: C1562-1EA
Hemocytometer	Fisher Scientific	Cat#: 02-671- 10
Ice bucket	VWR	Cat#: 10146-184
Ice	N/A	N/A
MojoSort™ Buffer (5×)	BioLegend	Cat#: 480017
MojoSort™ Magnet	BioLegend	Cat#: 480019
P10 micropipette tips	VWR	Cat#: 76322-132
P10 micropipette	VWR	Cat#: 76169-232
P1000 micropipette tips	VWR	Cat#: 76322-156
P1000 micropipette	VWR	Cat#: 76169-240
P20 micropipette tips	VWR	Cat#: 76322-134
P20 micropipette	VWR	Cat#: 76169-234
P200 micropipette	VWR	Cat#: 76169-238
P200 micropipette tips	VWR	Cat#: 76322-150
pH meter	VWR	Cat#: 76221-212
Pluronic™ F-127, 0.2 μm filtered (10% Solution in Water)	Thermo Fisher Scientific	Cat#: P6866
Sartorius Quintix213-1S Scale	VWR	Cat#: 611-2646
Streptavidin Magnetic Nanobeads	BioLegend	Cat#: 480016
μ-Slide 8 Well Glass Bottom: # 1.5H	ibidi	Cat# 80827
VWR Dura Shaker	VWR	Cat#: 10159-960
Water bath	VWR	N/A
Weigh paper	VWR	Cat#: 12578-165

MATERIALS AND EQUIPMENT

⚠ CRITICAL: Avoid repeated freeze-thaw cycles for reagents stored at −20°C or −80°C.

Essential 8 (E8) medium		
Reagent	Final concentration	Volume
E8 Basal Medium	N/A	490 mL
E8 Supplement (50×)	1×	5 mL
Penicillin Streptomycin (100×)	1% (v/v)	5 mL
Total	N/A	500 mL

⚠ CRITICAL: Complete E8 medium can be stored at 4°C for up to 2 weeks. Warm to 20°C–25°C prior to use. Do not warm to 37°C.

Neural Base Medium (NBM)		
Reagent	Final concentration	Volume
DMEM F-12 (1×)	N/A	482.5 mL
B-27 Supplement (50×)	1% (v/v)	5 mL
N2 Supplement (100×)	0.5% (v/v)	2.5 mL
Penicillin Streptomycin (100×)	1% (v/v)	5 mL
GlutaMAX (100×)	1% (v/v)	5 mL
Total	N/A	500 mL

⚠ CRITICAL: NBM can be stored at 4°C for up to 2 weeks.

Neural Induction Medium (NIM)		
Reagent	Final concentration	Volume
Noggin	50 ng/mL	12.5 μL
Dorsomorphin	0.5 μM	2 μL
NBM (1×)	N/A	50 mL
Total	N/A	50 mL

⚠ CRITICAL: NIM can be stored at 4°C for up to 2 weeks.

Neural Expansion Medium (NEM)		
Reagent	Final concentration	Volume
EGF	30 ng/mL	15 μL
FGF	30 ng/mL	15 μL
NBM (1×)	N/A	50 mL
Total	N/A	50 mL

⚠ CRITICAL: NEM can be stored at 4°C for up to 2 weeks.

Freezing Medium		
Reagent	Final concentration	Volume
Fetal Bovine Serum (FBS) (1×)	90% v/v	9 mL
Dimethyl Sulfoxide (DMSO)	10% v/v	1 mL
Total	N/A	10 mL

⚠ CRITICAL: Freezing medium can be stored at 4°C for up to 2 weeks.

Neural Differentiation Medium (NDM)		
Reagent	Final concentration	Volume
BDNF	20 ng/mL	10 μL
GDNF	20 ng/mL	10 μL
NBM (1×)	N/A	50 mL
Total	N/A	50 mL

⚠ CRITICAL: NDM can be stored at 4°C for up to 2 weeks.

Enzyme and Inhibitor (E/I) Stock Solution		
Reagent	Final concentration	Volume
D-(+)-Glucose	22.5 mM	112.5 mL
Sodium bicarbonate 7.5% Solution	26 mM	14.56 mL
EBSS Solution A 10×	1×	50 mL
EBSS Solution B 10×	1×	50 mL
DI water	N/A	273 mL
Hydrochloric acid (HCl)	N/A	As needed
Total	N/A	500 mL

⚠ CRITICAL: Prior to each dissociation, the pH of E/I Stock Solution should be adjusted to 7.4 with HCl. The E/I Stock Solution can be stored up to 3 months at 4°C.

Low Concentration Ovomucoid Solution		
Reagent	Final concentration	Volume/Weight
Bovine Serum Albumin	15 mg/mL	1 g
Ovomucoid inhibitor solution	15 mg/mL	20 mL
Sodium hydroxide (1N)	N/A	~330 µL
DPBS	N/A	46.6 mL
Total	N/A	67 mL

⚠ CRITICAL: Adjust the pH of the low concentration ovomucoid solution to pH 7.4 with 1N sodium hydroxide, filter sterilize using a 0.2 um filter. Aliquot and store at −20°C.

High Concentration Ovomucoid Solution		
Reagent	Final concentration	Volume/Weight
Bovine Serum Albumin	30 mg/mL	1 g
Ovomucoid inhibitor solution	30 mg/mL	20 mL
Sodium hydroxide (1N)	N/A	as needed
DPBS	N/A	rest
Total	N/A	33 mL

⚠ CRITICAL: Adjust the pH of the high concentration ovomucoid solution to pH 7.4 with 1N sodium hydroxide, filter sterilize using a 0.2 um filter. Aliquot and store at −20°C.

Astrocyte Medium		
Reagent	Final concentration	Volume
Astrocyte Basal Medium (1×)	N/A	480 mL
Fetal Bovine Serum (FBS) (1×)	2% v/v	10 mL
Astrocyte Growth Supplement (AGS) (1×)	1% v/v	5 mL
Penicillin/streptomycin solution (P/S) (1×)	1% v/v	5 mL
Total	N/A	500 mL

⚠ CRITICAL: Astrocyte Medium can be stored at 4°C for 30 days.

Tris Buffered Saline (TBS)		
Reagent	Final concentration	Weight/ Volume
Tris Base	50 mM	6.05 g
Sodium chloride (NaCl)	150 mM	8.76 g
DI water	N/A	800 mL
Hydrochloric acid (HCl)	N/A	As needed
Total	N/A	1 L

⚠ CRITICAL: Adjust pH to 7.5 with 1M HCl. TBS can be stored at 4°C for 3 months.

Cell Lysis Buffer		
Reagent	Final concentration	Amount/Volume
TBS (1×)	N/A	9.9 mL
Protease Inhibitor Tablet	N/A	1 tablet
Triton X-100	1% v/v	100 μL
Total	N/A	~10 mL

⚠ CRITICAL: Cell lysis buffer can be stored at 4°C for 10 days.

BrainPhys Neuronal Medium		
Reagent	Final concentration	Volume
BrainPhys Neuronal Base Medium	N/A	9.8 mL
SM1 Neuronal Supplement	N/A	200 μL
Total	N/A	10 mL

⚠ CRITICAL: BrainPhys Neuronal Medium can be stored at 4°C for 2 weeks.

Neuronal Calcium Imaging Solution		
Reagent	Final concentration	Amount/Volume
Fluo-4, AM	1 μM	4 μL
BrainPhys Neuronal Medium	N/A	10 mL
Pluronic™ F-127	0.02% v/v	20 μL
Total	N/A	~10 mL

⚠ CRITICAL: Neuronal Calcium Imaging Solution should be used immediately.

Astrocyte Calcium Imaging Solution		
Reagent	Final concentration	Amount/Volume
Fluo-4, AM	0.5 μM	2 μL
DMEM	N/A	10 mL
Pluronic™ F-127	0.02% v/v	20 μL
Total	N/A	~10 mL

⚠ CRITICAL: Astrocyte Calcium Imaging Solution should be used immediately.

STEP-BY-STEP METHOD DETAILS

Neural induction of hiPSCs

Timing: ~2 weeks

This step describes the neural induction of hiPSCs to allow for the robust generation of human neural progenitor cells (hNPCs). Neural induction is achieved through the inhibition of BMP and TGF-β signaling and the formation of embryoid bodies (EBs) (Chambers et al., 2009; Kim et al., 2011). After isolation of EB-derived neuroepithelial-like rosettes, hNPCs can be replated and maintained as proliferative, multipotent cells. Prior to neural induction working concentration of relevant reagents should be made as described in Table 1 and the necessary media should be made.

Note: HiPSCs should be grown for a minimum of two passages post-thaw prior to initiating differentiation. HiPSCs should be healthy, displaying their characteristic morphology.

1. Differentiation should be initiated when hiPSCs are 70–80% confluent (Figure 1A, left panels).
2. On day 0, aspirate the medium from the well.
3. Gently wash each well with 1 mL PBS. Then, add 1 mL of Accutase per well of a 6-well plate.
4. Incubate the plate with Accutase at 37°C for 5 min.
5. Wash the surface with 1 mL of DMEM to further dissociate the cells from the plate (blast cells with P1000 where necessary).
6. Transfer the cell suspension to a 15 mL conical tube. Take 10 μL of the cell suspension and count cells using a hemocytometer.
7. Centrifuge at 200 × g for 5 min at RT.
8. Aspirate the supernatant without disturbing the cell pellet.
9. Resuspend the cell pellet in E8 supplemented with 5 μM ROCKi so that the final cell concentration is 1 × 10^6 cells/mL.
10. Add 4 mL of cell suspension per well of 6-well cell-repellent plate. A total of 4 × 10^6 cells will be added to each well.

Note: For neural induction in suspension culture, the cells should be cultured in a 6-well cell repellent plate. To allow for appropriate cell aggregation, we do not recommend other cell culture formats be used.

11. Place the plate on an orbital shaker set at 95 rpm inside a 37°C, 5% CO_2 incubator. Aggregates in the form of embryoid bodies (EBs) should be observed 24 h post-plating (Figure 1A, right panels). Troubleshooting 1.
12. On day 1, carefully aspirate 3 mL of the medium from each well using a P1000 and replace with 3 mL of fresh E8 medium.
13. On day 2, carefully aspirate 2 mL of the medium from each well using a P1000 and replace with 2 mL of fresh E8 medium.
14. On day 3, carefully aspirate 3 mL of the E8 medium from each well and replace with 3 mL of NIM.

⚠ CRITICAL: Formation of EBs in E8 medium prior to neural induction allows for more consistent aggregate size and neural induction.

15. On days 4–8, carefully aspirate 2 mL of the medium from each well and replace with 2 mL of NIM.
16. On day 9, use a 5 mL serological pipette to gently transfer aggregates to a 15 mL conical and let the aggregates settle. Transfer aggregates to a Cultrex® Basement Membrane Extract (BME) coated 6-well plate, splitting aggregates at a 1:2 ratio.
17. Add NIM supplemented with 5uM of ROCKi so that the total volume in each well is 3 mL. Gently move the plate in several quick, short, back-and-forth, and side-to-side motions to disperse the EBs evenly across the cell culture surface.
18. On day 10, EBs should have settled and adhered to the Cultrex® BME-coated plate (Figure 1B, left panels). Change the medium with NIM on days 10–15. By day 12, the EBs should spread out on the Cultrex® BME-coated plates and neural rosette-like structures should be visible (Figure 1B, right panels). Troubleshooting 2.

Coating of plates with poly-L-ornithine (PLO) and laminin (Ln)

Timing: ~8 h

This step describes the coating of plates with poly-L-ornithine (PLO) and laminin (Ln) that will be used for hNPC culture. Prior to this step, the stock solutions of PLO and Ln should be made as described in Table 1.

19. To prepare PLO working solution, add 48 mL of PBS and 2 mL of PLO stock solution to a 50 mL conical.
20. Add 2 mL of the PLO working solution to each well of a 6-well plate.
21. Incubate at 37°C for 4 h.
22. Aspirate the PLO and wash each well twice with 2 mL PBS.
23. Thaw aliquots of Ln stock solution on ice.
24. To prepare Ln working solution, add 49.6 mL of PBS and 400 μL of Ln stock solution to a 50 mL conical.
25. Add 2 mL of the Ln working solution to each well of a 6-well plate.
26. Incubate at 37°C for 4 h.
27. Immediately prior to use, wash each well twice with 2 mL PBS.

Note: PLO/Ln coated plates can be stored at -20°C for 2 weeks with the Ln working solution remaining on the plate.

Neural Rosette selection

Timing: 2 days

This step describes the selective isolation of neural rosette clusters from adherent EBs. The isolated rosettes can be expanded as hNPCs in the presence of FGF and EGF (Koch et al., 2009). Before starting this step, the Neural Induction Medium (NIM) should be prepared. Rosette selection is adapted from the manufacturer's protocol for STEMdiff™ Neural Rosette Selection Reagent.

28. On day 16 of neural induction, aspirate NIM from the well and wash with 1 mL of DMEM/F-12.
29. Aspirate DMEM/F-12 and add 1 mL of STEMdiff™ Neural Rosette Selection Reagent.
30. Incubate at 37°C for 1.5 h.
31. Carefully aspirate the STEMdiff™ Neural Rosette Selection Reagent using a 1 mL pipette.
32. Using a P1000, firmly wash the surface with 1 mL of DMEM/F-12 to dissociate the rosettes from the plate.

Note: The DMEM/F-12 should be aimed directly at the rosettes in order to dissociate the neural rosettes from the culture surface.

33. Add the neural rosette suspension to a 15 mL conical tube using a 5mL serological pipette.
34. Repeat steps 32 and 33 until most of the rosettes have been isolated from the well. Do not over-select for rosettes. Troubleshooting 3.
35. Centrifuge rosette suspension at 300 × g for 5 min at RT.
36. Gently resuspend by pipetting cells in 3 mL NIM supplemented with 5 μM ROCKi 1–2 times.
37. Split cells at a 1:1 ratio. Add 3 mL of cell suspension to 1 well of a PLO/Ln coated 6-well plate. From herein, the cells are called hNPCs and this is considered passage 0.
38. Place the plate in the 37°C, 5% CO_2 incubator. Gently move the plate in several quick, short, back-and-forth, and side-to-side motions to spread cells evenly across the wells.
39. The next day, change the medium with 3mL NIM.
40. Change the medium with fresh NIM every day until cells reach 70–80% confluence. Flat adherent cells should appear with a morphology as shown in Figure 1C. Culture cells in NEM from passage 1.

HNPC expansion

Timing: 3–4 weeks

This step describes the expansion and routine passaging of hNPCs. HNPCs should be passaged when the cells reach 70–80% confluence. Healthy cell growth should require passaging every 3–4 days. This protocol describes expansion of hNPCs in 6-well plates. The protocol should be adjusted appropriately for expansion in other tissue culture plate formats.

41. When the cells reach 70–80% confluence (Figure 1C), aspirate the medium and add 1 mL of Accutase.
42. Incubate the plate with Accutase at 37°C for 5 min.
43. Wash the well with 1 mL of NEM to remove adherent cells (blast cells with P1000 where necessary).
44. Transfer cell suspension to a 15 mL conical tube. Take 10 μL of the cell suspension and count cells using a hemocytometer.
45. Centrifuge at 200 × g for 5 min at RT.
46. Aspirate the supernatant without disturbing cell pellet.
47. Resuspend cell pellet in NEM so that the final cell concentration is 1×10^6 cells/mL.
48. Plate the cells at a density of $2.5 \times 10^4/cm^2$ onto fresh PLO/Ln coated plates that have been washed twice with PBS prior to use.
49. Add the appropriate volume of NEM to each well so that the final volume per well is 3mL.
50. Place the plate in the 37°C, 5% CO_2 incubator. Gently move the plate in several quick, short, back-and-forth, and side-to-side motions to spread cells evenly across the wells.
51. Each passage increases the passage number by 1.
52. Cells should continue to display similar flat morphology as shown in Figure 1C. Cells should be characterized at passage 6 with >90% of the cells should expressing SOX1, SOX2, and NESTIN (Figure 1D). Troubleshooting 4.

Cryopreservation of hNPCs

Timing: 1 day

This step describes the cryopreservation of hNPCs. HNPCs should be passaged between passage 5 and 10 for use in large-scale differentiations. We recommend cryopreserving hNPCs on passage 5 after characterization. Before starting this step, prepare the Freezing Medium.

53. When the cells reach 70–80% confluence, aspirate the medium and wash once with PBS. Then, add 1 mL of Accutase.
54. Wash the well with 1 mL of NEM to remove adherent cells (blast cells with P1000 where necessary).
55. Transfer cell suspension to a 15 mL conical tube. Take 10 μL of the cell suspension and count cells using the hemocytometer.
56. Centrifuge at 200 × g for 5 min at RT.
57. Aspirate the supernatant without disturbing the cell pellet.
58. Resuspend the pellet in Freezing Medium so that the final cell concentration is 1×10^6 cells/mL.
59. Transfer 1 mL of cell suspension to a cryovial and transfer to a Mr. Frosty™ freezing container.
60. Place the Mr. Frosty™ in a −80°C freezer for 24 h before transferring the cryovial to a −150°C freezer.

Pause point: HNPCs can be stored at −150°C for 1 year.

Thawing of hNPCs

Timing: 3–4 days

This step describes the thawing of cryopreserved hNPCs. We recommend that hNPCs be expanded a minimum of 2 passages after thawing before proceeding with large-scale differentiation.

61. Quickly thaw a vial of hNPCs (1×10^6 cells/vial) in a bead bath and add cell suspension to 4 mL DMEM in a 15 mL conical.
62. Centrifuge cells at 200 × g for 5 min at RT.
63. Aspirate the supernatant without disturbing the cell pellet.
64. Resuspend cell pellet in 1 mL NEM.
65. Plate at an appropriate density (25–50% higher than density at which cells are routinely cultured; Figure 1E) and expand hNPCs on PLO/Ln coated plates as described in steps 41–51.

Coating of microcarriers (MCs) with vitronectin-derived peptide (VDP)

Timing: 2 days

In this protocol, a fully defined peptide substrate—termed vitronectin-derived peptide (VDP)—will be employed as a growth substrate for the large-scale differentiation of hNPCs in a microcarrier (MC)-based suspension culture system (Srinivasan et al., 2018; Varun et al., 2017). This step describes the coating of MCs with VDP. Although this protocol can be adapted to be used with PLO/Ln coated MCs, VDP provides a more reproducible and cost-effective substrate for the large-scale expansion and differentiation of hNPCs.

66. Sterilize weighing scale with 70% ethanol and transfer to tissue culture hood. Weigh 200 mg of sterile MCs and transfer to a 15 mL conical using a disposable spatula and weigh paper.
67. Add 5 mL PBS to 15 mL conical.
68. Using a P1000, mix the MCs in PBS by pipetting up and down.
69. Transfer 500 μL (approximately 20 mg) of MC suspension to a 1.5 mL Eppendorf tube.
70. Centrifuge the Eppendorf tube at 200 × g for 3 min.
71. Using a P1000, aspirate 50 μL PBS from the Eppendorf tube and replace with 50 μL VDP stock solution.
72. Using a P1000, mix the MCs well in VDP solution by pipetting up and down.
73. Incubate MCs with VDP for 48 h at 37°C.
74. Immediately before use, centrifuge the Eppendorf tube at 200 × g for 3 min.
75. Aspirate the supernatant without disturbing the MC pellet.
76. Gently add 1 mL PBS to resuspend the MCs. Do not pipette the MCs up and down vigorously.
77. Centrifuge the Eppendorf tube at 200 × g for 3 min.
78. Aspirate the supernatant without disturbing the MC pellet. Gently add 1 mL NEM.
79. Centrifuge the Eppendorf tube at 200 × g for 3 min.
80. Aspirate the supernatant without disturbing the MC pellet. Gently add 1 mL NEM. This is later added to the cell suspension prepared as described below.

Large-scale neural differentiation of hNPCs on VDP-coated MCs

Timing: ~30 days

This section describes the expansion of hNPCs on VDP-coated MCs as well as their subsequent differentiation to neurons and astrocytes through the removal of FGF and EGF and addition of brain-derived neurotrophic factor (BDNF) and glial derived neurotrophic factor (GDNF). Prior to starting this step, prepare the Neural Differentiation Medium (NDM).

81. Dissociate a healthy, confluent hNPC culture as described in steps 41–47. Resuspend the hNPCs at a concentration of 2×10^6 cell/mL in NEM.

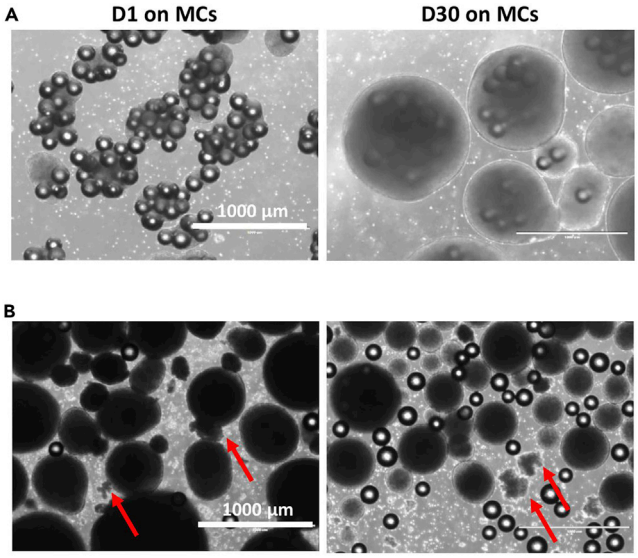

Figure 2. Expansion and differentiation of hNPCs on MCs

(A) Representative images of MC aggregate on day 1 (left panel) and day 30 (right panel) of differentiation.

(B) Representative images of poorly differentiating MC aggregates. Red arrows are pointing to patches of cells detaching from the MCs.

82. In a 15 mL conical, mix 2 × 10^6 cells in suspension, 130 µL washed VDP-coated MCs, 870 µL warmed NEM, and 2 µL ROCKi. The total volume of 1 well of a 6-well plate should be 2 mL.
83. Transfer contents of 15 mL conical to a single well of a 6-well cell-repellent plate. If seeding cells in more than one well of a 6-well plate, make sure to mix MCs and cells well and seed one well at a time using a 5mL serological pipette.
84. Place the plate in static conditions in the 37°C, 5% CO_2 incubator for 12 h.
85. Add 2 mL of NEM and 2 µL ROCKi to the well.
86. Place the plate on an orbital shaker set at 95 rpm in the 37°C, 5% CO_2 incubator.
87. After 24 h, use a P1000 to remove 3 mL of media and replace with 3 mL of fresh NEM.
88. Every day, use a P1000 to remove 2 mL of media and replace with 2mL of fresh NEM.

⚠ CRITICAL: While changing the media, swirl the aggregates to the middle of each well to ensure no cell aggregates are accidently removed.

89. Continue changing the media until the cells are confluent, approximately 4–5 days after seeding the MCs. There should be a thin layer of cells surrounding the MC aggregates (Figure 2A, left panel). The hNPCs should continue to express high levels of SOX1, SOX2, and NESTIN when grown on the MCs. Troubleshooting 5.
90. Once confluent, use a P1000 to remove 3 mL of NEM and replace with 3 mL of NDM.
91. For the next 30–35 days, each day use a P1000 to remove 2 mL of media and replace with 2 mL of NDM. The MC aggregates will continue to grow in size throughout the differentiation (Figure 2A, right panel). Troubleshooting 6.

Dissociation of cells from the MCs

Timing: ~5 h

This step describes the enzymatic dissociation of differentiated neural cells from the MC-based cultures into single-cell suspension. This protocol is a modification of a previously published protocol for the single-cell dissociation of organoid-based cultures (Sloan et al., 2018). Prior to starting this

step prepare the Enzyme and Inhibitor (E/I) Stock Solution, the Low Concentration Ovomucoid Solution and the High Concentration Ovomucoid Solution.

⚠ CRITICAL: This protocol is written for dissociation of 2 wells of 6-well plate of MC cultures. Dissociation of additional wells should be performed in parallel and not combined into a single 50 mL conical tube.

92. Label a 50 mL conical tube 'Low concentration ovomucoid inhibitor solution'. Add 27.8 mL E/I stock solution to the conical. Label another 50 mL conical tube 'High concentration ovomucoid inhibitor solution'. Add 5 mL solution to the conical.
93. Place both conicals upright in the 37°C, 5% CO_2 incubator with screw caps loosened to allow for equilibration. Alternatively, adjust the pH of E/I stock solution prior to use with 1N HCl and skip the equilibration step.
94. Prepare a 30U/mL papain enzyme solution by adding the appropriate volume of papain, 75 μL L-Cysteine stock solution, and 14.4 mL of E/I stock solution to a 50 mL conical.

Note: The volume of papain enzyme solution may vary according to enzyme stock concentration. The final concentration must be at 30 U/mL. The solution will be cloudy when the papain stock is added.

⚠ CRITICAL: Do not add the DNase I at this step. It will be added at a later step.

95. Incubate the papain enzyme solution at 37°C for 20 min until the solution becomes clear.
96. Filter the papain enzyme solution with a 0.2 μm filter into a new 50 mL conical.
97. After filter sterilization, add 94 μL DNase I stock solution to the papain enzyme solution.
98. Using a 25 mL serological pipette, transfer 2 wells of MCs to a 15 mL conical tube. Wash the well with 3 mL NBM to transfer any remaining aggregates left in the well.
99. Centrifuge the conical at 200 × g for 3 min.
100. Aspirate the supernatant and add 2 mL of Accutase per well.
101. Incubate for 5 min at 37°C.
102. Use a P1000 to gently break up the aggregates by pipetting up down several times.
103. Add 2 mL of NBM to the 15 mL conical.
104. Centrifuge the conical at 200 × g for 3 min.
105. Aspirate the supernatant and add 4 mL papain enzyme solution that was prepared in step 96.
106. Using a 5 mL serological pipette, transfer the aggregates in papain enzyme solution to 2 wells of a 6-well cell-repellent plate at 2 mL per well.
107. Place the plate on an orbital shaker set at 95 rpm in the 37°C, 5% CO_2 incubator. Incubate for 70 min.
108. During the incubation period, finish preparing the low and high concentration ovomucoid inhibitor solutions.
    a. For the low concentration ovomucoid inhibitor solution, thaw the low concentration ovomucoid solution on ice. To the 50 mL conical prepared in step 92, add 2 mL of low concentration ovomucoid solution and 188 μL DNase I.
    b. For the high concentration ovomucoid inhibitor solution, thaw the high concentration ovomucoid solution on ice. To the 50 mL conical prepared in step 92, add 1 mL of high concentration ovomucoid solution and 38 μL DNase I.
109. After incubation in papain enzyme, transfer cells from the 6-well cell-repellent plate to a 15 mL conical.
110. Centrifuge the conical at 300 × g for 5 min.
111. Aspirate supernatant and add 4 mL low concentration ovomucoid inhibitor solution prepared in step 108a.
112. Use a 5 mL serological pipette to gently pipette the aggregates up and down 15 times.

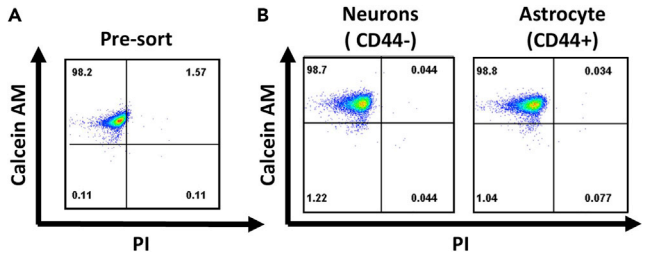

Figure 3. Assessment of cell viability after dissociation from MCs and MACS purification

(A) Representative flow cytometry analysis of Calcein AM and PI in cell populations after dissociation from MCs.

(B) Representative flow cytometry analysis of Calcein AM and PI in presumptive CD44- neuronal (left panel) and CD44+ (right panel) populations after MACS purification.

⚠ CRITICAL: Do not introduce bubbles while pipetting as this may lead to cell damage and death.

113. Allow undissociated aggregates to settle down and transfer cloudy cell suspension to a new 15 mL conical.
114. Repeat steps 111–113 with a P1000 until aggregates are broken up into single cell suspension. Triturate slightly more vigorously each round.
115. Put a 40 μm cell strainer over a new 15 mL conical and, using a P1000, slowly pass all the single cell-MC suspension obtained in step 114 through the filter.

Note: Change the cell strainer if it gets clogged with MCs. For higher yields, wash the cell strainer 1 to 2 times with low concentration ovomucoid inhibitor solution to ensure all single cells have passed through.

116. Centrifuge the single cell suspension at 300 × g for 5 min.
117. Aspirate the supernatant and resuspend in 6 mL low ovomucoid inhibitor solution.
118. In a new 15 mL conical tube, add 6 mL of high ovomucoid inhibitor solution prepared in step 108b.
119. Add the resuspended single cell suspension obtained in step 117 to the 15 mL conical with the high ovomucoid inhibitor solution prepared in step 118 dropwise.

Note: This step will allow for the removal of cell debris from the single cell suspension. When added slowly, the low ovomucoid cell suspension will stay on top of the high ovomucoid solution.

120. Centrifuge the conical at 300 × g for 5 min.
121. Aspirate the supernatant and resuspend in 10 mL of warm NDM supplemented with 5uM ROCKi with a P1000.

Note: Resuspend with 1 mL NDM first to ensure the pellet is thoroughly broken up prior to adding the remaining 9 mL NDM.

122. Incubate the cell suspension for 1 h in a 15 mL conical tube in a 37°C, 5% CO_2 incubator to allow the cells to recover from the dissociation.
123. Count the cells using a hemocytometer.
124. Take 1 × 10^6 cells and proceed with the cell viability assay as described in step 190 (Figure 3A). The remainder of the cells can be purified using magnetic activated cell sorting (MACS) as described in step 125.

Purification of neurons and astrocytes from neural cultures using magnetic activated cell sorting (MACS)

Timing: ~3 h

In this section, a MACS step employing an established astrocyte-specific cell surface marker, CD44 (Liu et al., 2004), is used to purify mixed neural populations into astrocytes and neurons.

125. Prepare 10 mL of 1× MojoSort™ Buffer by adding 2 mL of 5× MojoSort™ Buffer and 8 mL of sterile distilled water to a 15 mL conical tube.
126. Centrifuge cell suspension obtained in step 121 at 300 × g for 10 min.
127. Aspirate the supernatant and add 5 mL 1× MojoSort™ Buffer to the cell pellet without resuspending the pellet.
128. Centrifuge cells in 1× MojoSort™ Buffer for 300 × g for 10 min.

⚠ CRITICAL: The maximum number of cells that can sorted at one time should not exceed 2.5×10^8 cells. To that end, if more than 2.5×10^8 cells are isolated then the cells should be separated evenly into multiple 15 mL conical tubes.

129. Resuspend cell pellet with 1× MojoSort™ Buffer so that the final cell concentration is 1×10^8 cell/mL and add the cell suspension to a polypropylene tube.
130. Add 1 µL per 1×10^6 cells of Biotin-CD44 to the tube.
131. Gently flick the tube to mix the antibody and cell suspension.
132. Incubate conical on ice for 15 min.
133. Vortex Streptavidin nanobeads 5 times.
134. Add 1 µL Streptavidin nanobeads per 1×10^6 cells to the tube.
135. Gently flick the tube to mix the nanobeads and cell suspension.
136. Incubate on ice for 15 min.

⚠ CRITICAL: At this step, no more than 2.5 mL of 1× buffer, 250 µL Biotin-CD44 antibody, and 250 µL of Streptavidin Nanobeads should have been added. The magnet can sort a maximum of 3 mL of solution (~2.5×10^8 cells) at once. As such, if more than 2.5×10^8 cells are being purified then MACS sorting of parallel cell preparations staggered by 5 min should be performed.

137. Place the tube in the MojoSort™ magnet so that the solution is completely covered by the magnet. Loosely place a cap on top of the tube. It should not be sealed.
138. Leave the magnet in place for 5 min.
139. With the magnet still in place, decant the CD44- fraction into a new polypropylene tube labeled 'CD44- cells' in one motion.
140. Repeat steps 137–139 for higher purity of CD44- cells.

Note: The CD44+ cells from step 138 will be used in step 141. Do not combine the CD44+ cells left after a second round of MACS separation with the cells from step 138.

141. After the second round of MACS separation, add cells to a 15 mL conical labeled 'CD44-cells' and centrifuge at 300 × g for 5 min. Herein, these cells will be referred to as neurons.
    a. Aspirate the supernatant and resuspend the pellet in 10 mL warm NDM.
    b. Count the cells with a hemocytometer. Troubleshooting 7.
    c. Proceed to step 175 for replating of neurons or step 181 for cryopreservation of neurons.

142. Using the CD44+ fraction from step 138, resuspend the cells in 12 mL of warmed Astrocyte Medium supplemented with 5 μM ROCKi and transfer the cells to a 15 mL conical labeled 'CD44+ cells'. Herein, these cells will be referred to as astrocytes.
    a. Count the cells using a hemocytometer. Troubleshooting 7.
    b. Proceed to step 143 for replating and culturing of the astrocytes.

Astrocyte culture

Timing: ~2 weeks

In this section, we described the plating and expansion of MACS purified astrocytes. After culture, these astrocytes can be characterized for their expression of high levels of mature astrocytic markers (step 209) as well as properties characteristic of functionally mature astrocytes including production of Apolipoprotein E (ApoE; step 228) and appearance of robust calcium transients (step 235). This protocol describes the culture of astrocytes in 6-well plates. The protocol should be adjusted appropriately for expansion in other tissue culture plate formats.

143. Transfer 2.5×10^5 cells from the cell suspension obtained in step 142 to each well of a Cultrex® BME-coated 6-well plate.

Note: Because the Streptavidin Nanobeads might still be attached to the astrocytes, the cell counts obtained via the hemocytometer might be inaccurate. To that end, the user can elect to split the astrocyte cell suspension obtained in step 143 into empirically determined ratios onto Cultrex® BME-coated plates. We have found that evenly splitting the astrocyte cell suspension obtained in step 143 into 4 wells of a Cultrex® BME-coated plate will allow for robust attachment and growth.

144. Add appropriate amount of warmed Astrocyte Medium supplemented with 5 μM ROCKi so that the total volume per well is 3 mL.
145. Place the plate in the 37°C, 5% CO_2 incubator. Gently move the plate in several quick, short, back-and-forth, and side-to-side motions to spread cells evenly across the wells.
146. After 24 h replace the medium with 3 mL of fresh Astrocyte Medium. Troubleshooting 8.
147. Change the medium each day (~5 days) until the wells are 90% confluent.
148. When the cultures are 90% confluent aspirate the medium and add 1 mL of Accutase to each well.
149. Incubate in the 37°C, 5% CO_2 incubator for 5 min.
150. Add 2mL of Astrocyte medium to each well with Accutase.
151. Mechanically dissociate cells from the bottom of the well with a P1000 pipette.
152. Count the cells using a hemocytometer.
153. Transfer the cells to a 15 mL conical and centrifuge at 200 × g for 5 min.
154. Aspirate the supernatant and resuspend in 5 mL of warmed Astrocyte Medium.
155. Transfer 2.5×10^5 cells from the cell suspension to each well of a Cultrex® BME-coated 6-well plate.

Note: Cell plating density may vary with each cell line. To find the optimal seeding density, plate the cells at a gradient of cell density (e.g. 1×10^5, 2×10^5, 3×10^5) and use the density at which the cells become 90% confluent 3–4 days after passaging.

156. Add appropriate amount of warmed Astrocyte Medium supplemented with 5 μM ROCKi so that the total volume per well is 3 mL.
157. Place the plate in the 37°C, 5% CO_2 incubator. Gently move the plate in several quick, short, back-and-forth, and side-to-side motions to spread cells evenly across the wells.
158. Change the medium each day with 3 mL of warmed Astrocyte Medium.

159. When 90% confluent, passage the cells as describe in steps 148–156.
160. Astrocytes can be cryopreserved as described in step 161 or characterized as described in steps 209, 228, and 235.

Note: The astrocyte pellets during each passage will appear brown or darker in color because some of the streptavidin nanobeads will still be attached. Around passage 4, the nanobeads should be mostly removed. Because the attachment of the nanobeads to the cell surface of the astrocytes can interfere with downstream assays, we recommend proceeding with any characterization or experimental assays after passage 4 but prior to passage 10.

Cryopreservation of astrocytes

Timing: 1 day

This step describes the cryopreservation of astrocytes. We recommend cryopreserving astrocytes on passage 4 for use in subsequent experiments or assays.

161. When the cells reach 90% confluence, aspirate the medium and add 1 mL of Accutase.
162. Wash the well with 1 mL of DMEM to remove adherent cells (blast cells with P1000 where necessary).
163. Transfer cell suspension to a 15 mL conical tube. Take 10 μL of the cell suspension and count cells using a hemocytometer.
164. Centrifuge at 200 × g for 5 min at RT.
165. Aspirate the supernatant without disturbing the cell pellet.
166. Resuspend the pellet in Freezing Medium so that the final cell concentration is 1×10^6 cells/mL.
167. Transfer 1 mL of cell suspension to a cryovial and transfer to a Mr. Frosty™ freezing container.
168. Place the Mr. Frosty™ in a −80°C freezer for 24 h before transferring the cryovial to a −150°C freezer.

Thawing of astrocytes

Timing: 3–4 days

This step describes the thawing of cryopreserved astrocytes. We recommend that astrocytes be cultured for a minimum of 3 days before proceeding with characterization or other experimental assays.

169. Quickly thaw a vial of astrocytes (1×10^6 cells/vial) in a bead bath and add cell suspension to 4 mL DMEM in a 15 mL conical.
170. Centrifuge cells at 200 × g for 5 min at RT.
171. Aspirate the supernatant without disturbing cell pellet.
172. Resuspend cell pellet in 1 mL Astrocyte Medium.
173. Plate and expand astrocytes on Cultrex® BME-coated plates as described in steps 144–159. Troubleshooting 9.
174. Characterize astrocytes as described in steps 209, 228, 235.

Note: Allow the astrocytes to recover for a minimum of 3 days before proceeding the characterization assays.

Neuron culture

Timing: ~7–10 days

In this section, we described the plating of MACS purified neurons. After culture, these neurons can be characterized for their expression of high levels of mature post-mitotic neuronal markers (step 209) as well as characteristics of functionally mature neurons such as the appearance of spontaneous calcium transients (step 235). This protocol describes the culture of neurons in 24-well plates. The protocol should be adjusted appropriately for expansion in other tissue culture plate formats.

175. Transfer 1 × 10^6 cells from the cell suspension obtained in step 140 to each well of a Cultrex® BME-coated 24-well plate.

Note: Optimal plating density will be cell-line dependent but typically range 1–2 × 10^6 cells/well.

176. Add appropriate amount of warmed NDM supplemented with 5 μM ROCKi so that the total volume per well is 1 mL.
177. Place the plate in the 37°C, 5% CO_2 incubator. Gently move the plate in several quick, short, back-and-forth, and side-to-side motions to spread cells evenly across the wells.
178. After 24 h, use a P1000 to remove the medium and replace with 1 mL of fresh NDM. Troubleshooting 8.

Note: Neurons, especially 24–48 h post-plating, are very fragile and may be susceptible to peeling off the plate. While removing and adding the medium, it is important to use caution.

179. Using a P1000, change half the medium every other day until axonal projections appear (7–10 days; Figure 6A).
180. Proceed with experimental assays or characterization as described in steps 209 and 235.

Cryopreservation of neurons

Timing: 1 h

This step describes the cryopreservation of neurons without loss of viability or functionality.

181. Centrifuge the MACS-purified neurons obtained in step 140 at 300 × g for 10 min.
182. Aspirate the supernatant and resuspend in CryoStor so that the final concentration is 20 × 10^6 cells/mL.
183. Transfer 1 mL of cell suspension to a cryovial and transfer to a Mr. Frosty™ freezing container.
184. Place the Mr. Frosty™ in a −80°C freezer for 24 h before transferring the cryovial to a −150°C freezer.

Pause point: Neurons can be stored at −150°C for 2 years at least.

Thawing of neurons

Timing: 7–10 days

This step describes the thawing of cryopreserved neurons. We recommend that neurons be cultured for a minimum of 7–10 days before proceeding with characterization or other experimental assays.

185. Quickly thaw a vial of neurons in a bead bath. Transfer cell suspension to a 50mL conical and add 5 mL NDM dropwise. Swirl gently to mix and transfer to a 15 mL conical.
186. Centrifuge cells at 300 × g for 5 min at RT.
187. Aspirate the supernatant without disturbing the cell pellet.
188. Resuspend cell pellet in 1 mL NDM supplemented with 5 μM ROCKi.

189. Plate and culture neurons on Cultrex® BME-coated plates as described in steps 175–178. Troubleshooting 9.
190. Characterize neurons as described in steps 209 and 235.

Note: Allow the neurons to recover for a minimum of 7 days after thawing prior to assaying.

Assessment of viability of neuronal and astrocytic cell populations by flow cytometry

Timing: ~2 h

This section describes the protocol for assessing the viability of dissociated, MACS-purified cell populations with Calcein-AM and Propidium Iodide (PI). This assessment can also be performed upon thawing of cryopreserved cell populations.

191. Collect 1 × 10^6 cells in suspension and place in a 1.5 mL Eppendorf tube.
192. Centrifuge cells at 200 × g for 5 min at RT.
193. Aspirate the supernatant without disturbing the cell pellet.
194. Resuspend cell pellet in 500 μL of 50 nM Calcein AM diluted in PBS.
195. Incubate cells on ice, protected from light, for 30 min.
196. Wash the cells with 1 mL of PBS twice by centrifugation at 200 × g for 5 min at RT after each wash.
197. Resuspend the cells in 500 μL PBS.
198. Add 1μL of 1.5 mM stock PI. Mix by pipetting gently up and down with a P1000.

Note: For each sample, prepare single Calcein-AM and PI-stained along with unstained samples.

199. Analyze the cells with a flow cytometer and use the single- and un-stained samples to set gates and color compensation using FlowJo analysis software (Figure 3). Troubleshooting 10.

Characterization of neurons and astrocytes by flow cytometry

Timing: ~2 h

This step describes the characterization of MACS-purified populations by flow cytometry for expression of CD44. This protocol can be adapted to analyze cell populations for expression of other cell surface markers.

200. Collect 1 × 10^6 cells in suspension and place in a 1.5 mL Eppendorf tube.
201. Centrifuge cells at 200 × g for 5 min at RT.
202. Aspirate the supernatant without disturbing the cell pellet.
203. Resuspend cell pellet in 1 mL PBS.
204. Add 40 ul of PE mouse anti-human CD44 to the tube.

Note: For each sample, prepare a separate tube with the appropriate negative isotype control.

205. Incubate, protected from light, at 4°C for 1 h.
206. Wash the cells with 1 mL of PBS twice by centrifugation at 200 × g for 5 min at RT after each wash.
207. Resuspend the cells in 200 μL PBS.
208. Analyze the cells with a flow cytometer and use the negative isotype controls to set gates using FlowJo analysis software (Figures 4A and 6C). Troubleshooting 11.

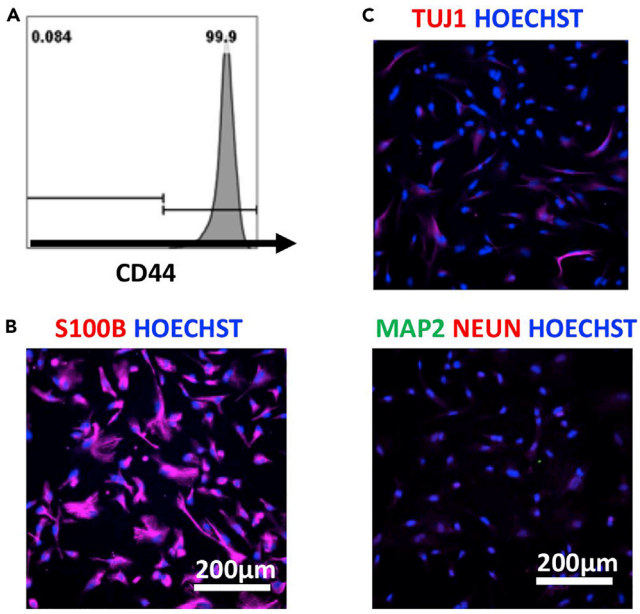

Figure 4. Characterization of MACS purified astrocytes

(A) Flow cytometry analysis showing astrocyte cell population is enriched for CD44.

(B) Immunofluorescence showing expression of astrocyte marker S100β in MACS purified astrocyte population. Scale bar = 200 µm.

(C) Immunofluorescence showing absence of neuron markers TUJ1, MAP2 and NEUN in MACS purified astrocyte population. Scale bar = 200 µm.

Characterization of neurons and astrocytes by immunofluorescence

Timing: 2 days

This section describes characterization of MACS-purified cell populations for neuronal (MAP2, NEUN, TUJ1), synaptic (vGlut1, PSD95), cortical (FOXG1, TBR1, CTIP2, SATB2) and astrocytic (S100β) markers. This protocol can be adapted to analyze cell populations for other markers. We recommend that cells be plated in 24-well plates for immunofluorescent assays. For assessing synaptic marker expression, cells can be plated on #1.5 coverslips to allow for confocal imaging.

209. Aspirate the medium from adherent cultures. For neurons, use a P1000 to aspirate.
210. Wash wells once with 500 µL PBS.
211. Aspirate PBS and add 300 µL of fixation buffer.
212. Incubate at RT for 15 min, protected from light.
213. Aspirate fixation buffer and wash wells twice with 500 µL PBS.
214. Aspirate PBS and add 300 µL of permeabilization buffer (Phosflow Perm Buffer III).
215. Incubate at 4°C for 30 min.
216. Aspirate permeabilization buffer and wash wells twice with 500 µL PBS.
217. Aspirate PBS and add 300 µL of primary antibody diluted in PBS at the concentration indicated in the key resources table.
218. Incubate primary antibody 12–24 h at 4°C, protected from light.
219. Aspirate primary antibody and wash wells twice with 500 µL PBS.
220. Aspirate PBS and add 300 µL of the corresponding secondary antibody diluted in PBS at the concentration indicated in the key resources table.
221. Incubate secondary antibody for 1 h at RT, protected from light.
222. Aspirate secondary antibody and wash wells twice with 500 µL PBS.

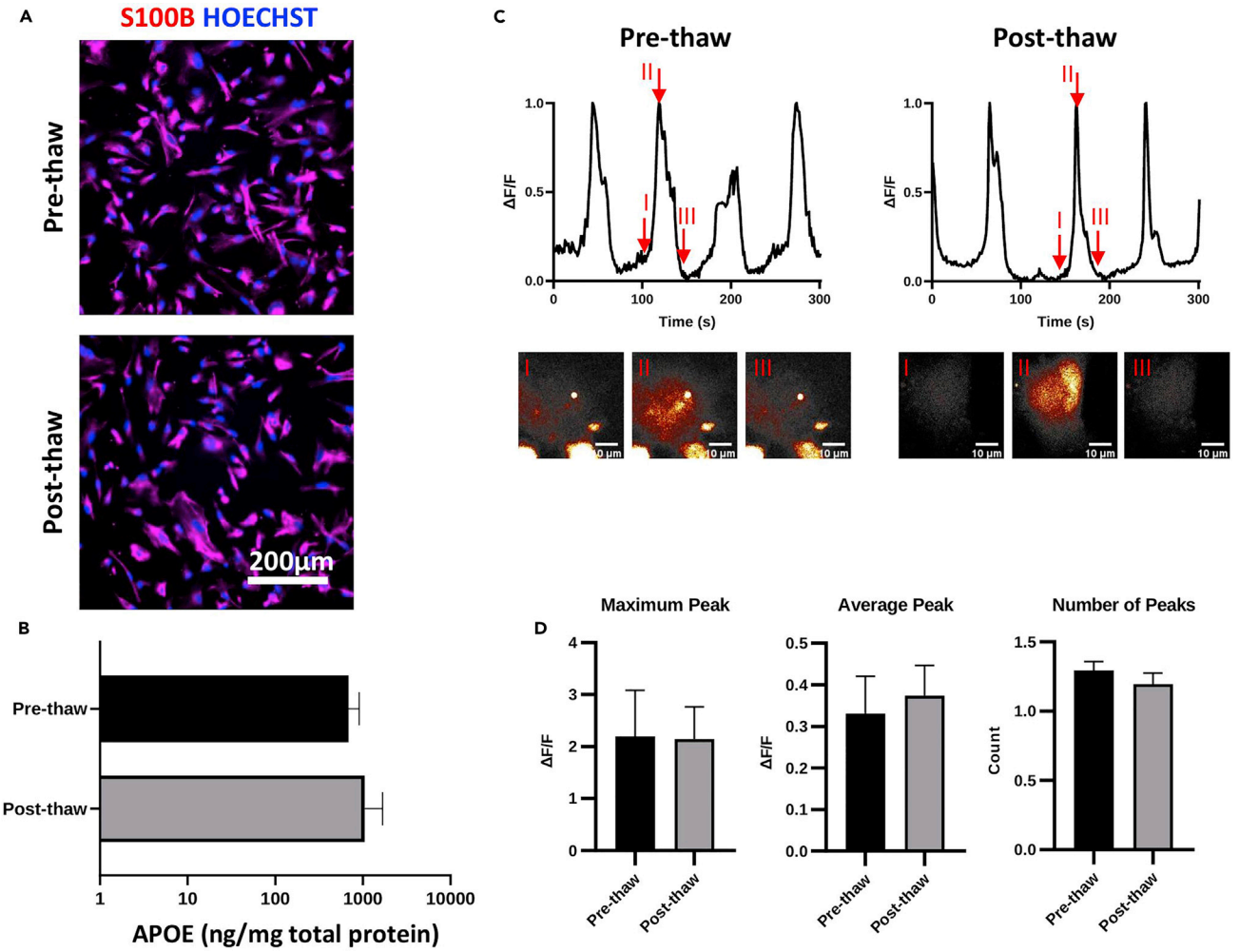

Figure 5. Characterization of MACS purified astrocytes before and after cryopreservation

(A) Immunofluorescence showing no difference in expression of astrocyte marker S100β in astrocyte cell population before and after cryopreservation. Scale bar = 200 µm.

(B) Quantification of APOE secretion before and after cryopreservation (mean ± S.E.M.; n = 24 from 4 independent differentiations).

(C) Analysis of spontaneous calcium transients shows maintenance of functionality in astrocytes after cryopreservation.

(D) Quantitative analysis of spontaneous calcium transients shows no statistical differences in functionality of astrocytes before and after cryopreservation (mean ± S.E.M.; n= 12 from 4 independent differentiations). Differences between groups were determined using unpaired two-sided t-test with $P < 0.05$ considered to be significant. Astrocytes were allowed to recover a minimum of 3 days after thawing before assaying.

223. Aspirate PBS and add Hoechst diluted 1:5000 in PBS.
224. Incubate for 10 min at RT, protected from light.
225. Aspirate Hoechst and wash wells twice with 500 µL PBS.
226. Leave 500 µL PBS in each well.

Pause point: Stained plates may be stored at 4°C, protected from light, for 1 week prior to imaging.

227. Image wells with a fluorescent microscope. (Figures 4B, 4C, 5A, 6B, 6D–6F, 7A, and 8C).

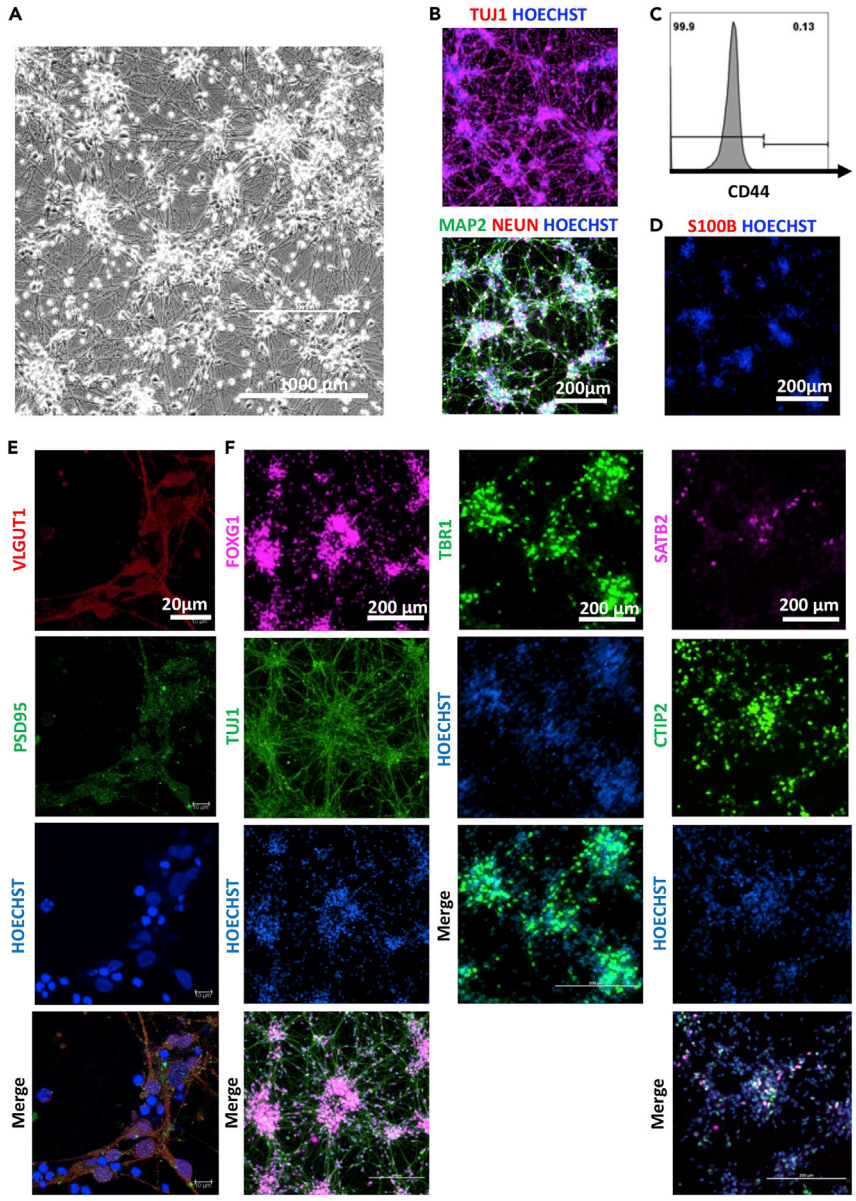

Figure 6. Characterization of MACS purified neurons

(A) Brightfield images of MACS purified neurons replated onto Cultrex® BME-coated plates.

(B) Immunofluorescence showing expression of neuron markers TUJ1, MAP2 and NEUN in MACS purified neuron population. Scale bar = 200 µm.

(C) Flow cytometry analysis showing neuron cell population is depleted for CD44.

(D) Immunofluorescence showing absence of astrocyte marker S100β in MACS purified neuron population. Scale bar = 200 µm.

(E) Immunofluorescence demonstrating expression of the presynaptic glutamate transporter VGLUT1 as well as post-synaptic protein PSD95. Scale bar = 20 µm.

(F) Immunofluorescence of neuronal populations for markers of the forebrain (FOXG1) as well as deep (TBR1, CTIP2) and superficial (SATB2) cortical layers. Scale bar = 200 µm.

Characterization of astrocytes for secretion of Apolipoprotein E (APOE)

Timing: ~1 week

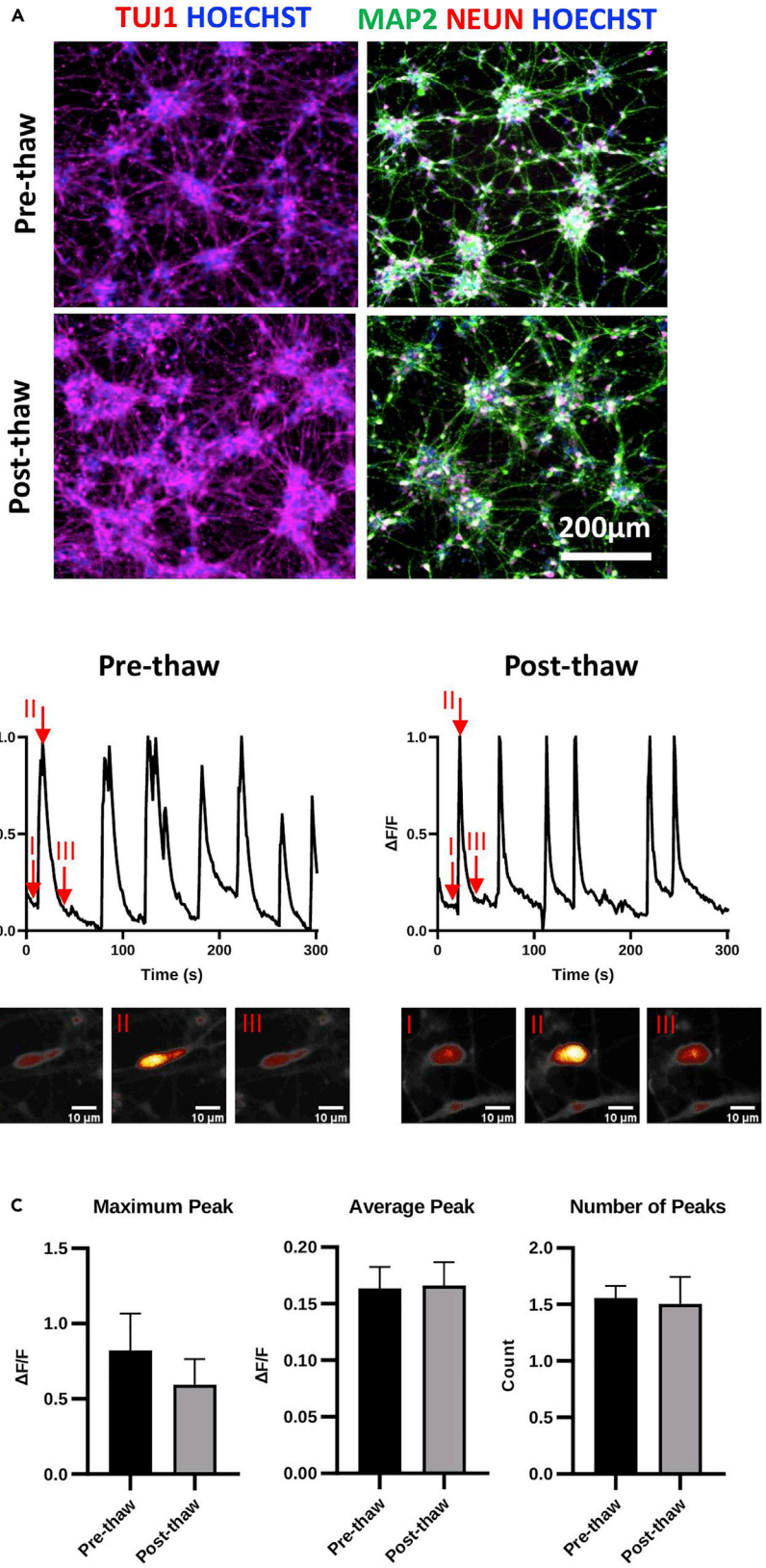
A
TUJ1 HOECHST
MAP2 NEUN HOECHST
Pre-thaw
Post-thaw
200μm
B
Pre-thaw
Post-thaw
ΔF/F
Time (s)
I
II
III
10 μm
C
Maximum Peak
Average Peak
Number of Peaks
ΔF/F
Count
Pre-thaw
Post-thaw

Figure 7. Characterization of MACS purified neurons before and after cryopreservation

(A) Immunofluorescence showing no difference in expression of neuron markers TUJ1, MAP2 and NEUN in neuron cell population before and after cryopreservation. Scale bar = 200 μm.

(B) Analysis of spontaneous calcium transients shows maintenance of functionality in neurons after cryopreservation.

(C) Quantitative analysis of spontaneous calcium transients shows no statistical differences in functionality of neurons before and after cryopreservation. Differences between groups was determined using unpaired two-sided t-test with $P < 0.05$ considered to be significant. Neurons were allowed to recover a minimum of 7 days after thawing prior to assaying.

This section describes the characterization of MACS-purified astrocytes for their ability to secrete robust amounts of APOE. APOE is a lipoprotein transporter that plays critical roles related to cholesterol transport, neuronal growth, synaptic plasticity, and cell membrane repair (Lane-Donovan and Herz, 2017; Suri et al., 2013). In the central nervous system, APOE is primarily generated and secreted by functionally mature astrocytes. This protocol describes the characterization of astrocytes plated in adherent cultures in 24-well plates. The protocol should be adjusted appropriately for expansion in other tissue culture plate formats. Prior to starting this step prepare the TBS and Cell Lysis Buffer.

228. Allow plated astrocytes to reach 60–70% confluence.
229. Aspirate the medium and replace 600 μL of fresh Astrocyte Medium.
230. Allow the cells to grow in 37°C, 5% CO_2 incubator for 6 days.
231. On day 6, collect the conditioned medium in a 1.5 mL Eppendorf tube.
    a. Centrifuge the condition medium at 200 × g for 5 min at RT.
    b. Transfer the supernatant to a fresh 1.5 mL Eppendorf tube.

Pause point: The supernatant can be stored at -80°C for 1 month.

    c. Using the manufacturer's protocol, quantify the APOE protein in the conditioned medium using the APOE ELISA.
232. Add 300 μL cell lysis buffer to each well.
    a. Use a P1000 to triturate the lysis buffer and mechanically lyse the cells.
    b. Transfer cell lysate to 1.5 mL Eppendorf tube and centrifuge at 12000 × g for 10 min at 4°C.
    c. Transfer the supernatant to a fresh 1.5 mL Eppendorf tube.

Pause point: The supernatant can be stored at -80°C for 1 month.

    d. Using the manufacturer's protocol, quantify the total protein concentration in the cell lysate using the Bradford Assay Kit.
233. For each well, normalize the APOE protein concentration to the total protein concentration (Figure 5B).

Characterization of calcium transients in MACS-purified astrocytes and neurons

Timing: 1 week

The section describes the use of fluorescent imaging for the assessment of calcium transients in MACS-purified astrocytes and neurons. A canonical property of both functionally mature astrocytes and neurons is the presence of spontaneous calcium transients (Ikegaya et al., 2005; Shigetomi et al., 2016). More specifically, astrocytes typically display slow, broad calcium transients while neurons exhibit rapid, frequent peaks of calcium transients. Prior to starting this step, prepare the BrainPhys Neuronal Medium, Neuron Calcium Imaging Solution, and Astrocyte Calcium Imaging Solution.

234. Culture neurons in NDM on Cultrex® BME-coated 24-well MatTek glass bottom plates for 7 days.

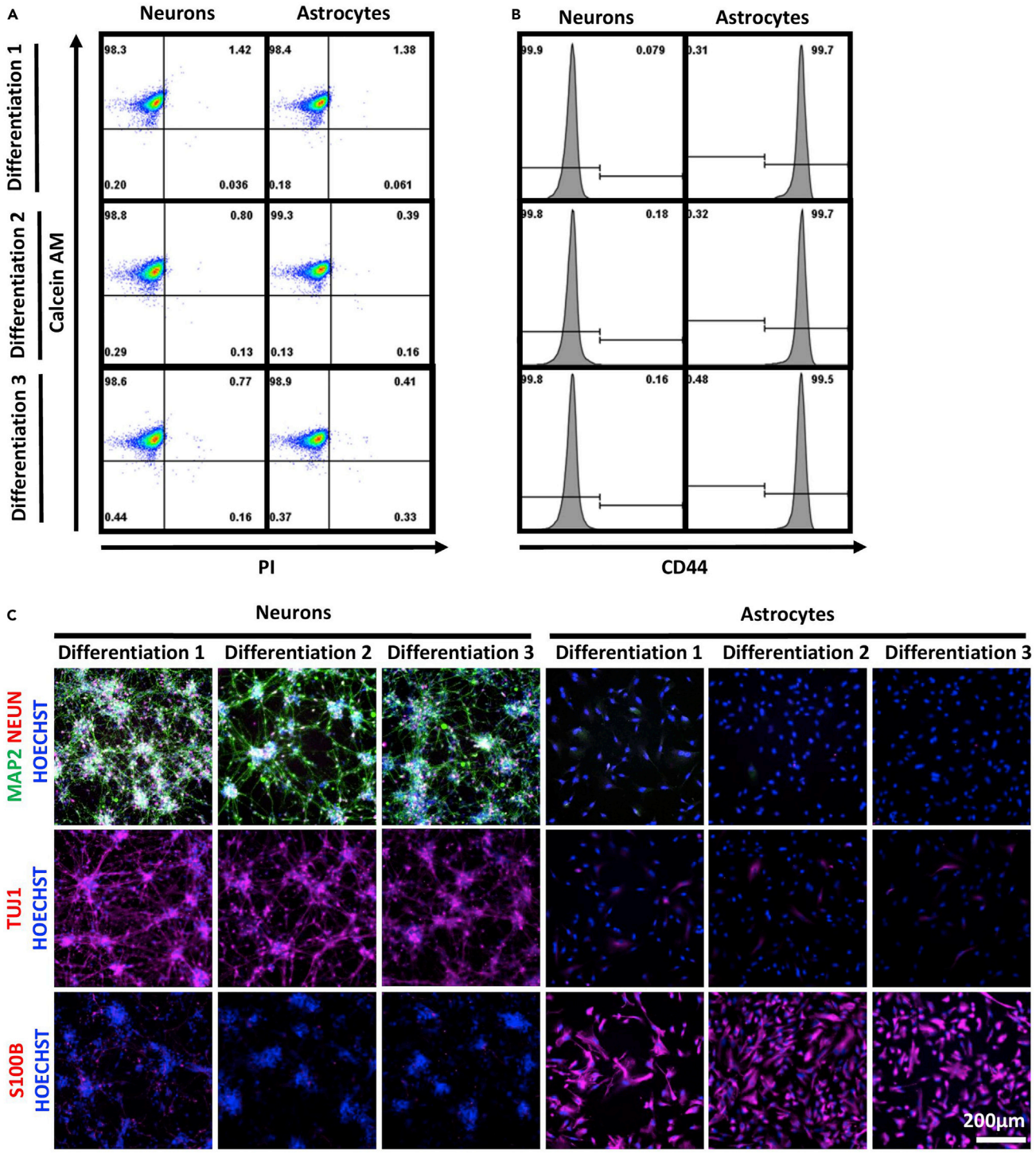

Figure 8. Comparison of marker expression across independent differentiations and MACS purifications

(A) Flow cytometry analysis of Calcein AM and PI.

(B) Flow cytometry analysis of CD44 in MACS purified neurons and astrocytes.

(C) Representative immunofluorescence staining of neuronal and astrocytic cultures from three independent differentiations and MACS purifications for neuronal markers MAP2, NEUN, and TUJ1 as well as astrocytic marker S100β.

Table 2. Parameters for calcium image analysis

Parameter	Astrocytes	Neurons
Intensity and Threshold Scaling Factor	3	4
Smoothing	3	4
Growing Z Threshold	1.2	1.2
Rising Time Uncertainty	3	5
Z-Score	10	20

a. On day 7, aspirate the NDM using a P1000 and replace with 1 mL of BrainPhys Neuronal medium.
b. Leave the neurons in BrainPhys media for 2 days.
c. On day 9, aspirate the BrainPhys-SM1 medium and add 1 mL of Neuron Calcium Imaging Solution to the well.
d. Incubate for 30 min at 37°C.
e. Aspirate the Neuron Calcium Imaging Solution and wash once with 1 mL of warm Tyrode's Solution. Leave in Tyrode's solution.
f. Incubate at RT for 15 min, protected from light.
g. Use a fluorescent microscope to image calcium transients at a frame rate of 1 per second and 20× magnification. Image for a minimum of 5 min at 37°C.

235. Culture astrocytes in Astrocyte Medium on Cultrex® BME-coated 24-well MatTek glass bottom plates for 4 days.

Note: Astrocytes should be 80–90% confluent by day 4.

a. On day 4, aspirate the Astrocyte Medium and wash the wells with 1 mL of PBS.
b. Aspirate the PBS and add 1 mL of Astrocyte Calcium Imaging Solution to the well.
c. Incubate for 15 min at 37°C.
d. Aspirate the Astrocyte Calcium Imaging Solution and wash once with 1 mL of warm Tyrode's Solution. Leave in Tyrode's solution.
e. Incubate at RT for 10 min, protected from light.
f. Use a fluorescent microscope to image calcium transients at a frame rate of 1 per second and 20× magnification. Image for a minimum of 5 min at 37°C.

236. Calcium imaging analysis.
a. Add the AQUA plugin to Fiji as described: https://github.com/yu-lab-vt/AQua.
b. Convert the .nd2 calcium imaging video to a 1024 pixel video from a 2048 pixel video. Save as .tiff file using Fiji.
c. Create a New Folder to save the results from analysis of each video.
d. Start Fiji. Open AQUA plugin.
e. Select New Project.
f. Select the video to be analyzed and the folder to save the results in. Click OK.
g. Run the program with the parameters listed in Table 2.
h. Export and save the results (Figures 5C, 5D, 7B, and 7C).

Note: For each video, the exported results will contain two files 'Aqua_Curve_Output.csv' and 'Aqua_Output_Excel.csv'. Visual images of the cells (Figures 5C and 7B) before, during, and after a calcium spike can be generated in Fiji with their respective ΔF/F graphs generated from the 'Aqua_Curve_Output.csv'. For quantification (Figures 5D and 7C), the average peak height and the maximum peak height can be extracted from the data contained in the 'Aqua_Output_excel.csv' file. The number of peaks fired (Figures 5D and 7C) can be determined by generating a 'Line Sparkline' for each event from the 'Aqua_Curve_Output.csv' file.

EXPECTED OUTCOMES

This protocol allows for the reproducible, large-scale generation of functionally mature neurons and astrocytes from hiPSCs. By combing a microcarrier (MC)-based differentiation, MACS-based purification strategies, and optimized cryopreservation methods, this protocol provides a scalable biomanufacturing system which allows for the generation of hiPSC-derived neurons and astrocytes in quantities necessary for any downstream application. Although we do not provide side-by-side comparisons to traditional 2-D approaches, the phenotypic characterization provided here and in previous publications (Brookhouser et al., 2021; Srinivasan et al., 2018) demonstrates that cells generated using MC-based approaches have similar molecular, transcriptomic, and functional properties as those generated using conventional monolayer approaches.

High quality, proliferating and well-maintained hiPSCs are required for this protocol (Figure 1A, left panels). The differentiation protocol begins by growing hiPSCs in 3-D aggregates in suspension cultures (Figure 1A, right panels). Neural induction is then achieved through the dual inhibition of BMP and TGF-β signaling and the replating of EBs to form neuroepithelial-like rosettes (Figure 1B). Human neural progenitor cells (hNPCs) can be isolated from the rosette structures and grown as proliferative, multipotent cells that express high levels of SOX1, SOX2, and NESTIN. HNPCs can also be cryopreserved without loss of viability or SOX1, SOX2, and NESTIN expression.

For large-scale neural differentiation, hNPCs are cultured in a microcarrier (MC)-based suspension culture system on a fully defined peptide substrate—termed vitronectin derived peptide (VDP). For large scale differentiations, VDP is more cost-effective and provides higher levels of reproducibility when compared to animal-derived substrates such as Cultrex® BME. HNPCs cultured on MCs will begin to form spherical aggregates with a thin layer of cells over the MCs and 3–5 MCs per aggregate. In addition, similar to when cultured on 2-D surfaces, hNPCs cultured on MCs should continue to proliferate and maintain high levels of SOX1, SOX2, and NESTIN expression (Figure 1D). To initiate neural differentiation, neurotrophic differentiation factors BDNF and GDNF are added to the medium for 30 days. Throughout the course of differentiation, the MC aggregates will continue to grow in size (Figure 2A, right panel). Unhealthy or poorly differentiated cells will appear in aggregates with rough edges or will exhibit low viability upon dissociation (Figure 2B).

After 30 days of neural differentiation, MC aggregates can be dissociated to single cells using a papain-based method. Upon dissociation, cells should display a high level of viability as measured by high levels of Calcein AM positivity and low levels of PI staining (Figure 3A). From a single 6-well plate, on average approximately 1–2 × 10^8 differentiated cells will be isolated from the MC culture. After dissociation, MACS enrichment can be used to separate the populations into neurons (CD44-) and astrocytes (CD44+) while maintaining high levels of cell viability (Figure 3B). After MACS sorting, approximately 95% of cell population will be the CD44- neuronal cell population that can either be replated or cryopreserved. Thus, a single differentiation of a 6-well of MCs should yield 1–2 × 10^8 purified neurons. The CD44+ astrocytic population, which constitutes 3–5% of the output fraction from MACS purification, is immediately replated and subsequently expanded over 4 passages to facilitate removal of the Streptavidin nanobeads. To that end, after 4 passages this initial population can be expanded to 1 × 10^8 purified astrocytes that can either be replated or cryopreserved.

MACS purified astrocytes can be replated and expanded in monolayer cultures. Flow cytometry analysis reveals that these astrocytic cultures are >95% CD44 positive (Figure 4A) and express high levels of S100β (Figure 4B). Importantly, these cultures are free from contaminating TUJ1/MAP2/NEUN-expressing neuronal cell populations (Figure 4C). Moreover, these cultures display spontaneous, slow calcium transients with long periods consistent with those observed in functionally mature astrocytes (Figures 5C and 5D). Lastly, astrocytes can be cryopreserved and thawed while maintaining high expression of astrocytic markers (Figure 5A), the ability to secrete robust amounts of APOE (Figure 5B), and their functional properties (Figures 5C and 5D).

MACS purified neurons can be replated as single cells in monolayer culture where axonal projections will begin to emerge after 2–3 days in culture (Figure 6A). Immunofluorescent staining demonstrates that the neuronal population expresses high levels of neuronal marker TUJ1 and mature neuron markers MAP2 and NEUN (Figure 6B). Additional analysis demonstrates that these neuronal cultures are free from CD44+ (Figure 6C) and S100β astrocytic populations (Figure 6D). In addition, fluorescent imaging revealed expression of the presynaptic glutamate transporter VGLUT1 and the post-synaptic protein PSD95, demonstrating the presence of excitatory glutamatergic synapses (Figure 6E). Furthermore, analysis of forebrain and cortical marker expression revealed that a high percentage of the neuronal population expressed the forebrain marker FOXG1 (Figure 6F). Characterization of specific neuronal subtypes demonstrated a heterogeneous mixture of deep-(TBR1, CTIP2) and superficial-layer (SATB2) cortical cells (Figure 6F). Finally, analysis of calcium transients reveals that replated MACS purified neurons are functionally mature displaying spontaneous, rapid, and frequent firing (Figures 7B and 7C). Importantly, MACS purified neurons can be cryopreserved and subsequently replated without any loss of neuronal marker expression (Figure 7A) or functionality (Figures 7B and 7C).

Although the data presented here was generated from healthy control hiPSCs, these methods have been applied to numerous independent hiPSC lines generated from healthy control and diseased patient samples (Brookhouser et al., 2021). More specifically, as shown in Figure 8 by flow cytometry and immunofluorescent analysis this protocol results in the generation of >95% NEUN+MAP2+ TUJ1+ neurons and >95% CD44+S100β+ astrocytes. In addition, we have shown that this protocol displays a high level of reproducibility between independent differentiations and MACS isolations (Figure 8; (Brookhouser et al., 2021)). In fact, our previous RNA-seq analysis revealed a high degree of transcriptional similarity between cells isolated from independent differentiations, MACS separations, and hiPSC lines (Brookhouser et al., 2021).

LIMITATIONS

It should be noted that some details of the protocol such as seeding densities, length of differentiation, and cell yield might vary depending on the hiPSC line used. As such, we highlight several instances in the protocol where specific culture conditions might need to be empirically determined and optimized.

Our previous transcriptomic analysis and related analysis (Figures 6E and 6F) has revealed that this protocol in its current form result in neuronal and astrocytic populations of largely cortical identity (Brookhouser et al., 2021). More specifically, this analysis revealed that these purified neurons express high levels of vesicular glutamate transporters (vGLUT) as well as markers of various cortical layers including TBR1, CTIP2 (BCL11B), BRN2 (POU3F2) and SATB2 (Figures 6E and 6F). It should be noted that a higher percentage of cells expressed markers of the deep-layer cortical neurons such as TBR1 and CTIP2 that markers of superficial-layer cortical cells such as SATB2 (Britanova et al., 2008; Saito et al., 2011). This trend is consistent with other studies that have shown that deep-layer cortical neurons tend to emerge early in differentiation than neurons of the superficial-layer (Pasca et al., 2015). We speculate that extending the differentiation time period in this protocol will increase the percentage of cells expressing deep-layer markers. It should also be noted that numerous medium formulations have been developed that result in the generation of neurons and astrocytes of various regional identities (e.g., basal forebrain cholinergic neurons, midbrain dopaminergic neurons, spinal motor neurons, striatal astrocytes; (Chang et al., 2021; Krencik and Zhang, 2011; Tao and Zhang, 2016)). To that end, we envision that the protocol described here can be employed with other differentiation mediums that lead to the generation of specific neuronal and astroglial subtypes. Along similar lines, our MACS-based protocol employs CD44, a marker of developmentally mature astrocytes (Liu et al., 2004), for the prospective purification of neuronal and astrocytic populations from heterogeneously differentiated cultures. However, the MACS-based purification described here can be employed in conjunction with other cell surface markers for the isolation of additional

neuronal and astrocytic subpopulations (Barbar et al., 2020; Garcia-Diaz et al., 2020; Pruszak et al., 2009; Samata et al., 2016; Yuan et al., 2011). Indeed, recent work has shown that cell surface markers such as CD49f can be employed to isolate astrocytes from heterogeneous cultures.

The protocol described here allows for the large-scale (~1 × 10^8 cells per 6-well pate) generation of purified neuronal and astrocytic populations without the need for specialized bioreactors. However, some applications may require the generation of the cell populations in larger quantities. To that end, we have previously shown that aspects of the MC-based culture system described in this protocol are compatible with a low shear rotating wall vessel (RWV) bioreactor (Srinivasan et al., 2018). Moving forward, this protocol can be adapted for use with other bioreactor systems (e.g., stirred suspension, vertical wheel bioreactors) or large-scale biomanufacturing paradigms (Hsu et al., 2018; Nogueira et al., 2021; Pandey et al., 2019).

Finally, we describe several biochemical, cellular, and functional assays by which the resultant neuronal populations can be characterized. We selected assays that do not require specialized equipment and can be easily employed by the typical researcher. With respect to neurons, their cellular identity can be confirmed using immunofluorescence analysis for expression of NEUN, MAP2, and TUJ1 whereas their functional properties can be assessed with calcium imaging. However, as described by us and others numerous additional assays can be employed such as Western blotting, transcriptomic analysis, microelectrode array (MEA) analysis, and patch clamp electrophysiology (Autar et al., 2022; Burke et al., 2020; Hyvarinen et al., 2019; Lin et al., 2016; Page et al., 2022). Along similar lines, with regards to purified astrocyte cultures we provide methods to assess their identity and functionality using immunofluorescence, flow cytometry, ELISA, and calcium imaging. In addition, as described by us and others these astrocytes can be characterized for additional properties including phagocytic capacity, responsiveness to inflammatory stimuli, and activation status (Barbar et al., 2020; Raman et al., 2020; Tcw et al., 2017).

TROUBLESHOOTING

Problem 1

EBs do not form in suspension culture. Step 11.

Potential solution

The optimal cell density for EB formation may vary with hiPSC lines. When using the protocol with a new hiPSC line, empirically determine the optimal cell density.

Ensure that ROCKi is added to the medium to aid in single cell survival. Be careful not to disturb the cells on the shaker for a minimum of 24 h.

Problem 2

EBs do not adhere to Cultrex® BME-coated plates. Step 17.

Potential solution

Allow the EBs to adhere for a minimum of 24 h prior to moving the plate. Be sure the EBs are evenly dispersed throughout the cell culture surface as aggregation of EBs into the center of the well can result in failure of EB attachment and rosette formation.

Problem 3

Rosettes do not detach from the plate. Step 34.

Potential solution

Increase the incubation time with the neural rosette selection reagent. Increase the washes after incubating with the neural rosette selection reagent. Be careful not to over select with the neural rosette selection reagent as that may result in poor hNPC quality.

Problem 4

HNPCs do no proliferate. Poor neural induction as shown by <75% of cells staining positive for SOX1, SOX2, and NESTIN. Step 52.

Potential solution

Ensure that healthy, pluripotent and karyotypically normal hiPSCs are used at the beginning of the differentiation. If cells do not adhere after each passage, ensure that the plates have been appropriately coated and that ROCKi is used with each passage. If cells display slow growth after each passage, the plating density might need to be optimized for each cell line. To find the optimal seeding density, plate the cells at a gradient of cell concentrations and use the density at which the cells become 70–80% confluent 3–4 days after passaging.

Problem 5

HNPCs do not adhere or grow on the MCs. Step 89.

Potential solution

If hNPCs do not adhere to the MCs, ensure that the MCs are appropriately and freshly coated with VDP. In addition, increase the amount of time cells are allowed to attach to the MCs in static conditions prior to placing the plate on the orbital shaker. However, extending time in static culture too long (>18 h) can lead to uneven seeding and poor differentiation.

Problem 6

Cells die during the differentiation. Step 91.

Potential solution

If cells die during the first few days of differentiation, this typically indicates a cell density issue. The MC seeding density might need to be optimized for each line. To find the optimal MC seeding density, plate the cells at various densities and use the density in which a thin layer of cells surround the MC aggregate approximately 4 days after seeding. If the issue persists, begin neural differentiation at different time points after seeding the hNPCs onto the MCs.

If cells die during the middle or end of differentiation, this typically indicates a cell quality issue. Ensure that hNPCs are between passages 6 and 12 before starting a differentiation. In addition, confirm that the hNPCs express high levels of SOX1, SOX2, and NESTIN. If the problem continues, remake the hNPCs.

Problem 7

Low cell yield after MACS purification. steps 141, 142.

Potential solution

Low cell yield can be indicative of poor differentiations. See troubleshooting problem 6.

Ensure that the cells during the dissociation are mechanically dissociated into single cell suspensions.

Ensure that the supernatant is being removed at each round of trituration during the dissociation. Excessive cell shearing may result in significantly fewer cells being purified.

Be careful not to leave any medium behind after decanting the CD44- fraction from the column. At this step, the cells are a high concentration so even a small volume could contain millions of cells.

Problem 8

Neurons and astrocytes do not adhere after MACS purification. steps 147, 179.

Potential solution

Low cell adhesion after MACS purification can be indicative of poor differentiation. See troubleshooting 6.

Ensure the cells are alive prior to plating by performing flow cytometry analysis for Calcein AM and PI. See troubleshooting 10.

Ensure that plates are appropriately coated with Cultrex® BME prior to cell seeding.

Some cell lines require astrocytes and neurons to be plated at higher densities. Plate cells at a gradient to find optimal seeding densities.

Problem 9

Astrocytes and neurons do not survive after thawing. steps 173, 189.

Potential solution

The viability of astrocytes and neurons post-thaw typically exceeds 95% and 75%, respectively, Calcein AM+PI- with very little variability. Lower viability indicates cells were not thawed or handled quickly enough. Cryopreservation and thawing must be performed quickly. Cell density of cryopreserved cells can also affect post-thaw viability. We recommend cryopreserving astrocytes and neurons at a density of 1×10^6 cells/vial and 20×10^6 cells/vial, respectively. However, if low cell viability is present after thawing we recommend cryopreserving and plating at higher densities.

Problem 10

Low cell viability after dissociation from the MCs. Step 199.

Potential solution

Typically, >90% of the cells are viable (i.e., Calcein AM+PI-) after dissociation (Figure 3A). Low viability after dissociation may indicate harsh trituration. Very low yield indicates quality of hNPCs was not good to start the differentiation. Another source of low cell survival after dissociation is that the MC aggregates were incubated too long. Vary the time of incubation in the Accutase (step 101) or papain enzyme solution (step 107). In addition, if the supernatant is not transferred between each round of trituration in step 113, the dissociated cells will be sheared and the cell viability will be significantly reduced. If viability is low (i.e., <80% Calcein AM+PI-) after dissociation from the MCs, we recommend restarting the differentiation and not proceeding with MACS purification.

Problem 11

Low cell purity after MACS isolation. Step 208.

Potential solution

Increasing the CD44 antibody concentration and incubation period can increase selectivity of MACS-based purification. Ensure that no more than 2.5×10^8 cells are sorted at a time. In addition, decreasing the concentration of cells during MACS isolation can increased purity and yield.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. David Brafman (David.Brafman@asu.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate any unique data sets or code.

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AUTHOR CONTRIBUTIONS

J.K., G.S., C.F., S.R., and N.B. designed the protocol, performed the experiments, and analyzed the data. A.E. performed the experiments. J.K., G.S., and C.F. wrote the manuscript. D.A.B. helped design the protocol, revised the manuscript, and supervised the study.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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