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Generation of high-purity human ventral midbrain dopaminergic progenitors for in vitro maturation and intracerebral transplantation

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We use this protocol and it's working

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Abstract

Generation of precisely patterned neural cells from human pluripotent stem cells (hPSCs) is instrumental in developing disease models and stem cell therapies. Here, we provide a detailed 16-d protocol for obtaining high-purity ventral midbrain (VM) dopamine (DA) progenitors for intracerebral transplantation into animal models and for in vitro maturation into neurons. We have successfully transplanted such cells into the rat; however, in principle, the cells can be used for transplantation into any animal model, and the protocol is designed to also be compatible with clinical transplantation into humans. We show how to precisely set the balance of patterning factors to obtain specifically the caudal VM progenitors that give rise to DA-rich grafts. By specifying how to perform quality control (QC), troubleshooting and adaptation of the procedure, this protocol will facilitate implementation in different laboratories and with a variety of hPSC lines. To facilitate reproducibility of experiments and enable shipping of cells between centers, we present a method for cryopreservation of the progenitors for subsequent direct transplantation or terminal differentiation into DA neurons. This protocol is free of xeno-derived products and can be performed under good manufacturing practice (GMP) conditions.

Materials

REAGENTS

- Accutase cell dissociation reagent (StemPro; Thermo Fisher Scientific, cat. no. A1110501)
- B-27 supplement minus vitamin A (Thermo Fisher Scientific, cat. no. 12587010) or CTS B-27 supplement minus vitamin A (Thermo Fisher Scientific, cat. no. A3353501; product will be released during 2017)
- Brain-derived neurotrophic factor (BDNF, recombinant human; Miltenyi, cat. no. 130-096-286)
- CHIR99021 (StemMACS, GSK3 inhibitor; Miltenyi, cat. no. 130-106-539)

CRITICAL :The patterning is highly sensitive to concentration and potency of the GSK3 inhibitor. If CHIR99021 from another supplier is preferred, the appropriate concentration range might vary from what we have suggested, and it should be tested through titration.

- Dibutyl-cyclic AMP (db-cAMP; Sigma-Aldrich, cat. no. D0627-1G)
- EDTA (0.5 M, pH 8.0; Thermo Fisher Scientific, cat. no. 15575020)
- N-[(3,5-difluorophenyl)acetyl]-l-alanyl-2-phenylglycine-1, 1-dimethylethyl ester (DAPT; R&D Systems, cat. no. 2634)
- Deionized sterile water (Milli-Q Integral Water Purification System for Ultrapure Water or similar)
- DMEM/F-12 (Thermo Fisher Scientific, cat. no. 21331020)
- DNase (Pulmozyme, dornase alfa; Roche, cat. no. 11899) or DNase I from bovine pancreas (Sigma-Aldrich, cat. no. DN25)
- D-PBS -Ca²⁺/-Mg²⁺ CTS (Thermo Fisher Scientific, cat. no. A1285601)
- D-PBS +Ca²⁺/+Mg²⁺ CTS (Thermo Fisher Scientific, cat. no. A1285801)
- FGF-8b (recombinant human, premium grade; Miltenyi, cat. no. 130-095-740)
- GDNF (recombinant human; R&D Systems, cat. no. 212-GD-010)
- Hanks Balanced Salt Solution (HBSS; Thermo Fisher Scientific, cat. no. 14175095)
- hPSC lines of choice. In this study, we have used the following human embryonic stem cell (hESC) lines: RC17 (Roslin Cells, cat. no. hPSCreg RCe021-A), H9 (WiCell, cat. no. hPSCreg WAe009-A), MShef7, Mshef13 (both from the University of Sheffield) and HS980, HS983a, HS999 and HS1001 (all from Karolinska Institutet)

CAUTION hPSCs should routinely be tested to ensure that they are authentic and free from mycoplasma infection.

- Human serum albumin solution (HSA, 10% (wt/vol); Irvine Scientific, cat. no. 9988)
- iPS-Brew XF (StemMACS; Miltenyi, cat. no. 130-104-368)
- L-Ascorbic acid (AA; Sigma-Aldrich, cat. no. A4403-100MG)
- L-Glutamine (200 mM; Thermo Fisher Scientific, cat. no. 25030081)
- Laminin-521 (Lam-521; 100 µg/ml; Biolamina, cat. no. LN-521)
- Laminin-111 (Lam-111; 100 µg/ml; Biolamina, cat. no. LN-111)
- N-2 supplement CTS (Thermo Fisher Scientific, cat. no. A1370701)
- Neurobasal CTS (Thermo Fisher Scientific, cat. no. A1371201)
- Noggin (recombinant human; Miltenyi, cat. no. 130-103-456)

CRITICAL Many neural differentiation protocols substitute recombinant Noggin with the small-molecule ALK inhibitor LDN-193189 to lower costs. However, we find that the use of LDN in place of Noggin results in contamination of the cultures with non-neural populations, and we recommend adhering to the use of Noggin in the protocol.

- Penicillin-streptomycin (10,000 U/ml, Thermo Fisher Scientific, cat. no. 15140122)

- Purmorphamine (StemMACS, SHH agonist; Miltenyi, cat. no. 130-104-465)
- SB431542 (StemMACS, TGF β inhibitor; Miltenyi, cat. no. 130-106-543)
- SHH-C24II (recombinant human, premium grade; Miltenyi, cat. no. 130-095-727)

CRITICAL SHH-C24II is a high-potency form of N-terminal SHH, carrying a cysteine to isoleucine–isoleucine substitution, which results in a lipophilic moiety and therefore a highly increased potency of the peptide. SHH-C24II should not be replaced by unmodified N-terminal SHH, as the lower activity of the latter will result in insufficient ventralization.

- Y-27632 dihydrochloride (StemMACS, ROCK inhibitor; Miltenyi, cat. no. 130-106-538)

EQUIPMENT

- Aluminum foil (VWR, cat. no. 89079-069)
- Biological safety cabinet, class II (Thermo Scientific Holten laminAir or similar)
- Bürker chamber (VWR, cat. no. 631-0921 or similar)
- Cell culture centrifuge (for 1.5-, 15- and 50-ml tubes; Beckman Coulter, model no. Allegra 21 or similar)
- Cell culture incubator set to 37 °C and 5% CO₂ (Thermo Fisher Scientific, model no. 3541 DH or similar)
- Countess II automated cell counter (Thermo Fisher Scientific, cat. no. AMQAX1000)
- Filter unit (0.22 μ m; VWR)
- Freezers operating at –20, –80 and below –140 °C (Thermo Scientific, ES Series Combination Lab Refrigerator/Freezer, Thermo Scientific TSE Series –86 °C, model no. 936, Panasonic Cryogenic ULT Freezer, model no. MDF-C2156VAN-PE or similar)
- Refrigerator operating at 4 °C Lam-521-coated plates Lam-111-coated plates Micro tube (1.5 ml; Sarstedt, cat. no. 72.692.005)
- Lam-521-coated plates
- Lam-111-coated plates
- Micro tube (1.5 ml; Sarstedt, cat. no. 72.692.005)
- Parafilm M (VWR, cat. no. 52858-032)
- Phase-contrast inverted microscope (Olympus, model no. CKX31SF or similar)
- Pipette controller (for pipetting volumes of 2–25 ml; Corning Stripettor Ultra Pipet Controller, model no. 4099 or similar)
- Pipette tips (for pipetting volumes of 0.5–1,000 μ l; TipOne Pipette Tips: 1250- μ l XL graduated tip, cat. no. S1112-1830; 200- μ l beveled tip, cat. no. S1111-1700; 10- μ l graduated tip, cat. no. S1111-3700)
- Pipettes (for pipetting volumes of 0.5–1,000 μ l; Thermo Scientific, Finnpiquette F2 variable-volume single-channel pipettes)
- Serological pipette (5 ml; Sarstedt, cat. no. 86.1253.001)
- Serological pipette (10 ml; Sarstedt, cat. no. 86.1254.001)
- Serological pipette (25 ml; Sarstedt, cat. no. 86.1685.001)
- Tissue-culture treated plastic plates (6-, 12-, 24- 48- and 96-well, Sarstedt, Greiner or Falcon plates)
- Cell culture flasks (25 cm², CellStar TC; Greiner Bio-One, cat. no. 690175)
- Tube (50 ml; Sarstedt, cat. no. 62.547.254)
- Tube (15 ml; Sarstedt, cat. no. 62.554.504)

REAGENT SETUP

I-Ascorbic acid

Make 200 mM stock aliquots in deionized sterile water and store them at $-20\text{ }^{\circ}\text{C}$ for up to 1 year. Thawed aliquots can be kept at $4\text{ }^{\circ}\text{C}$ for 1 month.

DNase

(DNase I from bovine pancreas) Dilute to 0.5% (wt/vol) in HBSS and filter the solution. Prepare aliquots and store stocks at $-20\text{ }^{\circ}\text{C}$ for up to 4 years. Thawed aliquots can be kept at $4\text{ }^{\circ}\text{C}$ for 1 week.

EDTA

Dilute EDTA to 0.5 mM in PBS ($-\text{Ca}^{2+}/-\text{Mg}^{2+}$) and store it at room temperature (RT; $20\text{--}25\text{ }^{\circ}\text{C}$) for up to 1 year.

iPS-Brew

To obtain complete medium, thaw StemMACS iPS-Brew XF, 50 $\times$ supplement at $4\text{ }^{\circ}\text{C}$ and add 10 ml to 500 ml of StemMACS iPS-Brew XF, basal medium. Mix well and divide the solution into aliquots in 50-ml tubes. The complete medium can be kept at $4\text{ }^{\circ}\text{C}$ for up to 2 weeks and can be stored at $-20\text{ }^{\circ}\text{C}$ for 3 months. To avoid degradation of the medium component, keep the medium protected from light by wrapping the tube in aluminum foil.

Recombinant human Noggin

Reconstitute in deionized sterile water to 1 mg/ml, and then dilute it further to 100 $\mu\text{g}/\text{ml}$ with 0.1% (wt/vol) HSA in PBS ($-\text{Ca}^{2+}/-\text{Mg}^{2+}$). Prepare aliquots and store them at $-20\text{ }^{\circ}\text{C}$ until the expiration date indicated on the vial label. Thawed aliquots can be kept at $4\text{ }^{\circ}\text{C}$ for 1 month.

Db-cAMP

Dissolve 1 g of db-cAMP in 40.7 ml of deionized sterile water to obtain a concentration of 50 mM. Filter-sterilize the solution, prepare aliquots and store them at $-20\text{ }^{\circ}\text{C}$ for up to 1 year. Thawed aliquots can be kept at $4\text{ }^{\circ}\text{C}$ for 1 month.

Recombinant human GDNF

Dilute in 0.1% (wt/vol) HSA in PBS ($-\text{Ca}^{2+}/-\text{Mg}^{2+}$) to obtain a concentration of 20 $\mu\text{g}/\text{ml}$. Prepare aliquots and store them at $-20\text{ }^{\circ}\text{C}$ for up to 3 months. Thawed aliquots can be kept at $4\text{ }^{\circ}\text{C}$ for 1 month.

BDNF

Reconstitute BDNF in deionized sterile water to 200 $\mu\text{g}/\text{ml}$, and then dilute it further to 20 $\mu\text{g}/\text{ml}$ with 0.1% (wt/vol) HSA in PBS ($-\text{Ca}^{2+}/-\text{Mg}^{2+}$). Prepare aliquots and store them at $-20\text{ }^{\circ}\text{C}$ until the expiration date indicated on the vial label. Thawed aliquots can be kept at $4\text{ }^{\circ}\text{C}$ for 1 month.

Recombinant human FGF-8b

Reconstitute FGF-8b in deionized sterile water to 1 mg/ml, and then dilute it further to 100 $\mu\text{g}/\text{ml}$ with 0.1% (wt/vol) HSA in PBS ($-\text{Ca}^{2+}/-\text{Mg}^{2+}$). Prepare aliquots and store them at $-20\text{ }^{\circ}\text{C}$ until the expiration date indicated on the vial label. Thawed aliquots can be kept at $4\text{ }^{\circ}\text{C}$ for 1 month.

Recombinant modified human SHH-C24II

Reconstitute SHH-C24II in deionized sterile water to 1 mg/ml, and then dilute it further to 100 $\mu\text{g}/\text{ml}$ with 0.1% (wt/vol) HSA in PBS ($-\text{Ca}^{2+}/-\text{Mg}^{2+}$). Prepare aliquots and store them at $-20\text{ }^{\circ}\text{C}$ until the expiration date indicated on the vial label. Thawed aliquots can be kept at $4\text{ }^{\circ}\text{C}$ for 1 month.

CHIR99021

Make 7- μl aliquots of CHIR99021 (10 mM) and store them at $-20\text{ }^{\circ}\text{C}$ for up to 6 months. These aliquots can be thawed and refrozen up to three times; exposure to light should be minimized to avoid degradation. Before CHIR99021 is applied in the differentiation medium, it can be diluted 1:100 in N2 medium: add 297 μl of N2 medium to a 1.5-ml tube and then add 3 μl of 10 mM CHIR99021 stock, and mix thoroughly with a pipette. Take caution to perform the dilutions using accurately calibrated pipettes, preferentially using the same pipette each time to

minimize any variation. Always use freshly prepared dilutions of CHIR99021 for each medium change, and never store aliquots at 4 °C.

DAPT

Reconstitute the powder in DMSO to obtain a concentration of 10 mM. Prepare aliquots and store them at –20 °C for up to 1 year. Thawed aliquots can be kept at 4 °C for 1 month.

Purmorphamine

Reconstitute the powder in DMSO to obtain a concentration of 10 mM. Prepare aliquots and store them at –20 °C for up to 1 year. Thawed aliquots can be kept at 4 °C for 1 month.

N2 medium

To prepare 40 ml of N2 medium, mix 19.6 ml of DMEM/F12 and 19.6 ml of neurobasal CTS medium and supplement it with 0.4 ml of N-2 supplement (final concentration = 1% vol/vol) and 0.4 ml of l-glutamine (final concentration = 2 mM). The addition of 80 µl of penicillin–streptomycin (final concentration = 0.2% (vol/vol)) is optional. N2 medium can be kept at 4 °C for up to 2 weeks.

B27 medium

To prepare 40 ml of B27 medium, supplement 38.7 ml of neurobasal CTS medium with 0.8 ml of B-27 supplement without vitamin A (final concentration = 2% (vol/vol)) and 0.4 ml of l-glutamine (final concentration = 2 mM). The addition of 80 µl of penicillin–streptomycin (final concentration = 0.2% (vol/vol)) is optional. B27 medium can be kept at 4 °C for up to 2 weeks.

Wash medium

To prepare 100 ml of wash medium, supplement 90 ml of DMEM/F12 with 10 ml of 10% (wt/vol) HSA (final concentration = 1%). Wash medium can be kept at 4 °C for up to 2 weeks.

EQUIPMENT SETUP

Lam-521-coated plates

Coat wells with Lam-521 (0.5 µg/cm²) in PBS (+Ca²⁺/+Mg²⁺) (total volume of 900 µl in a 12-well plate or 2 ml in a sixwell plate). If you are using only a few wells, PBS and Lam-521 are preferably mixed directly in the wells. In this case, shake the plate thoroughly to ensure homogeneous coating. For slow coating, wrap the plate in Parafilm and store it at 4 °C for 1–7 d and place the plate at 37 °C for 1–2 h before use. For urgent need, fast coating can be performed by placing the plate directly at 37 °C for 2 h.

CRITICAL If Lam-521 and PBS are premixed before coating, make sure to use polypropylene and not polystyrene vials to avoid adherence of laminin to the vial.

CRITICAL If the plates are stored for >24 h at 4 °C, make sure that they do not dry out.

Lam-111-coated plates

Coat wells with Lam-111 (1 µg/cm²) in PBS (+Ca²⁺/+Mg²⁺) (total volume of 55 µl in 96-well plates, 320 µl in 24-well plates or 4.5 ml in a T25 flask). If you are using only a few wells, PBS and Lam-111 are preferably mixed directly in the wells. In this case, shake the plate thoroughly to ensure homogeneous coating. For slow coating, wrap the plate in Parafilm and store it at 4 °C for 1–7 d and place the plate at 37 °C for 1–2 h before use. For urgent need, fast coating can be performed by placing the plate directly at 37 °C for 2 h.

CRITICAL (same as above)

Safety warnings

 For safety and hazards information, see the Safety Data Sheet (SDS).

Maintenance of hPSCs before start of differentiation

1w

- 1 Maintain the pluripotent hPSCs on Lam-521 (0.5 $\mu\text{g}/\text{cm}^2$) in iPS-Brew medium. Cells can be passaged every 7 days with [IM] 0.5 millimolar (mM) EDTA , followed by seeding at a density of 2,500 cells per cm^2 with ROCK inhibitor ([IM] 10 micromolar (μM) Y-27632) included in the medium for the first ⌚ 24:00:00 after plating.

1d



Note

CRITICAL STEP For low-density seeding of hPSCs, the addition of ROCK inhibitor to the medium for the first ⌚ 24:00:00 after replating will enhance the survival of the cells. If the hPSCs are passaged as bigger clusters or at high density, ROCK inhibitor is less critical to the cell survival. For this protocol, we recommend that ROCK inhibitor always be added to the medium when passaging the cells, for increased reproducibility and standardization of the replating steps.

- 2 Passage the cells 3–5 days before the planned start of differentiation, with the aim of having cultures covering 70–90% of the well area on the day of starting differentiation. Approximate seeding densities to use at passage are as follows: day –3, 25,000 cells per cm^2 ; day –4, 15,000 cells per cm^2 ; and day –5, 10,000 cells per cm^2 . A single well of a 12-well plate will suffice for most differentiation purposes.

Differentiation of hPSCs into mesDA progenitors

- 3 *Day 0: seeding of cells.* Before the differentiation is started, make sure that the hPSC colonies appear pluripotent by visual criteria (i.e., homogeneous-appearing colonies with clear borders and the absence of obvious differentiating zones). If you are using a hPSC culture system other than the one described here, manual removal of spontaneously differentiated colonies might be required. A desirable confluency of hPSC cultures is 70–90% of the well area at the start of differentiation.
- 4 Prepare an adequate volume of differentiation medium to start your differentiation: N^2 medium + Y-27632 ([IM] 10 micromolar (μM)) + SB431542 ([IM] 10 micromolar (μM)) + Noggin ([IM] 100 Mass Percent).



For patterning to ventral mesencephalic fates, also add

[IM] 300 Mass Percent Shh-C24II and [IM] 0.5 micromolar (μM) –

[IM] 1.0 micromolar (μM) CHIR99021 to the medium. For setting up the differentiation,

you will need 250 $\mu\text{L}/\text{cm}^2$ (500 μL per well in a 24-well plate and 6.25 mL for a T25 flask).

Note

CRITICAL STEP The concentration of CHIR99021 is highly sensitive and must be optimized for each cell line, but should normally be in the range of 0.6–1 μM . Before it is applied, dilute it 1:100 and add, e.g., 8 μL per 1 mL of N^2 medium to achieve a concentration of 0.8 μM . See Reagent Setup for correct handling of the compound.

- 5 Aspirate the iPS-Brew medium from the cells and wash once with PBS ($-\text{Ca}^{2+}/-\text{Mg}^{2+}$). Add a minimal volume of EDTA to the cells (i.e., 75 $\mu\text{L}/\text{cm}^2$). Incubate at 37 $^{\circ}\text{C}$ for 00:07:00 and rock the plate gently occasionally to ensure that all cells are submerged in EDTA. 7m
- 6 In the meantime, prepare a 15-ml tube with 10 mL wash medium .
- 7 After exactly 00:07:00 , remove the EDTA from the cells and immediately add 1 mL wash medium to the well and pipette off the colonies from all sides of the well using a P1000 pipette. Triturate the cells to yield homogeneously sized colonies while avoiding introduction of bubbles. The cell suspension should contain a mix of single cells and small cell clumps with two to six cells per clump. Transfer the colonies to the 15-ml tube containing the wash medium, and wash the wells again to collect any remaining colonies. The 15-ml tube should now contain a total of 10 mL cell suspension in wash medium. 7m
- 8 Spin down the cells at 400 x g, Room temperature, 00:05:00 and resuspend the cell pellet in 1 mL new wash medium . Perform two separate live-cell counts using a hemocytometer or an automated cell counter. If the cell suspension is too dense for accurate counting, it can be further diluted in wash medium. If the counts are very inconsistent, they should be redone. Calculate the mean achieved from the two counts and determine the concentration of cells per mL.
- 9 From the cell count, determine the total volume of cell suspension you will need to initiate your differentiation with 10,000 cells per cm^2 (i.e., to start differentiation in 6x

wells of a 24-well plate you will need a total of 120,000 cells and to start differentiation in a T25 flask you will need 250,000 cells). Transfer the needed volume of cell suspension to a new tube, and spin down the cells at 400 x g, Room temperature, 00:05:00 .

- 10 Aspirate the wash medium and resuspend the cells in premixed differentiation medium (from Step 4) to yield a cell suspension of 40,000 cells per ml. Aspirate the Lam-111 solution from the coated wells and seed the cell suspension onto the wells at a density of 10,000 cells per cm^2 (i.e., 250 $\mu\text{L}/\text{cm}^2$).

Note

CRITICAL STEP To ensure even seeding of cells, thoroughly shake the plate in all directions before placing the plate in the incubator. Dense areas of colonies will not result in good differentiation.

- 11 *Day 2: medium change.* Remove old medium and add new medium to the cells: N^2 medium + SB431542 (10 micromolar (μM)) + Noggin (100 Mass Percent) + 0.6 micromolar (μM) – 1.0 micromolar (μM) CHIR99021 + 300 Mass Percent Shh-C24II . Add 250 μL medium per cm^2 .

Note

CRITICAL STEP The N^2 medium can be kept for 2 weeks at 4 $^{\circ}\text{C}$, but the growth factors and small molecules must be freshly added for each medium change.

- 12 *Day 4: medium change.* Remove old medium and add new medium to the cells: N^2 medium + SB431542 (10 micromolar (μM)) + Noggin (100 Mass Percent) + 0.6 micromolar (μM) – 1.0 micromolar (μM) CHIR99021 + 300 Mass Percent Shh-C24II . Add 300 μL medium per cm^2 .

- 13 *Day 7: medium change.* Remove old medium and add new medium to the cells: N^2 medium + SB431542 (10 micromolar (μM)) + Noggin (100 Mass Percent) + 0.6 micromolar (μM) – 1.0 micromolar (μM) CHIR99021 + 300 Mass Percent Shh-C24II . Add 350 μL medium per cm^2 . At this stage, the colonies should be 70–100% confluent.



- 14 *Day 9: medium change.* Remove old medium and add new medium to the cells: N² medium + FGF8b (100 Mass Percent). Add 400 µL medium per cm².

Note

CRITICAL STEP The timing of FGF8b addition might need to be optimized when using a new cell line. For some cell lines, FGF8b should be added only at day 11 and onward, as it could interfere with the VM patterning. However, if added too late, a proper caudal VM patterning might not be obtained.

- 15 *Day 11: replat the cells.* Wash the cells twice with PBS (–Ca²⁺/–Mg²⁺) to get rid of dead and floating cells. Add a minimal volume of Accutase (i.e., 75 µl/cm²). Leave the plate at 37 °C for 00:10:00 and rock the plate gently when half of the time has passed to ensure that all cells are submerged in Accutase.

10m



- 16 While the cells are incubating, prepare a 15-ml tube with 10 mL wash medium .

- 17 After 00:10:00 , dissociate the cells with a 1-ml pipette to yield a single-cell solution. If the cells are not easily detaching from the well, leave the plate for up to 00:10:00 more at 37 °C . When the cells are dissociated, transfer them to the 15-ml tube of wash medium and use 1 mL wash medium to collect any residual cells from the well. Spin down the cells at 400 x g, Room temperature, 00:05:00 .

20m



- 18 After spinning, aspirate the medium without disturbing the cell pellet, resuspend the pellet in 1 mL N2 medium and then move the cell suspension to a 1.5-ml tube. Remove a 10 µL aliquot for counting and dilute it in N² medium to get an estimated cell concentration of ~0.5–2 × 10⁶ cells per ml in order to facilitate the counting (1 well of a 24-well plate will yield ~2–4 × 10⁶ cells on day 11, depending on the cell line). Perform two separate live cell counts using a hemocytometer or an automated cell counter. If the counts are very inconsistent, they should be re-done. Calculate the mean achieved from the two counts and determine the concentration of cells per ml.
- 19 Spin down the cells at 400 x g, Room temperature, 00:05:00 . In the meantime, prepare an adequate volume of B27 medium + Y-27632 (10 micromolar (µM)) + (





[M] 20 Mass Percent BDNF) + AA ([M] 0.2 millimolar (mM)) + FGF8b ([M] 100 Mass Percent) to resuspend the cells at a density of 1.4×10^6 cells per ml.

- 20 After the spin, resuspend the cells in the supplemented B27 medium. Aspirate the Lam-111 solution from the coated plates and directly seed the cells at a density of 800,000 cells per cm^2 (i.e.,  570 μL cell suspension per cm^2). To assess progenitor cell identity, seed cells on a separate 96- or 48-well plate for fixation and immunocytochemical analysis on day 14 or day 16.

Note

CRITICAL STEP To ensure even seeding of cells, thoroughly shake the plate in all directions before putting the plate in the incubator

- 21 *Day 14: medium change.* Remove old medium and add new medium: B27 medium + [M] 20 Mass Percent BDNF + AA ([M] 0.2 millimolar (mM)) + [M] 100 Mass Percent FGF8b . Add  600 μL medium per cm^2 .
- 22 On days 14–16, assess the progenitor cell identity by immunocytochemical analysis. The earlier time point can be used for pretransplantation QC if the cells are to be grafted on day 16. To further pinpoint the identity of the cells, RNA should be collected from the cells for qRT–PCR analysis on day 16.
- 23 On day 16, the patterning to mesDA progenitors is complete and you can proceed to freezing the cells, transplanting the cells (option A) or replating them for terminal differentiation (Option B).

STEP CASE

Preparation of cells for transplantation

9 steps

- 24 Prepare a vial with HBSS + DNase (if using Pulmozyme, dilute the stock product 1:6 in HBSS; if using bovine DNase, dilute the aliquotted stock 1:10 in HBSS). You will need a total of ~2 ml for the procedure.
- 25 Dissociate the cells to a single-cell suspension and wash as in "Differentiation of hPSCs into mesDA progenitors" Steps 15–17 (shown again in the next substeps). After centrifugation, resuspend the cells in  1 mL HBSS + DNase +  100 μL wash medium .

25.1 Wash the cells twice with PBS ($-Ca^{2+}/-Mg^{2+}$) to get rid of dead and floating cells. Add a minimal volume of Accutase (i.e., $75 \mu\text{L}/\text{cm}^2$). Leave the plate at 37°C for 00:10:00 and rock the plate gently when half of the time has passed to ensure that all cells are submerged in Accutase.

10m



25.2 While the cells are incubating, prepare a 15-ml tube with 10 mL wash medium .

25.3 After 00:10:00 , dissociate the cells with a 1-ml pipette to yield a single-cell solution. If the cells are not easily detaching from the well, leave the plate for up to 00:10:00 more at 37°C . When the cells are dissociated, transfer them to the 15-ml tube of wash medium and use 1 mL wash medium to collect any residual cells from the well. Spin down the cells at 400 x g, Room temperature, 00:05:00 . After centrifugation, resuspend the cells in 1 mL HBSS + DNase + 100 μL wash medium .

20m



26 Transfer the 1,100- μL cell suspension to a 1.5-ml tube and perform cell counting as in "Differentiation of hPSCs into mesDA progenitors" Step 18 (shown again in the next substep). In the meantime, spin down the cells at 400 x g, Room temperature, 00:05:00 .



26.1 After spinning, aspirate the medium without disturbing the cell pellet, resuspend the pellet in 1 mL N2 medium and then move the cell suspension to a 1.5-ml tube. Remove a 10 μL aliquot for counting and dilute it in N^2 medium to get an estimated cell concentration of $\sim 0.5\text{--}2 \times 10^6$ cells per ml in order to facilitate the counting (1 well of a 24-well plate will yield $\sim 2\text{--}4 \times 10^6$ cells on day 11, depending on the cell line). Perform two separate live cell counts using a hemocytometer or an automated cell counter. If the counts are very inconsistent, they should be re-done. Calculate the mean achieved from the two counts and determine the concentration of cells per ml.

27 Aspirate the medium carefully and resuspend the cells to a concentration of 37,500–75,000 cells per μL in HBSS + DNase. To obtain the correct volume, first add 10 μL less volume than needed, to take into account residual volume in the pellet. Then measure the volume with a pipette and adjust the volume as needed.

28 Leave cells On ice for transplantation. For transplantation into the adult rat striatum, we recommend grafting $\sim 150,000\text{--}300,000$ cells.





Note

CRITICAL STEP If cells are kept on ice for >3 h, the viability may be reduced. If many animals are to be transplanted, several cell preparations can be prepared to reduce the time the cells are on ice.