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iPSC Protocols

DA or Cortical Neuron Differentiation

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1 Introduction

1.1 Objectives

This protocol describes how to:

- Culture dopaminergic (DA) or cortical neural progenitor cells (NPCs)
- Generate DA or cortical neurons from DA or cortical NPCs, respectively

1.2 Protocol overview

By using a combination of small molecules that regulate multiple signalling pathways, we have adapted a method to guide human induced pluripotent stem cells (iPSCs) to a population of DA or cortical NPCs, which can be differentiated into an enriched population of functionally mature DA or cortical neurons, respectively, after 4 to 6 weeks in culture (Figure 1).

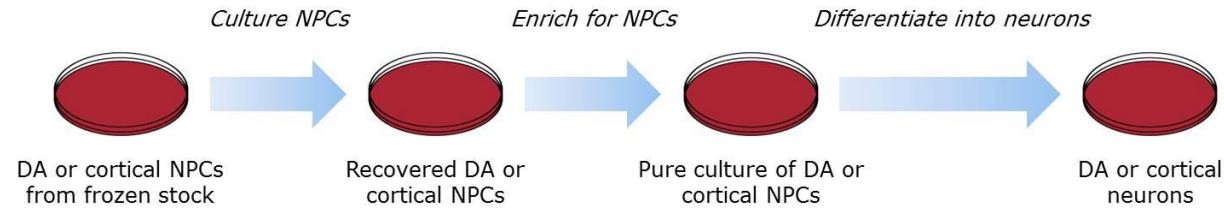


Figure 1. Protocol overview for the differentiation of DA or cortical neurons from DA or cortical NPCs, respectively.

1.3 Technical and safety considerations

The following information should be read before starting:

- NPCs and neurons must be handled within a Class II biosafety laminar flow hood to protect the worker from possible adventitious agents. McGill University Environmental Health and Safety (EHS) office regulations must be followed.
- The quality of materials used in this protocol is critical to its success. Refer to the Materials section for recommended suppliers and catalogue numbers.
- Work with 1 cell line at a time to avoid errors and having to leave cells unattended for too long during procedures.
- NPCs may be recovered from a frozen stock or generated from iPSCs.
- All media should be prepared fresh and only in the amount needed for that day. Excess media may be stored at 4°C for up to 10 days but it is preferable to use freshly prepared media.
- NPCs and neurons must be cultured on surfaces coated with poly-L-ornithine (PO) and laminin (except during 2-day suspension culture of NPCs to enrich for NPCs).

- Take extra precautions to maintain sterility:
  - Aspirate media using a 1000 µL tip on top of a 1-mL plastic serological pipet and change to a new tip frequently.
- Manipulate cells gently:
  - Add and aspirate media to vessels slowly and resuspend cells slowly. If possible, avoid adding media directly onto cells (e.g. dispense media onto the upper interior surface of the flask or onto the side of well of the plate).
  - Mix cells in a tube by pipetting slowly a few times or by gently inverting. Do not over-pipette cells.
- Maintain a stable culture environment for cells during incubation:
  - Culture vessels should be placed toward the back of the cell culture incubator shelf to maintain stable temperature and CO_2 levels when the door of the incubator is opened and closed.
  - When dissociating cells, use a different 37°C incubator than cell culture incubator to minimize opening and closing the door of the cell culture incubator. Note that the dissociation incubator should be sterile but does not require a CO_2 supply.
- DA and cortical NPC cultures must be monitored regularly to ensure optimal morphology and density prior to differentiation.

2 Materials

The quality of materials used in this protocol is critical to its success. The suppliers and catalogue numbers listed in this section allowed for successful preparation of DA or cortical neurons that expressed DA or cortical markers, respectively. There is significant lot-to-lot variability in the quality of materials which can negatively impact neuronal cultures. Note that specific lots may no longer be available.

Refer to the product datasheet from the supplier for further details on storage and preparation instructions.

2.1 Labware

Item	Supplier	Catalogue #
Conical tube, 15-mL	ThermoFisher	352097
Cryovial	Sarstedt	72.379
Culture dish, 100-mm	ThermoFisher	08772E
Culture dish, 60-mm	Eppendorf	0030701119
Culture flask, T25	ThermoFisher	12-556-009
Culture flask, T75	ThermoFisher	12-556-010
Culture plate, 12-well	ThermoFisher	0877229
Culture plate, 24-well	ThermoFisher	087721
Culture plate, 6-well	ThermoFisher	087721B
Culture plate, 96-well (flat-bottom)	Falcon	353219
Glass coverslip, 12-mm	ThermoFisher	12-545-80
Glass coverslip, Deckglaser	Paul Marlenfeld GmbH&Co.KG	0111520
Petri dish, 100-mm	ThermoFisher	FB0875712
Plastic serological pipet, 10-mL	Sarstedt	86.1254.001
Plastic serological pipet, 1-mL	Fisher	13-678-11B
Plastic serological pipet, 5-mL	Sarstedt	86.1253.001
Polypropylene microcentrifuge tube	Fisher	02-681-273

2.2 Culture reagents

Working aliquots of culture reagents that require storage at 4°C can be stored for up to 2 weeks.

Item	Supplier	Catalogue #	Stock conc.	Working conc.	Storage temp.
Antibiotic-Antimycotic	Gibco	15240-062	100x	1x	Stock: –20°C Working: 4°C
Ascorbic acid (AA)	Sigma	A5960-25G	200 mM	200 µM	Stock: –80°C Working: 4°C
B-27*	Gibco	17504044	50x	1x	Stock: –20°C Working: 4°C
BDNF	Peprotech	450-02	20 µg/mL	20 ng/mL	Stock: –80°C Working: 4°C
Compound E (γ-secretase inhibitor)*	STEMCELL Technologies	73954	0.1 mM	0.1 µM	Stock: –80°C Working: 4°C
db-cAMP	Carbosynth	ND07996	0.5 M	0.5 mM	Stock: –80°C Working: 4°C
DMEM/F12	Gibco	10565-018	1x	1x	4°C
DMSO	Fisher	BP231-1	100%	10%	Room temperature
EGF	Peprotech	AF-100-15	10 µg/mL	10 ng/mL	Stock: –80°C Working: 4°C
FBS	Gibco	12484-028	1x	1x	Stock: –80°C Working: Room temperature
FGF-b	Peprotech	100-18B	10 µg/mL	10 ng/mL	Stock: –80°C Working: 4°C
GDNF	Peprotech	450-10	20 µg/mL	20 ng/mL	Stock: –80°C Working: 4°C
Gentle Cell Dissociation Reagent	STEMCELL Technologies	07174	1X	1X	Room temperature
Laminin†	Invitrogen	23017-015	1 mg/mL‡	Culture vessel coating: 5 µg/mL Culture media: 1 µg/mL	Stock: –80°C Working: 4°C
	Sigma	L2020			

Item	Supplier	Catalogue #	Stock conc.	Working conc.	Storage temp.
MEM nonessential amino acid (NEAA) solution	Wisent	321-011-EL	100x	1x	4°C
Mitomycin C	Sigma	M4287	1 mg/mL	1 µg/mL	Stock: –80°C Working: 4°C
N-2*	Gibco	17502048	100x	1x	Stock: –20°C Working: 4°C
Neurobasal (NB) media	Life Technologies	21103-049	1x	1x	4°C
Poly-L-ornithine (PO)	Sigma	P3655	1 mg/mL	10 µg/mL	Stock: –20°C Working: 4°C
Purmorph-amine§	Sigma	SML-0868	2 mM	2 µM	Stock: –80°C Working: 4°C
StemPro Accutase Cell Dissociation Reagent	Thermo-Fisher	A11105 01	1X	1X	Stock: –20°C Working: 37°C
PBS	Wisent	311-010-CL	1X	1X	Room temperature
TGF-β3	Peprotech	100-36E	1 µg/mL	1 ng/mL	Stock: –80°C Working: 4°C
Valproic acid (VPA)	Sigma	P4543	0.5 M	0.5 mM	Stock: –80°C Working: 4°C
Y-27632 (ROCK inhibitor)	Selleckchem	S1049	10 mM	10 µM	Stock: –80°C Working: 4°C

*Light-sensitive reagent. Stock and working aliquots should be covered in aluminum foil to minimize exposure to light.

†Laminin from Invitrogen is slightly better than laminin from Sigma for culturing neurons for longer than 4 weeks.

‡The laminin stock concentration may vary from lot to lot. The exact concentration is labeled on the tube. Laminin stock solution must be aliquoted into polypropylene microcentrifuge tubes.

§The working concentration range of purmorphamine is very narrow. Prepare the stock solution as accurately as possible. When adding stock solution to culture media, use the smallest tip and a well-calibrated pipette.

2.3 Equipment

Item	Supplier	Catalogue #
Cell culture incubator	ThermoScientific	Steri-Cycle Model 370 Ref#20
Cell culture water bath	FisherScientific	IsoTemp GPD20
Centrifuge	Eppendorf 5702	022626001
Light microscope	Motic	AE2000
Cell counter	Logos Biosystems	LUNA-II Automated cell counter
Cell counting slide	Logos Biosystems	05181401

3 Protocol

3.1 Coating culture vessels

Materials:

- Culture vessels (flasks or plates; if using coverslips, add to plate wells)
- PO (1 mg/mL)
- Laminin (1 mg/mL)
- 1x PBS
- DMEM/F12
- Antibiotic-Antimycotic
- 37°C/5% CO_2 cell culture incubator

Procedure:

1. Prepare PO working solution by adding 500 µL of PO stock solution to 50 mL 1x PBS.
    - Thaw PO stock solution at 4°C.
    - Recommended volumes of PO working solution needed depending on the type of culture vessel are listed in Table 1.
    - Ensure transfer of all PO stock solution by rinsing tube with PBS twice.
    - PO working solution can be stored at 4°C for up to 2 months.
2. Apply recommended volume of PO solution to culture vessel (Table 1) and swirl to spread across surfaces.
    - **IMPORTANT:** Ensure culture surfaces are coated completely and evenly. Do not let surfaces dry. Uneven coating or evaporation of coating may affect cell distribution.
3. Incubate culture vessel at 37°C for at least 2 hours or overnight.
4. Aspirate PO solution from culture vessel and wash surfaces three times with 1x PBS.
5. Prepare laminin working solution by adding 100 µL of laminin stock solution and 200 µL of Antibiotic-Antimycotic to 20 mL of cold DMEM/F12.
    - **IMPORTANT:** Store laminin stock solution at –80°C and thaw at 4°C before using. At room temperature laminin easily adsorbs to plastic and tends to form aggregates.
    - Recommended volumes of laminin working solution needed depending on the type of culture vessel are listed in Table 1.
    - Laminin working solution can be stored at 4°C for up to 2 weeks.

6. Apply recommended volume of laminin solution to culture vessel (Table 1) and swirl to spread across surfaces.

    - **IMPORTANT:** Ensure culture surfaces are coated completely and evenly. Do not let surfaces dry. Uneven coating or evaporation of coating may affect cell distribution.

7. Incubate culture vessel at 37°C for 2 hours.
8. If plating cells on culture vessel immediately, aspirate laminin solution and proceed with plating. If not plating cells on culture vessel immediately, do not aspirate laminin solution and store culture vessel in a 37°C incubator for up to 3 days.

    - **IMPORTANT:** If using PO/laminin-coated culture vessels that have been stored (i.e. if not using immediately after coating procedure), check quality of coating before plating cells. Uneven coating or evaporation of coating may affect cell distribution.

Table 1. Recommended volumes of PO and laminin working solutions based on type of culture vessel.

Culture vessel	Volume of PO solution	Volume of laminin solution
T25 flask	3 mL/flask	3 mL/flask
T75 flask	8 mL/flask	8 mL/flask
6-well plate	2 mL/well	1 mL/well
12-well plate	1 mL/well	700 µL/well
24-well plate	500 µL/well	400 µL/well
60-mm dish	2 mL/dish	2 mL/well
100-mm dish	5 mL/dish	5 mL/dish

3.2 Culturing DA or cortical NPCs

Materials:

- Frozen cryovial of DA or cortical NPCs
- PO/laminin-coated T25 or T75 flasks
- 100-mm petri dish (NOT coated with PO/laminin)
- 15-mL conical tubes
- DMEM/F12
- Y-27632 (ROCK inhibitor)
- Gentle Cell Dissociation Reagent
- Media:

Media	Components
NPC media A	• DMEM/F12 • 1x B-27 • 1x N2 • 1x MEM NEAA solution • 1x Antibiotic-Antimycotic
NPC media B	• NPC media A • 10 ng/mL EGF • 10 ng/mL FGF-b
NPC media C	• NPC media B • 0.5 mM VPA
NPC media D (for DA NPC only)	• NPC media A • 2 µM purmorphamine
NPC media E	• NPC media A • 0.1 µM Compound E

- 37°C cell culture water bath
- 37°C/5% CO_2 cell culture incubator
- Centrifuge
- Light microscope

Procedure:

1. Thaw frozen cryovial of NPCs in 37°C water bath.

   - Transfer frozen cryovial from liquid nitrogen tank in small liquid nitrogen transfer vessel or dry ice.
   - Do not thaw frozen cryovial in your hand as you will not be able to maintain a constant temperature.
   - Thaw cryovial quickly by gently shaking the cryovial continuously until only a small, frozen cell pellet remains.
   - After cells have thawed, sterilize the outside of the cryovial with 70% ethanol.

2. Transfer cells to a 15-mL conical tube containing 5 mL DMEM/F12 and resuspend. Pellet cells by centrifuging tube at 1200 rpm (200 g) for 3 minutes.
3. Remove supernatant. Carefully resuspend cell pellet in 5 mL of NPC media A and transfer entire cell suspension to a PO/laminin-coated flask. Add ROCK inhibitor to media (1:1000). Incubate cells at 37°C.

   - T25 flasks are recommended for growing NPCs in order to maintain an ideal density. The best density for recovery from a frozen stock is 1.5 million to 2 million cells per T25 flask.
   - The number and size of flasks to use is determined by the size of the cell pellet (Table 2).

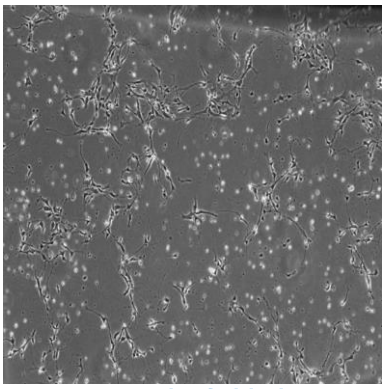
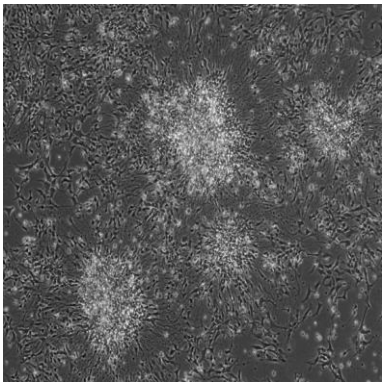
   Table 2. Number and size of flasks to use based on cell pellet size.

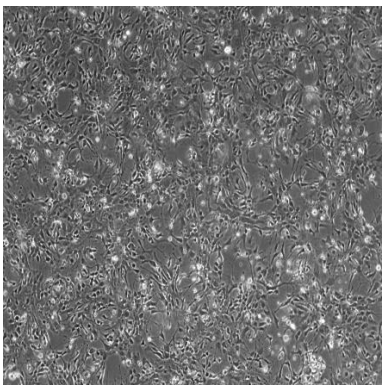
Pellet size	Number/size of flasks
< 1 mm	Thaw an additional cryovial of NPCs and add cells from both cryovials together on one T25 flask
1–1.5 mm	One T25 flask
> 1.5 mm	Two T25 flasks
~ 3 mm	One T75 flask

4. After 24 hours, assess cell morphology and density and follow recommendations as outlined in Table 3. Change media every other day. Continue to monitor cultures and follow recommendations as necessary.

   - Allow NPCs to recover for at least 1 week before passaging.
   - Initially, NPCs proliferate quickly and can be passaged 1:3. Eventually proliferation will slow down and cells should be passaged only 1:2 as low cell density may affect cell growth and morphology.

Table 3. Recommendations for NPCs after thaw depending on cell morphology and density.

Cell morphology and density	Recommendations
• Pre-neuronal morphology • Low density  Figure 2. Bright-field phase–contrast of OX1-19 DA NPCs after thaw.	• Change media to NPC media C to promote proliferation • To prevent neurogenesis only, continue using NPC media A and add 0.5 µM VPA • After 7 days of recovery, the cells are ready for differentiation
• Good morphology • Good density  Figure 3. Bright-field phase–contrast of SNCA DA NPCs after thaw.	• DA NPCs: Change media to NPC media D to promote the differentiation of aggregated naive progenitors into ventral progenitors • Cortical NPCs: Keep in NPC media A. • If cells continue to proliferate well (4–5-fold increase in cell number 1 week after recovery), split and/or freeze cells in FBS containing 10% DMSO • If proliferation slows down, change media to NPC media B

Cell morphology and density	Recommendations
• Lower proportion of NPCs present in culture (>30% undesired cells) • Good density  Figure 4. Bright-field phase–contrast of AIW002-02 DA NPCs after thaw.	• DA NPCs: Change media to NPC media D for 6 days • Cortical NPCs: Keep in NPC media A • Five to 7 days after thaw/recovery, aspirate media from cells and wash flask once with DMEM/F12. Add 2 mL Gentle Cell Dissociation Reagent and incubate at 37°C for 5 minutes. • Tap flask gently to detach NPCs (non-NPCs will remain attached) and transfer cell suspension to 15-mL conical tube. Add 5 mL DMEM/F12 to flask to collect residual detached cells and transfer cell suspension to tube. Pellet cells by centrifuging at 1200 rpm (200 g) for 3 minutes. • Remove supernatant. Resuspend cells in NPC media D (DA NPCs) or NPC media A (cortical NPCs) and transfer to a 100-mm petri dish (uncoated). Incubate cells at 37°C for 2 days. • Transfer media from dish containing floating NPC aggregates (Figure 5) to 15-mL conical tube. Let floating aggregates settle down to bottom of tube for 2 to 3 minutes. • Remove supernatant. Resuspend cells in NPC media E and transfer to a PO/laminin-coated T25 flask. Incubate cells at 37°C.

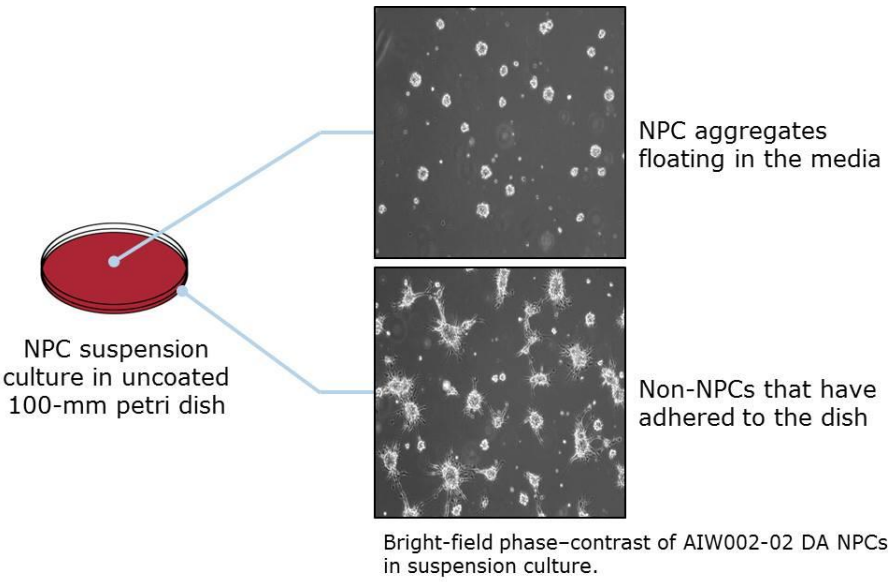


Figure 5. Bright-field phase–contrast of AIW002-02 DA NPCs in suspension culture. NPCs form aggregates that float in the media (top) while non-NPCs adhere to the dish (bottom).

5. Change media every 2 days. When cells reach confluence, split and/or freeze cells in FBS containing 10% DMSO.

   - Freeze NPCs at P1 to maintain cells as premature as possible upon recovery.
   - NPCs can be kept in culture until they are ready for differentiation.
   - NPCs are ready for differentiation when proliferation has slowed down and cells begin to develop a more neuronal phenotype (after approximately 3–4 passages).

3.3 Differentiating NPCs into DA or cortical neurons

Materials:

- DA or cortical NPC culture in T25 flask
- PO/laminin-coated T25 flask, 24-well plate (containing coated coverslips), and/or 96-well plate
- 15-mL conical tubes
- DMEM/F12 containing 1x Antibiotic-Antimycotic
- StemPro Accutase Cell Dissociation Reagent
- Y-27632 (ROCK inhibitor)
- Mitomycin C
- Media:

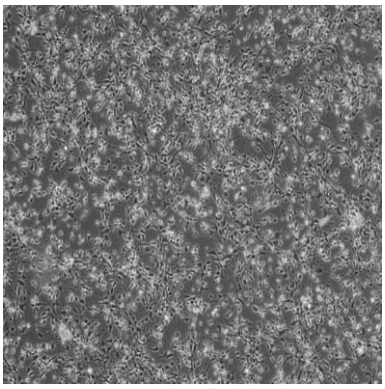
Media	Components
NPC media A + 0.1 µM Compound E	• DMEM/F12 • 1x B-27 • 1x N-2 • 1x MEM NEAA solution • 1x Antibiotic-Antimycotic • 0.1 µM Compound E
50% NB media	• 1:1 NB:DMEM/F12 • 1x B-27 • 1x N-2 • 1x Antibiotic-Antimycotic • 20 ng/mL BDNF • 20 ng/mL GDNF • 200 µM AA • 1 µg/mL laminin • 0.5 mM db-cAMP • 0.1 µM Compound E • 10 ng/mL TGF-β3
100% NB media	• NB media • 1x B-27 • 1x N-2 • 1x Antibiotic-Antimycotic • 20 ng/mL BDNF • 20 ng/mL GDNF • 200 µM AA • 1 µg/mL laminin • 0.5 mM db-cAMP • 0.1 µM Compound E

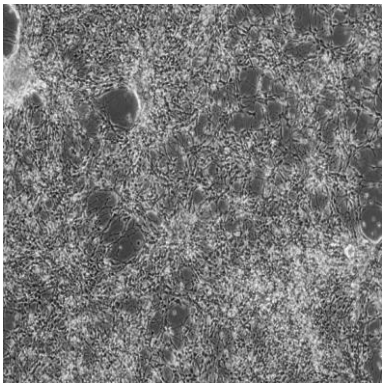
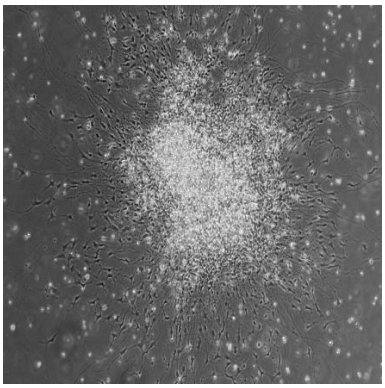
- 37°C cell culture water bath
- 37°C/5% CO_2 cell culture incubator
- Centrifuge
- Light microscope
- Cell counter and cell counting slide

Procedure:

1. Assess cell morphology and density of NPC cultures and follow recommendations as outlined in Table 4.

Table 4. Recommendations for NPCs after culturing depending on cell morphology and density.

Cell morphology and density	Recommendations
• Good morphology • Good density  Figure 6. Bright-field phase–contrast of OX1-19 DA NPCs cultured for 6 days.	NPCs are ready to start final differentiation. Proceed to step 2.

Cell morphology and density	Recommendations
• Good morphology • High density  Figure 7. Bright-field phase–contrast of SNCA DA NPCs cultured for 6 days.  Figure 8. Bright-field phase–contrast of AIW002-02 DA NPCs after NPC enrichment.	• Split culture 1:3 and resuspend cells carefully in 1 mL NPC media A containing 0.1 µM Compound E. Pipette cells up and down 3 to 5 times using a 1000 µL tip to obtain a single-cell suspension and transfer to a PO/laminin-coated flask. • After 5 to 6 days in culture, NPCs will be ready to start final differentiation. Proceed to step 2.

2. Rinse cells once with DMEM/F12 containing 1x Antibiotic-Antimycotic.
3. Add 3 mL of StemPro Accutase Cell Dissociation Reagent to the cells and incubate at 37°C for 3 to 5 minutes until the cells detach. Stop cell dissociation by adding 10 mL DMEM/F12 containing 1x Antibiotic-Antimycotic. Transfer cells to a 15-mL conical tube. Rinse flask with DMEM/F12 containing 1x Antibiotic-Antimycotic and transfer to tube containing cells. Pellet cells by centrifuging at 1200 rpm (200 g) for 3 minutes.

   - Incubation time for cell dissociation varies depending on NPC morphology and cell density (e.g. OX1-19 DA NPCs require 3 minutes of incubation while SNCA DA NPCs and AIW002-02 DA NPCs require 5 minutes). Monitor cells so that dissociation can be stopped as soon as all cells have detached. Do not dissociate cells for too long.
   - The activity of StemPro Accutase Cell Dissociation Reagent may decrease over time at 4°C (storage of leftover reagent).

4. Resuspend cells carefully in 1 mL 50% NB media using a 200 µL tip on top of a 1000 µL tip to obtain a single-cell suspension.

   - Do not over-pipette the suspension as this will destroy the premature neurons.
   - Resuspension technique varies depending on NPC morphology and cell density, and the activity of StemPro Accutase Cell Dissociation Reagent:
     - OX1-19 DA NPCs: Pipette up and down 3 times.
     - SNCA DA NPCs and AIW002-02 DA NPCs: Pipette up and down 5 times. Allow cell suspension to settle for 2 minutes and then transfer supernatant to a second tube. Add 500 µL 50% NB media to settled cells in first tube and pipette up and down.

5. Determine cell number using cell counter. Plate cells in 50% NB media containing ROCK inhibitor (1:1000) on a PO/laminin-coated culture vessel at the recommended density and media volume as outlined in Table 5.

Table 5. Recommended cell density and media volumes for plating DA or cortical neurons based on type of culture vessel.

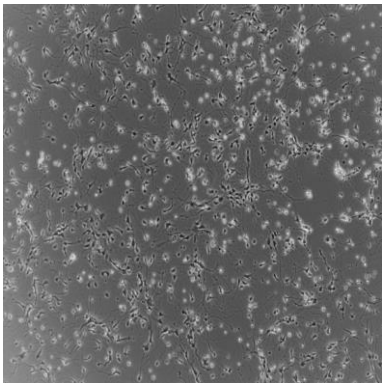
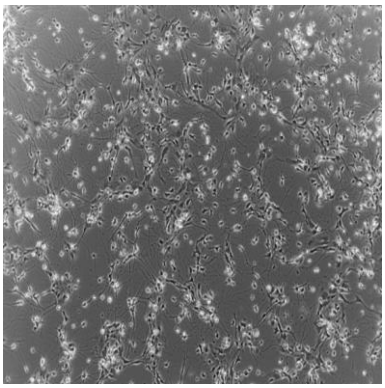
Culture vessel	Cell number and media volume
T25 flask	1.5 x 10^6–2.0 x 10^6 cells
96-well plate*	~15,000 cells/100 µL per well
24-well plate with coated coverslips	30,000–50,000 cells/500 µL per well

*Do not plate cells in outer wells to prevent evaporation of media. Add 100 µL 1x PBS to outer wells.

   - Work quickly to prevent the culture vessel surfaces from drying out.
   - Avoid plating aggregates. Allow aggregates in the cell suspension to settle to the bottom of the tube and use the supernatant for cell counting and plating.
   - If culture is determined to be purely neuronal, cells may be plated in 100% NB media containing ROCK inhibitor (1:1000).
   - If using coated coverslips, ensure they are completely attached to the bottom of the well after plating the cells. Cell attachment is reduced if coverslips are floating, resulting in plating failure.

6. After 48 hours, assess purity of neuronal cultures and follow recommendations as outlined in Table 6.

Table 6. Recommendations based on culture purity.

Culture purity	Recommendations
• Pure neuronal culture  Figure 9. Bright-field phase–contrast of OX1-19 DA NPCs directly differentiated to DA neurons.	• Treatment with Mitomycin C is not necessary because culture is already pure. • Change media to 100% NB media.
• Culture contains proliferating cells  Figure 10. Bright-field phase–contrast of SNCA DA NPCs differentiated to DA neurons.	• Add Mitomycin C (1:1000) and incubate for 1 hour at 37°C. • Change media to 100% NB media.

7. Change media 1 to 2 times per week using a half a media change.

   - Use half a media change to minimize exposure of cells to air (remove only half of the media volume so that plated cells remain covered by media). This technique reduces stress on the cells as media is aspirated and added. In addition, the remaining conditioned media contains secreted cytokines and growth factors that enhance neuronal survival and maturation.
   - DA and cortical neurons can survive in culture for 4 to 6 weeks using this protocol.