Institut-Hôpital neurologique de Montréal

Montreal Neurological Institute-Hospital

McGill

**EDDU Protocols**

# Induction of Dopaminergic or Cortical neuronal progenitors from iPSCs

Authors: Chen, Xiuqing; Rocha, Cecilia; Loignon, Martin; Peng, Huasheng; Rao, Trisha; Durcan, Thomas Martin

Version 2.0

EDDU-008-02

April 2020

# Induction of Dopaminergic or Cortical neuronal progenitors from iPSCs

Author(s): Chen, Xiuqing; Rocha, Cecilia; Loignon, Martin; Peng, Huasheng; Rao, Trisha; Durcan, Thomas Martin

| Version | Authors/Updated by | Date | Signature |
|---|---|---|---|
| v1.0 | Carol Xiuqing Chen<br>Cecilia Rocha<br>Martin Loignon<br>Huashang Peng<br>Trisha Rao | August 2, 2019 | |
| v2.0 | Carol Xiuqing Chen<br>Cecilia Rocha<br>Martin Loignon<br>Huashang Peng<br>Trisha Rao | April 1, 2020 | |

The involved functions approve the document for its intended use:

| Name | Function | Role | Date | Signature |
|---|---|---|---|---|
| Dr. Thomas Durcan | R&D | Associate Director, MNI Early Drug Discovery Unit (EDDU) | | |

# Table of Contents

# 1 Introduction

## 1.1 Objective

This protocol describes how to generate dopaminergic (DA) or cortical neural progenitor cells (NPCs).

## 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 (**Figure 1**).

iPSC cultures are first dissociated into single cells and then cultured in suspension to generate EBs. The EBs are re-plated and differentiated into neural rosettes. Finally, the rosettes are differentiated into DA or cortical NPCs. As an alternative method, dissociated iPSCs may be cultured under adherent conditions to generate a monolayer culture, which is then differentiated into DA or cortical NPCs. Since the EB and monolayer methods are the same except for a few key differences, the protocol describes the EB method and the modifications for the monolayer protocol are described in **Appendix 4.1**.

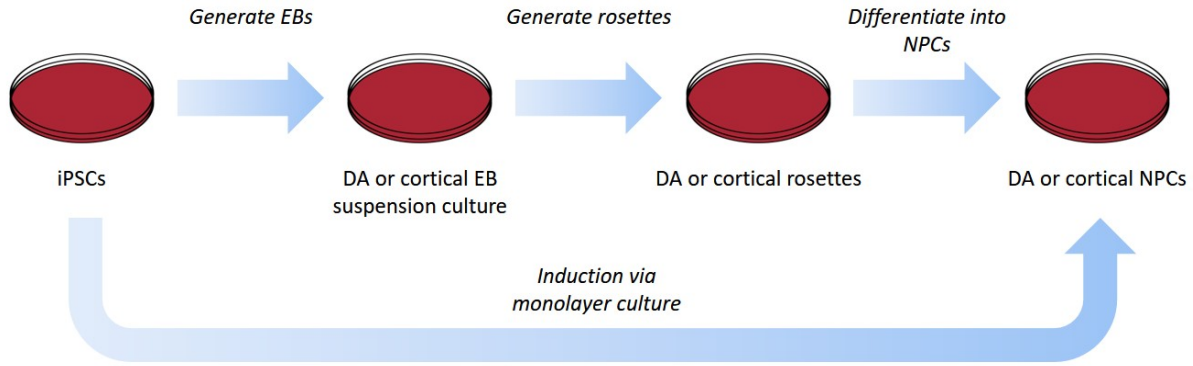


**Figure 1. Protocol overview for the generation of DA or cortical NPCs from iPSCs via EB suspension culture or via monolayer culture.**

DA or cortical NPC induction may be performed over 2 weeks or 3 weeks, depending on the desired type of NPCs (**Figure 2**). Typically, the induction is performed over 2 weeks, resulting in a culture of immature progenitors or a mixed cell population. To generate a more mature population of progenitors that are closer to a neuronal precursor phenotype and can be differentiated into neurons faster, the induction period is extended to 3 weeks. The additional week of culturing in induction media improves the cells to proliferate and more readily differentiate into neurons. Regardless of whether induction is performed over 2 or 3 weeks, the

first passage of the culture from induction media into NPC media is identified as P0. The cells may then be expanded by passaging at 1-week intervals (P1, P2, etc.), frozen down, or differentiated into final neurons.

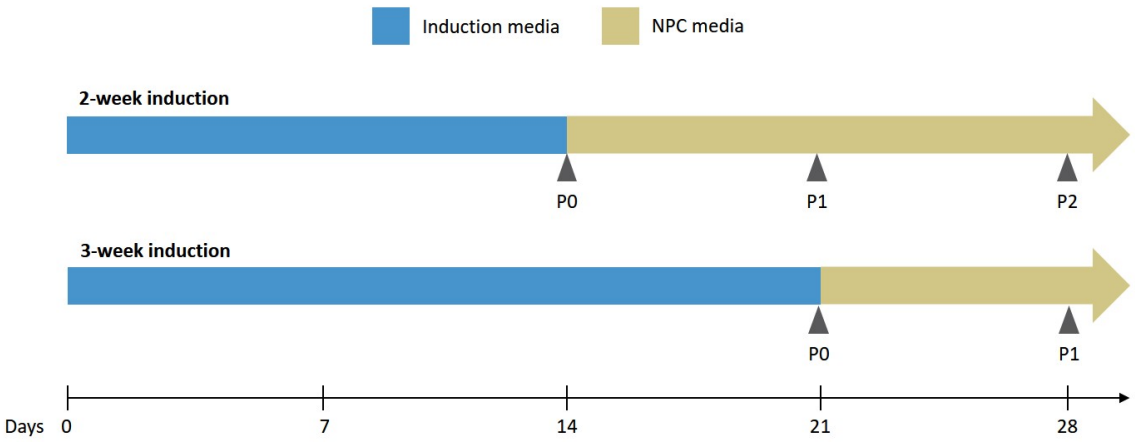


**Figure 2. Comparison 2-week and 3-week induction approaches. Following induction, cultures are passaged at 1-week intervals.**

DA or cortical NPCs can be further differentiated into an enriched population of functionally mature DA or cortical neurons, respectively.

## 1.3 Technical and safety considerations

The following information should be read before starting:

- All cells 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.
- High-quality iPSCs are essential for induction of NPCs. iPSCs should be recovered from a frozen stock and passaged at least twice before using in this protocol.
- EBs cultures consist of cell aggregates. Use a 5-mL glass pipet during resuspension and transfer of EB cultures to minimize breakage of cell aggregates.
- NPCs and neurons must be cultured on surfaces coated with poly-L-ornithine (PO) and laminin.

- 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 to avoid cross-contamination.
- 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 the plate well).
  - 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 the 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.
- 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 NPCs expressing DA or cortical progenitor markers, respectively. NPCs generated using this protocol can be further differentiated into DA or cortical neurons expressing DA or cortical neuronal 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 # |
|---|---|---|
| Cell scraper | Corning | 3010 |
| Conical tube, 15-mL | ThermoFisher | 352097 |
| Culture flask, T25 | ThermoFisher | 12-556-009 |
| Culture flask, T75 | ThermoFisher | 12-556-010 |
| Culture plate, 24-well | ThermoFisher | 087721 |
| Glass coverslip, 12-mm | ThermoFisher | 12-545-80 |
| Glass pipet, 5-mL | ThermoFisher | 13-678-27E |
| Low protein-binding filter, 0.2-µm | VWR | 28145-501 |
| Parafilm | ThermoFisher | 13-374-12 |
| 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 unless otherwise stated.

| Item | Supplier | Catalogue # | Stock conc. | Working conc. | Storage temp. |
|---|---|---|---|---|---|
| Antibiotic-Antimycotic | Gibco | 15240-062 | 100x | 1x | Stock: –20°C<br>Working: 4°C |

| Item | Supplier | Catalogue # | Stock conc. | Working conc. | Storage temp. |
|---|---|---|---|---|---|
| Bovine serum albumin (BSA; optional) | Invitrogen | 15260-037 | 75 mg/mL | 1 mg/mL | Stock: –20°C<br>Working: 4°C |
| B-27* | Gibco | 17504044 | 50x | 1x | Stock: –20°C<br>Working: 4°C |
| CHIR-99021 | Selleckchem | S2924 | 3 mM | 3 µM | Stock: –80°C<br>Working: 4°C |
| Compound E | STEMCELL Technologies | 73952 | 0.1 mM | 0.1 µM | Stock: –80°C<br>Working: 4°C |
| DMEM/F12 | Gibco | 10565-018 | 1x | 1x | 4°C |
| DMSO | Fisher | BP231-1 | 100% | 10% | Room temperature |
| Dorso-morphin homolog 1 (DMH1) | Selleckchem | S7146 | 4 mM | 2 µM | Stock: –80°C<br>Working: 4°C |
| FBS | Gibco | 12484-028 | 1x | 1x | Stock: –80°C<br>Working: Room temperature |
| FGF-8 | Peprotech | 100-25 | 100 µg/mL | 100 ng/mL | Stock: –80°C<br>Working: 4°C |
| Gentle Cell Dissociation Reagent† | STEMCELL Technologies | 07174 | 1x | 1x | Room temperature |
| Laminin | Sigma | L2020 | 1 mg/mL‡ | 5 µg/mL | Stock: –80°C<br>Working: 4°C |
| Matrigel Matrix hESC-qualified (monolayer method) | Corning Millipore | 354277 | 100x | 1x | Stock: −80°C<br>Working: 4°C§ |
| MEM nonessential amino acid (NEAA) solution | Wisent | 321-011-EL | 100x | 1x | 4°C |
| mTeSR1 5X supplement | STEMCELL Technologies | 05850 (component #05852) | 5x | 1x | Stock: −20°C<br>Working: 4°C |
| mTeSR1 basal media | STEMCELL Technologies | 05850 (component #05851) | 1x | 1x | 4°C |
| N-2* | Gibco | 17502048 | 100x | 1x | Stock: –20°C<br>Working: 4°C |

| Item | Supplier | Catalogue # | Stock conc. | Working conc. | Storage temp. |
|---|---|---|---|---|---|
| Noggin | Peprotech | 120-10C | 200 µg/mL | 200 ng/mL | Stock: –80°C<br>Working: 4°C |
| Poly-L-ornithine (PO) | Sigma | P3655 | 1 mg/mL | 10 µg/mL | Stock: –20°C<br>Working: 4°C |
| Phosphate-buffered saline (PBS) | Wisent | 311-010-CL | 1x | 1x | Room temperature |
| SB431542 | Selleckchem | S1067 | 10 mM | 10 µM | Stock: –80°C<br>Working: 4°C |
| Sonic hedgehog (SHH; C24II) | GenScript | Z03067 | 200 µg/mL | 200 ng/mL | Stock: –80°C<br>Working: 4°C |
| SHH[‖] | Peprotech | 100-45 | 200 µg/mL | 200 ng/mL | Stock: –80°C<br>Working: 4°C |
| VPA | Sigma | P4543 | 0.5 M | 0.5 mM | Stock: –80°C<br>Working: 4°C |
| Y-27632 (ROCK inhibitor) | Selleckchem | S1049 | 10 mM | 10 µM | Stock: –80°C<br>Working: 4°C |

[*]Light-sensitive reagent. Stock and working aliquots should be covered in aluminum foil to minimize exposure to light.
[†]The incubation time and temperature for Gentle Cell Dissociation Reagent may vary depending on cell morphology.
[‡]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.
[§]Matrigel working solution must be used immediately or stored at –20°C for later use.
[‖]SHH from Peprotech may be used as an alternative to SHH from GenScript when it is not available.

## 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 |

# 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 move back and forth and side to side 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 move back and forth and side to side 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 |

## 3.2 Generating DA or cortical NPCs from iPSCs

**Materials:**

- iPSC culture recovered from frozen and passaged at least twice (10-cm dish, 70%–80% confluent)
- T25 flask (NOT coated with PO/laminin)
- PO/laminin-coated T25 and T75 flasks
- Cell scraper
- 5-mL glass pipet
- 15-mL tubes
- DMEM/F12 containing 1x Antibiotic-Antimycotic
- Gentle Cell Dissociation Reagent
- Y-27632 (ROCK inhibitor)
- Media:

| Media | Components |
|---|---|
| mTeSR1 | Preparation instructions:<br>• Thaw mTeSR1 5x supplement at 4°C overnight.<br>• Add 100 mL of mTeSR1 5x supplement to 400 mL mTeSR1 basal media to obtain 500 mL of complete media. Mix well.<br>• Complete media is ready for use and does not require filtering. However, the complete media can be filtered using a 0.2-µm low protein-binding filter.<br>• Warm complete media at room temperature (do not warm at 37°C).<br>Storage instructions:<br>• Once supplement is thawed, use immediately or store at 4°C for up to 2 days.<br>• Complete media is stable when stored at 4°C for up to 2 weeks or at –20°C for up to 6 months. Prepare 40-mL aliquots in 50-mL conical tubes, seal with Parafilm, and store at –20°C. Thaw aliquots overnight at 4°C (do not warm in a 37°C water bath). Do not refreeze aliquots after thawing. |
| DA induction media | • DMEM/F12<br>• 1x N-2<br>• 1x B-27<br>• 1x MEM NEAA solution<br>• 1 mg/mL BSA (optional)<br>• 200 ng/mL Noggin<br>• 200 ng/mL SHH (C24II)<br>• 3 µM CHIR-99021<br>• 10 µM SB431542<br>• 100 ng/mL FGF-8 |

| Media | Components |
|---|---|
| Cortical induction media | • DMEM/F12<br>• 1x N-2<br>• 1x B-27<br>• 1x MEM NEAA solution<br>• 1 mg/mL BSA (optional)<br>• 10 µM SB431542<br>• 2 µM DMH1<br>• 100 ng/mL FGF-8 (optional) |
| NPC media A | • DMEM/F12<br>• 1x N-2<br>• 1x B-27<br>• 1x MEM NEAA solution<br>• 1 mg/mL BSA (optional) |

- Centrifuge
- 37°C/5% $CO_2$ cell culture incubator

**Procedure:**

1. On Day 1, assess iPSC culture to ensure that it is 70% to 80% confluent, cells are tightly packed with smooth edges, and there is rare, spontaneous differentiation.

    - Never use iPSCs just recovered from thaw to start the induction. Thawed iPSCs should be passaged at least twice prior to DA or cortical NPC induction. Keep in mind that high-quality iPSCs are essential for neuronal induction.

2. Aspirate media from iPSC culture and rinse with 5 mL of DMEM/F12 containing 1x Antibiotic-Antimycotic. Add 5 mL Gentle Cell Dissociation Reagent and incubate at 37°C for 4 to 6 minutes.

    - If culture is more than 80% confluent or if the colonies are too large or tightly packed, extend incubation time in Gentle Cell Dissociation Reagent to 5 to 7 minutes.

3. Aspirate the Gentle Cell Dissociation Reagent and wash cells with 5 mL of DMEM/F12 containing 1x Antibiotic-Antimycotic. Add 5 mL of DMEM/F12 containing 1x Antibiotic-Antimycotic to cells and gently detach the colonies with a cell scraper.

    - If cells have already detached from dish, add 5ml DMEM/F12 directly to dish without aspirating the Gentle Cell Dissociation Reagent, then gently detach the remaining colonies with a cell scraper.
    - The cells are rinsed to remove dying cells and residual Gentle Cell Dissociation Reagent from the dish.

4. Transfer the cell suspension to a 15-mL conical tube. Rinse the dish with an additional 5 mL of DMEM/F12 containing 1x Antibiotic-Antimycotic to collect any remaining cells and transfer to the tube.
5. Pellet cells by centrifuging at 1200 rpm (200 g) for 3 minutes. Remove supernatant and resuspend cells in 1 mL DA induction media (for DA NPCs), cortical induction media (for cortical NPCs), or mTeSR1 media (if cell line is sensitive to passaging). Gently pipette the cells up and down 1 to 3 times.

    - It is preferable to resuspend and plate cells in induction media at this stage. However, some iPSC lines may be sensitive to passaging and should be resuspended and plated in mTeSR1 media; after 24 hours, the media is changed to induction media. mTeSR1 media should be used regardless of whether the cells were previously cultured in mTeSR1 or Essential 8 media, as the cells will not form EBs well in Essential 8 media.
    - Do not over-pipette the cells as they are sensitive to shear forces.

6. Plate all cells in 5 mL DA induction media, cortical induction media, or mTeSR1 media, containing ROCK inhibitor (2 µM) on an uncoated T25 flask. Incubate cells at 37°C.

    - The T25 flask must be uncoated so that the cells do not attach and are able to grow in suspension.
    - Alternatively, plate only 80% of the cells on the uncoated T25 flask and proceed to the next step, and plate the remaining 20% of the cells on a Matrigel-coated T25 flask for induction via monolayer culture (see **Appendix 4.1** for procedure).

7. For cultures in mTeSR1 media, change media the next day (Day 2) to DA induction media (for DA NPCs) or cortical induction media (for cortical NPCs).
8. Two days after plating (Day 3), perform an 80% media change by tilting the flask slightly so that the EBs settle down into a corner, then remove 4 mL of media, leaving 1 mL of media in the flask. Add 4 mL of fresh induction media and incubate cells at 37°C.
9. Change the media every 2 days using an 80% media change. Monitor cultures for EB formation. If an EB attaches to the flask surface, gently detach the EB using fluid pressure by pipetting media onto the attached EB.

    - If the media turns yellow, change media daily.
    - If cells are at low density or do not proliferate well, BSA may be added to the media at a final concentration of 1 mg/mL to stimulate cell growth.

- If cortical EBs do not grow well or if large quantities are needed, FGF-8 may be added to the media at a final concentration of 100 ng/mL to stimulate cell growth.
- Examples of DA EBs at days 1 to 4 are shown in **Figure 3** and **Figure 4**.

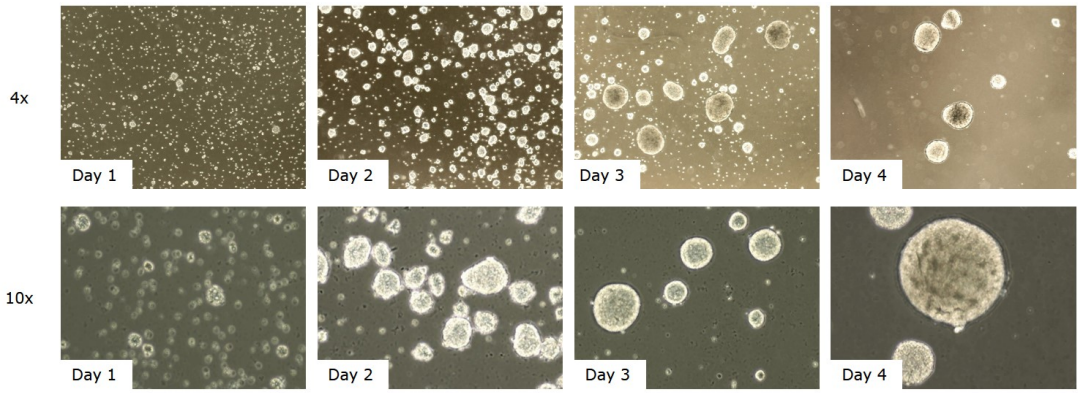


**Figure 3. Bright-field phase contrast of NCRM1 DA EBs at days 1 to 4 (top panel: 4x objective; bottom panel: 10x objective).**

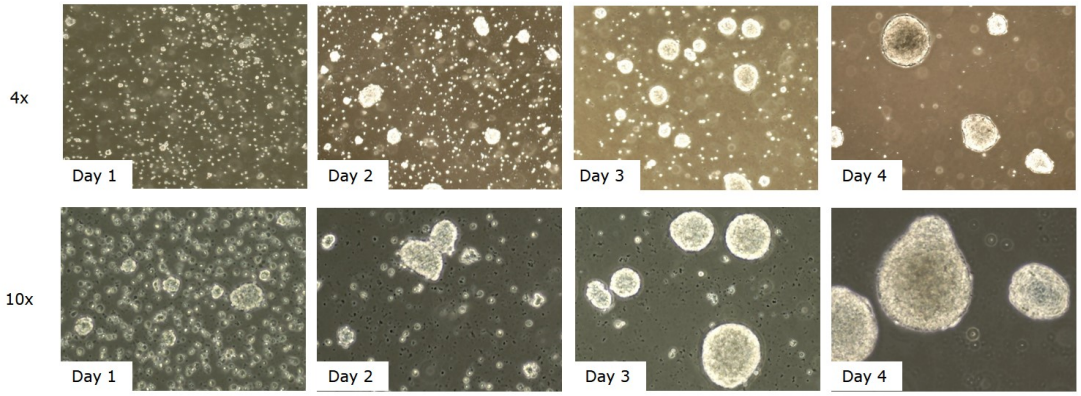


**Figure 4. Bright-field phase contrast of H9 DA EBs at days 1 to 4 (top panel: 4x objective; bottom panel: 10x objective.**

10. Seven days after plating (Day 8), gently transfer media containing floating EBs using a glass pipet to a 15-mL conical tube. Let floating EBs settle down to bottom of tube for 2 to 3 minutes.

    - Use a 5-mL glass pipet to minimize breakage of EBs. Do not pipette the EBs.

11. Remove supernatant. Gently resuspend cells in DA induction media (for DA NPCs) or cortical induction media (for cortical NPCs) containing ROCK inhibitor (2 µM) and transfer to a PO/laminin-coated T25 or T75 flask. Move the flask in several back-and-forth and side-to-side motions to distribute the EBs. Incubate the cells at 37°C.

- Use a 5-mL glass pipet to minimize breakage of EBs. Do not pipette the EBs.
- If EBs form large aggregates or aggregate chains, gently pipette 1 to 3 times to break up aggregates.
- 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 |
|---|---|
| <2 mm | One T25 flask |
| 2–4 mm | Two T25 flasks |
| >4 mm | One T75 flask |

12. After 24 hours (Day 9), change media to DA induction media (for DA NPCs) or cortical induction media (for cortical NPCs) without ROCK inhibitor. Continue to change media every 2 days.

    - If the media turns yellow, change media daily.
    - Rosettes typically appear 3 days after re-plating the EBs on PO/laminin-coated flasks (Day 12). Examples of DA rosettes 4 days after re-plating are shown in **Figure 5**.

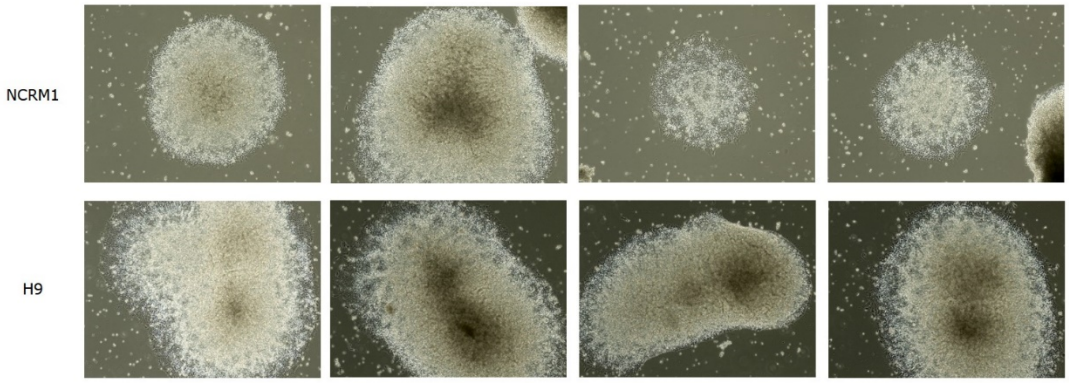


**Figure 5. Bright-field phase contrast of NRCM1 and H9 DA rosettes 4 days after plating (10x objective).**

13. On Day 15, aspirate media from rosettes and rinse with 5 mL of DMEM/F12 containing 1x Antibiotic-Antimycotic. Add 5 mL Gentle Cell Dissociation Reagent and incubate at room temperature for 5 to 7 minutes.

    - Do not incubate rosettes in Gentle Cell Dissociation Reagent at 37°C because this will cause all of the cells to detach, including non-desirable cell types on the bottom or the edge of rosettes.

14. Tap the side of the flask with the palm of your hand 3 to 6 times to detach the rosettes from the flask. Without removing the Gentle Cell Dissociation Reagent, add 5 mL of DMEM/F12 containing 1x Antibiotic-Antimycotic and transfer rosettes to a 15-mL conical tube. Rinse the flask with an additional 5 mL of DMEM/F12 containing 1x Antibiotic-Antimycotic to collect

any remaining rosettes and transfer to the tube. Pellet cells by centrifuging at 1200 rpm (200 g) for 3 minutes.

- Following dissociation of the rosettes, undesired cells will remain attached to the flask (**Figure 6**).

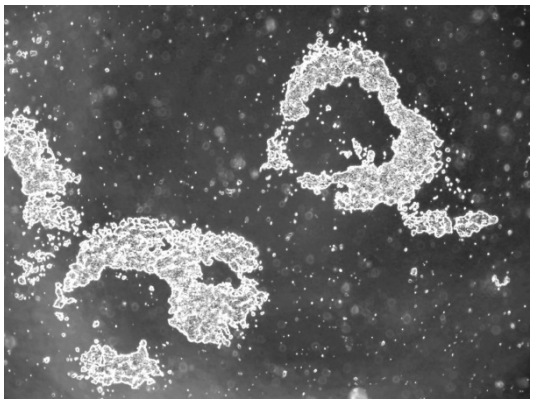

**Figure 6. Bright-field phase contrast of undesired cells remaining on flask after dissociation of AIW002-02 DA rosettes.**

15. Remove the supernatant and resuspend rosettes in 1 mL DA induction media (for mature DA NPCs), cortical induction media (for mature cortical NPCs), or NPC A media (for immature NPCs). Pipette up and down 3 to 5 times to break up the rosettes into small cell aggregates.

    - The choice between induction media and NPC media A depends on the desired type of NPCs:
        - To generate immature progenitors or a mixed population (2-week induction), resuspend rosettes in NPC media A.
        - To generate mature progenitors that are closer to a neuronal precursor phenotype and can be differentiated into neurons faster (3-week induction), resuspend rosettes in induction media.
    - Do not over-pipette the rosettes into a single-cell suspension. Larger cell aggregates or single cells will not differentiate well into NPCs.

16. Transfer cell aggregates to a PO/laminin-coated T25 or T75 flask and add 4 mL or 14 mL, respectively, of the same type of media. Incubate rosettes at 37°C.

    - The number and size of flasks to use is determined by the size of the cell pellet (Table 3).

    **Table 3. Number and size of flasks to use based on cell pellet size.**

| Pellet size | Number/size of flasks |
|---|---|
| <2 mm | One T25 flask |
| 2–3 mm | Two T25 flasks |
| 3–4 mm | One T75 flask |
| >4 mm | Two T75 flasks |

    - For rosettes plated in NPC media A (2-week induction), label cultures as P0.

17. Change the media every 2 days until Day 21, then proceed to **Section 3.3**.

    - If the media turns yellow, change media daily.
    - An example of DA NPCs 1 day after plating rosettes (Day 16) is shown in **Figure 7**.

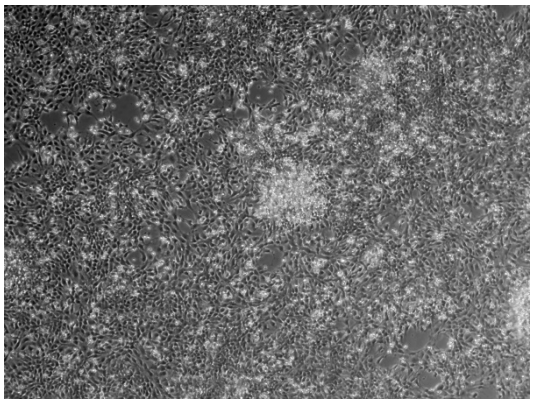

**Figure 7. Bright-field phase contrast of AIW002-02 DA NPCs 1 day after plating rosettes (Day 16).**

## 3.3 Expansion, cryopreservation, or final neuronal differentiation of DA or cortical NPCs

**Materials:**

- DA or cortical NPC culture at Day 21
- 15-mL tube
- DMEM/F12 containing 1x Antibiotic-Antimycotic
- Gentle Cell Dissociation Reagent
- 10% DMSO in FBS
- Media:

| Media | Components |
|---|---|
| NPC media A | • DMEM/F12<br>• 1x N-2<br>• 1x B-27<br>• 1x MEM NEAA solution<br>• 1 mg/mL BSA (optional)<br>• 100 ng/mL FGF-8 (optional) |
| NPC media B | • DMEM/F12<br>• 1x N-2<br>• 1x B-27<br>• 1x MEM NEAA solution<br>• 1 mg/mL BSA (optional)<br>• 0.1 µM Compound E<br>• 0.5 mM VPA |

**Procedure:**

1. Aspirate media from NPCs and rinse with 5 mL of DMEM/F12 containing 1x Antibiotic-Antimycotic. Add 5 mL Gentle Cell Dissociation Reagent and incubate at room temperature for 5 to 7 minutes.
2. Tap the side of the flask with the palm of your hand 3 to 6 times to detach the NPCs from the flask. Without removing the Gentle Cell Dissociation Reagent, add 5 mL of DMEM/F12 containing 1x Antibiotic-Antimycotic and transfer NPCs to a 15-mL conical tube. Rinse the flask with an additional 5 mL of DMEM/F12 containing 1x Antibiotic-Antimycotic to collect any remaining cells and transfer to the tube. Pellet cells by centrifuging at 1200 rpm (200 g) for 3 minutes.

3. Remove the supernatant and resuspend cells in the appropriate media according to the recommendations for expansion, cryopreservation, or final neuron differentiation outlined in **Table 4**.

**Table 4. Recommendations for expansion, cryopreservation, or differentiation.**

| Application | Recommendations |
|---|---|
| Expansion | • Resuspend and plate cells in NPC media A on a PO/laminin-coated T75 flask (label cultures as P1 for 2-week induction or P0 for 3-week induction).<br>• Change media every 2 days, or daily if the media turns yellow.<br>• Split cells once per week (see Table 3 for plating recommendations based on pellet size). Do not passage more than once per week, even if culture is over-confluent.<br>• If cells are at low density or do not proliferate well, BSA may be added to the media at a final concentration of 1 mg/mL to stimulate cell growth.<br>• If cells do not grow well or if large quantities are needed (e.g. for freezing), FGF-8 may be added to the media at a final concentration of 100 ng/mL to stimulate cell growth. |
| Cryopreservation | • Freeze cultures at P1 in FBS containing 10% DMSO.<br>– Label cryovials with NPC type (DA or cortical), passage number, induction time (2 weeks or 3 weeks), and induction method (EB or monolayer). |
| Initiate final neuron differentiation | • Change media to NPC media B and after 1 week proceed with protocol for DA or cortical neuron differentiation. |

- NPCs can be kept in culture until they are ready for differentiation. NPC cultures must meet specific criteria in terms of morphology and density before they can be differentiated into neurons.
- An example of DA NPCs (3-week induction) 1 day after splitting (Day 22; P1) is shown in **Figure 8**.

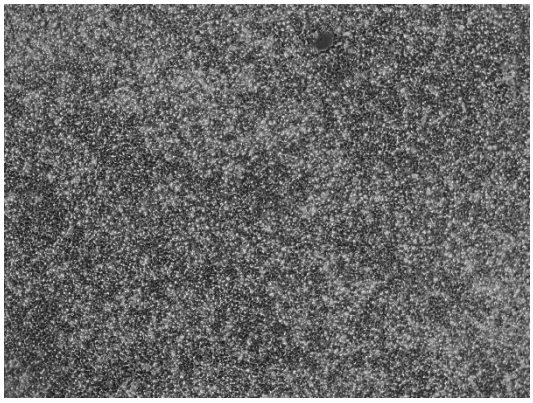

**Figure 8. Bright-field phase contrast of AIW002-02 DA NPCs (3-week induction) 1 day after splitting (Day 22; P1).**

# 4 Appendix

## 4.1 DA or cortical NPC induction via monolayer culture

To generate NPCs via adherent monolayer culture, follow the procedure in **Section 3.2** but make the modifications indicated in **Table 5**. Throughout the procedure, cells should be plated at 50% to 60% confluency and kept in culture for 6 to 7 days, even if cells reach confluence earlier. Cell density plays an important role in neuronal induction when using the monolayer culture method.

For NPCs generated using the EB method, there will not be enough cells at P0 for cryopreservation; therefore, cells are frozen at P1. However, for NPCs generated using the monolayer method, it is more likely that there will be enough cells at P0 for cryopreservation; therefore, cells can be frozen at P0 if possible, or at P1.

**Table 5. Comparison of steps that differ between the EB and monolayer culture methods for induction of DA or cortical NPCs.**

| Day of protocol | EB culture | Monolayer culture |
|---|---|---|
| 1 | • Plate ~80% of iPSCs from one 10-cm dish at 70%–80% confluence (>8 million cells) on an uncoated T25 flask. | • Plate ~20% of iPSCs from one 10-cm dish at 70%–80% confluence (2–3 million cells) on a Matrigel-coated T25 flask (see Appendix 4.2 for Matrigel coating procedure). |
| 8 | • Transfer EBs 1:1–2 and plate in induction media on a PO/laminin-coated T25 or T75 flasks. | • Dissociate cells at 37°C for 5-7 minutes.<br>• Split 1:3–5 and plate in induction media on PO/laminin-coated T25 or T75 flasks. |
| 15 | • Dissociate rosettes at room temperature for 5–7 minutes.<br>• Split and plate in NPC media A (2-week induction) or in induction media (3-week induction) on PO/laminin-coated T25 or T75 flasks. | • Dissociate cells at 37°C for 5-7 minutes.<br>• Split and plate in NPC media A (2-week induction) or in induction media (3-week induction) on PO/laminin-coated T25 or T75 flasks. |
| 21 | • NPCs are ready for expansion, cryopreservation (P1), or final neuron differentiation | • NPCs are ready for expansion, cryopreservation (P0 or P1), or final neuron differentiation |

An example of DA NPC induction via monolayer culture is shown in **Figure 9**.

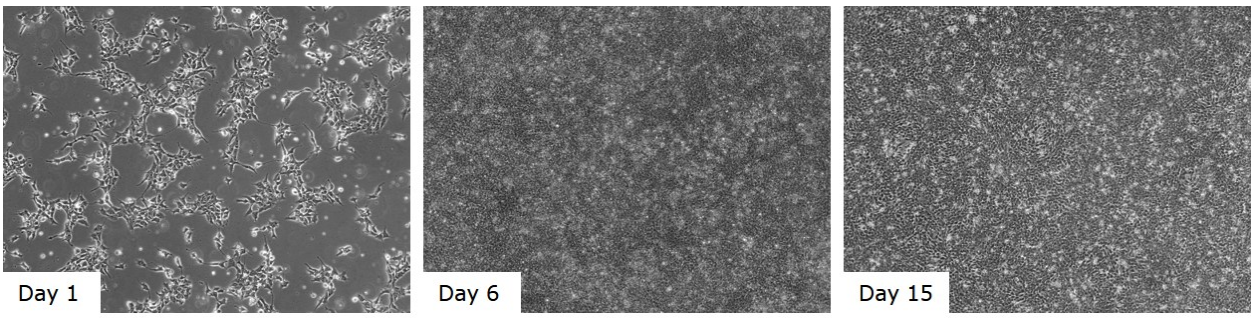


**Figure 9. Bright-field phase contrast of AIW002-02 iPSCs at days 1, 6, and 15 of DA NPC induction via monolayer culture.**

## 4.2 Coating culture vessels with Matrigel

**Materials:**

- Culture vessels (flasks or plates)
- Parafilm
- Matrigel (150-µL aliquot)
- DMEM/F12
- Antibiotic-Antimycotic
- 37°C/5% $CO_2$ cell culture incubator

**Procedure:**

1. Prepare Matrigel working solution on ice by adding 1 aliquot of Matrigel and 150 µL of Antibiotic-Antimycotic to 15 mL of cold DMEM/F12. Mix solution well.

    - **IMPORTANT:** Thaw Matrigel on ice. Keep DMEM/F12 with Antibiotic-Antimycotic on ice.
    - Recommended volumes of Matrigel working solution needed depending on the type of culture vessel are listed in **Table 6**.
    - Use immediately after preparation. Matrigel working solution can be stored at –20°C for up to 1 week. Thaw the Matrigel working solution on ice and use immediately. Do not re-freeze.

2. Immediately apply recommended volume of Matrigel solution to culture vessel (Table 6) and move back and forth and side to side 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.

**Table 6. Recommended volumes of Matrigel working solution based on type of culture vessel**

| Culture vessel | Volume of Matrigel solution |
|---|---|
| T25 flask | 3 mL |
| 10-cm dish | 5 mL |

3. Incubate culture vessel at 37°C for at least 1 hour.
4. If plating cells on culture vessel immediately, gently tilt culture vessel to one side to allow the excess Matrigel solution to collect in a corner. Remove the Matrigel solution with a serological pipet or by aspiration. Ensure coated surface is not scratched. Immediately add an appropriate volume of culture media to the culture vessel and then plate cells. If not plating cells on culture vessel immediately, do not aspirate Matrigel solution, seal with Parafilm, and store at 4°C for up to 7 days.

    - **IMPORTANT:** If using Matrigel-coated culture vessels that have been stored (i.e. if not using immediately after coating procedure), allow coated culture vessels to come to room temperature for 30 minutes and check quality of coating before plating cells. Uneven coating or evaporation of coating may affect cell distribution.