Institut-Hôpital neurologique de Montréal
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EDDU Protocols

Generation of OPCs and Oligodendrocytes from iPSCs

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Table of Contents

Introduction

Introduction

Objectives

- This protocol describes how to:
  - Generate and culture oligodendrocyte progenitor cells (OPCs) from iPSCs
  - Differentiate and culture functional myelinating oligodendrocytes

Introduction

Protocol Overview

By using a combination of small molecules and growth factors that regulate multiple pathways we adapted a method to induce and pattern spinal cord-like neural progenitor cells (sc-NPCs), to differentiate them into OPCs and to expand them within 75 days. OPCs can be expanded and stored or differentiated into fully functional myelinating oligodendrocytes within 20 days.

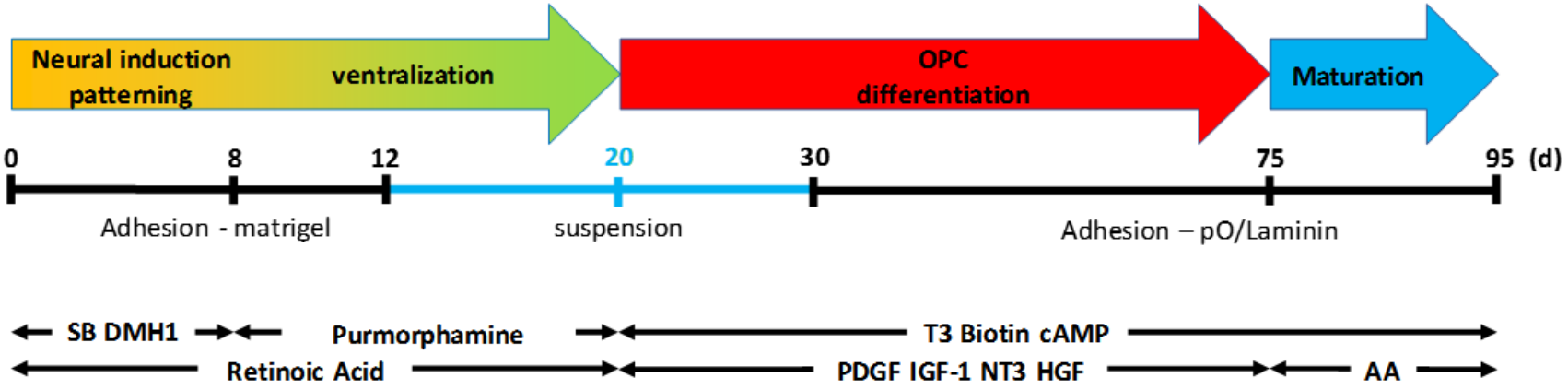


Figure 1. Schematic of the OPC differentiation protocol

Introduction

Technical and safety considerations

The following information should be read before starting:

- sc-NPCs, OPCs and oligodendrocytes must be handled within a Class II biosafety laminar flow hood to protect the worker from possible adventitious agents. 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.
- NPCs and OPCs 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. As a general rule, it is possible to create a basal medium to be stored for a maximum of 10 days into which appropriate molecules and growth factors can be added fresh when needed.
- OPCs and oligodendrocytes must be cultured on surfaces coated with poly-L-ornithine (PO) and laminin (except during the suspension phase to enrich for Olig-2 positive pre-OPCs)

Introduction

Technical and safety considerations (cont'd)

- **Take extra precautions to maintain sterility:**
  - Aspirate media using a 200 µL tip placed 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.
  - Avoid shaking plates when handling them; OPCs and oligodendrocytes tend to form layers that are loosely attached and can easily detach from the plate.
- **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.
- **OPC cultures must be monitored regularly to ensure optimal morphology and density prior to differentiation**.

Materials

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.

Materials

Labware

Item	Supplier	Catalogue #
Conical tube, 15-mL	Thermo Fisher	352097
Cryovial	Sarstedt	72.379
Culture flask, T25	Thermo Fisher	12-556-009
Culture flask, T75	Thermo Fisher	12-556-010
Culture plate, 6-well	Thermo Fisher	087721B
Culture plate, 96-well Black/Clear Flat Bottom	Falcon	353219
Petri-dish, 100-mm	Thermo Fisher	FB0875712
12 Well Chamber, removable	Ibidi	81201
Plastic serological pipette, 10-mL	Sarstedt	86.1254.001
Plastic serological pipette, 5mL	Sarstedt	86.1253.001
Plastic serological pipette, 1mL	Fisher	13-678-11B
Glass serological pipette, borosilicate disposable	FIsherbrand	13-678-27E
Polypropylene microcentrifuge tube	Fisher	02-681-273

*Table footnote

Materials

Culture reagents

Item	Supplier	Catalogue #	Stock conc.	Working conc.	Storage temp.
DMEM/F12 with GlutaMAX-I	Life Technologies	10565018	1X	1X	Stock:4°C Working: 4°C
Antibiotic-Antimycotic	Gibco	15240062	100X	1X	Stock: –20°C Working: 4°C
Laminin*	Sigma	L2020	1mg/mL†	5µg/mL	Stock: -80°C Working: 4°C
	Invitrogen	23017-015			
Accutase	Life Technologies	A11105-01	1X	1X	Stock: –20°C Working: 4°C
Y27632 (ROCK inhibitor)	Selleckchem	S1049	10mM	10µM	Stock: –80°C Working: 4°C
MEM Non-Essential Amino Acids	Wisent	321-011-EL	100X	1X	Stock: 4°C
2-Mercaptoethanol	Life Technologies	21985023	55mM	55µM	4°C
N2 Supplement	Gibco	17502-048	100X	1X	-20°C
B27 Supplement without vitamin A	Life Technologies	12587-010	50X	1X	-20°C

* 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 table continues on the next screen.

Materials

Culture reagents (cont'd)

Item	Supplier	Catalogue #	Stock conc.	Working conc.	Storage temp.
All-trans Retinoic Acid	Sigma-Aldrich	R2625	1mM	100nM	Stock: - 80°C Working: 4°C
SB431542	Selleckchem	S1067	10mM	10µM	Stock: - 80°C Working: 4°C
DMH1	Selleckchem	S7146	4mM	2µM	Stock: –80°C Working: 4°C
Puromorphamine	Sigma-Aldrich	SML-0868	2mM	2µM	Stock: - 80°C Working: 4°C
Recombinant human PDGF-AA	Peprotech	100-13A	10µg/mL	10ng/mL	Stock: - 80°C Working: 4°C
Recombinant human IGF-I, CF	Peprotech	100-11	10µg/mL	10ng/mL	Stock: - 80°C Working: 4°C
Recombinant human HGF	R&D Systems	294-HG-025	10µg/mL	5ng/mL	Stock: - 80°C Working: 4°C
Neurotrophin-3 (NT3)	Peprotech	450-03	10µg/mL	10ng/mL	Stock: - 80°C Working: 4°C
Insulin solution, human	Sigma-Aldrich	I2643	10mg/mL	25µg/mL	4°C
Biotin	Sigma-Aldrich	B4639	100µg/mL	100ng/mL	Stock: - 80°C Working: 4°C
Gentle dissociation reagent	Stem Cell	7174	1X	1X	RT

The table continues on the next screen.

Materials

Culture reagents (cont'd)

Item	Supplier	Catalogue #	Stock conc.	Working conc.	Storage temp.
Dibutyryl cAMP (db cAMP)	Carbosynth	ND07996	10mM	1µM	Stock: - 80°C Working: 4°C
3,3,5-Triiodo-l-thyronine (T3)	Sigma-Aldrich	T6397	60µg/mL	60ng/mL	Stock: - 80°C Working: 4°C
L-Ascorbic acid (AA)	Sigma-Aldrich	A5960	200mM	200µM	Stock: - 80°C Working: 4°C
Recombinant human FGF-2 basic	Peprotech	100-18B	10µg/mL	20ng/mL	Stock: - 80°C Working: 4°C
Heparin	Sigma-Aldrich	H3149	2mg/mL	2µg/mL	Stock: -80°C Working: 4°C
Poly-L-ornithine	Sigma-Aldrich	P3655	1mg/mL	10µg/mL	Stock: -20°C Working: 4°C
mTeSR1	StemCell	05851	1X	1X	Stock: -20°C Working: 4°C
mTesR Supplement	StemCell	05852	5X	1X	
Dibutyryl cAMP (db cAMP)	Carbosynth	ND07996	10mM	1µM	Stock: - 80°C Working: 4°C
3,3,5-Triiodo-l-thyronine (T3)	Sigma-Aldrich	T6397	60µg/mL	60ng/mL	Stock: - 80°C Working: 4°C
Matrigel Matrix hESC-qualified	Corning	354277	100X	1X	Stock: -80°C Working: thaw at 4°C

Materials

Equipment

Item	Supplier	Catalogue #
Cell culture incubator	ThermoScientific	SteriCycle Model 370 Ref#20
Cell culture water bath	Fisher Scientific	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

Protocol

Protocol: Coating of culture vessels

Materials

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

Protocol: Coating of culture vessels

Procedure

1. Prepare PO working solution by adding 500 µL of PO stock solution to 50mL 1x PBS.

Plate type	Volume of PO solution
T-25cm² flask	3ml/flask
T-75cm² flask	7ml/flask
6-well plate	1ml/well
12-well plate	1ml/well
24-well plate	0.5ml/well
60mm dish	2ml/well
100mm dish	7ml/dish

2. Apply recommended volume of PO solution to culture vessel and swirl to spread across surfaces.
3. Incubate culture vessel for at least 2 hours or overnight at 37°C.
4. Aspirate PO solution from culture vessel and wash surfaces three times with 1x PBS.

- Thaw PO stock solution at 4°C.
- Ensure transfer of all PO stock solution by rinsing tube with PBS twice.
- The PO working solution can be stored at 4°C for up to 2 months.

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

Protocol: Coating of culture vessels

Procedure

5. Prepare laminin working solution by adding 100µL of laminin stock solution and 200 µL of Antibiotic Antimycotic to 20 mL of cold DMEM/F12.

Plate type	Volume of laminin solution
T-25cm² flask	2,5ml/flask
T-75cm² flask	7ml/flask
6-well plate	1ml/well
12-well plate	500µl /well
24-well plate	300µl/well
60mm dish	2ml/well
100mm dish	7ml/dish

6. Apply recommended volume of laminin solution to culture vessel and swirl to spread across surfaces.
7. Incubate culture vessel at 37°C for 2 hours.

- **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.
- The laminin working solution can be stored at 4°C for up to 2 weeks

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

Protocol: Coating of culture vessels

Procedure

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.

Protocol: Preparation of iPSC cultures for Oligodendrocyte Differentiation

Materials

- Plate of 70-90% confluent iPSCs
- Matrigel-coated 6-well plate
- Gentle dissociation reagent
- 15-mL conical tubes
- 37°C/5% CO_2 cell culture incubator
- Media:

Media	Components
mTeSR1	• mTeSR1 Supplements • 10µM Y-27632 (ROCK inhibitor)
	OR
Essential - 8	• 10µM Y-27632 (ROCK inhibitor)

Protocol: Preparation of iPSC cultures for Oligodendrocyte Differentiation

Procedure

1. Aspirate media from cells and rinse with 5 mL of DMEM/F12 containing 1x Antibiotic-Antimycotic.
2. Add 5 mL Gentle Cell Dissociation Reagent and incubate at room temperature for 4 to 6 minutes or at 37°C for 3 to 5 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.
4. Transfer the detached cell aggregates 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 cell aggregates and transfer to the tube.
5. Pellet cell aggregates by centrifuging at 1200 rpm (200 g) for 3 minutes.

- Ensure that regions of differentiation have been removed by aspiration prior to passaging.
- Refer also to SOP EDDU_002_02.

- Take care to avoid breaking up cell aggregates.

Protocol: Preparation of iPSC cultures for Oligodendrocyte Differentiation

Procedure

6. Gently aspirate supernatant. Carefully pipette the cell aggregate mixture up and down 3 to 5 times with a glass pipet in complete media to break up the cell aggregates.
7. Plate 8 × 10^4 to 1 × 10^5 cells per well on a Matrigel-coated six-well plate that already contains 1.5 ml of mTeSR1 (or Essential 8) supplemented with 10 µM Y27632 per well.
8. Incubate the cells for 24 h at 37 °C, 5% CO2.
9. The next day, remove the old medium and add 2 ml of fresh mTeSR1 (or Essential 8) in each well.

- Do not create a single-cell suspension.

- This density of plated iPSCs is optimized to give a confluent well by day 8 and multilayered structures by day 12 of differentiation.

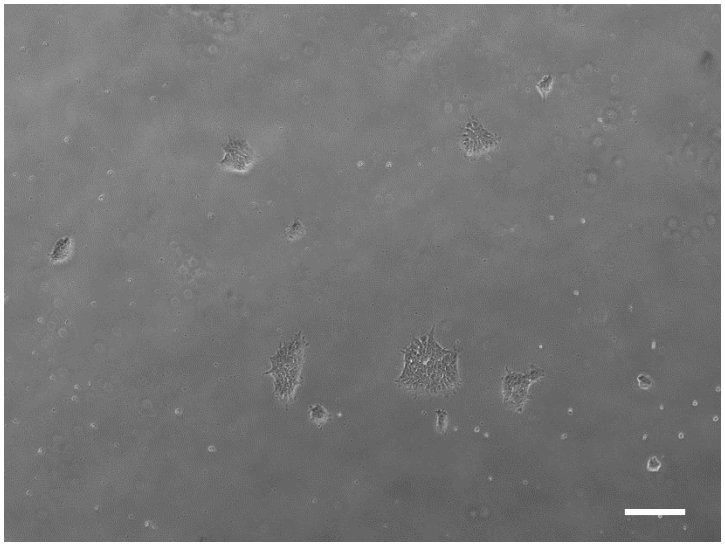
Protocol: Preparation of iPSC cultures for Oligodendrocyte Differentiation

Procedure

10. Incubate the cells for 1–2 days, until iPSC colonies reach a diameter of 100–250 µm.

Select a condition to view recommendations.

Too small colonies | Good size colonies | Too big colonies



- Colonies won't generate the proper number of cells by day 12 of differentiation
- Most of the colonies won't survive after neural induction

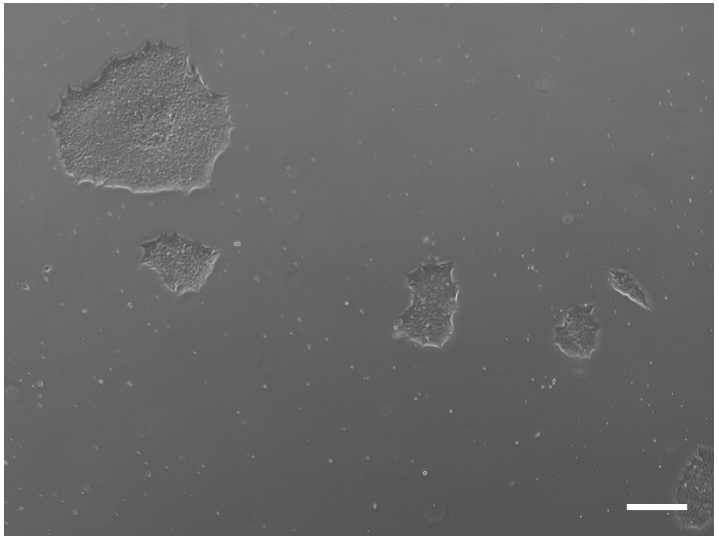
Protocol: Preparation of iPSC cultures for Oligodendrocyte Differentiation

Procedure

10. Incubate the cells for 1–2 days, until iPSC colonies reach a diameter of 100–250 µm.

Select a condition to view recommendations.

Too small colonies | **Good size** | Too big colonies



- This size ensures proper proliferation by day 12 of differentiation

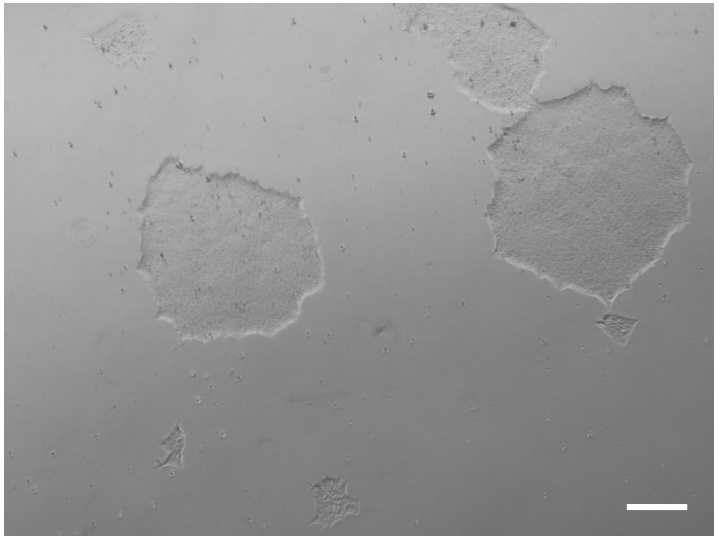
Protocol: Preparation of iPSC cultures for Oligodendrocyte Differentiation

Procedure

10. Incubate the cells for 1–2 days, until iPSC colonies reach a diameter of 100–250 µm.

Select a condition to view recommendations.

Too small colonies | Good size | **Too big colonies**



- Cells will over-proliferate by day 12 of differentiation
- Increased cell death due to higher consumption of nutrients

Protocol: Differentiation of OLIG2+ neural progenitors

Materials

- iPSCs cultured in 6-well plate
- 37°C/5% CO_2 cell culture incubator
- Accutase
- bFGF 20µg/mL

*It is very important for the differentiation of oligodendrocytes to avoid using vitamin A. The use of normal vitamin A-supplemented B27 may result in the arrest of cells in the OPC phase.

- Media:

Media	Components
Basal medium	• DMEM/F12 + Glutamax-I • 1X NEAA • 1X N2 • 1X B27 (w/o vit. A)* • 1X Anti-Anti • 55uM 2-mecaptoethanol • 2µg/mL Heparin • 25µg/mL Insulin
Neural induction/patterning medium	• Basal medium • 10µM SB431542 • 2µM DMH1 • 0.1µM Retinoic Acid
Ventralization medium	• Basal medium • 0.1µM Retinoic Acid • 1µM Puromorphamine

Protocol: Differentiation of OLIG2+ neural progenitors

Procedure

1. Once colonies are ~100–250 µm in diameter (see **Figure 2**), aspirate the old medium and induce differentiation by adding 2 ml of neural induction/patterning medium to each well. This is day 0.
2. Incubate the plate at 37 °C, 5% CO2, and perform media changes every day for 8 days, adding fresh RA, SB431542 and LDN193189 to the medium every day.

- Adding RA at a low concentration (100nM) from day 0 of the differentiation greatly improves the yield of OLIG2+ progenitors at day 12.

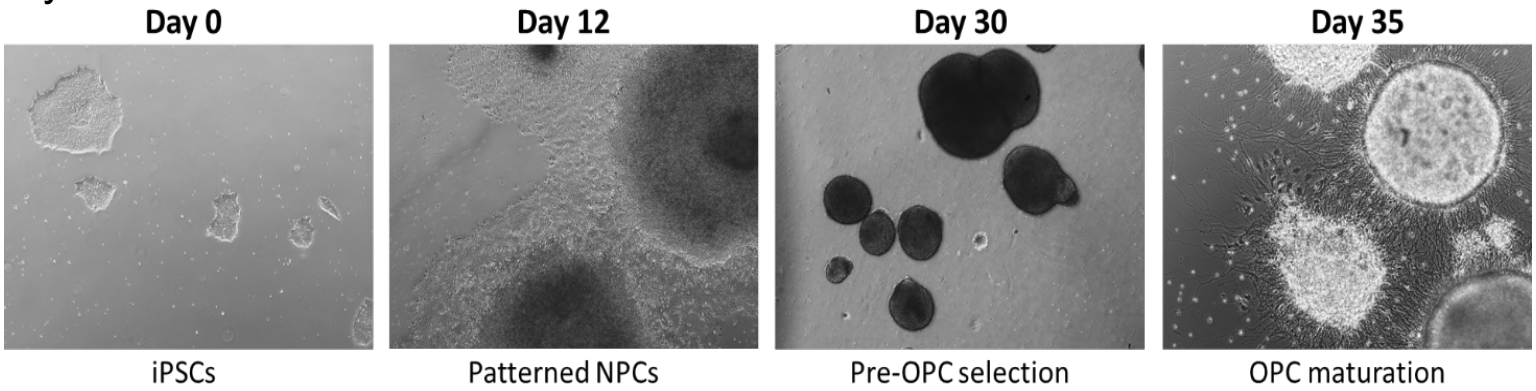


Figure 2. Brightfield image of cell morphology during the differentiation protocol

Protocol: Differentiation of OLIG2+ neural progenitors

Procedure

3. On day 8, switch to ventralization medium and incubate for 4 days, changing the medium daily, adding fresh RA and Purmorphamine to the medium every day.

PAUSE POINT: At this point cells can be frozen. Otherwise proceed with the aggregation and expansion of OLIG2+ cells.

- The medium will become yellowish owing to the rapid expansion of the cells and their increasing numbers. This is why it is important to change the media every day on the cells to provide the required nutrients.
- By day 8, cells should be confluent and PAX6 expression should be at its peak.
- By day 12, overconfluent cells piling up and 3D structures should be clearly visible. This is an important checkpoint before proceeding with the differentiation (see **Figure 2)**

Protocol: Differentiation of OLIG2+ neural progenitors

Procedure

4. Aspirate the medium and add 1 ml of prewarmed Accutase.
5. Incubate at 37 °C for 5 min (or until cells completely detach from the plate)
6. Dilute the Accutase solution by adding 2 ml of DMEM/F12 medium.
7. With a p1000 pipette, pipette the mixture 2–5 times to dissociate the culture into a single-cell suspension.
8. Transfer the cells to a 15-ml conical tube and dilute the solution further by adding another 5 ml of DMEM/F12.
9. Collect the cells by centrifugation at 1200rpm (200g) for 4 min at RT.

- In order to obtain a better single-cell suspension a double-tip can be used by attaching a p200 pipette tip in front of a p1000tip.

Protocol: Differentiation of OLIG2+ neural progenitors

Procedure

10. Resuspend the cell pellet in 1 ml of ventralization medium supplemented with 20ng/mL bFGF and distribute the single-cell suspension into a 100mm Petri dish containing 9mL of supplemented ventralization medium.
11. Incubate the plate for 2 days at 37 °C, 5% CO2.
12. Transfer the medium containing the cell aggregates into a 15-ml conical tube and centrifuge for 2 min at 100g at RT.
13. Remove three-quarters of the medium and add 6.5ml of fresh supplemented ventralization medium.
14. Return the aggregates to the same Petri dish and swirl the plate to redistribute the aggregates.
15. Repeat medium change steps every other day until day 20 of differentiation.

- The addition of bFGF maximizes the efficiency of sphere formation and promotes the differentiation of proliferating OPCs.

- According to the rate of expansion and the change in coloration of media, this can be performed twice a week.
- **PAUSE POINT:** At this point cells can be frozen as explained in the next section. Otherwise proceed with Differentiation of OPC and Oligodendrocytes in adherent cultures.

Protocol: Freezing of OLIG2+ spheres

Materials

- Spheres culture at day 20 in a 100mm Petri dish
- Cryovials
- 15-mL conical tube
- 5-mL glass pipet
- Accutase
- DMEM/F12
- Antibiotic-antimycotic
- 20% DMSO in FBS
- Centrifuge
- 37°C/5% CO_2 cell culture incubator

Protocol: Freezing of OLIG2+ spheres

Procedure

1. Collect the spheres in a 15-mL conical tube.
2. Wash the plate with 3-mL DMEM/F12 with Antibiotic-antimycotic.
3. Centrifuge the suspension at 1200rpm (200g) for 4 min.
4. Aspirate the medium and add 0.5-1 mL of prewarmed Accutase.
5. Incubate at 37 °C for 5 min (or until cells completely detach from the plate)
6. Dilute the Accutase solution by adding 2 ml of DMEM/F12 medium.
7. With a p1000 pipette, pipette the mixture 2–5 times to dissociate the culture into a single-cell suspension.

- In order to obtain a better single-cell suspension a double-tip can be used by attaching a p200 pipette tip in front of a p1000tip.

Protocol: Freezing of OLIG2+ spheres

Procedure

8. Incubate at 37°C for 5-10 min.
9. Gently aspirate the supernatant. Gently resuspend the cell pellet in FBS in half the volume needed to form 1 mL of cell aggregate mixture per cryovial.
10. Add an equal volume of 20% DMSO/FBS to the cell aggregate mixture to obtain a final DMSO concentration of 10%. Mix well.
11. Transfer 1 mL of cell aggregate mixture to each cryovial.
12. Place cryovials in a cryo-box and store at –20°C for 3 to 4 hours. Transfer cryo-box to –80°C and store overnight. Transfer cryovials to liquid nitrogen.

- In this case, each Petri dish will have enough spheres to freeze 3 cryovials so 1.5 mL should be added. This amount should be adjusted according to the amount of spheres generated.

- Do not store cryovials at –80°C for more than 3 days. OPCs are sensitive to changes in temperature. Long-term storage at –80°C reduces cell survival after thawing.

Protocol: Differentiation and expansion of OPC and differentiation into myelinating Oligodendrocytes

Materials

- Day 20 spheres or frozen cryovials of day 20 progenitors
- 37°C/5% CO_2 cell culture incubator
- 15-mL conical tube
- 5-mL glass pipette
- PO/laminin-coated vessels
- Basal medium

- Media:

Media	Components
PDGF medium (OPC medium)	• Basal medium • 10ng/mL PDGF-AA • 10ng/mL IGF-1 • 10ng/mL NT-3 • 5ng/mL HGF • 60ng/mL T3 • 100ng/mL Biotin • 1µM cAMP
AA medium (Differentiation medium)	• Basal medium • 200µM Ascorbic Acid • 60ng/mL T3 • 100ng/mL Biotin • 1µM cAMP

Protocol: Differentiation and expansion of OPC and differentiation into myelinating Oligodendrocytes

Procedure

1. On day 20, with a glass pipette, transfer the aggregates to a 15-ml conical tube and wait for 3 min for the aggregates to sink to the bottom of the tube.
2. Remove two-thirds of the medium and replenish it with PDGF medium.
3. Gently pipette five times up and down with a p1000 pipette.

- If the culture is started from frozen vials, plate the content of one vial in 10-mL of PDGF medium in a 100mm Petri dish. Proceed with **step 5**.
- **OPTIONAL:** 20ng/mL of bFGF can be added to the thawed culture to speed up sphere formation.

- It is important to break apart the aggregates that stick to each other through gentle pipetting.

Protocol: Differentiation and expansion of OPC and differentiation into myelinating Oligodendrocytes

Procedure

4. Return the aggregates to the same Petri dish.
5. Repeat medium change every other day until day 30 of differentiation.
6. On day 30, transfer the aggregates in a PO/laminin-coated vessel containing PDGF medium.
7. Incubate the plate at 37 °C, 5% CO2.

- Plating two spheres per cm^2 ensures that cells will migrate out of the spheres and fill the whole well around day 60 without the need for passaging the cells.
- For large production of OPCs an entire Petri dish of spheres can be plated in a T75 flask.

Protocol: Differentiation and expansion of OPC and differentiation into myelinating Oligodendrocytes

Procedure

8. Every other day carefully replenish two-thirds of the medium with fresh PDGF medium until day 75 of differentiation. After day 55 most of the cells should show a bipolar or tri-polar morphology typical of OPCs (see **Figure 3**).

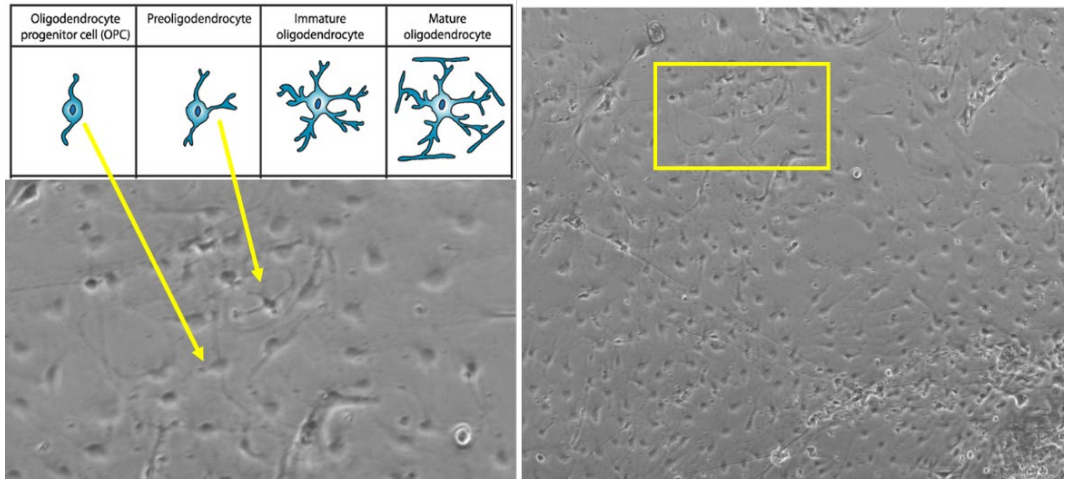


Figure 3. Morphology of OPCs at day 55 of differentiation

- **IMPORTANT:** Avoid sudden movements when you transfer the plate from the incubator, especially after day 40, as the cells can potentially detach from the plate, typically in the form of a sheet.
- After day 55, OPCs can be passaged in new PO/laminin-coated vessels in the presence of the ROCK inhibitor (Y27632).
- **PAUSE POINT:** Cells can be frozen at day 75.

Protocol: Differentiation and expansion of OPC and differentiation into myelinating Oligodendrocytes

Procedure

9. At day 75 carefully replenish two-thirds of the medium with fresh AA medium.
10. Repeat **step 9** every 2-3 days (or once a week) until day 95.

- Oligodendrocytes should have a typical multi-branched shape and should be positive to the nuclear marker SOX10 and the surface antigen O4 (see **Figure 4**).

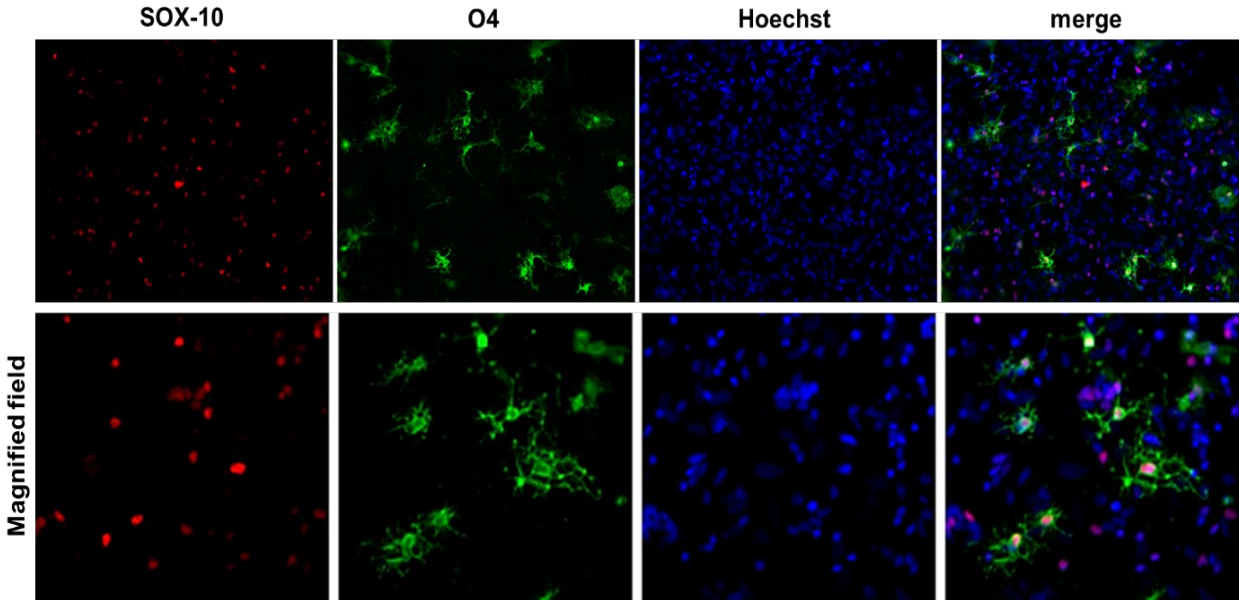


Figure 4. SOX10/O4-positive Oligodendrocytes at the end of the protocol

You have reached the end of the protocol.