EDDU Protocols

Motor Neuron Induction and Differentiation

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The involved functions approve the document for its intended use:

Name	Function	Role	Date	Signature
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1 Introduction

1.1 Objectives

This protocol describes how to:

- Generate motor neural progenitor cells (MNPCs)
- Generate motor neurons from MNPCs

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 MNPCs which are then further differentiated into an enriched population of functionally mature motor neurons (Figure 1).

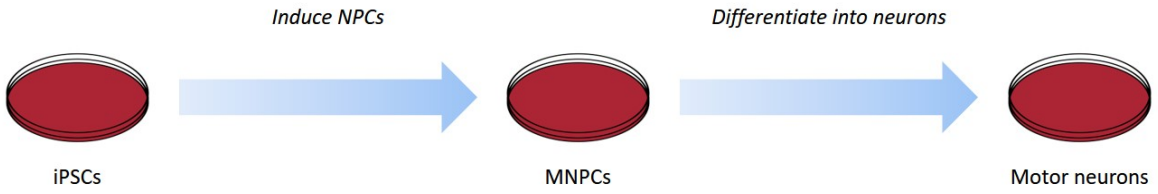


Figure 1. Protocol overview for the generation of MNPCs from iPSCs followed by the differentiation of MNPCs into motor neurons.

1.3 Technical and safety considerations

The following information should be read before starting:

- iPSCs, MNPCs, and motor 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.
- High-quality iPSCs are essential for induction of MNPCs. iPSCs should be recovered from a frozen stock and passaged at least once before using in this protocol. Genomic stability of the iPSCs should be confirmed prior to induction into neural progenitor cells.
- MNPCs and motor 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.

- Manipulate cells gently:
  - Add media to and aspirate media from culture 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).
  - 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.
- MNPC 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 MNPCs and motor neurons expressing motor neuron markers. 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
Cryovial	Sarstedt	72.379
Culture dish, 10-cm	BD Falcon	354277
Culture flask, T25	ThermoFisher	12-556-009
Culture flask, T75	ThermoFisher	12-556-010
Culture plate, 24-well	ThermoFisher	087721
Culture plate, 96-well (flat-bottom)	Falcon	353219
Glass coverslip, 12-mm	ThermoFisher	12-545-80
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 Working: 4°C
Ascorbic acid (AA)	Sigma	A5960-25G	200 mM	100 µM	Stock: –80°C Working: 4°C
B-27*	Gibco	17504044	50x	0.5x	Stock: –20°C Working: 4°C
BDNF	Peprotech	450-02	20 µg/mL	10 ng/mL	Stock: –80°C Working: 4°C
CHIR-99021	Selleckchem	S2924	3 mM	3 µM or 1 µM	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
CNTF	Peprotech	450-13	10 µg/mL	10 ng/mL	Stock: –80°C 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 Working: 4°C
FBS	Gibco	12484-028	1x	1x	Stock: –80°C Working: Room temperature
Gentle Cell Dissociation Reagent	STEMCELL Technologies	07174	1x	1x	Room temperature
GlutaMAX-I	Gibco	35050-061	100x (200 mM)	1x (2 mm)	Stock: Room temperature Working: 4°C
IGF-1	Peprotech	100-11	10 µg/mL	10 ng/mL	Stock: –80°C Working: 4°C
Laminin	Sigma	L2020	1 mg/mL†	5 µg/mL	Stock: –80°C Working: 4°C
Matrigel Matrix hESC-qualified	Corning Millipore	354277	100x	1x	Stock: −80°C Working: 4°C‡
N-2*	Gibco	17502048	100x	0.5x	Stock: –20°C Working: 4°C

Item	Supplier	Catalogue #	Stock conc.	Working conc.	Storage temp.
Neurobasal (NB) media	Life Technologies	21103-049	1x	1x	4°C
Phosphate-buffered saline (PBS)	Wisent	311-010-CL	1x	1x	Room temperature
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	0.5 µM or 0.1 µM	Stock: –80°C Working: 4°C
Retinoic acid (RA)[‖]	Sigma	R2625	1 mM	0.5 µM or 0.1 µM	Stock: –80°C Working: 4°C
SB431542	Selleckchem	S1067	10 mM	2 µM	Stock: –80°C Working: 4°C
StemPro Accutase Cell Dissociation Reagent	Gibco	A1110501	1x	1x	Stock: -20°C Working: 37°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.
[†]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.
[§]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.
[‖]RA is light- and air-sensitive. Stock and working aliquots should be covered in aluminum foil to minimize exposure to light. Always use a new aliquot of stock solution to prepare the final solution.

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
LUNA-II Automated cell counter	Logos Biosystems	L40002
Cell counting slide	Logos Biosystems	05181401

3 Protocol

3.1 Coating culture vessels with PO and laminin

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
24-well plate	500 µL/well	400 µL/well

3.2 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 2.
    - 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 2) 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 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.

Table 2. 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

3.3 Induction of iPSCs into MNPCs

Materials:

- iPSC culture recovered from frozen and passaged at least once (10-cm dish, 70%–80% confluent after culturing for 6–7 days)
- Matrigel-coated T25 flask
- PO/laminin-coated T25 and T75 flasks
- Cell scraper
- 15-mL tubes
- DMEM/F12 containing 1x Antibiotic-Antimycotic
- Gentle Cell Dissociation Reagent
- Y-27632 (ROCK inhibitor)
- Media:

Media	Components
Motor step 1 media	• 1:1 NB:DMEM/F12 • 1x Antibiotic-Antimycotic • 0.5x N-2 • 0.5x B-27 • 0.5x GlutaMAX-I • 100 µM AA • 3 µM CHIR99021 • 2 µM DMH1 • 2 µM SB431542
Motor step 2 media	• 1:1 NB:DMEM/F12 • 1x Antibiotic-Antimycotic • 0.5x N-2 • 0.5x B-27 • 0.5x GlutaMAX-I • 100 µM AA • 1 µM CHIR99021 • 2 µM DMH1 • 2 µM SB431542 • 0.1 µM RA • 0.5 µM purmorphamine

Media	Components
Motor step 3 media	• 1:1 NB:DMEM/F12 • 1x Antibiotic-Antimycotic • 0.5x N-2 • 0.5x B-27 • 0.5x GlutaMAX-I • 100 µM AA • 3 µM CHIR99021 • 2 µM DMH1 • 2 µM SB431542 • 0.1 µM RA • 0.5 µM purmorphamine • 0.5 mM VPA

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

Procedure:

1. 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 5 minutes.

    - Prior to dissociating culture, ensure regions of differentiation are removed. If regions of differentiation are observed, mark area with marker on bottom of dish. Remove regions of differentiation by aspiration with a 200-µL tip while frequently turning the dish to avoid exposing the cells to air. Regions of differentiation should not exceed 10% of the area of the dish.
    - If culture is more than 80% confluent or if the colonies are too large, extend incubation time in Gentle Cell Dissociation Reagent to 5 to 7 minutes.
    - The culture should be dissociated into small aggregates of cells. Avoid incubating the culture in dissociation reagent for too long as it could result in the culture dissociating into single cells, which are not ideal for the induction process.

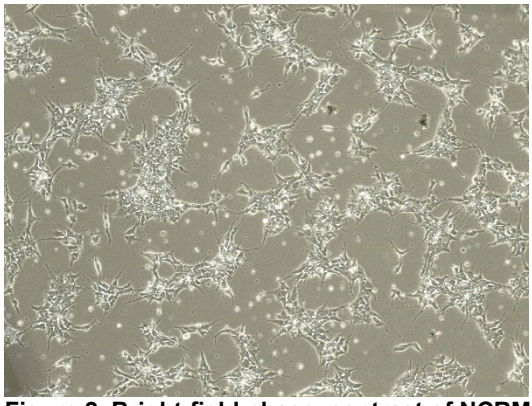
2. Aspirate the Gentle Cell Dissociation Reagent and rinse cells with 5 mL of DMEM/F12 containing 1x Antibiotic-Antimycotic. Aspirate the media and then add 5 mL of DMEM/F12 containing 1x Antibiotic-Antimycotic. Gently detach the colonies with a cell scraper.

    - The cells are rinsed to remove dying cells and residual Gentle Cell Dissociation Reagent from the dish.

3. Transfer cells 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.
4. Determine cell number using a cell counter. Transfer 2 million to 3 million cells to a fresh 15-mL tube and pellet cells by centrifuging at 1200 rpm (200 g) for 3 minutes.

5. Carefully aspirate supernatant. Resuspend cells in 1 mL motor step 1 media containing ROCK inhibitor (10 µM) and gently pipette cells up and down 3 to 5 times using a 1-mL pipet.
6. Transfer cells to a Matrigel-coated T25 flask and add 4 mL of motor step 1 media containing ROCK inhibitor (10 µM). Move the flask in several quick back-and-forth and side-to-side motions to distribute the cells. Place the cells in a 37°C incubator.
7. After 24 hours, assess cell morphology and density and follow the recommendations outlined in Table 3.

Table 3. Recommendations for iPSCs after plating in motor step 1 media depending on cell morphology and density.

Cell morphology and density	Recommendations
Cells have attached to the flask and are ~30% confluent  **Figure 2. Bright-field phase–contrast of NCRM1 iPSCs 1 day after plating in motor step 1 media.**	• Cells are at ideal density. Two days after plating, change media to motor step 1 media without ROCK inhibitor. • Continue to change media every other day.
Cells have not attached and/or there is a lot of cell death	• One day after plating, change media to motor step 1 media without ROCK inhibitor (10 µM) to remove dead cells. • Continue to change media every other day.
Cells are 100% confluent causing media to turn yellowish in colour	• Change media to motor step 1 media without ROCK inhibitor. • Continue to change media daily.

8. Six days after plating in motor step 1 media, cells should be 100% confluent. Aspirate media and rinse cells with 5 mL of DMEM/F12 containing 1x Antibiotic-Antimycotic. Add 2 mL Gentle Cell Dissociation Reagent and incubate at 37°C for 5 to 7 minutes.

    - If the cells have started to pile up and form aggregates, dissociate the cells using StemPro Accutase Cell Dissociation Reagent instead of Gentle Cell Dissociation Reagent.

- Tap the side of the flask with the palm of your hand to help detach the cells from the flask. If the cells remained attached to the flask, tap the flask more frequently and with slightly more force.

9. Without removing the Gentle Cell Dissociation Reagent, add 5 mL of DMEM/F12 containing 1x Antibiotic-Antimycotic and transfer cells to a 15-mL conical tube. Pellet cells by centrifuging at 1200 rpm (200 g) for 3 minutes.
10. Carefully aspirate supernatant. Resuspend cells in 1 mL motor step 2 media containing ROCK inhibitor (10 µM) and gently pipette cells up and down 3 to 5 times using a 1-mL pipet. Split cells 1:3 to 1:6 and plate in motor step 2 media on a PO/laminin-coated T75 flask. Move the flask in several quick back-and-forth and side-to-side motions to distribute the cells. Place the cells in a 37°C incubator.

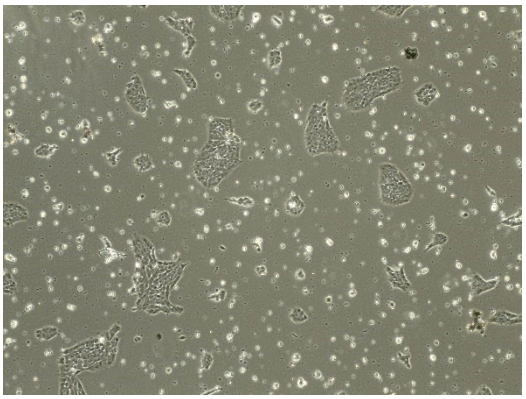
    - The number and size of flasks to use is determined by the size of the cell pellet (Table 4).

Table 4. Number and size of flasks to use based on cell pellet size.

Pellet size	Number/size of flasks
<1.5 mm	One T75 flask
1.5–2 mm	One T25 flask and one T75 flask
>2 mm	Two T75 flasks

11. After 24 hours, assess cell morphology and density and follow the recommendations outlined in Table 5.

Table 5. Recommendations for iPSCs after plating in motor step 2 media depending on cell morphology and density.

Cell morphology and density	Recommendations
Cells have attached to the flask and are ~50% confluent  **Figure 3. Bright-field phase–contrast of NCRM1 iPSCs 1 day after plating in motor step 2 media.**	• Cells are at ideal density. Two days after plating, change media to motor step 2 media without ROCK inhibitor. • Continue to change media every other day.
Cells have not attached and/or there is a lot of cell death	• One day after plating, change media to motor step 2 media without ROCK inhibitor (10 µM) to remove dead cells. • Continue to change media every other day.
Cells are 100% confluent causing media to turn yellowish in colour	• Change media to motor step 2 media without ROCK inhibitor. • Continue to change media daily.

12. Six days after plating in motor step 2 media (day 12 of protocol), cells should be 70% to 90% confluent and start to pile up (Figure 4). Repeat steps 8 to 11 in motor step 3 media.

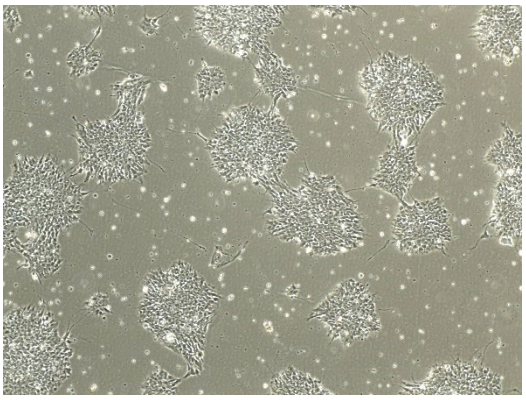


Figure 4. Bright-field phase–contrast of NCRM1 iPSCs 6 days after plating in motor step 2 media.

13. Six days after plating in motor step 3 medium (day 18 of protocol), cells should be 70% to 90% confluent and start to pile up (Figure 5). Proceed directly to motor neuron differentiation (section 3.4) or split and/or freeze MNPCs in FBS containing 10% DMSO.

- MNPCs can be passaged up to 5 more times.
- Freeze stocks of MNPCs from multiple T75 flasks. One T25 flask started from day 1 in motor step 1 media can be expanded to six to ten T75 flasks by day 18 in motor step 3 media, which can be frozen in 30 to 50 cryovials.
- MNPCs are ready for differentiation when cells have been cultured in motor step 3 media for 5 to 6 days.

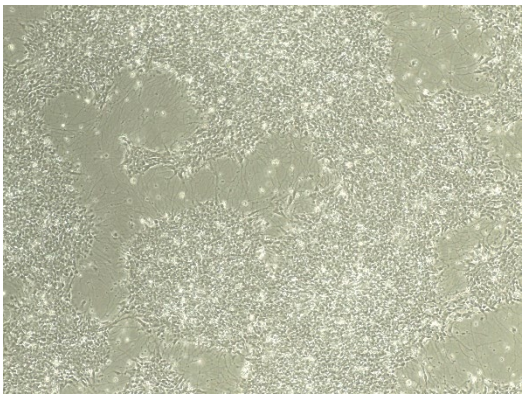


Figure 5. Bright-field phase–contrast of NCRM1 MNPs 6 days after plating in motor step 3 media.

3.4 Differentiating MNPCs into motor neurons

Materials:

- MNPC culture recovered from frozen or freshly prepared (T25 flask, 70%–80% confluent after culturing in motor step 3 media for 5–6 days)
- PO/laminin-coated T25 and T75 flasks
- PO/laminin-coated 24-well plate containing coverslips
- 15-mL tubes
- DMEM/F12 containing 1x Antibiotic-Antimycotic
- StemPro Accutase Cell Dissociation Reagent
- Y-27632 (ROCK inhibitor)
- Media:

Media	Components
Final motor neuron differentiation media	• 1:1 NB:DMEM/F12 • 1x Antibiotic-Antimycotic • 0.5x N-2 • 0.5x B-27 • 1x GlutaMAX-I • 100 µM AA • 0.5 µM RA • 0.1 µM purmorphamine • 0.1 µM Compound E • 10 ng/mL BDNF • 10 ng/mL CNTF • 10 ng/mL IGF-1

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

Procedure:

1. Aspirate media from MNPC culture and rinse cells with 5 mL DMEM/F12 containing 1x Antibiotic-Antimycotic. Add 2 mL StemPro Accutase Cell Dissociation Reagent and incubate at 37°C for 4 to 6 minutes or until the cells begin to detach from the flask.

    - Gently tap the side of the flask with the palm of your hand to help detach the cells from the flask.
    - Incubation time for cell dissociation varies depending on MNPC morphology and cell density. 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).

2. Without removing the StemPro Accutase Cell Dissociation Reagent, add 5 mL of DMEM/F12 containing 1x Antibiotic-Antimycotic to the flask. Transfer cells 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. Carefully aspirate supernatant and resuspend cells in 1 mL final motor neuron differentiation media containing ROCK inhibitor (10 µM). Gently pipette cells up and down 3 to 5 times with a 1-mL pipet.

    - Do not over-pipette the suspension as this will destroy the premature neurons.
    - Resuspension technique varies depending on MNPC morphology and cell density, and the activity of StemPro Accutase Cell Dissociation Reagent.

4. Determine cell number using a cell counter. Plate cells in final motor neuron differentiation media containing ROCK inhibitor (10 µM) on a PO/laminin-coated culture vessel at the recommended density and media volume as outlined in Table 6. Move the flask in several quick back-and-forth and side-to-side motions to distribute the cells. Place the cells in a 37°C incubator.

Table 6. Recommended cell density and media volumes for plating motor neurons based on type of culture vessel.

Culture vessel	Cell number and media volume
T25 flask	2 x 10^6 cells
T75 flask	6 x 10^6 cells
24-well plate with coated coverslips	30,000–50,000 cells/500 µL per well
96-well plate*	~15,000 cells/100 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 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.

5. Change media once per week to final motor neuron differentiation media (without ROCK inhibitor) 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.
    - MNPs should be fully differentiated into motor neurons after 2 weeks in final motor neuron differentiation media (Figure 6).

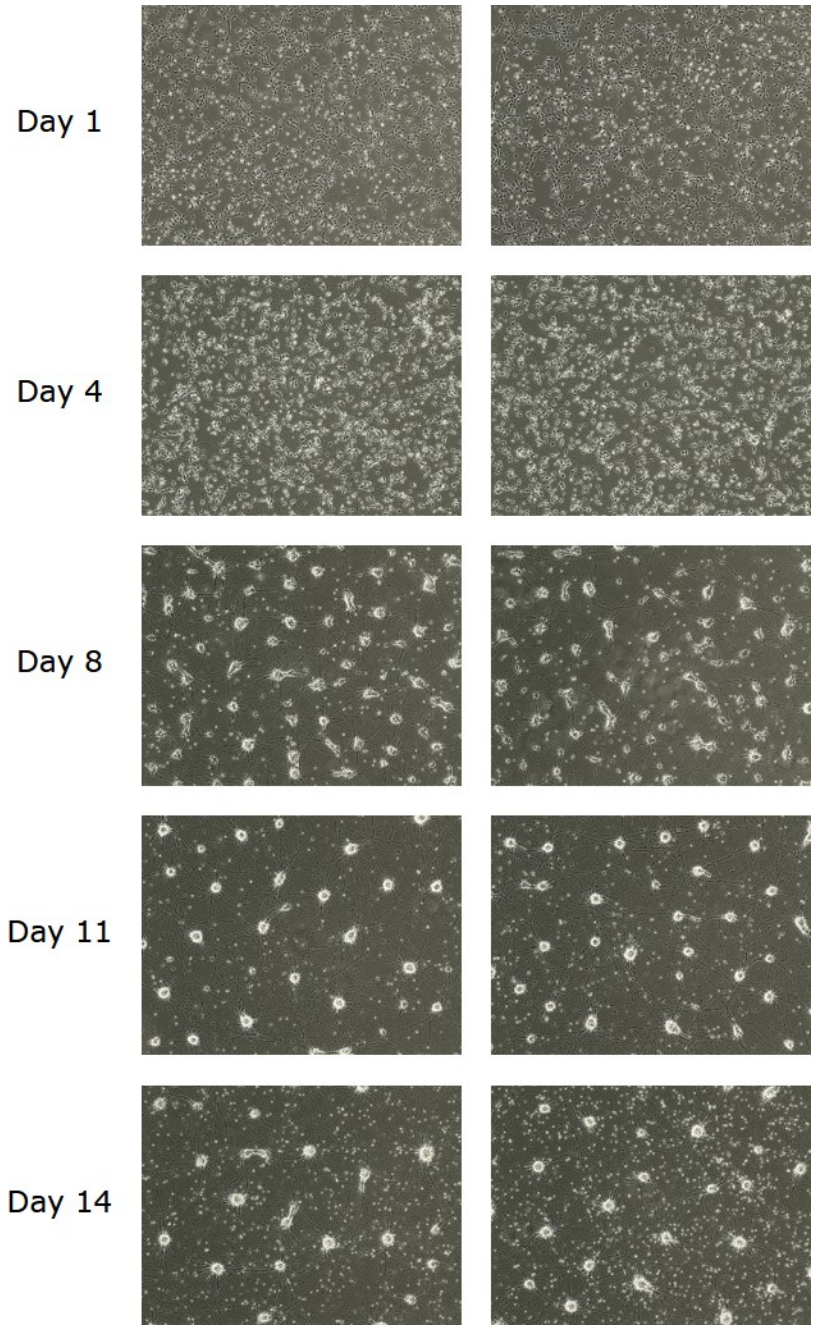


Figure 6. Bright-field phase–contrast of FA04 motor neurons at days 1, 4, 8, 11, and 14 after plating in final motor neuron differentiation media.

- Perform standard quality control tests on differentiated cells to confirm motor neuron characteristics (e.g. motor neuron marker expression). Expression of motor neuron markers by immunofluorescence after 2 weeks in final motor neuron differentiation media is shown in Figure 7.

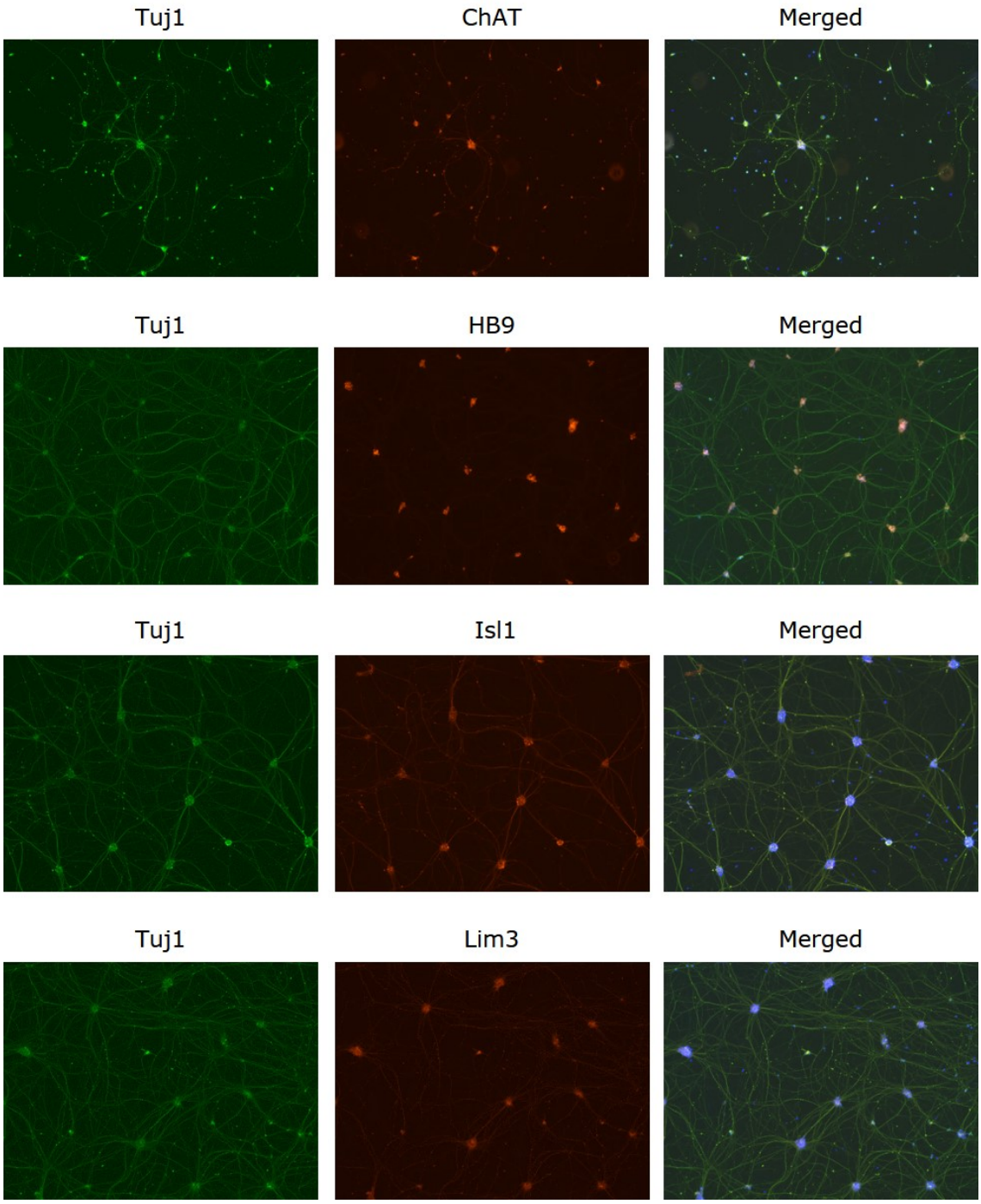


Figure 7. Immunofluorescence of Tuj1, ChAT, HB9, Isl1, and Lim3 in FA04 motor neurons at day 14 after plating in final motor neuron differentiation media.

- Motor neurons can survive in culture for 6 weeks using this protocol.