Direct iMN (diMNs) differentiation in monolayer from hiPSCs

*Please refer to tables for media compositions and reagent catalog numbers at the end of the document

Part 1: Human iPSC (hiPSC) culture

1.1 Expand hiPSC cultures using mTeSR1/matrigel (limited E8/Matrigel testing has been also been performed with similar results) method of iPSC expansion. "Groom" / clean the hiPSC cultures daily. hiPSC cultures devoid of differentiated cells is critical for consistent diMN cultures and avoiding batch-to-batch differentiation variability.
**NOTE: iPSCs should be cultured for minimal of two weeks before starting differentiation. Differentiation should not be done immediately after thawing iPSCs.*

1.2 Before start of differentiation hiPSCs should be plated on Matrigel coated plate at low density. Cells should be cleaned daily and should ideally between 30-40% confluence at the start of differentiation. On the iPSC passage before beginning the differentiation, passage the cells at a lower density. A well confluence of 30-40% is desired at day 3 – 6 post-passage of hiPSC culture.

Part 2: Stage 1 of differentiation - Neuroepithelial induction

Day 0

2.1 Prepare Stage 1 media as necessary (See table 1).

2.2 Pre-warm Stage 1 medium at room temperature.

2.3 "Groom" / clean the iPSCs before proceeding with the next step. Use standard "grooming" protocol by manually removing the differentiation with a sterile pipet tip. hiPSC cultures should contain as few or no (spontaneously) differentiated cells.

2.4 Aspirate and discard of the hiPSC media in each well (no rinse with stage 1).

2.5 Gently add 2.5 mL of Stage 1 media to cleaned undifferentiated iPSCs (~30%-40% confluence) in 6-well plate by slowly adding media to the side of the well.
**NOTE: It is important to start with low density iPSCs culture with little or no spontaneous differentiation. High density culture will result failure of the differentiation process)*

Day 1 - 5

2.6 Change media and feed cells with fresh 2.5 mL Stage 1 media daily if iPSCs are very confluent and/or every other day depending confluence

**NOTE: 5-20% cell death is observed after addition of Stage 1 media. It is important to monitor cells condition daily to insure a good yield at day 6 dissociation.*

Part 3: Laminin (mouse) or Matrigel (growth factor reduced) ECM coating

Day 6

3.1 Number of plates required for laminin or Matrigel (ECM) coating:
a. On Day 18 you should expect to have at least 0.5 – 1e6 cells/well of a 6 well plate so plan the required number of cells to coat with laminin / matrigel based on these estimates.
b. On day 6, each line typically yields 1.25e6 – 2.5e6 cells/well after dissociation. When plating, each well of a 6 well plate will require 7.5e5 cells/well and a 96 well plate requires 1.0e4 cells/well.

3.2 Number of plates coated should also account for any planned freeze down at day 12.
Laminin (mouse): Prepare laminin if necessary. Laminin can be stored at 4°C and re-used (see step 3.4). Add 1mL of stock laminin (1 mg/mL, Sigma L2020) to 19 mL PBS (+/+) to make 50 µg/mL working solution.
CRITICAL: Thaw the laminin stock at 4C overnight. **DO NOT** thaw the laminin in the waterbath. The laminin can gel during a quick thaw and produce inconsistent ECM coating. Expiration day is 14 days from production.

Matrigel (growth-factor reduced): Thin-film coating similar to hiPSC culture can be used (0.5 mg / 6 well plate).

3.3 Coat plates with 1X laminin solution. Add appropriate volume in a sterile tissue culture hood.
6 well plate – 1 mL/well
24 well plate –250 µL/well
96 well plate – 50 µL/well
**NOTE: It is important to swirl the 6 well plate and tap the 96 plate to insure coverage of the entire well with laminin*

3.4 Incubate plates in a 5% CO_2/37°C incubator for 2 – 24 hours. The minimum incubation time is 2 hours but the plates can be prepared the day before and incubated overnight.

3.5 Collect the laminin when ready to plate dissociated iPSC cells (**Step 2**). The used laminin can be collected and stored in a sterile conical tube for re-use. Store at 4°C for two more uses or two weeks, whichever comes first.
NOTE: *Ensure that the laminin coating solution doesn't dry out. (If laminin is aspirated before the cells are ready for plate down, add 0.5 mL or enough media to cover the wells and avoid drying out of the wells.)*

Part 4: Stage 2 of differentiation - MN precursor generation

Day 6-11

Preparation of a single cell suspension of neuroepithelial cells.
NOTE: *that these cells are now referred to as neuroepithelial cells. Day 6 cells may also be cryopreserved, see Step 6).*
4.1 Prepare stage 2 media as necessary (See **Table 2**). You may prepare enough media to feed each well every 1 – 2 days.

4.2 Pre-warm medium and accutase at room temperature, do not use a water-bath.

4.3 Rinse iPSCs once with 1 mL 1xPBS, adding slowly.

4.4 Add 1 mL of accutase per well.

4.5 Incubate in a 37°C incubator for 5 minutes.

4.6 Prepare a 50 mL conical tube - Label with cell identification information.

4.7 Remove cells from the incubator and add 1 mL 1xPBS to each well. Do not remove the accutase from the wells.

4.8 The cells typically do not lift off from the plate after the 5 minute incubation. At the 5 minute mark, after adding PBS to each well, use a serological pipette (10 mL size) and gently scrape/rinse the cells from the surface of the plate, similarly to iPSC passaging. You may transfer the contents from well-to-well for each 6 well plate collecting all cells along the way. Transfer collected cell suspension to the 50 mL conical tube at room temperature.

4.9 Wash each well again with 1 mL 1xPBS, scrape/collect cells, and transfer everything to the 50 mL conical tube. Repeat for all 6 well plates.

4.10 Centrifuge the cells at 200 x g for 5 minutes at room temperature.

4.11 Aspirate and discard the supernatant. Re-suspend the cells in 10-15mL of Stage 2 media depending on pellet size. Store at room temperature.

4.12 Remove a minimum of two samples (10µL) for counting. Dilute the cell suspension with 0.04% Trypan Blue/1xPBS to an appropriate dilution factor (ex. 1:20). Count viable and dead cells from each sample by counting the four corner squares using a hemacytometer.

4.13 Calculate the viable cell concentration as follows:

NOTE: *Steps 4.8-4.13 should be performed in fast pace to avoid long incubation of cells at room temperature or on ice. A cell viability of greater than 75% is typical*.

4.14 Re-suspend the cell suspension at a concentration of 3.75e5 cells/mL in Stage 2 media.

4.15 Plate cells at the appropriate density listed below onto the laminin-coated plates. Gently rock the plate(s) side-to-side to ensure even distribution. (Freeze down left over cells, (e.g., 5.0e6 cells/vial)

Medium Recommended starting guide for cell density and volume of plating (Stage 2 Medium)

- 6 well plate: 750,000 cells/well, 2 mL final volume.
- 24 well plate: 80,000 cells/well, 0.5 mL final volume.
- 96 well plate: 10,000 cells/well, 0.15 mL final volume.

4.16 On the days following the plating, monitor adherence and density of the single cells in neuronal cultures. Capture bright-field images to record. (Plates should not be moved around in incubator after plate down for 24 hrs. and no media change should be done on the next day.)
NOTE: Attachment from batch-to-batch and line-to-line is variable. Occasionally very little attachment has been observed on day 7 with some lines. We attribute this to either batch variability within ECM coating or the cell line. If this is the case, monitor for up to a week (day 13). If cells have not expanded, discard and repeat steps 1 – 4 with new iPSC cells).

4.17 Exchange spent media with fresh Stage 2 media as per recommended volumes every 2 days through day 11. Media may be exchanged every day if density is high.

NOTE: When removing media from 96 well plates one should "dump" the contents of the plate out(without having the plate touch any tissue paper or anything that plate is being dumped on.) instead of using aspiration. Aspiration has shown to lift cells from the surface. Also add media very gently to the side of the 96 well plate well using a multichannel pipette.

4.18 Return plate to incubator and culture at 37°C, 5% CO_2.

4.19 Carefully exchange all culture medium with fresh stage 2 media every other day until day 12.

Part 5: Stage 3 of differentiation - Terminal iMN maturation

Day 12 and greater
(Optional: cryopreservation at day 12, see step 6)

5.1 Prepare Stage 3 media as necessary (See Table 3).

5.2 Warm Stage 3 media at room temperature before use.

5.3 Exchange spent media with Stage 3 media.

5.4 Perform a full medium change every other day. Typical cell culture time frame varies from experiment to experiment but a minimum of 18 days is required for immature diMNS. 30 days is standard for more mature, physiologically active neurons. Cells can be matured for another 14-56 days with medium until cells are fixed or harvested for further analysis.
NOTE: Depending on the goal of the experiment, the time of differentiation is critical. For experiments that required high level cell imaging, etc. a timepoint of 18 days is recommended due to clumping at later stages. If maximum motor neuron maturity is required a longer time-point is recommended although a higher level of clumping will likely occur inhibiting the ability to observe cells in high detail.

5.5 Capture bright-field images to record every 2-3 days.

Part 6: Cryopreservation at day 12 of differentiation at the end of Stage 2 (optional)

Freezing
6.1 Day 6 and Day 12 diMNs can be cryopreserved as an optional step for future work. Cryostor CS10 (Stemcell Technologies) is the ideal cryopreservation media for diMNs.

6.2 On day 6 or day 12 cells are gently lifted with accutase

6.3 Centrifuge the cells at 200 x g for 5 minutes at 4°C. Resuspend cells in its appropriate media and count

6.4 After a quick spin down, Discard supernatant and re-suspend the cells in Cryostor at 5.0e6 cells/mL.

6.5 Aliquot 1 mL per cryovial. Freeze down with a controlled rate freezer or Mr. Frosty (standard method).

Thawing
For these steps plates should be coated with laminin using the steps 3.2 before thawing cells

6.6 Thaw cells using a Thawstar (BioCision) or standard water-bath method (gentile agitation in water until a small crystal is observed).

6.7 Transfer thawed cells from cryovial into a new 15 mL conical tube and slowly, dropwise add 5 mL of Stage 2 media for cells frozen at day 6 and stage 3 media for d12 for cells frozen at day 12 media to the cells.

6.8 Repeat for all vials of a given cell line. Centrifuge all conicals at 200 x g for 5 minutes at 4°C.

6.9 Combine cells and perform a cell count (see steps 4.12 – 4.13). Document viability and re-suspend the cells by repeating steps 4.14 – 4.19.

6.10 Plate cells immediately after determining cell number and viability. Avoid keeping cell at room temperature or on ice for long.

Part 7: Fixation and pellet collection

Fixation

7.a.1 Prepare a 4% PFA/1xPBS solution.

7.a.2 Remove and discard media. Gently rinse each well with 1xPBS and discard. Add 4% PFA/1xPBS to each well using the volumes below. Incubate for 15 minutes at room temperature.

7.a.3 Aspirate and discard the PFA. Rinse each well once with 1xPBS and discard. Add 1xPBS to each well, cover with appropriate adhesive lid or parafilm to reduce evaporation and store at 4°C.

Pellet

7.b.1 Perform the following steps on one 6 well plate at a time. Remove and discard media. Gently rinse each well with 1xPBS and discard.

7.b.2 Add 0.5 mL of 1xPBS to each well. Scrape each well with a cell scraper and combine into one tube. Mix and aliquot as necessary. Centrifuge at 200 x g for 5 minutes at 4°C. Remove the supernatant and discard. Snap freeze the cell pellets in LN2 or other appropriate freezing medium.

Media Tables

Table 1: Stage 1 media

Stage 1 (Day 0-Day 6)	Stock Concentration	Units	1X Concentration	Units	Volume 1000mL	Volume 500mL	Volume 250mL	Volume 100mL	Volume 50mL
IMDM	1X	mL	47.5%	mL	475mL	237.5mL	118.75mL	47.5mL	23.75mL
F12	1X	mL	47.5%	mL	475mL	237.5mL	118.75mL	47.5mL	23.75mL
NEAA	100X	mL	1%	mL	10mL	5mL	2.5mL	1mL	0.5mL
B27	50X	mL	2%	mL	20mL	10mL	5mL	2mL	1mL
N2	100X	mL	1%	mL	10mL	5mL	2.5mL	1mL	0.5mL
PSA	100X	mL	1%	mL	10mL	5mL	2.5mL	1mL	0.5mL
LDN193189	2	mM	0.2	µM	100µL	50µL	25µL	10µL	5µL
SB431542	10	mM	10	µM	1mL	500µL	250µL	100µL	50µL

CHIR99021	15	mM	3	µM	200µL	100µL	50µL	20µL	10µL

Table 2: Stage 2 media

Stage 2 (Day 6-Day 12)	Stock Concentration	Units	1X Concentration	Units	Volume 1000mL	Volume 500mL	Volume 250mL		
IMDM	1X	mL	47.5%	mL	475mL	237.5mL	118.75mL		
F12	1X	mL	47.5%	mL	475mL	237.5mL	118.75mL		
NEAA	100X	mL	1%	mL	10mL	5mL	2.5mL		
B27	50X	mL	2%	mL	20mL	10mL	5mL		
N2	100X	mL	1%	mL	10mL	5mL	2.5mL		
PSA	100X	mL	1%	mL	10mL	5mL	2.5mL		
LDN193189	2	mM	0.2	µM	100µL	50µL	25µL		
SB431542	10	mM	10	µM	1mL	500µL	250µL		
CHIR99021	15	mM	3	µM	200µL	100µL	50µL		
All-trans RA	10	mM	0.1	µM	10µL	5µL	2.5µL		
*SAG	100	mM	1	µM	10µL	5µL	25µL		

Table 3: Stage 3 media

Stage 3 (Day 12-DayXX)	Stock Concentration	Units	1X Concentration	Units	Volume 1000mL	Volume 500mL	Volume 250mL		
IMDM	1X	mL	47.5%	mL	475mL	237.5mL	118.75mL		
F12	1X	mL	47.5%	mL	475mL	237.5mL	118.75mL		
NEAA	100X	mL	1%	mL	10mL	5mL	2.5mL		
B27	50X	mL	2%	mL	20mL	10mL	5mL		
N2	100X	mL	1%	mL	10mL	5mL	2.5mL		
PSA	100X	mL	1%	mL	10mL	5mL	2.5mL		

Compound E	1	mM	0.1	µM	100uL	50uL	25µL		
DAPT	20	mM	2.5	µM	125µL	62.5µL	31.3µL		
db-cAMP	10	mM	0.1	µM	10µL	5µL	2.5µL		
All-trans RA	10	mM	0.5	µM	50µL	25µL	12.5µL		
*SAG	100	mM	0.1	µM	1µL	0.5µL	0.25µL		
Ascorbic Acid	500	µg/mL	200	ng/mL	400µL	200µL	100µL		
BDNF	10	µL/mL	10	ng/mL	1mL	500µL	250µL		
GDNF	10	µL/mL	10	Ng/mL	1mL	500µL	250µL		

Reagents Table

Materials	Catalog Numbers	Company
IMDM	12440061	LifeTech
F12	11765062	LifeTech
NeuroBasal	21103049	LifeTech
B27 (+vitamin A)	17504044	LifeTech
N2	1780240	LifeTech
NEAA	11140-50	Gibco
GlutaMax	35050061	LifeTech
Antibiotic antimycotic	15240062	LifeTech
CHIR99021	13122	Cayman Chemicals
LDN193189	S2618	Selleck

SB431542	1614	Tocris
SAG (Sonic Hedgehog Agonist)	11914	Cayman Chemicals
All-Trans Retinoic Acid	04-0021	Stemgent
BDNF	450-02	Peprotech
GDNF	450-10	Peprotech
Ascorbic acid	A4403	Sigma
Compund E	565790	calbiochem
DAPT	13917	Cayman Chemicals
db-cAMP	28745	Millipore
Accutase	SCR005	Millipore
Laminin (Mouse)	L2020	Sigma
Matrigel(Growth factor reduced)	354230	Corning
Poly-HEMA	P3932	SIGMA
6 well plates	08772-33	Fisher
Pre separation filters 30uM	130-041-407	Miltenyi Biotec (Macs)
96W Optical Bottom Plate, Black Polystyrene, Cell Culture Treated, with lid, Sterile	12-566-70	Fisher