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Differentiation of human cortical neurons (CNs) from induced pluripotent stem cells (iPSCs) V.1

Forked from Differentiation of human cortical neurons (CNs) from induced pluripotent stem cells (iPSCs) and their coculture with rat astrocytes

In 2 collections

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ABSTRACT

This protocol described the production of human cortical neurons from induced pluripotent stem cells. The differentiation protocol of cortical neurons is adapted and modified from Shi, Y. et al., 2012 (pharmacologically directed) and Zhang, Y. et al., 2013 (transcription factor directed), as previously described by Hedegaard et.al., 2020. These neurons exhibited molecular and functional hallmarks of cortical neurons.

MATERIALS

Reagents:

- Accutase (Stem Cell Tecnologies, CAT# 07920)
- AraC (Merck, CAT# C1768-100MG)
- B-27™ Plus Supplement (50X) (ThermoFisher Scientific, CAT# A3582801)
- B-27™ Supplement (50X), serum free (ThermoFisher Scientific, CAT# 17504044)
- B-Mercaptoethanol (ThermoFisher Scientific, CAT# 21985023)
- Cytosine β-D-arabinofuranoside (AraC) (Sigma-Aldrich, CAS# 147-94-4, SKU# C1768)
- Dispase II, powder (ThermoFisher Scientific, CAT# 17105041)
- Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, CAS# 67-68-5)
- DMEM/F-12, GlutaMAX™ supplement (ThermoFisher Scientific, CAT# 10565018)
- Doxycycline hyclate (Sigma-Aldrich, CAS# 24390-14-5, SKU# D9891-1G)
- Fibronectin human plasma (Sigma-Aldrich, SKU# F0895-1MG)

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- Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix (ThermoFisher Scientific, CAT# A1413202)
- Insulin solution from bovine pancreas (Sigma-Aldrich, CAS# 11070-73-8, SKU# I0516-5ML)
- Laminin from Engelbreth-Holm-Swarm murine sarcoma basement membrane (Sigma-Aldrich, CAS# 114956-81-9, SKU# L2020-1MG)
- L-Ascorbic acid (Sigma-Aldrich, CAS# 50-81-7, SKU# A4544-25G)
- LDN193189 hydrochloride (Sigma-Aldrich, CAS# 1062368-24-4, SKU# SML0559-5MG)
- Lenti-X concentrator kit (TaKaRa, CAT# 631231 & 631232)
- L-glutamine (CAT# 25030-024)
- Lipo 3K (Life Technologies, CAT# L3000001)
- Matrigel (CAT# 354277)
- MEM Non-Essential Amino Acids Solution (100X) (ThermoFisher Scientific, CAT# 11140050)
- N-2 Supplement (100X) (ThermoFisher Scientific, CAT# 17502048)
- Neurobasal (CAT# 21103049)
- non-essential amino acids (Life Technologies, CAT# 11140035)
- NT-3 human (Sigma-Aldrich, SKU# SRP3128-10UG)
- Optimem (Life Technologies, CAT#31985088)
- Penicillin-Streptomycin (P/S) (10,000 U/mL) (ThermoFisher Scientific, CAT# 15140122)
- Phosphate-buffered saline, pH 7.4 (PBS) (Life Technologies, CAT# 10010056)
- Poly-D-Lysine (ThermoFisher Scientific, CAT# A3890401)
- Poly-L-ornithine solution (Sigma-Aldrich, CAS# 27378-49-0, SKU# P4957-50ML)
- Puromycin (ThermoFisher Scientific, CAT# A1113803)
- Recombinant Human/Murine/Rat BDNF (BDNF) (PeproTech, CAT# 450-02)
- Recombinant Mouse FGF basic/FGF2/bFGF Protein (FGF) (Bio-Techne | r&d systems, CAT# 3139-FB)
- ROCK inhibitor Y-27632 (ROCKi) (CAT# 1254)
- SB 431542 (biotechne | tocris, CAT# 1614, CAS# 301836-41-9)
- Sodium pyruvate (CAT# 11360070)
- TrypLE (Life Technologies, CAT#12604013)
- ViralBoost (Alstem, VB100)

Preparing Neural Induction Medium (NIM):

1. Add to NMM base medium:

- 100 nM LDN193189 (1:10000)
- 10 mM SB431542 (1:1000)

Preparing Neural maintenance base medium (NMM):

- 1:1 mixture of N2 and B27 containing media.

Preparing N2 base medium:

- DMEM/F12 GlutaMAX
- 1X N2
- 5 mg/ml insulin
- 100 mM non-essential amino acids
- 100 mM B-mercaptoethanol
- 1 mM sodium pyruvate
- P/S (50 U or mg per ml)

Preparing B27 base medium (use within 3 weeks):

- Neurobasal
- 1X B27 plus with Vitamin A
- 2 mM L-glutamine

Preparing Ngn2 base medium (use within 1 week):

- Neurobasal
- 1X B27 with vitamin A
- 1X Glutamax
- P/S (50 U or mg per ml) (1:200)
- 1 mg/ml Doxycycline (1:1000)
- 10 ng/ml BDNF
- 10 ng/ml NT-3 (1:1000)
- 200 ng/ml Laminin (1:5000)
- 200 mM Ascorbic acid (1:1000)

Preparing Neural Freezing Medium (made fresh):

- NMM
- 10% DMSO
- 20 ng/ml FGF2

SAFETY WARNINGS

Part of this procotol involves working with lentiviruses during which stage extra precaution should be exercised. All materials in contact with lentiviruses must be decontaminated with chemgene in allocated space after use prior to further autoclaving.

BEFORE START INSTRUCTIONS

Sterile working techniques are an absolute must to ensure cell viability and vitality. This includes but not limited to filtering of all media to be used with 0.22µm filter, sterilisation of gloves, stripettes, falcons or any materials to be in contact with cells or cell media.

All growth factors should be added fresh on the day of intended use, or within 48 hours. Prior to use media must be warmed preferentially to 37ºC, or room temperature at the very least, as these cells are temperature-sensitive.

Differentiation of iPSCs into Neuronal Progenitor Cells (NPC..

1 **Day -2: Preparing plates for replating**

Two days before intending on starting the differentiation (Day -2), add 1 mL/well in a 6-well plate of Geltrex one day prior to replating the iPSCs to begin the differentiation.

Note

Geltrex should be prepared in DMEM/F12 basal medium based on manufacturer's dilution instructions and should be kept cold at all times.

Cells are typically replated the day before beginning the differentiation.

2 **Day -1: Replating iPSCs for differentiation**

Replating iPSCs for differentiation is identical to described in Protocol: Expansion and maintenance of human induced pluripotent stem cells (iPSCs), however, includes a cell counting step.

2.1 ***Prepare for splitting***

Follow steps described in **steps 6 and 7** of Protocol: Expansion and maintenance of human induced pluripotent stem cells (iPSCs).

2.2 ***Prepare for cell counting***

Prepare 99 µl of Phosphate Buffered Saline (PBS) into one Eppendorf per cell line for cell dilution.

2.3 ***Replate iPSCs***

As described in **step 7** of Protocol: Expansion and maintenance of human induced pluripotent stem cells (iPSCs), pausing when cell pellet is suspended in 1 mL of mTesR

media (i.e. mTesR plus their accompanying Supplement and 1% Penicilline/Streptomycin) + ROCKi (1:1000) to count cells.

2.4 ***Count cells (manually using a haemocytometer)***

2.4.1. Dilute cells by adding 1 µL of cell suspension to 99 µL of previously prepared PBS in an Eppendorf.
2.4.2. Mix thoroughly.
2.4.3. Take 10 µL of diluted cell mixture and add to a haemocytometer.
2.4.4. Using a microscope, focus on the grid lines of the hemocytometer with a 10X objective.
2.4.5. Manually count cells from all 4 all 4 sets of 16 corners of the haemocytometer using a tally counter.
2.4.6. Average cell count from each of the sets of 16 corner squares and multiply by 10,000 (10^4).
2.4.7. Multiply by 100 to correct for the dilution in **step 2.4.1.**
2.4.8. Calculate and plate cells based on the following optimal density for Day -1 plating: 1.5 millions cells per 6 well .

Note

Cell lines of different proliferative rates could be seeded at slightly different cell number to allow for similar starting date of differentiation.

2.4.9. Replate about 1.5 to 2 million cells per 6-well suspended in mTesR media+ ROCKi (1:1000) and swirl plate gently in a figure 8 motion.
2.4.10. Add more mTesR media + ROCKi (1:1000) to make up the total volume of 3 mL per well of a 6-well plate.

Differentiation of NPCs into Cortical Neurons (CNs)

3 **Day 0: Differentiation starts when cells reach 90-100% confluency**

3.1 Prepare Neural Induction Medium (NIM; see **Materials**).

3.2 Wash cells once with PBS, and add 2 mL of NIM media to each well.

4 **Day 1 – Day 12: Daily feed with NIM**

Replace medium every day and monitor morphological changes.

Note

Dense areas might appear brown after some time in NIM. Cells might peel to a certain extent from the edge of the wells.

5 **Day 11: Prepare plates for Day 12 passaging**

Coat new plates with 15 ug/mL fibronectin and incubate at 37°C overnight.

6 **Day 12: Splitting cells**

6.1 Prepare Neural Maintenance Medium (NMM)(see **Materials**).

6.2 Add 10 mL of NMM to a 15ml falcon (spinning tube) for each line to be passaged and pre-warmed to 37°C.

Note

Bigger falcon with larger NMM could be used if working with greater number of cells.

6.3 Add 10% medium volume of pre-warmed Dispase (10 mg/mL, sterilized, in PBS) directly into the well that still has medium in it.

6.4 Incubate at 37°C for 10 min at least.

Note

Some wells take longer (up to 30 min) for Dispase to lift the cell sheet. Check regularly.

6.5 In the well, break the cell sheet a couple of times with P1000 and keep the big clumps of cells as intact as possible.

6.6 Transfer the cells into 10 mL of NIM in 15-mL falcon tubes. Let the cells settle by gravity.

6.7 Remove the supernatant carefully without removing the cells at the bottom.

6.8 Add another 10 mL of NIM in 15-mL falcon tubes and repeat **steps 6.6 and 6.7.**

6.9 Split the cells 1:2 into 15 mg/mL fibronectin-coated plates with NIM and ROCKi (1:1000).

6.10 Allow cells to attach overnight.

Note

Colonies only attach on fibronectin-coating at this stage (can also pre-coat with 50 mg/mL poly-L-ornithine on Day 10 prior to fibronectin to enhance adhesion).

7 **Day 13 : Full media change**

1. Add to NMM base medium:
20 ng/ml FGF2

Note

If clumps of cells do not attached on Day 13, change medium, but keep the clumps in the plate as much as possible. They should attach after another 2 days.

8 **Day 15 : Full media change**

1. Add to NMM base media:
20 ng/ml FGF2

Note

Neural rosette structures should be obvious between Day 12 – 17.

9 **Day 16 : Prepare plates for splitting**

Wells are coated with fibronectin or 100 mg/mL poly-L-ornithine overnight followed by 10 mg/mL laminin for at least 2 hours (up to 24 hours).

10 **Day 17 : Splitting of NPCs**

Note

Culture should be relatively dense at Day 17 and so longer incubation with Disease might be necessary.

Cells could be split in 1:2 or 1:3.

Multiple wells of the same lines can be pooled into one tube and wash using more of NMM.

10.1 Repeat the splitting and washing steps as described in **step 6.**

10.2 Seed the cells on Poly-L-ornithine/Laminin/fibronectin-coated plates previously prepared in **step 9** and feed with media as described in **step 8**.

11 **Day 18 : Full media change**
NMM base media

12 **Day 20 – Day 26 : Full media change**
Change NMM media every 2 days.

13 **Day 26 : Freezing**
Cells can be frozen at 5-10 million cells/vial.

13.1 Add 1 mL of Accutase per well to be frozen.

13.2 Spin the cells down at 300 g for 5 mins.

13.3 Resuspend the cells slightly into clumps in Cryostore 10 or self-made freezing medium (see **Materials**).

Lentivirus packaging

14 This section describes the third-generation lentiviral packaging of plasmids to be used for

transduction of NPCs.

Plasmids to be packaged into lentiviruses:
1. FUdeltaGW-rtTA (rtTA; Addgene #19780)
2. pTet-O-Ngn2- puro (Ngn2; Addgene #52047)
3. pLV-TetO-hNGN2-eGFP-Puro (Ngn2-GFP; Addgene #79823)

14.1 *Prepare HEK293T cells*

14.1.1. Prepare DMEM/F12 medium supplemented with 10% FBS, 2 mM L-glutamine, and 50 U/mL Penicillin-Streptomycin.

14.1.2. Passage cells by single-cell passaging with TrypLE and seed at approximately 150,000 cells/cm^2, and at least 24 hours before plasmid transfection.

Note

Seeding density can vary depending on the desirable day for transfection at which point cells should be at 80-90% confluence.

14.2 *Day of transfection*

14.2.1 Pre-warm Optimem to room temperature.

14.2.2 Prepare Lipofectamine mix (Tube 1):
14.2.2.1. Add Lipo 3K to Optimem.
14.2.2.2. Flick to mix.
14.2.2.3. Incubate for 5-20 min.

14.2.3. Prepare DNA mix (Tube 2):
14.2.3.1. Prepare master mix of packaging vectors + P3000 in Optimem.
14.2.3.2. Add transgene-expressing lentiviral vector (e.g. pLEX).
14.2.3.3. Mix by briefly vortexing.

14.2.4. Add DNA mix (Tube 2) to Lipofectamine mix (Tube 1), mix by flicking tube 8 times
14.2.5. Wait ~ 20 minutes
14.2.6. Add DNA/Lipofectamine mixture to cells dropwise, and swirl plate immediately to evenly mix with fresh cell media without penicillin/streptomycin.

A	B	C	D	E	F	G	H	I	J	K
			Tube 1		**Tube 2**					
Dish	*Cell # (x 106)*	*Media*	*Optimem*	*Lipo 3K*	*Optimem*	*P3000*	*psPAX2*	*pMD2G*	*pAdVantage*	*Lenti vector*

	A	B	C	D	E	F	G	H	I	J	K
	1 x 6 well	2.5	3 mL	150 uL	1.9 uL	150 uL	5 uL	1.6 ug	0.6 ug	0.2 ug	2.4 ug
	1 x 10 cm	15	18 mL	900 uL	11 uL	900 uL	30 uL	10 ug	3.4 ug	1.4 ug	14.6 ug
	1 x 15 cm	45	36 mL	2.4 mL	30 uL	2.4 mL	80 uL	26.3 ug	9 ug	3.6 ug	39 ug

List and quantity of reagents and plasmids to be used for lentiviral packaging in **step 14.2.**

14.3 ***Day of transfection $^{+1}$***

Replace media with 3 mL of DMEM/FCS medium + 6uL ViralBoost (Alstem).

Note

From this stage onwards, all materials that are in contact with the cells or cell media contain viruses/viral particles and hence, would be treated with chemgene prior to autoclaving.

14.4 ***Day of transfection $^{+2}$: Collecting cell media***

Collect all cell media containing live viruses.

Note

Be careful to not disturb and collect unnecessary cell materials.

If no ViralBoost is used, smaller volumes of media can be collected (e.g.10ml for 10cm dish) daily and pool after 3 days (keep collections at 4°C until pooled).

14.5 ***Concentrating viruses***

This step was adopted from manufacturer's guideline for the TAKARA Lenti-X concentrator kit.

14.5.1. Lentivirus-containing supernatants are centrifuged briefly at 500g for 10 mins or filtered through a 0.45 µm filter.

Note

If filtering, use only cellulose acetate or polyethersulfone (PES) (low protein binding) filters.

Do not use nitrocellulose filters. Nitrocellulose binds surface proteins on the lentiviral envelope and destroys the virus.

14.5.2. Transfer clarified supernatant to a sterile container and combine 1 volume of Lenti-X Concentrator with 3 volumes of clarified supernatant.
14.5.3. Mix by gentle inversion. Larger volumes may be accommodated through the use of larger (i.e., 250 mL or 500 mL) centrifuge tubes.
14.5.4. Incubate mixture at 4°C overnight.
14.5.5. Centrifuge sample at 1,500g for 45 minutes at 4°C.
14.5.6. Carefully remove supernatant without disturbing the pellet. Residual supernatant can be removed with either a pipette tip or by brief centrifugation at 1,500g.
14.5.7. Gently resuspend the pellet in 1/100th of the original volume using complete DMEM.

Note

The pellet can be somewhat sticky at first, but will go into suspension quickly.

14.5.8. Immediately titrate sample into single-use aliquots and stored at -70°C.

Differentiation of NPCs into cortical neurons

15 The second part of the differentiation protocol beyond Day 26 was adapted and modified from Zhang, Y. et al., 2013. The protocol employs doxycycline-inducible overexpression system of *Ngn2* to direct excitatory cortical neuronal identity.

15.1 ***Prepare plates for replating***
Add 100 µg/mL of Poly-L-ornithinne onto plastic wells and incubate at 37ºC overnight.

15.2 ***Prepare media for thawing and replating***
15.2.1. Pre-warm spinning falcons containing 9 ml of Neurobasal.
15.2.2. Prepare Day 25 media (i.e. NMM) + ROCKi (1:1000) + 20 ng/mL FGF2.

15.3 ***Thawing and replating of Day 26 NPCs***

15.3.1. Thaw cryovial containing Day 26 NPCs in water bath until only a small component remains frozen.

15.3.2. Carefully transfer contents of cryovial to pre-warmed spinning tubes.

15.3.3. Centrifuge at 350g for 5 min.

15.3.4. While spinning, aspirate Matrigel and replace with Day 25 media (i.e. NMM) + ROCKi (1:1000) + 20 ng/ml FGF2.

15.3.5. Aspirate media from cell pellet in spinning falcon and replace with Day 25 media (NMM) + ROCKi (1:1000) + 20 ng/ml FGF2, slowly and gently resuspending the pellet.

15.3.6. Transfer an appropriate amount of cell suspension into previously prepared wells and swirl plate gently in a figure 8 motion.

15.4 ***Transduction of Day 26 NPCs***

15.4.1. Add *Ngn2* and *rTA* lentiviruses directly into the well with cell media immediately after depositing the cell suspension.

15.4.2. Replace medium with fresh NMM 8 hours later.

Note

Transducing cell while in suspension often results in the best transduction efficiency.

The exact amount of lentiviruses to be added needs to be titrated.

Too high viral load might negatively affect cell health; too low viral load results in low yield of cortical neurons.

15.5 ***Day 28 – Doxycycline induction***

The NMM media is replaced with freshly-made Ngn2 medium (see **Materials**) to induce doxycycline-dependent expression of *Ngn2* in successfully transduced neurons.

15.6 ***Day 30 – Antibiotic selection***

Full media change with freshly-made Ngn2 medium (see **Materials**) supplemented with 1 µg/mL puromycin to select for successfully transduced neurons.

Co-culture of iPS-cortical neurons with rat astrocytes

16 Resulting iPS-cortical neurons are co-cultured with commercially available rat astrocytes as

previously described in Hedegaard et.al., 2020. This coculture approach enhances both cortical intrinsic capacity to fire action potential and network-dependent synaptic activities.

16.1 *Rat Astrocyte Expansion*

16.1.1. Thaw and replate primary rat astrocytes onto uncoated T75 flasks according to manufacture's protocol, which is similar to **steps 15.2 and 15.3**. The only difference is the medium - use Astrocyte Maintenance Medium (AMM) (see **Materials**) for rat astrocytes.
16.1.2. Astrocytes are allowed to expand for 2 to 3 weeks till confluence.
16.1.3. Full media change twice a week.

16.2 *Splitting of Rat Astrocytes*

16.2.1. Astrocytes are split into 6 x uncoated T75 flasks.
16.2.2 Adherent astrocytes are washed with PBS once.
16.2.3. Add 10mL of Accutase per T75 flask and incubate the dish at 37°C for approximately 10-20 mins.
16.2.4. Check regularly for cell lifting.
16.2.5. Carefully transfer all of the dish content into a pre-warmed falcon.
16.2.6. Centrifuge at 350g for 5 mins.
16.2.7. While spinning, add fresh AMM to the new T75 flasks.
16.2.8. Aspirate media from cell pellet in spinning falcon and replace with AMM, slowly and gently resuspending the pellet.
16.2.9. Transfer an equal amount of cell suspension into previously prepared T75 flasks and swirl plate gently in a figure 8 motion.
16.2.10. Expand again for another 2 to 3 weeks till confluence.

16.3 *Freezing of rat astrocytes*

16.3.1. Lift confluent astrocytes using Accutase and centrifuge at 350g for 5 mins.
16.3.2. Remove the supernatant and resuspend cell pellet in AMM.
16.3.3. Freeze the cells at 2-5 million cells/vial in 90% v/v AMM + 10% v/v DMSO as P3 astrocytes.

16.4 *Co-culturing of cortical neurons with rat astrocytes*

16.4.1. P3 rat astrocytes are thawed and plated onto intended plates at 20,000 cells/cm^2 following **step 16.1.**

Note

20,000 cells/cm^2 astrocytes are plated 3 days prior to plating of cortical neurons (P3 rat astrocyte).

P3 rat astrocyte can continue to expand and hence, astrocyte plating density can be adjusted slightly depending on how early/late astrocyte plating is relative to that of cortical neurons.

16.4.2. Day 30 Cortical Neurons (**step 15.6.**) are lifted with Accutase and replated on top of the adherent astrocyte monolayer at the density of 100,000 cells/cm^2.
16.4.3. Upon centrifugation, cell pellet is resuspended in Ngn2 medium.
16.4.4.2 On Day 32, half media change is performed with fresh Ngn2 medium + 100 nM AraC.
16.4.3. From now on, half media change is done twice per week.