Differentiation of hPSCs to hypothalamic neurons

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ABSTRACT

This protocol is about Differentiation of hPSCs to hypothalamic neurons.

ATTACHMENTS

Differentiation_of_hPSCs_to_hypothalamic_neurons.pdf

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KEYWORDS

Differentiation, hPSCs, hypothalamic neurons

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ATTACHMENTS

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MATERIALS TEXT

Materials

- 1M CaCl2 **Sigma-aldrich Catalog #21115**
- L-Ascorbic acid **Sigma Aldrich Catalog #A4403**
- B-27 Supplement **Gibco - Thermo Fischer Catalog #17504044**
- Animal-Free Recombinant Human/Murine/Rat BDNF **peprotech Catalog #AF-450-02**
- CHIR99021 **Cell Guidance Systems Catalog #SM13-10**
- DAPT **Sigma Aldrich Catalog #D5942**
- DPBS, no calcium, no magnesium **Thermo Fisher Catalog #14190144**
- DMEM/F-12, GlutaMAX™ Supplement **Thermo Fisher Catalog #31331028**
- DMEM/F-12, HEPES, no phenol red **Thermo Fisher Catalog #11039021**
- DNase Vial (D2) **Worthington Biochemical Corporation Catalog #LK003170**
- Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix **Thermo Fisher Catalog #A1413202**

- GABA **Tocris Catalog #0344**
- GlutaMAX **Gibco - Thermo Fisher Catalog #35050038**
- Stemolecule LDN-193189 **Reprocell Catalog #04-0074**
- LM 22A4 **Tocris Catalog #4607**
- MEM Non-Essential Amino Acids Solution (100X) **Thermo Fisher Scientific Catalog #11140035**
- N2 supplement **Gibco - Thermo Fisher Catalog #17502048**
- Neurobasal-A Medium **Thermo Fisher Scientific Catalog #10888022**
- NKH 477 **Sigma Aldrich Catalog #N3290**
- PDS Kit Papain Vial **Worthington Biochemical Corporation Catalog #LK003176**

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PD 0332991 isethionate **Sigma Aldrich Catalog #PZ0199**

Penicillin-Streptomycin (10,000 U/mL) **Thermo Fisher Scientific Catalog #15140122**

Purmorphamine **Merck Millipore Catalog #540220**

SB 431542 hydrate **Sigma Aldrich Catalog #S4317**

MilliporeSigm Calbiochem Smoothened Agonist SAG **Fisher Scientific Catalog #56-666-01MG**

Sodium Bicarbonate 7.5% solution **Thermo Fisher Catalog #25080060**

StemFlex™ Medium **Thermo Fisher Scientific Catalog #A3349401**

Trypan Blue Stain (0.4%) for use with the Countess™ Automated Cell Counter **Thermo Fisher Scientific Catalog #T10282**

Stemolecule XAV939 **Reprocell Catalog #04-0046**

Y-27632 dihydrochloride (Rock Inhibitor) **Contributed by users Catalog #DNSK-KI-15-02**

Media and Reagents

StemFlex

Name	Volume
StemFlex basal medium	450 mL
StemFlex supplement	50 mL

N2B27

Name	Volume
Neurobasal-A	500 mL
DMEM/F12 with GlutaMAX	500 mL
Glutamax	10 mL
Sodium bicarbonate	10 mL
MEM Nonessential amino acids	5 mL
Ascorbic acid (200 mM)	1 mL
Penicillin-streptomycin	10 mL
Sterile filter, then add the following supplements:	
B27 supplement	10 mL
N2 supplement	5 mL

N2B27 + BDNF (maturation media)

Name	Volume
N2B27	500 mL
BDNF (100 µg/mL stock)	50 µL

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Trituration medium

Name	Volume
N2B27 + BDNF	30 mL
Y-27632 dihydrochloride (Rho kinase inhibitor; 10 mM stock)	30 µL
Dnase I (2 mg/mL stock; 5990 U/mg)	500 µL

Synaptojuice 1 (SJ1; enhanced maturation media)

Name	Volume
N2B27 + BDNF	500 mL
DAPT (50 mM stock)	50 µL
PD0332991 (20 mM stock)	50 µL
$CaCl_2$	185 µL
NKH477 (10 mM stock)	500 µL
CHIR99021 (20 mM stock)	50 µL
GABA (300 mM stock)	500 µL
LM22A4 (10 mM stock)	50 µL

Synaptojuice 2 (SJ2; enhanced maturation media)

Name	Volume
N2B27 + BDNF	500 mL
PD0332991 (20 mM stock)	50 µL
$CaCl_2$	185 µL
CHIR99021 (20 mM stock)	50 µL
LM22A4 (10 mM stock)	50 µL

SAFETY WARNINGS

For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet).

ABSTRACT

This protocol is about Differentiation of hPSCs to hypothalamic neurons.

BEFORE STARTING

Prepare Media and Reagents as described in section 'Materials'.

Thawing of human pluripotent stem cell (hPSC) lines:

1 Thaw an aliquot of 1:10 diluted Geltrex **On ice** or in the fridge.

2 Dilute aliquot 1:10 in ice-cold DMEM/F12 to a final concentration of 1:100.

3 2h

To coat plates, add 1:100 diluted Geltrex to TC dish/plate and incubate for **01:00:00** at **37 °C** , or **Overnight** at **4 °C** .

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Use **3 mL** for a 10 cm dish or **1 mL** for a well of a 6 well plate.

4

Aspirate Geltrex and rinse the dish/plate 1x with an equal volume of DPBS.

Note: do not let the dish/plate dry out.

5 Add pre-warmed hPSC culture media: StemFlex with **10 Micromolar (µM) Rock inhibitor** ; **10 mL** per 10 cm dish, **2 mL** per well of 6 well plate.

Note: the dish/plate is ready to receive cells for plating.

6 Take vial cells from liquid nitrogen to TC room on dry ice.

7 Dip bottom half of vial into **37 °C** water bath and swirl until partially thawed (approximately **00:01:00** - **00:03:00** , depending on volume of freeze). 4m

8 Thoroughly spray vial with **70 % ethanol** , and complete thaw by gently transferring pre-warmed hPSC culture media into the partially thawed cells.

9

Transfer cells into a 15 mL V-bottom polypropylene tube with pre-warmed wash media. Wash residual cells out of vial with **1 mL hPSC culture media** .

10

Spin cells at **160 x g, 00:03:00** .

11 Aspirate media.

12

Re-suspend the pellet with **10 mL hPSC culture media** and mix well by gently pipet it up and down.

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13 

Spin at **160 x g, 00:03:00** .

14 Aspirate media.

15 Re-suspend pellet with **1 mL hPSC culture media** . Dilute into appropriate volume depending on culture dishes/plates used.

16 Add and evenly distribute cells into dish/plate with pre-warmed hPSC culture media (from step 5).

17 

Transfer to incubator, cells should attach over a few hours.

18 Change media the following day to StemFlex without Rock inhibitor.

Note: Withdrawal of Rock inhibitor will result in a notable change in morphology, from 'spikey' look cells with thin processes, to a smoother appearance. Some cell death may also occur.

Hypothalamic Differentiation

19 Coat 6-well or 10 cm plates with Geltrex for differentiation as described above.

20 Nearly confluent hPSCs are dissociated and re-plated for differentiation

Note: Before induction of differentiation, hPSCs should lack obvious signs of differentiation or contamination, and be in a rapid growth phase.

21 

Aspirate culture medium and briefly and gently wash cells in **Room temperature** DPBS.

22 Add **37 °C** TrypLE to cell culture, **1 mL** per well in 6-well plate, **5 mL** per 10 cm plate.

23 

8m

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Incubate cell culture for **00:03:00** - **00:05:00** at **37 °C** . After a **3 minute** incubation, check to see if cells are detaching. Under a phase contrast microscope, the cells should start to round up and take on a phase-bright appearance, but not spontaneously detach from the plate. Once cultures adopt this appearance, gently suck up and dispel ~ **100 µl TrypLE solution** with a P1000 pipette against the cells. They should easily dislodge and leave a small area devoid of cells. If cells do not dissociate easily, extend TrypLE digestion for another minute and repeat this test.

Note: Take care to avoid over-digestion, which can cause cell death.

24 Gently aspirate TrypLE.

25 

To dissociate cells, add **1 mL** / **5 mL hPSC culture media** for a well of a 6 well/10 cm plate, and gently pipette this medium over the plate to detach cells and dissociate them to a single-cell suspension.

26 Collect cells in 15 ml V-bottom polypropylene tube, and adjust volume with hPSC culture media (Total volume = **10 mL**)

Note: This wash step dilutes residual TrypLE to slow further digestion.

27  8m

Spin at **160 x g** for **00:03:00** - **00:05:00** at **Room temperature** . Aspirate supernatant, re-suspend cells in **10 mL hPSC culture media** .

28  8m

Spin at **160 x g** for **00:03:00** - **00:05:00** at **Room temperature** . Aspirate supernatant, re-suspend cells in hPSC culture media

Note: These wash steps remove any remaining traces of TrypLE.

29 After re-suspending the cell pellet, adjust volume so that the suspension is visibly turbid, but not milky (approximately 1-5 x 10^6 cells/mL).

30 

In a 1.5 mL polypropylene tube, mix **10 µl** of this cell suspension with **10 µl Trypan blue** , transfer **10 µl**

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of that mixture onto cell counting slide. Count cells with automated cell counter

31 Plate cells onto Geltrex-coated plates in hPSC culture media at a concentration of 1 x 10^5 cells per cm^2 (corresponding to 9.5 x 10^5 cells per well of a 6-well plate, or 5.5 x 10^6 cells per 10 cm plate). This density corresponds to approximately 80% confluence the following day. Ensure that cells are evenly distributed across the plate by gently shaking the plate left to right, then top to bottom before and after transferring it to the incubator.

Note: If cells are sparser, wait until cells reach the desired density before starting the differentiation. Sparse or over-confluent cells will not pattern well.

32 

If cells plated for differentiation are evenly distributed over the plate and at a density of approximately 75%, start differentiation by washing cultures once with DPBS and adding Day 0 (D0) medium (see below). Every second day, make full medium changes as follows (**5 mL** - **6 mL** per 6-well plate, **10 mL** - **15 mL** per 10 cm plate):

Note: Observe cells daily for changes in morphology. From Days 0-2, the culture should reach confluence and cells should have a simple and uniform hPSC-like morphology. By Day 4, cultures are highly compacted and cells adopt a more rounded appearance. Between Days 4 and 8, the cultures take on a dense neuro-epithelial morphology with identifiable neural ridge-like structures. A neuro-epithelial morphology is still evident before passaging on Day 14.

32.1 ***Day 0 (D0):*** N2B27 + **[M]2 Micromolar (µM) XAV939** + **[M]100 Milimolar (mM) LDN-193189** + **[M]10 Micromolar (µM) SB431542**

32.2 ***Day 2 (D2):*** N2B27 + **[M]2 Micromolar (µM) XAV939** + **[M]100 Nanomolar (nM) LDN-193189** + **[M]10 Micromolar (µM) SB431542** + **[M]1 Micromolar (µM) SAG** + **[M]1 Micromolar (µM) Purmorphamine**

32.3 ***Day 4 (D4):*** N2B27 + **[M]1.5 Micromolar (µM) XAV939** + **[M]75 Nanomolar (nM) LDN-193189** + **[M]7.5 Micromolar (µM) SB431542** + **[M]1 Micromolar (µM) SAG** + **[M]1 Micromolar (µM) Purmorphamine**

32.4 ***Day 6 (D6):*** N2B27 + **[M]1 Micromolar (µM) XAV939** + **[M]50 Nanomolar (nM) LDN-193189** + **[M]5 Micromolar (µM) SB431542** + **[M]1 Micromolar (µM) SAG** + **[M]1 Micromolar (µM) Purmorphamine**

32.5 ***Day 8 (D8):*** N2B27 + **[M]0.5 Micromolar (µM) XAV939** + **[M]25 Nanomolar (nM) LDN-193189** + **[M]2.5 Micromolar (µM) SB431542** + **[M]5 Micromolar (µM) DAPT**

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32.6 ***Day 10 (D10):*** N2B27 + [M]**5 Micromolar (µM) DAPT**

32.7 ***Day 12 (D12):*** N2B27 + [M]**5 Micromolar (µM) DAPT**

32.8 ***Day 14 (D14):*** N2B27 + [M]**5 Micromolar (µM) DAPT**

Neuronal Maturation 24m

33 Coat plates with Geltrex for maturation as described above (Optional: use a [M]**0.02 %** final Geltrex concentration to facilitate neuronal attachment and long term culture).

34 On **Day 15**, neural progenitors generated above are dissociated and re-plated to encourage neurogenesis and neuronal survival and maturation.

Note: Plate cells based on different experiment requirements/layout.

35 

Wash cells gently with DPBS.

36 

Prepare a mixture of TrypLE and Papain by mixing **10 mL TrypLE** with 1 vial of Papain (140 U/vial). Papain aids in neuronal dissociation and will ensure significantly higher survival upon re-plating.

37 Add TrypLE with Papain to cells, **1 mL** per well in 6 well plate, **5 mL** per 10 cm plate.

38  11m

Incubate cell culture for **00:03:00** - **00:05:00** at **37 °C** . After **00:03:00** of incubation, check to see if cells are detaching. Under a phase contrast microscope, the cells should start to round up and take on a phase-bright appearance, but not spontaneously detach from the plate. Once cultures adopt this appearance, gently suck up and dispel ~ **100 µl TrypLE and Papain solution** with a P1000 pipette against the cells. They should easily dislodge and leave a small area devoid of cells. If cells do not dissociate easily, extend TrypLE digestion for another minute and repeat this test.

Note: Take care to avoid over-digestion, which can cause cell death and release of genomic DNA.

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39 Gently aspirate TrypLE and Papain solution.

40 

To dissociate cells, add **1 mL** / **5 mL trituration medium** for a well of a 6 well /10 cm plate, and gently pipette this medium over the plate to detach cells and dissociate them to a single-cell suspension.

41 Collect cells in 15 mL V-bottom polypropylene tube, and adjust volume with trituration medium (Total volume = **10 mL**).

42  8m

Spin at **160 x g, Room temperature** for **00:03:00** - **00:05:00** . Aspirate supernatant, re-suspend cells in **10 mL trituration medium** .

43  8m

Spin at **160 x g, Room temperature** for **00:03:00** - **00:05:00** . Aspirate supernatant, re-suspend cells in desired volume of trituration medium to enable plating at the desired density.

44 

In a 1.5 mL polypropylene tube, mix **10 µl cell suspension** with **10 µl Trypan blue** , transfer **10 µl of the mixture** onto cell counting slide. Count cells with automated cell counter.

> **Note:** If desired, cultures can be frozen at this point for later thawing as progenitors/immature neurons using the same procedure used for freezing hPSCs.

45 Plate cells onto Geltrex-coated plates in maturation media at a concentration of 1 x 10^5 cells per cm^2 for cells maturing in N2B27 + BDNF (corresponding to 9.5 x 10^5 cells per well of a 6 well plate, or 5.5 x 10^6 cells per 10 cm plate)

> **Note:** Plate cells at a density of 3 x 10^5 cells per cm^2 for cells maturing in the enhanced maturation media, Synaptojuice (SJ1/SJ2).

46 On **Day 16**, aspirate medium and feed with N2B27 + BDNF or Synaptojuice 1 (SJ1).

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47 On **Day 17**, aspirate medium and add twice the normal volume of Synaptojuice 1 (e.g. **4 mL** per well of a 6 well plate, **20 mL** per 10 cm plate) for neuronal maintenance for 1 week. This larger volume helps ensure that neurons are exposed to a relatively constant supply of nutrients. **After 1 week**, maintain the mature neurons on N2B27 + BDNF or Synptojuice 2 (SJ2).

48 Change [M]**75 %** of media volume every second day.

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