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Stem cell research

Generation of forebrain-specific cortical neurons in StemFlex Medium

This protocol was designed for the culture of human pluripotent stem cells (hPSCs) in Gibco™ StemFlex™ Medium to generate forebrain-specific cortical neurons for high-throughput applications. Figure 1 describes the workflow from hPSC spheroids to mature cortical neurons using reagents listed in the Appendix for both suspension and adherent culture pipelines. First, hPSCs cultured in StemFlex Medium are induced to form spheroids by the addition of 10 µM of the ROCK inhibitor Y-27632. Next, the spheroids are induced to develop a neural identity by the introduction of Neuronal Spheroid Induction Medium (Table 1). Following induction, neural spheroids are expanded using the Expansion Medium (Table 2). Expansion can be performed in suspension culture (3D) or with dissociated cells to form a monolayer culture (2D). Finally, the cultures are differentiated in Neuronal Maturation Medium to form mature cortical neurons (Table 3). This protocol is provided for differentiation from suspension cultures grown in StemFlex Medium. Guidance is provided for differentiating both whole spheroids and dissociated adherent culture.

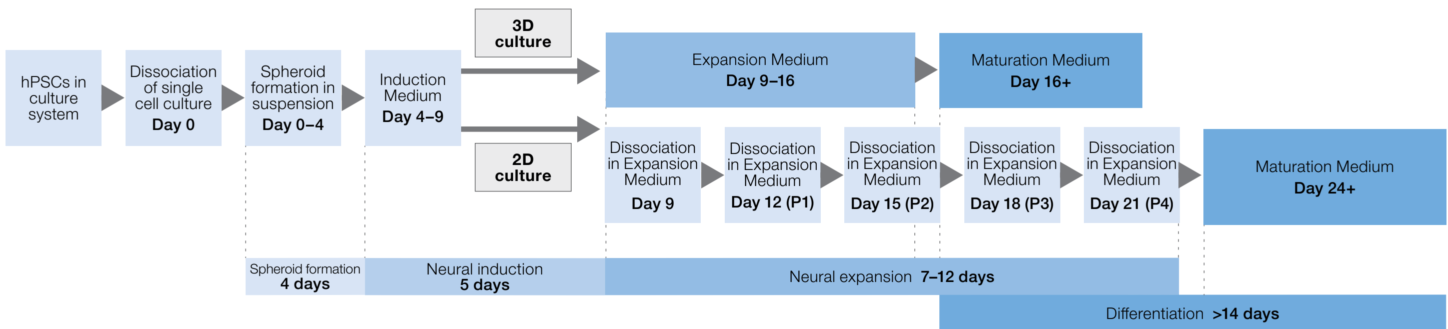

Figure 1. Schematic of the workflow for cortical neuron differentiation.

Table 1. Preparation of Neuronal Spheroid Induction Medium for 3D suspension culture grown in StemFlex medium.

Component	Quantity	Final concentration	Cat. No.
Essential 6™ Medium	149.6 mL	1X	A1516401
RepSox solution (10 mM)	15 µL	1 µM	Various suppliers
LDN-193189 solution (10 mM)	3 µL	0.2 µM	Various suppliers
XAV-939 solution (10 mM)	30 µL	2 µM	Various suppliers
N-2 Supplement (100X)	150 µL	0.1X	17502048
Gentamicin (10 mg/mL)	150 µL	10 µg/mL	15710064

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Table 2. Preparation of Neuronal Expansion Medium.

Component	Quantity	Final concentration	Cat. No.
DMEM/F-12 with GlutaMAX Supplement	48.5 mL	0.5X	10565018
Neurobasal Medium	48.5 mL	0.5X	21103049
N-2 Supplement (100X)	0.5 mL	0.5X	17502048
B-27 Supplement (50X)	1 mL	0.5X	17504044
GlutaMAX Supplement (100X)	0.5 mL	0.5X	35050061
MEM Non-Essential Amino Acids Solution (100X)	0.5 mL	0.5X	11140050
Insulin, human recombinant, zinc solution (4 mg/mL)	62.5 µL	2.5 µg/mL	12585014
Sodium pyruvate solution (100 mM)	0.5 mL	0.5 mM	11360070
RepSox solution (10 mM)	10 µL	1 µM	Various suppliers
DMH1 solution (10 mM)	10 µL	1 µM	Various suppliers
Gentamicin (10 mg/mL)	100 µL	10 µg/mL	15710064

Table 3. Preparation of Neuronal Maturation Medium.

Component	Quantity	Final conc.	Cat. No.
Neurobasal Plus Medium	95 mL	1X	A3582901
B-27 Plus Supplement (50X)	2 mL	1X	A3582801
Dopaminergic Neuron Maturation Supplement (50X)	2 mL	1X	A3147401
Antibiotic-Antimycotic (100X)	1 mL	1X	15240062

Note: Warm media in a 37°C water bath for 5–10 minutes prior to use. Do not warm media in a 37°C water bath for >10 minutes, as this may cause them to degrade.

Spheroid formation

Day 0: Passage hPSCs. Dissociate the adherent culture using prewarmed Gibco™ StemPro™ Accutase™ Cell Dissociation Reagent. Incubate the vessel with the StemPro Accutase reagent for 5 minutes at 37°C, then remove the cells from the well by flushing the medium over the well's surface several times and collecting them in a conical tube. Immediately add 3 mL StemFlex Medium per 1 mL of the cell suspension containing the Accutase reagent. Plate 3 x 10^5 cells/well in 2 mL of prewarmed StemFlex Medium containing 10 µM of the ROCK inhibitor Y-27632 in 6-well Thermo Scientific™ Nunclon™ Sphera™ plates. Maintain cells on an orbital shaker platform in a 37°C incubator under 5% CO_2. Incubate the cells uninterrupted for 96 hours.

Induction of neural identity

Day 4: After 4 days of uninterrupted culture in 6-well Nunclon Sphera plates, transfer the spheroids formed in each well into a 15 mL conical tube and allow the aggregates to settle to the bottom of the tubes (~5 minutes). After aspirating the spent medium, add 2 mL fresh DPBS with no calcium or magnesium to the aggregates and allow them to settle by gravity. Aspirate the spent medium, and leave the aggregates in a minimal volume (~100 µL) of liquid. Add 2 mL prewarmed Neuronal Spheroid Induction Medium to each tube. Transfer the spheroids along with the Neuronal Spheroid Induction Medium from each 15 mL tube to each well of a fresh 6-well Nunclon Sphera plate. Maintain the plate on an orbital shaker platform (70 rpm) in a 37°C incubator.

Days 6 and 8: Perform a 50% media change with prewarmed Neuronal Spheroid Induction Medium: tilt the plate at a 45° angle for 5 minutes, pipet off 1 mL from each well, and replace it with fresh Neuronal Spheroid Induction Medium. Incubate the plate on the orbital shaker platform at 70 rpm and 37°C for 48 hours.

Expansion of neural spheroids

Day 9: Tilt the plate at a 45° angle for 5 minutes and aspirate the spent Neuronal Spheroid Induction Medium—avoid removing any spheroids. Add 2 mL prewarmed Neuronal Expansion Medium to the spheroids in the 6-well plate. Maintain the plate on an orbital shaker platform (70 rpm) in a 37°C incubator for 48 hours.

Days 11, 13, and 15: Perform a 50% media change with Neuronal Expansion Medium: tilt the plate at a 45° angle for 5 minutes and pipet off 1 mL from each well, replace it with fresh Neuronal Expansion Medium. Return the plate to the orbital shaker platform and incubate it at 70 rpm and 37°C. Incubate the cells for 48 hours and repeat the process.

Maturation of neural spheroids

Day 16: Tilt the plate at a 45° angle for 5 minutes and aspirate the spent Neuronal Expansion Medium. Add 2 mL prewarmed Neuronal Maturation Medium to the spheroids in each well. Maintain the plate on an orbital shaker platform set at 65 rpm in a 37°C incubator.

Day 18 and onwards: Perform a 50% media change with Neuronal Maturation Medium: tilt the plate at a 45° angle for 5 minutes, pipet off 1 mL from each well, and replace it with fresh Neuronal Maturation Medium. Return the plate to the orbital shaker platform and incubate at 65 rpm in the 37°C incubator for 48 hours. Repeat the process for a minimum of 14 days to obtain a differentiated culture.

Preparation, expansion, and maturation of dissociated adherent culture

On day 9, to observe differentiation in an adherent culture, split and passage the spheroids in Modified Expansion Medium (Table 4). We recommend a minimum of four subsequent passages to obtain the maximum yield of mature neuronal cells.

Table 4. Preparation of Modified Expansion Medium.

Component	Quantity	Cat. No.
DMEM/F-12 with GlutaMAX Supplement	49 mL	10565018
Neurobasal Medium	49 mL	21103049
N-2 Supplement (100X)	1 mL	17502048
B-27 Supplement (50X)	1 mL	17504044

Day 9: For the preparation of laminin-coated plates, we recommend using a 1:100 dilution of Gibco™ rhLaminin-521 in distilled water. Thaw rhLaminin-521 overnight at 4°C and mix by inversion before dilution. Coat the culture vessels at 37°C under 5% CO_2 for >2 hours. We recommend using 12-well Thermo Scientific™ Nunc™ plates with Nunclon™ Delta surface treatment (Cat. No. 150628) as culture vessels.

Modified Expansion Medium passage: Dissociate the spheroids using prewarmed StemPro Accutase Cell Dissociation Reagent to obtain a single cell suspension. Plate 3 x 10^5 cells/cm^2 in 1 mL of prewarmed Modified Expansion Medium containing 10 µM Y-27632 in laminin-coated 12-well plates. Maintain the cells on a stationary platform in a 37°C incubator under 5% CO_2.

Day 10: Perform a 50% media change with Modified Expansion medium. Return the cells to the stationary platform in a 37°C incubator under 5% CO_2. Incubate and observe growth for the following 48 hours.

Days 12, 15, and 18: Repeat passages in Modified Expansion Medium. Observe growth and neurite development as the culture expands with each dissociation. Media changes may be required every 24–48 hours, depending on the expansion rate of the culture.

Day 21: Dissociate the spheroids using prewarmed StemPro Accutase Cell Dissociation Reagent to obtain a single cell suspension. Plate 1.5 x 10^5 cells/cm^2 in 200 µL of prewarmed Modified Expansion Medium containing 10 µM Y-27632 in 96-well plates coated with rhLaminin-521 and poly-D-lysine. (Plates precoated with poly-D-lysine can be used, or use Cat. No. A3890401 to double-coat with laminin.) Maintain the cells on a stationary platform in a 37°C incubator under 5% CO_2.

Day 22: Perform a 50% media change with Modified Expansion Medium. Return the cells to the stationary platform in a 37°C incubator under 5% CO_2. Incubate and observe growth for the following 48 hours.

Day 24: Neuronal Maturation Medium addition: Aspirate the spent Modified Expansion Medium. Add 200 µL of prewarmed Neuronal Maturation Medium to each well containing culture in the 96-well plate. Maintain in a 37°C incubator.

48 hours later: Perform a 50% media change with Neuronal Maturation Medium. Return the cells to the stationary platform in a 37°C incubator under 5% CO_2. Incubate the cells for 48 hours and repeat the process for a minimum of 14 days to obtain a differentiated culture.

Appendix

Media preparation

Refer to Tables 1–4 to prepare the media used in this protocol.

Note: Complete Neuronal Spheroid Induction Medium, Neuronal Expansion Medium, and Neuronal Maturation Medium can be stored at 2–8°C in the dark for up to 2 weeks.

Small molecule preparation

RepSox Solution (10 mM)

1. To prepare a 10 mM RepSox solution, aseptically mix the following components:
   - 10 mg RepSox
   - 3.48 mL DMSO
2. After dissolving, aliquot 50–100 µL into sterile tubes and store at –5°C to –20°C in the dark for up to 1 year. Thawed RepSox solution can be used once more after thawing and freezing.

LDN-193189 solution (10 mM)

1. To prepare a 10 mM LDN-193189 solution, aseptically mix the following components:
   - 10 mg LDN-193189
   - 2.08 mL distilled water
2. After dissolving, aliquot 50–100 µL into sterile tubes and store at –5°C to –20°C in the dark for up to 1 year. Thawed LDN-193189 solution can be used once more after thawing and freezing.

XAV-939 solution (10 mM)

1. To prepare a 10 mM XAV-939 solution, aseptically mix the following components:
   - 10 mg XAV-939
   - 3.2 mL DMSO
2. After dissolving, aliquot 50–100 µL into sterile tubes and store at –5°C to –20°C in the dark for up to 1 year. Thawed XAV-939 solution can be used once more after thawing and freezing.

DMH1 solution (10 mM)

1. To prepare a 10 mM DMH1 solution, aseptically mix the following components:
   - 10 mg DMH1
   - 2.63 mL DMSO
2. After dissolving, aliquot 50–100 µL into sterile tubes and store at –5°C to –20°C in the dark for up to 1 year. Thawed DMH1 solution can be used once more after thawing and freezing.

Y-27632 solution (10 mM)

1. To prepare a 10 mM Y-27632 solution, aseptically mix the following components:
   - 1 mg Y-27632
   - 0.31 mL distilled water
2. After dissolving, filter through a 0.22 µm filter, aliquot 50–100 µL into sterile tubes, and store at –5°C to –20°C in the dark for up to 1 year. Thawed Y-27632 solution can be kept at 4°C for up to 2 weeks.

Learn more at **thermofisher.com/stemflex**

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