# Feeder-free, Xeno-free Generation of Cortical Spheroids From Human Pluripotent Stem Cells

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**Method Article**

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# Abstract

Here, we show the generation of 3D human cortical spheroids \(hCS) from human induced pluripotent stem cells \(hiPSC) maintained in feeder-free, xeno-free conditions. This protocol describes hiPSC maintenance, generation of 3D cell aggregates in microwells, and cortical differentiation into hCS.

# Introduction

We have previously developed a method of differentiating human induced pluripotent stem cells \(hiPSC) into three-dimensional \(3D) cultures resembling the cerebral cortex \(1). These cultures, named human cortical spheroids \(hCS), contain cortical precursors, functional glutamatergic neurons and non-reactive astrocytes, and can be maintained for months to years in vitro \(2,3). In this method, hPSC are cultured on mouse embryonic fibroblast \(MEF) and knockout serum. To start differentiation, intact hiPSC colonies are lifted enzymatically and transferred in low attachment plates in media containing SMAD pathway inhibitors and growth factors. Here, we describe a method to derive hCS in xeno-free, feeder-free conditions using cell aggregation of hiPSC in microwells.

# Reagents

### FF hiPSC maintenance

| | |
|---|---|
| Essential 8™ medium | Life Technologies A1517001 |
| Vitronectin \(VTN-N) Recombinant Human | Life Technologies A14700 |
| DPBS \(no calcium, no magnesium) | Life Technologies 14190-144 |
| UltraPure™ 0.5 M EDTA, pH 8.0 | Life Technologies 15575-020 |

### Spheroid formation and neural induction

| | |
|---|---|
| Accutase | Innovative Cell Tec. AT-104 |
| Essential 6™ Medium | Life Technologies A1516401 |
| DMEM/F-12, HEPES | Life Technologies 11330-032 |

### Neural Media \(NM) components

| | |
|---|---|
| Neurobasal™-A Medium \(500 mL) | Life Technologies 10888-022 |
| B-27™ Supplement \(50X), minus vitamin A | Life Technologies 12587010 |
| GlutaMAX™ Supplement \(1:100) | Life Technologies 35050-061 |

### Stock Solutions

| Growth factors and small molecules | |
|---|---|
| Dorsomorphin \(2.5 µM); dissolved in DMSO | Sigma P5499-5MG |
| SB-431542 \(10 µM); dissolved in ethanol | Tocris 1614 |
| Y-27632 \(10 µM) | Selleckchem S1049 |
| EGF \(20 ng/mL) | R&D 236-EG |
| FGF2 \(20 ng/mL) | R&D 233-FB |
| BDNF \(20 ng/mL) | Peprotech 450-02 |
| NT-3 \(20 ng/mL) | Peprotech 450-03 |
| XAV-939 \(2.5 µM); dissolved in DMSO | Tocris 3748 |

### Equipment

| Cell culture dishes and plates | |
|---|---|
| AggreWell™800 | StemCell Technologies 34815 |
| Cell strainer, 40 µm | Corning 352340 |
| Ultra-low attachment culture plates \(100 mm) | Corning 3262 |
| Primaria™ tissue culture plates \(100 mm) | Corning 353803 |
| 6-well cell culture plates | Corning 3506 |

# Procedure

1. Maintenance of feeder-free \(FF) hiPSC • hiPSC are cultured on vitronectin in Essential 8™ \(E8) medium and are passaged every 4–5 days using EDTA. • For passaging hiPSC, coat wells of a 6-well plate by diluting 60 µl of vitronectin in 6 ml DPBS \(1:100 dilution) and adding 1 ml of diluted vitronectin solution per well and keep it at room temperature \(RT) for 1 hour. • Aspirate medium and rinse with 3–4 ml DPBS per well, add 1 ml of 0.5 mM EDTA and incubate for 7 minutes at RT. • Aspirate the EDTA solution and add 2 ml of pre-warmed E8 medium. • Remove cells by gently squirting medium and pipetting the colonies with a 5 ml serological pipette. Avoid the generation of bubbles. • Aspirate the residual vitronectin solution from the pre-coated dish and add 2 ml of pre-warmed E8 medium. • Mix the cell suspension by gently inverting several times, then transfer the appropriate volume into each well containing pre-warmed E8 medium according to the desired split ratio. • Gently place the plate into a 37°C, 5% $CO_2$ incubator. • No media change should be performed after the day of passage. Afterwards, replace medium daily \(2–2.5 ml per well). • Cultures should be checked regularly for Mycoplasma

contamination and the presence of genomic abnormalities. 2. \[Optional step] Differentiation day –2: DMSO pre-treatment • Two days prior to starting neural differentiation, and one day prior to spheroid formation, pre-treat hiPSC with 1% DMSO \(120 µl per 12 ml of E8 medium for one 100 mm culture dish). • This stage is optional, and based on ref \(4). 3. Differentiation day –1: Generation of 3D spheroids from hPSC maintained in FF • To generate spheroids, passage hPSC from a 6-well to a 100 mm culture dish, and culture them to 80–90% confluency. • Pre-warm E8 medium, Accutase, and DMEM/F-12 at RT. Supplement E8 medium with the ROCK inhibitor Y-27632 \(1:1000) to a final concentration of 10 µM. • To remove air bubbles from the AggreWell plate, add 1 ml per well of E8 supplemented with Y-27632 and centrifuge at 2,000 x g for 5 minutes in a swinging bucket rotor that is fitted with a plate holder. Check under the microscope to ensure bubbles have been removed from microwells. Set the plate in an incubator while preparing the single cell suspensions of hPSC. • Aspirate maintenance medium from the hPSC plates and rinse cells twice with DPBS \(no calcium, no magnesium). • Add 4 ml of Accutase per 100 mm culture plate and incubate for 7 minutes at 37°C, in a 5% $CO_2$ incubator. • Add pre-warmed E8 medium up to 10 ml volume and centrifuge the cell suspension at 200 x g for 4 minutes. Resuspend the pellet with E8 medium and count cell number. • Centrifuge the cell suspension at 200 x g for 4 minutes. Resuspend the pellet with pre-warmed EB medium supplemented with Y-27632 to obtain 3 million cells per 1 ml of medium. • Add 1 ml of this cell suspension to the previously prepared AggreWell plate, which contains 1 ml of E8 medium. Each well of AggreWell™800 plate contains 300 microwells, and one microwell will have 10,000 cells. • Centrifuge the AggreWell™800 plate at 100 x g for 3 minutes to distribute the cells in the microwells and incubate for 24 hours at 37°C, in a 5% $CO_2$ incubator. 4. Differentiation day 0: Dislodging and harvesting aggregated spheroids • Harvest the hPSC-derived spheroids from the microwells by firmly pipetting the medium in the well up and down with a 1 ml plastic tip that has been cut. • Place a 40 µm strainer on a 50 ml conical tube and pass the suspension of spheroids through the strainer. • Pipette 1 ml of DMEM/F-12 medium across the entire surface of the well to dislodge any remaining spheroids. Collect the spheroids and pass over the strainer as described. Repeat this step 3–5 times. • Invert the strainer, and place over a new 50 ml conical tube. Collect the spheroids by washing with Essential 6™ \(E6) medium for neural induction. • Observe the AggreWell™800 plate under the microscope to ensure that all aggregates have been removed from the wells. Repeat wash if necessary. 5. Neural induction and differentiation • Harvested spheroids are placed in ultra-low attachment 100 mm plates in E6 medium supplemented with 2.5 µM Dorsomorphin \(DM) and 10 µM SB-431542 \(SB). Optionally, 2.5 µM XAV-939 \(XAV) can be added for the first five days. Media changes are performed daily, except for day 1. • On day 6, E6 medium containing DM and SB is replaced with neural medium \(NM) supplemented with EGF2 \(20 ng/ml) and FGF2 \(20 ng/ml) for the 19 days with daily medium change in the first 10 days, and every other day medium changes for the subsequent 9 days. • To promote differentiation of the progenitors, FGF2 and EGF are replaced with 20 ng/ml BDNF and 20 ng/ml NT-3 starting at day 25 \(with media changes every other day). • From day 43 onwards only NM without growth factors is used for medium changes every four days or as needed. For additional experimental details, applications and troubleshooting, see ref \(3).

# References

\(1) Pasca, A. M. et al. Functional cortical neurons and astrocytes from human pluripotent stem cells in 3D culture. Nature methods 12, 671-678, doi:10.1038/nmeth.3415 \(2015). \(2) Sloan, S. A. et al. Human Astrocyte Maturation Captured in 3D Cerebral Cortical Spheroids Derived from Pluripotent Stem Cells. Neuron 95, 779-790 e776, doi:10.1016/j.neuron.2017.07.035 \(2017). \(3) Sloan, S. A., Andersen, J., Pasca, A. M., Birey, F. & Pasca, S. P. Generation and assembly of human brain region-specific three-dimensional cultures. Nat Protoc, doi:10.1038/s41596-018-0032-7 \(2018). \(4) Chetty, S. et al. A simple tool to improve pluripotent stem cell differentiation. Nature methods 10, 553-556, doi:10.1038/nmeth.2442 \(2013).

# Figures

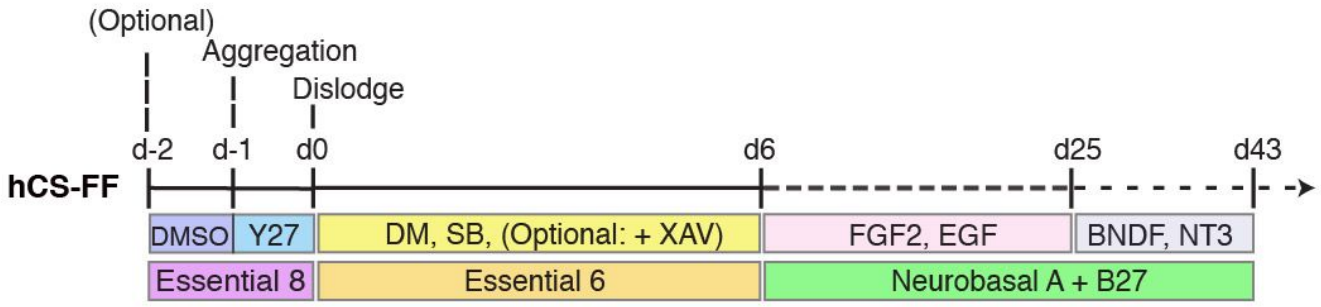


**Figure 1**

recipes_Feeder free_media.jpg recipes_Feeder free_media.jpg Schematic showing the protocol for generating hCS-FF from hPSC.