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Generation of hCS from hiPSC maintained in feeder-free conditions

Forked from Generation and assembly of human brain region-specific organoids

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1 *Works for me* This protocol may be deleted by the owner

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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.

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Yoon, S., Elahi, L.S., Pașca, A.M. et al. Reliability of human cortical organoid generation. *Nat Methods***16**, 75–78 (2019). https://doi.org/10.1038/s41592-018-0255-0

ATTACHMENTS

Generation of hCS from hiPSC maintained in feeder-free conditions.pdf

GUIDELINES

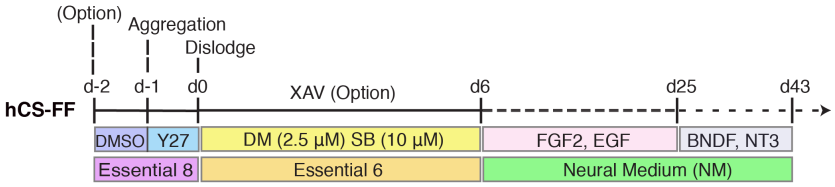

Figure 1. Schematic showing the protocol for generating hCS-FF from hiPSC

MATERIALS

NAME	CATALOG #	VENDOR
DPBS (no Ca, no Mg)	14190144	Thermofisher
Penicillin-Streptomycin (10,000 U/mL)	15140122	Thermo Fisher Scientific
Accutase®, 100 ml	AT104	Innovative Cell Technologies, Inc
Essential 8™ Medium	A1517001	Gibco, ThermoFisher
Recombinant Human/Murine/Rat BDNF	450-02	peprotech
Recombinant Human NT-3	450-03	peprotech
DMEM/F-12, HEPES	11330032	Thermo Fisher Scientific
Vitronectin (VTN-N) Recombinant Human Protein, Truncated	A14700	Thermo Fisher
Neurobasal™-A Medium	10888022	Thermo Scientific
Essential 6™ Medium	A1516401	Thermo Fisher Scientific
UltraPure™ 0.5 M EDTA pH 8.0	15575020	Thermo Fisher Scientific
B-27™ Supplement (50X) minus vitamin A	12587010	Thermo Fisher Scientific

NAME	CATALOG #	VENDOR
GlutaMAX™ Supplement	35050061	Thermo Fisher Scientific
Dorsomorphin	P5499-5MG	Sigma Aldrich
SB 431542	1614	Tocris
Y-27632	S1049	Selleckchem
Recombinant Human EGF Protein CF	236-EG	R&D Systems
Recombinant Human FGF basic/FGF2/bFGF (146 aa) Protein	233-FB	R&D Systems
XAV 939	3748	Tocris
Anti-Adherence Rinsing Solution	07010	Stemcell Technologies

MATERIALS TEXT

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 (1.2 µM); dissolved in DMSO	Tocris 3748

Equipment

Cell culture dishes and plates

AggreWell™800
Microwell culture plate
AggreWell™ 34811

Falcon® 40 µm Cell Strainer
Cell Strainer
Falcon 352340

100 mm Ultra-Low Attachment Culture Dish
Treated Culture Dishes
Corning® 3262

Primaria™ 100 mm x 20 mm Standard Cell Culture Dish
Enhanced Tissue Culture Surfaces
Corning® 353803

6-well Clear TC-treated Multiple Well Plates
96 Well Microplates
Costar® 3506

EQUIPMENT

NAME	CATALOG #	VENDOR
100 mm Ultra-Low Attachment Culture Dish	3262	Corning
Primaria™ 100 mm x 20 mm Standard Cell Culture Dish	353803	Corning
6-well Clear TC-treated Multiple Well Plates	3506	Corning
AggreWell™800	34811	Stemcell Technologies
Falcon® 40 µm Cell Strainer	352340	Corning

SAFETY WARNINGS

See SDS (Safety Data Sheet) for safety warnings and hazards.

BEFORE STARTING

See "Materials" for reagent setup.

Maintenance of feeder-free (FF) hiPSC

1 Human induced pluripotent stem cells (hiPSC) are cultured on vitronectin in Essential 8™ (E8) medium and are passaged **every 4–5 days** using EDTA.

2 For passaging hiPSC, coat wells of a 6-well plate by diluting **60 µl vitronectin** in **6 ml DPBS** (1:100 dilution) and adding **1 ml** of **diluted vitronectin solution** per well.

3 Keep it at **Room temperature** for **01:00:00** .

4 Aspirate medium and rinse with **3 ml** – **4 ml DPBS** per well.

5 Add **1 ml** of **0.5 Milimolar (mM) EDTA** .

6 Incubate for **00:07:00** at **Room temperature** .

7 Aspirate the EDTA solution and add **2 ml pre-warmed E8** .

8 Remove cells by gently squirting medium and pipetting the colonies with a **5 ml serological pipette**.

Avoid the generation of bubbles.

9 Aspirate the residual vitronectin solution from the pre-coated dish and add **2 ml pre-warmed E8 medium** .

10 Mix the cell suspensions by gently inverting several times, then transfer the appropriate volume into each well containing pre-warmed E8 medium according to the desired split ratio.

11 Gently place the plate into a **37 °C** , **5 %** CO_2 incubator.

12 **No media change should be performed after the day of passage.** Afterwards, replace medium daily (**2 ml** – **2.5 ml** per well).

Cultures should be checked regularly for Mycoplasma contamination and the presence of genomic abnormalities.

[Optional step] **Differentiation day –2: DMSO pre-treatment**

13 Two days prior to starting neural differentiation, and one day prior to spheroid formation, pre-treat hiPSC with **1 % volume DMSO** (120 µl per 12 ml of E8 medium for one 100 mm culture dish).

This stage is optional and based on reference:*Chetty, S. et al. A simple tool to improve pluripotent stem cell differentiation. Nature Methods 10, 553-556, doi:10.1038/nmeth.2442 (2013).*

Differentiation day –1: Generation of 3D spheroids from hiPSC maintained in FF

14 To generate spheroids, passage hiPSC from a 6-well to a 100 mm culture dish and culture them to **80–90 % confluency**.

15 Pre-warm E8 medium, Accutase, and DMEM/F-12 at **Room temperature** .

16 Supplement E8 medium with the ROCK inhibitor (Y-27632, 1:1000) to a final concentration of **10 Micromolar (µM)** .

17 Pre-treat wells with **500 µl Anti-Adherence Rinsing Solution** to each well and centrifuge at **1300 x g** for **00:05:00** in a swinging bucket rotor fitted with plate holders.

18 Check under a microscope to ensure that bubbles have been removed from microwells and rinse each well with **2 ml warm DMEM/F-12 medium** .

19 Remove the wash medium, and add **1 ml** per well of **E8 supplemented with Y-27632**. Set plate aside in an incubator while preparing the single cell suspensions of hiPSC.

20 Aspirate maintenance medium from the hiPSC plates and rinse cells **twice** with DPBS (no calcium, no magnesium).

21 Add **4 ml Accutase** per 100 mm culture plate.

22 Incubate for **00:07:00** at **37 °C** , **5 %** CO_2 incubator.

23 Add pre-warmed E8 medium **up to 10 ml** volume.

24 Centrifuge the cell suspension at **200 x g** for **00:04:00** .

25 Resuspend the pellet with E8 medium and count cell number.

26 Centrifuge the cell suspension at **200 x g** for **00:04:00** .

27 Resuspend the pellet with pre-warmed E8 medium supplemented with Y-27632 to obtain **3 million cells per 1 ml of medium**.

28 Add **1 ml** of this cell suspension to the previously prepared AggreWell plate, which contains 1 ml of E8 medium supplemented with Y-27632. Each well of AggreWell™800 plate contains 300 microwells, and one microwell will have 10,000 cells.

29 Centrifuge the AggreWell™800 plate at **100 x g** for **00:03:00** to distribute the cells in the microwells.

30 Incubate for **24:00:00** at **37 °C** , in a **5 %** CO_2 incubator.

Differentiation day 0: Dislodging and harvesting aggregated spheroids

31 Harvest the hiPSC-derived spheroids from the microwells by firmly pipetting medium in the well up and down with a 1 ml disposable tip that has been cut.

32 Place a 40 µm strainer on a 50 ml conical tube and pass the suspension of spheroids through the strainer.

33 Pipette **1 ml DMEM/F-12 medium** across the entire surface of the well to dislodge any remaining spheroids. Collect washes and pass over the strainer **until every spheroids are recovered** by checking under a microscope.

34 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.

35 Observe the AggreWell™800 plate under the microscope to ensure that all aggregates have been removed from the wells. Repeat wash if necessary.

Neural Differentiation

36 Harvested spheroids are placed in ultra-low attachment 100 mm plates in E6 medium supplemented with **[M]2.5 Micromolar (µM) Dorsomorphin (DM)** and **[M]10 Micromolar (µM) SB-431542 (SB)** .

Optionally, **[M]1.2 Micromolar (µM) XAV 939 (XAV)** can be added for the first five days.

Media changes are performed **daily, except for day 1.**

37 On **day 6**, E6 medium containing DM and SB is replaced with neural medium (NM) supplemented with EGF2 (**[M]20 ng/ml**) and FGF2 (**[M]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.

Neural Medium (NM) Composition	Volume (~ 500 ml)
Neurobasal™-A Medium	480 ml
B-27™ Supplement (50X), minus vitamin A	10 ml
GlutaMAX™ Supplement (1:100)	5 ml

Penicillin-Streptomycin (10,000 U/mL)	5 ml

38 To promote differentiation of the neural progenitors into neurons, FGF2 and EGF are replaced with [M]**20 ng/ml BDNF** and [M]**20 ng/ml NT-3** starting at **day 25** (with media changes every other day).

39 From **day 43** onwards only **NM without growth factors** is used for medium changes every four days or as needed.