Brain Organoid Formation

Important Note:
This protocol was created for iPSCs grown with Essential 8 and on Vitronectin-coated plates.

Equipment:
- ☐ Aggrewell 800 Plates (24-well)(Stemcell, Cat. 34811)
- ☐ 10cm Tissue Culture Treated Plates (VWR, Cat. 25382-701)
- ☐ Bright Field Microscope
- ☐ Incubator (Set at 5%CO2 and 37C)

Reagents:
- ☐ Anti-Adherence Rinsing Solution (AARS)(Stemcell, Cat. 07010)
- ☐ Essential 8 (ThermoFisher, Cat. A1517001)
- ☐ DMEM/F12 (VWR, Cat. 12001-600)
- ☐ Rock Inhibitor(Y-27632)(Tocris, Cat. 1254)
- ☐ Accutase (VWR, Cat. 10761-312)
- ☐ PBS (no CaMg)(VWR, Cat. 45000-434)
- ☐ GlutaMAX (ThermoFisher, Cat. 35050061)
- ☐ Penicillin/Streptomycin (VWR, 16777-164)
- ☐ Neurobasal-A (US Bio, N1020-02)

Preparing Media:
- ☐ ***Essential 6 (+) – For early fate induction in hCS, hSpS, and hSS***
  - Essential 6 (ThermoFisher, Cat. A1516401)
  - Essential 6 with Penicillin/Streptomycin (Pen/Strep) supplemented at a 1X concentration (or 100U/mL of Penicillin and 100ug/mL of Streptomycin)
- ☐ ***Neurobasal-A (+) – Base Media for hCS, hSpS, and hSS after Day 5***
  - Neurobasal-A
  - B27 Minus Vitamin A (1X)
  - Glutamax (1X)
  - Pen/Strep (1X)(or 100U/mL of Penicillin and 100ug/mL of Streptomycin)

Supplements:

Small Molecules/Proteins based on differentiation region:

hCS (Cortical)
- ☐ B27 Supplement (50X) minus Vitamin A (ThermoFisher, Cat. 12587010)
- ☐ Dorsomorphin (Sigma, Cat. P5499-25MG)
- ☐ SB-431542 (Selleck Chemicals, Cat. S1067)
- ☐ EGF (R&D Systems, Cat. 236-EG)

- FGF2 (R&D Systems, Cat. 233-FB)
- BDNF (PeproTech, Cat. 450-02)
- NT-3 (R&D Systems, Cat. 267-N3-005/CF)

hSS (Subpallial)

- B27 Supplement (50X) minus Vitamin A (ThermoFisher, Cat. 12587010)
- Dorsomorphin (Sigma, Cat. P5499-25MG)
- SB-431542 (Selleck Chemicals, Cat. S1067)
- XAV (StemCell Technologies, Cat. 72674)
- EGF (R&D Systems, Cat. 236-EG)
- FGF2 (R&D Systems, Cat. 233-FB)
- IWP2 (Selleck Chemicals, Cat. S7085)
- SAG (Sigma Millipore, Cat. 566660)
- BDNF (PeproTech, Cat. 450-02)
- NT-3 (R&D Systems, Cat. 267-N3-005/CF)

hSpS (Spinal)

- B27 Supplement (50X) minus Vitamin A (ThermoFisher, Cat. 12587010)
- N-2 (ThermoFisher, Cat. 17502048)
- Dorsomorphin (Sigma, Cat. P5499-25MG)
- SB-431542 (Selleck Chemicals, Cat. S1067)
- EGF (R&D Systems, Cat. 236-EG)
- FGF2 (R&D Systems, Cat. 233-FB)
- BDNF (PeproTech, Cat. 450-02)
- Retinoic Acid (RA)(Sigma, Catalog R2625)
- CHIR (Selleck Chemicals, Cat. S1263)
- SAG (Sigma Millipore, Cat. 566660)
- cAMP (Sigma, Cat. D0627)
- L-Ascorbic Acid (L-AA)(Fisher, Cat. 50-990-141)
- IGF (Peprotech, Cat. 100-11)

Procedure:

Differentiation Day –1: Generation of 3D spheroids from hiPSC maintained in Feeder Free Conditions

1. (*5-7 Days Before Starting*) Passage hiPSCs from a 6-well to a 100 mm culture dish, and culture them to 80–90% confluency.
2. Pre-warm E8 media (supplemented with Rock Inhibitor (Y-27632) at a final concentration of 10uM), Accutase, and DMEM/F-12 at room temperature.

a. Note: Please review the entire protocol to understand how much of each reagent you'll need.

3. Preparing Aggrewell Plate:
    a. Open AggreWell™ plates in a biosafety cabinet. NOTE: Do not expose AggreWell™ plates to organic solvents, including ethanol or isopropanol.
        i. Pre-treat wells with Anti-Adherence Rinsing Solution (AARS) as described below.
            1. Add 500uL AARS to each well to be used
            2. Centrifuge plate at 1300 x g for 5 minutes in a swinging bucket rotor fitted with plate holders. NOTE: Plates must be well balanced. Prepare a balance plate using a standard plate filled with water to match the weight and position of the AggreWell™ plate.
            3. Observe plate under a microscope to ensure that bubbles have been removed from microwells. If bubbles remain trapped in any microwells, centrifuge at 1300 x g for an additional 5 minutes.
            4. Aspirate AARS from the wells with an aspiration vacuum
            5. Add 1mL pre-warmed DMEM/F12 to rinse well
            6. Aspirate media from the well (repeat once)
            7. Aspirate last DMEM/F12 wash
            8. Add 1 mL warm E8 (+10uM Rock Inhibitor) to each well to be used and check under the microscope for bubbles.
            9. Set the plate in an incubator set at 37°C and 5% CO2 while preparing the single cell suspensions of hiPSCs
4. Aspirate existing media from the hiPSC plates and rinse cells once with 5mL of PBS (no CaMg).
5. Aspirate PBS and add 5 ml of Accutase per 100 mm culture plate and incubate for 7-10 minutes at 37°C, in a 5% CO_2 incubator.
    a. Check under a microscope to ensure that cells are releasing (bright borders between cells) after 7 minutes.
    b. Ideally, you want the cells to **all** (or at least 90%) be fully detached from the plate before proceeding. If this isn't the case, let cells continue to sit in accutase

while checking every minute to see if they're detaching (the maximum time in accutase should not exceed 10 minutes)

6. Pipette the cell suspension 3-5 times (or until you get a single cell suspension) on the plate with a serological pipette to ensure that any remaining clumps are fully dissociated and to dislodge any cells that are still attached.
7. Transfer cell suspension to a 50mL conical tube.
8. Add 5mL of E8 (+10uM Rock Inhibitor) per plate to rinse any remaining cells off from the plate.
9. Transfer to the same conical tube containing hiPSCs, and mix well
10. Remove some for counting and centrifuge the cell suspension at 200 x g for 5 minutes.
11. Count cells while spinning
12. Aspirate off supernatant to leave pellet behind
13. Resuspend the pellet with E8 media supplemented with Y-27632 to obtain **3 million** cells per 1.0 ml of media.
    a. In *general*, 1 well of a 6-well plate is about 1M cells, and an entire 10cm dish is about 10M cells (if the 10cm dish gives <7M cells, it was probably too early).
    b. In general, a fully confluent (90-100%) 10cm dish can yield about 4-5 AggreWells, and a full 6-well plate = 2-3 AggreWells.
14. Add 1 ml of this cell suspension to the previously prepared AggreWell plate, which contains 1 ml of E8 (+10uM Rock Inhibitor)(final volume 2mL).
    a. Note: Each well of AggreWell™800 plate contains 300 microwells.
15. Centrifuge the AggreWell™800 plate at 200 x g for 5 minutes to distribute the cells in the microwells and incubate for 24 hours at 37°C, in a 5% CO_2 incubator.

Differentiation day 0: Dislodging and harvesting aggregated spheroids.

Prep:

- ☐ Make Non-Attachment Plates
    a. To make Non-Attachment plates, add 5mL of AARS to a 10cm plate
    b. Shake well to make sure it covers the entire plate or well
    c. Remove AARS and add 1mL of DMEM/F12 to rinse
    d. Aspirate and add 10mL of appropriate media
- ☐ Prewarm media

a. Please see the organoid differentiation feeding schedule to know what media to use for your differentiation.

Procedure:

2. Add 10mL of pre-warmed media to a 10cm Ultra Low Attachment Plate(s)
3. Harvest the hiPSC-derived spheroids from the microwells by firmly pipetting the media in the well up and down with a 1000 ul plastic tip **that has been cut to make a wider bore size.**
4. Transfer spheroids to a 10cm Ultra Low Attachment plate that contains 10mL of media
    a. The number of aggrewells to 10cm plate depends on the type of differentiation:
        i. hCS – 1-2 aggrewells/10cm plate
        ii. hSS – 2-3 aggrewells/10cm plate
        iii. hSpS – ½ - 1 aggrewell/10cm plate
5. Pipette 1 ml of DMEM/F-12 media across the entire surface of the aggrewell to dislodge any remaining spheroids and add to plate.
6. Continue feeding organoids according to their feeding schedule.

Additional Tips

- When feeding organoids, it's best to perform media changes around same time each day (although do your best, it doesn't always have to be perfect) and a good goal is to minimize time organoids are kept outside of the incubator.
- From the ages Day 0 to Day 15, spheroids are very small. There are two ways to change media:
    a. (Recommended) Swirl dish gently and hold at 30 degree angle until spheroids aggregate on one edge. Carefully aspirate media from opposite end of plate, making sure to avoid aspirating small spheroids.
    b. When they are very small, carefully transfer spheroids + media to a 15mL conical tube and allow spheroids to settle by gravity (5-10 min). Aspirate the media above spheroids and carefully refill with appropriate cell culture media until the volume is about 12mL. Be careful not to disrupt the spheroids much at this time.

Protocol Updates:

Date	**Update Notes**

7/27/23 – Lexi	- The Additional Tips Section was updated. There was old material from the previous protocol. - Included the approximate ages of organoids where they are very small and difficult to feed - Included a detailed section above Supplements to list how to make each type of base media for each region.