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hiPSC Forebrain Organoid Differentiation

Modified from the Kadoshima protocol (PMID: 24277810)

1. **Purpose:** This document establishes the standard operating procedure for differentiating forebrain organoids from hiPSCs. This protocol can be used to generate dorsal or ventral forebrain organoids.

2. **Materials:**
   - PBS, pH 7.4 (Thermo Scientific 10010049)
   - Glasgow's Modified Eagle's Medium (GMEM) (Gibco™ 11710035)
   - KnockOut™ Serum Replacement (KSR) (Gibco™ 10828028)
   - Non-Essential Amino Acids Solution (100X) (Gibco™ 11140050)
   - Sodium Pyruvate (100 mM) (Gibco™ 11360070)
   - 2-Mercaptoethanol (Gibco™ 21985023)
   - Pen/Strep (Cytiva SV30010)
   - Fungizone (SV30078.01)
   - GlutaMAX™ Supplement (Gibco™ 35050079)
   - Chemically Defined Lipid Concentrate (Gibco™ 11905031)
   - N-2 Supplement (100X) (Gibco™ 17502001)
   - B-27™ Supplement (50X), minus vitamin A (Gibco™ 12587001)
   - B-27™ Supplement (50X), serum free (Gibco™ 17504001)
   - Characterized Fetal Bovine Serum (U.S.) (Cytiva SH30071.03)
   - Accutase (Innovative Cell Technologies AT104)
   - 96-well Round Bottom Plates, Ultra-Low Attachment (Corning 7007)
   - ROCK Inhibitor (ROCKi) (Tocris 1254 or StemCell Technologies 72304)
   - SB431542 (SelleckChem S1067)
   - IWR-1-endo (SelleckChem S7086)
   - SAG (BioGems 9128694)
   - Matrigel® hESC-Qualified Matrix, *LDEV-Free (Corning 354277)
   - Media Filter systems, 0.2 µm, low protein binding, Sterile
   - Reagent Reservoirs, Sterile

3. **Procedure**

   (From one well of 6-well plate or 60 mm plate at 70-80% confluency)

Day 0 Seeding (CDM1 + ROCK + IWR1 + SB)

Prepare **CDM 1 media** (recipe here).
Use prepared **CDM 1** to make fresh **CDM 1 media + ROCK + IWR1 + SB**.

Start with a plate (well) at 70-80% confluent.
Remove any differentiation colonies by aspiration 1hr before and add fresh mTeSR.

Wash with **3 ml PBS** (no calcium, no magnesium)
Add **3 ml of accutase** (5 ml for 10 cm plate; enzyme is at RT)
Incubate @ 37°C for 4-6 minutes (determine exact time by visual inspection for "brighter" cells, at sign of loss of adhesion).

Add **3 ml DMEM/F12** to the plate (5 ml for 10 cm)
Gently pipette off any attached cells using 5 ml pipette. Transfer to 15 ml conical.
Rinse plate with additional **3 ml DMEM/F12**, and add to 15 ml conical
Triturate 5-10 times with 5 ml pipet and take 10 ul sample to count.
Centrifuge 4' at 200g
While cells are spinning, count 10 ul sample while rest of cells are spinning

Resuspend in 1-2 ml of **CDM 1 + ROCKi + IWR1 + SB**
(Optional, additional trituration with 1ml pipet if during counting cells are still groups)

Plate **9,000** cells/well in Round or V-bottomed 96-well plates (100ul/well)
Need 900,000 cells in 10 ml CDM 1+ ROCK + IWR1 + SB (target = 90,000 cells/ml).
(If want 12 ml then 90,000x12, so need 1,080,000 cells)

Count = _____, _____, _____, _____

Ave Count = Count / 4 = _____

Cell Concentration = Ave Count x 2 (Trypan dilution) x 1x10^4 cells/ml

= _____cells/ml

Volume Needed = Cells Needed (900,000 cells) / Cell Concentration

= _____ml

Day 3 (CDM1 + ROCK + IWR1 + SB)

Add 100ul of medium to each well

Day 6 (CDM1 + IWR1 + SB)

Do a wash by setting the pipette to 100ul. Remove 80ul (there should be ~190ul in the well with evaporation) and add 100ul of CDM 1 medium + SB/IWR1 to each well.

Day 9 (CDM1 + IWR1 + SB)

Do a wash by setting the pipette to 100ul. Remove 80ul and add 100ul of CDM 1 medium + SB/IWR1 to each well, (same as for Day 6).

Day 12 (CDM1 + IWR1 + SB)

Do a wash by setting the pipette to 100ul. Remove 80ul and add 100ul of CDM 1 medium + SB/IWR1 to each well, (same as for Day 6).

Day 15 (CDM1) (Ventralize option)

Do a wash by setting the pipette to 100ul. Remove 80ul and add 100ul of CDM 1 medium + SB/IWR1 (Dorsal, default) or SAG (Ventral) (ventralizing recipe here)
Note: SAG treatment from day 15 to 21 (500nM for MGE and 30nM for LGE)

Day 18 (CDM2) (Ventralize option)

Fill each well in 24-well (low-attachment) with 400ul of CDM2 (with or without SAG) and transfer each EB in 100 ul of old medium (CDM1) to each well for a total of 500ul. (recipe here)

Day 21 (CDM2)

Remove 400ul and add 500ul of **CDM 2** medium (no SAG anymore).

Day 24 (Transfer)

Transfer the EBs to 6-well plates. Fill each well with 2ml of fresh medium and transfer each EB with 125ul of its old medium. Put 8 EBs/well—in total there will be 3ml in each well.

Leave stationary and as separated as possible.

Day 27 (CDM2)

Replace medium in 6 well plate (3ml/well)
Replace medium in 24 well plate (1ml/well)
Set ROCKER at 45 rpm.

Day 30 (CDM2)

Replace medium in 6 well plate (3ml/well)
Replace medium in 24 well plate (1ml/well)
Set ROCKER at 45 rpm.

Day 33 (CDM2)

Replace medium in 6 well plate (3ml/well)
Replace medium in 24 well plate (1ml/well)
Set ROCKER at 45 rpm.

Day 35 (CDM3) (Transfer Bioreactors)

Transfer to Bioreactor containing 100ml of **CDM 3 + matrigel** (recipe here)

Day 42 (CDM3)

Change medium to Bioreactor

Day 49 (CDM3)

Change medium to Bioreactor

Day 56 (CDM3)

Change medium to Bioreactor (remove Matrigel)

Day 63 (CDM3)

Change medium to Bioreactor

Day 70 (CDM4)

Change medium to Bioreactor with CMD4 (recipe here)

CDM1 - Cortical Differentiation Media 1

Induction Media (100ml, enough for 1 x 96-well plate for days 0-18)

Reagents	Volume	X	Target	Stock
Glasgow MEM	77ml		n/a	n/a
20% KSR	20ml	5	20%	100%
0.1mM NEAA	1ml	100	0.1mM	10mM AA (in MEM)
1mM pyruvate	1ml	100	1mM	100mM
0.1mM BME	182ul	550	0.1mM	55mM (in PBS)
Pen/Strep	1ml	100	1mM	100mM
Total Volume	**100ml**			
Filter sterile media				

Make fresh on day 0 and 3, (10ml, enough for 1x 96-well plate)

Reagents	Volume	X	Target	Stock
CDM 1 media	10ml		n/a	n/a
ROCK Inhibitor	40ul	250	20uM	5mM
IWR1	1ul	10000	3uM	30mM
SB431542	5ul	2000	5uM	10mM
Total Volume	**10ml**			

Induction Media (200ml)
(Enough for 2 x 96-well plate, day 0-18)

Reagents	Volume	X	Target	Stock
Glasgow MEM	154ml		n/a	n/a
20% KSR	40ml	5	20%	100%
0.1mM NEAA	2ml	100	0.1mM	10mM AA (in MEM)
1mM pyruvate	2ml	100	1mM	100mM
0.1mM BME	364ul	550	0.1mM	55mM (in PBS)
Pen/Strep	2ml	100	1mM	100mM
Total Volume	**200ml**			
Filter sterile media				

Make fresh on day 0 and 3, (20ml, enough for 2x 96-well plate)

Reagents	Volume	X	Target	Stock
CDM 1 media	20ml		n/a	n/a
ROCK Inhibitor	80ul	250	20uM	5mM
IWR1	2ul	10000	3uM	30mM
SB431542	10ul	2000	5uM	10mM
Total Volume	**20ml**			

CDM1 - Ventralizing Cortical Differentiation Media 1

Make fresh on day 15, (10ml, enough for 1x 96-well plate)
Dorsalize, default

Reagents	Volume	X	Target	Stock
CDM 1 media	10ml		n/a	n/a
IWR1	1ul	10000	3uM	30mM
SB431542	5ul	2000	5uM	10mM
Total Volume	**10ml**			

Make fresh on day 15, (10ml, enough for 1x 96-well plate)
Ventralize

Reagents	Volume	X	Target	Stock
CDM 1 media	10ml		n/a	n/a
SAG	5ul	2000	1uM	2mM

(1uM x 100ul fresh / 200 ul total volume = 500nM, true target)

Total Volume	**10ml**			

CDM2 - Cortical Differentiation Media 2

Differentiation Medium (200ml, enough for 2 x 96-well plate, day 18-35)

Reagents	Volume	X	Target	Stock
DMEM/F12	200ml		n/a	n/a
Glutamax	2ml	100		
N2 1%	2ml	100		
Lipid Conc 1%	2ml	100		
Fungizone 0.1%	200ul	1000		
PS	2ml	100		
Total Volume	**200ml**			
Filter sterile media				

Ventralize

Make fresh on day 18, (10ml, enough for 1x 96-well plate, Ventralization)

Reagents	Volume	X	Target	Stock
CDM 2 media	10ml		n/a	n/a
SAG	2.5ul	4000	500nM	2mM
Total Volume	**10ml**			

CDM3 - Cortical Differentiation Media 3

Maturation Medium (200ml, enough for 2 x 125ml Bioreactor flask, day 35-56)

Reagents	Volume	X	Target	Stock
DMEM/F12	180ml		n/a	n/a
10% FBS	20ml	10	10	100
1% lipids	2ml	100		
Glutamax	2ml	100		
N2	2ml	100		
Heparin	100ul	2000	5ug/ml	10mg/ml
Fungizone-AmphoB	200ul			
Pen/Strep	2ml			

Filter Sterilize media

Add 1% Matrigel 2ml (only up to day 56)

Maturation Medium (day 56-70)
Do NOT add Matrigel to CDM 3

CDM4 - Cortical Differentiation Media 4

Cortical Differentiation Medium 4, CDM4 (from day 70 onward)
(Extended culture Medium)

DMEM/F12	170ml
10% FBS	20ml
1% lipids	2ml
Glutamax	2ml
1% N2	2ml
B27	4ml (should be without Vitamin A)
Heparin	100ul of 10mg/ml (final 5ug/ml) (2000x)
0.1% Fungizone	200ul
PS	2ml

Cortical Differentiation Medium 4, CDM4 (from day 70 onward)
(Alternate Extended culture Medium)

DMEM/F12	180ml
5% FBS	10ml
1% lipids	2ml
Glutamax	2ml
1% N2	2ml
B27	4ml (should be with Vitamin A)
Heparin	100ul of 10mg/ml (final 5ug/ml) (2000x)
0.1% Fungizone	200ul
PS	2ml
14 ng/ml BDNF	28ul (Stock is 100ug/ml, 7143X; 7uL per 50mL flask)

ESM - Electrophysiology Support Media

Electrophysiology Support Media (200ml, enough for 2 x 10 CM dishes, 14 days before recording)

Reagents	Volume	X	Target	Stock
BrainPhys	194 ml		n/a	n/a
N-2 Supp.	2 ml	100	1%	100%
B-27 Supp.	4 ml	50	1%	50%
Total Volume	**200 ml**			
Filter sterile media				

Make fresh on day of feeding, (40ml, enough for 2 x 10 CM dishes)

Reagents	Volume	X	Target	Stock
ESM	40 ml		n/a	n/a
GDNF	40 ul	1000	20 ng/ml	20 ug/ml
BDNF	40 ul	1000	20 ng/ml	20 ug/ml
cAMP	400 ul	100	1 mM	100 mM
Ascorbic Acid	0.8 ul		200 nM	10 mM
Laminin	batch dependant		1 ug/ml	0.5-0.2 mg/ml
Total Volume	**40ml**			