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Generation of Three Dimensional Cerebral Brain Organoids from Human Pluripotent Stem Cells

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Fan Li[1,2], Ronni Kurzion[2,3,4], Guo-li Ming[2,4], Hao Wu[1,2]

[1]Department of Genetics, Epigenetics Institute, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA;
[2]The Institute for Regenerative Medicine, Perelman School for Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA;
[3]Neuroscience Graduate Program, University of Pennsylvania, Philadelphia, PA 19104, USA;
[4]Department of Neuroscience and Mahoney Institute for Neurosciences, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA

Wu Lab @ UPenn
Tech. support email: **haowu2@upenn.edu**

Fan Li
University of Pennsylvania

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Abstract

Human brain organoids serve as powerful in vitro models for studying brain development and disease, addressing limitations of traditional model organisms. Here, we present a scalable protocol for generating chimeric three-dimensional (3D) cerebral organoids (based on ref1-3) from a diverse panel of multi-ethnic human induced pluripotent stem cell (hiPSC) lines. These organoids self-organize into distinct brain regions, including a structured cerebral cortex with progenitor populations that give rise to cortical excitatory neurons. The system effectively recapitulates key aspects of human cortical development, including an inner layer of PAX6-positive radial glial cells, a middle layer of TBR2/EOMES-expressing intermediate progenitors, and an outer layer of CTIP2/BCL11B-positive immature neurons. This protocol provides a robust platform for modeling early-stage cortical development and disease, enabling deeper mechanistic insights and advancing therapeutic discovery.

Materials

M1 medium:
DMEM 385 mL
20% KOSR 100 mL
1X GlutaMAX 5 mL
1X MEM-NEAA 5 mL
0.1 mM 2-Mercaptoethanol 1 mL
Heparin solution (2 mg/mL to 2ug/mL, 0.2%) 500 uL
LDN (10 mM to 1 uM) 50 uL
SB (10 mM to 5 uM) 250 uL
IWR-1e (10 mM to 2uM) 100 uL
Trans-ISRIB (7 mM to 700 nM) 50 uL
Chroman 1 (5 mM to 50 nM) 5 uL
Emricasan (5mM to 2uM) 200 uL

F2 meidum:
DMEM 500 mL
1x N2 5 mL
1X GlutaMAX 5 mL
1X MEM-NEAA 5 mL
0.1 mM 2-Mercaptoethanol 1 mL
SB (10 mM to 1 uM) 250 uL
CHIR (5 mM) 1 uM, 100 uL

H3 meidum:

	A	B
	DMEM/F12	470 mL
	Neurobasal	470 mL
	1X N-2 supplement	10 mL
	B27 without vitamin A	20 mL
	1X GlutaMAX	10 mL
	1X MEM-NEAA	10 mL

	A	B
	0.1 mM 2-Mercaptoethanol	910 µL *2

hiPSC culture

1 **Coat 6-well plate** (coating should be done 1 day before)

1.1 Thoroughly thaw Matrigel on ice for up to 1 hour.

1.2 Prepare a 15 mL tube, add 6 mL cold DMEM/F12.

1.3 Add 100 µL Matrigel into DMEM/F12, or by adding cold DMEM/F12 and pipetting to dissolve Matrigel.

1.4 Mix by invert 15 mL tube 2-3 times. Transfer 1 mL to each well.

1.5 Tilt the plate to let the media cover the whole surface, and put plate into the incubator for up to 2 hours or overnight.

2 **Thawing hiPSCs**

2.1 Take 1 vial of hiPSCs (containing individual line or pooled lines) from liquid nitrogen tank and place it on dry ice.

2.2 Thaw the vial in a 37 °C water bath by holding it from the cap and shaking it in the water for 1 min, until thawed.

2.3 Bring the vial to a biosafety cabinet, dry the tube, and transfer 1 mL of hiPSCs to a 50 mL tube.

2.4 Suspend hiPSCs in 9 mL DMEM/F12 by dripping it slowly while periodically shaking the tube.

2.5 Centrifuge at 300 g for 4 min.

2.6 While waiting, add ROCKi into mTeSR Plus medium. We may need to dilute Trans-ISRIB, Chroman 1 and Emricasan in ahead and add these inhibitors 1:1000.

2.7 Aspirate the Matrigel from the coated well and replace with 1 mL mTeSR Plus medium (with 3 inhibitors).

2.8 When finished centrifuging, aspirate the supernatant by tilting the tube (the cells will stick to the bottom).

2.9 Resuspend hiPSCs in 1 mL mTeSR Plus medium (with 3 inhibitors), and transfer to the prepared well.

2.10 Fully change medium with mTeSR Plus every day until ~60% - 80% confluence, then passage.

3 **Passage hiPSCs**

3.1 Prepare new plate: Take the pre-coated plate into hood. Aspirate coating media. Add 2 mL mTeSR Plus to each well. Label line name, date, passage number, your name. Put aside.

3.2 Prepare a 15 mL tube:

3.3 Dissociation of hiPSCs from plate: Take the culture plate out, aspirate media. Add 1 mL TrypLE to each well. Put into incubator for 1-2 min. Check under microscopy. Pat heavily but steadily till > 80% cells are lifted. Add 2 mL DMEM/F12 into each well and immediately transfer them to the 15 mL tube gently.

3.4 Centrifuge at 300 g, 1 min.

3.5 Aspirate liquid. Resuspend cells in 1 mL mTeSR Plus and mix by hand. (Do not overdo it.)

3.6 Count cells: Prepare EP tube with 10 µL Trypan blue. Mix with 10 µL cells. Transfer 10 µL to counting chamber. Insert into cell counter.

3.7 Transfer appropriate cells to each new well (Depending on the cell density). Check under microscope.

3.8 Shake plates to evenly distribute cells. Check under microscope. Put plate into incubator.

Make Embryoid bodies

4 **Day 0:**

4.1 Prepare Aggrewell plate: Add 500 µL Anti-Adherence solution to one well. Put aside. Prepare balance plate for centrifuge: Add 2 mL DPBS/well to a 24-well plate. Put aside.

4.2 Dissociate, pellet, and resuspend cells as instructed above.

4.3 Count cells: Prepare EP tube with 10 µL Trypan blue. Mix with 10 µL cells. Transfer 10 µL to counting chamber. Insert into cell counter.

4.4 Calculate how much resuspended cell solution you need to achieve 3 million cells.

4.5 Aspirate Anti-Adherence solution. Wash with 1 mL mTeSR. Add 1 mL mTeSR + 3 inhibitors.

4.6 Transfer 3 million cells to Aggrewell. Mix by pipetting twice.

4.7 Centrifuge at 100 g, 3 min, RT.

4.8 Place Aggrewell plate in incubator.

Resuspend and culture the Embryoid Bodies

5 **Day 1:**

5.1 Prepare empty 6-well plate. Label with line name, date, media name. Add 2 mL M1 media. Put aside.

5.2 Cut p1000 tips.

5.3 Take Aggrewell out. Aspirate most but not all media. Immediately use an uncut p1000 to add 1 mL M1 to the well by circling around to resuspend EBs.

5.4 Use cut p1000 tip to pipet 1-2 times and transfer EBs to 6-well plate.

5.5 Put plate on shaker.

5.6 Wash Aggrewell with filtered ddH_2O and store it in a sterile place.

6 **Day 2:** Tilt the plate and let the EBs sink to bottom. Aspirate half of the media. Add 2 mL M1 media.

7 **Day 3-5:**
Aspirate half of the media. Add 2 mL M1 media.

Culture Cortical organoid

8 **Day 6:** Aspirate most of the media. Add 2 mL F2 media.

9 **Day 7 (embed into matrigel):**

9.1 Prepare cut p200, p1000 tips, EP tubes, low-attachment plate.

9.2 Swirl plate to gather EBs in the center. Transfer EBs with cut p1000 tip to EP tube. Split EBs to
less than 100/tube.

9.3 Allow EBs to settle. Aspirate the supernatant. Wash once with 1 mL F2 media.

9.4 Remove media to only 60 uL left. (Use an empty tube with 60 uL liquid as standard.)

9.5 Add 100uL Matrigel with cut p200 tip and mix with EBs by pipetting several times.

9.6 Immediately spread all Matrigel/EB mixture onto the center of the low-attachment 6-well plate.

9.7 Place plate in the incubator for at least 50 min to solidify.

9.8 Gently add 3 mL F2 media against the wall to Prepare cut p200, p1000 tips, EP tubes, low-attachment plate.

10 **Day 9-13:** Half change media with manually pipetting with F2 meidum.

11 **Day 14 (break EBs from matrigel):**

11.1 Use 10 mL pipette, suck all Matrigel cookie inside with normal speed, then aim at the bottom corner of the plate, pipette out with normal speed. Repeat 2-3 times till most EBs are out of Matrigel.

11.2 Transfer the medium to a new plate.

11.3 Remove most of the media by hand. Add 2 mL H3 into each well.

11.4 Put plate on shaker.

12 **Day 15 - 34:**
Remove most of the Matrigel. Add new H3.

13 **Day 35:** H3 + 0.5-1% Matrigel every 4 days.

Protocol references

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