Protocol for generation of cerebral organoids as described in Ormel *et al*. 2018 Nat. Comm.

The following protocol is an adaptation of the protocol published by Lancaster *et al.* 2014 Nature Protocols. Please refer to the last page of this protocol for a direct comparison between the 2 protocols. For further instructions and general principles behind the organoid generation please refer to Lancaster *et al.* 2014 Nature Protocols.

MATERIALS

REAGENTS

Cells

- iPSCs

Growth media and supplements

- mTeSR1 medium (Bio-Connect; 85857) or STEMFlex medium (Life Technologies; A3349401)
- DMEM-F12 (Life Technologies; 11320074)
- Knockout serum replacement (KOSR, Life Technologies; 10828028)
- Fetal Bovine Serum (FBS, Sigma Aldrich; F7524)
- L-Glutamine (Life Technologies; 25030024)
- MEM non-essential amino acids (MEM-NEAA, Life Technologies; 11140035)
- β-Mercaptoethanol
- bFGF (Prepotech; 100-18B)
- Heparin (Sigma Aldrich; H3149)
- ROCK inhibitor Y27632 (Y27, Axon Biochemicals; AXON1683)
- N2 supplement (Life technologies; 17502001)
- B27 without vitamin A supplement (Life Technologies; 12587001)
- B27 supplement (Life Technologies; 17504001)
- Penincillin-streptomycin (P/S, Life Technologies; 15140122)
- Neurobasal medium (Life Technologies; 21103049)
- Human Insulin solution (Sigma Aldrich; I9278)

Enzymes and other reagents

- Geltrex (Life Technologies; A1413202)
- PBS (Life Technologies; 14190169)
- 0.5M EDTA ultra pure (Life Technologies; 15575-038)
- Matrigel (Corning; 356234)
- Accutase (Tebu-Bio; AT-104)
- Tryphan Blue (Thermofisher; T10282)

Equipment (Besides general lab/cell culture equipment)

- 60mm petri dishes (Greiner Bio-One B.V.; 391-0487)
- Agrewell 800 plates (Bio-Connect; 34815)
- Countess II FL automated cell counter (Thermofisher; AMQAF1000)
- Counting chamber for Countess II FL (Thermofisher; C10283)
- 96 well plate ultra low attachment (Corning; 3473)
- Belly dancer (IBI scientific; BDRAA2205)
- Parafilm (Sigma Aldrich; P7793)

REAGENT SETUP

Feeder-free iPSC lines Culture iPSCs using standard procedures in a 5% CO2 incubator at 37°C. Briefly, maintain cells in 15mm dishes coated with Geltrex according to manufacturer's instructions with Stemflex or mTeSR1 medium. Passage cells by washing once with PBS and incubating with 0.5mM EDTA for 1 to 2 minutes at 37°C, gently aspirate the EDTA and spray dish with 1mL of culture medium supplemented with Y27 (1:1000) to generate small flakes and transfer an appropriate amount to a new dish. To start organoids is best to expand the iPSCs in 60mm dishes for higher yield.

Reconstitution and storage of growth factors and other additives

- **bFGF** To obtain 100ng/μl ressuspend 1mg vial in 1mL of sterile 5mM Tris (pH7.6). Transfer to 15mL tube and add 8mL of sterile 5mM Tris (pH7.6) 0.1% BSA. Aliquot in desired volume and store at -80°C. Working aliquots can be stored at -20C.
- **Y-27** Ressuspend 5mg vial in 3065μl of PBS to obtain 5mM working concentration. Aliquot at desired volume and store at -20 °C
- **Heparin** To obtain 5mg/mL working stocks dilute 100000 units in 99mL of sterile miliQ water. Make 500μl aliquots and store at -20°C.
- **β-mercaptoethanol dilution** Dilute β-mercaptoethanol 1:100 in DMEM-F12. Filter sterilize with 0.2μm filter.

Aliquoting Matrigel for Matrigel droplets Thaw matrigel on ice at 4°C overnight. Prepare ten microcentrifuge tubes in the sterile hood and gently mix matrigel in the vial before opening. Quickly aliquot 500 μL per tube and place them back on ice immediately. Store the aliquots at -20°C for up to a year. Avoid repeated freeze/thaw cycles. CRITICAL Matrigel will solidify at room temperature so it is important to minimize time at room temperature. Place 5mL pipettes or 1mL tips in the -20°C in advance and use them to prepare the aliquots. Additionally, if possible, place the microcentrifuge tubes in a -20°C or 4°C block while aliquoting.

CELL CULTURE MEDIA

Media should not be kept for longer than 2 weeks at 4°C. Therefore, is best to prepare according to the necessary volume or make aliquots and store at -20°C.

hES0 medium For 500mL of medium combine 100mL of KOSR, 15mL of FBS, 5mL of L-Glutamine, 5mL of MEM-NEAA, 3.5μl of 2-mercaptoethanol and add DMEM-F12 to a total volume of 500mL.

hES4 medium Add fresh bFGF (4ng/mL) to hES0 medium.

Neural induction medium For 500mL of medium combine 5mL of N2, 5mL of L-Glutamine, 5mL of MEM-NEAA, 500uL heparin, up to 500 mL with DmemF-12

Differentiation medium without Vitamin A For 250mL of medium combine 120mL of DMEM-F12 with 120mL of Neurobasal and add 1.25mL of N2, 2.5mL of L-Glutamine, 1.25mL of MEM-NEAA, 2.5mL of P/S, 2.5mL of B27 without RA, 87μl of β-mercaptoethanol dilution and 62.5μl of insulin.

Differentiation medium with Vitamin A Use same recipe as for "differentiation medium without Vitamin A" but replace B27 without RA by B27.

PROCEDURE

Making EBs TIMING 1 hour

1| Grow iPSC colonies in a 6cm dish until they are 80-90% confluent.

i Prepare Agrewell plate. Add 0.5mL of Agrewell rinsing solution per well to be used (add water to other wells) and centrifuge 5 minutes at 2000 rpm. Replace rinsing solution by 1mL of hES4 1:100

ii Prepare hES4 (2μL per 50mL) and divide in 1:100 (25mL) and 1:1000 (50mL) Y27

iii Remove StemFlex or mTeSR medium and wash with 2-3 ml of PBS. Remove PBS, add 1mL of EDTA and place in the incubator for 1-2 minutes. Gently aspirate the EDTA and add 1mL of Accutase for 4 minutes. Colonies should become loose when gently tapping the dish.

iv Prepare a 15mL tube per line with 5mL of hES4 1:1000.

v Quench Accutase reaction with 4mL of hES4 1:1000 medium. Collect the 6mL to 15mL tube to obtain a total of 10mL. Homogenize and take 10μL into an microcentrifuge tube for counting.

vi Centrifuge cells for 4 minutes at 200 rcf

vii In the meanwhile, add 10μL of tryphan blue to the microcentrifuge tube and mix gently. Load 10μL in a countess counting chamber and Count cells in Countess II FL cell counter. Register the number of live cells per mL to calculate the number of total live cells. **ALTERNATIVETLY** Count live cells manually on a Fuchs-Rosenthal chamber and calculate total live cell number accordingly.

viii Gently aspirate the medium without disturbing the pellet and ressuspend cells at 3.5x10^6 cells per mL in hES4 1:100 medium

ix Pipette 1 mL of single cell suspension per well in the Agrewell plate. Typically, 1 well will generate approximately 100 EBs.

x Place in incubator at 37˚C.

Growing EBs and germ layer differentiation TIMING 4 days

2| After 24 hours EBs with clear borders should be visible on the microwells. Carefully remove 1.5mL of medium and gently add 2mL of fresh hES4 1:100 without disturbing the EBs in the bottom

3| The following day flush EBs from Agrewell plate and single EBs in a ULA 96 well plate. Have a microscope inside the sterile hood and prepare in advance 6 cm petri dishes with 5mL of hES4 1:100 medium.

i. Carefully remove 1.5mL of medium from the wells.

ii. With the P1000 Spray the well with fresh medium in a circular movement. This should cause EBs to lift from the microwells. Gently pipette them to the 6 cm petri dish.

iii. Repeat with new medium as many times as necessary until all EB's have been removed. **CRITICAL** Always use fresh medium to avoid pipetting the EBs up and down on the microwell.

iv. Add 150μL of hES4 1:100 to the 96 well plates.

v. Transfer each EB to one well of the 96 well plate using a cut 200-uL pipette tip

4| Change medium every other day by removing 100μL and adding 150μL of fresh medium. **CRITICAL** Keep pipette tip at the of surface of the medium instead to in the bottom of the well to not aspirate EBs during medium change.

5| When EBs are approximately 320μm in diameter switch medium to hES0, typically at day 4. When switching media do a double medium remove the old medium as much as possible.

Induction of neuroectoderm **TIMING 8 days**

6| When EBs are approximately 330μm in diameter switch to NIM medium, typically at day 6. When switching media do a double medium remove the old medium as much as possible. Keep doing medium change every other day until day 13.

Matrigel embedment **TIMING 3 hours**

7|Thaw matrigel aliquots 1-2 hours in advance in iced water.

8| Prepare Parafilm dimples by laying a rectangle of Parafilm on an empty tray of 200μL tips and pressing with your fingertips over each whole to create a small dimple. Using a blade cut the Parafilm in squares of 4x4 dimples and place each square in a 6cm petridish.
CAUTION! Spray the tip tray with 70% Ethanol and put it under UV light in the hood for 30 minutes. Spray the Parafilm with 70% Ethanol before use and prepare the dimples using sterile chirurgical gloves. Once the 4x4 dimple squares are in the dishes place them under UV light for another 30 minutes.

9|Cut 200μL tips with a sterile knife over the first mark.
CRITICAL The edge of the tip should remain smooth so it does not cause damage to the tissue.

10| Transfer the embryoid bodies one by one to each dimple in a small droplet of medium. With an uncut tip remove the medium from the dimple carefully to not aspirate the tissue. Quickly add 30μL droplets of matrigel on top of each embryoid body and use a 10μL tip to center the tissue in the matrigel droplet. Place the plate at 37 ˚C for 15-20 minutes for matrigel polymerization

CRITICAL Do this step for a maximum of 16 dimples at once. The whole procedure should take 4-5 minutes for 16 dimples. If it takes longer it is best to do it for 8 or 4 instead. Avoid holding the bottom of the matrigel aliquot has it will polymerize quickly. When pipetting the matrigel do not press the second step on the pipette as this will create an air bubble on top of the matrigel.

11| Place 7mL of differentiation medium without vitamin A to the plate and gently remove the droplets from the Parafilm using the round side of curved forceps. Place the dish back in the incubator.

Expansion of neuroephitelial buds in stationary culture **TIMING 4 days**

12| Observe the embedded tissue after 24 hours, tissues should begin to form buds of neuroepithelial tissue.

13| After another 24 hours (day 15), feed the organoids with differentiation medium without vitamin A. Remove most of the medium and add another 7mL per plate.

14| Culture for another 48 hours.

Growth of organoids TIMING up to 6 months

15| Replace medium with 7mL of differentiation medium with vitamin A.

16| Place dishes in belly dancer in position 3,5 with slight tilt.

17| Refresh medium every other day until collection.

Comparison table between Lancaster *et al* 2014 Nature Protocols and Ormel *et al* 2018 Nat. Comm.

Description		Ormel et al. 2018	Lancaster et al. 2014
Embryoid bodies generation	medium	hES4 (-P/S; +FBS); Y27 (1:100)	hES4 (-P/S; +FBS); Y27 (1:100)
	plates	AggreWell 800, (300 microwells/well)	V- bottom ULS 96 well plate
	Volume	2 mL ($1.75x10^6$ cells per mL)	150 µL ($6x10^5$ cells/mL)
	cells per embryoid body (EB)	Approx. 11500 cells	9000 cells
Germ layer differentiation I	timing	up to day 4	up to day 4
	medium	hESC4 ; Y27 (1:100)	hESC4 ; Y27 (1:100)
	plates	flat bottom ULA 96 well plate	V- bottom ULA 96 well plate
	EB size	320 (+/- 40) µm	> 350-400 µm
Germ layer differentiation II	timing	Days 4 to 6	Days 4 to 6
	medium	hES0	hES0
	plates	flat bottom ULA 96 well plate	V- bottom ULA 96 well plate
	EB size	330 (+/- 37) µm	350-600 µm
Induction of neural ectoderm	timing	days 6 to 12	days 6 to 9
	medium	NIM with 0.1 µg/mL Heparin	NIM with 1 µg/mL Heparin
	plates	flat bottom ULA 96 well plate	flat bottom ULA 24 well plate
	EB size	330-570 µm	500-600 µm
Transfer to Matrigel	timing	Day 13	Day 11
	medium	Differentiation medium without RA	Differentiation medium without RA
	plates	60 mm petri dish	60 mm tissue culture dish
Transfer bioreactor	timing	Day 17	Day 15
	medium	Differentiation medium with RA	Differentiation medium with RA
	platform	Spinning bioreactor	Spinning bioreactor
	speed	27.5 rpm	25 rpm