

## Production of Phenotypically Uniform Human Cerebral Organoids from Pluripotent Stem Cells

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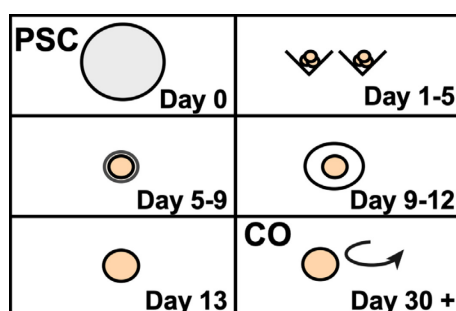
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**[Abstract]** Recent advances in stem cell technology have allowed researchers to generate 3D cerebral organoids (COs) from human pluripotent stem cells (hPSCs). Indeed, COs have provided an unprecedented opportunity to model the developing human brain in a 3D context, and in turn, are suitable for addressing complex neurological questions by leveraging advancements in genetic engineering, high resolution microscopy, and tissue transcriptomics. However, the use of this model is limited by substantial variations in the overall morphology and cellular composition of organoids derived from the same pluripotent cell line. To address these limitations, we established a robust, high-efficiency protocol for the production of consistent COs by optimizing the initial phase of embryoid body (EB) formation and neural induction. Using this protocol, COs can be reproducibly generated with a uniform size, shape, and cellular composition across multiple batches. Furthermore, organoids that developed over extended periods of time (3–6 months) showed the establishment of relatively mature features, including electrophysiologically active neurons, and the emergence of oligodendrocyte progenitors. Thus, this platform provides a robust experimental model that can be used to study human brain development and associated disorders.

### Graphic abstract:

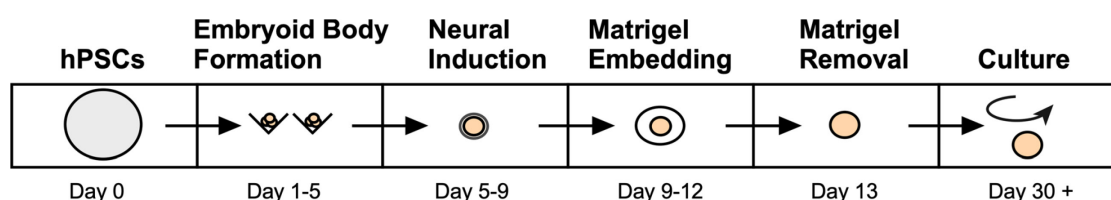


### Overview of cerebral organoid development from pluripotent stem cells

**Keywords:** Stem cells, Organoids, Cerebral organoids, Brain organoids, 3D culture, Neural development, Neuroscience

**[Background]** Recent advancements in the *in vitro* development of 3D cerebral organoids (COs) derived from human pluripotent stem cells (hPSCs) have provided an unprecedented opportunity to

model the developing human brain and relevant complex diseases in an experimentally tractable system. Indeed, this approach has allowed researchers to study early brain development and the consequences of alterations associated with various human neurological disorders, such as Alzheimer's, blindness, Autism Spectrum Disorder (ASD), and Zika virus infection (Lancaster and Knoblich, 2014b; Quadrato *et al.*, 2016; Di and Kriegstein, 2017; Huch *et al.*, 2017; Amin and Pasca, 2018; Rossi *et al.*, 2018; Chen *et al.*, 2019). In addition, several groups have applied COs to study and establish preclinical models of human brain cancers such as glioblastoma multiforme (Drost and Clevers, 2018; Linkous *et al.*, 2018). In recent years, numerous protocols have emerged to facilitate the development of region specific-COs by controlling the underlying cell signaling pathways with exogenous growth factors and small molecule inhibitors to guide cell fate changes as the organoid matures (Lancaster *et al.*, 2013; Mariani *et al.*, 2015; Jo *et al.*, 2016; Qian *et al.*, 2016; Birey *et al.*, 2017; Quadrato *et al.*, 2017; Watanabe *et al.*, 2017; Pollen *et al.*, 2019; Velasco *et al.*, 2019; Yoon *et al.*, 2019). However, due to the fact that human whole-brain organoids are largely produced by intrinsic self-patterning and do not rely on controllable exogenous factors, stochastic differentiation often leads to cellular diversity, which is amplified with extended culture. Unfortunately, the considerable variability between individual organoids obtained using whole-brain differentiation platforms can therefore limit the utility of these COs for studying disease mechanisms or the development of potential therapeutics. Here, we describe our robust protocol for efficiently and reproducibly generating mature, uniform human COs (Figure 1). By optimizing an established protocol for the creation of self-patterned whole-brain organoids (Lancaster and Knoblich, 2014a; Lancaster *et al.*, 2013), we successfully generated phenotypically uniform forebrain organoids with reproducible cell-type compositions.



**Figure 1. Overview of the developed method to generate human COs from pluripotent stem cells**

## **Materials and Reagents**

1. 96-well V-bottomed non-binding plates (Greiner Bio-One, catalog number: 651970)
2. 24-well clear flat-bottomed ultra-low attachment plates (Corning, catalog number: 3473)
3. 4-well cell culture plates (ASI, catalog number: TP9004)
4. 50 ml and 15 ml conical centrifuge tubes (Corning, Falcon, catalog numbers: 352096, 352070)
5. Low-retention microcentrifuge tubes (1.5 ml and 0.6 ml, Thermo Fisher Scientific, catalog numbers: 3451 and 3446)
6. 50 ml sterile disposable reagent reservoirs (Corning, catalog number: 4870)
7. DMEM/F-12, HEPES (Thermo Fisher Scientific, Gibco, catalog number: 11330032, store at 4 °C)

8. KnockOut™ Serum Replacement – Multi-Species (Thermo Fisher Scientific, Gibco, catalog number: A3181502, store at -20°C)
9. MEM Non-Essential Amino Acid Solution (100×) (Thermo Fisher Scientific, Gibco, catalog number: 11140050, store at 4°C)
10. 2-Mercaptoethanol (1,000×) (Thermo Fisher Scientific, Gibco, catalog number: 21985023, store at 4 °C)
11. Animal-Free Recombinant Human FGF-basic (Peprotech, catalog number: AF-100-18B, store at -80°C)
12. TrypLE™ Express Enzyme (1×) (Thermo Fisher Scientific, Gibco, catalog number: 12604013, stored at room temperature [22°C] in the dark)
13. Neural Basal Medium (Thermo Fisher Scientific, Gibco, catalog number: 21103049, store at 4°C)
14. Y27632 ROCK Inhibitor (Cedarlane, catalog number: S1049-10MG, store at -80°C)
15. GlutaMAX™ Supplement (Thermo Fisher Scientific, Gibco, catalog number: 35050-061, store at 4°C)
16. Heparin sodium salt (Sigma-Aldrich, catalog number: H4784, store at -20°C)
17. Insulin solution human (Sigma-Aldrich, catalog number: I9278, store at 4°C)
18. N-2 Supplement (100×) (Thermo Fisher Scientific, Gibco, catalog number: 17502001, aliquots of 500 µl, store at -20°C)
19. B-27™ Supplement (50×), minus vitamin A (Thermo Fisher Scientific, Gibco, catalog number: 12587010, aliquots of 500 µl, store at -20°C)
20. B-27™ Supplement (50×), serum-free (Thermo Fisher Scientific, Gibco, catalog number: 17504044, aliquots of 500 µl, store at -20°C)
21. Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix (Corning, catalog number: 356231, aliquots of 500 µl, store at -20°C)
22. D-PBS<sup>-/-</sup>, 1×, without calcium and magnesium (Wisent Bioproducts, catalog number: 311-425-CL, store at 4°C)
23. Embryoid Body Media (EB Media) (see Recipes)
24. Neural Induction Media (see Recipes)
25. Cerebral Organoid Differentiation Media without Vitamin A (CDM-A) (see Recipes)
26. Cerebral Organoid Differentiation Media with Vitamin A (CDM+A) (see Recipes)

## **Equipment**

1. Nalgene™ Square PETG media bottles (250 ml) (Gibco, Thermo Fisher Scientific, catalog number: 2019-0250)
2. Pipettes (5 ml, 10 ml, 25 ml, 50 ml), micro-pipettes (10 µl, 20 µl, 200 µl, 1,000 µl)
3. Multi-channel pipette (Eppendorf, model: Research plus 12 channel pipette, catalog number: ES-12-300)
4. Water bath

5. Centrifuge
6. Phase contrast microscope
7. Hemocytometer
8. 37°C, 5% CO<sub>2</sub> cell culture incubator
9. Orbital shaker that can be installed inside the incubator (such as Thermo Fisher, catalog number: 88881101)
10. Blade and scalpel

## **Procedure**

### **A. Embryoid Body Production: Singularizing and Plating Human Embryonic Stem Cells (hESCs)**

1. Prepare EB Media without growth factors and prewarm to 37°C using a water bath. EB Media (without growth factors) can be stored for up to 1 week at 4°C, and once warmed, the stock should be used or discarded.
2. Wash hESCs, which have reached 70–80% confluence, twice with 3 volumes of D-PBS<sup>-/-</sup>.  
*Note: This protocol was established using hESCs cultured in mouse embryonic fibroblast (MEF) conditioned media supplemented with bFGF to a final concentration of 4 ng/ml (Sivitilli et al., 2020).*
3. Add 1 ml TrypLE and incubate for 5 min at room temperature (22°C).
4. Gently pipette up and down to dissociate colonies to a single-cell suspension.
5. Neutralize TrypLE with 4 volumes (*i.e.*, 4 ml) EB media and add the resulting suspension to a 15-ml Falcon tube.
6. Use a hemocytometer to count the cells. Avoid using an automated cell counter, which can result in inaccurate cell numbers because hESCs are prone to clumping.
7. Centrifuge cells at 150 × *g* for 5 min at room temperature (22°C).
8. During centrifugation, prepare EB Media by supplementing with 4 ng/ml bFGF and 50 μM Y-27632 (ROCK inhibitor). For this, use 15 ml EB media with 1.5 μl bFGF (from a 40 ng/μl stock) and 7.5 μl Y-27632 (from a 100 mM stock).
9. Resuspend single hESCs to a final concentration of 80,000 cells/ml in the freshly prepared EB Media supplemented with bFGF and Y-27632.
10. Transfer the resuspended cells to a sterile disposable reagent reservoir and add 150 μl cell suspension (12,000 cells) to each well of a 96-well V-bottomed non-binding plate using a multi-channel pipette.
11. Place the plate at 37°C in a CO<sub>2</sub> incubator for 2 days.

### **B. Embryoid Body Production: Feeding**

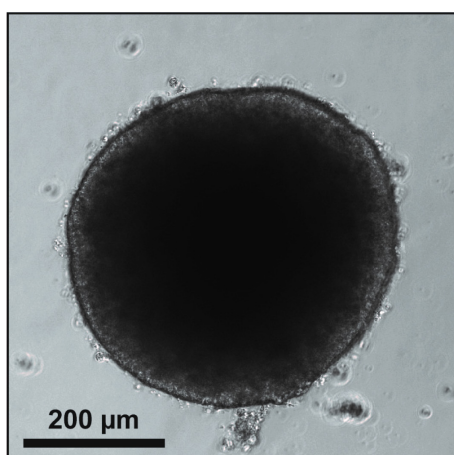
1. Prepare EB Media fresh daily and prewarm to 37°C using a water bath.
2. On day 2, feed the EBs with EB media supplemented with 2 ng/ml bFGF. To reach the required concentration, mix 15 ml EB media with 0.75 μl bFGF (40 ng/μl).

3. Remove 135  $\mu$ l media from each well of the incubated 96-well plate using the multi-channel pipette.
4. Transfer the EB media (supplemented with 2 ng/ml bFGF) into a sterile disposable reagent reservoir and add 150  $\mu$ l EB media using the multi-channel pipette.
5. Return the plate to the CO<sub>2</sub> incubator for a further 2 days.

*Note: Due to evaporation, wells toward the peripheral edge of the 96-well plate may have a slightly lower volume. This is normal and EBs should still form correctly.*

#### C. Embryoid Body Production: Assessment Checkpoint

On day 5, measure the EB size using a brightfield microscope to determine whether the EBs are ready for neural induction (Figure 2). If the size of the EBs is 350–600  $\mu$ m, proceed to Procedure D (Neural Induction); if the size is below 350  $\mu$ m, then repeat Procedure B; if the size is above 600  $\mu$ m, we do not recommend continuing with the protocol. Restart the protocol and double check cell counting in Procedure A to ensure accuracy when seeding EBs and start another batch.



**Figure 2. Representative EB derived from H9 hESCs on Day 5**

#### D. Neural Induction: Transfer to Neural Induction

1. Prepare Neural Induction Media fresh for each differentiation and prewarm an aliquot to 37°C using a water bath. Neural Induction Media can be stored for 1 week at 4°C. Once warmed, Neural Induction Media should not be reused.
2. Choose EBs that meet the size and visual criteria (Figure 2).
3. Cut 2–3 mm off the extremity of a 200- $\mu$ l filtered pipette tip using a razor blade to make a wider opening.
4. Prepare a 24-well ultra-low attachment plate by adding 500  $\mu$ l prewarmed Neural Induction Media to each well.
5. Using a dissecting scope and a cut 200  $\mu$ l pipette tip, pick up the EB and transfer to a 24-well ultra-low attachment plate. Set the pipette to 20  $\mu$ l, place the end at the bottom of the well and

pipette up the EB. A maximum of 2 EBs per well can be added; additional EBs increase the likelihood of further aggregation.

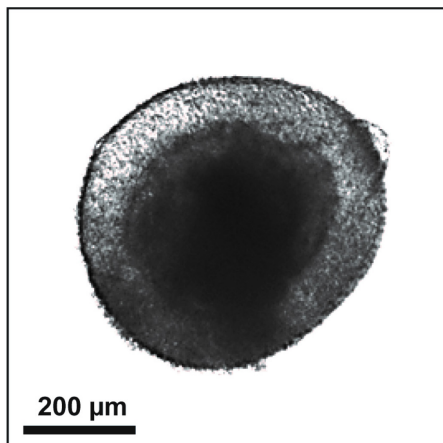
6. Place the plate in a CO<sub>2</sub> incubator for 2 days.

#### E. Neural Induction: Feeding

1. Prewarm Neural Induction Media to 37°C using a water bath.
2. Feed the EBs by adding an additional 500 µl Neural Induction Media to each well.
3. Return the plate to a CO<sub>2</sub> incubator for a further 2 days.

#### F. Neural Induction: Assessment Checkpoint

1. Use a brightfield microscope to examine the neuralized EBs for optical clearing in the outer 100 µm of the EB (Figure 3). The outer ring structure should be prominent and marked by a stark change in opacity as compared with the central region of the EB. This is a critical checkpoint; EBs that do not have the correct organization of the neuroepithelial ring will not form organoids.



**Figure 3. Neuroectoderm formation in an EB derived from H9 hESCs at Day 9**

#### G. Neuroepithelial Expansion: Transfer to Solid Phase Matrigel Scaffold

1. Thaw Growth Factor Reduced Matrigel on ice for 2 h. Once thawed, Matrigel can be used for up to 6 h but must be kept on ice. Matrigel cannot be refrozen once thawed.
2. Prepare CO Differentiation Media without Vitamin A (CDM-A) fresh for each differentiation and prewarm an aliquot to 37°C using a water bath. CDM-A can be stored for 2 weeks at 4°C. Once warmed, CDM-A should not be reused.
3. Prepare a box of wide-bore tips by cutting 3–4 mm off the extremity of a 200 µl filtered pipette tip using a razor blade to make a wider opening. Pick up the EBs one by one using a 200 µl cut tip and transfer to the center of each well of the 4-well plate (1 EB per well).
4. Using an uncut 200 µl tip, draw 25 µl to aspirate the remaining Neural Induction Media from each well. It is critical to remove all media from the EB in the 4-well plate; failure to do so will

prevent the Matrigel from attaching to the plate surface and prevent removal of the Matrigel in Procedure J.

5. Cover the neuralized EB with a drop of cold Matrigel (30  $\mu$ l) using an uncut 200  $\mu$ l tip.
6. Position the EB in the center of the Matrigel droplet using a 10  $\mu$ l tip. Ensure that the EB is not resting on the bottom of the plate.
7. Once a plate has been filled (4 EBs), transfer the full plate to a CO<sub>2</sub> incubator for 10 min at 37°C to polymerize the Matrigel.
8. After 10 min, carefully add 500  $\mu$ l prewarmed CDM-A and return to a CO<sub>2</sub> incubator for a further 2 days.

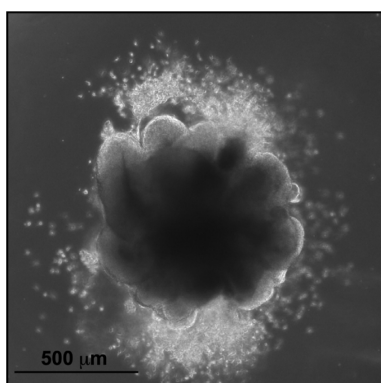
*Note: Be consistent with the amount of media collected when transferring the EB. We recommend collecting EBs with 25  $\mu$ l media to create a droplet in the 4-well plates.*

#### H. Neuroepithelial Expansion: Feeding

1. Prewarm an aliquot of CDM-A to 37°C using a water bath.
2. Carefully aspirate all media and add 500  $\mu$ l CDM-A to each well of the 4-well plate.
3. Return the plate to the incubator for a further 2 days.

#### I. Neuroepithelial Expansion: Assessment of Checkpoint

1. Use a brightfield microscope to examine the COs for ring structures in the peripheral regions of the organoid (Figure 4). COs should be approximately 500–700  $\mu$ m in diameter and have multiple ring structures throughout; this is a critical checkpoint, those without ring structures will not form organoids.



**Figure 4. Representative hCOs derived from H9 hESCs on day 13 prior to Matrigel extraction**

#### J. Transfer to Spinning Culture: Extraction from Solid Phase Matrigel

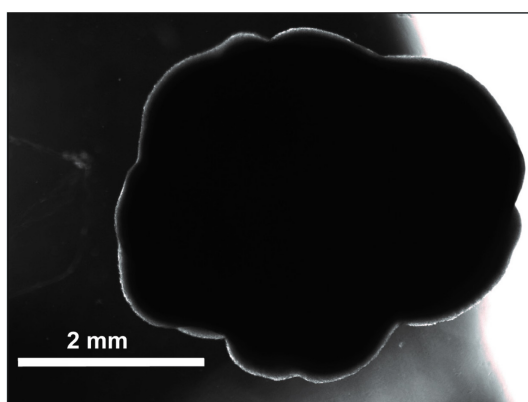
1. Prepare CO Differentiation Media (CDM) fresh and prewarm an aliquot to 37°C using a water bath. CDM can be stored for 2 weeks at 4°C. Once warmed, CDM should not be reused.
2. Prepare wide-bore pipette tips by cutting 4–5 mm off the extremity of 200  $\mu$ l pipette tips to make a wider opening.



3. Prepare a 6-well plate with 3 ml prewarmed CDM per well.
4. Using a dissection microscope, carefully cut around the COs implanted in the Matrigel droplet; the goal here is to remove as much Matrigel as possible without damaging the CO. Excess Matrigel remaining on the CO at this stage will result in formation of cyst-like structures in the spinning culture.
5. Use a cut 200  $\mu$ l tip to transfer the COs to the 6-well plate containing CDM; a maximum of 4 hCOs per well is acceptable.
6. Once all the COs have been transferred, place the 6-well plate on an orbital shaker (ThermoFisher Cat: 88881101) in a 37°C incubator at a speed of 80–90 rpm (rpm may vary if a different orbital shaker model is used).

K. Transfer to Spinning Culture: Feeding in Spinning Culture

1. Prewarm CDM using a 37°C water bath.
2. Pause the orbital shaker and collect the plate containing the COs.
3. Carefully aspirate 2/3 of the total media volume from each well; be mindful not to aspirate the COs with the media since they are in suspension.
4. Add 3 ml prewarmed CDM to each well.
5. Return the plate to the orbital shaker in the incubator and initialize (Figure 5).



**Figure 5. Representative hCO in a spinning culture derived from H9 hESCs at 12 weeks**



## Recipes

### 1. Embryoid Body Media (EB Media) (Table 1)

**Table 1. EB Media Composition.** A list of components, stock and final concentrations (Con.), and volumes for working stocks are indicated.

Components	Stock Con.	Final Con.	/50 ml medium
DMEM/F12			39.5 ml
KOSR			10 ml
MEM:NEAA	100×	1×	500 $\mu$ l
2-mercaptoethanol	100×	1×	50 $\mu$ l

### 2. Neural Induction Media (prepared fresh every week) (Table 2)

**Table 2. Composition of Neural Induction Media (prepared fresh every week).** Once warmed, Neural Induction Media should not be reused. A list of components, stock and final concentrations (Con.), and volumes for working stocks are indicated.

Components	Stock Con.	Final Con.	/50 ml medium
DMEM/F12			48.5 ml
GlutaMax	100×	1×	500 $\mu$ l
MEM:NEAA	100×	1×	500 $\mu$ l
N2	100×	1×	500 $\mu$ l
Heparin	10 mg/ml	0.001 mg/ml	5 $\mu$ l

### 3. Cerebral Organoid Differentiation Media without Vitamin A (CDM-A) (Table 3)

**Table 3. Composition of Cerebral Organoid Differentiation Media without Vitamin A.** A list of components, stock and final concentrations (Con.), and volumes for working stocks are indicated.

Components	Stock Con.	Final Con.	/50 ml medium
DMEM/F12			24 ml
Neural Basal Media			24 ml
MEM:NEAA	100×	0.5×	250 $\mu$ l
GlutaMax	100×	1×	500 $\mu$ l
B27-Vit A	50×	0.5×	500 $\mu$ l
N2	100×	0.5×	250 $\mu$ l
2-mercaptoethanol	1,000×	1×	50 $\mu$ l
Insulin	9.5–11.5 mg/ml	2–3 $\mu$ g/ml	12.5 $\mu$ l

#### 4. Cerebral Organoid Differentiation Media with Vitamin A (CDM+A) (Table 4)

**Table 4. Composition of Cerebral Organoid Differentiation Media with Vitamin A.** A list of components, stock and final concentrations (Con.), and volumes for working stocks are indicated.

Components	Stock Con.	Final Con.	/100 ml medium
<b>DMEM/F12</b>			48 ml
<b>Neural Basal Media</b>			48 ml
<b>MEM:NEAA</b>	100×	0.5×	500 µl
<b>GlutaMax</b>	100×	1×	1 ml
<b>B27 (with Vit A)</b>	50×	0.5×	1 ml
<b>N2</b>	100×	0.5×	500 µl
<b>2-mercaptoethanol</b>	1,000×	1×	100 µl
<b>Insulin</b>	9.5–11.5 mg/ml	2–3 µg/ml	25 µl

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#### Competing interests

The authors declare that they have no conflicts of interest.

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