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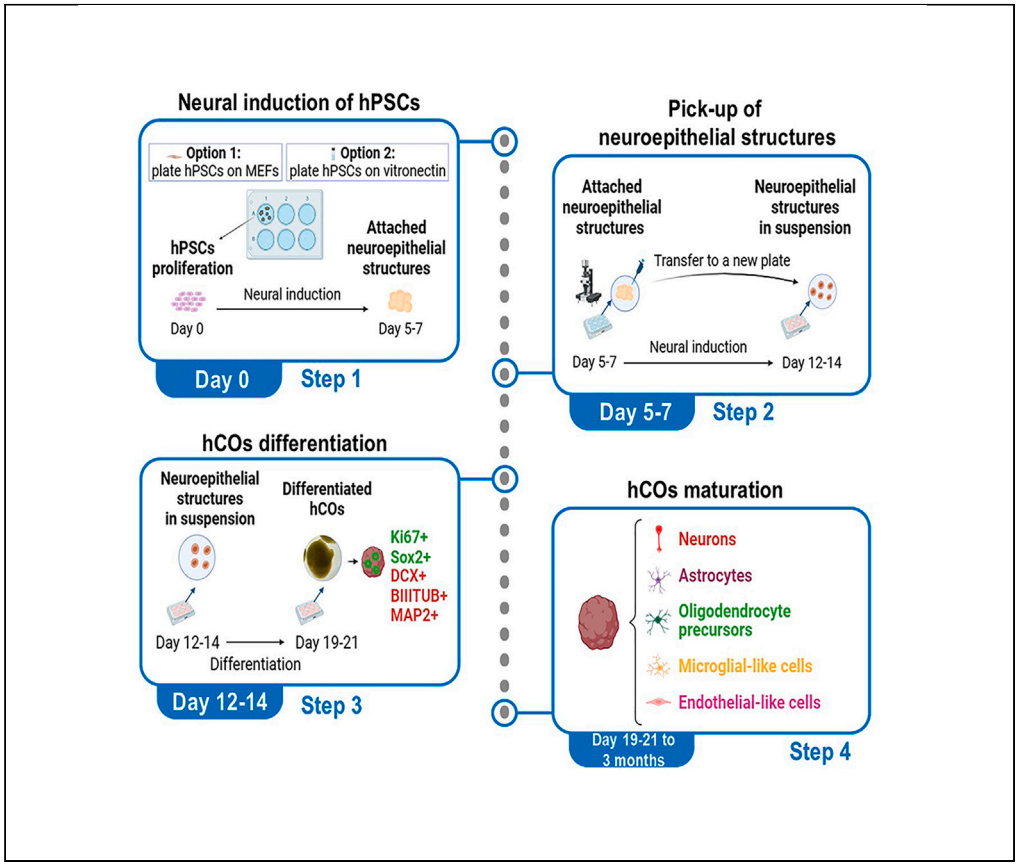
Protocol for generating human cerebral organoids from two-dimensional cultures of pluripotent stem cells bypassing embryoid body aggregation



Human cerebral organoids (hCOs) provide an excellent model for the study of human brain development and disease. Here, we present a protocol to obtain hCOs directly from two-dimensional (2D) pluripotent stem cell (PSC) cultures, avoiding cell dissociation and posterior embryoid body (EB) aggregation. We describe steps for subjecting 2D cultures to a neural fate and subsequently developing hCOs. We then detail the evaluation of different cellular types.

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Highlights

Steps for generating hCOs from 2D colonies of PSCs bypassing EB aggregation

Instructions for highly efficient neuroepithelial induction

Guidance on the characterization of hCOs with cellular diversity

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Protocol for generating human cerebral organoids from two-dimensional cultures of pluripotent stem cells bypassing embryoid body aggregation

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SUMMARY

Human cerebral organoids (hCOs) provide an excellent model for the study of human brain development and disease. Here, we present a protocol to obtain hCOs directly from two-dimensional (2D) pluripotent stem cell (PSC) cultures, avoiding cell dissociation and posterior embryoid body (EB) aggregation. We describe steps for subjecting 2D cultures to a neural fate and subsequently developing hCOs. We then detail the evaluation of different cellular types.

For complete details on the use and execution of this protocol, please refer to González-Sastre et al.[1]

BEFORE YOU BEGIN

Before carrying out any experiment, it is necessary to obtain all required institutional permissions and approvals. The work conducted in the present study was approved by the ISCIII Ethics Committee (Ref. CEI-PI93-2020, CEI-PI76_2023 and CEI-PI77_2023) and then authorized by the Spanish National Committee of Guarantees for the Use and Derivation of Human Cells and Tissues.

Since the first human cerebral organoid (hCO) was generated[2,3] many efforts have been made in order to refine the protocols. Among the different strategies carried out, it is worth highlighting the non-guided protocols and the guided protocols. The former produce organoids that generate different brain regions spontaneously[2–4] while the latter employ different patterning factors in order to generate organoids from a specific brain region.[5–11]

In this work, we describe a protocol for the generation of hCOs directly from two-dimensional (2D) colonies of pluripotent stem cells (PSC)[1] using certain factors during neural induction. This is an efficient protocol as almost 100% of the neuroepithelial structures generated give rise to hCOs. It is also a simple protocol since we avoid cell dissociation and aggregation of embryoid bodies (EBs), and it does not require embedding in matrigel. hCOs development is robust since they present several

ventricular zones (VZs) containing different types of progenitors which give rise to mature neurons and glial cells. Moreover, we detected a variety of cell types in the hCOs including oligodendrocyte precursors, astrocytes, microglia-like cells and endothelial-like cells.

We provide a comprehensive list of materials and required equipment in the key resources table. The below protocol describes the generation of hCOs directly from two-dimensional PSC culture. We use both embryonic stem cells (ESCs) (AND-2) and induced pluripotent stem cells (iPSCs) (IC-Ctrl1-F-iPS-4F-1 and IC-Ctrl2-F-iPS-4F-1) with comparable results.

Institutional permissions

The work conducted in the present study was approved by the ISCIII Ethics Committee (Ref. CEI-PI93-2020, CEI-PI76_2023 and CEI-PI77_2023) and then authorized by the Spanish National Committee of Guarantees for the Use and Derivation of Human Cells and Tissues.

Media preparation

Timing: 15–30 min

For all the media described below see materials and equipment.

1. This protocol can be carried out following two different approaches for human PSC (hPSC) culture.
   a. Option 1: Grow hPSCs over MEFs. Prepare MEFs medium, MEFs freezing medium and hESC medium.
   b. Option 2: Grow hPSCs over vitronectin. Prepare Essential 8 medium.
2. The following steps concerning hCOs generation require three different media: neural induction (NI) medium, differentiation medium and maintenance medium.

   Note: Avoid long-term storage at 4°C. For NI medium, differentiation medium and maintenance medium, we recommend first preparing the N2 medium and the neurobasal medium and adding freshly the different compounds required for each medium.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit monoclonal anti-Ki67 clone SP6 (1:250)	Thermo Fisher Scientific (Invitrogen)	Cat#MA5-14520; RRID:AB_10979488
Rabbit polyclonal anti-Oct3/4 (1:300)	Santa Cruz Biotechnology	Cat#SC-9081; RRID:AB_2167703
Rabbit polyclonal anti-Sox2 (1:1000)	Merck (Millipore)	Cat#AB5603; RRID:AB_2286686
Goat polyclonal anti-Doublecortin (DCX) clone C-18 (1:100)	Santa Cruz Biotechnology	Cat#SC-8066; RRID:AB_2088494
Mouse monoclonal anti-β-III-Tubulin clone TUJ1 (1:500)	BioLegend	Cat#801202; RRID:AB_2313773
Mouse monoclonal anti-MAP2 clone HM-2 (1:200)	Merck (Sigma-Aldrich)	Cat#M4403; RRID:AB_477193
Rabbit polyclonal anti-Synapsin 1 (Syn1) (1:200)	Merck (Millipore)	Cat#AB1543; RRID:AB_2200400
Mouse monoclonal anti-GFAP clone 4A11 (1:1000)	BD Biosciences (BD Pharmingen)	Cat#556327; RRID:AB_396365
Rabbit monoclonal anti-S100β clone EP1576Y (1:100)	Abcam	Cat#AB52642; RRID:AB_882426
Rabbit polyclonal anti-IBA-1 (1:400)	FUJIFILM Wako Pure Chemical Corporation	Cat#019-19741; RRID:AB_839504
Mouse monoclonal anti-CNPase clone 11-5B (1:200)	Merck (Millipore)	Cat#MAB326; RRID:AB_2082608
Rabbit monoclonal anti-VEGFR2 (KDR) clone 55B11 (1:100)	Cell Signaling Technology	Cat#2479; RRID:AB_2212507
Rat monoclonal anti-CD31 clone MEC 13.3 (1:50)	BD Biosciences (BD Pharmingen)	Cat#553370
Donkey polyclonal anti-rabbit Alexa Fluor 448 (1:500)	Thermo Fisher Scientific (Invitrogen)	Cat#A21206; RRID:AB_2535792
Donkey polyclonal anti-rabbit Alexa Fluor 555 (1:400)	Thermo Fisher Scientific (Invitrogen)	Cat#A31572; RRID: AB_162543

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Donkey polyclonal anti-mouse Alexa Fluor 448 (1:500)	Thermo Fisher Scientific (Invitrogen)	Cat#A21202; RRID:AB_141607
Donkey polyclonal anti-mouse Alexa Fluor 555 (1:400)	Thermo Fisher Scientific (Invitrogen)	Cat#A31570; RRID:AB_2536180
Donkey polyclonal anti-goat Alexa Fluor 555 (1:400)	Thermo Fisher Scientific (Invitrogen)	Cat#A21432; RRID:AB_2535853
Goat polyclonal anti-rat Alexa Fluor 555 (1:400)	Thermo Fisher Scientific (Invitrogen)	Cat#A21434; RRID:AB_2535855
Chemicals, peptides, and recombinant proteins		
Hoechst 33258 (1:500)	Thermo Fisher Scientific (Invitrogen)	Cat#H3569; RRID:AB_2651133
KnockOut DMEM	Thermo Fisher Scientific (Gibco)	Cat#10829-018
KnockOut serum replacement	Thermo Fisher Scientific (Gibco)	Cat#10828-028
L-glutamine	Corning	Cat#25-005-CI
MEM non-essential amino acids 100×	Thermo Fisher Scientific (Gibco)	Cat#11140-050
Penicillin/streptomycin	Corning	Cat#30-002-CI
2-mercaptoethanol	Thermo Fisher Scientific (Gibco)	Cat#M6250
Fibroblast growth factor 2	Thermo Fisher Scientific (PeproTech)	Cat#AF-100-18B
Y-27632 dihydrochloride	Biogen (Tocris)	Cat#129830-38-2
DMEM:F12 with GlutaMAX	Thermo Fisher Scientific (Gibco)	Cat#31331-028
D-glucose	Merck	Cat#104074
N-2 supplement (100×)	Thermo Fisher Scientific (Gibco)	Cat#17502-048
AlbuMAX	Thermo Fisher Scientific (Gibco)	Cat#11020-021
HEPES (1 M)	Thermo Fisher Scientific (Gibco)	Cat#15630-056
Neurobasal medium	Thermo Fisher Scientific (Gibco)	Cat#21103-049
B27 supplement without vitamin A (50×)	Thermo Fisher Scientific (Gibco)	Cat#12587-010
B27 supplement with vitamin A (50×)	Thermo Fisher Scientific (Gibco)	Cat#17504-044
L-ascorbic acid	Merck (Sigma-Aldrich)	Cat#A4544
SB-431542	Biogen (Tocris)	Cat#301836-41-9
Noggin	Thermo Fisher Scientific (PeproTech)	Cat#120-10C
CHIR99021	Biogen (Tocris)	Cat#252917-06-9
Insulin solution human	Merck (Sigma-Aldrich) or Gibco (Thermo Fisher Scientific)	Cat#I9278 or Cat#12585-014
Mitomycin C	Merck (Sigma-Aldrich)	Cat#M0503
DPBS 10×	Corning	Cat#20-031-CV
Vitronectin	STEMCELL Technologies	Cat#07180
Dimethyl sulfoxide	Merck (Sigma-Aldrich)	Cat#D2438
Fetal bovine serum	Thermo Fisher Scientific (Gibco)	Cat#10437-028
DMEM	Corning	Cat#10-013-CV
Essential 8 basal medium	Thermo Fisher Scientific (Gibco)	Cat#A1517001
Essential 8 supplement	Thermo Fisher Scientific (Gibco)	Cat#A1517001
CTS PSC cryomedium	Thermo Fisher Scientific (Gibco)	Cat# A4238801
Experimental models: Cell lines		
Human iPSCs (IC-Ctrl1-F-iPS-4F-1 and IC-Ctrl2-F-iPS-4F-1)	Banco Nacional de Líneas Celulares of Instituto de Salud Carlos III	RRID: CVCL_UN04 and RRID: CVCL_UN05 https://www.isciii.es/servicios/biobancos/banco-nacional-lineas-celulares
Human ESCs (AND-2)	Banco Nacional de Líneas Celulares of Instituto de Salud Carlos III	RRID: CVCL_B527 https://www.isciii.es/servicios/biobancos/banco-nacional-lineas-celulares
MEFs (E13, E14)	N/A	N/A
Software and algorithms		
Adobe Photoshop CS6	N/A	N/A
Leica LAS v.4.0	N/A	N/A
LAS X 3.7.4.23463	N/A	N/A
CellChat	R package	https://github.com/sqjin/CellChat
BioRender	BioRender	www.biorender.com
Other		
BB15 CO_2 incubator	Thermo Fisher Scientific	Cat#51023126
Biological safety cabinet	Telstar Bio II Advance Plus	N/A

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
6-well tissue culture plate	Falcon (Corning)	Cat#353046
Ultra-low attachment 6-well plates	Corning	Cat#3471

MATERIALS AND EQUIPMENT

MEFs Medium

Reagent	Final concentration	Amount
DMEM	N/A	492.5 mL
FBS	10%	50 mL
L-Glutamine	2 mM	5 mL
Penicillin/Streptomycin	0.5×	2.5 mL
Total	N/A	**550 mL**

Store at 4°C for up to 2 weeks.

MEFs Freezing Medium

Reagent	Final concentration	Amount
MEFs Medium	N/A	40 mL
FBS	10%	5 mL
Dimethyl Sulfoxide	10%	5 mL
Total	N/A	**50 mL**

Store at 4°C for up to 2 weeks.

hESC Medium

Reagent	Final concentration	Amount
Knockout DMEM	N/A	463.3 mL
KnockOut Serum Replacement	20%	120 mL
L-Glutamine	2 mM	6 mL
Non-Essential Amino Acids	1×	6 mL
Penicillin/Streptomycin	0.5×	3 mL
2-Mercaptoethanol	50 μM	1.2 mL
Fibroblast Growth Factor 2	10 ng/mL	300 μL
Y-27632 dihydrochloride	5 μM	200 μL
Total	N/A	**600 mL**

Store at 4°C for up to 2 weeks.

Essential 8 Supplement Medium

Reagent	Final concentration	Amount
Essential 8 Basal Medium	N/A	98 mL
Essential 8 Supplement	1×	2 mL
Total	N/A	**100 mL**

Store at 4°C for up to 2 weeks.

N2 Medium

Reagent	Final concentration	Amount
DMEM:F12 with GlutaMAX	N/A	468.5 mL
D-Glucose	6 mg/mL	10 mL

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Continued		
Reagent	Final concentration	Amount
N2 Supplement	1×	5 mL
AlbuMAX	2.6 mg/mL	6.5 mL
HEPES	5 mM	2.5 mL
Penicillin/Streptomycin	1×	2.5 mL
Non-Essential Amino Acids	1×	5 mL
Total	N/A	**500 mL**
Store at 4°C for up to 2 weeks.		

Neurobasal Medium without Vitamin A		
Reagent	Final concentration	Amount
Neurobasal	N/A	97 mL
B27 Supplement without Vitamin A	1×	2 mL
L-Glutamine	2 mM	1 mL
Total	N/A	**100 mL**
Store at 4°C for up to 2 weeks.		

Neurobasal Medium with Vitamin A		
Reagent	Final concentration	Amount
Neurobasal	N/A	97 mL
B27 Supplement with Vitamin A	1×	2 mL
L-Glutamine	2 mM	1 mL
Total	N/A	**100 mL**
Store at 4°C for up to 2 weeks.		

Neural Induction Medium		
Reagent	Final concentration	Amount
N2 Medium	N/A	50 mL
Neurobasal Medium without Vitamin A	N/A	50 mL
L-Ascorbic Acid	0.1 mM	100 µL
SB-431542	10 µM	100 µL
Noggin	50 ng/mL	50 µL
CHIR99021	3–6 µM*	15–30 µL*
Total	N/A	**100 mL**
Store at 4°C for up to 2 weeks.		

Alternatives: It is possible to use a range from 3 to 6 µM CHIR, therefore it would be convenient to perform a CHIR dose-response experiment for each cell line used.

Neural Differentiation Medium		
Reagent	Final concentration	Amount
N2 Medium	N/A	50 mL
Neurobasal Medium without Vitamin A	N/A	50 mL
Insulin	1.3 µg/mL	26.6 µL
Total	N/A	**100 mL**
Store at 4°C for up to 2 weeks.		

Neural Maintenance Medium		
Reagent	Final concentration	Amount
N2 Medium	N/A	50 mL
Neurobasal Medium with Vitamin A	N/A	50 mL
Insulin	1.3 μg/mL	26.6 μL
Total	N/A	**100 mL**
Store at 4°C for up to 2 weeks.		

STEP-BY-STEP METHOD DETAILS

Neural induction directly from adherent hPSC colonies

Timing: 5–7 days

Before initiating the neural induction (See Figure 1A), culture hPSCs on MEFs or vitronectin (both options have similar results), using the following steps.

1. Plate over MEFs: mitotically inactivate MEFs by 12-h treatment with 1 μg/mL mitomycin C under standard conditions.
   a. Plate at a density of approximately 4000-5000 cells/cm^2 per well of a 6-well plate in MEFs medium.
2. Plate in vitronectin: dilute vitronectin for coating at a ratio of 1:50 in PBS1× (final concentration vitronectin 5 μg/mL).
   a. Add 750 μL of vitronectin diluted in PBS1× per well of a 6-well plate.
   b. Treat plates with vitronectin for 1-h at 18°C–24°C.

   Note: Plates can be used after removal of the vitronectin.

3. Plate the hPSCs on MEFs in hESC medium or on vitronectin in E8 medium.

   Note: Plate the PSCs as cell aggregates, plating too many cells will likely result in colonies that are too big or too numerous, which will hinder proper differentiation. Allow the PSCs to proliferate until they reach a maximum confluence of 80%

   Note: Transfer colonies from one well of the 6-well plate to 6 wells of a new 6-well plate using a 1000 μL pipette with standard 1000 μL tips. No enzymes are required for this process.

4. Maintain hPSCs in hESC medium or E8 medium until isolated colonies present small-medium size (200–400 μm diameter) and approximately 20–40% of confluency.
5. Colony assessment.
   a. Check the hPSC colonies using a bright-field microscope (e.g., See Figure 1B).
   b. Ensure that the colonies show no signs of cell differentiation or cell death. Optimal colonies should not be too large and present delimited edges. Cells should display large nucleus and minimal cytoplasm and should express the cell cycle marker Ki67 and the pluripotent markers Oct4 and Sox2 (Figure 1C).
6. Prepare NI medium by combining the reagents listed in the recipe.

   Note: Different concentrations of CHIR (vehicle = DMSO) were tested on the colonies. Only with the 3 μM concentration the colonies formed neuroepithelial structures. It is important to note that this final concentration will depend on the experimental cell type. It is possible to use a range from 3 to 6 μM, therefore it would be convenient to perform a CHIR dose-response experiment for each cell line used.

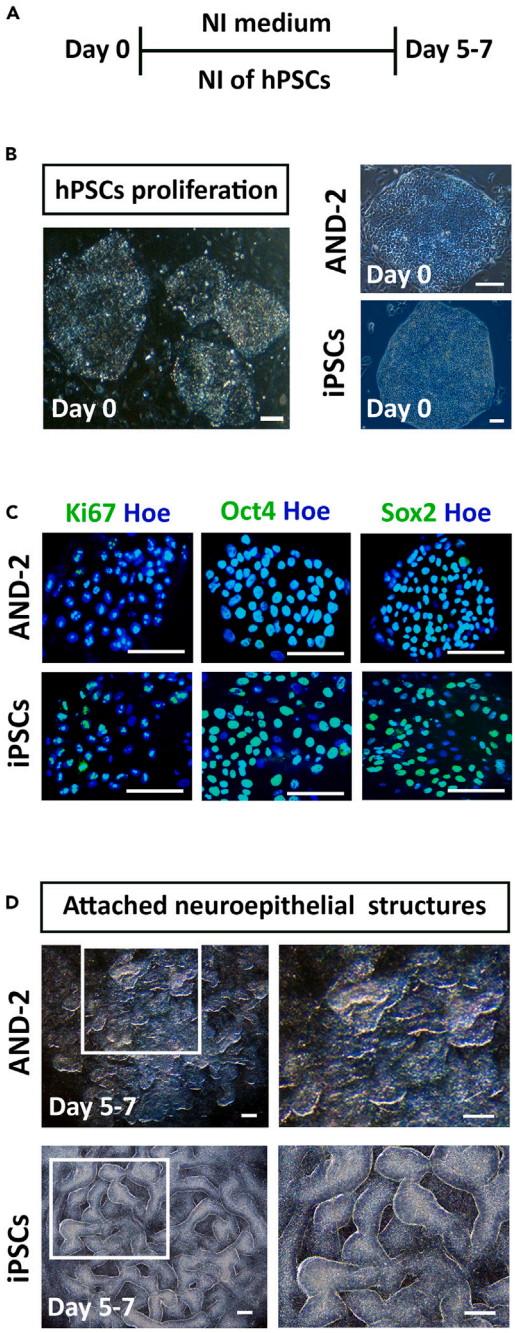

Figure 1. Characterization of hPSCs and formation of neuroepithelial structures

(A) Schematic diagram presenting the timeline and media used to generate 3D neuroepithelial structures from 2D hPSC colonies.

(B) Representative bright-field images showing hPSC colonies of proliferating hESCs (AND-2 cell line) and hiPSCs. Scale bar = 100 μm.

(C) Immunocytochemistry staining of hPSC colonies labeled with Ki67 (cell cycle marker), Oct4 and Sox2 (pluripotent markers) (green). Cell nuclei were identified by Hoechst 33258 staining (blue). Scale bar = 100 μm.

(D) Representative bright-field images showing 3D attached neuroepithelial structures (day 5-7). White box indicates zoomed image. Scale bar = 100 μm. NI, Neural Induction; hPSC, human Pluripotent Stem Cell; iPSC, induced Pluripotent Stem Cell; Hoe, Höechst.

7. Remove the hESC or E8 medium from the plate with hPSCs and add 3 mL of NI medium (day 0) to each well of a 6-well plate.
8. Renew NI medium each day until approximately day 5-7.

Note: Colonies begin to differentiate towards neural fate around day 2-3, producing tissue remodeling. About day 5-7 they form 3D neuroepithelial structures (Figure 1D), however it will depend on the experimental cell type used.

Pickup of the neuroepithelial structures

Timing: 5–7 days

In this phase of the protocol, it is crucial to select the correct neuroepithelial structures in order to obtain suitable hCOs.

9. Collect proper neuroepithelial structures using a 200 μL pipette (using normal pipette tips structures detach easily, enzyme use is not required) and transfer them into a new 6-well plate (Figure 2A). Neuroepithelial structures look like continuous layers of cells as shown in Figure 2B.

Note: For this protocol, neuroepithelial structures were transferred to an ultralow-adherence plate but normal plates can be also valid, however, we recommend checking daily that the structures do not adhere to the plate.

Note: After collecting the neuroepithelial structures for the first time, add new NI medium to the well and allow new neuroepithelial structures to form. This process can be repeated until the culture is exhausted or begins to show signs of senescence.

Note: Pay special attention in the case of neuroepithelial structures generated from iPSCs, select them as soon as possible, it is not advisable to leave them in culture for more than 1–2 days after they appear.

⚠ CRITICAL: Neuroepithelial colonies could start being senescent or mis-differentiated colonies could derive into non-neural colonies or colonies with 2D rosettes, avoid all these undesired tissues which will not give rise to hCOs (Figure 2C).

10. Maintain the neuroepithelial structures in NI medium for 5 days more, until they acquire a rounded shape and present dense nuclei with optically translucent areas along the entire periphery (neuroepithelial layer) (Figure 2D).

⚠ CRITICAL: At this point it is important to discard those structures that do not present neuroepithelium, are fused or do not show smooth edges (Figure 2E).

Note: With this protocol 97% of the structures formed present the neuroepithelium. This protocol bypasses EB formation, avoiding dissociation and aggregation steps, which facilitates manipulation and avoids cell disruption. We did not use Matrigel either, which facilitates the subsequent maintenance and medium change of the organoids, all these modifications greatly simplify the methodology.

Differentiation of the hCOs

Timing: 5–7 days

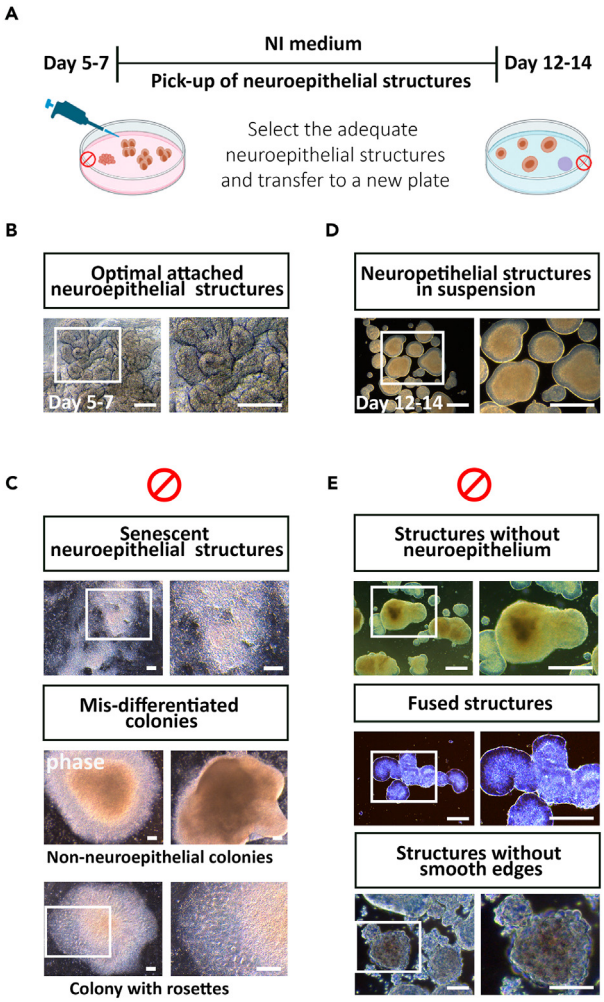

Figure 2. Selection and transfer of attached neuroepithelial structures to obtain neuroepithelial structures in suspension

Created with BioRender.com.

(A) Schematic diagram presenting the timeline and media used to obtain neuroepithelial structures in suspension and graphical representation of the structures picking up.

(B) Representative bright-field images showing optimal attached 3D neuroepithelial structures. White box indicates zoomed image. Scale bar = 100 μm.

(C) Examples of bright-field images showing structures that should be avoided ranging from senescent neuroepithelial structures to mis-differentiated structures that form undesired lineages such as non-neural colonies or colonies with 2D rosettes. White box indicates zoomed image. Scale bar = 100 μm.

(D) Representative bright-field images showing optimal neuroepithelial structures in suspension, presenting peripheral neuroepithelium. White box indicates zoomed image. Scale bar = 100 μm.

(E) Examples of bright-field images showing structures that should be avoided ranging from structures without neuroepithelium, fused structures or structures without smooth edges. Scale bar = 100 μm. NI, Neural Induction.

This phase of the protocol gives rise to differentiated hCOs in which neural precursors will form the ventricular zone (VZ) and later differentiate into mature neurons.

11. Change NI medium to differentiation medium (Figure 3A).

> *Note:* During this stage hCOs increase significantly in size, we recommend leaving approximately 10 hCOs per well of a 6-well plate. If this recommendation is followed, change the medium every 1–2 days.

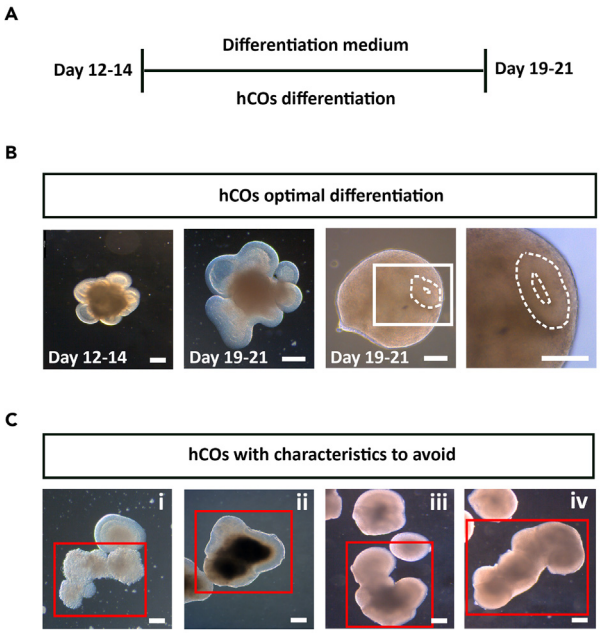

Figure 3. Differentiation of hCOs

(A) Schematic diagram presenting the timeline and media used to obtain differentiated hCOs.

(B) Representative bright-field images showing the characteristics of hCOs at day 12-14 presenting several neuroepithelial buds and morphology change at day 19-21 (approximately, some hCOs may still show buds by this age) where they become more rounded and present VZs (marked with white dashed lines). White box indicates zoomed image. Scale bar = 200 μm.

(C) hCOs with un-desired characteristics (red boxes) that will probably end up in cell death, ranging from hCOs without smoothed edges (i), hCOs with darker central zones (ii), too large hCOs(iii) or fused hCOs (iv). Scale bar = 200 μm. hCOs, human cerebral organoids.

12. Allow hCOs to grow and become more rounded and compact (Figure 3B).

Note: Usually, clear zones with rounded or oval shapes may be observed in phase contrast (white dashed lines in Figure 3B). These VZs formed by neural precursors indicate a correct differentiation.

13. During this phase hCOs tend to aggregate, it is possible to gently pipette these aggregates to separate hCOs or cut with a needle.

⚠ CRITICAL: At this point it is important to discard those hCOs without optimal characteristics like hCOs without smooth edges (Figure 3Ci), hCOs with darker central zones (Figure 3Cii), too large hCOs (Figure 3Ciii) or fused hCOs (Figure 3Civ).

Maturation of the hCOs

Timing: Up to 3 months

This last phase of the protocol allows hCOs to be maintained in culture for 3 months approximately, at this point hCOs keep growing and more neurons are maturated. In addition, with this protocol it is possible to obtain other cell types present in the human brain such as oligodendrocyte precursors, astrocytes, microglia-like cells and endothelial-like cells.

14. Change differentiation medium to maintenance medium at day 19–21 (Figure 4A).

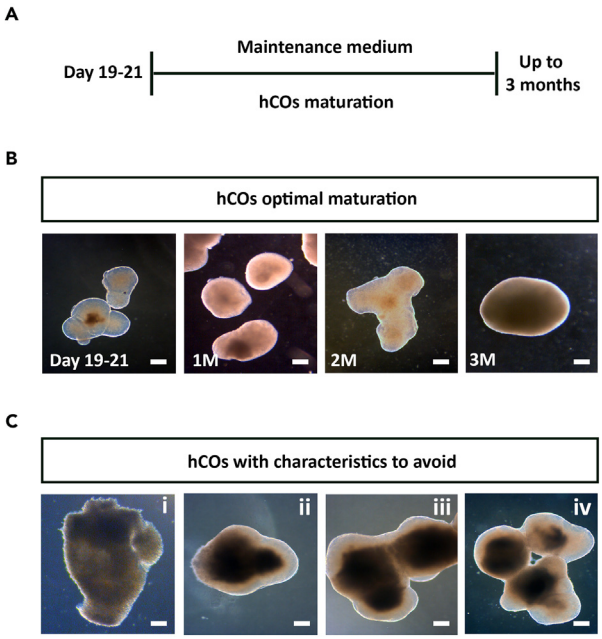

Figure 4. Maturation of hCOs

(A) Schematic diagram presenting the timeline and media used to obtain mature hCOs.

(B) Representative bright-field images showing the characteristics of hCOs at different times during maturation (day 19-21, 1 month (1M), 2 months (2M) and 3 months (3M)). Scale bar = 200 μm.

(C) hCOs with un-desired characteristics that will probably end up in cell death, ranging from hCOs without smoothed edges (i), hCOs with darker central zones (ii), too large hCOs (iii) or fused hCOs (iv). Scale bar = 200 μm. hCOs, human cerebral organoids.

Note: At this point, clear VZs may not be observed since hCOs present higher cell density.

15. The correct maintenance of hCOs requires checking the optimal characteristics and changing medium very often (every 1–2 days) (Figure 4B).

Note: Pay particular attention to the color of the medium during this stage. When the hCOs reach a larger size, the medium containing the pH indicator phenol red will turn yellowish, indicating medium acidification, and the medium should be changed more frequently. It is also possible to leave a smaller number of hCOs per well of a 6-well plate.

16. During this phase hCOs tend to aggregate, it is possible to gently pipette these aggregates to separate hCOs or cut with needle.

⚠ CRITICAL: At this point it is important to discard those hCOs without optimal characteristics like hCOs without smooth edges (Figure 4Ci), hCOs with darker central zones (Figure 4Cii), too large hCOs (Figure 4Ciii) or fused hCOs (Figure 4Civ).

EXPECTED OUTCOMES

The main outcome of the protocol described is the efficient production of hCOs directly from 2D cultures of PSCs. hCOs are an enormously valuable model for studying brain development and the pathologies that affect it. This protocol not only avoids the traditional steps of dissociation and aggregation of the PSCs to form embryoid bodies, but also increases the efficiency of hCOs production, being close to 100%. To achieve a direct neural induction (NI) of the PSC colonies, they were subjected to a NI medium enriched with different factors that promote dual-SMAD inhibition in combination with the addition of CHIR99021. This protocol also turns out to be simple and highly

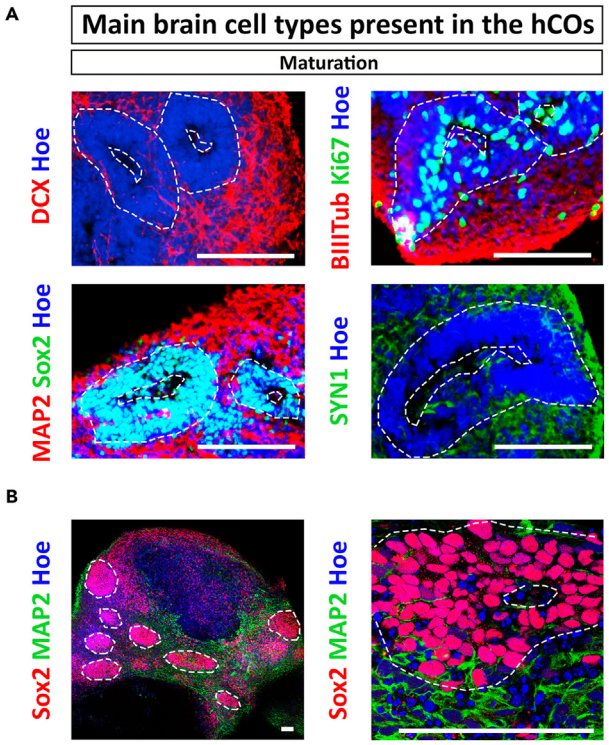

Figure 5. Characterization of the main cell types in the hCOs at maturation

(A) hCOs immunostained for cell cycle marker Ki67 (green) and neural precursor marker Sox2 (green), different neuronal markers (DCX, βIIITubulin and MAP2) (red) and synaptic marker (SYN1) (green). Cell nuclei were identified by Höechst 33258 staining (blue). VZs are marked with white dashed lines. Scale bar = 100 μm.

(B) Whole-mount immunohistochemistry of hCOs stained for Sox2 (red) and MAP2 (green). Cell nuclei were identified by Höechst 33258 staining (blue). VZs are marked with white dashed lines. Scale bar = 100 μm. hCOs, human cerebral organoids.

reproducible since hCOs generated present multiple VZs with neural precursors that give rise to mature neurons and glial cells. Interestingly, the hCOs also present the main brain cell types, which is of great importance in the field to better mimic the human brain *in vivo*.

These hCOs present VZs which stain positive for cell cycle marker (Ki67) and radial glia marker (Sox2) as soon as day 19-21, however these zones reach their maximum size by 2 months of maturation (Figure 5A). Growing radially outwards from these VZs are neurons of varying degrees of maturation which stained for migrating neuroblasts marker (doublecortin+; DCX+), differentiated neurons marker (βIIITubulin), mature neurons marker (MAP2; microtubule associated protein 2) and for the synaptic vesicle marker (Synapsin I; SYN1), showing that neurogenesis and neuronal differentiation are taking place during hCOs development. Additionally, whole-mount 3D reconstruction shows that each hCO presents several VZs which proves that the protocol is robust (Figure 5B).

Another important outcome of this protocol is that hCOs contain additional cell types present in the human brain (Figure 6A) such as shown by positive staining of the astrocyte marker GFAP (glial fibrillary acidic protein) and S100β (S100 protein subunit beta) detected at 2 months of maturation (Figure 6B). At 1 month of maturation there was presence of positive cells for the oligodendrocyte marker CNPase (2′,3′-cyclic nucleotide 3′-phosphodiesterase) (Figure 6C) and IBA1+ microglial-like cells (Figure 6D).

Further details on RT-qPCR analysis for genes associated with these cell types can be found in González-Sastre et al.[1] hCOs showed increased expression of *OLIG2* (oligodendrocyte transcription

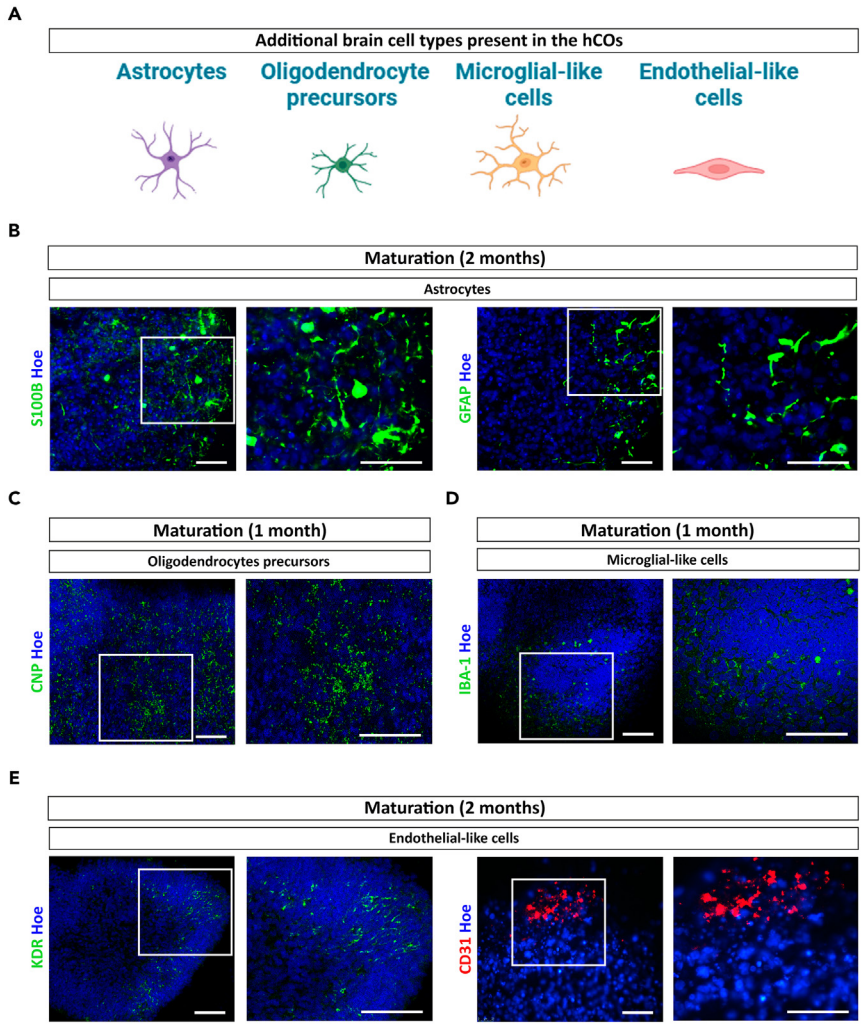

Figure 6. Characterization of additional cell types in the hCOs

(A) Graphical representation of the additional brain cell types present in the hCOs.

(B) hCOs immunostained for astrocytes markers S100β and GFAP (green) at 2 months of maturation. Cell nuclei were identified by Höechst 33258 staining (blue). White box indicates zoomed image. Scale bar = 100 μm.

(C) hCOs immunostained for oligodendrocyte precursor marker CNP (green) at 1 month of maturation. Cell nuclei were identified by Höechst 33258 staining (blue). White box indicates zoomed image. Scale bar = 100 μm.

(D) hCOs immunostained for microglial-like cell marker IBA1 (green) at 1 month of maturation. Cell nuclei were identified by Höechst 33258 staining (blue). White box indicates zoomed image. Scale bar = 100 μm.

(E) hCOs immunostained for endothelial-like cell marker KDR (green) and CD31 (red) at 2 months of maturation. Cell nuclei were identified by Höechst 33258 staining (blue). White box indicates zoomed image. Scale bar = 100 μm.

hCOs, human cerebral organoids.

factor 2) and *NG2* (neuron-glial antigen 2, also known as chondroitin sulfate proteoglycan 4, *CSPG4*) during differentiation stage, which subsequently decreased during hCOs maturation. The expression of *PDGFRA* (platelet-derived growth factor alpha receptor) declined over time in hCOs across both differentiation and maturation stages, suggesting that OPCs are progressing towards a more mature lineage. To further assess this, we analyzed the gene expression of *O4* (marker of pre-oligodendrocytes), *GALC* and *PLP1* (markers of mature oligodendrocytes), all of which showed an increased expression during differentiation stage and 1 month of hCOs maturation.

Additionally, we investigated the expression of myelin-related proteins associated with myelinating oligodendrocytes such as *MOG* (myelin oligodendrocyte glycoprotein) and *MBP* (myelin basic

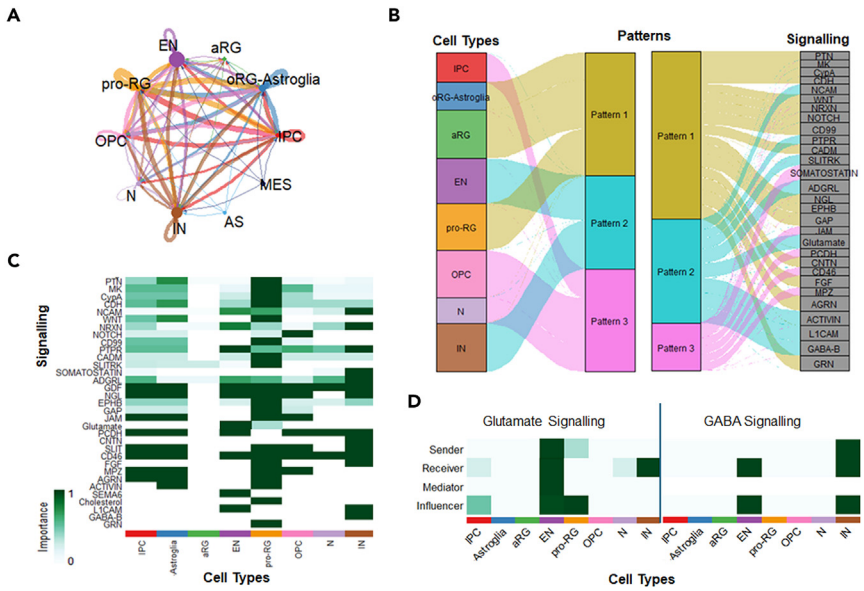

Figure 7. Functional heterogeneity found in the cell types of the hCOs

The communication networks were inferred with CellChat and the scRNAseq data of hCOs generated from AND-2 with 60 days in culture.

(A) Network showing the global cell communications are represented by circles with sizes proportional to the number of cells in each cluster, and with edges where colors represent the signaling pathways and the width the communication probability. The loop indicates the autocrine pathway of the cell cluster.

(B) The alluvial plot shows how each cell type coordinates with certain patterns of signaling pathways to respond to signals.

(C) Heatplot showing the correspondence between cell type and the signaling pathways to drive signals. The thickness of the flow indicates the contribution of the cell group to each signaling pathway.

(D) Heatmap showing the relative importance of each cell type as sender, receiver, mediator or influencer in the glutamate and GABA signaling pathways. EN, Excitatory Neurons; IN, Interneurons, N, Neurons, OPC, Oligodendrocyte Precursors Cells; AS, Astrocytes; oRG, outer Radial Glia; proRG, proliferative Radial Glia; MES, Mesodermal; aRG, apical Radial Glia; IPC, Intermediate Precursor Cells.

protein) both of which exhibited a progressive increase over time in culture, with significant differences at 2 months of hCOs maturation. The gene encoding for IBA-1, *AIF1* (allograft inflammatory factor 1) increased its expression at 1 month of maturation. Furthermore, hCOs expressed *CX3CR1* (microglia marker), *P2RY12* (marker strongly expressed in resting microglia) and *CD68* (marker strongly upregulated in reactive microglia) during neural induction, differentiation and maturation stages. Ultrastructural characterization, as detailed in Mateos-Martínez et al.,[12] confirmed the presence of astrocytes, oligodendrocyte precursors, and microglia-like cells.

Finally, we detected the presence of positive cells for the vascular endothelial growth factor receptor 2 (VEGFR2, also known as KDR) and CD31 (Figure 6E) at 2 months of maturation.

Further information for the cells found in the hCOs can be found in González-Sastre et al.[1] and in Mateos-Martínez et al.[12] from the scRNA-seq data deposited at National Center for Biotechnology Information Gene Expression Omnibus (GEO) with the numbers GEO: GSE242329, GSE266667. Although extensive analyses of the scRNAseq results from this protocol, are reported previously, we present in Figure 7 the analysis done with the computational tool CellChat[13] to infer and analyze cell-cell communication networks from the single-cell transcriptomic data of hCOs with 60 days in culture (GEO: GSE242329) to uncover the coordinated responses among the different cell types found in the hCOs.

LIMITATIONS

One of the limitations of hCOs is that they cannot be kept in culture long enough to match the development time of the brain. However, although this protocol does not make use of bioreactors or agitation, our methodology is fully compatible with these approximations in order to maintain organoids in culture for longer periods of time. Currently, another major limitation in the field is to obtain all the cell types present in the brain. Although with the protocol described here it is possible to find astrocytes, oligodendrocyte precursors, microglia-like cells and endothelial-like cells, representation of these cell types needs to be encouraged. To this end, we believe that our protocol is compatible with the use of factors to promote these cell lineages. Another approach could be the co-culture of hCOs with the desired cell type.

TROUBLESHOOTING

Problem 1

PSC culture can be made following two different approaches (related to step 1).

Potential solution

Plate PSC over MEFs or over vitronectin.

Problem 2

PSC colonies should present 20–40% confluency and present optimal characteristics (related to step 5).

Potential solution

Check the confluence of the colonies, size and morphology using a bright-field microscope. Identify colonies with no signs of differentiation or cell death. If there are still problems identifying proliferating and non-differentiated colonies, perform an immunocytochemistry assay for cell cycle marker Ki67 and pluripotent marker Oct4 and Sox2. If any colony starts to differentiate, it is possible to aspirate it in order to eliminate it, leaving only the optimal colonies.

Problem 3

Generation of neuroepithelial structures from PSCs will depend on the experimental cell line used (related to step 6).

Potential solution

Perform a CHIR dose-respond experiment for each cell line used and determine which is the optimal CHIR concentration.

Problem 4

Selection of non-appropriate neuroepithelial structures (related to step 9).

Potential solution

Identify those structures which will not develop hCOs. Use a bright-field microscope and avoid misdifferentiated colonies or senescent colonies as shown in Figure 2.

Problem 5

Maintenance of structures that will not give rise to hCOs (related to step 10).

Potential solution

Using a bright-field microscope and avoid structures without neuroepithelium, structures without smooth edged or fused structures as shown in Figure 3.

Problem 6

Fusion of hCOs or maintenance of non-optimal hCOs that will end up in cell death (related to step 13 and 16).

Potential solution

Gently pipette the aggregates of hCOs or cut them with a needle until they are separated. Using a bright-field microscope, discard those hCOs without smooth edges, darker central zones, too large or fused hCOs as shown in Figures 3 and 4.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Isabel Liste (iliste@isciii.es).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Rosa González-Sastre (rosa.gsastre@isciii.es).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate new coding data.

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AUTHOR CONTRIBUTIONS

R.G.-S. performed the experiments, analyzed and interpreted data, and generated the figures. R.G.-S., R.C., P.M.-M., and L.M. generated the hCOs. B.A. generated the MEFs. L.M. generated the tables. I.L. supervised hCOs generation, designed the study, and interpreted the results. V.L.-A. performed the analysis and interpreted the scRNA-seq data. R.G.-S., R.C., P.M.-M., E.L.-B., A.L., V.L.-A., and I.L. wrote the manuscript in consultation with all authors. All authors provided feedback in editing the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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