EDDU Protocols

Generation and profiling of Forebrain Spheroids

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Version 1.0

EDDU-015-01
May 2020

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1 Introduction

1.1 Objectives

This protocol describes how to generate forebrain spheroids from iPSCs. Forebrain spheroids are formed in stationery cultures, they contain cortical neurons and astrocytes and can be maintained in cultures for several months allowing the study of neuronal progenitors and mature cells. Thus, Forebrain spheroids provide a 3D model to study cortical development and brain activity.

1.2 Protocol overview

By using a combination of small molecules that regulate multiple signalling pathways, we adapted an earlier method (Pasca et al., 2015) to guide human induced pluripotent stem cells (iPSCs) into forming forebrain spheroids in stationary cultures.

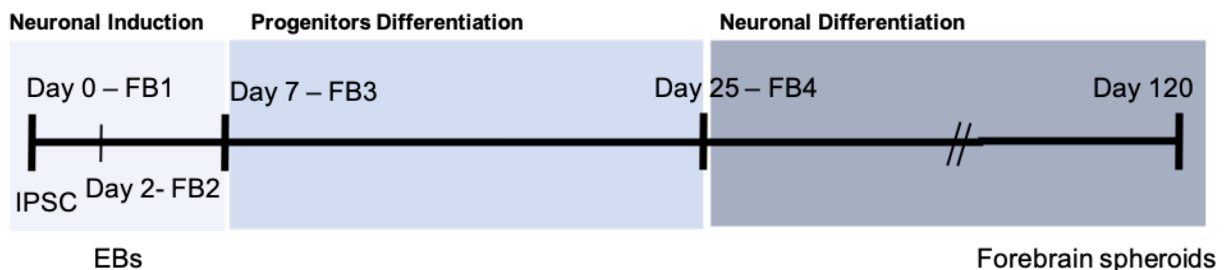

Figure 1. Protocol overview of Forebrain spheroid generation. iPSCs are cultivated under low attachment conditions to allow the formation of 3D structures called embryoid bodies (EBs). After several days in culture using different media (FB1. FB2, FB3 and FB4, please see Table 4) at specific time points forebrain spheroids are obtained and can be maintained for several months.

1.3 Technical and safety considerations

The following information should be read before starting:

- iPSCs and forebrain spheroids must be handled within a Class II biosafety laminar flow hood to protect the worker from possible adventitious agents. Appropriate Environmental Health and Safety (EHS) office regulations must be followed at all times.
- The quality of materials used in this protocol is critical to its success. Refer to the Materials section for recommended suppliers and catalogue numbers.
- Work with one cell line at a time to avoid errors and having to leave cells unattended for too long during procedures.
  It is advised to use freshly prepared media for every step of the procedure. In case of excess media preparation, the media can be stored at 4°C and used up to 7 days later.
- All cut tips used to transfer spheroids must be autoclaved.
- iPSC cultures must be monitored regularly for sterility, optimal morphology, spontaneous differentiation and density prior to spheroid generation. For additional information please check Troubleshooting section.

2 Materials

Refer to the product datasheet from the supplier for further details on storage and preparation instructions.

2.1 Labware

Item	Supplier	Catalogue #
100-mm culture dishes	Thermo-Fisher	08772E
100-mm petri dishes	Thermo-Fisher	FB0875712
10-mL plastic serological pipets	Sarstedt	86.1254.001
12-mm glass coverslips	Thermo-Fisher	12-545-80
12-well culture plates	Thermo-Fisher	0877229
15-mL conical tubes	Thermo-Fisher	352097
1-mL plastic serological pipets	Fisher	13-678-11B
5-mL plastic serological pipets	Sarstedt	86.1253.001
60-mm culture dishes	Eppendorf	0030701119
6-well culture plates (low attachment)	Corning co-start	CLS3471
96-well culture plates (low attachment U-bottom)	Corning	CLS7007
Centrifuge	Eppendorf 5702	022626001
Cryovials	Sarstedt	72.379
Polypropylene microcentrifuge tubes	Fisher	02-681-273

2.2 Culture reagents

Item	Supplier	Catalogue #	Stock conc.	Working conc.	Storage temp.
Antibiotic-Antimycotic	Gibco	15240-062	100x	1x	Stock: –20°C Working: 4°C
B27 without vitamin A	Gibco	12587010	50x	1x	Stock: –80°C Working: Room temperature
BDNF	Peprotech	450-02	20 µg/ml	20 ng/ml	Stock: –80°C Working: 4°C

Item	Supplier	Catalogue #	Stock conc.	Working conc.	Storage temp.
2-Mercaptoethanol	Gibco	21985023	55mM	0.1nM	4°C
DMEM/F12	Gibco	10565042	1x	1x	4°C
DMSO	Fisher	BP231-1	100%	10%	Room temperature
Dorsomorphin	Tocris	3093-10mg	10 mM	10uM	
EGF	Peprotech	AF-100-15	10 µg/ml	10 ng/ml	Stock: –80°C Working: 4°C
FBS	Gibco	12484-028	1x	1x	Stock: –80°C Working: Room temperature
FGF-b	Peprotech	100-18B	10 µg/ml	10 ng/ml	Stock: –80°C Working: 4°C
Gentle Cell Dissociation Reagent	Stem Cell Technologies	07174	1X	1X	Room temperature
GlutaMax	Gibco				
Knockout Serum (KOSR)	Gibco	10828028	100%	20%	Stock: –20°C Working: Room temperature
MEM nonessential amino acid (NEAA) solution	Wisent	321-011-EL	100x	1x	4°C
Neurobasal (NB) media	Life Technologies	21103-049	1x	1x	4°C
NT3					
PBS	Wisent	311-010-CL	1x	1x	Room temperature
PFA	Fisher Scientific	50-980-487	16%	4%	Room temperature
Pen/Strep	Wisent	450-200-EL	100x	1x	Stock: –80°C Working: 4°C
SB-431542	Selleckchem	S1067	10 mM	10uM	Stock: –80°C Working: 4°C
Sucrose	Fisher	BP-220-1		20%	Room temperature
Y-27632 (ROCK inhibitor)	Selleckchem	S1049	10 mM	10 µM	Stock: –80°C Working: 4°C

2.3 Equipment

Item	Supplier	Catalogue #
Cell culture incubator	ThermoScientific	Steri Cycle Mod370 Ref#20
Cell culture water bath	FisherScientific	IsoTemp GPD20
Centrifuge	Eppendorf	5702
Light microscope	Motic	AE2000
Cell counter	Logos Biosystems	Luna-II Automated cell counter
Luna cell counter slide	Logos Biosystems	#05181401

3 Protocol

3.1 Generation of Forebrain Organoids

Materials: refer to **section above on culture reagents.**

- Media:

Table 1. Forebrain generation media

Day	0	2-6	7-25	26
Stage	**Making EBs**	**Neuronal induction**	**Progenitors differentiation**	**Neuronal differentiation**
Media	**FB1**	**FB2**	**FB3**	**FB4**
Main medium	DMEM/F12	DMEM/F12	Neurobasal	Neurobasal
Serum	20% KOSR	20% KOSR	-	-
Supplement	-	-	B27 without vitamin A 50x	B27 without vitamin A 50x
Rock inhibitor	Y-27632 20 µM (500x)	-	-	-
GlutaMax	GlutaMax (100x)	GlutaMax (100x)	GlutaMax (100x)	GlutaMax (100x)
MEM-NEAA	MEM-NEAA (100x)	MEM-NEAA (100x)		
2-mercapto	2-mercapto 0.1 nM	2-mercapto 0.1 nM		
Antibiotics	Anti-Anti (100x)	Anti-Anti (100x)	Pen/Strep (100x)	Pen/Strep (100x)
Inhibitors		Dorsomorphin 10 µM (2000x)		
		SB-431542 10µM (1000x)		
Factors			FGF-b 20 ng/mL (1000x)	BDNF 20 ng/mL (1000x)
			EGF 20 ng/mL (1000x)	NT3 20 ng/mL (500x)

Procedure:

1. **iPSC Culture:** iPSCs cultures must be monitored regularly to ensure sterility, optimal morphology, spontaneous differentiation and density prior to forebrain spheroid generation (**Figure 2A-C**). We recommend mycoplasma testing once a month and before making frozen stocks. Also, make sure to passage them twice after thawing, and remove spontaneous differentiation (**Figure 2C**) as much as possible before starting your experiment. Please refer to our iPSC Culture SOP for detailed information on this topic: EDDU-002-02.

2. **Day 0 - EB Preparation:** The first step in the protocol consists of generating embryoid bodies (EBs) from iPSCs, a process that normally begins within 24h of seeding. EBs should present well delimited outer membrane (**Figure 2 E and F**).

Option A: Growth of EBs in 6 well dishes

- Grow iPSCs in 10cm plates (**Figure 2B**). Make sure to passage them twice after thawing and remove spontaneous differentiation as much as possible.
- Once 70% confluency is reached, detach cells with 3mL of gentle cell dissociation reagent for 7-10 minutes at 37°C.
- Collect cell suspension into a 15mL centrifuge tube and wash 10 cm plate with 5mL of prewarmed DMEM-F12 media twice to collect remaining cells.
- Centrifuge cells at 1,200 rpm for 3 minutes.
- Resuspend cells in prewarmed DMEM-F12 media and count the total cell number.
- Plate 10^6 cells per well in low attachment 6-well plates. Add 4mL of FB1 media 1 per well (**Table 3**).

Option B: Growth of EBs in 96 well dishes

In the 6 well plate format, 2 million cells are added into one well and EBs will be generated from random quantities of cells. EBs of different sizes will thus be formed. In contrast, with 96 well dishes, in each well a defined number of cells is added followed by a centrifugation to collect the cells in the bottom of each well. Cells will subsequently start to cluster, leading to the formation of one EB. If homogenous size in your spheroids is required, it is recommended to generate EBs in 96 well plates with. Spheroids generated in 96 wells can however be smaller than the spheroids produced when you seed cells directly onto 6-well plates, which can affect the number of cells present in your spheroids at the end point of your experiment. However, steps involved in the development and maintenance of the EBs in the 96 well dishes are the same as described above for the 6 well plates

- You will need 10^6 cells in 10 mL FB1 media for one 96-well plate. Thus, prepare your cell solution of 10,000 cells per 100 µl per well in fresh FB1 media. This method is preferred if you require homogeneous EBs and for more homogenous spheroid size.
- Plate 100 µl of cell suspension per well using a multichannel pipet, centrifuge at 200 g for 10 minutes at RT and incubate at 37°C. Alternatively, if automation is available, seeding can be performed in an automated manner.
- Check that cells are clustered in the center of the well **(Figure 2D).**

3. **Day 1 - Check EB formation:** The first step of the protocol consists of generating EBs from iPSCs and this process normally occurs within 24h. EBs should present with a well delineated outer membrane (**Figure 2 E and F**). If no EBs form within 24h, longer periods of time (48h-72h) can be used but this will depend on the cell line and in some cases EBs may simply not form indicating issues with the cells or reagents used. Make sure to use good quality iPSC culture (lower amount of spontaneous differentiation and that are at least 2 passages post thawing) with fresh media and consider testing higher concentrations of ROCK Inhibitor (500x instead of 1000x).

4. **Day 3-6 - Neuronal induction:** The first week of this protocol is critical for optimal spheroid generation. Make sure to follow the steps and plan accordingly so that media changes are done at the appropriate time points. To ensure optimal cell survival, use fresh media that is prewarmed to room temperature prior to manipulation.

   - Change half of the conditioned media to Forebrain media 2 (FB2 media - see table above) every other day. To do so, tilt plate around a 30-40° angle to let EBs collect in corner of each well, allowing half the media to be aspirated carefully from the top of the well to avoid aspirating the EBs. Add the same volume of FB2 media back into each well that was removed.

5. **Day 7-24 - Progenitors differentiation:** After the first week, spheroids can be passed through a cell strainer (40μm) to wash off cells that did not form EBs. It is easier to observe any spheroid shedding of cell death with clean media (**Figure 2F**). A healthy spheroid has a continuous well delineated outer membrane with a dense darker interior. Budding can be observed around Day 10 (**Figure 2 and 3D**) and is no longer observed after Day 25. If spheroids present with a discontinuous membrane with blebbing and growth, remove the unhealthy spheroids from culture (**Figure 2 G and F**).

   - Change half of the conditioned media to Forebrain media 3 (FB3 media- see table above) every other day in the first 5 days, then every 4 days after that. To do so, tilt plate 30-40° angle to let spheres fall and aspirate half of the volume from top of the well to avoid aspirating the forebrains. Add the same volume of FB3 media.

6. **Day 25 - Neuronal differentiation:**

   - Change half of the conditioned media to Forebrain media 4 (FB4 media- see table above and **Figure 1**) every other day in the first 5 days. At Day 30, forebrain organoids that are generated in 96-well plates can be transferred to low attachment 6-well plates using cut tips autoclaved. Add 4mL of FB4 media per well. Change half of the conditioned media every 4 days. In order to do so, tilt plate around 30-40° angle to let spheres fall, and using a multichannel pipet, aspirate 2mL of media from the top of the well to avoid aspirating the forebrains. Add 2mL of FB4 media. Plates are placed back in the incubator after the transfer.

Note: It is normal to observe fusion of multiple spheres in stationary cultures (**Figure 2**). If this is a problem to the desired analysis, spheroids can be transferred to 12 well plates, or 96 well formats should be implemented (check alternative procedure at **Step 1**).

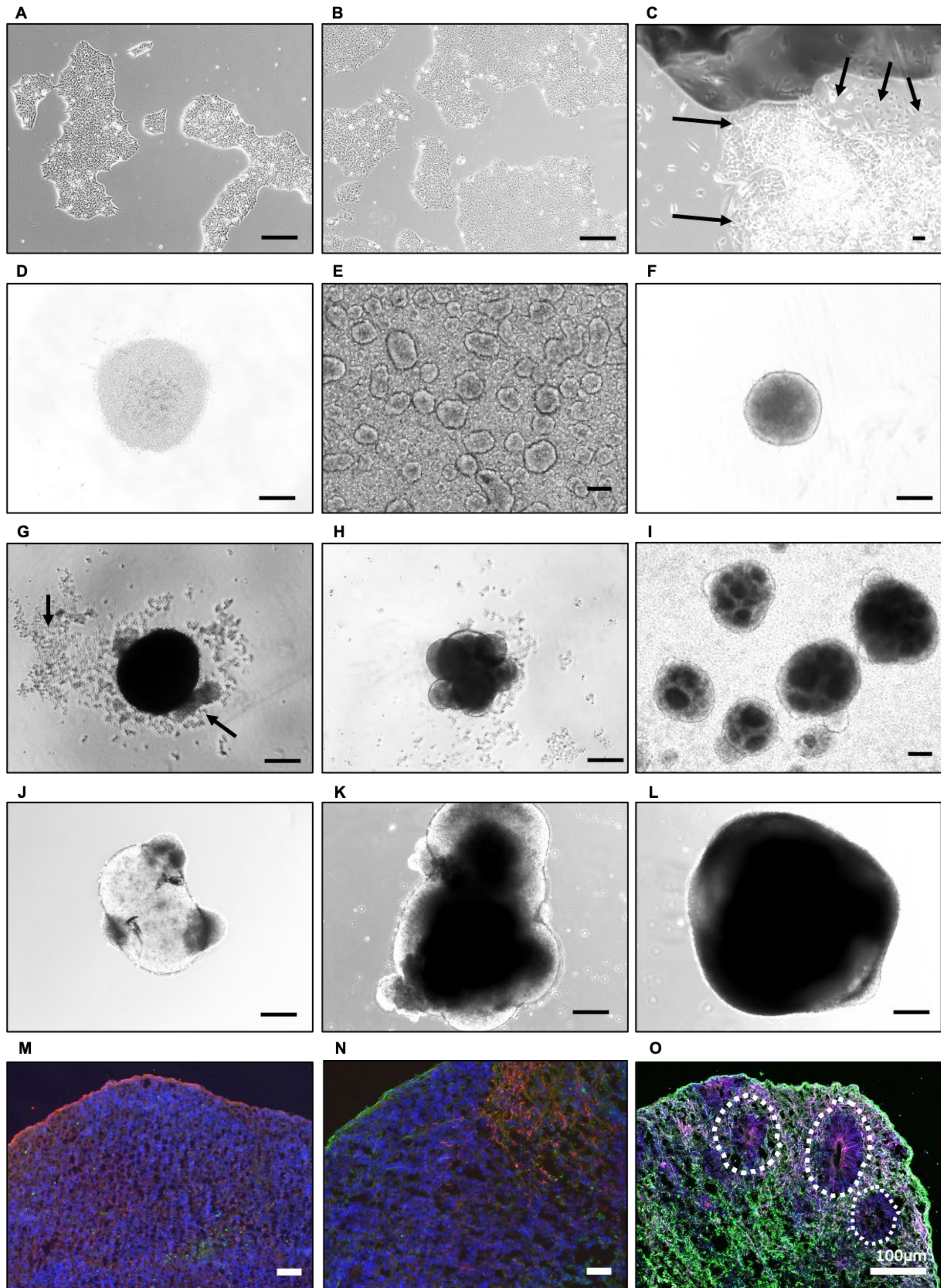

Figure 2. Forebrain spheroids generation. Images represent iPSC culture (A-C) and forebrain spheroids at different stages of development (D-L). High quality iPSC culture is required for forebrain spheroid obtention, cells must be healthy with well-defined clusters (A and B). Spontaneous differentiation can be removed from culture but if undesired cells are still observed (arrows in C) culture must not be used for forebrain spheroid production. The first step to form forebrain spheroids is to form EBs (E and F). At day 1, EBs can usually be observed (E). Outer membrane should be well defined (F, Day 3). When iPSCs are plated into 96 well plates, one cluster of cells should be obtained after centrifugation (D, Day 0). As cultures grow older, forebrain spheroids grow significantly in size and different shapes are then observed. A healthy culture has very low amounts of cells that are not incorporated into the EBs (G and H). In G, lots of cells are detected and that might reflect low quality of iPSC culture. It is recommended to start with healthier batch. In H, EB is not a single sphere presenting budding. As forebrain organoid grow in 6 well plates some spheroids might fuse (I-K, day 13-15). This is not an issue but if spheroids contain a clear content this is a sign of poor development (J). Overall, healthy forebrain organoids present well-delimited outer membrane and dense content (I and L, days 13 and 30). Scale bars for A-L are 200µm. Different cell types can be detected by immunofluorescence, images show presence of astrocytes (M) and neurons (N and O). In M, we observe GFAP (astrocyte marker, green) and Tuj1 (neuronal marker, red) positive cells in 60 days-old forebrain spheroids. Confocal images of neuronal markers (Tuj1 in green and MAP2 in magenta) in 60 days-old forebrain spheroids showing corticogenesis in ventricular-like zones delimited by dashed circles in white. Scale bar for M-O is 100µm.

3.2 Cryopreservation and Cryosectioning of Forebrain Spheroids

Materials: OCT, PBS, PFA 4% and sucrose 20%.

Procedure:

1. Gently collect forebrain spheroids using a cut tip and transfer them to 15 mL conical tube. Maximum of 5 spheres per tube.
2. Let forebrain spheroids sink to the bottom of the tube and aspirate media with pipette without touching the spheres.
3. Add 10 mL of PBS to wash off remaining media. Do not centrifuge.
4. Inside a chemical hood, remove PBS and add 5 mL of PFA 4% and incubate for 1h at RT.
5. Remove PFA solution and dispose accordingly.
6. Wash forebrain spheroids twice with 10 mL of PBS for 5 minutes per wash.
7. Add 10 mL of 20% sucrose (in 1x PBS) and incubate spheres at 4°C overnight.
8. Using a plastic cryomold embed spheres using OCT (Optimal cutting temperature compound) and avoid bubbles.
9. Place molds containing spheres in cooler containing dry ice to slowly freeze samples. Place at -20°C overnight, transfer to -80°C for long term stock.
10. Set cryostat optimal temperature (this need to be tested and might vary according to sample).
11. Place sample in the cryostat 20 minutes prior to manipulation to equilibrate the temperature.
12. Attach sample block on holder using OCT.
13. Use cryostat to trim samples. Suggested thickness for forebrain spheroids is 8-10µm.
14. Let samples attach to slides for 1 hour at room temperature.
15. Samples can be used for immunohistochemistry or placed at -20°C overnight, then transfer to -80°C for long term stock.

3.3 Immunofluorescence

Materials: PBS, BSA, NDS (normal donkey serum) and Triton X-100.

Procedure:

1. Wash samples in PBS 1x and draw a circle on the slide around spheroids using hydrophobic barrier pen.
2. Prepare Blocking Solution containing 5% NDS, 0.05% BSA and 0.2% Triton in PBS.
3. Incubate samples in blocking solution for one hour at room temperature or overnight in humidified chamber.
4. Prepare solution of primary antibodies in blocking solution.
5. Incubate for two hours at room temperature or overnight at 4°C in humid chamber.
6. Wash three times for 10 minutes with PBS 1x.
7. Prepare solution of secondary antibodies in blocking solution in humid chamber.
8. Incubate for two hours at room temperature and protect it from light.
9. Wash three times for 10 minutes with PBS 1x.
10. Proceed with nuclear labeling if needed.
11. Wash three times for 5 minutes with PBS 1x.
12. Apply anti-fading mounting media and carefully place coverslip on tissue. Slides can be stored at 4°C.

4 Reference

Pasca, A.M., Sloan, S.A., Clarke, L.E., Tian, Y., Makinson, C.D., Huber, N., et al. (2015). Functional cortical neurons and astrocytes from human pluripotent stem cells in 3D culture. *Nat Methods* 12(7), 671-678. doi: 10.1038/nmeth.3415.