Chapter 17

Establishment of In Vitro Brain Models for AON Delivery

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Abstract

Progress in stem cell biology has made it possible to generate human-induced pluripotent stem cells (hiPSC) that can be differentiated into complex, three-dimensional structures, where the cells are spatially organized. To study brain development, Lancaster and colleagues developed an hiPSC-derived three-dimensional organoid culture system, termed cerebral organoids, that develop various discrete, although interdependent, brain regions. Here we describe in detail the generation of cerebral organoids using a modified version of the culture protocol.

Key words Cerebral organoid, Disease modeling, Induced pluripotent stem cells

1 Introduction

Many brain disorders are hereditary diseases with a known genetic cause, which allowed scientists to generate animal models to study disease progression, understand disease mechanisms, and perform therapeutic intervention studies [1, 2]. However, (1) mice are different from humans, and it is difficult to translate results from animal experiments into clinical application; (2) the genetic cause of many diseases is not yet known; (3) many disease-causing genes are mainly expressed in the cells that are affected; (4) for many of them, there are no (humanized-)mouse models available; (5) there is governmental and public pressure to advance the development of alternative model systems to replace animal studies. This emphasizes the need for patient-derived disease models that bridge the translational gap between animal models and human clinical trials. Progress in stem cell biology has made it possible to generate human induced pluripotent stem cells (hiPSCs) [3] that can be differentiated into the important cell types of the brain, neurons, and astrocytes [4, 5]. The disadvantage of these 2D models is that they are descriptive at a cellular level, but they fail to adequately provide the details that could be derived from a more complex, three-dimensional structure, where the cells are spatially organized

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[6]. In 2013, Lancaster and colleagues developed a hiPSC-derived three-dimensional organoid culture system, termed cerebral organoids, that develop various discrete, although interdependent, brain regions [7]. These organoids recapitulate many features of human cortical development, including a progenitor zone organization with abundant outer radial glial stem cells [8].

Here we describe the generation of cerebral organoids using a modified version of the Lancaster protocol [7, 9]. In short, feeder-free cultured hiPSCs were dissociated and replated in neural induction medium in a non-adherent cell culture plate, and differentiated for 100 days (Fig. 1). Cryosections of these organoids can be used for immunofluorescence studies. Organoids can be used for many different purposes including disease modeling, studying disease mechanisms, or analyzing therapeutic interventions (using for example antisense oligonucleotides) at any given time point.

2 Materials

2.1 Neuroectodermal Differentiation

1. mTeSR™1.
2. Matrigel.
3. Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12 Nutrient Mixture (DMEM/F12).
4. ACCUTASE™.
5. STEMdiff™ Neural Induction Medium (NIM).
6. Y-27632.
7. v-bottom shape 96-well plate.

2.2 Neurospheres

1. Neurosphere medium: DMEM/F12 and Neurobasal medium 1:1, 1:200 N2 supplement, 1:100 B-27 supplement (without vitamin A), 1:100 L-glutamine, 0.05 mM non-essential amino acids (MEM-NEAA), 100 U/ml penicillin, 100 μg/ml streptomycin, 1.6 mg/l insulin, 0.05 mM β-mercaptoethanol.
2. Wide orifice pipette tips.
3. Organoid embedding sheet (or parafilm and a 200 μl tip box).

2.3 Organoids

1. Brain organoid medium: DMEM/F12 and Neurobasal medium 1:1, 1:200 N_2 supplement, 1:100 B27 supplement w/o vitamin A, 1:100 L-glutamine, 0.05 mM MEM-NEAA, 100 U/ml penicillin, 100 μg/ml streptomycin, 1.6 mg/l insulin, 0.5 μM dorsomorphin, 5 μM SB431542, 0.05 mM β-mercaptoethanol.
2. Spinner flask or 6-well plates.
3. Bioreactor or shaker.

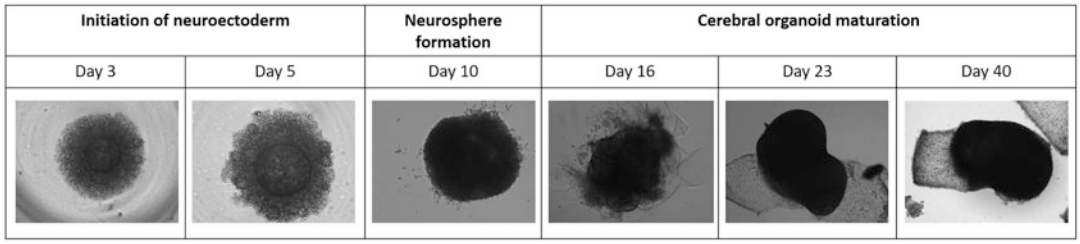

Fig. 1 Cerebral organoids during the various stages of organoid culturing. Organoids are cultured using a modified version of the Lancaster protocol. After 5 days of neuroectodermal differentiation, the neurospheres are embedded in Matrigel and cultured in the neurosphere medium in a 6-well plate for 5 days. For cerebral organoid maturation, the embedded neurospheres are transferred into a spinner flask and can be used for downstream applications if needed

2.4 Fixation and Embedding

1. Wide orifice pipette tips.
2. Dulbecco's Phosphate Buffered Saline (DPBS).
3. 4% paraformaldehyde (PFA).
4. 30% sucrose in distilled water.
5. Embedding mold.
6. Optimum cutting temperature compound (OCT).

2.5 Cryosectioning and Immunofluorescent Staining

1. PLL-coated glass cryoslides.
2. Barrier pen.
3. PBS-glycine: 200 mM of glycine in DPBS.
4. Blocking Solution: 5% goat or horse serum, 0.1% Triton X-100, 200 mM glycine in Dulbecco's Phosphate-Buffered Saline.
5. Immunobuffer solution: 1% goat or horse serum, 0.1% Triton X-100 in Dulbecco's Phosphate-Buffered Saline.
6. Prolong Diamond Antifade Mounting (+DAPI).

3 Methods

3.1 Neuroectodermal Differentiation

1. Culture hiPSCs under feeder-free conditions in mTeSR™1 in a culture dish coated with Matrigel. For neuroectodermal differentiation one 100 mm cell culture dish is required (*see* **Note 1**).
2. When the hiPSCs are ready for passaging, wash the hiPSCs with 10 ml pre-warmed (37 °C) DMEM/F12.
3. Remove DMEM/F12, add 2 ml pre-warmed ACCUTASE™ and incubate for 5 min at 37 °C and 5% CO_2, allowing cells to detach (*see* **Note 2**).

4. Pipette the cell suspension up and down 3–5 times using a 1-ml micropipette to make a single-cell suspension and collect the suspension in a 15-ml tube (*see* **Note 3**).
5. Add 6 ml of pre-warmed DMEM/F12, wash the culture dish and collect the suspension in the same 15-ml tube as in **step 4**.
6. Centrifuge at 300 × *g* for 5 min at room temperature.
7. Remove supernatant and resuspend the cell pellet in 1 ml of NIM supplemented with 10 μM Y-27632 and count the cells using a cell counter.
8. Dilute the cell suspension in NIM supplemented with 10 μM Y-27632 to 4.5×10^5 cells per ml and add 100 μl per well in a non-adherent, v-bottom shape, 96-well plate (*see* **Note 4**).
9. Centrifuge the plate at 500 × *g* for 3 min at room temperature and incubate at 37 °C and 5% CO_2.
10. Change medium by carefully removing 50 μl NIM from the top of the wells, without disturbing the embryoid bodies, and by adding 50 μl of fresh NIM. Medium changes should be done daily for the next 5 days (*see* **Note 5**).

3.2 Neurosphere Embedding

1. Use an 1-ml micropipette with a wide orifice pipet tip to place the neurospheres on a silicone organoid embedding sheet (*see* **Notes 6** and **7**).
2. Carefully remove the medium from the well, without disturbing the neurosphere, and expel it back to dislodge the neurosphere from the bottom of the well.
3. Use a wide orifice tip to collect the neurosphere and transfer it to the embedding sheet.
4. Carefully remove as much liquid from the embedding sheet as possible.
5. Use an ice-cold tip to add a drop of Matrigel onto each neurosphere.
6. Use an ice-cold tip to place the neurosphere in the center of the Matrigel droplet.
7. Incubate at 37 °C and 5% CO_2 for 15 min.
8. Carefully wash the neurospheres from the embedding sheet into a 100-mm culture dish by flushing them with the neurosphere medium and incubate at 37 °C and 5% CO_2. The total volume of neurosphere medium in the dish is 10 ml (*see* **Note 8**).
9. Add 2 ml of fresh neurosphere medium on day 2.
10. Use the embedded neurospheres 4 days after the embedding for the next step in the protocol.

3.3 Organoids

1. Carefully transfer the embedded neurospheres into a spinner flask containing 100 ml of pre-warmed organoid medium using a 2-ml serological pipette (*see* **Note 9**).
2. Place the spinner flask on the magnetic stirring platform in the incubator and use a stirring program at 25 rpm.
3. Culture the organoids on the magnetic stirring platform at 37 °C and 5% CO_2 for up to 100 days (*see* **Notes 10** and **11**).
4. Change the medium weekly by removing 50 ml of the organoid medium and adding 50 ml of fresh organoid medium (*see* **Note 12**). To refresh the medium, remove the spinner flask and let the organoids sink to the bottom for 5 min. Then carefully remove the medium, without disturbing the organoids.

3.4 Fixation and Embedding

1. Collect the organoids with an 1-ml micropipette with a wide orifice pipet tip and transfer them in a 60-mm dish or in a 6-well plate (*see* **Note 6**).
2. Wash the organoids with 5 ml of prewarmed Dulbecco's Phosphate-Buffered Saline.
3. Use a wide orifice tip to transfer each organoid separately into a 1.5-ml Eppendorf tube with 500 µl of 4% PFA. Incubate the organoids for 30 min at room temperature (*see* **Notes 13** and **14**).
4. Remove the 4% PFA solution and wash the organoids twice with 1 ml DPBS for 5 min (*see* **Note 15**).
5. Remove DPBS and add 1 ml of 30% sucrose in distilled water per tube to dehydrate the organoids and incubate the organoids at 4 °C overnight (*see* **Note 16**).
6. Fill a Peel-A-Way embedding molds with 400 µl of optimum cutting temperature compound (until the middle) and use an inoculation loop to place the organoid in the center of the mold. Label the rim of the mold with the sample name (*see* **Note 17**).
7. Snap-freeze the organoid-containing mold with ethanol on dry-ice and store at −80 °C until further use.

3.5 Cryosectioning and Immunofluorescent Staining

1. Section cryoprotected frozen organoids into 16- to 20-µm-thick slices on PLL-coated glass cryoslides using a cryostat (*see* **Notes 18** and **19**).
2. Thaw (if they were frozen) and dry the slides for 30 min at room temperature.
3. Draw a hydrophobic barrier around each section using a barrier pen.
4. To quench the PFA-induced autofluorescence wash the slides twice with 200 µL of PBS-glycine for 3 min.

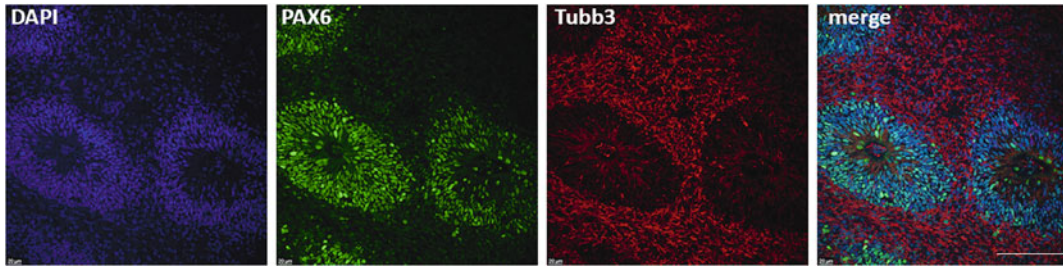

Fig. 2 Immunofluorescent staining of a cortical plate structure. Cortical plate structure in cerebral organoids stained with DAPI (blue), the neural progenitor marker PAX6 (green), and the neural marker TUBB3 (red). The scale bar represents 100 μm

5. Block nonspecific binding by adding 100 μl of Blocking Solution to the section for 1 h at room temperature.
6. Add 100 μl of primary antibody diluted in immunobuffer solution and incubate the slides overnight at 4 °C (*see* **Note 20**).
7. Wash three times with DPBS for 5 min.
8. Incubate the sections with the secondary antibody in immunobuffer solution for 1.5–2 h at room temperature (*see* **Note 21**).
9. Wash the slides three times in PBS.
10. Put a drop of Prolong Diamond Antifade Mountant (+DAPI) on the section and put a coverslip on top. Leave overnight at room temperature.
11. Store in fridge at 4 °C until performing microscopy. An example of a cortical plate structure in cerebral organoids stained with the neural progenitor marker PAX6 and the neural marker TUBB3 can be seen in Fig. 2.

4 Notes

1. Change mTeSR™1 daily and passage hiPSCs after 5–7 days. hiPSCs are ready to passage when the majority of the colonies are large, compact, and have centers that are dense compared to their edges. Only use undifferentiated, high-quality hiPSCs that are fully characterized according to the latest human pluripotent stem cell registry guidelines (https://hpscreg.eu/).
2. 1 ml ACCUTASE™ per 25 cm^2 surface area.
3. ACCUTASE™ is a cell detachment solution of proteolytic and collagenolytic enzymes and does not need to be neutralized.
4. Make sure that the cells are equally distributed in the suspension by mixing the tube regularly.

5. There is more evaporation from the four corner wells. Do not use these wells or add extra medium daily (up to 100 μl total culture volume).
6. The wide orifice tip can be replaced by a cut tip. The cut on the tip should be done by using sterile scissors. You can effectively sterilize scissors in an autoclave, but tools can also be sterilized in alcohol or a flame.
7. The silicone organoid embedding sheet can be replaced by placing parafilm over an empty 200-μl tip box and using a finger to make small holes.
8. A maximum of 20 neurospheres per 100-mm cell culture dish.
9. A maximum of 20 organoids per culture flask.
10. When spinner flasks are not available, non-adhesive culture plates on a rotating platform can be used. Use a shaking program of 75 rpm.
11. If an orbital shaker and a 6-well plate are used, do not transfer more than 10 organoids per well. Moreover, monitor the organoids regularly to reduce the chance of them sticking together. If organoids stick together, you can easily separate these by using a sterile pipet tip as a knife.
12. Medium changes need to be done more often when there is a color change of the medium. Check the organoids regularly and replace the medium as done in **step 3** in Subheading 3.3 when needed. For a 6-well plate, a volume of 3-ml medium per well can be used and medium should be changed twice a week and/or when there is a color change of the medium.
13. For a big batch of organoids, fixation can be done in a 6-well plate.
14. For larger organoids (>2 mm), a 4% PFA incubation at 4 °C overnight is recommended.
15. You can store the organoids in 1 ml DPBS at 4 °C for up to 7 days.
16. After the addition of 30% sucrose solution, the organoids should float at the surface, and by the next day, the organoids should sink down to the bottom of the tube. You can keep the organoid in 30% sucrose for up to a month. The recommended time for organoids larger than 4 mm is 5–7 days.
17. To better visualize the organoid while cryosectioning, add Trypan-blue diluted 1:50 in DPBS before embedding the organoids in OCT (and after 30% sucrose) for 15 min at room temperature. The outer area of the organoid will be colored blue.
18. Up to 100 sections can be obtained from one organoid.
19. The slides can be stored at −80 °C.

20. Put a wet tissue inside the box to prevent the slides from drying out.
21. Keep in the dark from here on.

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