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Scalable Generation of Mature Cerebellar Organoids from Human Pluripotent Stem Cells and Characterization by Immunostaining

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Abstract

The cerebellum plays a critical role in the maintenance of balance and motor coordination, and a functional defect in different cerebellar neurons can trigger cerebellar dysfunction. Most of the current knowledge about disease-related neuronal phenotypes is based on postmortem tissues, which makes understanding of disease progression and development difficult. Animal models and immortalized cell lines have also been used as models for neurodegenerative disorders. However, they do not fully recapitulate human disease. Human induced pluripotent stem cells (iPSCs) have great potential for disease modeling and provide a valuable source for regenerative approaches. In recent years, the generation of cerebral organoids from patient-derived iPSCs improved the prospects for neurodegenerative disease modeling. However, protocols that produce large numbers of organoids and a high yield of mature neurons in 3D culture systems are lacking. The protocol presented is a new approach for reproducible and scalable generation of human iPSC-derived organoids under chemically-defined conditions using scalable single-use bioreactors, in which organoids acquire cerebellar identity. The generated organoids are characterized by the expression of specific markers at both mRNA and protein level. The analysis of specific groups of proteins allows the detection of different cerebellar cell populations, whose localization is important for the evaluation of organoid structure. Organoid cryosectioning and further immunostaining of organoid slices are used to evaluate the presence of specific cerebellar cell populations and their spatial organization.

Introduction

The emergence of human pluripotent stem cells (PSCs) represents a excellent tool for regenerative medicine and disease modeling, because these cells can be differentiated into most cell lineages of the human body[1,2]. Since their discovery, PSC differentiation using diverse approaches has been reported to model different diseases, including neurodegenerative disorders[3,4,5,6].

Recently, there have been reports of 3D cultures derived from PSCs resembling human cerebral structures; these are called brain organoids[3,7,8]. The generation of these structures from both healthy and patient-specific PSCs provides a valuable opportunity to model human development and neurodevelopmental disorders. However, the methods used to generate these well-organized cerebral structures are difficult to apply for their large-scale production. To produce structures that are large enough to recapitulate tissue morphogenesis without necrosis inside the organoids, protocols rely on the initial neural commitment in static conditions, followed by encapsulation in hydrogels and subsequent culture in dynamic systems[3]. However, such approaches may limit the potential scale-up of organoid production. Even though efforts have been made to direct PSC differentiation to specific regions of the central nervous system, including cortical, striatal, midbrain, and spinal cord neurons[9,10,11,12], the generation of specific brain regions in dynamic conditions is still a challenge. In particular, the generation of mature cerebellar neurons in 3D structures has yet to be described. Muguruma et al. pioneered the generation of culture conditions that recapitulate early cerebellar development[13] and recently reported a protocol that allows for human embryonic stem cells to generate a polarized structure reminiscent of the first trimester cerebellum[7]. However, the maturation of cerebellar neurons in the reported studies requires the dissociation of the organoids, sorting of cerebellar progenitors, and coculture with feeder cells in a monolayer culture system[7,14,15,16]. Therefore, the reproducible generation of the desired cerebellar organoids for disease modeling under defined conditions is still a challenge associated with culture and feeder source variability.

This protocol presents optimal culture conditions for 3D expansion and efficient differentiation of human PSCs into cerebellar neurons using single-use vertical wheel bioreactors (see **Table of Materials** for specifications), hereafter called bioreactors. Bioreactors are equipped with a large vertical impeller, which in combination with a U-shaped bottom, provide a more homogeneous shear distribution inside the vessel, allowing gentle, uniform mixing and particle suspension with reduced agitation speeds[17]. With this system, shape and size-controlled cell aggregates can be obtained, which is important for a more homogeneous and efficient differentiation. Moreover, a larger number of iPSC-derived organoids can be generated in a less laborious manner.

The main feature of the organoids, which are 3D multicellular structures usually formed from stem cells, is the self-organization of different cell types that forms specific shapes like those seen in human morphogenesis[18,19,20]. Therefore, organoid morphology is an important criterion to be evaluated during the differentiation process. Cryosectioning of organoids and further immunostaining of organoid slices with a specific set of antibodies allow for the spatial visualization of molecular markers to analyze cell proliferation, differentiation, cell population identity, and apoptosis. With this protocol, by immunostaining organoid cryosections, an initial efficient neural commitment

is observed by the 7th day of differentiation. During differentiation, several cell populations with cerebellar identity are observed. After 35 days in this dynamic system, the cerebellar neuroepithelium organizes along an apicobasal axis, with an apical layer of proliferating progenitors and basally located postmitotic neurons. During the maturation process, from days 35–90 of differentiation, distinct types of cerebellar neurons can be seen, including Purkinje cells (Calbindin$^+$), granule cells (PAX6$^+$/MAP2$^+$), Golgi cells (Neurogranin$^+$), unipolar brush cells (TBR2$^+$), and deep cerebellar nuclei projection neurons (TBR1$^+$). Also, a nonsignificant amount of cell death is observed in the generated cerebellar organoids after 90 days in culture.

In this system, human iPSC-derived organoids mature into different cerebellar neurons and survive for up to 3 months without the need for dissociation and feeder coculture, providing a source of human cerebellar neurons for disease modeling.

Protocol

1. Passaging and maintenance of human iPSCs in monolayer culture

1. Preparation of plates
    1. Thaw the basement membrane matrix (see **Table of Materials**) stock at 4 °C and prepare 60 µL aliquots. Freeze the aliquots at -20 °C.
    2. To coat the wells of a 6 well plate, thaw one aliquot of the basement membrane matrix on ice. Once thawed add 60 µL to 6 mL of DMEM-F12. Gently resuspend by pipetting up and down.
    3. Add 1 mL of diluted basement membrane matrix solution to each well of a 6 well plate and incubate at RT for at least 1 h before passaging or store at 4 °C for up to 1 week.
2. Passaging of iPSC colonies with EDTA
    1. Maintain iPSCs in monolayer culture in 6 well plates in the incubator at 37 °C, 95% humidity, and 5% CO_2.
    NOTE: In this protocol, three distinct human iPSC lines were used: F002.1A.13[21], human episomal iPSC line (iPSC6.2)[22], and commercially obtained iPS-DF6-9-9T.B (see **Table of Materials**).
    2. Before passaging, incubate the stored plates (step 1.1) at room temperature (RT) for 15 min and prepare the mTesR1 medium (**Table 1**).
    3. Aspirate the solution from the plate using a serological pipette and immediately add 0.5 mL of mTeSR1 medium to each well.
    4. Aspirate the spent medium from the well containing iPSCs and wash once using 1 mL of 0.5 mM EDTA per well.
    5. Add 1 mL of 0.5 mM EDTA to each well and incubate at RT for 5 min.
    6. Aspirate EDTA and remove the cells from the wells by gently adding mTeSR1 medium and pipetting the colonies using a P1000 micropipette. Collect the cells in a conical tube.
    NOTE: Do not pipette cells up and down more than 3x.
    7. Add 1 mL of cell suspension (diluted 1:4) to each well so that each well contains 1.5 mL of medium after the cell suspension is added. Return cells to the incubator at 5% CO_2, 37 °C.
    8. Replace the spent medium daily and passage every 3 days when 75%–80% confluence is achieved.

2. Seeding of human iPSCs in the bioreactor

1. Incubate iPSCs grown as monolayers in mTeSR1 supplemented with 10 µM of ROCK inhibitor Y-27632 (ROCKi). Add 1 mL of supplemented medium to each well from a 6 well tissue culture plate and incubate for 1 h at 37 °C, 95% humidity, and 5% CO_2.

   NOTE: ROCKi is used to protect dissociated iPSCs from apoptosis[23] .

2. After 1 h of incubation, aspirate the spent medium from each well and wash 1x with 1 mL of 1× PBS per well.

3. Add 1 mL of the cell detachment medium (see **Table of Materials**) to each well of a 6 well plate and incubate at 37 °C for 7 min until cells detach easily from the wells with gentle shaking.

4. Pipette the cell detachment medium up and down with a P1000 micropipette until the cells detach and dissociate into single cells. Add 2 mL of complete cell culture medium to each well to inactivate enzymatic digestion and pipette the cells gently into a sterile conical tube.

5. Centrifuge at 210 × *g* for 3 min and remove the supernatant.

6. Resuspend the cell pellet in culture medium (i.e., mTeSR1 supplemented with 10 µM of ROCKi) and count the iPSCs with a hemocytometer using trypan blue dye.

7. Seed 15 × 10^6 single cells in the bioreactor (maximum volume of 100 mL) with 60 mL of mTeSR1 supplemented with 10 µM of ROCKi at a final cell density of 250,000 cells/mL.

8. Insert the vessel containing the iPSCs in the universal base unit placed in the incubator at 37 °C, 95% humidity, and 5% CO_2.

   NOTE: The bioreactor stirring is maintained for 24 h by setting the universal base unit control to 27 rpm to promote iPSC aggregation.

3. Differentiation and maturation of human iPSC-derived aggregates in cerebellar organoids

1. Define the day of single cell seeding as day 0.

2. On day 1, collect 1 mL of the iPSC aggregates sample using a serological pipette. Maintain the bioreactor under agitation as before by placing the universal base unit with the bioreactor containing the aggregates in a sterile flow prior to collecting the sample. Plate the cell suspension in an ultra-low attachment 24 well plate. Check that iPSC-derived aggregates are formed.

3. Acquire images with an optical microscope using a total magnification of 40x or 100x to measure aggregate diameter.

4. Measure the area of the aggregates in each image using FIJI software.

   1. Select "**Analyze** | **Set Measurements**" from the menu bar and click on "**Area**" and "**OK**".

   2. Select "**File** | **Open**" from the menu bar to open a stored image file. Select the line selection tool presented in the tool bar and create a straight line over the scale bar presented in the image. Select "**Analyze** | **Set scale**" from the menu bar.

   3. In **"Known distance**" add the expanse of the image's scale bar in µm. Define the "**Unit of length**" as µm. Click on "**Global**" to maintain the settings and "**OK**". Select **Oval Selection** in the tool bar.

   4. For each aggregate delineate the area with the oval tool. Select "**Analyze** | **Measure**". Calculate their

diameter based on measured area, considering that aggregates are approximately spherical using

$$\text{diameter} = \sqrt{4A/\pi}$$

with A as the area of the aggregate.

5. When the average diameter of the aggregates is 100 µm, replace 80% of the spent medium with fresh mTeSR1 without ROCKi. When aggregates reach 200–250 µm in diameter, replace all the spent medium with gfCDM (**Table 1**), letting the organoids settle at the bottom of the bioreactor.

   NOTE: If the average aggregate diameter exceeds 350 µm do not start the differentiation protocol. Repeat the seeding of single cells. Generally, it takes around 1 day for the aggregate to reach an average diameter of 100 µm.

6. Insert the bioreactor containing the aggregates in the universal base unit placed in the incubator at 37 °C, 95% humidity, and 5% CO_2 .

7. Decrease the bioreactor agitation to 25 rpm.

8. On day 2, repeat steps 3.2, 3.3, and 3.4 to evaluate the aggregate diameter. Add 30 µL of FGF2 (final concentration, 50 ng/mL) and 60 µL of SB431542 (final concentration, 10 µM) to 60 mL of gfCDM differentiation medium (**Table 1**). Replace all spent medium from the bioreactor with the supplemented gfCDM. Repeat step 3.6.

   NOTE: SB431542 is crucial to inhibit mesendodermal differentiation, inducing neural differentiation[24] . FGF2 is used to promote the caudalization of the neuroepithelial tissue[25] .

9. On day 5, repeat steps 3.2, 3.3, 3.4, and 3.8.

   NOTE: Aggregate size should increase during the differentiation protocol. However, the diameter is only critical when the differentiation starts, because this parameter could influence the efficacy of differentiation.

10. On day 7, repeat steps 3.2, 3.3, and 3.4. Dilute FGF2 and SB431542 to 2/3: Add 20 µL of FGF2 and 40 µL of SB431542 to 60 mL of gfCDM differentiation medium. Replace all spent medium from the bioreactor with supplemented gfCDM. Repeat step 3.6 and increase bioreactor agitation to 30 rpm.

11. On day 14, repeat steps 3.2, 3.3, and 3.4. Add 60 µL of FGF19 (final concentration, 100 ng/mL) to 60 mL of gfCDM differentiation medium. Replace all spent medium from the bioreactor with gfCDM supplemented with FGF19. Repeat step 3.6.

    NOTE: FGF19 is used to promote polarization of mid-hindbrain structures[26] .

12. On day 18, repeat steps 3.2, 3.3, 3.4, and 3.11.

13. On day 21, repeat steps 3.2, 3.3, and 3.4. Replace all spent medium from the bioreactor with complete neurobasal medium (**Table 1**). Repeat step 3.6.

    NOTE: Neurobasal medium is a basal medium used to maintain the neuronal cell population within the organoid[7] .

14. On day 28, repeat steps 3.2, 3.3, and 3.4. Add 180 µL of SDF1 (final concentration, 300 ng/mL) to 60 mL of complete neurobasal medium. Replace all spent medium from the bioreactor with complete neurobasal medium supplemented with SDF1. Repeat step 3.6.

    NOTE: SDF1 is used to facilitate the organization of distinct cell layers[27] .

15. On day 35, repeat steps 3.2, 3.3, and 3.4. Replace all spent medium from the bioreactor with complete BrainPhys medium (**Table 1**). Repeat step 3.6.

NOTE: BrainPhys is a neuronal medium that supports synaptically active neurons[28].

16. Replace 1/3 of the total volume every 3 days with complete BrainPhys medium until day 90 of differentiation.

4. Preparation of organoids for cryosectioning and immunohistochemistry

1. Collection of organoids for immunostaining
    1. Collect 1 mL of sample of medium containing organoids with a serological pipette from the bioreactor to a 15 mL conical tube.
    NOTE: Organoids should be collected at different timepoints to evaluate the efficacy of differentiation, including days 7, 14, 21, 35, 56, 70, 80, and 90.
    2. Remove the supernatant and wash once with 1 mL of 1× PBS.
    NOTE: Do not centrifuge the organoids. Let the organoids settle at the bottom of the tube by gravity.
    3. Remove the supernatant and add 1 mL of 4% paraformaldehyde (PFA). Incubate at 4 °C for 30 min. Remove the spent PFA and add 1 mL of 1× PBS.
    4. Store the organoids in 1 mL of 1× PBS at 4 °C until processing for cryosectioning.
    NOTE: Store the organoids in 1x PBS for no more than 1 week after fixation.
2. Preparation of organoids for cryosectioning
    1. Remove the supernatant from the stored organoids. Add 1 mL of 15% sucrose (w/v, diluted in 1× PBS), mix well by gentle swirling, and incubate overnight at 4 °C.
    2. Prepare a solution of 15% sucrose/7.5% gelatin (**Table 2**) and maintain at 37 °C during preparation to avoid gelatin to solidify.
    3. Remove the 15% sucrose solution, add 1 mL of 15% sucrose/7.5% gelatin to the organoids, and quickly mix by gentle swirling. Incubate at 37 °C for 1 h.
    4. Add 15% sucrose/7.5% gelatin solution to a plastic container up to half of its volume. Wait for solidification at RT.
    5. After a 1 h incubation, carefully place a sucrose/gelatin drop containing the organoids on the solidified gelatin with a Pasteur pipette. Leave to solidify at RT for about 15 min. Make sure to avoid bubble formation.
    6. Place 15% sucrose/7.5% gelatin on top of the organoids until the container is filled. Wait for complete solidification at RT.
    7. After solidification, incubate 20 min at 4 °C.
    8. Cut the gelatin into a cube containing the organoids in the center and fix the gelatin cube on a piece of cardboard with a drop of O.C.T. compound.
    9. Place 250 mL of isopentane in a 500 mL cup and fill an appropriate container with liquid nitrogen. Using forceps and thick gloves, carefully place the cup containing isopentane on the surface of liquid nitrogen and cool the isopentane to -80 °C.
    10. When -80 °C is reached, place the gelatin cube into the cup containing isopentane until it freezes, keeping the temperature at -80 °C. Depending on the size of the cube, it might take 1–2 min.

NOTE: Avoid temperatures below -80 °C or excessive freezing time, because it might cause cracking of the cube.

11. When frozen, quickly store the gelatin cube at -80 °C and store until cryosectioning.

3. Cryosectioning of organoids

1. Turn on the cryostat and define both specimen (OT) and cryochamber (CT) temperatures at -25 °C.
2. When both temperatures stabilize, fix the gelatin cube containing the organoids on the specimen by using O.C.T. compound.
3. Define section thickness at 12 µm.
4. Cut the cube and collect 3–4 slices on adhesion microscope slides (see **Table of Materials**).
5. Store at -20 °C until use.

4. Immunostaining of organoids slices

1. Place the microscope slides containing organoid sections in a copling jar with 50 mL of prewarmed 1x PBS, holding up to 10 slides back-to-back.
   NOTE: All organoid sections should be submerged with liquid.
2. Incubate for 45 min at 37 °C to degelatinize slides.
3. Wash 1x with 50 mL of 1× PBS for 5 min at RT: Transfer the slides to a copling jar containing fresh 1× PBS.
4. Transfer the slides to a copling jar containing 50 mL of freshly prepared glycine (**Table 2**) and incubate for 10 min at RT.
5. Transfer the slides to a copling jar containing 50 mL of 0.1% triton (**Table 2**) and permeabilize for 10 min at RT.
6. Wash with 1× PBS for 5 min 2x.
7. Prepare the immunostaining dish with 3 mm paper soaked in 1× PBS. Dry slides with a tissue all around the slices and place them onto 3 mm paper. With a Pasteur pipette, cover the whole surface of the slides with blocking solution (**Table 2**) with ~0.5 mL per slide. Incubate for 30 min at RT.
8. Remove excess blocking solution and dry the slides with a tissue all around the slices. Place 50 µL of the primary antibody (**Table 3**) diluted in blocking solution over the sections and cover with the coverslips. Place the slices in a previously prepared immunostaining dish. Incubate overnight at 4 °C.
9. Transfer the slides to a copling jar with 50 mL of TBST (**Table 2**), let the coverslips fall, and wash with TBST for 5 min 3x.
10. Place 50 µL of the secondary antibody diluted in blocking solution over the sections and cover with the coverslips. Place the slices in the previously prepared immunostaining dish. Incubate for 30 min at RT, protected from light.
11. Transfer the slides to a copling jar again and wash with 50 mL of TBST for 5 min 3x.
12. Dry the slides with a tissue all around the slices and place the slices in a previously prepared immunostaining dish. Add 0.5 mL of DAPI solution over the whole surface of the slides with a Pasteur pipette. Incubate for 5 min at RT.
13. Repeat step 4.4.9.
14. Carefully dry the slides with a tissue. Add 50 µL of mounting medium drop by drop along the slide

and then carefully lower a coverslip onto each slide, slightly bending it to avoid bubbles.

Representative Results

The protocol was initiated by promoting cell aggregation using the 0.1 L bioreactors (**Figure 1A**). Single cell inoculation of the iPSCs was performed, with 250,000 cells/mL seeded in 60 mL of medium with an agitation speed of 27 rpm. This was defined as day 0. After 24 h, the cells efficiently formed spheroid-shaped aggregates (day 1, **Figure 1B**), and the morphology was well-maintained until day 5, with a gradual increase in size, demonstrating a high degree of homogeneity in aggregate morphology and size over time. (**Figure 1B**). A quantitative analysis by microscopy also revealed normal distribution of aggregate sizes by day 1 (**Figure 1C**). The aggregate size is an important physical parameter capable of prompting the cells to differentiate toward different lineages[29,30]. For this reason, based on the aggregate size reported in previous studies to induce an efficient neural[31,32] and cerebellar commitment[21], the generated aggregates were maintained in mTeSR1 medium at 25 rpm until they reached the desired diameter before starting differentiation (~200 µm). At day 2, the average diameter was 221.0 ± 54.4 µm (mean ± SD) for the F002.1A.13 cell line and 212.1 ± 42.1 µm for the iPSC6.2 cell line. As such, both cell lines attained the optimal aggregate size at this timepoint (**Figure 1C**).

Defining the day on which the seeding of iPSCs was performed as day 0, at day 2, after achieving the desired aggregate diameter, neural commitment was induced by simultaneously using SB431542, FGF2, and insulin, promoting neuroectodermal differentiation, as well as a moderate caudalization necessary for mid-hindbrain patterning. Afterwards, FGF19 and SDF1 were added to the culture at days 14 and 28, respectively, to promote the generation of different cerebellar progenitors. For the first days of neural induction, a rotation speed of 25 rpm was used, which was increased to 30 rpm after 7 days to avoid the accumulation and clumping of bigger aggregates (**Figure 2A**). During differentiation, organoids showed a more pronounced epithelization similar to neural tube-like structures with luminal space (**Figure 2B**). Additionally, the evaluation of organoid diameter distribution demonstrated a homogeneous size distribution during the initial cerebellar commitment until day 14 (**Figure 2B**).

Immunofluorescence analysis supports that an efficient neural commitment of the iPSC-derived organoids is already achieved by day 7 of differentiation after adding FGF2 and SB431542. The cryosections of organoids revealed many structures reminiscent of the neural tube staining for PAX6 and NESTIN, with most cells within the organoids expressing progenitor marker NESTIN at days 7 and 14 of differentiation (**Figure 2C**). Afterwards, FGF19 and SDF1 promoted the generation of continuously proliferating progenitor layers ($PAX6^+$) and an efficient neuronal differentiation was achieved, as demonstrated by the expression of TUJ1, neuron-specific class III beta-tubulin, by days 21 and 35 (**Figure 2C**). In addition, an efficient cerebellar differentiation was also observed after 21 days in the 0.1 L VW bioreactors, demonstrated by the presence of two different cell populations: granule cell progenitors ($BARLH1^+$ cells, **Figure 3A**), and Purkinje cell progenitors ($OLIG2^+$ cells, **Figure 3B**). After 35 days in culture, different cell populations within the organoids appeared to be organized into distinct layers. Various flat-oval structures within the organoids were observed with $BARHL1^+$ dorsal cerebellar progenitors as a continuous layer on the superficial side of the organoid (**Figure 3C**,**D**) and $SOX2^+$ in the luminal region of these oval

structures (**Figure 3D**). In addition, TUJ1^{+} newborn neurons appeared to migrate towards the surface, reestablishing the radial alignment on the outer surface of the organoid (**Figure 3E**).

After the generation of cerebellar progenitors, further maturation was promoted using BrainPhys medium[28] supplemented with neurotrophic factors BDNF and GDNF. Immunofluorescence staining of organoid cryosections was used to detect distinct subtypes of cerebellar neurons. Purkinje cells, GABAergic neurons expressing the calcium-binding protein calbindin (CALB, **Figure 3F**), were detected in the cerebellar organoids after the maturation protocol. In addition, another major cerebellar neuronal type, granule cells, was identified as a subset of cells coexpressing PAX6 and MAP2 (**Figure 3G**). Interestingly, a pool of PAX6^{+} progenitors not expressing MAP2 was maintained until 80 days of differentiation. Other types of cerebellar neurons were also detected, including unipolar brush cells expressing TBR2 (**Figure 3H**), and deep cerebellar nuclei projection neurons expressing TBR1 (**Figure 3I**). In addition to efficient cerebellar differentiation and maturation, this 3D dynamic culture system using the PBS 0.1 L VW bioreactors allowed organoids to remain viable for up to 90 days, without significant cell death and necrosis (**Figure 3J**).

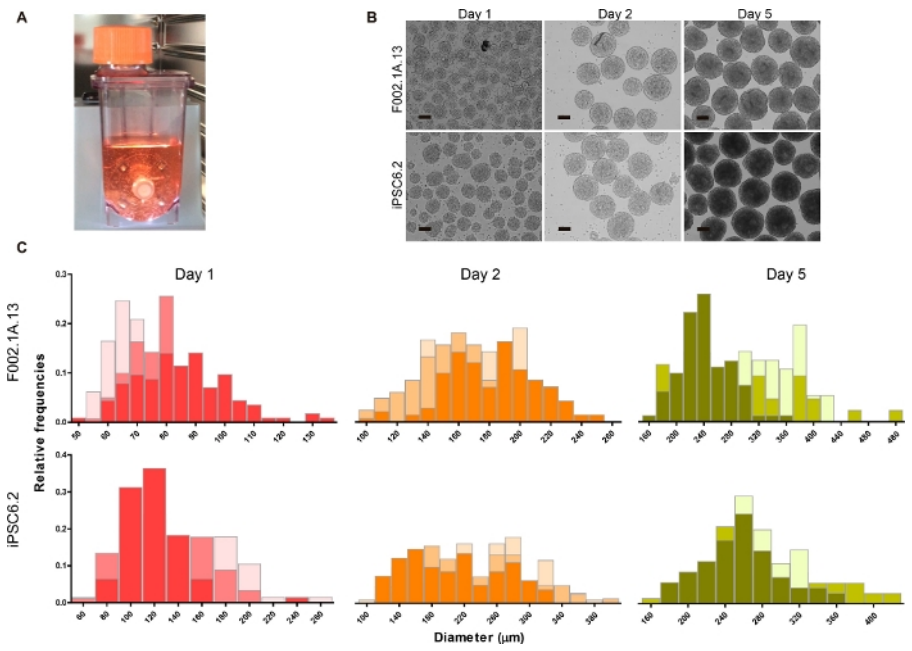

Figure 1: Generation of size-controlled aggregates using scalable bioreactors. (**A**) Design features of the bioreactor. (**B**) Brightfield photomicrograph showing aggregates from two different iPSC lines on days 1, 2, and 5. Scale bar = 100 µm. (**C**) The size distribution of floating aggregates from different iPSC lines in the bioreactors. Please click here to view a larger version of this figure.

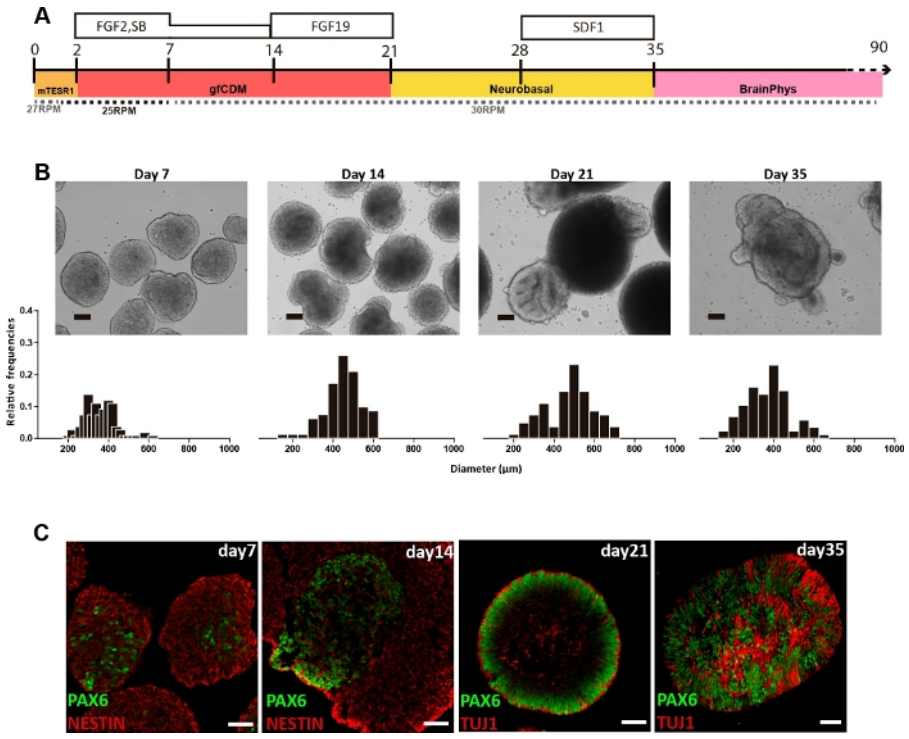

Figure 2: Generation of human iPSC-derived organoids using 0.1 L bioreactors. (**A**) Schematic representation of the culture procedure to induce differentiation of iPSCs to cerebellar organoids. Cells were seeded at a density of 250,000 cells/mL and an agitation speed of 27 rpm was used to promote cell aggregation. During the first days of differentiation, aggregates were maintained at an agitation speed of 25 rpm. Afterwards, to avoid the accumulation of bigger aggregates, the agitation speed was increased to 30 rpm. (**B**) Characterization of organoid shape and size. Brightfield photomicrographs showing iPSC-derived organoids during cerebellar differentiation in the 0.1 L VW bioreactors. Scale bar = 100 µm. The distribution of organoid diameters demonstrates that the culture maintained homogeneous organoids sizes along the differentiation protocol. (**C**) Efficient neural induction in iPSC-derived organoids. Immunofluorescence for NESTIN, PAX6, and TUJ1 during cerebellar differentiation. Scale bar = 50 µm. Please click here to view a larger version of this figure.

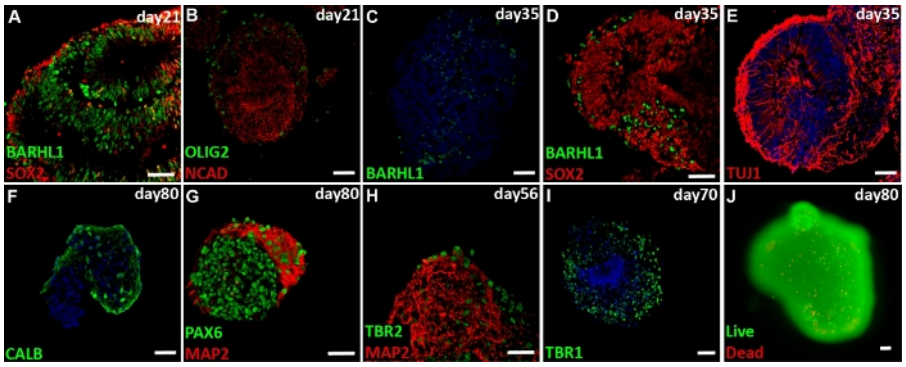

Figure 3: Efficient cerebellar differentiation and maturation in human iPSC-derived organoids. (**A**-**E**) Efficient cerebellar commitment. Immunostaining analysis for BARHL1, SOX2, OLIG2, NCAD, and TUJ1 markers at indicated timepoints of the cerebellar differentiation protocol. (**F**-**I**) Efficient maturation of human iPSC-derived cerebellar organoids. Immunofluorescence showing different types of cerebellar neurons, including Purkinje cells (CALB, F), granule cells (PAX6 and MAP2, G), unipolar brush cells (TBR2), and deep cerebellar nuclei projections neurons (TBR1). (**J**) High cell viability after cerebellar maturation. Live/dead (calcein-AM, green and propidium iodide, red) staining of organoids showed high cell viability and no evidence of necrotic areas after 80 days in the bioreactors. Scale bar = 50 µm. Please click here to view a larger version of this figure.

Media preparation	**mTeSR1** Final volume: 500 mL	1. Thaw mTeSR1 5× supplement at room temperature (RT) or at 4 °C overnight and mix with basal medium 2. Store complete mTeSR1 medium at 4 °C for up to 2 weeks or prepare 40 mL aliquots and store at -20 °C 3. Pre-warm complete mTeSR1 at RT before use
	gfCDM (growth factor-free chemically defined medium) Final volume: 60 mL	30 mL Ham's F12 30 mL IMDM 600 µL chemically defined lipid concentrate (1 % v/v) 2.4 µL monothioglycerol (450 µM) 30 µL apo-transferrin (stock solution at 30 mg/mL in water, final concentration: 15 µg/mL) 300 mg crystallization-purified BSA (5 mg/mL) 42 µL insulin (stock concentration at 10 mg/mL, final concentration: 7 µg/mL) 300 µL P/S (0.5% v/v, 50 U/ml penicillin/50 µg/ml streptomycin)
	Neurobasal Final volume: 60 mL	60 mL of Neurobasal medium 600 µL N2 supplement 600 µL Glutamax I 300 µL P/S (0.5 % v/v).
	Complete BrainPhys Final volume: 60 mL	60mL of BrainPhys 1.2 mL NeuroCult SM1 Neuronal Supplement 600 µL N2 Supplement 12 µL BDNF (final concentration: 20 ng/mL) 12 µL GDNF (final concentration: 20 ng/mL)

		300 µL Dibutyryl-cAMP (stock concentration: 100 mg/mL in water, final concentration: 1 mM) 42 µL ascorbic acid (stock concentration: 50 µg/mL in water, final concentration: 200 nM)
Stock solutions of growth factors and small molecules	**Basic fibroblast growth factor (bFGF/FGF2)** Stock concentration: 100 µg/mL	1. Reconstitute in 5 mM Tris, pH 7.6, at a concentration of 10 mg/mL 2. Dilute with 0.1 % BSA in PBS (v/v) to a final stock concentration of 100 µg/mL
	Stromal cell-derived factor 1 (SDF1) Stock concentration: 100 µg/mL	1. Reconstitute in water at a concentration of 10 mg/mL 2. Dilute with 0.1 % BSA (v/v) in PBS to a final stock concentration of 100 µg/mL.
	Brain-derived neurotrophic factor (BDNF) Stock concentration: 100 µg/mL	
	Glial cell-derived neurotrophic factor (GDNF) Stock concentration: 100 µg/mL	
	Fibroblast growth factor 19 (FGF19) Stock concentration: 100 µg/mL	1. Reconstitute in 5 mM sodium phosphate, pH 7.4, at a concentration of 10 mg/mL 2. Dilute with 0.1 % BSA in PBS (v/v) to a final stock concentration of 100 µg/mL
	ROCK inhibitor Y-27632 Stock concentration: 10mM	Reconstitute in DMSO at a concentration of 10 mM.
	SB431542 Stock concentration: 10mM	
	Insulin Stock concentration: 10 mg/mL	1. Reconstitute 10 mg of insulin in 300 µL of 10 mM NaOH 2. Carefully add 1 M NaOH until the solution becomes clear-transparent

		3. Fill to 1 mL with sterile water.

Table 1: Stock solutions and media preparation. Listed are all the components and volumes used to prepare media for the iPSCs maintenance and differentiation protocol, as well as stock solutions of growth factors and small molecules. For stock solutions, all stock concentration and protocols for reconstitution are listed.

Gelatin/Sucrose Final concentration: 7.5%/15% w/w	1. Weigh 15 g of sucrose and 7.5 g of gelatin in a sterile Schott Glass Bottle and mix well 2. Pre-warm the PBS 1× at 65 °C 3. Add pre-warmed PBS 1× to a final weight of 100 g and mix well 4. Place the Schott Glass Bottle in a heating plate at 65 °C and shake until the gelatin melts 5. Incubate at 37 °C until the solution stabilizes
Glycine Final concentration: 0.1 M	Add 0.37 g glycine to 50 mL of freshly-prepared PBS 1×.
Triton solution Final concentration: 0.1 % w/v	1. Prepare a 10 % Triton X-100 stock: 5 g of Triton X-100 in 50 mL of PBS 1× 2. Add 0.5 mL of Triton X-100 stock to 50 mL of PBS 1×.
TBST 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05 % w/v Tween-20	20 mL Tris 1 M 30 mL NaCl 5 M 5 mL Tween-20 (10 % stock: 5 g of Tween-20 in 50 mL water) Fill to 1 L with water.
Blocking Solution	Add 5 mL of fetal bovine serum (FBS, final concentration: 10 % v/v) to 50 mL of TBST.
DAPI solution	Add 15 µL of DAPI stock solution (1 mg/mL) to 10 mL of destilated water
Mowiol	1. Add 2.4 g of Mowiol to 6 g of glycerol and shake for 1 h in a pre-warmed plate at 50 °C 2. Add 6 mL of distilled water and shake for 2 h 3. Add 12 mL of Tris 200 mM (pH 8.5) and shake for 10 min 4. Centrifuge at 5,000 × g for 15 min 5. Aliquot and store at -20 °C.

Table 2: Solutions for preparation of organoids for cryosectioning and immunostaining. Listed are all the components and volumes used to prepare the solutions used in the preparation of organoids for cryosectioning and immunostaining.

Antibody	Host species	Dilution
BARHL1	rabbit	1/500
CALBINDIN	rabbit	1/500
MAP2	mouse	1/1000
N-CADHERIN	mouse	1/1000
NESTIN	mouse	1/400
OLIG2	rabbit	1/500
PAX6	rabbit	1/400
SOX2	mouse	1/200
TBR1	rabbit	1/200
TBR2	rabbit	1/200
TUJ1	mouse	1/1000

Table 3: Primary antibodies. The primary antibodies, clone, and optimized dilutions used for immunostaining are listed.

Discussion

The need for large cell numbers as well as defined culture conditions to generate specific cell types for drug screening and regenerative medicine applications has been driving the development of scalable culture systems. In recent years, several groups have reported the scalable generation of neural progenitors and functional neurons[32, 33, 34], providing significant advances in the development of new models for neurodegenerative disorders. Nonetheless, the recapitulation of some critical events of embryonic development is still lacking, and the maintenance of the generated functional neurons in suspension for long periods of time has not yet been achieved[34]. Presented here is a dynamic 3D culture system able to generate iPSC-derived neural organoids with cerebellar identity, and to further promote maturation into functional cerebellar neurons under chemically-defined and feeder-free conditions in dynamic culture.

Before starting cerebellar differentiation, it is critical to maintain the quality of the human iPSCs. Thus, in order not to compromise the differentiation, no more than three passages of iPSCs should be performed from thawing to bioreactor inoculation. An important step in the differentiation protocol is to evaluate the aggregate size. The aggregate size has a critical role in inducing differentiation towards a specific cell lineage[29]. Besides that, there is a minimum size threshold that appears to favor differentiation[35]. As already reported, the optimal iPSC-derived aggregate diameter to promote an efficient neural commitment[31, 32] and cerebellar differentiation[21] is a ~200 µm diameter.

Additionally, in this dynamic protocol, the agitation speed used in the first days of culture is crucial to control the

aggregate diameter and neural induction. The culture started at 27 rpm, which is sufficient to promote iPSCs aggregation and to avoid the formation of larger aggregates (diameters above 350 µm should be avoided). The agitation used to promote cell aggregation after single cell seeding could be increased to 30 rpm without affecting cell viability; however, higher agitation speeds are expected to produce smaller aggregates. Depending on the iPSC line, 24 h after cell seeding using 27 rpm, two different scenarios are expected: the aggregates formed present smaller diameters (<200 µm) or have reached a range of sizes between 200–300 µm. If aggregates are larger than 350 µm at 24 h after cell seeding, differentiation should not be performed, and the cell seeding should be repeated, because the efficiency of the differentiation will be very low. If aggregates are smaller than 200 µm, the spent medium should be replaced with iPSC maintenance medium, and the agitation speed reduced to 25 rpm. With this adjustment, aggregate diameter is expected to increase from day 1 to day 2, probably due to the merging of individual aggregates promoted by the decrease in the agitation speed. In case of aggregates with sizes between 200–300 µm, the spent medium should be replaced with differentiation medium, and neural induction with FGF2 should be started after 2 days in culture. At this point, the agitation speed should also be slightly reduced to prevent excessive cell death, because cells are more sensitive to shear stress in the presence of differentiation medium. Additionally, the population homogeneity could be analyzed using the coefficient of variation (CV), which measures the variability by correlating standard deviation with the mean of aggregate diameters, according to the equation

$$Cv = \delta/\mu$$

in which δ represents the standard deviation of the aggregate diameter and μ is the average diameter. In this dynamic system, the observed average CV was 12.5 ± 3.3% (mean ± SD) for the F002.1A.13 cell line and 19.0 ± 0.37% for the iPSC6.2 cell line at day 2. Thus, in this system, a homogeneous size population with a CV below 0.2 (< 20% of variation) should be expected. After 7 days of differentiation, the average aggregate diameter ranged from 300–360 µm, and the agitation speed was increased to 30 rpm to prevent aggregates to settle at the bottom of the 0.1 L VW bioreactor.

The differentiation of cerebellar organoids until day 35 and the analysis of aggregate size in static conditions were recently reported[21] . The authors showed that 3D aggregates formed and maintained in plates (e.g., Aggrewell) until day 7 of differentiation were homogeneous in size and shape[21] . However, after transferring the aggregates to ultra-low attachment 6 well culture plates, the aggregates started to vary in size and morphology[21] . On day 35 in static conditions, some of the 3D aggregates reached 1,000 µm for different cell lines, which limited the diffusion of nutrients and oxygen. In contrast, using our dynamic conditions, aggregates did not reach more than 800 µm in diameter by day 35, with improved mass transfer due to the constant agitation of the medium promoted by the vertical wheel. Furthermore, the aggregate sizes were maintained until the end of the maturation process, showing an aggregate diameter of 646.6 ± 104.2 µm by day 90, the longest culture performed in 0.1 L VW bioreactors.

Efficient cerebellar induction was induced by sequential addition of SB431542, FGF2, FGF19, and SDF1 in this 3D dynamic system. The protocol starts with the combination of SB431542, which is a transforming growth factor beta (TGF-ß)-receptor blocker that inhibits mesendodermal differentiation, and FGF2, which has a major effect in

the caudalization of neuroepithelial tissue[25]. Therefore, the addition of these two molecules during the first days of culture is essential to promote the cell differentiation to the mid-hindbrain, the territory that gives rise to the cerebellar tissue. After initial induction to mid-hindbrain tissue, it is necessary to add FGF19 for promoting the spontaneous generation of mid-hindbrain structures with dorsal-ventral polarity, as well as the generation of different cerebellar progenitors[36, 26]. SDF1 facilitates the organization of distinct layers of cerebellar progenitors, as seen at the developmental stage in which cerebellar neurogenesis occurs[27]. Until day 35, these molecules can promote the organization of cerebellar organoids that can recapitulate human cerebellar development, which corresponds to the first trimester cerebellum. After the organization of cerebellar progenitors into different layers, a defined neuronal medium was used to promote their maturation[28]. Other media used to maintain neuronal cells could also be tested, but lower efficiencies are anticipated. Thus, in this protocol, BrainPhys was used to promote the differentiation of cerebellar-committed cells into cerebellar neurons, because it has been reported to better mimic the healthy neuronal environment and to support neurophysiological activity of the generated neurons[28].

Using these dynamic conditions, a more efficient diffusion of nutrients, oxygen, and growth factors can be achieved. However, some limitations are associated with the agitation used in the differentiation protocol. Some shear stress can be introduced by the agitation process, which can affect the survival, proliferation, and differentiation of cells. Therefore, during the maturation step, in which the cells are more sensitive, the culture must be carefully monitored.

The differentiation of cerebellar organoids reminiscent of human embryonic cerebellar development has already been reported[7]. However, further maturation of these embryonic cerebellar organoids into cerebellar neurons using 3D cultures remains a challenge. The generation of functional cerebellar neurons was only achieved by coculturing with granule cells from various sources[4, 7, 15]. This protocol successfully upscaled cerebellar commitment of human iPSCs; in addition, this is the first protocol for the differentiation of different cerebellar neurons in a 3D culture system without coculturing with feeder cells. Specifically, the following cell types can be produced in our dynamic culture system: Purkinje cells (Calbindin$^+$), granule cells (PAX6$^+$/MAP2$^+$), unipolar brush cells (TBR2$^+$), and deep cerebellar nuclei projection neurons (TBR1$^+$), which were maintained in suspension for as long as 3 months.

The scalable generation of cerebellar organoids represents a valuable tool for studying the embryonic development of the cerebellum and the pathological pathways involved in the degeneration of this organ. Furthermore, high-throughput screening for molecules that restore cerebellar function may be performed using organoids obtained with this scalable system. Overall, this method satisfies an unmet need for a scalable protocol for the generation of high-quality cerebellar organoids that may be important for a variety of biomedical applications.

Disclosures

Authors YH and SJ are employees of PBS Biotech. The author BL is CEO and co-founder of PBS Biotech, Inc. These collaborating authors participated in the development of the bioreactors used in the manuscript. This does not alter the authors' adherence to all the policies of the journal on sharing data and materials. All other authors declare no conflict of interest.

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