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

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Author Questionnaire

1. **Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **yes**

   If **Yes**, can you record movies/images using your own microscope camera?

   yes

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **no**

3. **Filming location:** Will the filming need to take place in multiple locations? **no**

Introduction

1. Introductory Interview Statements

Videographer: Interviewee headshots are required. Take a headshot for each interviewee.

Authors: While filming the interview portion, our videographer will also photograph you for the **JoVE Dedicated Author Webpage**. Please look at this **example**. For questions about the author profile pages and pictures, please contact **author.liaison@jove.com**.

Authors: Please memorize the interview statements prior to your filming day.

1.1. **Teresa Silva:** We present for the first time a new approach for the reproducible and scalable generation of human iPSC-derived cerebellar organoids under chemically defined conditions using single-use bioreactors **[1]**.

1.1.1. INTERVIEW: Named author says the statement above in an interview-style shot while looking slightly off-camera.

1.2. **Teresa Silva:** The generation of high-quality cerebellar organoids may be important for disease modeling and drug screening **[1]**.

1.2.1. INTERVIEW: Named author says the statement above in an interview-style shot while looking slightly off-camera.

Answers have been edited for consistency with journal style guidelines.

Introduction of Demonstrators on Camera

1.3. **Teresa Silva:** Demonstrating the procedure will be Ph.D. student Diogo Nogueira and postdoctoral researcher Euguenia Bekman **[1][2].**

1.3.1. INTERVIEW: Author saying the above.

1.3.2. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera.

Protocol

2. Seeding the Bioreactor

2.1. To obtain cells for seeding the bioreactor, add 1 milliliter of cell detachment medium to each well of a 6-well plate of human iPSCs *(pronounce I-P-S-sees)* **[1]**. Incubate the plate at 37 degrees Celsius for 7 minutes, until gentle shaking easily detaches the cells from the well **[2]**.

2.1.1. Talent adds cell detachment medium to 6-well plate.
2.1.2. Talent removes plate from incubator and shakes gently.

2.2. Using a P1000 micropipette, pipette the cell detachment medium up and down until the cells dissociate until single cells **[1]**. Next, add 2 milliliters of complete cell culture medium to each well; this will inactivate enzymatic digestion **[2]**.

2.2.1. Talent pipettes medium up and down.
2.2.2. Talent adds cell culture medium to each well.

2.3. Then, use the pipette to gently transfer the cells to a sterile conical tube **[1]**. Centrifuge the tube at 210 times gravity for 3 minutes **[2]**.

2.3.1. Talent uses pipette to transfer cells to a conical tube.
2.3.2. Talent places tube in centrifuge.

2.4. Remove the supernatant **[1]**, and resuspend the cell pellet in culture medium **[1B-added]**. Count the cells using a hemocytometer and tryptan blue dye **[2]**.

2.4.1. Talent removes supernatant.

2.4.1. (B) Added shot: Resuspends cell pellet.
2.4.2. Talent counts cells in microscope using hemocytometer.

2.5. Seed the bioreactor vessel with the iPSCs, at a density of 250,000 cells per milliliter **[1-TXT]**. Insert the bioreactor vessel into the universal base unit, in an incubator at 37 degrees Celsius, 95 percent humidity, and 5 percent carbon dioxide **[2]**. To promote iPSC aggregation, set the agitation rate of the base control unit to 27 rpm **[3]**.

2.5.1. Talent adds iPSC cell suspension and medium to bioreactor. **TEXT: 15 × 10^6 single cells; 60 mL mTeSR1 + 10 µM ROCKi**
2.5.2. Talent places bioreactor vessel in base unit.
2.5.3. Talent adjusts agitation rate on base control unit.

3. Differentiation and Maturation of Aggregates

3.1. On day 1, with day 0 being the day of seeding the bioreactor, place the bioreactor and base unit in a sterile flow hood **[1]**. Then, use a serological pipette to collect a 1-milliliter sample of the cell suspension **[2]**.

3.1.1. Talent sets up bioreactor in sterile flow hood.
3.1.2. Talent uses a pipette to collect a sample from the bioreactor. *Videographer: This is one of the most important steps for viewers to see.*

3.2. Plate the cell suspension in an ultralow attachment 24-well plate **[1]**. Use a microscope to confirm that iPSC-derived aggregates have formed **[2]**. If so, capture images of the aggregates at 40x or 100x **[3].**

3.2.1. Talent adds cell suspension to 24-well plate.
3.2.2. SCOPE: iPSC-derived aggregates (JOVE_#61143_09.08.20_3.2.2) - Talent captures images of aggregates.

3.3. Continue culturing the iPSCs in the bioreactor until the average diameter of the aggregates is 100 micrometers **[1]**. Then, replace 80 percent of the medium with fresh mTeSR1 *without* ROCK inhibitor **[2]**.

3.3.1. LAB MEDIA: *Figure 1. Video editor, show only Figure 1B.*
3.3.2. Talent replaces medium in bioreactor. *Videographer: This is one of the most important steps for viewers to see.*

3.4. When the aggregates reach 200 to 250 micrometers in diameter, let all the organoids settle at the bottom of the bioreactor **[1]**. Then, replace all the spent medium with gfCDM differentiation medium **[2]**

3.4.1. Talent places the bioreactor in the sterile flow hood. NOTE: Author left a note to reuse 3.1.1, so this may have not been filmed.
3.4.2. Talent replaces all the medium in the bioreactor. NOTE: Author left a note to reuse 3.3.2, so this may have not been filmed.

3.5. Place the base unit and bioreactor in the incubator, and decrease agitation to 25 rpm **[1]**.

3.5.1. Talent places base unit and bioreactor in incubator and adjusts agitation.

4. Preparing Organoids for Cryosectioning

4.1. After removing the supernatant from the stored organoids, add 1 milliliter of 15 percent sucrose to the pellet, and mix well by swirling gently **[1]**. After incubating the organoids overnight at 4 degrees Celsius, remove the sucrose solution **[2]**. Then, add 1 milliliter of 15 percent sucrose, 7.5 percent gelatin, and quickly mix by gentle swirling **[3]**.

4.1.1. Talent adds sucrose solution and mixes by swirling.
4.1.2. Talent removes sucrose solution.
4.1.3. Talent adds sucrose/gelatin solution and mixes by swirling.

4.2. While the organoids are incubating at 37 degrees Celsius, fill a plastic container halfway with the 15 percent sucrose, 7.5 percent gelatin solution, and allow it to solidify **[1]**.

4.2.1. Talent adds sucrose/gelatin solution to plastic container.

4.3. After the organoids have been incubating for 1 hour, use a Pasteur pipette to place a drop of the organoid-sucrose-gelatin mixture on top of the solidified sucrose and gelatin **[1-TXT]**.

4.3.1. Talent places a drop of the organoid mixture on the solidified sucrose-gelatin.
TEXT: Note: Avoid bubble formation.

4.4. After waiting 15 minutes for the drop to solidify, fill the remainder of the plastic container with more 15 percent sucrose, 7.5 percent gelatin **[1]**. Allow it to solidify completely at room temperature, and then incubate it for 20 minutes at 4 degrees Celsius **[2]**.

4.4.1. Talent fills container with sucrose-gelatin.
4.4.2. Talent places container of solidified sucrose-gelatin in refrigerator.

4.5. Cut the gelatin into a cube, with the organoids in the center **[1]**. Fix the cube to a piece of cardboard with a drop of O.C.T. compound **[2]**.

4.5.1. Talent takes the gelatin out of the container, cuts gelatin into a cube and shows the organoids inside the gelatin cube.
4.5.2. Talent attaches cube to a piece of cardboard.

4.6. Then, place 250 milliliters of isopentane in a 500-milliliter cup **[1]**. Fill an appropriate container with liquid nitrogen **[2]**. Using forceps and thick gloves, carefully place the cup of isopentane on the surface of the liquid nitrogen **[3]**.

4.6.1. Talent pours isopentane into a cup.
4.6.2. Talent fills a container with liquid nitrogen.
4.6.3. Talent places cup of isopentane on the surface of the liquid nitrogen.

4.7. **Teresa Silva**: Isopentane and liquid nitrogen are hazardous reagents. The use of these two reagents requires personal protective equipment, including thick gloves, and an adequate ventilation. **[1].**

4.7.1. INTERVIEW: Named talent says the statement above in an interview-style shot. *Videographer: Since this is a warning statement, have the talent look more directly at the camera compared to other interview statements.*

4.8. When the isopentane has cooled to negative 80 degrees Celsius, place the gelatin cube in the isopentane until it freezes **[1]**. A well-frozen cube produces a metallic sound when lightly tapped with forceps, indicating that it is ready to be stored **[2-added]**.

4.8.1. Talent places gelatin cube in isopentane, showing thermometer at -80.

4.8.2. Added shot: Talent gently hits the cube with forceps to hear the metallic sound. NOTE: Seems like sound it important here, consider leaving it in.

5. Immunostaining

5.1. Wash the microscope slides containing the organoid sections with 50 milliliters of 1x PBS for 5 minutes **[1]**. Then, transfer the slides to a coplin jar containing fresh 1× PBS **[2]**.

5.1.1. Talent washes slides with PBS.
5.1.2. Talent transfers slides to jar. NOTE: Recorded multiple takes.

5.2. Transfer the slides to a coplin jar containing 50 milliliters of freshly prepared glycine, and incubate for 10 minutes at room temperature **[1]**. Then, transfer the slides to a coplin jar containing 50 milliliters of 0.1 percent triton, and permeabilize for 10 minutes at room temperature **[2]**.

5.2.1. Talent transfers slides to jar. NOTE: reuse 5.1.2.
5.2.2. Talent transfers slides to another jar. NOTE: reuse 5.1.2.

5.3. Wash the slides twice with 1× PBS, for 5 minutes each time **[1]**.

5.3.1. Talent washes slides with PBS. NOTE: reuse 5.1.1.

5.4. Prepare the immunostaining dish with 3-millimeter paper soaked in 1× PBS **[1]**. Dry the slides with a tissue all around the slices, and place them on the 3-millimeter paper **[2]**.

5.4.1. Talent places soaked paper in immunostaining dish. *Videographer: This is one of the most important steps for viewers to see.*
5.4.2. Talent dries the slides and places them on the paper. Note: Take 3 – organoids zoom.

5.5. With a Pasteur pipette, cover each slide with 0.5 milliliters of blocking solution **[1]**. After incubating for 30 minutes at room temperature, remove excess blocking solution, and dry the slides with a tissue all around the slices **[2]**.

5.5.1. Talent adds blocking solution to slides. *Videographer: This is one of the most important steps for viewers to see.*

5.5.2. Talent removes blocking solution and dries slides with a tissue.

5.6. Place 50 microliters of diluted primary antibody on each slide, and cover them with coverslips **[1]**. Place the slides in the previously prepared immunostaining dish **[2]**, and incubate them at 4 degrees Celsius **[3]**.

5.6.1. Talent places primary antibody on sections and covers them with coverslips. *Videographer: This is one of the most important steps for viewers to see.* NOTE: Recorded multiple takes.

5.6.2. Talent places slides in immunostaining dish. NOTE: Recorded multiple takes.

5.6.3. Talent places slides in refrigerator.

5.7. After overnight incubation, transfer the slides to a coplin jar with 50 milliliters of TBST, letting the coverslips fall off **[1]**. Then wash the slides 3 times with TBST for 5 minutes each time **[2]**.

5.7.1. Talent places slides in jar. NOTE: Recorded multiple takes.

5.7.2. Talent begins washing the slides in TBST, showing coverslip falling down. NOTE: Recorded multiple takes.

5.8. Place 50 microliters of diluted secondary antibody on each slide, and cover with the coverslips **[1]**. Place the slides in the previously prepared immunostaining dish. Incubate for 30 minutes at room temperature, protected from light **[2]**.

5.8.1. Talent adds secondary antibody to sections and covers them with coverslips. - NOTE: Reuse 5.6.1

5.8.2. Talent places slides in the immunostaining dish. NOTE: Reuse 5.6.2

5.8.3. ~~Talent places slides in a dark location to incubate~~.

5.9. Transfer the slides to a coplin jar again and wash them three times with 50 milliliters of TBST, for 5 minutes each time **[1]**.

5.9.1. Talent transfers slides to jar and begins washing them. NOTE: Reuse 5.7.1. and 5.7.2.

5.10. Using a Pasteur pipette, add 0.5 milliliters of DAPI solution over the whole surface of each slide, and incubate for 5 minutes at room temperature **[1]**.

5.10.1. Talent adds DAPI to each slide.

Results

6. Results: Efficient Cerebellar Differentiation and Maturation

6.1. After 24 hours in the bioreactor, iPSCs efficiently formed spheroid-shaped aggregates **[1]**. The morphology was well-maintained until day 5, with a gradual increase in size, demonstrating a high degree of homogeneity **[2]**.

6.1.1. LAB MEDIA: Figure 1. *Video editor, show Figure 1B only, and emphasize the two images on the left.*

6.1.2. LAB MEDIA: Figure 1. *Video editor, show Figure 1B only, and emphasize the two images on in the middle.*

6.2. A quantitative analysis by microscopy also revealed normal distribution of aggregate sizes by day 1, and both cell lines attained the optimal aggregate size by day 2 **[1]**.

6.2.1. LAB MEDIA: Figure 1. *Video editor, show Figure 1C only.*

6.3. After the desired aggregate diameter was achieved, neural commitment was induced and then generation of different cerebellar progenitors was promoted **[1]**.

6.3.1. LAB MEDIA: Figure 2. *Video editor, show Figure 2A only.*

6.4. During differentiation, organoids showed a more pronounced epithelization, similar to neural tube-like structures with luminal space. Additionally, the organoid diameter distribution was homogeneous during the initial cerebellar commitment, until day 14 **[1]**.

6.4.1. LAB MEDIA: Figure 2. *Video editor, show Figure 2B only.*

6.5. Immunofluorescence analysis supports that an efficient neural commitment of the iPSC-derived organoids **[1]** is already achieved by day 7 of differentiation **[2]**.

6.5.1. LAB MEDIA: Figure 2. *Video editor, show Figure 2C only.*

6.5.2. LAB MEDIA: Figure 2. *Video editor, show Figure 2C only, and emphasize the photo labeled day 7.*

6.6. Immunostaining also demonstrated efficient cerebellar commitment **[1]**, and efficient maturation of cerebellar organoids **[2]**. Furthermore, after 80 days in the bioreactor, live-dead staining of organoids showed high cell viability and no evidence of necrotic areas **[3]**.

6.6.1. LAB MEDIA: Figure 3. *Video editor, emphasize 3A through 3E.*

6.6.2. LAB MEDIA: Figure 3. *Video editor, emphasize 3F through 3I.*

6.6.3. LAB MEDIA: Figure 3. *Video editor, emphasize 3J.*

Conclusion

7. Conclusion Interview Statements

7.1. **Teresa Silva:** This technique represents an important tool for studying pathological pathways involved in the degeneration of the cerebellum, observed in cerebellar ataxias, and for evaluating the therapeutic effect of new drugs **[1].**

7.1.1. INTERVIEW: Named author says the statement above in an interview-style statement while looking slightly off-camera.