Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE62756R1
Full Title:	Generation of human brain organoids for mitochondrial disease modeling
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TITLE:

Generation of Human Brain Organoids for Mitochondrial Disease Modeling

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KEYWORDS:

brain organoids; iPSCs; mitochondrial disease; bioenergetic profiling

SUMMARY:

We describe a detailed protocol for the generation of human induced pluripotent stem cell-derived brain organoids and their use in modeling mitochondrial diseases.

ABSTRACT:

Mitochondrial diseases represent the largest class of inborn errors of metabolism and are currently incurable. These diseases cause neurodevelopmental defects whose underlying mechanisms remain to be elucidated. A major roadblock is the lack of effective models recapitulating the early-onset neuronal impairment seen in the patients. Advances in the technology of induced pluripotent stem cells (iPSCs) enable the generation of three-dimensional (3D) brain organoids that can be used to investigate the impact of diseases on the development and organization of the nervous system. Researchers, including these authors, have recently introduced human brain organoids to model mitochondrial disorders. This paper reports a detailed protocol for the robust generation of human iPSC-derived brain organoids and their use in mitochondrial bioenergetic profiling and imaging analyses. These experiments will allow the use of brain organoids to investigate metabolic and developmental

dysfunctions and may provide crucial information to dissect the neuronal pathology of mitochondrial diseases.

INTRODUCTION:

Mitochondrial diseases represent the largest class of inborn errors of metabolism[1]. They are caused by genetic mutations disrupting different mitochondrial processes, including oxidative phosphorylation (OXPHOS)[2], respiratory chain assembly, mitochondrial dynamics, and mitochondrial DNA transcription or replication[3]. Tissues with energy requirements are particularly affected by mitochondrial dysfunction[4]. Accordingly, patients with mitochondrial diseases typically develop early-onset neurological manifestations.

There are currently no treatments available for children affected with mitochondrial diseases[5]. A major hindrance for drug development of mitochondrial diseases is the lack of effective models recapitulating the human disease course[6]. Several of the currently studied animal models do not exhibit the neurological defects present in the patients[7]. Hence, the mechanisms underlying the neuronal pathology of mitochondrial diseases are still not fully understood.

Recent studies generated iPSCs from patients affected by mitochondrial diseases and used these cells to obtain patient-specific neuronal cells. For example, genetic defects associated with the mitochondrial disease, Leigh syndrome, have been found to cause aberrations in cellular bioenergetics[8,9], protein synthesis[10], and calcium homeostasis[9,11]. These reports provided important mechanistic clues on the neuronal impairment occurring in mitochondrial diseases, paving the way for drug discovery for these incurable diseases[12].

Two-dimensional (2D) cultures, however, do not enable the investigation of the architectural complexity and regional organization of 3D organs[13]. To this end, the use of 3D brain organoids derived from patient-specific iPSCs[14] may allow researchers to gain additional important information and thereby help to dissect how mitochondrial diseases impact the development and function of the nervous system[15]. Studies employing iPSC-derived brain organoids to investigate mitochondrial diseases are beginning to uncover the neurodevelopmental components of mitochondrial diseases.

Spinal cord organoids carrying mutations associated with the mitochondrial disease, mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes syndrome (MELAS), showed defective neurogenesis and delayed motor neuron differentiation[16]. Cortical organoids derived from patients with the mitochondrial disease, Leigh syndrome, showed reduced size, defects in neural epithelial bud generation, and loss of cortical architecture[17]. Brain organoids from Leigh syndrome patients showed that the disease defects initiate at the level of neural progenitor cells, which cannot commit to mitochondrial metabolism, causing aberrant neuronal branching and morphogenesis[18]. Thus, neural progenitors may represent a cellular therapeutic target for mitochondrial diseases, and strategies promoting their mitochondrial function may support the functional development of the nervous system.

The use of brain organoids might help uncover the neurodevelopmental components of mitochondrial diseases. Mitochondrial diseases are mainly considered as early-onset neurodegeneration[5]. However, neurodevelopmental defects are also present in patients

affected by mitochondrial diseases, including developmental delay and cognitive impairment[19]. Patient-specific brain organoids may help address these aspects and elucidate how mitochondrial diseases may impact human brain development. Mitochondrial dysfunction could also play a pathogenetic role in other more common neurological diseases, such as Alzheimer's disease, Parkinson's disease, and Huntington's disease[4]. Hence, elucidating the impact of mitochondrial defects in neurodevelopment using brain organoids might also be instrumental for the study of those diseases. This paper describes a detailed protocol for generating reproducible brain organoids that can be used for conducting disease modeling of mitochondrial diseases.

PROTOCOL:

NOTE: The use of human iPSCs may require an ethical approval. iPSCs used in this study were derived from healthy control individuals following local ethical approval (#2019-681). All cell culture procedures must be performed under a sterile cell culture hood, carefully disinfecting all reagents and consumables before transferring under the hood. Human iPSCs used for differentiation should have a passage number below 50 to avoid potential genomic aberrations that may occur upon extensive culture. The pluripotent state of the cells should be validated before organoid generation, for example, by monitoring the expression of pluripotency-associated markers such as NANOG or OCT4. Mycoplasma tests should be conducted weekly to ensure mycoplasma-free cultures.

1. Generation of brain organoids

1.1. Culture of human iPSCs

1.1.1. Culture human iPSCs under feeder-free conditions in iPSC medium (see the **Table of Materials**) on coated 6-well plates and keep them in a humidified tissue culture incubator at 37 °C and 5% CO_2.

NOTE: The carryover of feeder cells may hamper the organoid differentiation. Passage the cells at least once in feeder-free conditions.

1.1.2. Passage the iPSCs at 80% confluency using enzyme-free detachment medium in ratios ranging from 1:4 to 1:12. To increase cell survival, add 10 µM Rho-associated protein kinase (ROCK) inhibitor (Y27632) after each splitting.

1.2. Dissociate the iPSCs (80% confluency)—Day 0.

1.2.1. Prepare Cortical Differentiation Medium I (CDMI) (**Table 1**). Prewarm CDMI medium at room temperature (22–25 ˚C) before adding it to the cells.

1.2.2. Wash the wells containing the iPSCs with phosphate-buffered saline (PBS) to remove dead cells and debris.

1.2.3. Add 500 µL of prewarmed Reagent A (**Table of Materials**) to each well and incubate for 5 min at 37 ˚C. Check under the microscope to ensure cell detachment.

1.2.4. Add 1 mL of iPSC medium to dilute reagent A to neutralize its activity.

1.2.5. Use a 1000 µL pipette to dissociate the cells by pipetting up and down and transfer the cell suspension to a 15 mL centrifuge tube.

1.2.6. Gently centrifuge the iPSCs at 125 x *g* for 5 min at room temperature (22–25 ˚C).

1.2.7. Carefully aspirate the supernatant to avoid disturbing the cell pellet.

1.2.8. Resuspend the pellet with 1 mL of CDMI to obtain a single-cell suspension, and count the cell number.

1.2.9. Prepare the seeding medium with 9,000 iPSCs per 100 µL in CDMI supplemented with 20 µM ROCK inhibitor, 3 µM WNT-catenin inhibitor (IWR1), and 5 µM SB431542.

1.2.10. Add 100 µL of seeding medium per well to a 96-well v-bottom plate.

1.2.11. Keep the plate in a humidified tissue culture incubator at 37 °C and 5% CO_2.

1.3. Neurosphere generation

1.3.1. On day 1, observe that round cell aggregates (neurospheres) with defined smooth borders are forming. Note the dead cells around the aggregates. Continue to culture in the incubator at 37 ˚C and 5% CO_2.

1.3.2. On day 3, agitate the plate by tapping on the sides three times to detach dead cells.

1.3.3. Add 100 µL of CDMI supplemented with 20 µM ROCK inhibitor, 3 µM IWR1, and 5 µM SB431542 to each well.

1.3.4. Return the plate to the incubator at 37 °C and 5% CO_2.

1.3.5. On day 6, carefully remove 80 µL of the supernatant medium from each well. Avoid touching the bottom of the well.

1.3.6. Add 100 µL of CDMI supplemented with 3 µM IWR1 and 5 µM SB431542 to each well. Return the plate to the incubator at 37 °C and 5% CO_2.

1.3.7. Repeat steps 5 and 6 every 3 days until day 18.

1.4. Transfer of neurospheres

1.4.1. On day 18, prepare Cortical Differentiation Medium II (CDMII) (**Table 1**) and add 10 mL to a 100 mm ultra-low attachment cell culture plate.

1.4.2. Use a 200 µL pipette with the tip cut off to transfer the round neurospheres from the 96-well plate to the 100 mm ultra-low attachment cell culture plate.

NOTE: Be gentle to avoid damaging the neurospheres by making sure the opening of the tip is wide enough and that the aggregates are not aspirated too quickly.

1.4.3. Remove 5 mL of medium from the plate containing the neurospheres and add 5 mL of fresh CDMII.

NOTE: This procedure helps to reduce the amount of CDMI medium that may have carried over from the transfer of neurospheres.

1.4.4. Place the plate on an orbital shaker at 70 rpm inside a humidified tissue culture incubator at 37 °C and 5% CO_2.

NOTE: Visually inspect the neurospheres the next day. Increase the speed of the orbital shaker if the neurospheres are clumped together or attached to the bottom of the plate.

1.4.5. Every 3 days, carefully aspirate the supernatant medium and replace it with fresh CDMII. Leave a small amount of the medium to prevent the neurospheres from drying out.

1.4.6. On day 35, prepare Cortical Differentiation Medium III (CDMIII) (**Table 1**).

NOTE: The matrix component should be dissolved in cold CDMIII.

1.4.7. Aspirate the medium from the plate and add 10 mL of cold CDMIII.

NOTE: It is more effective to use cold medium so that the matrix component can coat the organoids without forming clumps.

1.4.8. After changing the medium, place the plate back on an orbital shaker at 70 rpm inside a humidified tissue culture incubator at 37 °C and 5% CO_2.

1.4.9. Change the medium every 3–5 days depending on the rate of growth, as indicated by the color of the medium.

1.4.10. On day 70, prepare Cortical Differentiation Medium IV (CDMIV) (**Table 1**). Use CDMIV medium until the desired age of organoids is reached. During this period, keep the plate on an orbital shaker set at 70 rpm inside a humidified tissue culture incubator (37 °C and 5% CO_2).

1.4.11. Change the medium every 3–5 days, depending on the growth rate.

2. Immunostaining of brain organoids

2.1. Tissue preparation

2.1.1. Prepare 4% paraformaldehyde (PFA) solution, and place it under a safety hood.

NOTE: Wear personal safety equipment when handling PFA.

2.1.2. Collect brain organoids and gently transfer them with a blunt-tipped 3 mL plastic Pasteur pipette to a 6-well plate filled with PFA.

NOTE: Use organoids older than 40 days to allow the visualization of structures with higher cellular complexity.

2.1.3. Keep the organoids in the PFA solution for 1 h at room temperature.

2.1.4. Carefully remove the PFA with a 3 mL plastic Pasteur pipette, and wash the fixed organoids three times using PBS.

2.1.5. Store the fixed organoids at 4 °C in PBS until further use.

2.2. Preparation of brain organoid slices

2.2.1. Prepare a 3% agar solution and heat slowly until liquefied.

2.2.2. Place the mold (the cut end of a 10 mL syringe) on a piece of absorbent filter paper (smooth side up). Place a droplet of agar onto it.

2.2.3. Quickly take a single organoid out of the 6-well plate with a spatula and remove excessive PBS with filter paper.

NOTE: Be careful not to touch the organoid directly with filter paper.

2.2.4. Place the organoid onto the agar droplet.

2.2.5. Repeat this procedure with up to three organoids.

NOTE: Work fast to avoid solidification of the agar during this step.

2.2.6. Refill the mold with agar until all the organoids are fully covered.

2.2.7. Wait until the agar begins to solidify, and then gently transfer the entire mold containing the organoids, including the absorbent filter paper, onto a cooling element.

NOTE: If a cooling element is unavailable, store the organoids for a few minutes in a refrigerator at 4 °C.

2.2.8. In the meantime, prepare for the slicing procedure: place a razor blade (cleaned with acetone and washed with double-distilled water) into the holder of the vibratome, mount on the bath, and fill it with PBS.

2.2.9. Remove the mold from (solidified) agar and use a scalpel to trim it to form a cube.

2.2.10. Attach the agar cube containing the organoids on the carrier plate of the vibratome with adhesive gel (see the **Table of Materials**), and place it in the bath containing PBS.

2.2.11. Adjust the vibratome (see the **Table of Materials**) to cut slices at a thickness of 150 µm.

NOTE: Vibratome settings (proper angle, amplitude, frequency, and velocity of the blade) may be similar to those used for slicing fixed brain tissue derived from early postnatal animals. However, the ideal settings depend strongly on the type of the vibratome and must be determined in a first step to prevent distortion or even ripping of the tissue while cutting.

2.2.12. Start the cutting procedure. Use a glass pipette or a spatula to gently transfer each freshly cut slice into a 24-well plate filled with PBS.

2.2.13. Store the plate containing slices at 4 °C (for up to a few days) until further processing.

2.2.14. Transfer the slices out of the plate with a glass pipette or a spatula onto microscope slides. Use a minimum of 2 slices per slide.

2.2.15. Carefully remove the agar and excess PBS with a syringe.

2.2.16. Allow the slices to dry until they adhere to the slides.

NOTE: While microscope slides can be stored in plastic chambers filled with PBS at 4 °C, they should be stained as soon as possible after the slicing procedure.

2.3. Immunohistochemical staining

2.3.1. Prepare the blocking solution (**Table 1**).

2.3.2. Use a PAP pen to draw a hydrophobic border around the slices on the slide to help keep all the solutions on the slides.

2.3.3. Carefully add the blocking solution on the slide, and incubate for 1 h at room temperature (22–25 °C). To avoid destroying the tissue, do not add the solution directly on top of the slices.

2.3.4. Aspirate the blocking solution and apply the desired primary antibody diluted in the blocking solution.

2.3.5. Incubate the slide overnight in a humidified chamber at 4 ˚C.

2.3.6. Rinse the slide three times with 1x PBS for 10 min each.

2.3.7. Incubate the slices with the specific secondary antibody diluted in the blocking solution and perform Hoechst staining (1:2,500) for 1 h at room temperature in the dark.

NOTE: Remember to perform negative controls to confirm there are no non-specific binding or auto-fluorescence.

2.3.8. Rinse three times with 1x PBS for 10 min each in the dark.

2.3.9. Add one drop of mounting medium to the slice, place a coverslip on the edge of the drop, and slowly lay the coverslip down toward the slice to avoid air bubbles.

2.3.10. Allow the slide to rest overnight at room temperature. Apply nail polish on the border of the coverslip to further seal the slide. For long-term storage, store at 4 ˚C.

2.4. Documentation of staining

2.4.1. To scan large images for stitching, utilize a motorized upright wide-field microscope equipped with (see the **Table of Materials** for details) a high-quality objective; DAPI filter set (e.g., excitation (EX): 340–380 nm, dichroic mirror (DM): 400 nm, barrier filter (BA): 435–485 nm); fluorescein isothiocyanate filter set (e.g., EX: 465–495 nm, DM: 505 nm, BA: 515–555 nm); Alexa594 filter set (e.g., ET 575/40; T 600 LPXR; HC 623/24); digital camera; high-performance acquisition software allowing for automated stitches and stack operations.

2.4.2. For image handling, use an image processing program capable of generating 8-bit tif-files, cropping stitches, adjusting contrast and brightness, merging the channels (e.g., blue, green, and red), and adding scale bars.

2.4.3. To scan details, use a motorized confocal laser scanning microscope equipped with a high-quality objective, a UV laser (EX: 408 nm), an Argon laser (EX: 488 nm), a Helium-Neon laser (EX: 543), imaging software for a confocal microscope.

2.4.4. For image handling of details, use an image processing program capable of generating maximum-intensity projections of confocal z-stacks (e.g., optical sections of 0.6 µm each), generating 8-bit tif-files, adjusting contrast and brightness, merging the channels (e.g., blue, green, and red), adding scale bars.

2.4.5. Use a graphic editor to arrange the figures.

3. Bioenergetic profiling of brain organoids

3.1. Preparation of organoids for bioenergetic profiling

3.1.1. Prepare the papain and DNase solution following the manufacturer’s protocol.

3.1.2. Transfer 3–5 organoids into a 6-well plate. Wash them two times with prewarmed **PBS**.

3.1.3. Add 2 mL of prewarmed activated papain solution containing DNase. Using a blade, cut the organoids into small pieces.

3.1.4. Place the plate onto an orbital shaker set at 27 rpm inside a cell culture incubator (at 37 °C, 5% CO_2), and incubate for 15–20 min.

NOTE: The time of incubation depends on the organoid stage. Early-stage organoids can be used as they are. For organoids older than 3 months, it is recommended to cut the organoids into 2–3 pieces before dissociation and incubate the pieces at a rocking speed set at 27 rpm for 15–20 min at 37 °C. This procedure can help remove necrotic tissue that may be present in the later-stage organoids.

3.1.5. Collect the digested tissues into a 15 mL tube and add 5 mL of organoid culture medium CDMIV (**Table 1**).

3.1.6. Triturate the tissue with a 10 mL plastic pipette by pipetting up and down 10–15 times. Let the undissociated tissue settle down to the bottom of the tube.

3.1.7. Carefully transfer the cell suspension to a 15 mL tube, avoiding any pieces of undissociated tissue. Filter the solution through a 40 µm cell strainer (e.g., polystyrene round-bottom tubes with cell strainer caps).

3.1.9. Pellet the cells by centrifuging at 300 x *g* for 5 min at room temperature.

3.1.10. Assess the cell number and quality using trypan blue.

3.1.11. Plate the desired number (~20,000/well) of cells onto coated 96-well microplates. Change the medium 6–8 h after plating to Neuronal Medium (**Table 1**).

3.1.12. Incubate the coated 96-well microplate in a CO_2 incubator (37 °C, 5% CO_2) for 4 days.

3.2. Bioenergetic profiling

3.2.1. On day 3 after replating the dissociated cells, add 200 µL of calibration solution into each well of the bottom part of the 96-well microplate, and place the top green sensor cartridge onto the hydrated microplate.

NOTE: Place the sensor cartridge on top of the microplate in the correct orientation, and ensure that the calibrant solution covers all the sensors.

3.2.2. Incubate the hydrated 96-well microplate in a non-CO_2 incubator at 37 °C overnight.

3.2.3. Turn on the analyzer to allow the instrument to stabilize at 37 °C overnight.

3.2.4. On day 4 after replating, inspect the disassociated organoid culture on the 96-well microplate under the microscope to ensure that the cells appear as a confluent monolayer.

3.2.5. Prepare Assay Medium (**Table 1**).

3.2.6. Remove Neuronal Medium from all wells with a pipette without touching the bottom of the well to prevent cell damage. Alternatively, carefully invert the whole plate and then dry it on clean paper. Work quickly to avoid cell death.

3.2.7. Wash the cells twice with prewarmed 200 µL of Assay Medium. Add Assay Medium to a final volume of 180 µL per well. Incubate the 96-well microplate in a non-CO_2 incubator at 37 °C for 1 h.

3.2.8. Prepare 10 µM solutions of mitochondrial inhibitors in Assay medium. Note that the final concentration after injection is 1 µM.

3.2.9. Load the sensor cartridge placed in the hydrated microplate with 10x solutions of the mitochondrial inhibitors.

3.2.9.1. Add 18 µL of mitochondrial inhibitor 1 into port A.

3.2.9.2. Add 19.8 µL of mitochondrial inhibitor 2 into port B.

3.2.9.3. Add 21.6 µL of mitochondrial inhibitor 2 into port C.

3.2.9.4. Add 23.4 µL of mitochondrial inhibitor 3 into port D.

3.2.10. Place the loaded cartridge in the hydrated microplate in a non-CO_2 incubator at 37 °C until the start of the assay.

3.2.11. Set up a running protocol in the instrument's software (**Table 2**).

3.2.12. Press **START**. Take the loaded cartridge from the non-CO_2 incubator and place it into the analyzer for calibration.

NOTE: Make sure the plate is inserted in the correct orientation and without the lid.

3.2.13. Once the calibration step ends, remove the calibration plate. Take the 96-well microplate from the non-CO_2 incubator and place it into the analyzer. Click on **CONTINUE** to start the measurements.

3.2.14. When the run is finished, remove the 96-well cell culture microplate from the analyzer and collect the medium from all the wells without disturbing the cells.

NOTE: The medium can be stored at -20 °C and used later for measuring the amount of lactate released by the cells in the medium using an appropriate lactate assay kit.

3.2.15. Wash the cells with 200 µL of 1x PBS in each well.

3.2.16. After removing the PBS, freeze the plate at -20 °C.

NOTE: The frozen plate can be used to quantify cells, proteins, or DNA in each well of the microplate. This quantification will be needed for normalizing the obtained bioenergetic rates. Follow the manufacturer´s instructions for cell, protein, or DNA quantification assays.

REPRESENTATIVE RESULTS:

The protocol described here facilitates the robust generation of round organoids **(Figure 1A)**. The generated organoids contain mature neurons that can be visualized using protein markers specific for axons (SMI312) and dendrites (microtubule-associated protein 2 (MAP2)) **(Figure 1B)**. Mature organoids contain not only neuronal cells (MAP2-positive) but also glial cells (e.g., positive for the astrocyte marker S100 calcium-binding protein B (S100ß)) **(Figure 1B)**.

By analyzing sliced brain organoids using confocal microscopy, it is possible to identify and monitor the detailed distribution and organization of different cell types and cellular structures. This could provide new insight into how mitochondrial diseases might affect nervous system development. For example, it is possible to monitor neuronal axons (SMI312-positive) and dendrites (MAP2-positive) **(Figure 2A)** or the mutual occurrence of neuronal cells (MAP2-positive) and glial cells (S100ß-positive) **(Figure 2A)**. Confocal images may also help to investigate in more detail the distribution and organization of neural progenitors ((sex determining region Y) box-2 (SOX2)-positive) with respect to neurons (beta-III tubulin (TUJ1)-positive) **(Figure 2B)**. Finally, brain organoids can be stained for mitochondria-specific markers (such as the outer mitochondrial membrane protein, translocase of outer membrane 20 kDa subunit (TOM20)) **(Figure 2C)**.

The described protocol enables researchers to perform bioenergetic profiling of brain organoids. Using this procedure, it is possible to measure both mitochondrial metabolism using the oxygen consumption rate (OCR) **(Figure 2D)** and the glycolytic metabolism using the extracellular acidification rate (ECAR) **(Figure 2E)**. Bioenergetic profiling allows monitoring how cells may modify their OCR and ECAR profiles in response to a sequential administration of mitochondrial inhibitors.

First, the ATP synthase inhibitor, oligomycin, can be applied. Oligomycin causes a drop in the OCR profile **(Figure 2D)**, and therefore, identifies the OCR needed for ATP production. Upon oligomycin treatment, there may also be a compensatory increase in ECAR **(Figure 2E)**, suggesting that the cells can upregulate glycolysis to prevent the metabolic stress caused by the reduction in mitochondrial metabolism. The subsequent double application of the proton ionophore, carbonyl cyanide-p-trifluoromethoxyphenylhydrazon (FCCP), causes the loss of the mitochondrial membrane potential. As the oxygen molecules are now free to move, this causes a rapid increase in OCR **(Figure 2D)**.

These changes in the OCR profile identify the maximal respiration capacity of the cells. The final administration of rotenone plus antimycin A causes a block of the electron transport, and therefore, a steep decrease in OCR **(Figure 2D)**. ECAR may show fluctuation after treatment with FCCP and rotenone plus antimycin A **(Figure 2E)**, depending on the residual glycolytic capacity of the cells. The OCR and ECAR profiles may be dramatically altered in brain organoids derived from mitochondrial patients.

FIGURE AND TABLE LEGENDS:

Figure 1: Generation of brain organoids from human iPSCs. (**A**) Schematic representation of the protocol used to produce brain organoids with corresponding transmission images. Day 0 corresponds to the dissociation of iPSCs and seeding in a 96-well plate with V-bottom using CDMI supplemented with a ROCK inhibitor, a WNT inhibitor, and SB431542. At day 18, neurospheres are transferred from the 96-well plates to 100 mm cell culture dishes with CDMII supplemented with N2. From this point onwards, the cultures are positioned on an orbital shaker. At day 35, the medium is switched from CDMII to CDMIII, which also contains a dissolved matrix component (**Table 1**). From day 70 onwards, CDMIII is switched to CDMIV supplemented with B27. A representative neurosphere image was taken at day 12 using a microscope camera with 10x magnification. An early organoid image was taken at day 22 using a microscope camera at 4x magnification. A mature organoid image was taken at day 40 using a microscope camera with 4x magnification. (**B**) The overall structure and cellular organization of brain organoids can be visualized using wide-field microscopy. Representative stitched wide-field images are shown to visualize the relationships between dendrites (MAP2-positive) and axons (SMI312-positive), and between neuronal cells (MAP2-positive) and presumed astrocytes (S100ß-positive). The cells were counterstained with Hoechst to reveal the nuclei. All images were taken using 78 day-old brain organoids. The right column shows the overlay of three (**A**) or two (**B**) channels (merge). Scale bars = 500 µm. Abbreviations: iPSC = induced pluripotent stem cell; CDM = Cortical Differentiation Medium; ROCK = Rho kinase; MAP2 = microtubule-associated protein 2; S100ß= S100 calcium-binding protein B.

Figure 2: Visualization and bioenergetic profiling of brain organoids for mitochondrial disease modeling. The detailed organization and architecture of organoids can be analyzed using confocal microscopy. All images were taken using 78 day-old brain organoids and counterstained with Hoechst to reveal the nuclei. The right column shows the overlay of three channels (merge). Scale bars = 50 µm. (**A**) Representative extended-focus projections (44–48 optical planes, 0.6 µm each) addressing the interplay between dendrites (MAP2-positive, arrowheads) and axons (SMI312-positive, arrows), and between neuronal cells (MAP2-positive, arrowheads) and presumed astrocytes (S100ß-positive, arrows). (**B**) Representative extended-focus projections (14–31 optical planes, 0.6 µm each) showing the distribution of neurons (TUJ1-positive, arrowheads) with respect to neural progenitors (SOX2-positive, arrows). (**C**) Representative extended-focus projections (20 optical planes, 0.6 µm each) showing the distribution within neurons (TUJ1-positive, arrowheads) of mitochondria (visualized using antibodies against the outer mitochondrial membrane protein TOM20, arrows). (**D**) Mitochondrial respiration of brain organoids can be monitored based on the profile of the OCR after sequential administration of different mitochondrial inhibitors (see text for details). (**E**) Glycolytic activity of brain organoids can be monitored based on the ECAR upon sequential administration of mitochondrial inhibitors (see text for details). For bioenergetic profiling, approximately 10–15 brain organoids were dissociated to obtain enough cells for replating onto the 96-well microplate. The bars indicate the SEMs based on the results obtained in two independent experiments. Abbreviations: MAP2 = microtubule-associated protein 2; S100ß = S100 calcium-binding protein B; TUJ1 = beta-III tubulin; SOX2 = (sex determining region Y) box-2; TOM20 = translocase of outer membrane 20 kDa subunit; OCR = oxygen consumption rate; ECAR = extracellular acidification rate; Oligom. = oligomycin;

FCCP = carbonyl cyanide-p-trifluoromethoxyphenylhydrazon; R = rotenone; AntA = Antimycin A; SEMs = standard error of means.

Table 1: Details of media and solutions used for organoid generation.

Table 2: Protocol setup for bioenergetic profiling. Description of the steps and their length in minutes using the Seahorse Wave Desktop software. Abbreviations: FCCP = carbonyl cyanide-p-trifluoromethoxyphenylhydrazon; Rot = rotenone; Anti A = Antimycin A.

DISCUSSION:

This paper describes the reproducible generation of human iPSC-derived brain organoids and their use for mitochondrial disease modeling. The protocol described here is modified based on a previously published work[20]. One major advantage of the present protocol is that it does not require the manual embedding of each organoid into a scaffolding matrix. In fact, the matrix solution is simply dissolved into the cell culture medium. Moreover, there is no need to employ expensive bioreactors, as organoids can be cultured in standard tissue culture 6-well plates positioned onto an orbital shaker inside the incubator. This procedure also enables the parallel cultivation of several plates containing different organoids derived from various individual lines, thereby increasing the throughput of the experiments and allowing the monitoring of potential differences emerging in the growth profiles of different organoids. We tested this protocol using different iPSCs derived from healthy controls and individuals affected by mitochondrial diseases, with consistent results.

For mitochondrial disease modeling, it is essential to use different markers to visualize the morphology and organization of the mitochondrial network. This procedure enables the investigation of whether mitochondrial number, morphology, or distribution might be altered in brain organoids derived from patients with mitochondrial diseases. The presence and organization of neural progenitors within the brain organoids could be of crucial importance for modeling mitochondrial disorders. We recently discovered that mutations causing the mitochondrial disease, Leigh syndrome, disrupt the cellular architecture and distribution of neural progenitor cells within patient-derived brain organoids[18].

For performing bioenergetic profiling, we have adapted a method that was previously described for assessing the bioenergetics of pluripotent stem cells[21]. A recent protocol described how to carry out bioenergetic profiling of organoids derived from mouse small intestine, human colon, and colorectal tumors[22]. However, those organoids are quite small compared to brain organoids, and therefore, a different protocol, such as the one reported here, is needed for brain organoids. We recently employed this protocol for assessing the bioenergetic profile of human brain organoids carrying mutations in the surfeit locus protein 1 gene (*SURF1)* that causes the severe mitochondrial disease, Leigh syndrome[18]. We found that the OCR profile is particularly affected in Leigh syndrome organoids, as shown by a significant decrease in the basal OCR level, the ATP production rate, and the maximal respiration rate[18].

In conclusion, we present here a detailed protocol for the robust generation of human brain organoids and describe how to perform experiments that would be important for the investigation of the disease mechanisms underlying mitochondrial diseases. Human brain

organoids may also be of critical importance for elucidating the mitochondrial diversity in the human brain and its role in human health and diseases[23]. It is important to clarify that brain organoids generated with currently available protocols, including the one described here, still bear limitations. These include, for example, the lack of vascularization and the absence of microglia population[24]. These aspects need to be taken into consideration to interpret the results correctly.

For example, the lack of vasculature and microglia could limit compensatory mechanisms that may be in place *in vivo*. Patient-derived brain organoids might thus exhibit defects that are stronger than the ones observed in patients[17-18]. Moreover, despite a general reproducibility of this protocol[20], line-to-line heterogeneity can be observed. To this end, when performing disease modeling studies, it is always important to systematically quantify the uniformity of control and patient organoids by assessing the patterns of morphology (size, layer) and the distribution of molecular markers across different organoids.

Finally, it is not possible to generate brain organoids from a single iPSC, limiting the feasibility of large-scale genetic screening with CRISPR/Cas9. Given the pace of research, it is likely that some of the current limitations of the protocol described here will soon be overcome. Optimized protocols will become available. These 3D models of mitochondrial diseases will hopefully enable the eventual discovery of implementable therapies for mitochondrial diseases, which are detrimental, and for incurable diseases with highly unmet medical needs.

ACKNOWLEDGMENTS:

We thank Miriam Bünning for technical support. We acknowledge support from the Deutsche Forschungsgemeinschaft (DFG) (PR1527/5-1 to A.P.), Spark and Berlin Institute of Health (BIH) (BIH Validation Funds to A.P.), the United Mitochondrial Disease Foundation (UMDF) (Leigh Syndrome International Consortium Grant to A.P.), University Hospital Duesseldorf (Forschungskommission UKD to A.P.), and the German Federal Ministry of Education and Research (BMBF) (e:Bio young investigator grant AZ 031L0211 to A.P.). Work in the laboratory of C.R.R. was supported by the DFG (FOR 2795 "Synapses under stress", Ro 2327/13-1).

DISCLOSURES:

The authors declare no competing financial or non-financial interests.

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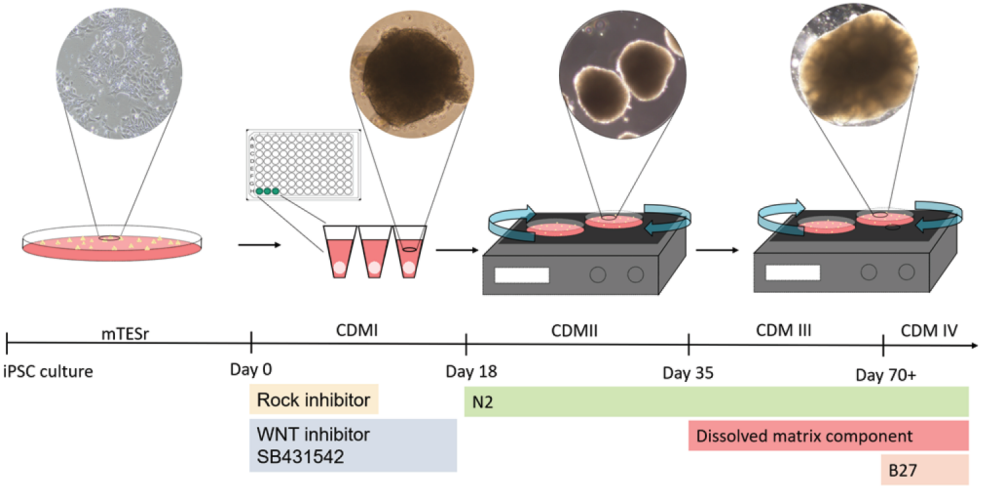
A



B

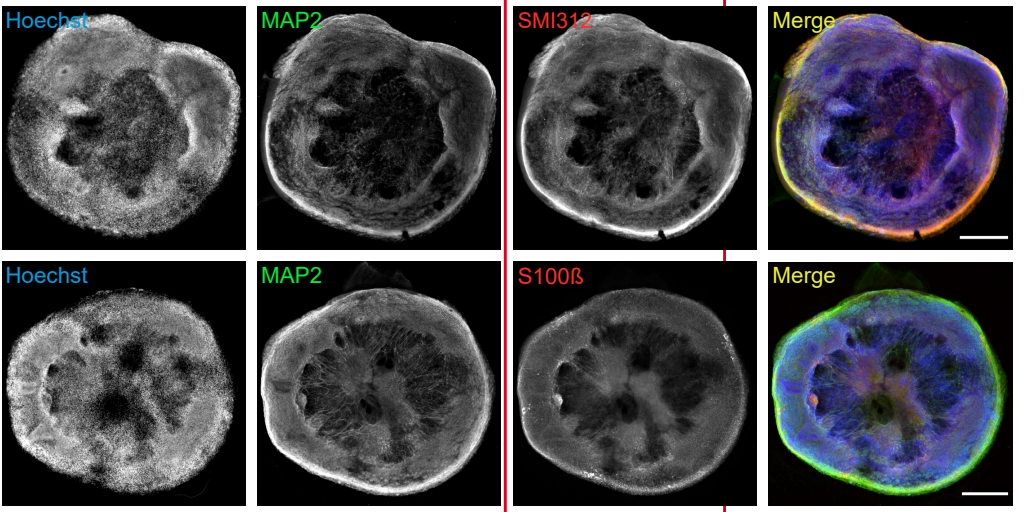
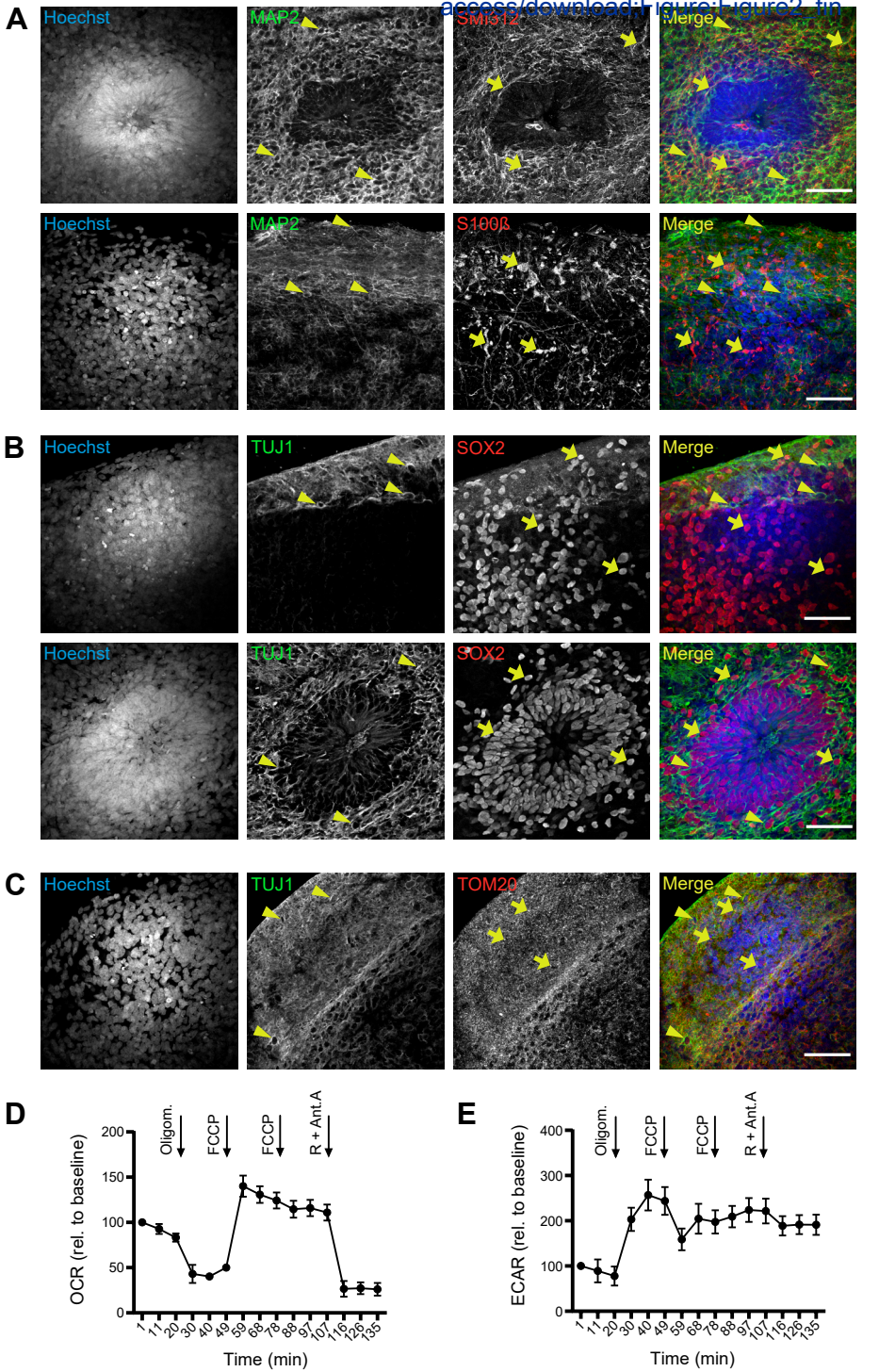


Figure 2



Media Composition

CDMI (Day 0-18)			**Final conc.**
Glasgow-MEM	Gibco	11710-035	[1:1]
Knockout Serum Replacement (KSR)	Gibco	10828010	20%
MEM-NEAA (MEM non-essential amino acid solution)	Gibco	11140-050	0.1 mM
Sodium Pyruvate	Gibco	11360070	1 mM
2-mercaptethanol	Gibco	31350-010	0.1 mM
Penicillin and streptomycin	Gibco	15140-122	100 U/mL and 100 µg/mL

CDMII (Day 18-35)			**Final conc.**
DMEM/F12	Gibco	31330038	[1:1]
Glutamax	Gibco	35050-061	2 mM
N-2 Supplement (100x)	Gibco	17502-048	1%
Chemically Defined Lipid Concentrate	Gibco	11905031	1%
Penicillin and streptomycin	Gibco	15140-122	100 U/mL and 100 µg/mL

CDMIII (Day 35-70)			**Final conc.**
DMEM/F12	Gibco	31330038	[1:1]
Glutamax	Gibco	35050-061	2 mM
N-2 Supplement (100x)	Gibco	17502-048	1%
Chemically Defined Lipid Concentrate	Gibco	11905031	1%
Penicillin and streptomycin	Gibco	15140-122	100 U/mL and 100 µg/mL
Fetal Bovine Serum (FBS)	Gibco	10270-106	10%
Heparin	Merck	H3149-25KU	5 µg/mL
Matrigel	Corning	356231	1%

CDMIV(Day 70+)			**Final conc.**
DMEM/F12	Gibco	31330038	[1:1]
Glutamax	Gibco	35050-061	2 mM
N-2 Supplement (100x)	Gibco	17502-048	1%
Chemically Defined Lipid Concentrate	Gibco	11905031	1%
Penicillin and streptomycin	Gibco	15140-122	100 U/mL and 100 µg/mL
Fetal Bovine Serum (FBS)	Gibco	10270-106	10%
Heparin	Merck	H3149-25KU	5 µg/mL
Matrigel	Corning	356231	2%
B-27 supplement with Vitamin A 50x	Gibco	17504044	2%

Neuronal Medium			**Final conc.**
DMEM/F12	Gibco	31330038	[1:1]
N-2 supplement	Gibco	17502048	[1x]
B-27 supplement with vitamin A 50x	Gibco	17504044	[1x]
L-Ascorbic acid	Sigma Aldrich	A92902	[200 µM]

db-cAMP (dibutyryl cyclic adenosine monophosphate)	Sigma Aldrich	D0627	500 µM
BDNF (brain-derived neutrotrophic factor)	MACS Miltenyi	130-093-811	[10 ng/mL]
GDNF (glial cell line-derived neurotrophic factor)	MACS Miltenyi	130-096-290	[10 ng/mL]
Human TGF-β3 (transforming growth factor-beta3)	MACS Miltenyi	130-094-007	[1 ng/mL]

Assay Medium			**Final conc.**
Seahorse XF DMEM medium	Seahorse Bioscience	103680-100	500 mL
Sodium Pyruvate	Gibco	11360070	1 mM
L-Glutamine	Lonza	BEBP17-605E	2 mM
Glucose	Sigma Aldrich	50-99-7	10 mM

Blocking Solution			**Final conc.**
PBS-Tween			[1:1] 0.1% Tween
Donkey Serum	Sigma Aldrich	D9663	10%
Triton-X	Merck	X100-5ML	1%

Table 2

Initialization	Baseline (X3)	Oligomycin Injection (X3)
Calibrate	Mix (04:00)	Mix (04:00)
Equilibrate (12:00)	Wait (02:00)	Wait (02:00)
	Measure (03:00)	Measure (03:00)

FCCP Injection (X3)	FCCP Injection (X3)	Anti A + Rot Injection (X3)
Mix (04:00)	Mix (04:00)	Mix (04:00)
Wait (02:00)	Wait (02:00)	Wait (02:00)
Measure (03:00)	Measure (03:00)	Measure (03:00)

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hhu Heinrich Heine Universität Düsseldorf

Department of General Pediatrics, Neonatology, and Cardiology
Children's Hospital, Building 13.41, Level 3, Room 29

Vidhya Iyer, Ph.D.
Review Editor
JoVE

May 17, 2021

Dear Dr. Iyer,

On behalf of all authors, I thank you for considering our review manuscript and for sending it for revision. The input provided by the reviewers was indeed critical and it allowed us to significantly improve our manuscript.

We have responded to all the editorial concerns and points raised by the reviewers, and prepared a new version of the manuscript R1. We used the red font to indicate the revised portions in the manuscript.

Our point-by-point response to the reviewers' comments is provided below in blue font.

We hope that you will now be willing to consider publishing our work in *JoVE* and look forward to your reply.

Sincerely yours,
Alessandro Prigione

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Point-by-point response

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

We proofread the text.

2. Please provide an email address for each author.

We included the email addresses.

3. Please revise the following lines to avoid overlap with previously published work: 44-46, 288-290, 298-302, 317-323, 335-336, 339-346, 355-360, 363-364, 367-372

We revised the text.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: Matrigel; Accutase; falcon tube; Glasgow-MEM; Knockout Serum Replacement (KSR); Glutamax; Benchkote®; Superfrost Plus slides; Trition-X; Nikon Eclipse 90i; Nikon, Duesseldorf, Germany; DS-Q1Mc camera; Nikon, Duesseldorf, Germany; NIS-Elements Advanced Research 3.2 software; Nikon, Duesseldorf, Germany; ImageJ software; NIH, Bethesda, MD (although this is open-source; mention only name “ImageJ”); Nikon Eclipse C1, Nikon, Duesseldorf, Germany; Nikon, Duesseldorf, Germany; Silver Version software; Nikon Instruments, Japan; Affinity Designer; Serif Europe Ltd, Nottinghamshire, UK; Worthington papain solution; Worthington DNase solution; Matrigel-coated XF96 cell culture microplate; XFe96 FluxPak; XF Calibrant Solution; Seahorse XFe96 Analyzer; Seahorse XF DMEM medium; XF Assay Medium; XFe96 sensor cartridge; CyQUANT®etc

We modified the text to use generic terms.

5. Please consider providing solution composition only in Tables in separate .xls or .xlsx files uploaded to your Editorial Manager account. These tables can then be referenced in the protocol text. Remove the composition from lines 112-115; 224; 310-312

We modified the text and included the information in separate excel tables.

6. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We revised the text.

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions

should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

We revised the text.

8. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We revised the text to include as much details as possible.

9. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

We simplified the steps.

10. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a one line space between each protocol step and then highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. PLEASE LEAVE A ONE-LINE SPACE BETWEEN THE STEPS AND NOTES. AFTER ENSURING YOU HAVE ONLY 2-3 ACTIONS PER STEP AND NOTES ARE SEPARATED FROM STEPS, HIGHLIGHT ONLY STEPS (NOT NOTES) TO ENSURE YOU DO NOT EXCEED 3 PAGES OF HIGHLIGHTED TEXT.

We revised the text. We highlighted in yellow the 3 pages for inclusion in the video.

11. Please consider separating representative results from discussion so that it’s a little easier to read. The legends section will then go between the results and discussion sections.

We reorganized the sections as suggested.

12. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (italics). Volume (bold) (Issue), FirstPage–LastPage (YEAR).] For 6 and more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate the journal names.

We revised the reference format.

13. Please sort the Materials Table alphabetically by the name of the material.

We revised the Materials table and sorted the names alphabetically.

Reviewers' comments:

Reviewer #1:
Comments
The protocol will be a good addition to evolving field of brain organoids. I only have several questions to the authors.
- Reproducibility in independent cell lines. Would like to see how consistent this protocol would be in 2-3 different hPSC lines.

We included a sentence regarding the reproducibility in different human iPSC lines derived from controls and patients (lines 567-568).

- Some labs use feeder cells (MEF, SNL) to maintain hPSCs. Could you comment if the presence of feeders might render the process of organoid formation ineffective. If so, any recommendation to users?

We included a note regarding feeder cultures (lines 121-122).

- How uniform these organoids are? Could you quantify patterns of morphology (size, layer) and molecular markers in each organoids (-30?) and demonstrate uniformity?

We did not systematically quantify the uniformity of the organoids. We included a section in the discussion to address this point (lines 598-602).

- For future study, could you comment if it could be possible to generate organoids from a single cell of hPSC per well? It would be useful for genetic screening with CRISPR. I guess that current protocol still needs further optimization though. It would be encouraging for users to be shown the direction of the evolution of brain organoid technology.

We included a section in the discussion to address this point (lines 602-604).

Reviewer #2:
Manuscript Summary:
In this manuscript, titled "Generation of human brain organoids for mitochondrial disease modeling," the authors describe their technique in generating iPSC-derived brain organoids designed to study mitochondrial disorders and their neurodevelopmental consequences. The authors have adequately described in detail their technique, as well as disclosed the materials needed for this protocol. The major advantage to this proposed protocol is that the organoids are not required to be manually embedded in a scaffolding matrix. Another advantage of these organoids, aside from morphological study, is that they can be used to assess mitochondrial metabolism in terms of oxygen consumption rate and extracellular acidification rate, which the authors demonstrated well. Taking the above-mentioned into account, we recommend the production of video and publication of this manuscript, with a minor revision.

Major Concerns:
None.

Minor Concerns:
1. Please add number of organoids (n=?) used to plot Figures 2D and E to the figures and to the figure legends.

We defined the number of organoids used for Figures 2D-E (lines 551-553).

2. Please discuss the limitations of your protocol. One limitation for example is the absence of vasculature and microglia in the produced brain organoids. Discuss how the limitations affect the interpretation of results.

We included a discussion on how these limitations may affect the interpretation of results (lines 594-598).

3. Minor grammatical errors throughout the manuscript would need to be corrected such as consider revising the sentence on page lines 78-81 (too lengthy).

We corrected the typos and grammatical errors and we revised the mentioned sentence (lines 87-92).

Reviewer #3:
Manuscript Summary:
Manuscript of Li et al. entitled "Generation of human brain organoids for mitochondrial disease modeling" provides detailed protocol for the robust and reproducible generation of human iPSC-derived brain organoids and for their use in mitochondrial bioenergetics profiling and in-depth imaging analyses. Derivation of brain organoids is already well known and common procedure in many laboratories, however efficacy of the presented protocol is unquestioned, as for the first time it is adjusted to screen bioenergetics in brain organoids. The authors, are well experienced, working with modeling of mitochondrial disorders with induced pluripotent stem cells and patient-derived brain organoids - thus provided sufficient introduction and unbiased discussion for the presented protocol, especially regarding the use of this protocol to investigate metabolic and developmental dysfunctions in human 3D culture model. Couple of minor concerns are below.

Major Concerns:
No major concerns

Minor Concerns:
1) Page 2 lines 82-84. The sentence regards reconstruction - to long difficult to follow.

We revised the sentence (lines 93-96).

2) Page 9, lines 402-403. "Interconnection" between neuronal and glial cells cannot be seen on the level of magnification presented in the manuscript. I would rather say "mutual occurrence"

We modified the text as suggested (line 488).

3) In discussion please refer to other tools than bioengineering profiling and in-depth imaging used for analyzing the role of mitochondria in health, development and disease as presented in recent review Liput at al., Dev Neurobiol. 2021 Mar 16. doi: 10.1002/dneu.22818.

We included the mentioned reference (reference 15). Thanks for bringing this to our attention.

3) Page 10. In the Fig1 legend: "dissolved Matrigel" should be mentioned in description

We included a sentence in the legend of Figure 1 to clarify this point (lines 522-523) and included a more clear description of when the matrix component (i.e. Matrigel) is added to the cultures (lines 203-210).

Reviewer #4:
Manuscript Summary:
This manuscript describes in detail the generation of brain organoids for the modelling of mitochondrial disorders. bestie teh generation of the organoids they describe in detail how to assess the bioenergetic level of these brain organoids,

Major Concerns:
There are no main concerns. The protocol is clearly written and contains all necessary details in order to reproduce the protocol.