FINAL SCRIPT: APPROVED FOR FILMING

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Title: Modeling Mitochondrial Disease Using Brain Organoids: A Focus on Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-Like Episodes

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **NO**

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**

4. **Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **Yes**

Current Protocol Length
Number of Steps: 23
Number of Shots: 56

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

INTRODUCTION:

1.1. **Shihori Kawano:** The scope of our research is to understand the pathophysiology of a mitochondrial disease, MELAS, and to find drugs to treat it.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:5.4*

1.2. **Shihori Kawano:** The challenge is to retain the capacity for high-throughput assays, even though the technique contains organoid formation.

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

CONCLUSION:

1.3. **Chika Saegusa:** Our results suggest that patient-derived iPSC-based organoid models represent a useful platform for studying MELAS mechanisms and for drug screening.

1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:5.2*

1.4. **Chika Saegusa:** Our conventional methods, with the short culture time and cost-effectiveness offer practical tools for investigating the pathophysiology of MELAS.

1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

1.5. **Masato Fujioka:** We would like to search for the potential drugs for MELAS by screening compounds with patient-derived brain organoids in the future.

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

Videographer: Obtain headshots for all authors available at the filming location.

Testimonial Questions (OPTIONAL):

Videographer: Please capture all testimonial shots in a wide-angle format with sufficient headspace, as the final videos will be rendered in a 1:1 aspect ratio. Testimonial statements will be presented live by the authors, sharing their spontaneous perspectives.

How do you think publishing with JoVE will enhance the visibility and impact of your research?

1.6. **Shihiori Kawano, Medical student , Kitasato University School of Medicine**: (authors will present their testimonial statements live)

Can you share a specific success story or benefit you've experienced—or expect to experience—after using or publishing with JoVE? (This could include increased collaborations, citations, funding opportunities, streamlined lab procedures, reduced training time, cost savings in the lab, or improved lab productivity.)

1.7. **Shihiori Kawano, Medical student , Kitasato University School of Medicine** : (authors will present their testimonial statements live)

Ethics Title Card

This research has been approved by the Ethics Committee at Kitasato University School of Medicine

2. Generation of Cerebral Organoids and Early Differentiation

Demonstrator: Shihori Kawano

2.1. To begin, remove the medium from induced pluripotent stem cell culture plates when they are approximately 70 to 80 percent confluent **[1].** Wash the cells with PBS **[2]**. Add 350 microliters of enzyme solution to the cells and incubate **[3-TXT]**.

2.1.1. WIDE: Talent removing the medium from induced pluripotent stem cell culture plates.

2.1.2. Talent washing the cells with phosphate-buffered saline. **Videographer's NOTE:** 2.1.2 is mis-slated as 2.2.2 (filename: A004_11031226_C022.mov)

2.1.3. Talent adding 350 µL enzyme solution to the culture plates. **TXT: Incubation: 37°C, 5 min**

2.2. Add 800 microliters of STEMFit *(Stem-fit)* medium to stop the reaction **[1]**. After transterring the cell suspension to a fresh tube **[2]**, centrifuge it at 190 *g* for 5 minutes at 22 degrees Celsius **[3]**. Next, resuspend the pellet in 1 milliliter of differentiation medium **[4-TXT]**. **NOTE:** VO added for the extra shot

2.2.1. Talent adding 800 microliters of STEMFit medium to the culture plates.

2.2.1a - Added shot - Collecting medium into tube (filename: A004_11031236_C025.mov)

2.2.2. Talent placing the cell suspension in the centrifuge and starting the run.

2.2.3. Talent resuspending the pellet in 1 milliliter of differentiation medium with inhibitors. **TXT: Differentiation Medium: 30 µM ROCK inhibitor, 5 µM SB431542, and 2.5 µM IWP-2**

2.3. After counting the cells, seed 3,000 cells in 100 microliters into each well of a U-shaped, 96-well, low-attachment plate **[1]**. Incubate for 6 days at 37 degrees Celsius with 5 percent carbon dioxide **[2]**.

2.3.1. Talent seeding cells into a 96-well, low-attachment plate.

2.3.2. Talent placing the plate inside the incubator.

2.4. After incubation, use a wide-bore pipette to transfer the organoids from the 96-well plate into a 10-centimeter dish **[1]**. Pipette 50 microliters of supplemented DMEM/F12 *(D-M-E-M-F-Twelve)* medium to each well of a new 96-well plate **[2-TXT]**.

2.4.1. Talent transferring organoids into a 10-centimeter dish using a wide-bore pipette.

2.4.2. Talent adding 50 microliters of supplemented DMEM medium into each well of a new 96-well plate. **TXT: DMEM/F12: % N2, 1% glutamine substitute, 1% NEAA, 2.5 µM IWP-2, 5 µg/mL heparan sulfate, and 1% PS**

2.5. Collect the organoids into 2-milliliter tubes **[1]**. Remove the medium carefully without aspirating the organoids and add 1.5 milliliters of supplemented DMEM/F12 medium **[2-TXT]**.

2.5.1. Talent transferring organoids into 2 milliliter tubes.

2.5.2. Talent carefully removing the medium and adding fresh Dulbecco's Modified Eagle Medium/F12. **TXT: Repeat wash 2 x**

2.6. After pipetting out the last wash, add 500 microliters of fresh medium **[1]**. Then transfer the organoids to a 10-centimeter dish **[2].** Add 5 milliliters of medium to facilitate picking each organoid **[3]**.

2.6.1. Talent adding 500 microliters of fresh medium to tubes containing organoids.

2.6.2. Talent transferring the organoids to a 10-centimeter dish.

2.6.3. Talent adding 5 milliliters of medium.

2.7. Transfer all organoids into each well of a 96-well plate using a micropipette set to 50 microliters**[1]**.

2.7.1. Talent picking up one organoid with a micropipette set to 50 microliters.

2.8. Collect the organoids into 2-milliliter tubes and wash 3 times with DMEM/F12 **[1]**. Then resuspend the organoids in differentiation medium **[2]**.

2.8.1. Talent transferring organoids into 2 milliliter tubes and adding DMEM/F12 to the tubes.

2.8.2. Talent resuspending the organoids in differentiation medium with supplements.
AND
TEXT ON PLAIN BACKGROUND:
Differentiation Medium
1:1 DMEM/F12 and neurobasal
1% N2 supplement
2% B27 without vitamin A
100 µM 2-mercaptoethanol (ME)
2.5 µg/mL insulin, 1% glutamine substitute
0.5% NEAA
1% basement membrane extract
Video Editor: Please play both shots side by side

2.9. Transfer approximately 10 organoids per well into a 6-well plate and adjust the final volume of medium in each well to 2 milliliters **[1]**. Culture the plate statically for 6 days at 37 degrees Celsius with 5 percent carbon dioxide **[2]**.

2.9.1. Talent transferring approximately 10 organoids into each well of a 6-well plate and adjusting volume to 2 mL/well.

2.9.2. Talent placing the 6-well plate inside the incubator.

2.10. On the fifteenth day, collect the organoids into 15-milliliter tubes **[1].** After letting the tubes to settle, add fresh differentiation medium supplemented with B27 *(B-Twenty-seven)* and vitamin A after pipetting out the supernatant **[2]**.

2.10.1. Talent transferring organoids into 15 milliliter tubes.

2.10.2. Talent adding fresh differentiation medium with vitamin A supplement.

2.11. Transfer approximately 10 organoids per well into a 6-well plate **[1]**. Culture the organoids statically for 15 days at 37 degrees Celsius with 5 percent carbon dioxide **[2-TXT]**.

2.11.1. Talent transferring organoids into a 6-well plate.

2.11.2. Talent placing the plate into the incubator. **TXT: Replace medium every 5 - 7 days until Day 30**

3. Dissociation of Cerebral Organoids and Seeding for 2D Neuronal Culture

3.1. Coat the culture plates with the dilute poly-L-ornithine solution **[1-TXT].** Incubate the plates overnight at 37 degrees Celsius **[2]**. The next day, wash the wells 3 times for 5 minutes each, with PBS **[3]**.

3.1.1. Talent coating culture plates with the diluted poly-L-ornithine. **TXT: polu-L-Ornithine : Diluted 1: 5 in water**

3.1.2. Talent placing the coated plates in the incubator.

3.1.3. Talent adding PBS to the wells.

3.2. Now add diluted laminin solution to the wells **[1-TXT]**. Incubate the plates for 3 hours at 37 degrees Celsius or overnight **[2]**.

3.2.1. Talent adding laminin solution to the wells. **TXT: Laminin : Diluted 1:1000 in PBS**

3.2.2. Talent placing the plates in the incubator for incubation.

3.3. Centrifuge the organoid suspension at 200 *g* for 5 minutes **[1]**. Remove the supernatant then add 1 milliliter of dispersion solution and mix well **[2]**.

3.3.1. Talent centrifuging the organoid suspension.

3.3.2. Talent removing the supernatant.

~~3.3.3. Talent adding 1 mL dispersion solution.~~

~~3.3.4. Shot of the mixture being pipetted up and down to mix.~~ **NOTE**: 3.3.3 AND 3.3.4 do not have any VO. Must be here by mistake - Wrong content - "take 2 real" should not be used (A004_11031439_C053.mov)

3.4. Carefully layer the isolation solution beneath the suspension **[1]**. Then centrifuge the suspension at 200 *g* for 5 minutes **[2]**.

3.4.1. Talent layering isolation solution beneath the cell suspension.

3.4.2. Talent placing the tube in a centrifuge.

3.5. Now, pipette 1 milliliter of neuronal medium into the tube **[1]**. Mix the solution and pass the suspension through a 70-micrometer strainer into a 50-milliliter tube **[2]**.

3.5.1. Talent adding neuronal medium and mixing the suspension.
AND
TEXT ON PLAIN BACKGROUND:
Neuronal Medium:
Neurobasal with 1% B27
0.25% glutamine substitute
1% Penicillin-Streptomycin solution
Video Editor: Please play both side by side on split screen

3.5.2. Talent passing the suspension through a 70-micrometer strainer into a 50 milliliter tube.

3.6. After counting the cells, seed the cell suspension at a density of 100,000 cells per square centimeter on poly-L-ornithine and laminin-coated plates **[1]**. Replace the medium every 4 to 7 days until Day 60 **[2]**.

3.6.1. Talent seeding cells into coated plates.

3.6.2. Talent replacing the medium in culture plates.

4. Fixation and Collection of Cerebral Organoids and Cells for Immunofluorescence and RT-qPCR

4.1. To fix the organoids for immunofluorescence, first harvest the organoids into microtubes **[1]**. Rinse the organoids with PBS **[2]**. Then fix the organoids in 4 percent paraformaldehyde for 30 minutes at room temperature **[3]**.

4.1.1. Talent transferring organoids into labeled microtubes.

4.1.2. Talent pipetting PBS into tubes with the organoids.

4.1.3. Talent adding paraformaldehyde to the tubes and leaving them at room temperature.

4.2. Post fixation, wash the fixed organoids 3 times with PBS **[1]**. Store the organoids at 4 degrees Celsius until use **[2]**.

4.2.1. Talent pipetting PBS into tubes with the organoids.

4.2.2. Talent placing the microtubes with fixed organoids in cold storage.

4.3. Thirty days after seeding for 2D-neuronal culture, remove the culture medium from the plates **[1].** Then wash the cells with PBS **[2].** Fix the cells with 4 percent paraformaldehyde for 15 minutes at room temperature **[3]**.

4.3.1. Shot of the culture medium being pipetted out of the cell-culture plate.

4.3.2. Talent pipetting PBS into the plates.

4.3.3. Talent adding paraformaldehyde to the plates.

4.4. Pipette out the paraformaldehyde **[1].** Then wash the cells with PBS 3 times **[2]**. Store the fixed cells at 4 degrees Celsius until use **[3]**.

4.4.1. Talent removing paraformaldehyde .

4.4.2. Talent adding PBS to the plates.

4.4.3. Talent placing culture plates with fixed cells in cold storage.

4.5. To collect the organoids for RT-qPCR, transfer the organoids into microtubes **[1]**. Pipette PBS into the tubes to wash the organoids **[2]**.

4.5.1. Talent transferring organoids into labeled microtubes.

4.5.2. Talent pipetting PBS into tubes with the organoids.

4.6. Now add 350 microliters of lysis buffer to the organoids **[1].** Then snap-freeze them in liquid nitrogen **[2]**. Store the tubes at minus 80 degrees Celsius until use **[3]**.

4.6.1. Talent adding 350 µL lysis buffer to the tubes.

4.6.2. Talent immersing tubes in liquid nitrogen.

4.6.3. Talent transferring the frozen organoids to a minus 80 degrees Celsius freezer.

Results

5. Results

5.1. Brain organoids containing FOXG1 *(fox-G-One)*-positive neurons were successfully induced from the healthy control iPSC *(i-P-S-C)* line 414C2 *(Four-One-Four-C-Two)* on Day 30 **[1]**, and 2D-cultured neural networks were obtained on Day 60 **[2]**.

5.1.1. LAB MEDIA: Figure 2A,D,E. Video editor: Highlight the images corresponding to 414C2 in A and E and the column for the same in D

5.1.2. LAB MEDIA: Figure 3A.

5.2. Heteroplasmy levels of the m.3243A>G *(M-Dot-Three-Two-Four-Three-A to- G)* variant were confirmed by qPCR **[1]**. Line 2-8 *(Two -To-Eight)* had low heteroplasmy and lines 2-6 *(Two-to-six)* had high heteroplasmy **[2]**.

5.2.1. LAB MEDIA: Figure 4.

5.2.2. LAB MEDIA: Figure 4. *Video editor: Sequentially highlight the bar labelled 2-8 (LH) and then the bar labeleld 2-6 (HH)*

5.3. Brain organoids derived from the low heteroplasmy line 2-8 exhibited sizes and morphologies similar to those from healthy controls **[1]**.

5.3.1. LAB MEDIA: Figure 2A-C. *Video editor: Highlight the organoid images for the 2-8 line and 414C2 line in 2 A and the column for the same in B and C*

5.3.2. LAB MEDIA: Figure 2C. Video editor: Highlight the bar for 2-8 showing circularity.

5.4. Organoids generated from the high heteroplasmy line 2-6 displayed reduced size of 0.3 square millimeter **[1]** and irregular morphology with a circularity value of 0.82 **[2]**.

5.4.1. LAB MEDIA: Figure 2B. *Video editor: Highlight the bar for 2-6 showing reduced organoid size.*

5.4.2. LAB MEDIA: Figure 2C. *Video editor: Highlight the bar for 2-6 showing circularity*.

5.5. Most neurons induced from the 414C2 line and the 2-8 line were FOXG1-positive on Days 30 and 60 **[1]**. FOXG1-positive neurons were rarely detected in organoids or 2D-cultured neurons derived from the 2-6 line **[2]**.

5.5.1. LAB MEDIA: Figure 2D,E and Figure 3A,B. *Video editor: Highlight the images and columns for 414C2 and 2-8 lines.*

5.5.2. LAB MEDIA: Figure 2D,E and Figure 3C. *Video editor: Highlight the images and columns for 2-6 lines*.

- **induced**

Pronunciation link: https://www.merriam-webster.com/dictionary/induced
IPA: /ɪnˈdjuːst/
Phonetic Spelling: in-dyoo-st

- **pluripotent**

Pronunciation link: https://www.merriam-webster.com/dictionary/pluripotent
IPA: /plʊˈrɪpətənt/ or /ˌplʊˈrɪpəˌtənt/
Phonetic Spelling: ploo-RIP-uh-tuhnt

- **stem cell**

Pronunciation link: https://www.merriam-webster.com/dictionary/stem%20cell
IPA: /ˈstɛm ˌsɛl/
Phonetic Spelling: STEM sell

- **organoid**

Pronunciation link: https://www.merriam-webster.com/dictionary/organoid
IPA: /ˈɔːrgəˌnɔɪd/ (American)
Phonetic Spelling: OR-guh-noid

- **centrifuge**

Pronunciation link: https://www.merriam-webster.com/dictionary/centrifuge
IPA: /ˈsɛntrəˌfjuːdʒ/
Phonetic Spelling: SEN-truh-fyoohj

- **neurobasal**

Pronunciation link: No confirmed link found
IPA: /ˌnjʊroʊˈbeɪsəl/
Phonetic Spelling: noo-roh-BAY-suhl

- **heparan** (as in heparan sulfate)

Pronunciation link: https://www.merriam-webster.com/dictionary/heparan
IPA: /ˈhɛpəˌræn/
Phonetic Spelling: HEP-uh-ran

- **heteroplasmy**

Pronunciation link: https://www.merriam-webster.com/dictionary/heteroplasmy
IPA: /ˌhɛtəroʊˈplæzmi/
Phonetic Spelling: HEH-tuh-roh-PLAZ-mee

- **paraformaldehyde**

Pronunciation link: https://www.merriam-webster.com/dictionary/paraformaldehyde
IPA: /ˌpærəˌfɔːrˈmældəˌhaɪd/
Phonetic Spelling: par-uh-FOR-mal-duh-hyde

- **microliter** (µL)

Pronunciation link: https://www.merriam-webster.com/dictionary/microliter
IPA: /ˈmaɪkroʊˌliːtər/
Phonetic Spelling: MY-kroh-lee-ter