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Title: Three-Dimensional Motor Nerve Organoid Generation

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Author Questionnaire

1. Microscopy: Does your protocol demonstrate the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or similar? **N**

2. Software: Does the part of your protocol being filmed demonstrate software usage? **N**

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **35**

Introduction

1. Introductory Interview Statements

REQUIRED:

1. **Tatsuya Osaki**: This protocol facilitates investigation of the mechanisms underlying the development and diseases of axon bundles using motor nerve organoids derived from human iPS cells outside of the body **[1]**.

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

REQUIRED:

2. **Siu Yu A. Chow**: By simple the culture of neuronal spheroids in custom-made micro-culture chips, unidirectional axon bundles can be spontaneously generated and be used for various downstream experiments **[1]**.

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

OPTIONAL:

3. **Jiro Kawada**: Motor nerve organoids can facilitate the screening and testing of drugs for motor neuron diseases, including ALS, by providing a more physiological model than previous in vitro systems **[1]**.

    3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can cut for time*

Protocol

2. Photolithographic SU-8 Mold Fabrication

2.1. In a fume hood and wearing the appropriate PPE (P-P-E), dispense 3 milliliters of SU-8 (S-U-eight) 2100 onto an acetone-cleaned silicon wafer **[1-TXT]** and place the wafer in the center of a spin coater **[2]**.

2.1.1. WIDE: Talent adding SU-8 to wafer

2.1.2. Wafer being placed in center of spin coater NOTE: no slate

2.2. Fix the wafer by vacuum **[1]** and spin the wafer at 500 revolutions per minute for 10 seconds to coat the SU-8 uniformly across the wafer surface **[2]**.

2.2.1. Wafer being fixed

2.2.2. Wafer being spun

2.3. Then spin the wafer at 1500 revolutions per minute for 30 seconds with an acceleration of 300 revolutions/second to obtain a 150-micron-thick layer of photoresist on the wafer **[1]**.

2.3.1. Talent setting spin coater NOTE: included in 2.2.1-2 shot

2.4. After soft baking, set the photomask to the mask aligner **[1-TXT]** and expose the wafer to ultraviolet light for 60 seconds **[2]**.

2.4.1. Talent setting photomask to mask aligner **TEXT: Soft bake: 10 min at 50 °C, 7 min at 65 °C, 45 min 95 °C**

2.4.2. Shot of wafer, then UV light being turned on over wafer

2.5. After baking on a hot plate, develop the wafer for 10-20 minutes in photoresist developer **[1-TXT]** with agitation on an orbital shaker **[added-1a]**, changing the developing solution once during the process **[2]**.

2.5.1. Talent adding developer to wafer **TEXT: Hot plate bake: 6 min at 65 °C, 13 min at 95 °C**

Added shot: 2.5.1 a agitating solution on orbital shaker

2.5.2. Talent changing solution

3. Polydimethlysiloxane (PDMS) Microfluidic-Based Tissue Culture Chip Fabrication

3.1. To prepare the bottom layer of the chip for tissue culture, pour freshly prepared PDMS (P-D-M-S) onto the wafer to the desired thickness **[1-TXT]** and degas the mixture in a vacuum chamber **[2]**.

3.1.1. WIDE: Talent pouring PDMS onto wafer, with PDMS container visible in frame **TEXT: See text for PDMS preparation details**

3.1.2. Talent placing dish into chamber

3.2. Cure the PDMS in an oven for at least 3 hours at 60 degrees Celsius **[1]**. After cooling, use a blade to cut the cured PDMS from the wafer **[2]**.

3.2.1. Talent placing dish into oven

3.2.2. PDMS being cut from wafer

3.3. Then use uncured PDMS with baking to bond a medium reservoir to the PDMS bottom layer to obtain the assembled PDMS tissue culture chip **[1-TXT]**.

3.3.1. The bottom PDMS layer being attached to reservoir, with PDMS container visible in frame **TEXT: See text for reservoir preparation details**

4. PDMS Chip Preparation for Motor Nerve Organoid (MNO) Formation

4.1. To prepare the PDMS chip for motor nerve organoid formation, sterilize the chip and a 76- x 52-millimeter microscope glass in a Petri dish containing 70% ethanol for at least 1 hour **[1]**.

4.1.1. WIDE: Talent adding chip and/or glass to dish, with ethanol container visible in frame

4.2. At the end of the incubation, place the chip on the wet microscope glass **[1]**. After overnight drying, the chip should adhere to the glass **[2]**.

4.2.1. Chip being placed onto glass

4.2.2. Chip being lifted, showing adherence to glass

4.3. Place a 30-microliter droplet of diluted basement membrane matrix in DMEM-F12 (D-M-E-M-F-twelve) on one side of the inlet of the channel **[1]** and aspirate the solution from the other side of the inlet to coat the surface of the microchannel with the matrix **[2-TXT]**.

4.3.1. Droplet being added to side of inlet *Videographer: Important step*

4.3.2. Solution being aspirated *Videographer: Important step* **TEXT: Do not aspirate too much solution to avoid bubble contamination**

4.4. Then incubate the PDMS chip with the coating solution in a Petri dish for 1 hour at room temperature **[1-TXT]**.

4.4.1. Shot of the coated PDMS chip along with the Petri-dish **TEXT: Alternative: Incubate overnight at 4 °C**

5. Induced Pluripotent Stem (iPS) Cell Differentiation

5.1. To induce 3D iPS (eye-P-S) cell differentiation into motor neurons, seed 4 x 10^4 iPS cells/well into the appropriate number of wells of a 96-well U-bottom plate in 100 microliters of feeder- and serum-free cell culture medium supplemented with 10-micromolar Y-27632 (Y-two-seven-six-three-two) **[1-TXT]**.

5.1.1. WIDE: Talent adding cells to well(s), with medium and rock inhibitor containers visible in frame *Videographer: Important step* **TEXT: See text for all medium and solution preparation details**

5.2. The next day, replace the supernatant in each well with 100 microliters of KSR (K-S-R) medium supplemented with 10-micromolar SB431542 (S-B-four-three-one-five-four-two) and 100-nanomolar LDN-193189 (L-D-N-one-nine-three-one-eight-nine) **[1]**.

5.2.1. Medium being added to well(s), with medium, SB431542, and LDN-193189 containers visible in frame

5.3. On days 2 and 3, replace the supernatants with 100 microliters of KSR medium supplemented with 10 micromolar SB431542, 100-nanomolar LDN-193189, 5-micromolar DAPT (D-A-P-T), 5-micromolar SU5402 (S-U-five-four-oh-two), 1-micromolar retinoic acid, and 1-micromolar smoothened agonist **[1]**.

5.3.1. Medium being added to well(s), with medium, SB431542, LDN-193189, DAPT, SU5402, RA, and SAG containers visible in frame

5.4. On days 4 and 5, replace the supernatants with a mixture of 75% of KSR medium and 25% of N2 medium supplemented with 10-micromolar SB431542, 100-nanomolar LDN-193189, 5-micromolar DAPT, 5-micromolar SU5402, 1-micromolar retinoic acid, and 1-micromolar smoothened agonist **[1]**.

5.4.1. Medium being added to well(s), with medium, SB431542, LDN-193189, DAPT, Su5402, RA, and SAG containers visible in frame

5.5. On days 6 and 7, replace the supernatants with a mixture of 50% of KSR medium and 50% of N2 medium supplemented with 5-micromolar DAPT, 5-micromolar SU5402, 1-micromolar retinoic acid, and 1-micromolar smoothened agonist **[1]**.

5.5.1. Medium being added to well(s), with medium, DAPT, SU5402, RA, and SAG containers visible in frame

5.6. On days 8 and 9, replace the supernatants with a mixture of 25% of KSR medium and 75% of N2 medium supplemented with 5-micromolar DAPT, 5-micromolar SU5402, 1-micromolar retinoic acid, and 1-micromolar smoothened agonist **[1]**.

5.6.1. Medium being added to well(s), with medium, DAPT, SU5402, RA, and SAG containers visible in frame

5.7. On days 10 and 11, replace the supernatants with N2 medium supplemented with 5-micromolar DAPT, 5-micromolar SU5402, 1-micromolar retinoic acid, and 1-micromolar smoothened agonist **[1]**.

5.7.1. Medium being added to well(s), with medium, DAPT, SU5402, RA, and SAG containers visible in frame

5.8. On day 12, replace the supernatants in each well with 100 microliters of maturation medium supplemented with 20 nanograms/milliliter of brain-derived neurotrophic factor per well **[1]**.

5.8.1. Medium being added to well(s), with medium and BDNF containers visible in frame

6. MNO Formation

6.1. To induce motor nerve organoid formation, replace the coating solution in the PDMS chip with 150 microliters of maturation medium supplemented with 20 nanograms/milliliter of brain-derived neurotrophic factor **[1]** and use a wide-bore micropipette tip to gently add motor neuron spheroids from one well of the 96-well plate into the inlet of the microchannel of the chip **[2]**.

6.1.1. WIDE: Talent adding medium to chip, with medium and BDNF containers visible in frame *Videographer: Important/difficult step*

6.1.2. Talent adding cells to chip, with 96-well plate visible in frame *Videographer: Important/difficult step*

6.2. Place a small reservoir of sterile water near the tissue culture chip in the dish to prevent medium evaporation **[1]** and place the dish in a 37-degree Celsius and 5% carbon dioxide incubator **[2]**.

6.2.1. Reservoir being placed into dish

6.2.2. Talent placing dish into incubator

6.3. Every 2-3 days, replace half of the exhausted culture medium from the center of the medium reservoir **[0-added]** with fresh maturation medium supplemented with 20 nanograms/milliliter of brain-derived neurotrophic factor **[1]**.

Added: 6.3.0 this step is removing medium not adding. NOTE: Shot as 6.3.1 but should be 6.3.0

6.3.1. Shot of empty central reservoir, then medium being added to reservoir *Videographer: Important step*

6.4. Axons will grow from the motor neuron spheroid into the channel **[1]**, spontaneously assembling into a single bundle over a period of 2-3 weeks to form a motor nerve organoid **[2-TXT]**.

6.4.1. LAB MEDIA: Figure 5A middle image

6.4.2. LAB MEDA: Figure 6B without dashed line and scissors **TEXT: MNO can be cultured >1 mo in device**

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?
4.3., 5.1., 6.1., 6.3.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?
6.1.

Results

7. Results: Representative Large-Scale Radio Array Lunar Surface Imaging Simulation

7.1. Motor neurons differentiate within 12-14 days in 3D differentiation procedures **[1]**.

7.1.1. LAB MEDIA: Figure 4A

7.2. Importantly, more than 60% of the cells express the motor neuron marker HB9 (H-B-nine) during the differentiation **[1-TXT]** and approximately 80% of the cells in the motor neuron spheroid are SMI32 (S-M-eye-thirty-two)-positive **[2-TXT]**.

7.2.1. LAB MEDIA: Figure 5A SMI-32/Hoechst image and Figure 5B TUJ1/HB9/Hoechst image *Video Editor: please emphasize green signal in Figure 5A image* **TEXT: HB: homeobox**

7.2.2. LAB MEDIA: Figure 5A SMI-32/Hoechst image and Figure 5B TUJ1/HB9/Hoechst image *Video Editor: please emphasize pink signal in Figure 5B image* **TEXT: SMI32: selective neurofilament**

7.3. With the microchannels serving as physical guides **[1]**, axons elongate from the motor neuron spheroid to form a bundle by axo-axonal interaction within 24 hours of introduction to the spheroid **[2]**.

7.3.1. LAB MEDIA: Figure 6A *Video Editor: please emphasize microchannel*

7.3.2. LAB MEDIA: Figure 6A *Video Editor: please emphasize spheroid*

7.4. The axons reach the center of the microchannel within the next 3-4 days **[1]**, extending to the other end of the microchannel after an additional 10 days **[2]**.

7.4.1. LAB MEDIA: Figure 6A *Video Editor: please emphasize middle of channel*

7.4.2. LAB MEDIA: Figure 6A *Video Editor: please emphasize non-spheroid end of microchannel*

7.5. Motor nerve organoids can be collected from the chip for biological analysis by detaching the PDMS from the microscope glass **[1]**.

7.5.1. LAB MEDIA: Figure 6B

7.6. The axon bundles and cell bodies can then be dissected and isolated under a microscope using a surgical knife or tweezers **[1]**.

7.6.1. LAB MEDIA: Figure 6B *Video Editor: please add/emphasize dotted line and scissors*

7.7. In axon bundles of motor nerve organoids, dendritic maker proteins are not detected by western blotting **[1]**.

7.7.1. LAB MEDIA: Figure 6C *Video Editor: please emphasize lack of Dendritic marker in Axon lane*

Conclusion

8. Conclusion Interview Statements

8.1. **Joel Hernandez**: Make sure that the spheroids are completely dropped to the bottom of the culture chip. Visual examination from the bottom and sides can help determine the spheroid locations within the chip **[1]**.

8.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (6.1.)

8.2. **Yui Nakanishi**: The isolated axon bundles can be examined by various additional methods and large quantities of axons can be obtained and used for biochemical assays that require substantial materials **[1]**.

8.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can cut for time*