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Title: In Vitro Neuromuscular Junction Induced from Human Induced Pluripotent Stem Cells

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Author Questionnaire

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

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

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

Protocol Length
Number of shots: **26**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Megumu K. Saito**: This human neuromuscular junction induction system can be used to induce the formation of pre- and post- synaptic components, including motor neurons, skeletal muscle, and Schwann cells **[1]**.

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

REQUIRED:

1.2. **Chuang-Yu Lin**: Not only can a mature complex NMJ structure be obtained in single culture dish from a single starting cell population, but the resulting NMJ also possesses the ability to contract **[1]**.

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

OPTIONAL:

1.3. **Megumu K. Saito**: This method has potential for studying pathological mechanisms of muscle contraction disability diseases, such as SMA, and for therapeutic compound screening **[1]**.

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

OPTIONAL:

1.4. **Chuang-Yu Lin**: The differentiated NMJ is a very thick complex tissue and is difficult to visualize. By demonstrating the procedure, we can instruct readers how to identify specific structures **[1]**.

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

Protocol

2. Induced Pluripotent Stem Cell (iPSC) Culture

~~2.1. **[1-TXT] [2].**~~

~~2.1.1. WIDE: Talent adding cells to well(s), with medium container visible in frame **TEXT: See text for all medium and solution preparation details**~~

~~2.1.2. Talent placing plate into incubator~~

2.2. Wash the cells with 3 milliliters of PBS **[1]** before adding 1 milliliter of cell detachment solution to a Petri dish **[2]**.

2.2.1. Well being washed, with PBS container visible in frame NOTE: 2.2.1 and 2.2.2 are both in 2.1.1.

2.2.2. Talent adding solution to dish, with solution container visible in frame

2.3. After 10 minutes at 37 degrees Celsius, add 3 milliliters of primate embryonic stem cell medium to each well **[1]** and gently pipette three times to dissociate the cells from the coverslips **[2]**.

2.3.1. Talent adding medium to well(s), with medium container visible in frame Videographer NOTE: 2.3.1 - 2.3.2: Combined into one shot. Exposure is slightly darker than other shots in the sequence, so please fix in post if required. Filename: A007_08051256_C062.mov

2.3.2. Medium being pipetted

2.4. Next, pool the detached stem cell-containing supernatants into a 50-milliliter conical tube for centrifugation **[1-TXT]** and resuspend the stem cell pellet in 3 milliliters of fresh primate embryonic stem cell medium supplemented with 10-micromolar Y27632 (Y-two-seven-six-three-two) ROCK (rock) inhibitor for counting **[2]**.

2.4.1. Talent adding supernatant to tube(s) **TEXT: 5 min, 160 x g, 4 °C** Videographer NOTE: This content can be found in shot 2.3.1 - 2.3.2

(A007_08051256_C062.mov), as well in separate shots (Filenames: A007_08051300_C063.mov or A007_08051301_C064.mov)

2.4.2. Shot of pellet if visible, then medium being added to tube, with medium and Y27632 containers and hemocytometer visible in frame

2.5. Then dilute the cells to a 2 x 10^5 cells/milliliter of medium supplemented with ROCK inhibitor concentration **[1]** and return 2 milliliters of cells to each coverslip in each well of the extracellular matrix-coated 6-well plate **[2]**.

2.5.1. Talent adding medium to tube, with medium and inhibitor containers visible in frame *Videographer: Important step* Videographer NOTE: 2.5.1 and 2.5.2 are combined into one shot (Filename: A007_08051317_C066.mov)

2.5.2. Talent adding cells to well(s) *Videographer: Important/difficult step*

3. iPSC Differentiation

3.1. After 24 hours, replace the supernatant in each well with 2 milliliters of fresh primate embryonic stem cell medium supplemented with 1 microgram/milliliter of doxycycline **[1]** and return the plate to the cell culture incubator for 24 hours **[2]**.

3.1.1. WIDE: Talent adding medium to well(s), with medium and doxycycline containers visible in frame *Videographer: Important step*

3.1.2. Talent placing plate into incubator *Videographer: Important step*

3.2. At the end of the incubation, replace the supernatants with 2 milliliters of differentiation medium supplemented with doxycycline per well **[1]** and return the plate to the cell culture incubator for 10 days **[2-TXT]**.

3.2.1. Talent adding medium to well(s), with medium and doxycycline containers visible in frame

3.2.2. Talent placing plate into incubator **TEXT: Refresh medium daily**

3.3. At the end of the incubation, replace the supernatants with 2 milliliters of myogenic differentiation medium supplemented with doxycycline per well **[1]** and return the plate to the cell culture incubator for an additional 10 days **[2-TXT]**.

3.3.1. Talent adding medium to well(s), with medium and doxycycline containers visible in frame

3.3.2. Talent placing plate into incubator **TEXT: Refresh medium daily**

3.4. At the end of the incubation, replace the supernatants with 2 milliliters of neuromuscular junction medium per well **[1]** and return the plate to the cell culture incubator for 30 days **[2-TXT]**.

3.4.1. Talent adding medium to well(s), with medium and doxycycline containers visible in frame

3.4.2. Talent placing plate into incubator **TEXT: Refresh medium every 3-4 d**

3.5. At the end of the incubation, visualize the differentiated neuromuscular junctions by inverted microscopy **[1]**.

3.5.1. Talent at microscope, looking at cells

4. Neuromuscular Junction (NMJ) Muscle Contraction and Curare Treatment

4.1. To trigger the myotube contractions, add 25-millimolar calcium chloride to each well of differentiated neuromuscular junction cells **[1]** and, within 1-2 minutes of treatment, place the plate on the stage of an inverted microscope **[2]**.

4.1.1. WIDE: Talent adding CaCl2 to well(s), with CaCl2 container visible in frame *Videographer: Important step*

4.1.2. Talent placing plate onto microscope stage, with monitor visible in frame *Videographer: Important step*

4.2. Using live cell microscopy, record a movie of the myotube contraction for 20 seconds **[1]**.

4.2.1. Talent starting recording, with monitor visible in frame *Videographer: Important step*

4.3. At the end of the recording, add 300 nanograms/milliliter of curare to the culture medium **[1]** and record the movie for another 20 seconds **[1]** before opening the movie file in an appropriate motion vector analysis software program for analysis **[2]**.

4.3.1. Talent adding curare to well(s), with curare container visible in frame *Videographer: Important step*

4.3.2. SCREEN: Movie acquisition: 00:03-00:09

4.3.3. SCREEN: Movie analysis: 00:04-00:19 *Video Editor: please speed up*

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?

2.5., 3.1., 4.1.-4.4.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?
2.5. The well attachment of stem cells on coverslip influences the outcome of differentiation. We can prepare more batches of samples to increase the success possibility.

Results

5. Results: Representative NMJ Phenotypic, Morphological, and Functional Characterization

5.1. Immunofluorescence staining **[1]** of the neurofilaments synaptic vesicles **[2]** and acetylcholine receptors can be performed to identify the neurons **[3]**.

5.1.1. LAB MEDIA: Figures 1B-1D
5.1.2. LAB MEDIA: Figures 1B-1D *Video Editor: please emphasize green signal in Figure 1D*
5.1.3. LAB MEDIA: Figures 1B-1D *Video Editor: please emphasize red signal in Figure 1D*

5.2. Motor neurons can be identified **[1]** by Tuj1 (T-U-J-one) and Islet 1 staining **[2-TXT]**, while post-synaptic myotubes can be identified by staining for myosin heavy chain **[3]**.

5.2.1. LAB MEDIA: Figures 1G-1I *Video Editor: please emphasize green signal in Figure 1G*
5.2.2. LAB MEDIA: Figures 1G-1I *Video Editor: please emphasize red signal in Figure 1H* **TEXT: Tuj1: class III beta-tubulin**
5.2.3. LAB MEDIA: Figure 1G-1I *Video Editor: please emphasize blue signal in Figure 1I*

5.3. Schwann cells can be labeled with S-100 antibody **[1]**.

5.3.1. LAB MEDIA: Supplementary Figure 2 *Video Editor: please emphasize green signal in Supplementary Figure 2A*

5.4. In these scanning electron microscopy images, the morphology of mature neuromuscular junctions can be observed **[1]**, with clear visualization of the expanded axon terminals **[2]**, axons **[3]** and muscle fibers **[4]**.

5.4.1. LAB MEDIA: Figures 2A and 2B
5.4.2. LAB MEDIA: Figures 2A and 2B *Video Editor: please emphasize Axon terminal texts/regions indicated by axon terminal text*
5.4.3. LAB MEDIA: Figures 2A and 2B *Video Editor: please emphasize Axon texts/regions indicated by axon text*
5.4.4. LAB MEDIA: Figures 2A and 2B *Video Editor: please emphasize Mf texts/regions indicated by Mf text*

5.5. Transmission electron microscopy can be used to visualize the mature ultrastructure of the neuromuscular components **[1]**, including the pre-synaptic axon terminal with

synaptic vesicles **[2]** and the post-synaptic region, which is separated by the synaptic cleft **[3]**.

5.5.1. LAB MEDIA: Figures 2C-2E
5.5.2. LAB MEDIA: Figures 2C-2E *Video Editor: please emphasize Sv texts/regions indicated by Sv text and red arrow heads*
5.5.3. LAB MEDIA: Figures 2C-2E *Video Editor: please emphasize Sc texts/regions indicated by Sc text and pink arrows*

5.6. Junctional folds mark the junction between the neuron and muscle fibers **[1]**.

5.6.1. LAB MEDIA: Figures 2C-2E *Video Editor: please emphasize Jf text below yellow dotted line*

5.7. Here mature axon terminals that contain synaptic vesicles are shown **[1]**.

5.7.1. LAB MEDIA: Figures 2C-2E *Video Editor: please emphasize yellow arrows*

5.8. Taken together, these morphological features indicate that the neuromuscular components were well induced and matured **[1]**.

5.8.1. LAB MEDIA: Figures 2C-2E

5.9. Functional assessment of the cells reveals that prominent neuromuscular junction motion signals can be induced by calcium chloride **[1]** and that these contractions can be interrupted by curare, confirming that the motor neuron signals are transmitted through the neuromuscular junction to trigger muscle contraction **[2]**.

5.9.1. LAB MEDIA: Supplemental Movie 1: 00:00-00:05
5.9.2. LAB MEDIA: Supplemental Movie 1: 00:00-00:07

Conclusion

6. Conclusion Interview Statements

6.1. **Chuang-Yu Lin**: The cell seeding density influences the efficiency of the NMJ formation. It can be modified to adapt the protocol according to the experimental purposes **[1]**.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (2.5.)

6.2. **Megumu K. Saito**: This NMJ culture contains multiple cell types and NMJ components and can be used to study the interactions between these cells during NMJ development or in response to specific disease pathologies **[1]**.

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