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Title: Sarcomere Shortening of Pluripotent Stem Cell-Derived Cardiomyocytes Using Fluorescent-Tagged Sarcomere Proteins

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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 similar? **N**

2. Software: Does the part of your protocol being filmed demonstrate software usage? **Y**
Videographer: All screen capture files provided, do not film

3. Interview statements: Considering the Covid-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

☒ Interviewees wear masks until the videographer steps away (≥6 ft/2 m) and begins filming. The interviewee then removes the mask for line delivery only. When the shot is acquired, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

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

Protocol Length
Number of Shots: **36**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Nawin Chanthra**: It is difficult to assess sarcomere function in pluripotent stem cell-derived cardiomyocytes due to their underdeveloped and disorganized sarcomeres. Using fluorescent-tagged sarcomere proteins, we can now assess sarcomere shortening **[1]**.

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

REQUIRED:

1.2. **Razan E. Ahmed**: We can visualize sarcomeres and assess their in vitro function using stem cell-derived cardiomyocytes and fluorescent-tagged sarcomere proteins. Using AAV-based transduction, we can expand the method to any patient-derived iPSCs **[1]**.

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

OPTIONAL:

1.3. **Hideki Uosaki**: This method can be extended to the development of cardiomyopathy therapies, as it can be used to directly assess disease-related sarcomere dysfunction in vitro **[1]**.

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

OPTIONAL:

1.4. **Hideki Uosaki**: This method is useful in the cardiology field, including pediatric cardiology studies. However, it can also be applied to the study of skeletal muscle dysfunctions, such as myopathy **[1]**.

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

Protocol

2. Human Pluripotent Stem Cell Differentiation

2.1. On day minus 4 of differentiation induction, coat a 6-well plate with 0.5 micrograms/square-centimeter of laminin 511-E8 (five-one-one-E-eight) diluted in PBS for a 30-minute incubation at 37 degrees Celsius and 5% carbon dioxide **[1]**.

2.1.1. WIDE: Talent adding laminin to well(s), with laminin container visible in frame

2.2. Nearing the end of the incubation, treat the human induced pluripotent stem cells with recombinant trypsin for 3 minutes at 37 degrees Celsius **[1]**.

2.2.1. Talent adding trypsin to plate, with trypsin container visible in frame

2.3. When the cells have detached, harvest cells to medium supplemented with 10% FBS for counting **[1]** and resuspend the cells at a 1.25 x 10^5 cells/2 milliliters of AK02N (A-K-zero-two-N) medium supplemented with laminin 511-E8 and ROCK (rock) inhibitor after centrifugation **[2]**.

2.3.1. Talent transferring cells from plate to medium container visible in frame
2.3.2. Shot of pellet if visible, then medium being added to tube, with medium, laminin, and container visible in frame

2.4. Next, aspirate the coating solution from each well of the 6-well plate **[1]** and seed 2 milliliters of cells into each well **[2]**. Return the plate to the cell culture incubator **[3-TXT]**.

2.4.1. Solution being aspirated
2.4.2. Talent adding cells to well(s)
2.4.3. Talent placing plate into incubator **TEXT: Refresh medium on d -3 and -1;** LAB MEDIA: AR21-5 d-3 x4 24w 6D_1647.tif

2.5. After 4 days, the cells reach to 80% confluency **[1]**. Replace the supernatant in each well with 2 milliliters of RPMI plus B27 (R-P-M-eye plus B-twenty-seven) without insulin medium supplemented with GSK3 (G-S-K-three)-inhibitor per well to induce the differentiation **[2]**.

2.5.1. LAB MEDIA: AR21-5 d0 x4 6D_1671.tif
2.5.2. Talent adding medium to well(s), with medium and CHIR99021 containers

visible in frame

2.6. After two days of differentiation, gently replace the medium with 2 milliliters of RPMI plus B27 without insulin medium supplemented with porcupine inhibitor per well **[1]**. The cells should have reached 100% confluency **[2]**.

2.6.1. Talent gently adding medium to well, with medium and WntC59 containers visible in frame
2.6.2. LAB MEDIA: AR21-5 d2 x4 6D_1690.tif

2.7. On day 4 of differentiation, gently replace the supernatants with 2 milliliters or RPMI plus B27 without insulin per well **[1]**. The cells should remain at a high density and some may have begun to float **[2]**.

2.7.1. Talent gently adding medium to well, with medium container visible in frame
2.7.2. LAB MEDIA: AR21-5 x4 6D_1700.tif

2.8. On days 7 and 9 of differentiation, replace the supernatant in each well with 2 milliliters of RPMI plus B27 with insulin supplemented with puromycin for selective pluripotent stem cell-derived cardiomyocyte culture **[1]**. At this time point, beating cells should be observed within the cultures **[2]**.

2.8.1. Talent adding medium to well(s), with medium and puromycin containers visible in frame
2.8.2. LAB MEDIA: AR21-5 d9 x20 6D_1740.avi

3. Patterned Pluripotent Stem Cell-Derived Cardiomyocyte (PSC-CM) Culture

3.1. To set up a patterned pluripotent stem cell-derived cardiomyocyte culture, first use mending tape to remove any dust from the surface of a custom made PDMS (P-D-M-S) stamp **[1]** and briefly submerge the stamp in 70% ethanol **[2-TXT]**.

3.1.1. WIDE: Talent removing dust from stamp
3.1.2. Talent submerging stamp into ethanol, with ethanol container visible in frame **TEXT: See text for stamp fabrication details**

3.2. Use an air duster to remove the ethanol from the stamp surface **[1]** and treat the surface with 5-10 microliters of 0.5% MPC (M-P-C) polymer in ethanol **[2-TXT]**.

3.2.1. Ethanol being blown off stamp
3.2.2. Talent adding MPC to stamp, with MPC container visible in frame **TEXT: MPC: 2-methacryloyloxyethyl phosphorylcholine**

3.3. When the polymer has completely dried, place the stamp onto a polymer coverslip in a 35-millimeter imaging dish **[1]** and place a weight onto the stamp **[2]**.

3.3.1. Talent placing stamp into dish
3.3.2. Weight being placed onto stamp

3.4. After 10 minutes, use a light microscope to confirm that the pattern has been transferred onto the coverslip **[1]** and wash the dish and coverslip two times with PBS **[2]**.

3.4.1. LAB MEDIA: 25um stamp_1765.tif
3.4.2. Talent washing dish, with PBS container visible in frame

3.5. After the second wash, coat the coverslip with 0.5-1 microgram/square centimeter of laminin 511-E8 diluted in 0.1% gelatin for a 2-4-hour incubation at room temperature **[2]**.

3.5.1. Talent adding laminin to coverslip, with laminin and gelatin containers visible in frame

3.6. On day 10 of differentiation, wash the cells two times with PBS **[1]** followed by treatment with 1 milliliter of recombinant trypsin per well for 3 minutes at 37 degrees Celsius **[2]**.

3.6.1. Talent washing cells, with PBS container visible in frame
3.6.2. Talent adding trypsin to well(s), with trypsin container visible in frame

3.7. After counting, resuspend the cells at a 2.5-5 x 10^5 cells/milliliter of RPMI plus B27 with insulin supplemented with puromycin concentration **[1]** and seed 1 milliliter of cells onto the plate for an overnight incubation at 37 degrees Celsius **[2]**.

3.7.1. Talent adding medium to cells, with medium container and hemocytometer visible in frame
3.7.2. Talent adding cells to dish

3.8. The next morning, replace the supernatant with fresh RPMI plus B27 with insulin supplemented with puromycin **[1]** and return the cells to the cell culture incubator, refreshing the medium 2-3 times/week until days 21-28 for imaging **[2]**.

3.8.1. Medium being added to dish, with medium container visible in frame
3.8.2. Talent placing plate into incubator

4. Time-Lapse Sarcomere Imaging

4.1. For time-lapse imaging of the sarcomere shortening in the PC-CSM cultures, apply oil to the 100X objective lens of a fluorescent confocal microscope **[1]** and select live-imaging conditions in the associated software **[2]**.

4.1.1. WIDE: Talent adding oil to lens
4.1.2. Talent selecting live imaging, with monitor visible in frame

4.2. To obtain good representative data, select the highest framerate, set the shutter to open, and apply a 4 x 4 binning and a crop of the acquisition area to achieve the shortest intervals between images during the time-lapse imaging **[1-TXT]**.

4.2.1. SCREEN: Time lapse sarcomere imaging 1: 00:14-00:52 *Video Editor: please speed up* **TEXT: e.g., ≥20 ms or 50 fps framerate**

4.3. If the beating rate of the cells is low, evoke the cells by electrical field stimulation **[1]** and start the time-lapse recording, taking care that the imaged field remains in focus during the imaging **[2]**.

4.3.1. Stimulus being applied
4.3.2. SCREEN: Time lapse sarcomere imaging 1: 00:53-01:02

4.4. At the end of the imaging period, save the time-lapse images into an appropriate folder **[1-TXT]**.

4.4.1. SCREEN: Time lapse sarcomere imaging 1: 01:03-01:33 *Video Editor: please speed up*

5. Time-Lapse Imaging Analysis

5.1. To analyze the time-lapse images, open a series of time-lapse images into ImageJ **[1]** and adjust the brightness and contrast of the images until the sarcomere pattern can be clearly observed **[2]**.

5.1.1. WIDE: Talent opening images, with monitor visible in frame
5.1.2. SCREEN: Screen-5.1.2-5.3.1: 00:00-00:20 *Video Editor: please speed up*

5.2. In the **More tools** menu, select **SarcOptiM** (sark-optee-M) and press ctrl-shift-P and the 1-micron button on the toolbar to use the instructions to calibrate the program **[1]**.

5.2.1. SCREEN: Screen-5.1.2-5.3.1: 00:30-00:30

5.3. Then draw a line across the region of the sarcomere to be used to measure the sarcomere shortening and click **SingleCell AVI** (A-V-eye) to begin the sarcomere shortening analysis **[1]**.

5.3.1. SCREEN: Screen-5.1.2-5.3.1: 00:40-00:55

Results

6. Results: Representative Sarcomere Shortening Evaluation

6.1. Time lapse imaging of sarcomere-labeled patterned pluripotent stem cell-derived cardiomyocytes **[1]** can be used to measure sarcomere shortening at different time points **[2]**.

6.1.1. LAB MEDIA: Figures 1B and 1C *Video Editor: please emphasize Figure 1B images*
6.1.2. LAB MEDIA: Figures 1B and 1C *Video Editor: please emphasize data line in Figure 1C*

6.2. To overcome the sarcomere disorganization typically observed in patterned pluripotent stem cell-derived cardiomyocyte cultures, specific PDMS stamps can be used to culture patterned pluripotent stem cell-derived cardiomyocytes in a stripe pattern **[1]**, which promotes an elongated cell shape and a more organized sarcomere pattern **[2]** compared to cells cultured in a non-pattern area **[3]**.

6.2.1. LAB MEDIA: Figures 2B and 2C
6.2.2. LAB MEDIA: Figures 2B and 2C *Video Editor: please emphasize Figure 2C images*
6.2.3. LAB MEDIA: Figures 2B and 2C *Video Editor: please emphasize Figure 2B image*

6.3. Patterned cultures also promote a better cell contraction and provide a smooth sarcomere length profile **[1]** compared to non-pattern cultured cells **[2]**.

6.3.1. LAB MEDIA: Movie 3
6.3.2. LAB MEDIA: Movie 2

6.4. AAV-transduced patterned pluripotent stem cell-derived cardiomyocytes express sarcomeric GFP signals along the patterned pluripotent stem cell-derived cardiomyocytes as early as three days post-transduction **[1]** and can be also be used to measure sarcomere contraction profiles **[2]**.

6.4.1. LAB MEDIA: Figures 3C and 3D *Video Editor: please emphasize images*
6.4.2. LAB MEDIA: Figures 3C and 3D *Video Editor: please emphasize data lines*

6.5. Further, the addition of a blasticidin-resistant gene during transduction can be used to facilitate patterned pluripotent stem cell-derived cardiomyocyte selection to a purity of over 90% **[1]**.

6.5.1. LAB MEDIA: Figure 4C *Video Editor: please emphasize red dotted shape in right graph*

Conclusion

7. Conclusion Interview Statements

7.1. **Razan E. Ahmed**: To facilitate an easier and higher quality image analysis, it is essential to identify an area with sarcomeres that move linearly and to obtain images at the highest framerate **[1]**.

7.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (4.2.)

7.2. **Hideki Uosaki**: Using this method, we can assess the role of mutations in sarcomere proteins to study sarcomere dysfunction in live cardiomyocytes derived from disease-specific, patient-derived iPS cells **[1]**.

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