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Title: Analyzing the alpha-Actinin Network in Human iPSC-Derived Cardiomyocytes Using Single Molecule Localization Microscopy

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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? **Y**

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

Protocol Length
Number of Shots: **32**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Oleksandra Chabanovska**: This method can be used for the quantitative evaluation of sarcomere maturation in iPSC-derived cardiomyocytes **[1]**.

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

REQUIRED:

1.2. **Oleksandra Chabanovska**: Using this super-resolution-based approach, it is possible to detect even subtle alterations in sarcomere organization, which is not possible with conventional confocal imaging **[1]**.

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

Protocol

2. Photoactivated Localization Microscopy (PALM) Image Acquisition Setup

2.1. At least three hours before use, switch on the microscope **[1]** and bring the sample **[2]** to room temperature **[3]**.

2.1.1. WIDE: Talent turning on microscope *Videographer: Important step*

2.1.2. Talent placing sample at room temperature *Videographer: Important step*

2.1.3. Added shot: Sample getting at room temperature

2.2. When the microscope is ready, add 300 microliters of PALM (palm) imaging buffer to a well of labeled cells **[1-TXT]** and insert the chamber slide into the stage holder of the microscope **[2]**.

2.2.1. Talent adding buffer to well, with buffer container visible in frame **TEXT: See text for buffer preparation details**

2.2.2. Talent inserting slide into holder

2.3. Select the 1.57 NA 100x oil objective **[1]** and the PALM mode in the imaging software **[2]** and activate the TIRF (turf) settings **[3-TXT]**.

2.3.1. Talent selecting objective

2.3.2. Talent at computer, selecting mode

2.3.3. SCREEN: 2.3.3: 00:02-00:04 **TEXT: TIRF: total internal reflection fluorescence**

2.4. Set the number of frames to 5000-10,000, the UV laser power to 0.1% and the 647 laser to 0.2%, and the gain level to 50-100 **[1]**.

2.4.1. SCREEN: 2.4.1 *Video Editor: can speed up*

2.5. When all of the acquisition parameters have been set, switch on the laser illumination and select a target cell **[1-TXT]**.

2.5.1. SCREEN: 2.5.1 **TEXT: Increase gain level if signal intensity too low**

3. PALM Image Acquisition

3.1. To acquire an image, reduce the gain to 0 **[1]** and increase the 647 laser power to 100% **[2]**.

3.1.1. WIDE: Talent reducing gain, with monitor visible in frame
3.1.2. SCREEN: 3.1.2: 00:00-00:05

3.2. Bleach the target cell for about 5 seconds **[1]**.

3.2.1. SCREEN: 3.2.1

3.3. Then increase the gain to 50 and initiate the PALM image acquisition **[1-TXT]**.

3.3.1. SCREEN: 3.3.1 **TEXT: Optional: Steadily enhance UV laser power to increase signal intensity and fluorophore blinking**

4. PALM Data Reconstruction

4.1. For reconstruction of the PALM data, at the end of the acquisition, open the data in ImageJ **[1]** and open the **Thunderstorm** plugin **[2]**.

4.1.1. WIDE: Talent opening data in ImageJ, with monitor visible in frame
4.1.2. SCREEN: 4.2.1: 00:00-00:06

4.2. Select **Run analysis** in the plugin and open the **Camera setup** menu **[1]**.

4.2.1. SCREEN: 4.2.1: 00:06-00:11

4.3. Enter the pixel size and the electromagnetic gain **[1]**.

4.3.1. SCREEN: 4.3.1: 00:00-00:12

4.4. In the **Run analysis menu**, set the B-spline order to 3, the B-spline scale to 2, the peak intensity threshold to stf (S-T-F) Wave.F1, the fitting radius to 3, the initial sigma to 1.6, the magnification to 5, the update frequency to 50, and the lateral shifts to 2 and click **OK [1]**.

4.4.1. SCREEN: 4.4.1: 00:00-00:24

5. Reconstructed PALM Image Post Processing

5.1. After reconstruction, select **Sigma** in the **Plot histogram menu [1]**, and use the **Rectangle** tool to select a region of interest, excluding possible artefacts **[2]**.

5.1.1. WIDE: Talent selecting Sigma, with monitor visible in frame
5.1.2. SCREEN: 5.1.2: 00:06-00:12

5.2. Add the region of interest to the filter and add **And uncertainty less than 25** to the region of interest values **[1]**.

5.2.1. SCREEN: 5.2.1: 00:00-00:16 *Video Editor: can speed up*

5.3. In the **Remove duplicates** tab, enter a distance threshold of 10 nanometers **[1]**.

5.3.1. SCREEN: 5.3.1: 00:00-00:10

5.4. In the **Merging** tab, set the maximum distance to 20, the maximum frames per molecule to 0, and the maximum off frames to 1 **[1]**.

5.4.1. SCREEN: 5.4.1: 00:00-00:12

5.5. In the **Drift correction** tab, select **Cross correlation** and set the **Number of bins** and the **Magnification** to 5 **[1]**.

5.5.1. SCREEN: 5.5.1: 00:00-00:12

5.6. Then save the final PALM image and export the post processed data **[1]**.

5.6.1. SCREEN: 5.6.1: 00:00-00:18 *Video Editor: can speed up*

6. Sarcomere Filament Analysis

6.1. To analyze the sarcomere length, import the reconstructed PALM image of interest into ImageJ **[1]** and use the line tool to draw a line between the selected sarcomere structures perpendicular to the z-disc to measure the shortest distance between the actinin filaments **[2]**.

6.1.1. WIDE: Talent importing image, with monitor visible in frame *Videographer: Important step*
6.1.2. SCREEN: 6.1.2: 00:00-00:10

6.2. In the **Analysis** menu, select **Plot profile** and acquire the length between the two peaks **[1-TXT]**.

6.2.1. SCREEN: 6.2.1: 00:00-00:19 *Video Editor: can speed up* **TEXT: Measure ≥20 sarcomeres**

6.3. To analyze the z-disc thickness, convert the reconstructed PALM image into an 8-bit mode image and open the **Ridge detection** plugin **[1]**.

6.3.1. SCREEN: 6.3.1: 00:00-00:09

6.4. Set the line width to 20, the high contrast to 230, the low contrast to 10, the sigma to 0.79, the lower threshold to 25.84, and the minimum line length to 20 **[1]**.

6.4.1. SCREEN: 6.4.1

6.5. Select **Estimate width**, **Extend line**, and **Display results [1]**.

6.5.1. SCREEN: 6.5.1: 00:00-00:09

6.6. Then click **OK** and use the **Mean line width** from results table for further analyses **[1]**.

6.6.1. SCREEN: 6.6.1: 00:00-00:10

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?
2.1., 6.1.

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 Human Induced Pluripotent Stem Cell (iPSC)-Derived Cardiomyocyte alpha-Actinin Network Analysis

7.1. iPSC (eye-P-S-C)-derived cardiomyocyte **[1]** and neonatal cells exhibit a similar alpha-actinin pattern **[2]** with irregular, disarranged sarcomere structures **[3]**.

7.1.1. LAB MEDIA: Figure 2A
7.1.2. LAB MEDIA: Figure 2A *Video Editor: please emphasize iPSC-derived cardiomyocyte image*
7.1.3. LAB MEDIA: Figure 2A *Video Editor: please emphasize neonatal cardiomyocyte image*

7.2. Quantitative assessment **[1]** demonstrates that the length **[2]** and thickness of the alpha-actinin filaments are almost identical between the two groups of cells **[3]**, indicating a premature developmental state of iPSC-derived cardiomyocytes **[4]**.

7.2.1. LAB MEDIA: Figures 2A and 2B
7.2.2. LAB MEDIA: Figures 2A and 2B *Video Editor: please emphasize iPSC and neonatal data clusters in length graph*
7.2.3. LAB MEDIA: Figures 2A and 2B *Video Editor: please emphasize iPSC and neonatal data clusters in thickness graph*
7.2.4. LAB MEDIA: Figures 2A and 2B

7.3. In contrast, adult mature cardiomyocytes exhibit a regular sarcomere network **[1]** with a slightly increased sarcomere length **[2]** and a reduced z-disc thickness **[3]**.

7.3.1. LAB MEDIA: Figures 2A and 2B *Video Editor: please emphasize adult cardiomyocyte image*
7.3.2. LAB MEDIA: Figures 2A and 2B *Video Editor: please emphasize adult data cluster in length graph*
7.3.3. LAB MEDIA: Figures 2A and 2B *Video Editor: please emphasize adult data cluster in thickness graph*

7.4. A comparison of conventional confocal imaging **[1]** and PALM reveals no significant difference in sarcomere length **[2]**. However, a profound reduced z-disc thickness is detected when iPSC-cardiomyocytes are subjected to PALM imaging **[3]**.

7.4.1. LAB MEDIA: Figure 2C
7.4.2. LAB MEDIA: Figure 2C *Video Editor: please add bracket and "n.s." text over data*

clusters in length graph OR emphasize length graph

7.4.3. LAB MEDIA: Figure 2C *Video Video Editor: please add bracket and * text over data clusters in thickness graph OR emphasize thickness graph*

7.5. A gain in resolution is observed PALM when is applied **[1]**, which is supported by the corresponding intensity plots **[2]**.

7.5.1. LAB MEDIA: Figures 2D and 2E *Video Editor: please emphasize PALM image*

7.5.2. LAB MEDIA: Figures 2D and 2E *Video Editor: please emphasize PALM graph*

7.6. Notably, sarcomere structures **[1]** acquired using a low-quality buffer appear to be thicker **[2]** compared to structures captured under optimal imaging conditions **[3]**.

7.6.1. LAB MEDIA: Figure 3A

7.6.2. LAB MEDIA: Figure 3A *Video Editor: please emphasize Low buffer quality data cluster and/or add/emphasize asterisks*

7.6.3. LAB MEDIA: Figure 3A *Video Editor: please emphasize High buffer quality data cluster*

7.7. This lack of data accuracy is due to the reduced blinking properties of the fluorophore **[1]**, resulting in fewer detected photons per localization event **[2]**.

7.7.1. LAB MEDIA: Figure 3C

7.7.2. LAB MEDIA: Figure 3C *Video Editor: please emphasize grey histogram in Detected photons graph*

7.8. Moreover, localization precision is decreased **[1]**, lowering the overall resolution of the reconstructed PALM image **[2]**.

7.8.1. LAB MEDIA: Figure 3C *Video Editor: please emphasize grey Localization uncertainty graph*

7.8.2. LAB MEDIA: Figure 3C

7.9. In addition, sample drift can affect the precise localization of the fluorescent molecules, resulting in blurry images **[1]**.

7.9.1. LAB MEDIA: Figure 3D *Video Editor: please emphasize excessive sample drift graph*

Conclusion

8. Conclusion Interview Statements

8.1. **Oleksandra Chabanovska**: To acquire proper PALM images, it is very important to allow thermal equilibration of the entire imaging system to avoid excessive sample drift during imaging **[1]**.

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

8.2. **Oleksandra Chabanovska**: Following this procedure, other cellular structures, such as mitochondria, can be imaged by PALM to acquire additional parameters of cardiomyocyte maturation **[1]**.

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