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To the Editor
"*Journal of Visualized Experiments*"
Jaydev Upponi, Ph.D.
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22.06.2020

Submission of our revised manuscript entitled
"Analyzing the α-actinin network in human iPSC-derived cardiomyocytes by application of single molecule localization microscopy"
(previous title: "Analyzing the structural maturation of human iPSC-derived cardiomyocytes using photoactivated localization microscopy)

Dear Dr. Upponi,

we appreciate the opportunity for revision of our manuscript and hereby re-submit it for publication in *Journal of Visualized Experiments*.
The editor and the reviewers have provided us with valuable suggestions to significantly further improve our work.
As suggested by the reviewers, we have performed additional experiments showing the sarcomere organization of adult cardiomyocytes, which allows better evaluation of the structural maturation of iPSC-derived cardiac cells.
In this regard, we have added new subfigures and modified the text in the results and discussion section. Further, we have included data showing the improved lateral resolution of PALM imaging for more accurate data acquisition.
Below please find our point-by-point responses to the issues raised by the editor and the reviewers that, we believe, are fully addressed in our revised manuscript. We therefore are highly confident that the manuscript can now be accepted for publication.

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Sincerely,

Prof. Robert David, PhD

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version. Please use American English throughout. done

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points. done

3. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol. done

4. Please revise and expand the Introduction to include all of the following with citation:

a) A clear statement of the overall goal of this method

b) The rationale behind the development and/or use of this technique

c) The advantages over alternative techniques with applicable references to previous studies

d) A description of the context of the technique in the wider body of literature

e) Information to help readers to determine whether the method is appropriate for their application

We have modified the introduction section to fulfill the criteria mentioned above, e.g. including a statement about the overall goal of the presented protocol.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. done

6. The Protocol should contain only action items that direct the reader to do something. done

7. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. done

8. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step. done

9. Please ensure you answer the “how” question, i.e., how is the step performed? done

10. What kind of cells are used in the protocol? Significance?

We have used a human iPSC cell line, differentiated into cardiomyocytes. Adult and neonatal cardiomyocytes were isolated from NMRI mice. Since our protocol focuses on labelling and image analysis of the sarcomere network rather than on cell differentiation and isolation, we briefly explained the cell culture procedure and refer to previous publications that described cell cultivation and isolation in more detail (Protocol section 1.-3.)

11. 2.4.1- Is this 10 x g or 10, 000 x g? To avoid misunderstanding, we have removed the period.

12. Is thunderstorm plugin open access? Yes, the thunderstorm plugin is open source software.

13. Please use complete sentences to describe the action. done

14. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. done

15. Please ensure the result are described with respect to your technique presented; you performed an experiment, how did it help you to conclude what you wanted to and how is it in line with the title. done
16. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]." No copyright permission is needed.
17. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
done
18. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials. Please sort the materials table in alphabetical order. done

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The manuscript provides a clear protocol, and the authors demonstrate that the protocol yields high resolution images of cardiomyocytes (CMs) which can subsequently be analyzed for parameters such as sarcomere length and z- disc thickness. The authors also demonstrate why adherence to the protocol is required to obtain high resolution images. However, the authors do not adequately contextualize the relevance and need for the protocol. Revisions to address such shortcomings are suggested and listed below.

Major Concerns:

1. *Reference to previous studies which demonstrate differences in sarcomere length and z-disk width in wild type vs. disease iPSC-derived CMs (such as Pioner et. al. Cardiovascular Research, 2020) as well as studies that demonstrate changes in sarcomere length as a result better maturation (such as Carson et. al. ACS Appl Master Interfaces, 2017) would justify using these parameters to assess maturation.*

   We appreciate the reviewer's suggestion and have added information why z-Disc thickness and sarcomere length are appropriate parameters to evaluate maturation, including respective references (line 81-85.)

2. *The authors claim that the comparison of sarcomere lengths for iPSC-derived CMs and neonatal CMs demonstrates the lack of maturation of the iPSC-derived CMs. Measurement of sarcomere length in mature CMs should be included to demonstrate that mature cardiomyocytes do have longer sarcomere lengths and that sarcomere length can therefore be used to assess maturation.*

   The reviewer is right that data from fully mature cardiomyocytes would allow better evaluation of the developmental state of iPSC-derived CMs. Therefore, we have included additional data of z-Disc thickness and sarcomere length measured in adult mature cardiomyocytes obtained from murine heart tissue (Figure 2A, B).

3. *The need for techniques such as PALM to investigate the sarcomere structure can be made stronger by citing previous studies that study the role of sarcomere structure/composition in cardiac disease such as Zaunbrecher et. al. Circulation, 2019*

   We thank the reviewer for his advice. We have included a statement in the discussion section to further highlight the importance of PALM for investigating the role of specific proteins for sarcomere function (412-414)

4. *In the introduction a lack of functional maturity of iPSC-CMs is mentioned, but a significant drawback of this method is the inability to collect functional parameters concerning cell contractility. Preemptively addressing this by citing studies that already accomplish this such as Miklas et. al. Circulation Research, 2020 and Pioner et. al. Stem Cell Reports, 2016 is needed to help clarify that this technique is addressing the need to improve the investigation of sarcomere structure instead of function.*

   The reviewer is right that the presented method does not allow acquisition of functional parameters, like contractility. We have added a paragraph describing these drawbacks and provide information about studies that have acquired both structural and functional data, although with much lower resolution (line 468-475)

5. *Comparison of sarcomere length and z-disk thickness measurements made via PALM vs. conventional/standard techniques are required to support the assertion that PALM yields more accurate analysis.*

   According to the reviewer´s suggestion, we have performed additional experiments to show the difference between conventional confocal imaging and PALM-based image acquisition. We have included these data in Figure 2 and modified the results and discussion section. Figure 2 C shows sarcomere length and z-Disc thickness acquired with both imaging techniques. Further we highlight the improved resolution of PALM by indicating the full width at half maximum of fluorescence intensity profile plots (Figure 2 D,E) (line 327-334, line 427-431)

Minor Concerns:

6. *In step 3.4.3 the authors suggest taking "5.000 to 10.000 frames." The use of a decimal/period makes it unclear as to whether the authors means "five to ten" or "five thousand to ten thousand" frames.*

   We have acquired 5000-10000 frames per PALM image. To avoid misunderstandings, we have removed the period.

7. *For step 5.1.2 it may be appropriate to mention specifically to draw the line perpendicular to the z-discs so that peaks in the resulting plot represent the shortest distance between the z-discs*

   As suggested by the reviewer, we have added the information regarding line drawing (line 287-288).

8. *In the zoomed in images in Figure 2 indicators such as arrows to indicate a representative measurement of sarcomere length and z-disc thickness would make it clearer what exactly is being measured, especially for the z-disc thickness as the image by itself does not make it clear whether the short/thin or longer/wider dimension of the z-disc is being measured.*

   We appreciate the reviewers´ suggestions. As Figure 2 does not contain any zoomed images, the reviewer´s comment obviously refers to Figure 3. We have now indicated representative measurements of sarcomere length by red lines. Also, filament structures used for calculating z-Disc thickness were labelled in green.

Reviewer #2:

Manuscript Summary:
Assessing the maturation level of iPSC derived cardiomyocytes (CMs) is essential for using and comparing CM models and the results obtained using them. The authors present a method for accurately imaging the CM key structural aspects, and measure sarcomere lengths and z-disc widths. The methods are generally well described. The process and results are easy to understand and follow.
I have only a few minor remarks to make.

Minor Concerns:

1. *Title: The study presents a method for analyzing the structural properties of human iPSC-derived CMs, which can be of use when assessing the maturity level of these cells, among other uses. The title in its current form is slightly misleading, as it suggests that CM maturity is analyzed, while the study does not include mature cell types or compare the results with previous studies. The title of the study should reflect this.*

   We agree with the reviewer that CM maturity is difficult to evaluate without a direct comparison with mature CM. Therefore, we have included data of z-Disc thickness and sarcomere length of

mature cardiomyocytes derived from adult mice (Figure 2) Also, we have changed the title that now focuses on analysis of the α-actinin sarcomere network.

2. *Introduction: The authors set a high bar for the scientific community when requiring identicalness: "researchers have failed to generate iPSC-derived CM, matching identical phenotypical and functional properties as their native counterparts." Animal models are very far from the humans in drug screening assays but are nevertheless used. Progress in the iPSC-CM field has certainly not been as fast as many would have hoped. However, "all models are wrong, but some are useful". The authors could revise this statement e.g. by indicating aspects of iPSC-CMs that hamper their in vitro use*.

   As suggested by the reviewer, we have modified the respective statement (line 61-62)

3. *The disorganized sarcomere structure not only causes reduced ability of cell contraction, but also influences the entire contraction dynamics: the uniaxial contraction of mature cells is replaced by the radial contraction and contraction focal points in immature cells. This is briefly mentioned by the authors in the second to last paragraph in discussion, but the orientation issue should be raised in introduction as well.*

   We appreciate the reviewers comment. We have added information about the orientation of sarcomere filaments in immature CM and their impact on cell contraction dynamics (line67-70).

4. *Protocol/table of materials: The study does not indicate the origin of the iPSC-CM cell line and the animal from which the neonatal cells are excised from. Although the study aims to present the method and not the cells, hIPSC-CM culture and differentiation protocol should be documented in some way as well, as the measurement results are compared with literature values.*

   We thank the reviewer for his advice. Origin of the iPSC cell line is now given in the table of materials. Origin of neonatal and adult cardiomyocytes is mentioned in the protocol section (2.1, 3.1). As already stated by the reviewer, our protocol focuses on the imaging technique rather than on cell culture methods. Thus, we briefly introduced cultivation and isolation of hiPSC-CM and native CM and refer to previously published studies that describe the process of isolation and cultivation in more detail.

5. *Results: Please indicate in text the N and measure of variation in text, as now it is mentioned only in figure captions.*

   We have added the respective information in the results section.

6. *Discussion: In line with my previous remark regarding the title, I would suggest rewording the last paragraph to better fit the study. While the measured parameters are very important aspects in assessing sarcomere properties, CM structural maturity consists of more aspects, such as*

remodeling of nuclei and other organelles, development of T-tubules, increase in myofibril content.

We have modified the title and discussion section according to the reviewer's suggestions. We now describe our method as a tool to particularly characterize the maturation state of the sarcomere/α-actinin network in CM, rather than a tool to evaluate structural maturation in general (line 327).

Reviewer #3:

Manuscript Summary:
The manuscript describes a microscopy approach to monitoring cardiomyocyte sarcomeres with high resolution. The sarcomere images are very clear and the procedural details are present. There are concerns however that reduce enthusiasm for publication

Major Concerns:

1. *There is no detail about the cardiomyocytes used for the study. What is the source of the neonatal cells? What is the source of the hiPSC-CMs?*

   We thank the reviewer for his comment. We have added information regarding source of neonatal mice and origin of hiPSC-CM in the protocol section (1.-3.) and table of materials. Since our protocol focuses on the imaging technique, we briefly described the process of isolation and cultivation of cells and refer to previous studies that provide more detailed information of cardiac differentiation and cell isolation.

2. *Also, there is no application of the procedure to study maturation-the process that this approach is suppose to enable study of.*

   We appreciate the reviewer´s comment. We did not intend to provide a strategy in order to improve the maturation of iPSC-derived CM. Instead, the purpose of the study was to demonstrate our method as tool to evaluate the sarcomere maturation. To further support this conclusion, we included data of mature CM for the direct comparison of z-Disc thickness and sarcomere length between neonatal, adult and iPSC-CM (Figure 2). These data show that our approach is suitable to characterize the sarcomere network at different developmental stages and thus in the future can be applied to analyze the efficiency of protocols/strategies aiming to improve sarcomere maturation.

Minor Concerns:

3. *It is not clear how this new microscopy approach is an improvement over using FFT analysis of phase contrast or confocal images. There are other methods available to determine maturation of hiPSC-CMs that do not require fixation and processing. Further, there are live cell staining approaches using fluorescent labelled actin and actinin to probe maturation in living cells.*

We agree with the reviewer that several methods are available to analyze the maturation process of iPSC-CM. However, compared to conventional confocal imaging, PALM gives significantly improved spatial resolution that allows increased data accuracy. This is in particular important when subtle alterations of the sarcomere network need to be detected.
Also, the reviewer is right that live cell imaging with fluorescently labelled actinin also represents a technique that can be applied to obtain structural and functional data, although spatial resolution is much reduced. Due to the long acquisition process our microscopy approach is less appropriate for live cell imaging. Yet, our approach provides the possibility to be combined with previous time lapse recordings in order to correlate PALM data with functional parameters like contraction capacity. We have included this information in the discussion section and mentioned the drawbacks of our method. (line 457, 468-475)