Rebuttal letter: Live-cell Imaging of Single-Cell Arrays (LISCA) – a versatile technique to quantify cellular kinetics

Anita Reiser, Daniel Woschée, Simon Maximilian Kempe, Joachim Oskar Rädler

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Dear Dr. Nam Nguyen,

herewith we resubmit our revised manuscript „Live-cell Imaging of Single-Cell Arrays (LISCA) – a versatile technique to quantify cellular kinetics“ for publication in JoVe.

We would like to thank all reviewers for their valuable time and effort reviewing our paper, and their constructive feedback, which has significantly improved the manuscript.

The reviewers wished clarifications and additional information, which we now have included in the revised manuscript. In the following, we have answered the reviewers' comments point-by-point and indicated the changes made. We have revised the manuscript and the protocol for clarity and correct language, as suggested by the reviewers. Also the editorial comments regarding language and formatting have been addressed, and we have revised the protocol section to comply with the editorial policy.

We believe that the revised version of our manuscript is now suitable for publication in JoVe.

Sincerely,

Anita Reiser, Daniel Woschée, Simon M. Kempe and Joachim O. Rädler

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. Please define all abbreviations at first use.

> **Answer:** The manuscript has been thoroughly proofread. Spelling and grammar issues have been corrected. Abbreviations have been defined at first use.

2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Sylgard 184 Silicone Elastomer Kit, Lipofectamine 2000, Opti-MEM,

> **Answer:** Commercial language has been removed from the manuscript.

3. 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. Please include all safety procedures and use of hoods, etc.

> **Answer:** The protocol section has been adapted to only contain imperative tense. Text that cannot be written in imperative tense has been canceled, written as a "Note" or moved to the discussion section. Unnecessary "Notes" have been removed.

4. After including a one line space between each protocol step, highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.

> **Answer:** A one-line space has been added between protocol steps. Protocol text for inclusion in the protocol section of the video has been marked yellow.

5. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.

> **Answer:** The scale bars have been inserted and indicated appropriately. The scale labels cannot be removed from Figure 3 because Figure 3 is copied from another publication.

Reviewer #1

Minor Concerns

1. Maybe the authors can add some more thoughts and discuss how this procedure might be expanded for the usage of ratiometric biosensors e.g. FRET-based probes.

> **Answer:** FRET-based probes are discussed now at the end of the article.

2. The authors might also add some discussion on how the nature/geometry of the micropatterning might impact cell signaling, function, and fate.

> **Answer:** The effect of micro-pattern on cell viability has been studied by us and others. In short we did not observe adverse effects using viability assays and also did not find changes in division times. We have addressed this issue in the text.

Reviewer #2

Major Concerns

1. The author should give a relation with the pattern size and the cell scale for other cell lines.

   Answer: Lines 367-370 of the corrected manuscript give examples of suitable pattern sizes for other cell lines.

2. What is the distribution of cell number on single pattern?

   Answer: The cells are seeded out in concentrations such that occupancy of less than 70% of adhesion sites is achieved. In this case we only find cell number occupancies 0, 1 or 2 in accordance with a Poisson distribution. See also Röttgermann *et al.*, **Soft Matter**, 2014,**10**, 2397-2404 (Reference 18 of the manuscript).

3. Lines 139-161: any guidance on how to optimize the seeing procedure for a specific type of cells?

   Answer: Lines 375-388 of the corrected manuscript give some advice on how to adapt the protocol to other conditions, e.g. other cell types.

4. What is the speed of perfusion? Does the shear stress affect the attachment of cells? How to optimize this parameter (criterion)?

   Answer: The liquid exchange is performed manually. For this reason the generated flow rate and the resulting shear stress cannot be controlled precisely. The liquid exchange has to be done very carefully. We have not observed any cell detachment or morphological changes due to the liquid exchange. We do not expect any long term effects of the two short periods of liquid exchange.

5. I do not think thresholding is a suitable method for the segmentation. For example, there are two cells attached on the patter on the letter bottom corner in Figure 4. But they are segmented into one mask. This will lead to false results in fluorescence analysis. The authors may want to try those segmentation methods that are widely used like active contour, watershed and machine learning method.

   Answer: Active contour and watershed require the cell position as input and, thus, cannot be used as primary segmentation method. Since our goal is automated cell recognition, the input should not be provided interactively by the user but by another segmentation method. Due to the heterogeneous appearance of cells in phase-contrast images, we do not expect that active contour or watershed could outperform the current thresholding algorithm.

   It is planned, however, to detect cell contours and multiple occupancies with machine learning in future versions of PyAMA. In the mean time, the pre-selection may contain some false positives, which need to be deselected manually. Lines 432-434 of the corrected mansucript clarify this.

   To avoid confusing the reader, we have edited Figure 4 to not include a double occupancy.

Minor Concerns

1. There are a few typos that need further type-editing.

   Answer: Various typos have been corrected in the corrected manuscript.

Reviewer #3

Comments

1. Is the presented methodology adaptable to other, smaller cell types such as prokaryotes?

   Answer: The methodology has been developed for studies on eukaryotes and cannot be easily adapted to bacteria. Bacteria are too small and will not array as single cells on pattern, even if smaller patterns could be made.

2. Is there any control of viability for the cells incubated in the microarrays (growth, dyes indicating metabolic activity)?

   Answer: Controls can (and should) be run in parallel or in separate experiments. We have measured cell division times and viability in the past. We included notes regarding control experiments in the revised text.

3. What kinds of tubing and syringes were used? Please indicate and be as precise and extensive with you description of the used materials.

   Answer: No special syringes are needed but we have added the used type of syringe to the material list. The used tubing and luer connectors are listed in the material list and a reference with details for the perfusion system fabrication is added to the protocol section.

Quality guidelines

1. *Are there any other potential applications for the method/protocol the authors could discuss?*
   → Is the presented method also adaptable to establish and track microbial cultures (bacteria, yeasts) or even distinguish between different types of cells that a cultivated in co-culture? Please shortly discuss this aspect.

   Answer: The methodology has been developed for studies on eukaryotes and cannot be easily adapted to bacteria. However, different types of eukaryote cells can be easily studied. Also experiments in co-culture are feasible. A prominent example are cell-killing assays, where the adhesion sites are filled with target cells and killer cells are added (see for example: Chatzopoulou, E.I., C.C. Roskopf, F. Sekhavati, T.A. Braciak, N.C. Fenn, K.-P. Hopfner, F.S. Oduncu, G.H. Fey, and J.O. Rädler. 2016. Chip-based platform for dynamic analysis of NK cell cytolysis mediated by a triplebody. Analyst. 141: 2284–2295.). We added these applications to the discussion.

2. *Are appropriate controls suggested?*
   → As stated before, it would be very important to check for cell viability or their general physiological status to exclude that gene expression heterogeneities originate from the incubation environment.

   Answer: As described in lines 437-440 of the corrected manuscript, single-cell arrays present a uniform microenvironment to all cells, thus excluding an impact of the cell surroundings on heterogeneities in cell behaviour.

3. *Are all the critical steps highlighted?*
   → No, this could be improved. Please highlight which steps of the protocol are particularly important for successful, and more importantly, meaningful experiments and results.

   Answer: The protocol section has been revised to clarify critical steps.

4. *Is there any additional information that would be useful to include?*
   → Yes, please specify the machines and devices that have been used in detail.

   Answer: The table of used materials has been revised and completed.

Conclusion

1. Control experiments are needed to exclude effects of the cultivation environment on cell physiology.

   Answer: We recommend viability testing when using new cell lines for the first time. A corresponding sentence is inserted into the corrected manuscript.

Reviewer #4

1. The authors should mention novel ways of picking up label-free kinetic signals from single-cells to put their solution into a wider context. See for example: Sztilkovics et al. Scientific Reports volume 10, Article number: 61 (2020). Please give a fair comparison, the advantages/disadvantages of employing fluorescence should be discussed.

   Answer: The LISCA method was developed to extend the use of fluorescence markers for automated analysis of time courses of fluorescence signals from single cells. There are many other techniques that allow single-cell readouts. The referee mentions the elegant approach by Sztilkovics *et al.* measuring adhesion forces and adhesion energies of single cells. However, these mechanical measurements are difficult to compare to fluorescence readout. The applications of Sztilkovics *et al.* and the LISCA approach are almost orthogonal, and a comparison of such completely different methods would rather confuse the reader. However, we mention in the discussion now the possibility of using Fluid_FM, e.g. for subsequent analysis.

2. At the end of the manuscript I miss at least 2-3 sentences about the possibility of picking up the cells for further investigations. Computer controlled micropipette, FluidFM BOT could be mentioned and discussed. These are both highly relevant techniques to pick up the interesting cells from the planar array. (See the above paper and references therein.)

   Answer: The idea of picking cells up at the end of time-lapse studies for further analysis, e.g. single-cell sequences, is very ambitious but well justified. We included this aspect in the discussion and mention the possibility of applying FluidFM Bot.

Reviewer #5

Major Concerns

1. The protocol is unclear. Many steps of the protocol are incomplete or not well described.

   Answer: The protocol has been revised and clarified in the corrected manuscript.

2. The english is also bad in many sentences, what makes it even harder.

   Answer: Language issues have been addressed in the corrected manuscript.

3. It is unclear whether the contents of this manuscript are included in other publications by the same authors.

   Answer: Lines 45–48 of the corrected manuscript indicate publications in which the µPIPP patterning is described.

Minor Concerns

1. Line 85. ml must be mL ; it is not clear the meaning of PLL(20kDa)-g[3.5] ; the composition of the PLL-PEG solution is not clearly described.

   Answer: The unit “ml” has been corrected into “mL”. “PLL(20 kDa)-g[3.5]-PEG(2 kDa)” is the official name provided by the manufacturer.

2. Line 94. Should say: "For the fabrication of PDMS stamps, mix the PDMS monomer with ..."

Answer: The corresponding protocol section has been revised. The suggested amendment is not necessary any more.

3. Line 99. The whole point 3.1 is unclear.

   Answer: The point 3.1 has been clarified in the corrected manuscript.

4. Line 107. Why is it talking about channels? What protection foil?

   Answer: These questions have been clarified in the corrected manuscript.

5. In order to understand the protocol, it is critical to have details on the equipments used. However some details are lacking, for example, what plasma cleaner ir used?

   Answer: The missing entries in the Table of Used Materials have been completed, including details on the plasma cleaner.

6. Line 115. ...plasma cleaner under a biosafety cabinet..." must by "...in the safety cabinet..."

   Answer: The preposition has been corrected in the corrected manuscript.

7. Lines 115 - 118. The English is bad, the text is hard to understand.

   Answer: The protocol step has been clarified in the corrected manuscript.

8. Line 119 - 121. The English is bad, the text is hard to understand.

   Answer: The protocol step has been clarified in the corrected manuscript.

9. Line 122. Still, I did not understand what the foild is....

   Answer: The protocol step has been clarified in the corrected manuscript.

10. Line 122. What is a six channels sticky slide?

    Answer: Sticky slides are established microscopy slides that allow manufacturing custom microstructured surfaces. Details on properties and usage of the six-channel sticky slides used in this protocol can be obtained from the table of used materials and from the manufacturer's website. A more detailed definition would go beyond the scope of the article.

11. Line 170. It is unclear which perfusion system they are using. The table of materials is unclear.

    Answer: The perfusion system is in-house fabricated using the listed material. We inserted this information in the protocol section with a reference where the assembly of the tube system is described in more detail.

12. Line 176. They do not explain how to keep the sterile conditions, what is highly relevant for the experiments.

    Answer: The connection of the tubes to the slide has to be done in a biosafety cabinet. We added this information to the protocol section.

13. Line 191. "...pre-warmed heating chamber..." To what temperature?

    Answer: The protocol step has been clarified in the corrected manuscript.

14. Line 228. 300 uL seems like a very large volume... The channels dimensions are not described. What volume may hold each channel?

    Answer: The volume of 300 µL was chosen to ensure a total volume exchange of the liquid in the channel. The channel volume is 30 µL, the Luer reservoirs have an additional volume of 120 µL, and there is the extra dead volume of the tubing.

15. For what is used the NIS element software?

    Answer: NIS Elements is provided by the manufacturer of the microscope (Eclipse Ti-E) and is used for controlling the microscope, acquiring microscopy images and exporting the images to the TIFF format. Depending on his microscope, a researcher using LISCA may or may not use NIS Elements for microscopy.

16. Lines 426 - 429. It is unclear what the authors mean in this paragraph.

    Answer: The paragraph has been clarified in the corrected manuscript.

17. Line 489. FACS must be defined.

    Answer: The term “FACS” has been replaced by “flow cytometry” in the corrected manuscript.

18. Woschée, D. et al. Single Cell Fluorescence Acquisition on Micropatterned Surfaces (to be published). Seems to be the same publication.

    Answer: This reference refers to another manuscript in preparation, which is concerned with technical aspects of various image analysis algorithms and their performance. To avoid confusion, we removed this citation.

19. I also miss a clear scheme describing the fabrication of the Single-Cell Arrays

    Answer: A scheme of the microarray fabrication by µPIPP is added as new Figure 3.