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Dear Sir, 8/10/18

We appreciate the comments of the reviewers, and have addressed them in detail, as summarized below.

Detailed reply:

*Reviewer #1:*

*Manuscript Summary:*

*Manuscript describes the use of PLA to visualize and quantify MST1/MST2 heterodimerization in fixed cell lines.*

*Major Concerns:*

*None*

We thank the reviewer for his/her remarks.

*Minor Concerns:*

*Both abstracts state that the technique is used to visualize and quantify with MST1/MST2 heterodimerization, however, quantification is not discussed in this manuscript.*

We now provide information regarding analytic methods which can be used for quantification analysis.

*Reviewer #2:*

*Major Concerns:*

*1) The authors refer to PLA signals as interactions or heterodimers. However, as the authors also point out on Row 33-35, the isPLA assay measures proximity between proteins and not directly the interactions per se. Homodimers (Mst1/Mst1 and Mst2/Mst2) co-localized in the same region of the cell may potentially also be able to generate an Mst1/Mst2 isPLA signal if they are close to each other. To improve the manuscript the authors should:*

*A: Update the text to reflect that isPLA visualizes proximity between the protein isoforms and not directly interactions. This topic should also be brought up in the discussion.*

We thank the reviewer for raising this important point. We have modified the Long Abstract, Legends and Discussion to indicate that PLA signals arise from protein proximity, not necessarily dimerization.

*B: To better understand the data and to support the conclusion of how well the Mst1/Mst2 isPLA design exclusively measures heterodimers, an additional control that compares signals from samples with low or high ratios of heterodimers vs homodimers would be desirable in the representative results. Perhaps, H-RAS induced heterodimerization described by the authors in a recent article (PMID 27238285 , Ref 4) could be utilized? Or if there are cells that are known to have high or low levels of Mst1/Mst2 dimers compared to homodimers?*

We appreciate the reviewer’s point, but would prefer not to include additional panels at this point. It is not known currently which cells have more or less MST heterodimers. While it is true that these can be induced in HEK293 with HRAS and we could show that again, this article is designed to demonstrate the practical aspects of the PLA procedure and we think the images presented do a reasonable job in this regard. If the reviewer insists, we can also provide additional experiments that address the quantitative aspects of PLA, but that was not our initial intent.

*2) The description of the isPLA technique on Row 38-43 is a bit unclear needs to be clarified. The DNA strands on the secondary antibodies are not complementary to each other as may be stated on row 38? They are complementary to connector-oligos that are added in the ligation step. The connector-oligos can then be ligated into a complete circle in situ if the secondary antibodies are in close proximity, and this circle serves as the template for the rolling circle amplification. Please update this section according to information available in isPLA reviews and at Sigma-Aldrich.*

We have corrected this error.

*Minor Concerns:*

*3) In the paper there are two different concentrations of TX-100 mentioned in the permeabilization buffer 0.1% (Row59) vs 0.5% (Row71). Please specify which one is used when.*

We have corrected this error.

*4) May be advantageous to change "Antibody diluent" to "Duolink antibody diluent" to avoid confusion (Row 90)*

We have removed the term “Duolink” from all sections except “Materials” and spell out the composition of reagents which can be used instead of those included in the Duolink kit.

*Reviewer #3:*

*Manuscript Summary:*

*The protocol describes the uses of Proximity Ligation antibodies to detect the location of the interaction of two specific proteins in cells. The protocol is quite detailed and easy to follow.*

*Major Concerns:*

*There seems to be a missing negative control, namely a 2-protein assay in which the proteins should not be interacting (such as perhaps ERK/MST1)*

This experiment has been redone and a new replacement panel, with more negative controls (Fig. 1), included.

*I watched the video. It seems to me the video would be much more valuable with some images of how the cells look at different stages*

*of the process. As it is, it just conveys multiple pipette steps.*

We now add scheme of the experiment and add parts of this scheme on titles of the video congruent to the step of the experiment.

*A point that does come across somewhat is the different instruments being used, such as the multiple well plate.*

*Those ideas could be brought out better as well by focusing on them.*

We now add description of chamber slides into discussion.

*Reviewer #4:*

*Manuscript Summary:*

*The Authors describe a protocol for Proximity Ligation Assay (PLA) using Duolink PLA Kit (Sigma). The protocol described is in the most part identical to the published protocol by the vendors, with some additional points of emphasis.*

*Major Concerns:*

*The methodology described add little insight beyond the published protocol by Sigma.*

*The use of pERK/ERK antibodies in Figure 1B ("As a positive control, we also used ERK and pERK antibodies that are expected to be in close proximity") is misleading, a positive signal could be achieved in such case regardless of proximity between different ERK molecules as the 2 antibodies would likely be able to bind the same molecule at distinct epitopes.*

We agree, and have reworded the relevant section to avoid confusion. In fact, we use ERK/pERK simply as a strong positive control since the epitopes are on the same protein.

*Minor Concerns:*

*Several grammatical and spelling errors throughout the manuscript.*

We have corrected these errors.

*Changes to be made by the Author(s) regarding the written manuscript:*

*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.  
2. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.  
3. Figure 1: Please include scale bars. Please place the panel label at the upper left corner as in Panel G.  
4. Please remove references from the Long Abstract.  
5. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.  
6. 1.1: What are the culture conditions? How are the cells split?  
7. What are the volumes and concentrations?  
8. Please use commas in numbers to denote thousands instead of periods.  
9. 4.2: Please specify the volume of one drop.  
10. 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: Duolink II, etc.*

All changes regarding text has been made. With respect to point 10, we have removed the term Duolink from all sections except the Materials section, and there also list non-commercial alternatives.

*Changes to be made by the Author(s) regarding the video:*

*1. Please remove the University splash logo before the title card.*

*2. Please increase the homogeneity between the written protocol text and the protocol video. The steps are not exactly in the same order and the details given are not the same.*

*3. Please use SI abbreviations for time in the video: h instead of hrs for hours, etc.*

*4. There are many formatting and grammatical errors in the text of the video:*

*5. 3:18 – 0.5% Triton X-100, etc.*

*6. Please remove Duolink references in the video.*

*7. Please ensure that the L in the microliter abbreviation is capitalized throughout.*

*8. 0:00-0:04 - The opening splash with the logo will have to be removed from the beginning of the video. It can be moved to the end of the video, if the authors wish to show it.*

*9. Text/formatting issues*

*• 9:13 - The "www.JOVE.com" should be removed from this card, since it wasn't produced by JoVE. The rest of the credits can and should stay.*

*• 8:09 - A chapter title card should be added here that reads "Representative Results".*

*• 8:43 - A chapter title card should be added here that reads "Conclusion".*

*10. Frame size/proportions issues*

*• Almost the entire video is letterboxed. Future submissions should have the video filling the entire frame for the entire run of the video. Since the live action video is at a 16:9 aspect ratio, I would strongly recommend setting the entire video to a 16:9 aspect ratio.*

*• 0:42-1:33 - There are thin black borders on various sides of the frame that should be eliminated. The white background should be extended to fill the frame.*

All changes regarding video has been made.

Sincerely,

Jonathan Chernoff