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Detection of Heterodimerization of Protein Isoforms using an in situ Proximity Ligation Assay.

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TITLE:
Detection of Heterodimerization of Protein Isoforms using an *in situ* Proximity Ligation Assay

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KEYWORDS:
PLA, MST, signal transduction, dimerization, protein-protein interaction, Hippo pathway

SUMMARY:
Here, we show how to use a Proximity Ligation Assay (PLA) to visualize MST1/MST2 heterodimerization in fixed cells with high sensitivity.

ABSTRACT:
Regulated protein-protein interactions are a guiding principle for many signaling events, and the detection of such events is an important element in understanding how such pathways are organized and how they function. There are many methods to detect protein-protein interactions in cells, but relatively few can be used to detect interactions between endogenous proteins. One such method, the proximity ligation assay (PLA), has several advantages to recommend its use. Compared to other common methods of protein-protein interaction analysis, PLA has relatively high sensitivity and specificity, can be performed with minimal cell manipulation, and, in the protocol described herein, requires only two target-specific antibodies derived from different species (*e.g.*, from mouse and rabbit) and one specialized reagent: a set of secondary antibodies that are covalently linked to specific oligonucleotides that, when brought in close proximity of one another, create an amplifiable platform for *in situ* PCR or rolling circle amplification. In this presentation, we show how to apply the PLA technique to visualize changes in MST1 and MST2 proximity in fixed cells. The technique described in this manuscript is particularly applicable for the analysis of cell signaling studies.

INTRODUCTION:
Disruption of MST1/Hippo signaling has been connected to developmental disorders and carcinogenesis[1]. In mammals, the kinases MST1 and MST2 activate (phosphorylate) MOB1 and LATS1/2, the latter of which then phosphorylates and inactivates the transcriptional co-activator Yes-associated protein (YAP)[2]. In its active (unphosphorylated) form, YAP has oncogenic activity, enhancing transcription of cell proliferation genes; conversely, when YAP is inactivated by the Hippo pathway, cell proliferation is suppressed and apoptosis promoted[3]. In

tissues, MST1 and MST2 exist mainly as active homodimers, but oncogenic stimuli can increase levels of MST1/MST2 heterodimers, and such heterodimers are inactive[4]. However, how MST1/MST2 heterodimerization is regulated remains poorly understood. Both homo- and heterodimers are mediated *via* interactions between C-terminal coiled-coil regions of MST1 and MST2 known as SARAH domains[5]. Using an *in situ* PLA demonstrated in this article, we show the presence of MST1/MST2 heterodimers in Human Schwann Cells (HSC) and Human Embryonic Kidney cells (HEK-293). PLA has an advantage over other protein/protein interaction detection methods because it allows the detection of endogenous protein-protein interactions, which can be identified and quantified without the need of transgene expression or the use of epitope tags[6].

Signaling transduction pathways are largely controlled by the conditional association of component proteins. For example, stimulation of most receptor tyrosine kinases leads to their homo- or hetero-dimerization and subsequent association with additional intracellular signaling proteins, which themselves form further complexes. The purpose of the PLA method is to visualize proximity between the proteins in cells, provided that the proteins are less than 30-40 nm apart. Protein proximity is usually detected by first incubating the cells with appropriate primary antibodies raised in different species (*e.g.*, rabbit and mouse) against each interacting protein, then adding species-specific secondary antibodies, pre-coupled to short DNA probes. If the DNA probes are in close proximity, a specific linking DNA oligonucleotide can simultaneously bind both of these probes, forming a platform for amplification by *in situ* PCR or by rolling circle mechanism. Fluorescent tags added to the amplification reaction allow visualization of the interacting proteins, which appear as fluorescent dots that can be readily quantified and localized to particular regions in the cell[7-10].

PROTOCOL:

1. Preparation of Solutions

1.1. Prepare fixative solution: 4% paraformaldehyde (PFA) in 1x PBS. For 10 mL, take 2.5 mL of 16% PFA and add 7.5 mL of 1x PBS.

Hazards: PFA is carcinogenic at low doses. Fumes and skin contact are hazardous. Store at -20 °C.

1.2. Prepare permeabilization solution: 0.1% Triton X-100 in 1x PBS. For 100 mL of solution, add 100 µL of Triton X-100 into 100 mL of 1x PBS. Store at room temperature (RT).

1.3. Prepare Wash Buffer: 1x TBST. For 1 L, take 100 mL of 10x TBS, 890 mL of dH_2O, and 10 mL of Tween 20 (10%).

1.4. Prepare blocking solution as supplied by kit. Alternatively, use 1x PBS solution containing 2% BSA.

1.5. Prepare the antibody diluent as supplied by kit. Alternatively, use 1x PBS solution containing 1% BSA.

2. Plating of Cells

Note: In this study, we used immortalized Human Schwann Cells (iHSC) and Human Embryonic Kidney 293 cells (HEK 293); however, this method can be used for many types of adherent cells.

2.1. Culture HEK 293 in DMEM supplemented with 10% FBS, 0.2% Glucose, 2 mM L-Glutamine, 100 U/mL penicillin G and 100 µg/mL streptomycin on tissue culture-treated plates at 37 °C in 5% CO_2. Maintain iHSC in 10% FBS/DMEM supplemented with Pen/Strep and 2 µM forskolin. Routinely test cells for mycoplasma. No cell line authentication was performed.

2.2. Coat a 16-well chamber slide with 50 µL of 10 µg/mL natural mouse laminin or 0.01% poly-L-lysine solution and incubate for 30 min at 37 °C in 5% CO_2. Split cells using 0.04% trypsin-EDTA.

2.3. Plate iHSC and HEK-293 in 100 µL of medium at 80% confluence (15,000-25,000 cells/well).

2.4. Incubate the cells for 12 -24 hours at 37 °C in a humidified, 5% CO_2 incubator.

3. Fixation and Permeabilization

3.1. Remove the medium from the wells and wash with 100 µL of 1x PBS. Aspirate with a micropipette to minimize the risk of removing the sample.

3.2. Fix cells by adding 50 µL of 4% PFA per well and incubate for 10 min at RT, without agitation. Cells are sensitive to detachment, so avoid pipetting the solutions directly onto the cells.

3.3. Wash the cells with 0.05% TBST three times for 5 min each. Aspirate with a micropipette to minimize the risk of removing the sample.

3.4. Treat the cells with permeabilization solution (0.1% Triton x-100 in 1x PBS) for 10 min without agitation at RT.

3.5. Wash cells with TBST three times for 5 min each with agitation.

4. Blocking

4.1. Tap off the TBST (very gently with a micropipette). Visualize the slide in a microscope to see if cells remain attached.

4.2. Add one drop (50-60 µL) of 1x Blocking Solution to each sample.

4.3. Pre-heat a humidity chamber (empty tip box with water) at 37 °C and incubate the slides in it for 1 h at 37 °C.

5. Primary Antibodies

5.1. Dilute the primary antibodies (1:100) in the Antibody Diluent (see **Table of Materials**). Prepare 40 µL antibody solution per well.

5.2. Remove blocking solution from the slides by tapping off the liquid. Do not allow cells to dry.

5.3. Vortex and add 40 µL of the antibody solutions to each well.

5.4. Incubate 1 h at 37 °C in a preheated humidity chamber.

6. Proximity Ligation Assay Probes

Note: PLA probes are provided as part of a kit (see **Table of Materials**). The choice of probes will depend on the species of primary antibodies used to detect the proteins of interest.

6.1. Dilute the two PLA probes (anti-Mouse MINUS and anti-Rabbit PLUS) 1:5 in the Antibody Diluent. For each sample, prepare 40 µL of PLA probe. Incubate the mixture for 20 min at RT.

6.2. Tap off the primary antibody solution from the slides. Wash the slides twice for 5 min each with Wash Buffer at RT.

6.3. Gently tap of the Wash Buffer from the slides and add the diluent PLA probe solution to wells (40 µL/well).

6.4. Incubate the slides in a pre-heated humidity chamber for 1 h at 37 °C.

7. Ligation

Note: The *in situ* Detection Reagents Red, Ligase, and Ligation Solution are provided as part of a kit (see **Table of Materials**).

7.1. Use the *in situ* Detection Reagents Red.

7.2. Immediately before use, vortex and dilute the required volumes of the 5x ligation stock 1:5 in high purity water.

Note: Do not store diluted reagents.

7.3. Tap off the PLA probe solution from the slides. Wash the slides in 1x Wash Buffer twice for 5 min each at RT.

7.4. Immediately before addition to the samples, vortex and add Ligase to the Ligation solution at 1:40 dilution and vortex again.

7.5. Tap off the Wash Buffer from the slides and add the Ligation/Ligase solution to each well (40 µL/well).

7.6. Incubate the slides in a pre-heated humidity chamber for 30 min at 37 °C.

8. Amplification

Note: The amplification stock is provided as part of a kit (see **Table of Materials**). Reagents are light sensitive; thus avoid exposing the slides to light.

8.1. Vortex and dilute the required volumes of the 5x Amplification stock 1:5 in high purity water and mix.

8.2. Tap off the Ligation/Ligase solution from the slides. Wash the slides in 1x Wash Buffer twice for 2 min each at RT.

8.3. Vortex and add the polymerase to the Amplification solution at 1:80 dilution and vortex again.

8.4. Tap off Wash Buffer from the slides and add the Amplification/Polymerase solution to each well (40 µL/well). Incubate the slides in a pre-heated humidity chamber for 100 min at 37 °C.

9. Preparation for Imaging

Note: These are light sensitive reagents. Keep the slides protected from light.

9.1. Tap off the Amplification-Polymerase solution from the slides and wash twice for 10 min each in 1x Wash Buffer at RT.

9.2. Remove the chambers and silicone around the wells from the slide completely. Scrape off the remaining bits of silicone with a razor. Draw a grid on the slide separating each well.

9.3. Add ~40 µL of the mounting medium with DAPI. Be careful to avoid trapping air bubbles under the cover slip. The edges of the cover slip can be sealed with nail polish.

9.4. Wait for at least 20 min before performing image analysis using a confocal microscope.

Note: The protocol can be paused here.

9.5. After imaging, store the slides at -20 °C in the dark.

REPRESENTATIVE RESULTS

We used the PLA assay to test the interaction between MST1 and MST2 in HEK-293 and iHSC. The cells were fixed, permeabilized, and stained with various antibodies, followed by *in situ* amplification according to the PLA protocol (**Figure 1**). To document the level of MST1/MST2 heterodimerization, cells were stained with MST1 and MST2 antibodies (**Figures 1A, 1C, and 1G**). As a positive control, we also used ERK and pERK antibodies that are expected to be in close proximity in cells with activated ERK (**Figure 1B and 1F**). Cells lacking MST1 and MST2 show no PLA signal (**Figure 1C**). Cells stained with only one of the two antibodies likewise show no PLA signal (**Figure 1G**). These results suggest that HEK-293 cells and iHSC cells contain MST1/MST heterodimers, consistent with previous findings from our laboratory[4]. As an additional negative control we incubated cells with ERK and MST2 primary antibodies to show specificity of signals (**Figures 1D and 1H**).

To confirm levels of MST1 and MST2 expression, extracts from WT and MST1/MST2 knockout HEK-293 cells, respectively, were analysed by immunoblot with the indicated antibodies (**Figure 1I**).

FIGURE AND TABLE LEGENDS

Figure 1: **Representative PLA data**. (A-H) Cells were fixed and stained with the indicated antibodies followed by the PLA procedure. Blue: DAPI, RED: PLA signal. Photomicrographs were obtained with a Leica SP5 confocal microscope. (I) Immunoblot for MST1 and MST2. Scale bar is 10 µm.

DISCUSSION

We found it useful to use glass chamber slides for this experiment, as it is very convenient to perform experiment with several (14-16) cell lines and there is no need to change the sample every time during microscopy analysis. A few complications may arise, such as an increased risk for cross-contamination with antibodies. Therefore, we suggest washing every well individually instead of using a Coplin jar, despite the increased duration of the experiment. In addition, removal of silicone insert is a delicate affair and must be done with care and patience. Even with meticulous technique, it is sometimes possible to see small amounts of silicone debris after removal the insert. For this reason, one must remove the silicone insert scrupulously, using a razorblade if necessary, as too much unremoved silicone can alter the confocal distance during microscopy. It can also be useful to draw lines as borders of wells after removing the silicon insert to help find cells under microscope.

It is imperative to use non-cross reactive primary antibodies that each recognize only one member of the heterodimer (*e.g.,* the MST1 antibodies should not also recognize MST2 and *vice-versa*). Also, it is important to remember to use different tips to avoid cross-contamination

of primary antibodies and PLA probes, to avoid touching the bottom of the well and to avoid pipetting directly over samples. We found that for iHSC, it is better to coat chamber slides with 0.01% poly-L-lysine solution, and for HEK-293 it is better to coat with mouse 10 µg/mL laminin.

As with any procedure, there are some limitations of this method. The PLA assay, while a powerful method for all the reasons listed above, is limited by the specificity and sensitivity of the antibodies. Furthermore, antibody concentrations and cell culture conditions should be finely tuned before laboratory experiments are set up to reduce the costs of the assays. In that regard, an alternative to reduce the cost of the assays is to use uncoated 35-mm glass-bottom dish instead of chamber slides.

An advantage of the assay is that intensity and number of fluorescent dots can be quantified using various computer software programs such as the Blob-finder software, the Duolink Image Tool, and th Olink Bioscience.

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DISCLOSURES

The authors declare that they have no competing financial interests.

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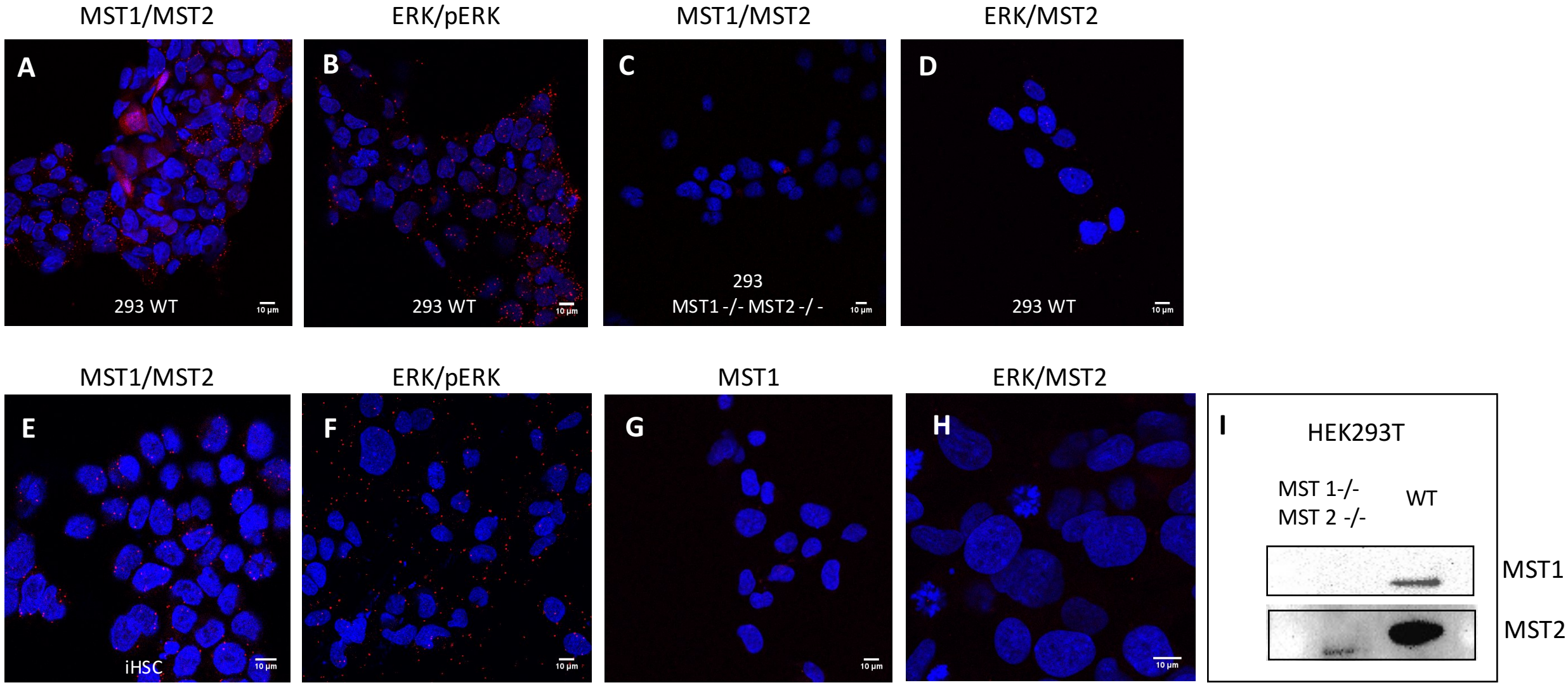
Figure



Name of Material/ Equipment	Company
Chamber slides	Thermo Fisher Scientific
PLA probe Anti-Mouse Minus	Sigma-Aldrich
PLA Probe Anti-Rabbit PLUS	Sigma-Aldrich
Wash Buffers, Flurescence	Sigma-Aldrich
Mounting Medium with DAPI	Sigma-Aldrich
Detection Reagents Red	Sigma-Aldrich
p44/42 MAPK (Erk1/2) Antibody	Cell Signaling
Phospho-p44/42 MAPK (Erk1) (Tyr204	Cell Signaling
MST2 antibody	Cell Signaling
Krs-2 (RJ-5)	Santa Cruz
16% Paraformaldehyde	Electron microscopy s
Triton x100	Fisher BioReagents
Confocal microscopy	Leica TCS SP8, 63x

Catalog Number	Comments/Description	
178599	16 well, glass slide	
DUO92004	Contains 1x Blocking solution, 1x antibod	
DUO92002	Contains 1x Blocking solution, 1x antibod	
DUO82049	Contains Wash Buffer A and Wash Buffer	
DUO82040		
DUO92008	Contains 5x Ligation, 1x Ligase, 5x Amlific	
9102s		
5726s	pERK antibody	
3952s		
sc-100449	MST1 antibody	
15710	Dilute to 4% PFA in PBS for fixing solutior	
BP 151-500	To prepare 0.1% Triton x-100 in 1xPBS fo	
	Image analysis with ImageJ software	

ly Diluent

ly Diluent

B

cation Red, 1x Polymerase

n

r Permeabilization solution

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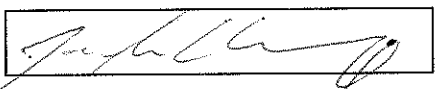
Signature:	
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Dear Sir, 8/10/18

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.
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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