Dear Dr. DSouza,

We are submitting a revision of the manuscript JoVE60982 "*In situ* detection of RNP complex assembly in the *C. elegans* germline using Proximity Ligation Assay". We appreciate your attention to our manuscript and thank the reviewers for their comments, which guided the changes in this manuscript (marked in blue in the revised version). We hope that you find this manuscript ready for publication.

We have provided a line by line response to both the editorial and peer-review comments in blue text below.

Best regards.
Nicholas Day and Ekaterina Voronina

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

Revised text is in blue.

• **Protocol Language:**
1) Please re-write steps 1.1-3.2 in the protocol section to be in the imperative voice/tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible. For example: " Worms are kept on NGM plates that are seeded with the OP50 strain" should be " Keep worms on NGM plates that are seeded with the OP50 strain…".

We have revised steps 1.1-3.2 so that they are written in the imperative voice/tense as suggested. Based on feedback from the protocol detail section below, we have included additional steps for section 2 to provide more detail on preparation of a synchronous culture. These new steps are also written in the imperative voice/tense.

2) Please split up long steps so that each steps contains 3-4 actions in 4 or fewer sentences (e.g., several steps in section 12).

We have revised several steps in section 12, including steps 12.1.1, 12.3, 12.5, 12.6, 12.7, and 12.8, where excessive details were split either into additional sub-steps, or into separate notes. Steps 7.2 and 8.2 were also split after adding other details that were recommended by the reviewers.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific**

details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples:
1) 1.1: Mention worm age, and strain. How were the plates seeded?

The NOTE that precedes section 1 has now been revised to include information about the strains used for the protocol. The 3xFLAG::DLC-1; GFP strain is designated as a negative control while the 3xFLAG::DLC-1; OMA-1::GFP strain is the experimental strain. The age at which worms are passaged as described in step 1.1 is now indicated as adult. Section 2 is revised to include a more detailed description of how to prepare and seed the synchronous larval cultures.

• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.
1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

The revised protocol has now been highlighted with content that should be filmed and now excludes the NOTE sections.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

The revised Discussion section now addresses these points.

• **Tables:** Please remove the embedded Table from the manuscript. All tables should be uploaded to the Editorial Manager site in the form of Excel files. A description of the table should be included with the Figure legends.

The embedded table has been removed from the manuscript and is now included as an excel file named “Table 1_Summary of PLA Results”.

• **References:** Please spell out journal names.

The references provided in this manuscript are provided in the JoVE citation style format that is available through Endnote.

• **Commercial Language:**JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Duolink, Adobe Photoshop.
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

The "Duolink" trademark names are now removed. Each reagent that contained the Duolink name and is listed for the first time in the text is now followed by "(see table of materials)". "Adobe Photoshop" is now given the generic name "image processing software" and is followed with the "(see table of materials)" direction.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

The figures and tables in this manuscript are original and have not been published previously.

Comments from Peer-Reviewers:

Reviewers' comments:
Reviewer #1:

In this manuscript, Day and colleagues present a protocol to perform the proximity ligation assay (PLA) on dissected germlines of the nematode C. elegans. The authors do an excellent job of

providing a detailed method and also suggest a downstream analysis protocol for use in the widely used Fiji/imageJ image analysis software. The approach is applied using the interaction between DLC-1 and OMA-1, and highlights how the PLA method can identify interactions in vivo that might be hard to detect using other methods.

Overall, I found the manuscript well written and the protocol easy to follow. I only have some minor questions/comments that would be useful for the authors to address/discuss in a revised version of the manuscript:

1) Have the authors attempted this protocol using other regions of the animal in addition to extruded germlines?

We have found that this protocol also works with extruded and unobstructed embryos. We have included this detail in the NOTE that precedes section 3 as well as the Discussion.

Does the method would work well for intact (i.e non extruded) germlines, or embryos, or somatic tissues?

We have looked at embryos and gonad arms that still remain inside the cuticle after dissection and found that PLA does not produce a signal in these tissues. We believe that PLA would not work on intact or un-dissected worm tissues using the current protocol, and now note this in the Discussion.

Would different fixation methods need to be employed or protocols normally used for whole-mount IF apply here? Perhaps some guidelines on what parameters to optimize based on their experiences getting this technique to work on extruded germlines would be useful for those looking to apply this protocol in slightly different contexts in the worm.

We thank this reviewer for this suggestion and have revised the Discussion section of the manuscript accordingly. Although we have not tested PLA after a whole-mount IF approach, using the protocol that produces reliable IF staining is a suitable place to start for optimization of PLA as these conditions allow tissue accessibility for antibodies.

2) It would be great to incorporate use of the negative controls in the write-up of the protocol itself. I was looking for these as I was reading the protocol, and did not see description of recommended negative controls (i.e use of DLC-1::FLAG with GFP control strain) until the discussion section.

We thank the reviewer for pointing this out. In the revised manuscript, we designated what negative control and experimental strains were used during the protocol in the NOTE that precedes section 1.

Is this the main control needed to assess background?

Yes, the 3xFLAG::DLC-1; GFP alone strain is intended to be a control that assesses background.

Would one also need a reciprocal control (GFP-tagged candidate with FLAG alone) or is this considered redundant? Have the authors tried this reciprocal control?

The GFP alone control serves as a negative control as we do not expect GFP to interact with nematode proteins and the resulting signal is treated as background. Our approach is to set up PLA experiments that use the same pair of tags (3xFLAG and GFP) for each pair of potentially interacting partners. Given that 3xFLAG::DLC-1 and GFP alone are expressed throughout the germline, PLA on the GFP alone strain is a control that establishes the background that results from the pair of FLAG and GFP antibodies. This background is evenly distributed throughout the germline, making it an ideal control to use when studying PLA at different regions of the germline. We consider a reciprocal control redundant and have not tested that. Given the small size of the 3xFLAG peptide sequence (24 AA), obtaining expression of 3xFLAG alone in the worm might be challenging.

Reviewer #2:

Manuscript Summary:
This manuscript provides a detailed and relatively clear protocol to utilize the Proximity Ligation Assay (PLA) technique in the C. elegans gonad in order to identify spatiotemporal, in vivo protein-protein interactions (PPIs) of two proteins. Importantly, the manuscript includes specific details regarding how to conduct the required image analysis after PLA and quantification of the PPIs using publicly available software (FIJI/ImageJ). This article will be useful for many researchers conducting experiments in the C. elegans gonad. The steps listed in the procedure are generally presented in a clear fashion and easy to follow as written. More details on the authors recommended negative controls should be included.

Major Concerns:

1. The authors need to be more detailed from the start regarding what their "3xFlag::DLC-1; GFP" worm strain is. The reviewer is confused as to what is tagged with GFP in this particular strain. This comes up, for example in Line 400 and in Figure 2.

The 3xFLAG::DLC-1; GFP strain is the negative control that is used for this protocol. In this strain, GFP is expressed by itself and is not fused to any worm protein. Therefore, it is used to test for any background PLA that results from spurious proximity of 3xFLAG-tagged target protein and diffusely-distributed GFP. For clarification, we have revised the NOTE section that precedes section 1 of the protocol. Here we introduce the strain for the first time as 3xFLAG::DLC-1; GFP alone. After this first instance, it is referred later in the text as 3xFLAG::DLC-1; GFP.

2. What negative controls do the authors specifically recommend?

As a negative control, we recommend using the same primary antibodies on a pair of proteins that is not expected to interact. In the case of the article, our negative control is un-tagged GFP, which is not an endogenous protein in the worm and is not expected to interact with 3xFLAG-tagged DLC-1.

Providing these controls can help other researchers in their ability to conduct the protocol. Also, it is suggested that the specific negative controls to be included in the experiment be mentioned much earlier in the protocol (they come only in Line 312).

In the revised manuscript, we introduce the control in the NOTE section that precedes section 1, and note that this strain is available upon request.

Minor Concerns:

1- The sentence comprising lines 42-44 is not clear in regards to how the transparent cuticle allows for visualization of PPIs throughout worm development.

We have rewritten the sentence.

2- Lines 75-77 consist of a sentence that is unclear and should be rewritten for clarity sakes.

We have clarified the description of BiFC.

3- Line 132: The authors should list the specific worm strains they are using in this protocol so that readers can obtain them if they are interested in appropriate controls.

The NOTE section that precedes section 1 of the protocol has been revised to now include the strain information and the fact that the strains are available upon request. Readers are now also directed to the table of materials for specific details about the strain designation and genotypes.

4- Line 145: Inclusion of the reference name and location where one can find the bleaching protocol is important versus just presenting the numerical reference at the end of the sentence.

Citations in this manuscript are presented in the JoVE citation style format, however this particular reference has now been revised to include the lead author name, year, and journal.

5- Line 158: Authors should provide reference to standard gonad staining protocol

The revised manuscript now contains a reference that describes the standard gonad staining protocol.

6- Line 167: What solution should be used to "rinse the worms 2-3 times until all bacteria are removed"?

The revised manuscript now contains steps 3.1 and step 3.2, which describe this washing step in greater detail. For clarity here, the worms should be rinsed 2-3 times in 1x M9 + levamisole media.

7- Lines 173-174: More information regarding the specific microscope slides utilized should be included, as the reviewer cannot locate these slides when searching using the provided Thermo Fisher catalog number at the end of the manuscript submission document.

The slides are custom-ordered through a Fisher Scientific representative given the description and the catalog number.

8- Line 199: More details should be included in step 4.3 in how you let the fixative run off slide. For example, "Tilt slide at a 45degree angle against the paper towel to allow fixative to run off slide onto paper towel"

This step is now rephrased to include more detail. This is an important detail that is now addressed in other steps, including: 4.5, 5.1, 6.2, 7.2.1, and 8.2.1.

9- Lines 214-216: What comprises "Proper dilution" of an antibody for best PLA results? Add more information about what is the ideal dilution to use and how the reader can go about determining the ideal dilution.

This text in the NOTE has been rephrased to direct the reader to Discussion for notes on optimizing antibody dilution. The revised Discussion now goes into greater detail on how obtain the optimal dilution of primary antibody. In brief, determining the ideal dilution for PLA will start with the dilution that is typically used for immunofluorescence. The users can proceed with testing this dilution with PLA to establish that these antibody concentrations do not produce excessive signal in the negative control.

How does the typically antibody dilution used for immunofluorescence relate to the antibody dilution needed for the PLA experiment? Some strategies to help the reader could be included here.

The antibody dilution used for PLA is either the same as what is used for immunofluorescence or even higher. This has been included in the Discussion section as well as more detail about what are some possible outcomes for PLA that result from too low or high of a primary antibody dilution.

10- Line 227 (and other places in protocol): Why must the washes be done in a Coplin jar?

The protocol from the manufacturer says that all wash steps should be performed in a staining jar. The immunofluorescence protocol used in the lab also performs washes in a Coplin jar to provide sufficient volume for the washes.

Can the washes be done just by adding buffers to cover the sample area on slide, as per standard antibody protocol, or must it be done in a Coplin jar? I think this is important to point out if it only works with Coplin jar washes and why the authors have chosen to provide this method as the method of choice for the wash steps.

We have not experimented with adding buffers to cover the sample area as a way to wash the sample. Washing in Coplin jars is preferred because a greater volume of buffer will help to improve the efficiency of the washes. Further, washing in Coplin jars reduces the chance of damaging or washing off the samples immobilized on the slide as it minimizes the need to pipette wash buffer directly onto the sample. A NOTE has been added to step 4.3, where a Coplin jar is mentioned first, to emphasize its usage in the protocol.

11- Lines 433 and 439: The way the description is written it implies that the oocytes are not part of the gonad. "In each image, the gonad is outlined with dashed lines, while the oocytes are outlined with dotted lines". These sentences could be clarified.

We have revised the image descriptions for clarity.

12- Line 436: Including details as to why the authors have utilized the GFP antibody dilution at 1:40,000 here for the PLA versus the 1:200 dilution for the immunostained gonads would be beneficial to the reader.

To be clear, line 436 in the initial manuscript says that “GFP antibody was used at a 1:4000 dilution”. The 1:4000 dilution of GFP antibody was optimal for PLA reactions, however it was too dilute for immunofluorescence. The optimal dilution for the immunostain was 1:200, which gave the best signal. This discrepancy is addressed in the Discussion, as the sensitivity of the PLA reaction is much greater than what is observed by IF. In the case that the reviewer was concerned about the 1:40,000 dilution, this was performed as a part of a 10-fold greater dilution of the FLAG and GFP antibodies that were used for PLA. This comparison showed a reduction in PLA density, however the difference between the control and the experiment was still significantly different and suggests that PLA is still sensitive with a higher dilution of antibody.

Reviewer #3:

Manuscript Summary:
This manuscript describes an alternative approach to examining protein-protein interactions in

fixed tissues. The Proximity Ligation Assay (PLA) has been used by many groups/systems, but there does not appear to be any published reports of its use in C. elegans and as such, this manuscript is timely and should encourage the field to adopt this approach where appropriate. A detailed and clearly written PLA experimental protocol is outlined as well as an example of the data that can be generated using approach. Importantly there is a strong emphasis on image/data analysis using open source software.

Minor Concerns:

Introduction:
Some comment should be made about super-resolution microscopy which offers superior resolution compared to older imaging approaches discussed.

We have included a comment on super-resolution microscopy being able to more stringently test for protein colocalization; however it would still be of limited power in detecting PPIs of diffusely-distributed proteins.

Protocol
The discussion of negative controls comes at the end of the manuscript, but reference to negative controls is mentioned throughout, so I would be good to quickly define what are the best negative controls when first mentioned and then have the wider discussion latter.

The revised manuscript now contains more detail about the negative control much earlier in the text, in the NOTE that precedes section 1 of the protocol.

Line 202: On the slides, the edges of the area containing the dissected germlines should be wiped dry so the addition of the next reagent is held by surface tension around the sample.

This is an important detail that is now included in the revised manuscript. Besides step 4.4, it has also been included in steps 4.2, 4.5, 5.1, 6.2, 7.2.1, and 8.2.1.

Line 215: providing some idea of dilution ranges to be tested would help a novice interpret this important point.

The text in the NOTE that precedes step 5.1 has been revised. Readers are referred to the Discussion where we discuss antibody dilution in more detail.

Discussion:
The cost of the PLA methodology is a major consideration, so some comments about the relative costs of PPI approaches would be useful.

This is a valuable point to discuss about PLA. Some comments have been included in the second paragraph of the Discussion.

Figure 2: include zoomed-in images to highlight the PLA foci in control and samples.

Figure 2C-D of the updated manuscript now contains zoomed in images. The DAPI channels are now moved up to 2Cii and 2Dii. The PLA channels are now moved down to 2Ciii and 2Diii. The dashed green boxes in 2Ciii and 2Diii denotes the location of the zoomed in images in 2Civ and 2Div.

Reviewer #4:

Manuscript Summary:
The manuscript describes an important methodology referred to as Proximity Ligation Assay (PLA) which allows in situ detection of protein-protein interactions. It is well-written and the PLA is clearly justified using an interesting example of interaction between DLC-1 and OMA-1 proteins.