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TITLE:

In Situ Detection of Ribonucleoprotein Complex Assembly in the *C. elegans* Germline using Proximity Ligation Assay

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SUMMARY:

This protocol demonstrates use of the proximity ligation assay to probe for protein-protein interactions in situ in the *C. elegans* germline.

ABSTRACT:

Understanding when and where protein-protein interactions (PPIs) occur is critical to understanding protein function in the cell and how broader processes such as development are affected. The *Caenorhabditis elegans* germline is a great model system for studying PPIs that are related to the regulation of stem cells, meiosis, and development. There are a variety of well-developed techniques that allow proteins of interest to be tagged for recognition by standard antibodies, making this system advantageous for proximity ligation assay (PLA) reactions. As a result, the PLA is able to show where PPIs occur in a spatial and temporal manner in germlines more effectively than alternative approaches. Described here is a protocol for the application and quantification of this technology to probe PPIs in the *C. elegans* germline.

INTRODUCTION:

Over 80% of proteins are estimated to have interactions with other molecules[1], which emphasizes how important PPIs are to the execution of specific biological functions in the cell[2]. Some proteins function as hubs facilitating assembly of larger complexes that are necessary for cell survival[1]. These hubs mediate multiple PPIs and help organize proteins into a network that facilitates specific functions in a cell[3]. Formation of protein complexes is also affected by biological context, such as the presence or absence of specific interacting partners[4], cell signaling events, and developmental stage of a cell.

C. elegans is commonly used as a model organism for a variety of studies, including development. The simple anatomy of this animal is comprised of several organs, including the gonad, gut, and transparent cuticle, which facilitates the analysis of worm development. The germline residing in the gonad is a great tool to study how germline stem cells mature into gametes[5] that develop into embryos and eventually the next generation of progeny. The distal tip region of the germline contains a pool of self-renewing stem cells (**Figure 1**). As stem cells leave the niche, they progress into the meiotic pachytene and eventually develop into oocytes in the young adult stage (**Figure 1**). This program of development in the germline is tightly regulated through different mechanisms, including a post-transcriptional regulatory network facilitated by RNA-binding proteins (RBPs)[6]. PPIs are important for this regulatory activity, as RBPs associate with other cofactors to exert their functions.

There are several approaches that can be used to probe for PPIs in the worm, but each has unique limitations. In vivo immunoprecipitation (IP) can be used to isolate protein-protein complexes from whole worm extracts; however, this approach does not indicate where the PPI occurs in the worm. In addition, protein complexes that are transient and only form during a specific stage of development or in a limited number of cells can be difficult to recover by co-immunoprecipitation. Finally, IP experiments need to address the concerns of protein complex reassortment after lysis and non-specific retention of proteins on the affinity matrix.

Alternative approaches for in situ detection of PPIs are co-immunostaining, Förster resonance energy transfer (FRET), and bimolecular fluorescence complementation (BiFC). Co-immunostaining relies on simultaneous detection of two proteins of interest in fixed worm tissue and measurement of the extent of signal colocalization. Use of super-resolution microscopy, which offers greater detail than standard microscopy[7], helps to more stringently test protein colocalization beyond the diffraction-limited barrier of 200–300 nm[8]. However, co-immunostaining using both conventional and super-resolution microscopy works best for proteins with well-defined localization patterns. Additionally, it becomes much less informative for diffusely distributed interacting partners. Measuring for co-localization of signals based on overlap does not provide accurate information about whether the proteins are in complex with each other[9,10].

Furthermore, co-immunoprecipitation and co-immunostaining of protein-protein complexes are not quantitative, making it challenging to determine if such interactions are significant. FRET and BiFC are both fluorescent-based techniques. FRET relies on tagging proteins of interest with fluorescent proteins (FPs) that have spectral overlap at which energy from one FP (donor) is transferred to another FP (acceptor)[11]. This nonradiative transfer of energy results in fluorescence of the acceptor FP that can be detected at its respective wavelength of emission. BiFC is based on reconstitution of a fluorescent protein in vivo. It entails splitting GFP into two complementary fragments, such as helices 1–10 and helix 11[12], which are then fused to two proteins of interest. If these two proteins interact, the complementary fragments of GFP become close enough in proximity to fold and assemble, reconstituting the GFP fluorophore. Reconstituted GFP is then directly observed as fluorescence and indicates where a PPI has occurred.

As such, both FRET and BiFC depend on large fluorescent tags that can disrupt the function of the tagged protein. In addition, FRET and BiFC require abundant and comparable expression of the tagged proteins to obtain accurate data. FRET may not be suitable for experiments where one partner is in excess of the other, which can lead to high background[13]. Overexpression in BiFC experiments should also be avoided, as this can induce nonspecific assembly[14] that results in increased background. Both techniques require optimization of expression and imaging conditions of the tagged proteins, which may prolong the time required to complete experiments.

The proximity ligation assay (PLA) is an alternative approach that can address the limitations of the techniques mentioned above. PLA takes advantage of primary antibodies that recognize the proteins of interest (or their tags). These primary antibodies are then bound by secondary antibodies containing oligonucleotide probes that can hybridize with one another when within a 40 nm (or shorter) distance[15]. The resulting hybridized DNA is amplified through a PCR reaction, which is detected by probes that complement the DNA. This results in foci that are visualized by a microscope. This technology can detect PPIs in situ in complex tissues (i.e., the worm gonad), which is organized as an assembly line containing cells at various stages of development and differentiation. With PLA, PPIs can be directly visualized in a fixed worm gonad, which is advantageous for investigating whether PPIs occur during a specific stage of development. PLA offers greater resolution of PPIs as opposed to co-localization-based assays, which is ideal for making precise measurements. If used, super-resolution microscopy has the potential to provide finer detail about the location of PLA foci within a cell. Another advantage is that the foci resulting from PLA reactions can be counted by an ImageJ-based analysis workflow, making this technique quantitative.

The LC8 family of dynein light chains was first described as a subunit of the dynein motor complex[16] and hypothesized to serve as a cargo adapter. Since its initial discovery, LC8 has been found in multiple protein complexes in addition to the dynein motor complex[17-20]. Scanning for protein sequences that contain the LC8 interaction motif[19] suggests that LC8 may have many interactions with a wide array of different proteins[17-22]. As a result, LC8 family proteins are now considered hubs that help promote the assembly of larger protein complexes[19,22], such as assemblies of intrinsically disordered proteins[21].

One *C. elegans* LC8-family protein, dynein light chain-1 (DLC-1), is widely expressed across many tissues and not enriched in specific subcellular structures[23,24]. Consequently, identification of biologically relevant in vivo partners of DLC-1 in *C. elegans* is challenging for a number of reasons: 1) co-immunoprecipitation does not indicate the tissue source where the interaction occurs; 2) limited expression of particular partners or transient interactions may hinder the ability to detect an interaction by co-immunoprecipitation; and 3) diffuse distribution of DLC-1 leads to non-specific overlap with potential partner proteins by co-immunostaining. Based on these challenges, PLA is an ideal approach for testing in vivo interactions with DLC-1.

It has been previously reported that DLC-1 directly interacts with and serves as a cofactor for the RNA-binding proteins (RBPs) FBF-2[23] and GLD-1[25]. Our work supports the model of DLC-1 serving as a hub protein and suggests that DLC-1 facilitates an interaction network that spans beyond dynein[19,22]. Using a GST pulldown assay, a new DLC-1-interacting RBP named OMA-1 has been identified[26]. OMA-1 is important for oocyte growth and meiotic maturation[27] and functions in conjunction with a number of translational repressors and activators[28]. While FBF-2 and GLD-1 are expressed in the stem cells and meiotic pachytene regions, respectively, OMA-1 is diffusely expressed in the germline from the meiotic pachytene through the oocytes[27] (**Figure 1**). This suggests that DLC-1 forms complexes with RBPs in different regions of the gonad. It has also been found that the direct interaction between DLC-1 and OMA-1 observed in vitro is not recovered by an in vivo IP. The PLA has been successfully used as an alternate approach to further study this interaction in the *C. elegans* germline, and results suggest that PLA can be used to probe many other PPIs in the worm.

PROTOCOL:

NOTE: This protocol uses *C. elegans* strains in which potential interacting partners are both tagged. It is strongly recommended that a negative control strain be used, in which one tagged protein is not expected to interact with another tagged candidate interaction partner. Here, GFP alone was used as a negative control to assess background, as DLC-1 is not expected to interact with GFP in the worm. GFP-tagged OMA-1 was used as the experimental strain, as preliminary data suggest an interaction with DLC-1. Nematode strains co-expressing control and test proteins with 3xFLAG-tagged DLC-1 are referred to in this text as 3xFLAG::DLC-1; GFP and 3xFLAG::DLC-1; and OMA-1::GFP (strains available upon request; more information in **Table of Materials**), respectively. Here, the 3xFLAG and GFP tags are used; however, other tags may be substituted as long as their antibodies are compatible with the PLA kit reagents.

1. Animal care

1.1. Keep worms on nematode growth medium (NGM) plates that are seeded with the OP50 strain of *E. coli* and maintain at 24 °C for optimal expression of GFP.

1.2. Passage adult worms every 2–3 days to propagate worms and keep them well-fed.

2. Preparation of synchronous culture

2.1. Synchronize worms by bleaching a plate of well-fed, gravid hermaphrodites. A bleaching protocol is described in Porta-de-la-Riva et al.[29]. Let the embryos hatch overnight in a centrifuge tube at 24 °C while rotating end over end in 10 mL of M9 minimal media (M9) buffer. This will produce a culture of arrested L1 larvae.

2.2. Incubate the tube of arrested L1 stage larvae on ice for 10 min, then top off the tube with ice cold 1x M9.

2.3. Use a centrifuge to pellet the larvae at 600 x *g* for 5 min at 4 °C. Carefully aspirate the supernatant so that only 1–2 mL of supernatant remains.

2.4. Re-suspend the pellet of larvae and use a micropipette to transfer 2 µL of suspended larvae culture to a glass slide. Count how many larvae are present to determine the density of the larvae culture, which will help guide seeding of the worms in step 2.5.

NOTE: A density of 10–15 L1 larvae/1 µL works well for seeding.

2.5. Use a micropipette to transfer the volume of larvae culture needed to seed approximately 100–120 L1 stage larvae on a 60 mm OP50 plate. For example, seed 10 µL of a larvae culture that has a density of 10 L1 larvae/1 µL of culture.

NOTE: Do not exceed a volume of 40 µL to seed the larvae, or excess liquid will disrupt the OP50 lawn. If the culture volume exceeds 40 µL, repeat steps 2.3–2.4 to further reduce the volume and increase the density of larvae culture.

2.6. Grow worms at 24 °C. Record the time when L1s are seeded on plate and periodically check the stage of development to identify the ideal time for dissection.

NOTE: At 52 h after seeding of L1s, worms cultured at 24 °C are typically in the young adult stage, which is the ideal stage for dissection for gonad-targeted PLA. However, the actual time at which the synchronized worms reach young adult stage may vary among strains and incubation temperature.

3. Dissection/gonad extrusion

NOTE: Dissection to extrude the gonad is necessary for gonad-targeted PLA to work successfully. This approach can also release embryos, which also work using this protocol for PLA (see discussion for more information). After dissection, both the negative control and experimental samples are fixed and treated for PLA together in parallel. It is also suggested that an additional set of samples be prepared for the purpose of fluorescent co-immunostaining[23] to demonstrate expression patterns of the protein partners of interest.

3.1. Pick 30–40 young adult worms into a watch glass dish containing 500 µL of 1x M9 + levamisole (2.5 mM final concentration). After collecting the worms, carefully remove and discard most of the media to remove bacteria that is transferred along with the worms.

3.2. Add in fresh 500 µL of 1x M9 + levamisole and use the pipette to gently draw up and dispense the media to rinse the worms. Carefully remove and discard most of the media to clear bacteria that is transferred along with the worms.

3.2.1. Repeat this step 2x–3x until all bacteria are removed. After washes are completed, leave worms in about 100 µL of media to keep hydrated.

NOTE: Do not let the worms sit too long in media (|7–8 min), as this will impair the extrusion of gonads during dissection. Perform washes under aid of dissecting microscope to monitor removal of media so that worms are not lost.

3.3. Using a glass or polyethylene pipette, transfer worms to a 25 mm x 75 mm microscope slide coated with 0.001% poly-L-lysine (slides used in this procedure have an epoxy coated perimeter, leaving three workspaces, 14 mm x 14 mm each). Remove excess media so that approximately 10–15 µL of media remains.

3.4. Under the aid of a dissecting microscope and using two 26½ gauge needles, place one needle over the other so that the ends form a pair of scissors. Using needles oriented in this fashion, cut worms behind the pharynx to release the germlines. Dissect all worms within 5 minutes.

NOTE: More detail on how to perform dissections can be found in a previous publication by Gervaise and Arur[30].

3.5. After all worms are dissected, gently place a 22 mm x 40 mm coverslip over the slide so that it is perpendicular to the slide. The ends of the coverslip should hang off the slide.

3.6. Freeze the slides on a pre-chilled aluminum block maintained on dry ice for at least 20 min. Gently place a chilled pencil on top of the coverslip to prevent the coverslip from becoming loose due to ice expansion.

4. Fixation/blocking

4.1. When ready for fixation, flick off coverslips with a pencil or other blunt-edged tool and immediately dip the slide into a jar containing fresh, ice-cold methanol (chilled to -20 °C) for 1 min.

4.2. Gently wipe the edges of the slide that surround the sample so that the next reagent is held by surface tension around the sample. Apply 150 µL of fixative (2% formaldehyde in 100 mM KH_2PO_4, pH = 7.2) for 5 min at RT.

NOTE: We have also tested a methanol/acetone fixation procedure[31,32] and found that it is compatible with the PLA reaction.

4.3. Touch the slide to a paper towel at a perpendicular 90° angle to let the fixative run off the slide and absorb into the paper towel. Block slides 2x for 15 min at RT in a Coplin jar with 50 mL of 1x PBS/1% Triton X-100/1% bovine serum albumin (PBT/BSA).

NOTE: Coplin jars or other types of staining jars are recommended for this blocking step and the washing steps below in sections 6–9. These provide sufficient volumes for efficient exchange of blocking or washing buffer with the sample.

4.4. Block slides with a PBT/BSA solution containing 10% normal goat serum. Gently wipe edges that surround the slide and apply 100 µL of the solution to the slide. Incubate for 1 h at RT in a humid chamber.

NOTE: This step is highly recommended for staining with the αFLAG primary antibody. The humid chamber is constructed by securing glass pipettes with tape in the tray for the slides to lay on as they incubate. Dampened task wipes (**Table of Materials**) are placed in the tray to raise the internal humidity of the tray to prevent evaporation. The lid and tray are covered in foil to protect the samples from light during the light-sensitive steps.

4.5. Place slide on a paper towel to let PBT/BSA/10%NGS solution run off the slide and gently wipe the edges of the slide. Use the blocking reagent (**Table of Materials**) to block slides. Apply one drop to the 14 mm x 14 mm space. Incubate slides for 1 h at 37 °C in a humid chamber.

5. Primary antibody incubation

NOTE: To obtain the best PLA results and minimal background, the dilution factor of the primary antibodies may require optimization (see discussion for more details). Additionally, the primary antibodies should be raised in different hosts that match the specificity of the secondary antibodies used for PLA.

5.1. Place slide on a paper towel to let blocking reagent run off the slide and gently wipe the edges. Use the antibody diluent (**Table of Materials**) to dilute the primary antibodies. Apply 40 µL of primary antibody solution per 14 mm x 14 mm space.

5.2. Incubate slides in a humid chamber overnight at 4 °C.

6. PLA probe (secondary antibody) incubation

NOTE: For steps 6–9, use wash buffers A and B at RT. If the buffers are stored at 4 °C, then let them warm to RT prior to using.

6.1. Wash slides 2x for 5 min with 50 mL of 1x wash buffer A (**Table of Materials**) at RT in a Coplin jar. Set the Coplin jar on an orbital shaker set to 60 rpm.

6.2. Place slide on a paper towel to let wash buffer run off the slide and gently wipe the edges. Prepare a 40 µL solution containing PLUS and MINUS probes (diluted 1:5 with antibody diluent). Apply the solution to each 14 mm x 14 mm space.

6.3. Incubate slides in a humid chamber for 1 h at 37 °C.

7. Ligation

7.1. Wash slides 2x for 5 min with 50 mL of 1x wash buffer A at RT in a Coplin jar. Set the Coplin jar on an orbital shaker set to 60 rpm.

7.2. Dilute the ligation buffer (**Table of Materials**) 1:5 with ultrapure water. Use this buffer to dilute the ligase (**Table of Materials**) 1:40 to prepare a working stock of ligation solution.

7.2.1. Place the slide on a paper towel to let the wash buffer run off the slide and gently wipe the edges. Apply 40 µL of the ligation solution to each 14 mm x 14 mm space.

7.3. Incubate slides in a humid chamber for 30 min at 37 °C.

8. Amplification

NOTE: Using detection reagents with red fluorophores (**Table of Materials**) results in the least amount of background in *C. elegans* tissue.

8.1. Wash slides 2x for 5 min with 50 mL of 1x wash buffer A at RT in a Coplin jar. Set the Coplin jar on an orbital shaker set to 60 rpm.

8.2. Dilute the amplification red buffer (**Table of Materials**) 1:5 with ultrapure water. Use this buffer to dilute the polymerase (**Table of Materials**) 1:80 to prepare a working stock of amplification solution and protect from light.

8.2.1. Place the slide on a paper towel to let the wash buffer run off the slide and gently wipe the edges. Apply 40 µL of the amplification solution to each 14mm x 14 mm space.

8.3. Incubate slides in a humid chamber for 1 h and 40 min at 37 °C. Make sure the humid chamber is covered with foil to protect the samples from light.

9. Final washes

9.1. Wash slides 2x for 10 min with 50 mL of 1x wash buffer B (**Table of Materials**) at RT in a Coplin jar. Set the Coplin jar on an orbital shaker set to 60 rpm.

9.2. Wash slides 1x for 1 min with 50 mL of 0.01x wash buffer B at RT in a Coplin jar. Set the Coplin jar on an orbital shaker set to 60 rpm. This buffer is prepared by diluting wash buffer B with ultrapure water.

10. Coverslip mounting

10.1. Let the excess wash buffer run off the slide onto a paper towel and wipe off any residual buffer remaining on the epoxy-coated perimeter of the slide.

10.2. Add 10 µL of mounting medium (**Table of Materials**) to sample and gently lay a coverslip on top, allowing for the mounting medium to spread out.

10.3. Paint around the edge of coverslip with nail polish to seal the coverslip and slide. Be gentle with application of nail polish to avoid moving the coverslip, which will damage the germlines. Let the nail polish harden for at least 20 min at RT, while slides are protected from light, before viewing it under a microscope.

10.4. Store slides in a dark container or slide holder, as PLA-labeled samples are light-sensitive. The manufacturer suggests that slides can be stored at -20 °C for long-term storage or at 4 °C for short-term storage. Slides prepared using this protocol will last at least 2 months when stored at -20 °C.

11. Image acquisition

11.1. For the purpose of quantification, use a confocal microscope to capture images of extruded germlines that are in clear view, undamaged, and unobstructed. Capture a z-stack of the germline that spans the whole germline in the z-plane and generate a maximum projection image to use for quantitation.

NOTE: Confocal microscopy is ideal for obtaining and quantifying PLA images with less background compared to those obtained using an epifluorescent microscope.

11.1.1. If the germline does not fit in one field of view, capture the overlapping fields of view as necessary to image the whole germline. Maximum projections of these images can be stitched together in FIJI.

11.2. Be sure to keep imaging conditions the same between control and experimental samples to set a fair and proper threshold for identification of foci during image analysis.

NOTE: Record at least 8–10 germlines from each sample per replicate to facilitate statistical analysis of PLA quantification. At least three biological replicates are recommended for PLA to obtain reliable and consistent quantitative results.

12. Image analysis and quantification using FIJI/ImageJ

NOTE: The following workflow is based on images acquired using the 40x objective on a confocal microscope, in which images are saved in the .czi format. These .czi images and their accompanying metadata, including dimensions, can be accessed and opened in FIJI/ImageJ for further analysis. It should be checked whether FIJI accepts the format of confocal files from the confocal available to the specific user. If not, images in the .tiff format can be alternatively used

for analysis, but the user will need to set the scale of the image manually in FIJI/ImageJ (**Analyze | Set scale**). It is recommended that all negative control images be analyzed together first to establish the level of background.

12.1. Start the analysis workflow by analyzing all negative control images first, then move on to the experimental samples. Open a maximum projection image in FIJI/ImageJ to analyze (**Figure 2A**). If using a .czi file, a **Bio-Formats Import Options** box will be prompted.

12.1.1. Include the following options to open your image: view stack with **Data Browser**; color mode = **Colorized.** A window with the image should now open with a slide bar to toggle between different channels captured by confocal (e.g., DAPI or PLA).

12.1.2. If images need to be stitched, create duplicates of each channel from each image by right-selecting the image with the mouse and selecting **Duplicate** to open the **Duplicate** window. Specify only the channel number (c) that corresponds to the PLA or DAPI channel (e.g., **2**) and uncheck the box for **Duplicate Hyperstack**.

12.1.3. With both images to be stitched open, select **Plugins | Stitching | Deprecated | 2D stitching**. A **Stitching of 2D Images** window will open. Select which images will be used for stitching and use the default parameters that are preset in the window, then select **Ok**. The resulting image will be an assembled grayscale image.

NOTE: If the sub-images do not perfectly align, adjusting parameters (i.e., increasing the number of peaks to be checked from **5** to **500** or changing the fusion method from **Linear Blending** to **Max. Intensity)** may help to obtain the desired image. While other stitching tools are available, this approach retains the dimensionality of the image, which is important for quantification.

12.2. Open the ROI (Region of Interest) manager by pressing **T** on the keyboard. A new window named **ROI Manager** will open.

12.3. Select the polygon tool from the FIJI toolset box. Drop points around the germline to outline it and generate a ROI (**Figure 2B**). Connect the last dot to the first to generate a complete ROI.

NOTE: For darker images, it is helpful to adjust the contrast to improve visibility of the germline (which is reversible) so that it can be outlined more accurately.

12.3.1. Immediately after completing the ROI, go to the ROI manager and select **Add [t]** to store the ROI. It is imperative that any changes to the ROI including adding/removing points or movement of the ROI and points be updated (select **Update** from ROI manager) before proceeding to the next step, or they will be lost. Further detail on manipulation of ROIs and their points can be found in the FIJI/ImageJ User Guide.

12.4. Once the ROI orientation is set, it can be saved for later reference by selecting the ROI name in the ROI manager followed by **More | Save… |** (**name file and specify destination for where to save**). Saved ROIs can be opened in the ROI manager by selecting **More | Open |** (**select the file**).

12.5. Measure the area inside the ROI by selecting the ROI name from the ROI manager, then selectting the **Measure** button on the ROI manager. A **Results** window will open with information about the ROI, including the area that it covers in the image (μM^2) (inset of **Figure 2B**). Record this information in an Excel spreadsheet for subsequent calculations.

NOTE: Make sure that the scale of the image has been set properly so that the proper dimensions of the ROI are collected. The types of measurements reported in the **Results** box can be modified by going to **Analyze | Set Measurements… .**

12.6. Open a duplicate image of only the PLA channel by right-selecting the PLA image and selecting **Duplicate** (**Figure 2C**). A **Duplicate** window will open. Specify only the channel number (c) that corresponds to the PLA channel (e.g., **2**) and uncheck the box for **Duplicate Hyperstack**. Duplication of this image is recommended so that the original image does not get modified.

NOTE: These options will only show up when viewing the images containing multiple channels on FIJI/ImageJ. An alternate approach is to split the channels **Image | Color | Split Channels**, but this will modify your original image file.

12.7. With only the image of PLA channel selected, go to **Image | Adjust | Threshold**. A **Threshold** window for the image will open. Select **Default** as the threshold method, **Red** as the color, and check the **Dark background** box. Using the upper track on the window, slide the bar towards the right until all PLA foci are distinctly highlighted in the image.

12.7.1. Record the value in the box to the right of the upper track and take note of what value was used to set the threshold. Select **Apply** in the **Threshold** window to finalize the threshold, and the image will convert to a wselecte background with only the threshold foci visible as black dots (**Figure 2D**). Test the threshold on several negative control images to ensure that it is appropriate for capturing all PLA foci from image to image before quantitation.

NOTE: A threshold value between 30–40 is a good starting point for identifying PLA in the germline; however, the ideal value may vary depending on background.

12.8. To quantify the PLA foci, apply the ROI generated from steps 12.3–12.3.1 to the threshold image by selecting the ROI name from the ROI manager window. The outline of the ROI should appear on the image in the same location as it was from the source image (**Figure 2E**).

12.8.1. Go to **Analyze | Analyze Particles**. The **Analyze Particles** window will open and select the following parameters: size (micron^2) = **0-Infinity**, circularity = **0.00-1.00**, show = **Nothing**. Check the **Summarize** box.

12.8.2. Select **Ok**, and a **Summary** table will appear with information about the ROI (i.e., the total count of PLA foci, total area occupied by the PLA foci inside in the ROI, average size of the PLA foci, and percentage of area occupied by PLA foci relative to the size of the ROI) (inset of **Figure 2E**). Record these measurements in an excel spreadsheet.

12.9. Repeat steps 12.1–12.8 for several negative control images using the same threshold.

12.9.1. Once all negative control images have been analyzed, repeat steps 12.1–12.8 for all experimental samples using the same threshold that was determined by the negative control to identify and quantify PLA foci.

REPRESENTATIVE RESULTS:

Co-immunostaining of both 3xFLA: G::DLC-1; GFP and 3xFLAG::DLC-1; OMA-1::GFP germlines with FLAG and GFP antibodies revealed their patterns of expression in the germline (**Figure 3Aii-iii,2Bii-iii**). While GFP was expressed throughout the germline (**Figure 3Aiii**), OMA-1::GFP expression was restricted to the late pachytene and oocytes (**Figure 3Biii**)[27]. FLAG immunostaining shows that 3xFLAG::DLC-1 was expressed throughout the germline in both strains (**Figure 3Aii,2Bii**). By co-immunostaining, the overlap between 3xFLAG::DLC-1 and OMA-1::GFP is indistinguishable from that between 3xFLAG::DLC-1 and GFP (negative control).

Since these experiments tested for interactions between DLC-1 and OMA-1, the region of interest for PLA quantification in the germline encompassed the late pachytene through the oocytes in all germlines examined (**Figure 2B**), as this is the region of OMA-1 expression (**Figure 1, Figure 3Biii).** 3xFLAG::DLC-1; OMA-1::GFP germlines appeared to have a greater quantity of PLA foci within this region compared to the 3xFLAG::DLC-1; GFP germlines (**Figure 3Ciii-iv,2Diii-iv**). Quantification of PLA revealed that the number of PLA foci present in 3xFLAG::DLC-1; OMA-1::GFP germlines was significantly greater than 3xFLAG::DLC-1; GFP (**Figure 3Ciii-iv,2Diii-iv; Table 1**). Further, even with 10x higher dilution of GFP and FLAG antibodies, the difference between the control and experimental PLA was still significantly different; however, the overall density and average size of foci were reduced (**Table 1**).

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of *C. elegans* germline. The distal tip region contains the stem cell pool, which is followed by meiotic pachytene, where cells have switched from mitosis to meiosis. Cells that exit the meiotic pachytene develop into oocytes, with the most mature oocyte at the proximal end. The region shaded in green, which spans from the late meiotic pachytene through all the oocytes, represents the OMA-1 pattern of expression.

Figure 2: Representative images of workflow for germline PLA quantification. The germline used in this figure is a representative 3xFLAG::DLC-1; GFP germline from Figure 3C. (**A**) Image of merged PLA and DAPI channels opened in FIJI/ImageJ. (**B**) The polygon tool in FIJI is used to outline and define the region of interest (ROI) in the germline (yellow line with wselecte boxes) that are quantified, and the area of the ROI (μM^2) is measured (inset of B). (**C**) A single image of the PLA channel is obtained by duplicating or splitting the original image in (A,B). (**D**) The threshold is carefully set to distinctly highlight all PLA foci in the PLA image. The same threshold must be applied to all experimental and control images that will be analyzed together. (**E**) With the ROI selected in the threshold image, the **Analyze Particles** function will return a table of results that includes the total count of foci included inside the ROI (inset of E). Images are snapshots from FIJI/Image J: **Plugins | Utilities | Capture Image**. Scale bars = 10 μM.

Figure 3: Representative images of germlines following co-immunostaining or PLA. (**A,B**) The expression patterns of tagged proteins in 3xFLAG::DLC-1; GFP (Ai-iv) and 3xFLAG::DLC-1; OMA-1::GFP (Bi-iv) were evaluated in dissected, fixed, and immunostained gonads. Anti-FLAG antibody was used at a 1:1000 dilution, while anti-GFP antibody was used at a 1:200 dilution, which is optimal for immunofluorescence images. DNA was stained by DAPI, and the individual channel is shown in grayscale for better contrast (**Aiv,Biv**). In each image, the stem cells and meiotic pachytene are outlined with dashed lines, while the oocytes are outlined with dotted lines. Images were acquired with an epifluorescent microscope. Scale bars = 10 μM. (**C,D**) PLA in the extruded gonads of 3xFLAG::DLC-1; GFP (**Ci,ii,iii,iv**) and 3xFLAG::DLC-1; OMA-1::GFP (**Di,ii,iii,iv**). Anti-FLAG antibody was used at a 1:1000 dilution, while anti-GFP antibody was used at a 1:4000 dilution. DNA was stained by DAPI, and both the individual DAPI (**Cii,Dii**) and PLA channels (**Ciii,iv, Diii,iv**) are shown in grayscale for better contrast. The green, dashed box (**Ciii,Diii**) denotes the location of the zoomed-in PLA images (**Civ,Div**). In each image, the stem cells and meiotic pachytene are outlined with dashed lines, while the oocytes are outlined with dotted lines. Images were acquired with a confocal microscope. Scale bars = 10 μM. (A,B,C,D) were all assembled with image processing software (see **Table of Materials**).

Table 1: Summary of PLA results. Table reporting a summary of PLA quantification at two dilutions of primary antibody. The differences in average PLA density or average size of PLA foci for OMA-1::GFP between both antibody titrations were not significant (p-value not shown). The same comparison was also applied to GFP, which also resulted in no significant difference (p-value not shown). The p-values were determined using a two-tailed/equal variance *t*-test.

DISCUSSION:

When studying PPIs in the *C. elegans* germline, the higher resolution offered by PLA compared to co-immunostaining allows visualization and quantification of locations where interactions occur in the germline. It was previously reported that DLC-1 directly interacts with OMA-1 using an in vitro GST pulldown assay[26]; however, this interaction was not recovered by an in vivo pulldown. The fluorescent co-immunostaining of 3xFLAG::DLC-1; OMA-1::GFP germlines shows an overlap in the expression patterns for DLC-1 and OMA-1; however, there is no indication of where their interactions occur in the germline, and the overlap itself is not greater than that between 3xFLAG::DLC-1 and GFP that is not fused to any protein (negative control). Using in

situ PLA, it was found that DLC-1 does interact with OMA-1 in the germline, which suggests that PLA may be more sensitive for detection of PPIs compared to other approaches. Through this approach we continue to expand upon the emerging role of DLC-1 as an RBP cofactor. This work demonstrates the capability of PLA to detect PPIs in the germline and establishes a reference for future users exploring the interactions between proteins of their own interest.

PLA offers users the ability to test for PPIs with comparable sensitivity without the drawbacks associated with other techniques such as FRET and BiFC. Biologically relevant levels of protein expression may not be optimal for FRET and BiFC. Also, the function of potential interaction partners may be affected by the large tags used in both approaches. Furthermore, FRET assays require a specialized microscopy set-up that may not be readily available. PLA may also be a cost-effective approach to study PPIs compared to other techniques. Users only need to obtain PLA reagents and access to a confocal microscope for imaging in addition to the reagents needed for immunostaining. Image analysis is performed using the open-source program FIJI/ImageJ, which is available to any user at no cost. Users that have no experience with FRET or BiFC may find PLA to be a suitable alternative. The protocol presented here only contains several additional steps beyond a typical immunostaining procedure, making this technique virtually accessible to any user with immunostaining experience.

Extrusion of the gonad by dissection is important for PLA to work successfully. Tissues that are retained inside of the worm cuticle are not labeled by PLA using this protocol. It has been further found that extruded embryos are effectively labeled by this PLA protocol. This suggests that other tissues that are released during dissection, such as the gut, are also likely to be compatible with PLA. It has been found that PLA produces robust signals on gonad as well as embryo samples prepared with two fixation protocols that are often used for immunostaining. This suggests that additional fixation procedures used in the field may be compatible with PLA but will need to be individually evaluated by the user.

Determining the optimal dilution of primary antibodies is critical for successful PLA. It is best to start is with the dilution that has been optimized for immunofluorescence. This is typically achieved by titrating the primary antibody in an immunofluorescence experiment to find the optimal dilution where there is low background and a high, specific signal. Once the optimal dilutions for immunofluorescence have been established, these same dilutions can be tested in a PLA assay that compares the signal produced by a pair of potential interactors to the signal produced by a control pair of non-interacting proteins.

In the case in which abundant signal is observed in the control sample, further dilution of primary antibodies is required. It has been found that the optimal primary antibody dilutions for PLA are at least the same or even more dilute than what is used for immunofluorescence. For example, immunofluorescence images in **Figure 3A,B** are representative of a 1:1000 dilution of anti-FLAG and a 1:200 dilution of anti-GFP. However the antibody dilutions in PLA images in **Figure 3C,D** were 1:1000 of anti-FLAG and 1:4000 of anti-GFP. The dilution of anti-GFP antibody used in PLA is greater than what was used for immunofluorescence, suggesting that PLA is much more sensitive. It was found that diluting antibodies 10-fold further resulted in

a reduction of PLA density as well as the size of DLC-1/OMA-1 foci (**Table 1**). Despite this reduction, the difference in PLA density between the negative control and DLC-1/OMA-1 was still significantly different. This suggests that PLA is still very sensitive with higher dilutions of primary antibody; however, the prevalence of detectable interactions will be underestimated.

By contrast, too low of an antibody dilution might have two kinds of detrimental consequences. First, it may produce significant background signal in the negative control. Second, PLA foci produced by the interacting partner proteins might merge and overlap, making them difficult to resolve in a max projection image. This leads to an underestimation of PLA foci number and density during image analysis. PLA signal is a balance of detecting spurious proximity between non-interacting partners and detecting every instance of real PPIs that occur in the sample. As a result, incorporation of a negative control where two proteins do not interact is essential for determining the level of background in PLA experiments. Omission of a primary antibody in a PLA experiment has been used as a negative control in other reports[9,10]; however, this approach cannot account for nonspecific interactions or nonspecific antibody binding that may impact the result in the experimental PLA. GFP was used here as a negative control, since no direct interaction between DLC-1 and GFP was expected. It was found that the negative control did have some background signal. This further supports the importance of a negative control for a PLA assay when evaluating the experimental data.

Once PLA-optimized dilutions are established, these dilutions can be used to test across an array of different worm strains that contain different pairs of interaction partners tagged with the same affinity tags. It is important to use the same pair of primary antibodies to ensure a fair comparison of resulting PLA signals, as variation in antibody affinity can affect the outcome of a PLA experiment. Another report on PLA suggests optimizing dilution of the PLA secondary antibodies[10]; however, this is not recommended. Higher dilutions of secondary antibodies may reduce the efficacy of the other downstream PLA steps that depend on recognition of PLUS and MINUS probes that are conjugated to the secondary antibodies.

ACKNOWLEDGEMENTS:
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DISCLOSURES:
The authors have no conflicts of interest.

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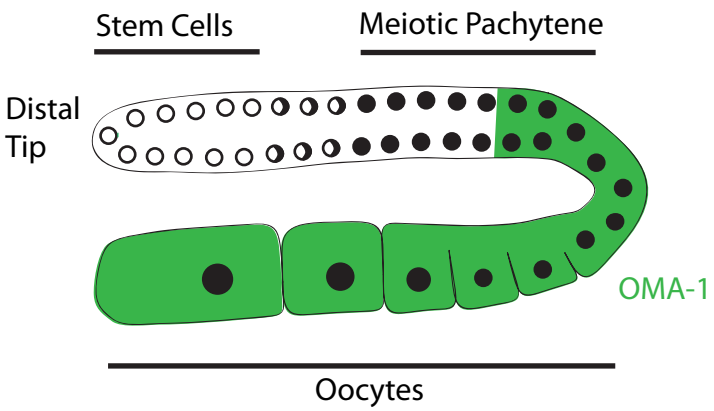
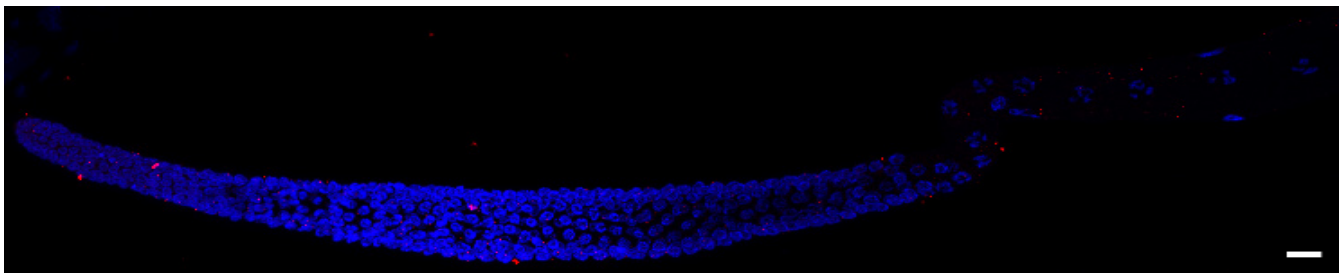


fig

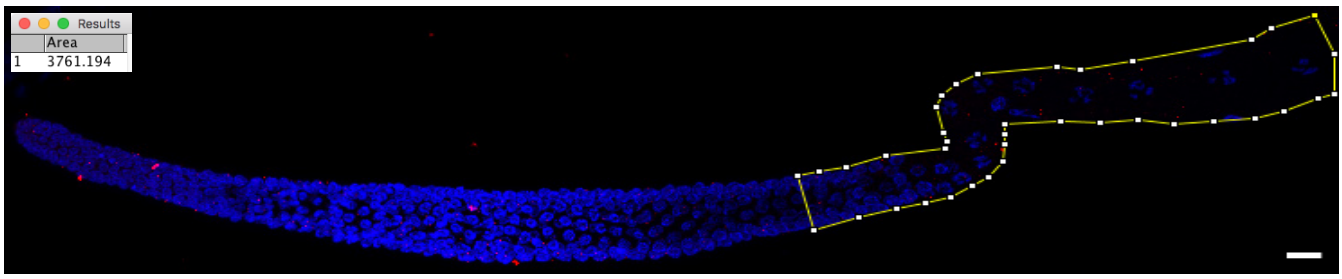
Open image in FIJI

A.



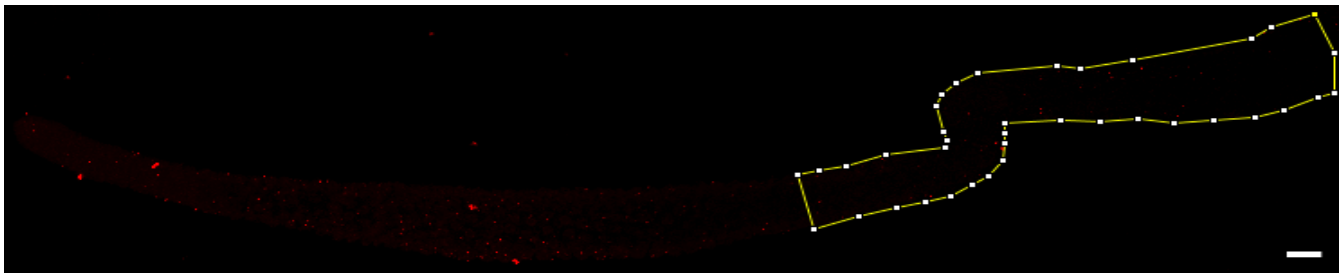
Draw ROI and measure area

B.



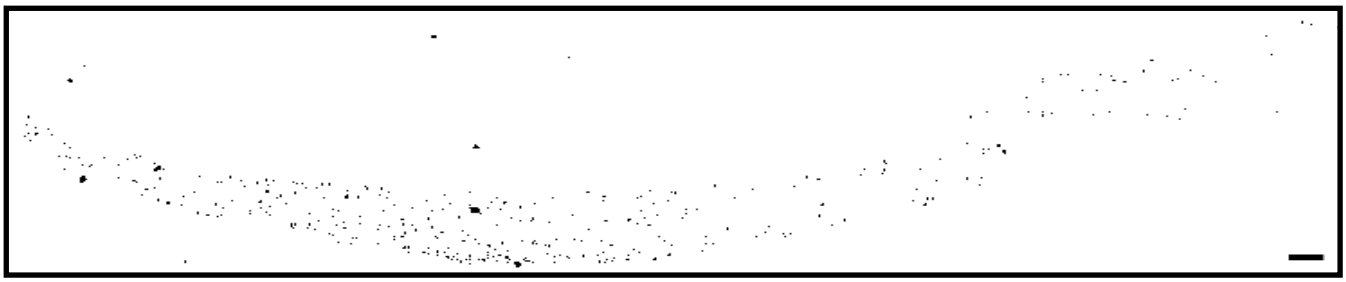
Duplicate PLA channel

C.



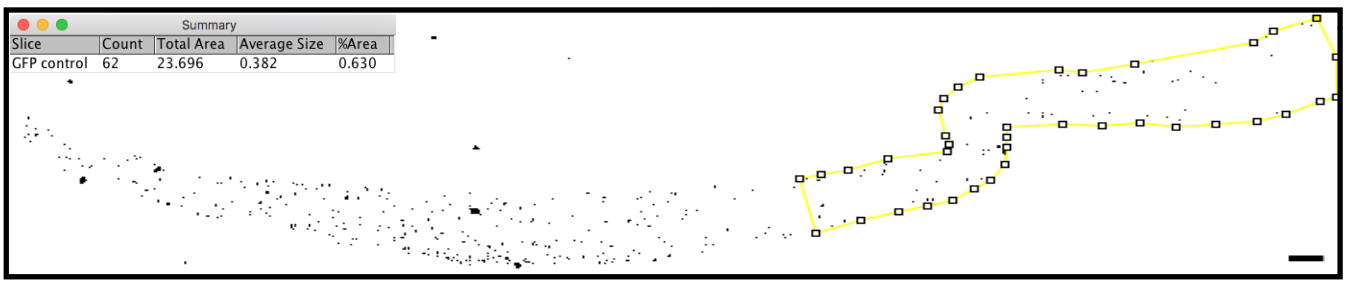
Set threshold

D.



'Analyze Particles' inside ROI

E.



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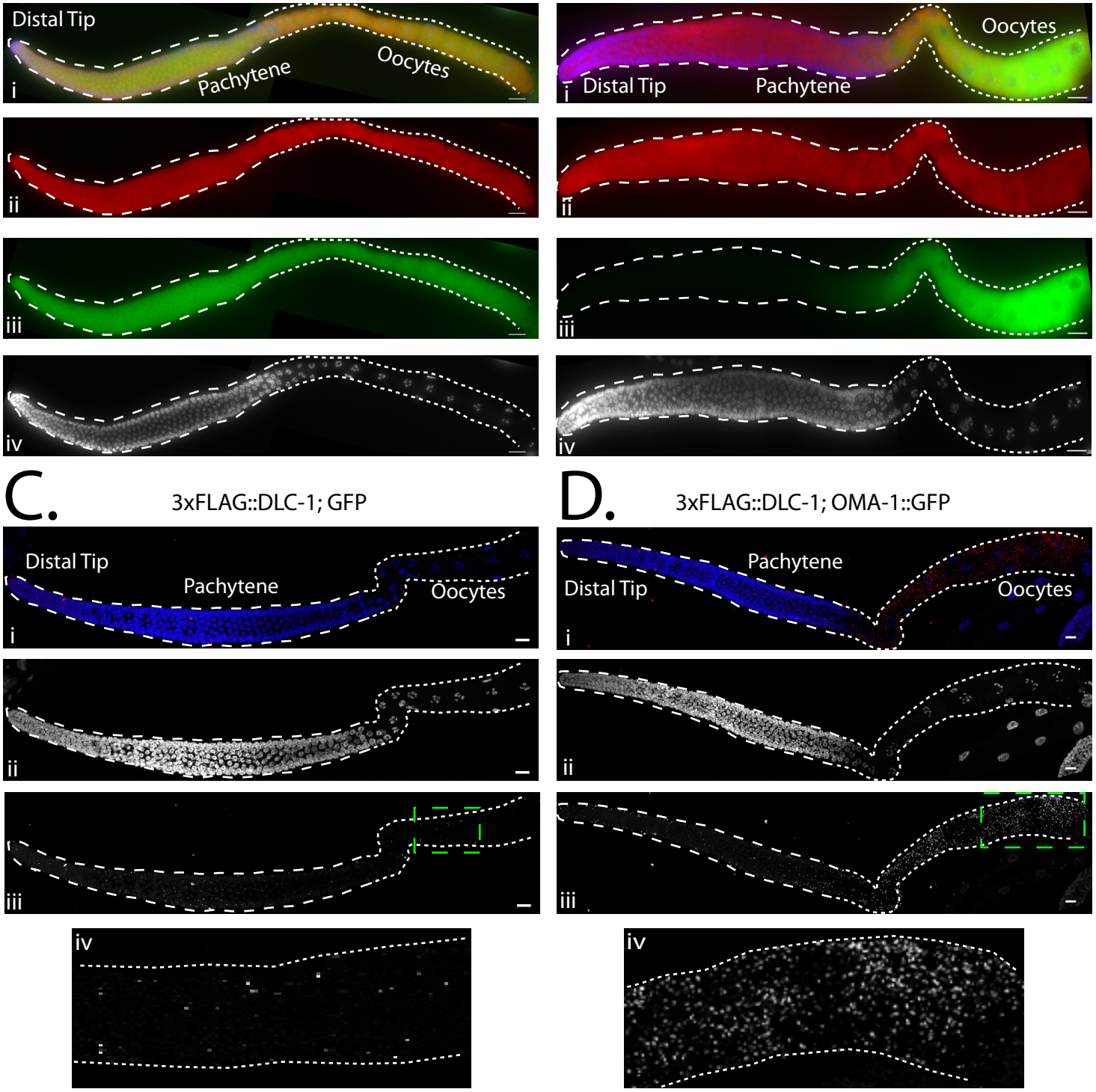

Table 1

Click here to access/download;Table;Table 1_Summary of PLA Results.xlsx

Antibody Dilution	Strain Tested	Average PLA Density (foci/μM^2) X 10^{-2}	T test	Average Size of PLA Foci (μM^2)	T test
αFLAG (1:1000), αGFP (1:4000)	3xFLAG::DLC-1; GFP	3.9±1.4	*P* =1.917E-05	0.52±0.127	*P* =0.057
	3xFLAG::DLC-1; OMA-1::GFP	9.1±2.7		1.8±2.08	
αFLAG (1:10,000), αGFP (1:40,000)	3xFLAG::DLC-1; GFP	3.2±2.4	*P* =3.395E-04	0.51±0.1	*P* =0.019
	3xFLAG::DLC-1; OMA-1::GFP	7.7±3		0.7±0.24	

Table of Materials

Name of Material/Equipment	Company	Catalog Number	Comments/Description
16% paraformaldehyde solution	Electron Microscopy services	15710	used to make 4% working solution
1M KH_2PO_4	Sigma	P0662	Prepare a 1M working stock
1x M9	various	various	prepared as 10x stock used at 1x; see
1x PBS	various	various	see wormbook.org for protocol
26.5 Gauge Needle	Exel International	26402	Needles used for dissection
BSA	Lampire	7500802	
Centrifuge Tubes	Thermo Scientific	05-529C	50ml Oak ridge centrifuge tube used f
Confocal Microscope	Zeiss	880	
Coplin Jar	PolyLab	62101	
Coverslip to Freeze Sample	Globe Scientific	1411-10	22x40mm, No. 1
Coverslip to Seal Slide	Globe Scientific	1404=15	22x22mm, No. 1.5
DAPI Mounting Medium for Immunofluorescence	Vector	H-1200	
Ligase	Sigma-Aldrich	DUO82029	Duolink 1x Ligase, Comes as part of th
Amplification red buffer	Sigma-Aldrich	DUO82011	Duolink 5x Amplification Red buffer, C
Ligation Buffer	Sigma-Aldrich	DUO82009	Duolink 5x Ligation buffer, Comes as p
Antibody Diluent	Sigma-Aldrich	DUO82008	Duolink antibody diluent,Comes with
Blocking Solution	Sigma-Aldrich	DUO82007	Duolink blocking solution, Comes with
Mounting Medium for PLA	Sigma-Aldrich	DUO82040	Duolink In Situ mounting medium with
MINUS Probe	Sigma-Aldrich	DUO92004	Duolink In Situ Probe Anti-Mouse MIN
PLUS Probe	Sigma-Aldrich	DUO92002	Duolink In Situ Probe Anti-Rabbit PLUS
Wash Buffer A	Sigma-Aldrich	DUO82046	Duolink In Situ wash Buffer A
Wash Buffer B	Sigma-Aldrich	DUO82048	Duolink In Situ wash Buffer B
Polymerase	Sigma-Aldrich	DUO82030	Duolink Polymerase, Comes as part of
Epifluorescent Microscope	Leica		DFC300G camera, DM5500B microsco
Goat anti-mouse Alexa 594	JacksonImmuno	115-585-146	Use at 1:500
Goat anti-rabbit Alexa 488	JacksonImmuno	111-545-144	Use at 1:200
Image Processing Software	Adobe		Adobe Photoshop + Illsutrator CS3
Glass Pipette	Corning	7095B-5X	
Levamisole	ACROS Organics	187870100	Prepare a 250mM working stock

Methanol	Fisher Scientific	A454	
Mouse anti-FLAG	Sigma	F1804	Use at 1:1000 for immunofluorescenc
Nailpolish	L.A. colors	CNP195	
Nematode Growth Medium (NGM)	various		See wormbook.org for protocol
Normal Goat Serum	JacksonImmuno	005-000-121	
Polyethylene Pasteur Pipette	Globe Scientific	135030	
Poly-L-Lysine	Sigma-Aldrich	P1524	Prepared as 0.1% stock solution in wa
Petri Dishes	Tritech	PD7060	60 mm diameter
Rabbit anti-GFP	Thermo Fisher	G10362	Use at 1:200 for immunofluorescence
Slides	Thermo Fisher	30-2066A-Brown	Three-square 14x14mm autoclava
Sodium Hypochlorite solution	Fisher Scientific	SS290-1	
task wipes	Kimtech	34120	4.4x8.4 inch task wipes
Trays (242x241x20mm)	Thermo Fisher	240845	Used to make humid chamber
Triton X-100	ACROS Organics	327372500	
Ultrapure water	Milli-Q		Ultrapure water obtained from Milli-C
Watchglass	Carolina Biological	742300	
-20°C freezer			
-80°C freezer			
Aluminum Foil			
OP50 strain *E. coli*			
Orbital Shaker			
Tape			

Nematode strains used in this study (both available upon request)

Genotype	Strain name	Transgene
mntSi13[pME4.1] II; unc-119(ed3) III; teIs1 [pRL475]	UMT 376	*dlc-1 prom::3xFLAG::dlc-1::dlc-1 3'UTR; oma-1 prom::oma-1::GFP* ; Reference 24
mntSi13[pME4.1] II; mntSi21[pXW6.22] unc-119(ed3) III	UMT 422	*dlc-1 prom::3xFLAG::dlc-1::dlc-1 3'UTR; gld-1 prom::ceGFP::fbf-1 3'UTR + unc-119(+)* ; Reference: this

wormbook.org for protocol

for synchronization

ıe Duolink In Situ Detection Reagents Red kit DUO92008
Comes as part of the Duolink In Situ Detection Reagents Red kit DUO92008
part of the Duolink In Situ Detection Reagents Red kit DUO92008
DUO92004 and DUO92002, Note: A 1x PBS/1% BSA solution can also be used as a substitute to dilute the antibody.
ı DUO92004 and DUO92002
h DAPI
NUS

the Duolink In Situ Detection Reagents Red kit DUO92008
ope

:e and PLA, pre-block with normal goat serum recommended

ter, stored at -20C, and diluted 1:100 in water to coat slides.

, 1:4000 for PLA
ble slides with bars are custom-ordered through Fisher Scientific. Poly-L-Lysine added to slides in the lab

Integral Water Purification System

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.