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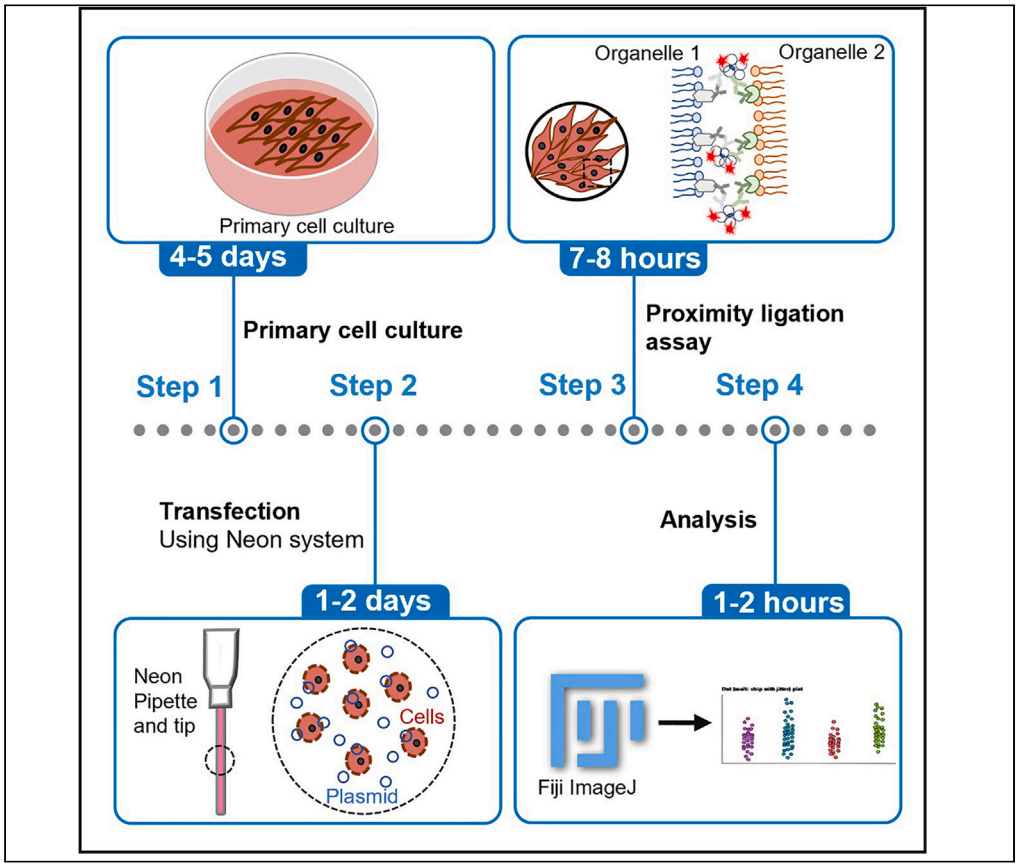
Protocol for measuring interorganelle contact sites in primary cells using a modified proximity ligation assay



Interorganelle contact sites regulate lipid metabolism, organelle dynamics and positioning, as well as apoptosis and autophagy. Here, we present a proximity ligation assay (PLA) protocol for measuring the association of two organelles in fixed cells. We describe steps for primary cell culture, primary cell transfection, and the assay itself. We then detail procedures for manual and image J-based analysis of PLA foci. This protocol optimizes the use of assay products and improves the identification of PLA foci labeling actual contact sites.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Primary fibroblast cell culture and maintenance

Transient transfection of primary cells using the Neon transfection system

Modified proximity ligation assay to measure the close association of two organelles

Manual and image J-based analysis of PLA foci

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Protocol for measuring interorganelle contact sites in primary cells using a modified proximity ligation assay

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SUMMARY

Interorganelle contact sites regulate lipid metabolism, organelle dynamics and positioning, as well as apoptosis and autophagy. Here, we present a proximity ligation assay (PLA) protocol for measuring the association of two organelles in fixed cells. We describe steps for primary cell culture, primary cell transfection, and the assay itself. We then detail procedures for manual and image J-based analysis of PLA foci. This protocol optimizes the use of assay products and improves the identification of PLA foci labeling actual contact sites.

For complete details on the use and execution of this protocol, please refer to Ilamathi et al. (2023).[1]

BEFORE YOU BEGIN

This protocol details a step-by-step procedure to measure interorganelle contact sites in any cell type using a modified proximity ligation assay. While this procedure can be used with any cell type, we usually use it with primary human fibroblasts, which are large and flat cells that are easy to image by confocal microscopy. As these cells are however challenging to grow and transfect, we also provide a protocol for their transfection using Neon transfection. We have also tested this assay in mouse embryonic fibroblasts, Huh7 hepatocarcinoma, JEG-3 placenta, and A549 lung adenocarcinoma cells.[1,2]

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Calnexin 1:200	Millipore	MABF2067
TOM20 1:250	Abcam	ab186735; RRID:AB_2889972
IP3R1 1:200	Novus Biologicals	NBP2-22458
VDAC1 1:200	Sigma-Aldrich	MABN504; RRID:AB_2716304

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mouse IgG isotype control 1:200	Invitrogen	08-6599
Fluorescent-tagged secondary antibody 1:500	Jackson ImmunoResearch	715-605-150; RRID:AB_2340862 (Anti-mouse 647), 711-165-152; RRID:AB_2307443 (Anti-Rabbit cy3)
Chemicals, peptides, and recombinant proteins		
Dulbecco's modified Eagle's medium	Wisent	319-005-CL
Trypsin with EDTA	Wisent	325-042-CL
Penicillin-Streptomycin	Wisent	SV30010
Fetal bovine serum	Corning	35-077-CV
L-glutamine solution	Wisent	609-065 EL
1X phosphate-buffered saline, sterile	Wisent	311-425-CL
Paraformaldehyde	Sigma-Aldrich	P6148
Bovine serum albumin	Sigma-Aldrich	A7906
Triton X-100	Sigma-Aldrich	9002-93-1
Epredia Immu-Mount	Fisher Scientific	9990412
DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride)	Thermo Fisher Scientific	D1306
Critical commercial assays		
Duolink *in situ* green kit mouse/rabbit	Sigma-Aldrich	DUO92014
Duolink *in situ* PLA probe anti-rabbit PLUS	Sigma-Aldrich	DUO92002
Duolink *in situ* PLA probe anti-mouse MINUS	Sigma-Aldrich	DUO92004
Duolink *in situ* wash buffers, fluorescence	Sigma-Aldrich	DUO82049
Neon Transfection System 10 µL Kit	Thermo Fisher Scientific	MPK1025
Experimental models: cell lines		
Primary human fibroblasts (controls and DRP1 mutants)	Skin biopsies (Research Ethics Board of the Children's Hospital of Eastern Ontario (DRP1 mutants))	N/A
Recombinant DNA		
mCherry	Addgene	54517
mCherry-tagged CLIMP63	Addgene	136293
Software and algorithms		
ImageJ	National Institutes of Health	https://imagej.nih.gov/ij/
R	www.r-project.org/	R4.0.5
RStudio	http://www.rstudio.com/	Version 2023.03.0 + 386
Other		
Fisherbrand Cover Glasses	Fisher Scientific	1254580
Neon transfection system	Thermo Fisher Scientific	10431915
Dry bath		
Confocal microscope	Leica Microsystems	Leica SP8

MATERIALS AND EQUIPMENT

- Primary cell culture media.

Reagent	Final concentration	Amount
Dulbecco's Modified Eagle Medium (DMEM) supplemented with 4.5 *g*/L glucose, sodium pyruvate, L-glutamine, and phenol red	N/A	440 mL
200 mM L-Glutamine	6 mM	5 mL
Fetal bovine serum (FBS)	10%	50 mL
Penicillin and Streptomycin (10,000 U/mL)	100 U/mL	5 mL
Total	**N/A**	**500 mL**

Storage conditions: 4°C, up to 4 weeks.

- Trypsin with EDTA, cell culture grade.

Storage conditions: 4°C, up to 3 weeks.

- 4% Paraformaldehyde (PFA).

4 g of Paraformaldehyde is added to 70 mL of 1X PBS and mixed in the magnetic stirrer. The temperature is then slowly increased to 65°C and left for stirring until the paraformaldehyde is dissolved completely. The solution can then be cooled down to 25°C and the pH adjusted to 7.4–7.5 with 1 N NaOH. The volume is then adjusted to 100 mL with PBS. PFA solution can be stored at 4°C for short-term storage (1–2 months) and −20°C for long-term storage (1 year).

- Phosphate buffered saline (PBS), pH 7.4–7.5.

Chemicals	Concentration	Amount (g) for 1 L
NaCl	137 mM	8
KCl	2.7 mM	0.2
Na_2HOP_4	10 mM	1.44
KH_2PO_4	2 mM	0.24

Adjust the pH and make up the volume to 1 L with water. Autoclave the PBS and store at 25°C for up to 2 years or until a white precipitate is observed at the bottom of the bottle.

- Neon transfection kit, including the Neon tube, Neon pipette tip, electrolytic buffer (E), and cell suspension buffer (R and T).

  Note: A homemade transfection buffer can be used as an alternative to the company-supplied electrolyte and suspension buffers. This sucrose-based buffer[3] (250 mM sucrose and 1 mM MgCl2 in D-PBS. Filter-sterilize and store at 4°C) can be used as a replacement for both buffers.

- Duolink Proximity ligation assay kit- It includes a blocking solution, antibody diluent, wash buffer A and B, probe (+/-), ligation buffer and enzyme, amplification enzyme, and its buffer.

  Note: A higher volume of probes can be purchased individually.

- DAPI- A stock solution (5 mg/mL) is prepared in water and stored at 4°C up to 6 months Cover the tubes with aluminum foil to protect them from the light.
- Primary antibodies from different animal species that face the cytosolic side of the organelles of interest.
- Fluorophore-coupled secondary antibodies.
- Permeabilization buffer (0.2% Triton X-100 in PBS). Store at 4°C for up to a month.
- Home-made blocking buffer (1% BSA and 0.1% Triton X-100 in PBS). Store at 4°C for up to a month.
- Nuclease-free high-purity water or 0.22 micron filtered autoclaved MilliQ water.
- Mounting solution (Immu-Mount).

STEP-BY-STEP METHOD DETAILS

Preparation of primary cell culture media

Timing: 1 h

1. Warm DMEM, L-glutamine, FBS, and Penicillin-Streptomycin mix to 37°C before culture medium preparation.
2. To 500 mL of DMEM, add 6 mM Glutamine, 10% FBS, and 100 U/mL Penicillin and streptomycin.
3. The medium can be immediately used or stored at 4°C until further use.

⚠ CRITICAL: It is ideal to maintain separate consumables (e.g., pipette tips and Pasteur pipette) and solutions (e.g., PBS) for primary cell culture. This avoids any cross-microbial contamination and saves time to grow the primary cells. All the steps, including media preparation, cell culture, and transfection, must be performed in a sterile environment.

Primary cell culture

Timing: 4–5 days

4. Rapidly hand-thaw frozen human primary fibroblasts and resuspend them in 10 mL of primary cell culture medium.
5. Centrifuge the cells at 600 × *g* for 5 min at 25°C.
6. Aspirate the medium and
   a. add 1 mL of fresh medium to the cell pellet.
   b. Once cells are resuspended, grow them in an appropriate cell culture plate.

⚠ CRITICAL: Primary fibroblast cells grow better when they have neighboring cells nearby, that is, when cell density is relatively high in the culture plate. So, reviving the cells in 6-well plates rather than a 100 mm dish is better when the cell pellet size is small (~10 μL).

7. Incubate the plate at 37°C with 5% CO_2.
8. Once the cells reach 75%–80% confluency,
   a. remove the medium,
   b. wash once with PBS, detach cells with trypsin (300 μL for 6-well or 1 mL for 100 mm plate) for 5 min 37°C.
   c. Pellet the cells at 600 × *g* for 5 min at 25°C.
9. The cells can then be resuspended in DMEM and transferred to a new plate for further expansion, or the pellet can be used for Neon transfection (below).

Transfection of primary cells

Timing: 1–2 days

Transfection of primary cells with a plasmid or siRNAs is challenging with transfection lipid reagents. We have found that the Neon transfection system is much more efficient to transfer the plasmid/siRNA into primary fibroblast cells relative to standard lipid transfection reagents (unpublished work). Neon transfection is an electroporation-based transfection method to transiently create pores in the plasma membrane. The Neon transfection kit has different transfection buffers for adherent and suspension cells. In this procedure, cells are suspended in a transfection buffer, electroporated with the desired DNA, then transferred to cell culture plates or coverslips and grown for 24–48 h before analysis.

10. Cells from step 6 are resuspended in 10 μL of solution R from the Neon transfection kit or home-made buffer.[3] Each coverslip will require 2–3 × 10^4 cells but a larger number of cells can be transfected to plate several coverslips at the same time. We typically transfect 30 × 10^4 cells at a time (in 10 μL).

⚠ CRITICAL: Upon transfection, 5%–10% of cell death is often observed, depending on the protein overexpressed or knocked down. It is important to have around 70% cell confluency while seeding the transfected cells on the coverslip. A low cell density will increase cell death and impact cell growth and transfection efficiency, whereas high cell density affects the quality of imaging.

11. Add 1 μg of the plasmid to transfect to the cell suspension (30×10^4 cells/10 μL). Gently tap the tube to mix the plasmid with the cells.

⚠ CRITICAL: The concentration of plasmid DNA needs to be optimized. The concentration of plasmid used for transfection might vary with the plasmid type and protein. 1 μg of plasmid DNA is a good starting point, but the concentration can be either increased or decreased depending on the cellular response and transfection efficiency. It is, however, challenging to find transfected cells if a very low amount is used (<100 ng). On the other hand, higher expression of the plasmid might result in off-target effects.

12. Place the Neon Tube into the Neon pipette station.
    a. The golden electrode should touch the golden side ball plunger in the pipette station.
    b. A click sound suggests the proper placement of the tube into the pipette station (refer to Methods video S1 for the Neon transfection procedure).
13. Add 3 mL of electrolytic buffer E or homemade buffer[3] to the Neon Tube.

Note: Store the remaining buffers (buffer E and buffer R) at 4°C after use.

14. Double-press the pipette to push out the clamp.
    a. Insert the pipette clamp into the tip and push downward until there is a click sound.
    b. Slowly release the pressure from the pipette button.
    c. Make sure there is no gap between the tip and the pipette edge (refer to Methods video S1).
15. Press once the pipette and carefully aspirate the cell suspension containing plasmid DNA with the help of Neon transfection 10 μL tip (refer to Methods video S1).

⚠ CRITICAL: Avoid bubbles while aspirating the samples. The presence of air bubbles generally causes a program error during transfection.

16. Set the program for the transfection in the Neon instrument program. For primary fibroblasts, the following parameters work well without causing much cell death.

Parameter	For primary fibroblast
Pulse voltage	1,400 V
Pulse width	20 ms
Pulse number	2

Note: Generally, transfection voltage, pulse width, and pulse time need to be optimized based on the type of cells transfected.

17. Insert the pipette with the cell sample into the Neon tube until there is a click sound (refer to Methods video S1).
18. Press "start" on the program to initiate the electroporation.
19. Once the electroporation is finished, the Neon instrument screen will display "complete" (refer to Methods video S1).
20. Prepare 24-well plates with coverslips (Diameter: 12 mm; thickness: 0.13–0.16 mm).

a. Collect transfected cells in the falcon tube containing the cell culture medium (500 μL/well of 24-well plate).
b. Transfected cells are seeded on the uncoated coverslips.

Note: While preparing multiple coverslips for each of the plasmids, cells can also be transfected in bulk and grown for 16 h in a 12-well plate before seeding them in the 24-well plates containing coverslips.

21. Repeat the transfection for other samples.

Note: Separate tips must be used for each cell type and plasmid DNA. Tips can be reused following UV sterilization. Tips can be reused for transfecting other cell types by sterilizing them in 70% ethanol followed by UV sterilization.[3]

Methods video S1: Video of transfection using Neon system.

22. Keep transfected cells 16 h in the incubator at 37°C with 5% CO_2.

Note: If the cells are either stressed or exhibit cell death after 24 h, the medium can be replaced with fresh medium and left in the incubator for another 24 h.

23. 24–48 h post transfection,
a. Wash the coverslips with PBS once.
b. Fix cells by adding 500 μL of 4% paraformaldehyde (in PBS) for 10–15 min at 25°C.

⚠ CRITICAL: Paraformaldehyde should be warmed to 25°C before use as fixing with cold PFA affects mitochondrial structure.[4]

24. Wash fixed cells once with PBS and store in PBS at 4°C. Coverslips can be stored for 6–12 months as long as they do not get dried out or cells detach.

Pause point: Coverslips can be stored at 4°C, and proximity ligation assay can be performed later.

Proximity ligation assay

Timing: 7–8 h

Proximity ligation assay (PLA) measures the proximity of two proteins. In a PLA reaction, antibodies are used to recognize each protein. Species-specific oligonucleotide-coupled secondary antibodies (PLA probes) then bind to the primary antibodies. The oligonucleotides are then ligated together when the two proteins are in close proximity (<40 nm) and this closed circular DNA is amplified through a rolling circle mechanism. The amplified DNA is finally labeled by specific fluorescently tagged oligonucleotides. This method can be used to detect the close proximity between two organelles by choosing surface proteins on the organelles of interest that functionally interact with each other. For example, IP3R1 on the endoplasmic reticulum (ER) membrane interacts with VDAC1 on the mitochondrial outer membrane, which can be used as a PLA pair (Figure 1).[5] Alternatively, abundant resident proteins homogenously distributed on the surface of the two organelles can be used to detect the contact sites between the organelles.[1] For example, TOMM20 on the mitochondrial outer membrane and calnexin on the ER can be used to identify global changes in mitochondria-ER contact sites (Figure 2). The PLA probes and amplification reagents can be bought from Sigma-Aldrich as a Duolink kit.

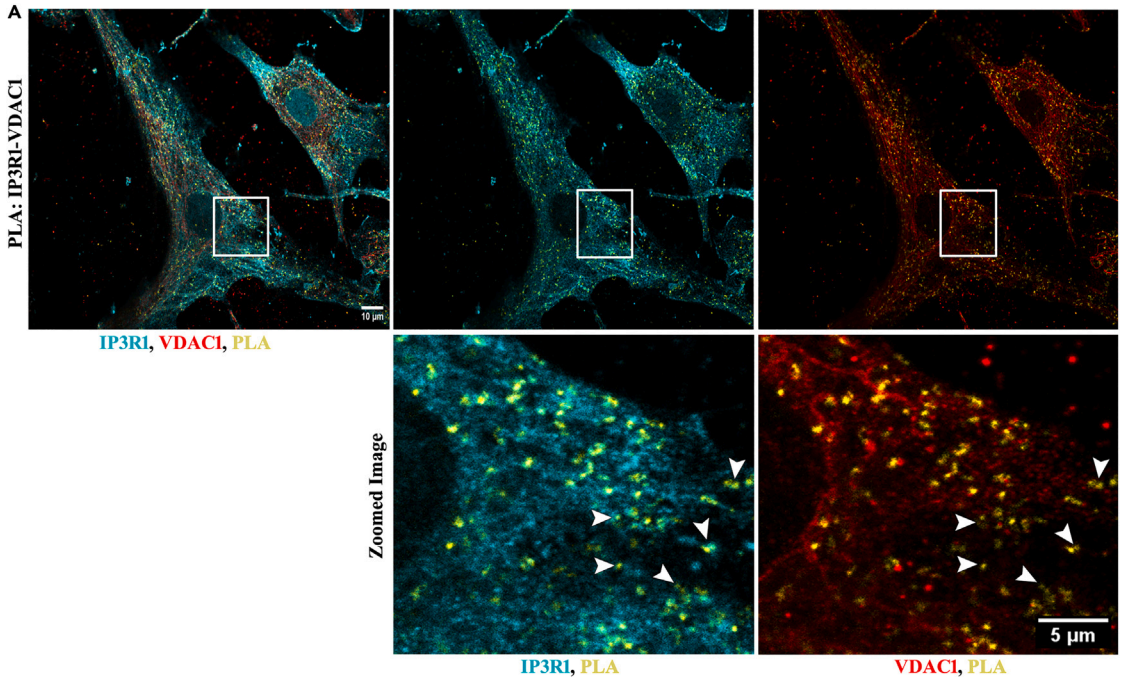

Figure 1. Images of IP3R1 (endoplasmic reticulum)-VDAC1 (mitochondria) PLA foci in primary cells

PLA is influenced by the antibody pair chosen. Antibodies that non-specifically label organelle result in non-specific PLA foci as shown by the white arrowheads. Boxed regions are enlarged in the bottom panels. Scale: 10 µm for actual image, 5 µm for the magnified image.

⚠ CRITICAL: To perform PLA, it is important to choose two antibodies that specifically recognize the cytosolic domain of proteins on the organelles of interest. These antibodies must be validated and specifically label the organelle of interest without background/off-target staining. Also, two antibodies must be from different animal sources.

Note: It is important to include negative controls where a coverslip will be labeled with a non-specific IgG antibody from a similar animal source than one of the primary antibodies.

25. Permeabilize the fixed cells with 50 µL of permeabilization buffer (PBS containing 0.2% Triton X-100) for 15 min at 25°C.

Note: Use 200 µL of permeabilization buffer (or enough to cover the coverslip) if permeabilizing directly in the 24-well plate.

Optional: Coverslips can be maintained in this homemade coverslip holder (Figure 3) where coverslips are placed on top of Eppendorf tube lids that minimize the use of the reagents. Using this setup, it is possible to use a volume as small as 30 µL for all antibody and PLA steps since, as the edge of the coverslip does not touch anything, the liquid stays as a droplet on top of the coverslip because of surface tension. If using a different system, such as a multi-well plate, to incubate the coverslips or while utilizing large-sized coverslips compared to one mentioned here (refer to key resources table) or when samples are mounted on the slides directly, adjust volumes accordingly.

26. Wash the coverslips three times with PBS.

Note: Excess solution is removed from the coverslips by tilting them sideways on absorbent paper with the cell side facing upwards.

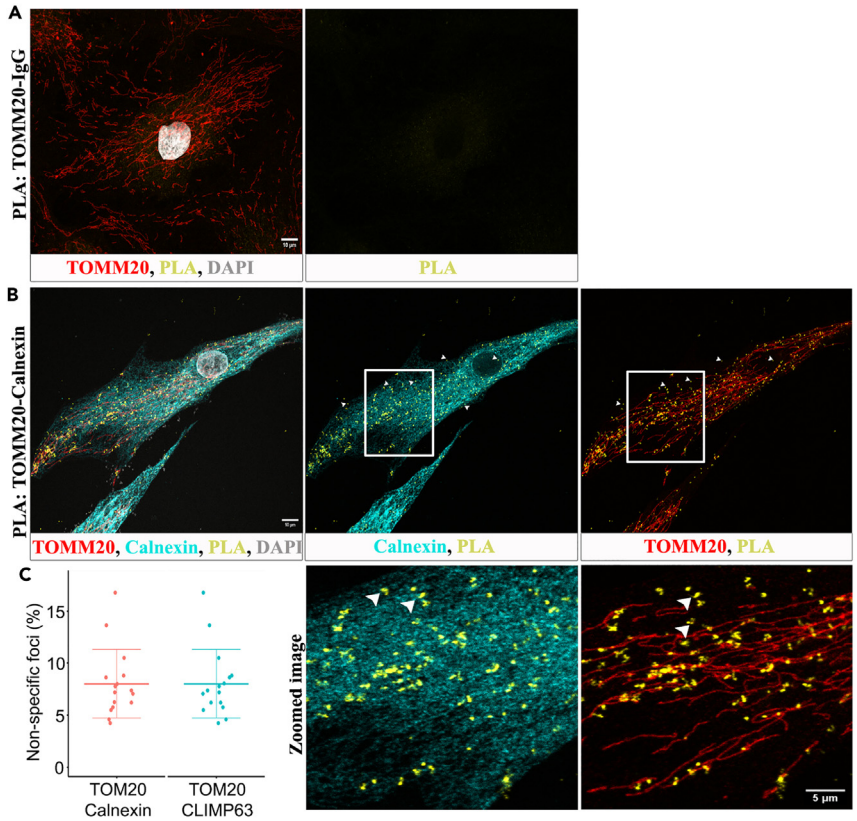

Figure 2. Images of PLA foci in transfected cells

(A) Control PLA using IgG mouse antibody and TOMM20 (mitochondria). The PLA image demonstrates the specificity of the assay.

(B) PLA for TOMM20 (mitochondria) and calnexin (endoplasmic reticulum). Boxed regions are enlarged in the bottom panels. White arrowheads show non-specific foci. Scale bar: 10 µm for the actual image and 5 µm for zoomed images.

(C) Percentage of non-specific PLA foci (Calnexin-TOMM20, CLIMP63-TOMM20) in primary human fibroblast. Each point represents an individual cell. Bars show the average ± SD.

27. Incubate the coverslips with a drop of Duolink blocking solution for 1 h at 37°C.

Note: Coverslips can be either maintained in a humidity chamber or a dry heating block in the homemade coverslip holder (Figure 4).

28. Prepare 30 µL of primary antibodies per coverslip.
    a. Dilute primary antibodies recognizing the organelles of interest (ER and mitochondrial protein here) in the antibody diluent provided with the PLA kit.

Note: Alternatively, a homemade blocking buffer can be used (refer to materials and equipment section).

29. Remove the blocking solution.
    a. Add the primary antibodies and.
    b. incubate for 1 h at 25°C.
30. Remove the primary antibodies and.
    a. wash twice the coverslips with 50 µL of wash buffer A for 5 min at 25°C.

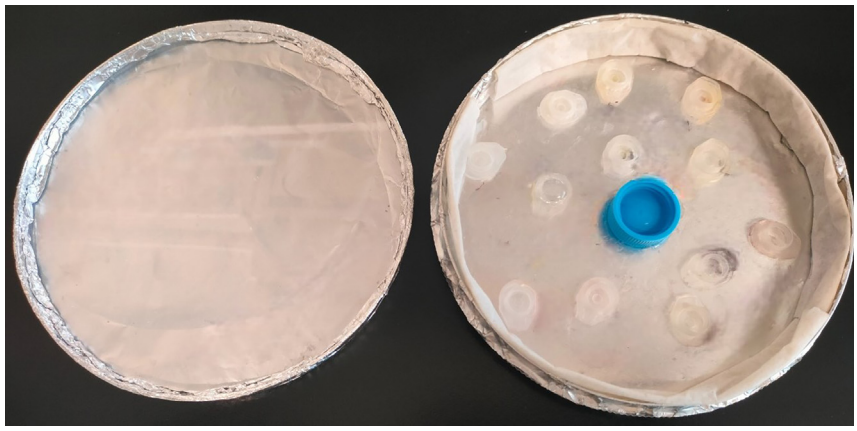

Figure 3. Homemade coverslip support
Eppendorf tube lids are glued to the bottom of a 15 cm Petri dish. The dish and its lid are covered with aluminum foil to protect coverslips from the light. To maintain the humidity and prevent the evaporation of the PLA solutions, wet tissue is placed along the wall of the dish and a centrifuge tube cap filled with water is placed inside the dish.

Note: The PLA kit includes a 1X wash buffer powder (A and B) sufficient to prepare 1 L in autoclaved MilliQ or sterile water. Store at 4°C for long-term storage as specified by the manufacturer (https://www.sigmaaldrich.com/SE/en/technical-documents/protocol/protein-biology/protein-and-nucleic-acid-interactions/duolink-fluorescence-user-manual). Prepare aliquots and bring them to 25°C before use.

31. Prepare Plus and Minus probes in the antibody diluent at the ratio of 1:5. Une 30 µL probe solution (plus and minus) for each coverslip.

Reagents	Volume per coverslip, µl
Probe Plus	6
Probe Minus	6
Antibody diluent	18

Note: To minimize the use of PLA kit components, we use a volume of 30 µL per coverslip for probing, ligation, and amplification steps. Volumes will need to be adjusted if using a different system.

⚠ CRITICAL: Prepare required volume of probes just before use. It is not advisable to store the probe solution.

32. Add 30 µL probe solution and incubate coverslips at 37°C for 1 h.
33. Wash the coverslips twice with 50 µL wash buffer A for 5 min each at room temperature.
34. Prepare 1X ligation buffer by diluting at a ratio of 1: 5 in sterile MilliQ/ nuclease-free ultrapure water.

Reagents	Volume per coverslip, µl
5X ligation buffer	6
Sterile water	24

Figure 4. Coverslips are maintained at the appropriate temperature in a homemade coverslip holder using a dry heating block

35. Prepare the ligation reaction mix just before addition to the coverslips. Maintain ligase at −20°C in the freezer block.

Reagents	Volume per coverslip, µl
1X ligation buffer	29.25
Ligase	0.75

⚠ CRITICAL: Prepare required volume of ligation solution just before use. It is not advisable to store the ligation solution. Add ligase to the ligation solution just before use.

36. Remove the wash buffer and add 30 µL of ligation mix per coverslip.
37. Incubate the coverslips at 37°C for 30 min.
38. Wash the coverslips with 50 µL of wash buffer A twice for 5 min each at 25°C.
39. Prepare 1X amplification buffer in sterile ultrapure water.

⚠ CRITICAL: Buffer preparation and amplification steps must be performed in the dark. Keep the diluted buffers protected from light.

Reagents	Volume per coverslip, µl
5X Amplification buffer	6
Sterile water	24

⚠ CRITICAL: The amplification reaction mix must be prepared just before use, and the polymerase must be maintained in a freezing block until diluted in the buffer.

Reagents	Volume per coverslip, µl
1X Amplification buffer	29.625
Polymerase	0.375

⚠ CRITICAL: Prepare the required volume of amplification solution just before use. It is not advisable to store the prepared amplification solution. Add polymerase to the ligation solution just before use.

40. Remove the wash buffer and add the amplification reaction mix.
41. Incubate the coverslips at 37°C for 1 h 40 min protected from light.
42. Wash the coverslips twice with 50 μL of 1X wash buffer B for 10 min each at 25°C.
43. Wash coverslips once with 50 μL of 0.01X wash buffer B for a minute.

Note: Use wash buffer B warmed to 25°C.

44. Prepare fluorophore-coupled secondary antibodies that recognize the primary antibodies that were used for the PLA reaction.
    a. Make sure that these antibodies have excitation and emission spectrums that are distinct from the PLA signal.
    b. Dilute the secondary antibodies (as mentioned in KRT) 1:500 in either homemade blocking buffer or blocking solution supplied by company.
    c. Add 50 μL of secondary antibody to the coverslips and incubate for 1 h at 25°C.

Note: For cells that can be stably transfected or transduced, an alternative to this step is to use cells stably expressing ER or mitochondria-targeted fluorophores. For instance, Huh7.5 cells stably expressing mito-mTurquoise2 have successfully been used for this purpose.[2]

Note: There are always non-specific PLA foci, and labeling the organelles helps to identify the genuine points of contact between the two organelles. (Refer to troubleshooting 1).

45. Tap off the excess of antibody mix and wash the coverslips with PBS three times at 25°C.
46. Dilute DAPI (1 μg/mL) in Duolink blocking solution or homemade blocking buffer.
    a. Add 50 μL to each of the coverslips.
    b. incubate for 10 min at 25°C.
47. Wash the coverslips with PBS three times at 25°C.
48. Add a drop of mounting solution on a microscopy slide and mount the coverslips on the drop (with cells facing the solution).

Note: Avoid air bubbles while adding mounting solution and placing coverslips on the slides.

49. Acquire Images using standard confocal microscopy with a 63X oil-objective lens.

Note: Slides can be kept at 4°C for in the slide storage box up to 6 months, or until they dry out.

Note: For flat cells like primary fibroblasts, single z-plane images covering the maximal cytoplasmic plane will be sufficient to identify all PLA foci. This can be achieved by either making sure that mitochondria are properly focused or using the plane where the DAPI staining is the strongest. However, when using cells that have a significant thickness, it is advisable to use z-stacking to avoid missing foci from different focal planes.

EXPECTED OUTCOMES

When transfecting primary cells using this method, we typically get 30%–35% transfection efficiency. For the PLA, the control IgG normally show no foci although some background foci can be observed for some less specific antibodies (Figure 2). For a real PLA pair, we typically observe 50–300 PLA foci,

depending on the PLA pair (Figures 1 and 2). PLA foci not localized to the target organelles should remain below 10%.

QUANTIFICATION AND STATISTICAL ANALYSIS

PLA images can be either analyzed manually or using the Analyze particle tool in Fiji Image J software which can be downloaded for free here: https://imagej.net/software/fiji/downloads.

Note: Analysis can be done on individual cells or different regions of interest in a frame. Here, we separated individual cells using polygonal tool in image J prior to the analysis.

Manual

Note: We generally use manual analysis when the number of PLA foci is below 100.

1. Open Images in Fiji Image J.
2. Merge the PLA channel with the ER and mitochondria channels (or any other organelles of interest).
3. PLA foci colocalizing with ER and mitochondria are considered "true interactions".
4. PLA foci on neither of the organelles of interest are considered false positive foci.

Analysis using Image J

Note: There are multiple ways to count PLA foci by Image J. This method is useful when there are more than 100 PLA foci.

5. Smooth the PLA channel using a median filter (value chosen based on the image noise level, normally 1 or 2).

Note: This step is used to remove noise in the image and smooth the contour of the PLA foci.

6. Convert the image into 8-bit grayscale (Image > Type > 8-bit).
7. Convert the 8-bit image into a binary image using the threshold function (Image > Adjust > Threshold).
   a. Use the default method.
   b. Manually adjust the threshold value to represent the actual image.
8. Create a mask of the binary image, creating filled outlines of the PLA foci (Edit > Selection > Create Mask).
9. Separate clustered foci using the watershed tool (Process > Binary > Watershed).
10. Use the Erode function to remove small foci that represent background signal. (Process > Binary > Option > set iteration (10) and count (5) > Do: Erode).
11. Count particles using Analyze particles (Analyze > Analyze particles > default settings).

Note: Use the option Size (Pixel^2) to exclude any remaining small background particles.

12. Manually count Non-specific PLA foci that are negative for mitochondria and ER labeling. Remove these non-specific foci from the total particle count.

Statistical analysis

13. Count total true positive PLA foci per cell as above in control and test samples.

Note: It is ideal to count PLA foci in a minimum of 10–15 cells per experiment. At least 3 independent experiments are required for statistical analysis.

14. Depending on the number of test samples, compare PLA foci/cells in the test sample to the control by a two-way t-test or one-way ANOVA, Tukey post-hoc test (more than one test sample).
15. A p-value less than 0.05 is considered statistically significant with a 95% confidence interval.

LIMITATIONS

Primary human fibroblasts are excellent tools for studying cell biology. Their large size and the fact that they are very flat cells make them ideal for microscopy studies. In addition, they do not contain the genetic alterations present in malignant or immortalized cells. It is also possible to study specific disease-causing mutations by using patient-derived primary fibroblasts. However, reviving, culturing and handling primary cells is challenging and requires a lot of patience, especially for untrained hands. Primary fibroblasts also have a restricted maximal passage number (~20) and it is preferable to use them at low passage numbers for investigations to avoid cellular aberrations generally observed in "older" cells.

PLA is a straightforward method for measuring the proximity between two organelles that does not require the use of electron microscopy. However, the antibody pairs used to detect contact sites have a significant impact on the results. It is critical to identify protein combinations that preferentially mark organelles without non-specific binding. It is also critical to keep in mind that this method locates contact points between organelles based on the proteins' proximity (40 nm, according to the Sigma-Aldrich manual). Also, though any uniformly dispersed organelle surface proteins could be chosen, it should be kept in mind that those contact sites are not necessarily related to any functional role such as calcium or lipid signaling.

TROUBLESHOOTING

Problem

The presence of multiple non-specific PLA foci that are negative for either of the organelles (Step 45) (Figure 1).

Potential solution

- Always include an antibody control where one of the protein-specific antibodies is replaced with Ig control from similar species.
- Initially, perform pilot immunofluorescence studies to identify the best antibodies specifically labeling the organelles studied. It is better to avoid antibodies that show high background or off-target staining.
- Table 1 shows the list of antibody pairs that we have tested to quantify ER-mitochondria contact sites in different cells.

Table 1. List of antibody pairs that we have tested to quantify ER-mitochondria contact sites in different cells

Rabbit antibody	Mouse antibody	Use	Reliability	Cells tested
TOMM20 (Mitochondria, Abcam, # ab186735, 1:250)	Calnexin (ER, Millipore, # MABF2067, 1:200)	Mitochondria-general ER interaction	Reliable	Primary fibroblast, Mouse embryonic fibroblast,
IP3R1 (ER, Novus Biologicals, # NBP2-22458, 1:200)	VDAC1 (Mitochondria, Sigma-Aldrich, #MABN504, 1:200)	Mitochondria-general ER interaction	Not fully reliable, high non-specific background staining	Primary fibroblast
IP3R1 (ER, Novus Biologicals, # NBP2-22458, 1:200)	VDAC1 (Mitochondria, Abcam, # ab14734, 1:100)	Mitochondria-general ER interaction	Partially reliable	Hepatocarcinoma Huh7-derived cells
PTPIP51 (Mitochondria, Sigma-Aldrich, # HPA009975, 1:100)	VAPB (ER; Proteintech # 66191-1-Ig, 1:100)	Mitochondria-general ER interaction	Reliable	Hepatocarcinoma Huh7-derived cells
TOM20 (Mitochondria, Abcam, # ab186735,1:250)	CLIMP63(ER, ENZO, # ENZ ABS669, 1:200)	Mitochondria- rough ER interaction	Reliable	Primary fibroblast
SYNJ2BP (Mitochondria, Sigma-Aldrich, # HPA000866, 1:200)	RRBP1(ER, Thermo Fisher Scientific, # MA5-18302, 1:200)	Mitochondria- rough ER interaction	Reliable	Primary fibroblast, Hepatocarcinoma Huh7-derived cells

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Marc Germain (marc.germain1@uqtr.ca).

Technical contact

Further information on technical aspects should be directed to Marc Germain (marc.germain1@uqtr.ca).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate new datasets.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2024.102915.

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AUTHOR CONTRIBUTIONS

Conceptualization, H.S.I., S.B., and M.G.; methodology, H.S.I., S.B., L.C.-C., and M.G.; writing – original draft, H.S.I. and M.G.; writing – review and editing, all the authors; supervision, M.G.; funding acquisition, L.C.-C. and M.G.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Ilamathi, H.S., Benhammouda, S., Lounas, A., Al-Naemi, K., Desrochers-Goyette, J., Lines, M.A., Richard, F.J., Vogel, J., and Germain, M. (2023). Contact sites between endoplasmic reticulum sheets and mitochondria regulate mitochondrial DNA replication and segregation. iScience *26*, 107180. https://doi.org/10.1016/j.isci.2023.107180.

2. Wesley Freppel, A.A., Nouhi, Z., Mazeaud, C., Gilbert, C., Tremblay, N., Barragan Torres, V.A., Sow, A.A., Laulhé, X., Lamarre, A., Rodrigue-Gervais, I.G., et al. (2023). Flaviviruses alter endoplasmic reticulum-mitochondria contacts to regulate respiration and apoptosis. Preprint at bioRxiv. https://doi.org/10.1101/2023.03.09.531853.

3. Brees, C., and Fransen, M. (2014). A cost-effective approach to microporate mammalian cells with the Neon Transfection System. Anal. Biochem. *466*, 49–50. https://doi.org/10.1016/j.ab.2014.08.017.

4. Hobro, A.J., and Smith, N.I. (2017). An evaluation of fixation methods: Spatial and compositional cellular changes observed by Raman imaging. Vib. Spectrosc. *91*, 31–45. https://doi.org/10.1016/j.vibspec.2016.10.012.

5. Szabadkai, G., Bianchi, K., Várnai, P., De Stefani, D., Wieckowski, M.R., Cavagna, D., Nagy, A.I., Balla, T., and Rizzuto, R. (2006). Chaperone-mediated coupling of endoplasmic reticulum and mitochondrial Ca2+ channels. J. Cell Biol. *175*, 901–911. https://doi.org/10.1083/jcb.200608073.