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Published in:
Journal of Visualized Experiments

DOI:
10.3791/60172

Video Article

In Situ Monitoring of Transiently Formed Molecular Chaperone Assemblies in Bacteria, Yeast, and Human Cells

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URL: https://www.jove.com/video/60172
DOI: doi:10.3791/60172

Keywords: Biology, Issue 151, Proximity Ligation Assay, Chaperone, J-domain protein, Hsp70, human, bacteria, yeast, *E. coli*, *S. cerevisiae*, protein interaction, proteostasis

Date Published: 9/2/2019

Citation: Alberts, N., Mathangasinghe, Y., Nillegoda, N.B. In Situ Monitoring of Transiently Formed Molecular Chaperone Assemblies in Bacteria, Yeast, and Human Cells. *J. Vis. Exp.* (151), e60172, doi:10.3791/60172 (2019).

Abstract

J-domain proteins (JDPs) form the largest and the most diverse co-chaperone family in eukaryotic cells. Recent findings show that specific members of the JDP family could form transient heterocomplexes in eukaryotes to fine-tune substrate selection for the 70 kDa heat shock protein (Hsp70) chaperone-based protein disaggregases. The JDP complexes target acute/chronic stress induced aggregated proteins and presumably help assemble the disaggregases by recruiting multiple Hsp70s to the surface of protein aggregates. The extent of the protein quality control (PQC) network formed by these physically interacting JDPs remains largely uncharacterized in vivo. Here, we describe a microscopy-based in situ protein interaction assay named the proximity ligation assay (PLA), which is able to robustly capture these transiently formed chaperone complexes in distinct cellular compartments of eukaryotic cells. Our work expands the employment of PLA from human cells to yeast (*Saccharomyces cerevisiae*) and bacteria (*Escherichia coli*), thus rendering an important tool to monitor the dynamics of transiently formed protein assemblies in both prokaryotic and eukaryotic cells.

Video Link

The video component of this article can be found at https://www.jove.com/video/60172/

Introduction

A vast amount of genomic information remains uninterpretable due to our incomplete understanding of cellular interactomes. Conventional protein-protein interaction detection methodologies such as protein co-immunoprecipitation with/without chemical cross-linking and protein co-localization, though widely used, pose a range of disadvantages. Some of the main disadvantages include poor quantification of the interactions and the potential introduction of non-native binding events. In comparison, emerging proximity-based techniques provide an alternative and a powerful approach for capturing protein interactions in cells. The proximity ligation assay (PLA)[1], now available as a proprietary kit, employs antibodies to specifically target protein complexes based on the proximity of the interacting subunits.

PLA is initiated by the formation of a scaffold consisting of primary and secondary antibodies with small DNA tags (PLA probes) on the surface of the targeted protein complex (**Figure 1**, steps 1-3). Next, determined by the proximity of the DNA tags, a circular DNA molecule is generated via hybridization with connector oligonucleotides (**Figure 1**, step 4). The formation of the circular DNA is completed by a DNA ligation step. The newly formed circular piece of DNA serves as a template for the subsequent rolling circle amplification (RCA)-based polymerase chain reaction (PCR) primed by one of the conjugated oligonucleotide tags. This generates a single-stranded concatemeric DNA-molecule attached to the protein complex via the antibody scaffold (**Figure 1**, step 6). The concatemeric DNA molecule is visualized using fluorescently labeled oligonucleotides that hybridize to multiple unique sequences scattered across the amplified DNA (**Figure 1**, step 7)[2]. The generated PLA signal, which appears as a fluorescent dot (**Figure 1**, step 7), corresponds to the location of the targeted protein complex in the cell. As a result, the assay could detect protein complexes with high spatial accuracy. The technique is not limited to simply capturing protein interactions, but could also be utilized to detect single molecules or protein modifications on proteins with high sensitivity[1,2].

Hsp70 forms a highly versatile chaperone system fundamentally important for maintaining cellular protein homeostasis by participating in an array of housekeeping and stress-associated functions. Housekeeping activities of the Hsp70 chaperone system include de novo protein folding, protein translocation across cellular membranes, assembly and disassembly of protein complexes, regulation of protein activity and linking different protein folding/quality control machineries[3]. The same chaperone system also refolds misfolded/unfolded proteins, prevents protein aggregation, promotes protein disaggregation and cooperates with cellular proteases to degrade terminally misfolded/damaged proteins to facilitate cellular repair after proteotoxic stresses[4,5]. To achieve this functional diversity, the Hsp70 chaperone relies on partnering co-chaperones of the JDP family and nucleotide exchange factors (NEFs) that fine-tune the Hsp70's ATP-dependent allosteric control of substrate binding and

release[3,6]. Further, the JDP co-chaperones play a vital role in selecting substrates for this versatile chaperone system. Members of this family are subdivided into three classes (A, B and C) based on their structural homology to the prototype JDP, the *E. coli* DnaJ. Class A JDPs contain an N-terminal J-domain, which interacts with Hsp70, a glycine-phenylalanine rich region, a substrate binding region consisting of a Zinc finger-like region (ZFLR) and two β-barrel domains, and a C-terminal dimerization domain. JDPs with an N-terminal J-domain and a glycine-phenylalanine rich region, but lacking the ZFLR, fall into class B. In general, members of these two classes are involved in chaperoning functions. Members falling under the catchall class C, which contains JDPs that only share the J-domain[4], recruit Hsp70s to perform a variety of non-chaperoning functions. The important role of JDPs as interchangeable substrate recognition "adaptors" of the Hsp70 system is reflected by an expansion of the family members during evolution. For example, humans have over 42 distinct JDP members[4]. These JDPs function as monomers, homodimers and/or homo/hetero oligomers[4,5]. Recently, a functional cooperation via transient complex formation between class A (e.g., *H. sapiens* DNAJA2; *S. cerevisiae* Ydj1) and class B (e.g., *H. sapiens* DNAJB1; *S. cerevisiae* Sis1) eukaryotic JDPs was reported to promote efficient recognition of amorphous protein aggregates in vitro[7,8]. These mixed class JDP complexes presumably assemble on the surface of aggregated proteins to facilitate the formation of Hsp70- and Hsp70+Hsp100-based protein disaggregases[7,8,9,10]. The critical evidence to support the existence of these transiently formed mixed class JDP complexes in eukaryotic cells was provided with PLA[8].

PLA is increasingly employed for assessing protein interactions in metazoa, primarily in mammalian cells. Here, we report the successful expansion of this technique to monitor transiently formed chaperone complexes in eukaryotic and prokaryotic unicellular organisms such as the budding yeast *S. cerevisiae* and the bacterium *E. coli.* Importantly, this expansion highlights the potential use of PLA in detecting and analyzing microbes that infect human and animal cells.

Protocol

1. HeLa Cell Preparation

1. Prepare the following materials: PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4), pH 7.4; DMEM, supplemented with 10% FCS and 1% Pen-Strep; 4% paraformaldehyde in PBS; 0.5% Triton-X100 in PBS; TBS-T (150 mM NaCl, 20 mM Tris, 0.05% Tween), pH 7.4; 0.0001% sterile poly-L-Lysine solution; 10-well diagnostic slides; a humid chamber; tissue paper; and Coplin slide-staining jars.
   NOTE: To ensure optimal fixation efficiency, paraformaldehyde solutions should be prepared fresh before every experiment.
2. Prepare a humid chamber by covering the bottom of a closed box with wet tissues. Place the humid chamber at 37 °C before starting the experiment, to ensure that the chamber temperature is at 37 °C while incubating the enzymatic reactions.
3. Culture HeLa cells in T25 flasks, in 5 mL of DMEM (High Glucose, Glutamate and Sodium Pyruvate supplemented) supplemented with 10% FCS and 1% Pen-Strep, in a 37 °C CO_2 incubator containing 5% CO_2. Dissociate adherent cells using 0.05% Trypsin-EDTA. After addition of fresh DMEM to dissociated cells, count cells using a cell counting chamber and grow on diagnostic slides inside a humid chamber.
4. Sterilize diagnostic slides by UV-irradiation in a sterile cell culture hood for 30 min.
5. Add 100 µL of sterile filtered 0.0001% poly-L-lysine to each well required for the experiment. Incubate for 30 min. Wash away excess poly-L-lysine by washing each well 3x with 50 µL ultrapure water.
6. Trypsinize the HeLa cells and add approximately 15,000 cells to each well. If necessary, dilute cells to at least 30-50 µL of DMEM per well.
7. Grow cells in a humid chamber within a 37 °C 5% CO_2 incubator for approximately 24 h. Cells should be 60%–80% confluent before commencing the PLA.
   NOTE: Too high a confluency decreases the absorption of reagents, decreasing the obtained signal at the end of the protocol.
8. Remove medium by placing a tissue paper at the edge of the well. Wash wells 3x with 50 µL of PBS.
   NOTE: Cells are subject to detaching when liquids are added harshly. This can be prevented by not letting the wells dry completely before adding new liquid and by adding the new liquid gently to the edge of the well.
9. Fix cells by adding 50 µL of freshly prepared 4% paraformaldehyde in PBS to each well. Incubate for 10 min at room temperature.
10. Wash slides 3x in PBS. Perform washes in a Coplin slide-staining jar containing 100 mL of PBS. For each wash, incubate for 5 min at room temperature without shaking.
11. Permeabilize the cell membrane by submerging the slides in 100 mL of 0.5% Triton-X100 in PBS in a Coplin slide-staining jar. Incubate for 10 min at room temperature without shaking.
12. Wash slides 3x in TBS-T. Perform washes in a Coplin slide-staining jar containing 100 mL of TBS-T. For each wash, incubate for 5 min at room temperature without shaking.
13. After the last wash, remove excess buffer with a tissue paper. At this point the cells are ready for the Proximity Ligation Assay protocol that will be discussed in section 4.

2. *S. cerevisiae* Cell Preparation

1. Prepare the following materials: 100 mM KPO_4, pH 6.5, referred to as Wash Buffer; 37% formaldehyde; 4% paraformaldehyde in 100 mM KPO_4, pH 6.5; 1.2 M sorbitol in 100 mM KPO_4, pH 6.5; lyticase solution (500 µg/mL lyticase, 20 mM β-mercaptoethanol, 100 mM KPO_4, pH 6.5); 0.0001% poly-L-Lysine solution; 1% Triton-X100 in 100 mM KPO_4, pH 6.5; 10-well diagnostic slides; a humid chamber (prepared as in step 1.2); tissue paper; and Coplin slide-staining jars.
   NOTE: To ensure optimal fixation efficiency, paraformaldehyde solutions should be prepared fresh before every experiment.
2. Grow an overnight culture in non-selective Yeast Extract, Peptone and Dextrose (YPD) medium at 30 °C while shaking.
   NOTE: Depending on experimental requirements *S. cerevisiae* cells can be grown in Synthetic Complete (SC) medium or selective Synthetic Minimal (SM) medium instead.
3. Dilute the stationary culture to an OD_{600} of 0.1 in 20 mL medium. Grow the cells at 30 °C while shaking until the OD_{600} reaches 0.5.
4. Transfer the 20 mL culture to a 50 mL centrifuge tube. Pellet the cells by centrifugation at 665 x *g* for 3 min. Remove the supernatant. Resuspend the cells in 5 mL of fresh medium.
5. Fix the cells by adding 550 µL of 37% formaldehyde to the culture. Incubate at room temperature for 15 min.
6. Pellet the cells by centrifugation at 665 x *g* for 3 min. Remove the supernatant. Resuspend the pellet in 1 mL of freshly prepared 4% paraformaldehyde in Wash Buffer. Incubate for 45 min at room temperature.

7. During the incubation, prepare the diagnostic slides by adding 100 µL of 0.01% poly-L-lysine solution to each well. Incubate the slides for 30 min at room temperature.
8. After 30 min, wash away excess poly-L-lysine with ultrapure water and let the slides air dry. The dry slides are ready for use.
9. Wash cells twice with 1 mL of Wash Buffer. Perform washes by centrifugation of the cells at 665 x *g* for 3 min. Remove the supernatant and resuspend the cells in Wash Buffer.
10. Pellet the cells by centrifugation at 665 x *g* for 3 min. Remove the supernatant. Resuspend the cells in 1 mL of 1.2 M sorbitol in Wash Buffer.
11. Pellet the cells by centrifugation at 665 x *g* for 3 min. Remove the supernatant. Resuspend the pellet in 250 µL of freshly prepared Lyticase solution to digest the cell wall. Incubate the cells in Lyticase solution for 15 min at 30 °C while shaking.
12. After digestion, wash cells 3x by centrifugation at 665 x *g* for 3 min and remove the supernatant. Resuspend the cells in 250 µL of 1.2 M sorbitol in Wash Buffer.
    NOTE: Because cells are fragile after cell wall digestion, resuspend them very carefully to not damage the cells.
13. Add 20 µL of resuspended cells to the poly-L-lysine coated slides. Allow them to attach to the slides for 30 min. Wash away non-adherent cells by washing the wells 3x with 50 µL of Wash Buffer.
14. Permeabilize the cell membrane by washing 3x with 50 µL of 1% Triton-X in Wash Buffer.
    NOTE: At this point the cells are ready for the proximity ligation assay protocol that will be discussed in section 4.

3. *E. coli* Cell Preparation

1. Prepare the following materials: PBS-T (140 mM NaCl, 2 mM KCl, 8 mM K_2HPO_4, 1.5 mM KH_2PO_4, 0.05% Tween-20), pH 7.4; lysozyme solution (2 mg/mL lysozyme, 25 mM Tris-HCl pH 8.0, 50 mM Glucose, 10 mM EDTA); 0.0001% poly-L-Lysine solution; 99% ice-cold methanol; 99% room temperature methanol; 99% acetone; 10-well diagnostic slides; a humid chamber (prepared as in step 1.1.1); tissue paper; and Coplin slide-staining jars.
2. Grow an overnight culture in Luria-Bertani (LB) medium at 30 °C while shaking.
3. Dilute the stationary culture to an OD_{600} of 0.02 in fresh LB medium. Grow the cells at 30 °C while shaking until the OD_{600} reaches 0.4 for log phase cells.
4. Approximately 15 min before cells reach an OD_{600} of 0.4, prepare poly-L-lysine coated slides by adding 100 µL of 0.0001% poly-L-lysine to each well. Incubate the slides for 30 min at room temperature.
5. After 30 min wash away excess poly-L-lysine with ultrapure water and let slides air dry. The dry slides are ready for use.
6. When the cells reach an OD_{600} of 0.4, transfer 1 mL of the culture to a sterile microcentrifuge tube and pellet cells at 2,650 x *g* for 2 min.
7. Resuspend cells in 50 µL of LB medium.
8. Fix cells by adding 1 mL of ice-cold 99% methanol. Mix very gently by hand. Incubate cells for 30 min at -20 °C.
9. After fixation add 20 µL of the cells to the poly-L-lysine coated slides. Let the slides air dry for 30 min.
10. Add 50 µL of freshly prepared lysozyme solution to each well to digest the cell wall. Incubate in a humid chamber for 30 min at 25 °C.
11. Remove the lysozyme solution from the wells by adding a tissue paper to the edge of the well. Wash slides 3x in 100 mL of PBS-T. Perform each wash in a Coplin slide-staining jar for 30 s, without shaking.
12. Remove wash buffer from the slides by tapping the slides on a tissue paper.
13. Permeabilize the cell membranes by adding 50 µL of 99% methanol to each well. Incubate for 1 min at room temperature.
14. Remove methanol by placing a tissue paper to the edge of the well.
15. Add 50 µL of 99% acetone to each well. Incubate for 1 min.
16. Remove excess acetone by placing a tissue paper to the edge of the well. Allow slides to air dry. At this point the cells are ready for the proximity ligation assay protocol that will be discussed in section 4.

4. Proximity Ligation Assay

1. Prepare the following materials: Blocking Buffer; Antibody Dilution Buffer; Wash Buffer 'A' – (10 mM Tris, 150 mM NaCl, 0.05% Tween-20) pH 7.4; Wash Buffer 'B' – (200 mM Tris, 100 mM NaCl) pH 7.4; Anti-Rabbit Secondary Antibody PLUS; Anti-Mouse Secondary Antibody MINUS; 5x Ligation Buffer; ligase; 5x Amplification Buffer (Orange: λ_{ex} 554 nm; λ_{em} 576 nm); polymerase; ultrapure water; and mounting medium containing DAPI.
   NOTE: The PLA detection reagents are also available in the variants Green (λ_{ex} 495 nm; λ_{em} 527 nm), Red (λ_{ex} 594 nm; λ_{em} 624 nm), FarRed (λ_{ex} 644 nm; λ_{em} 669 nm) or Brightfield (horseradish peroxidase (HRP) conjugated).
2. Block the cells by adding a drop of Blocking Buffer to each well. Incubate for 30 min at 37 °C in a humid chamber.
3. Prepare antibody solutions by diluting stock antibodies in Antibody Dilution Buffer. For each well 40 µL of antibody solution is required.
4. Remove blocking buffer by placing a tissue paper at the edge of the well. Add 40 µL of antibody diluted in Antibody Dilution Buffer to each well. Incubate for 60 min in a humid chamber at 37 °C or overnight at 4 °C.
5. Remove antibody solution from the wells by placing a tissue paper at the edge of the well. Wash slides 2x in 100 mL of Wash Buffer 'A' in a Coplin slide-staining jar for 5 min, without shaking.
6. During the wash steps, dilute 5x secondary antibody probes, anti-rabbit PLUS & anti-mouse MINUS (the species specificity of the probes depends on the primary antibodies used), in Antibody Dilution Buffer. Prepare 40 µL of antibody solution per well.
7. Add 40 µL of secondary antibody solution to each well. Incubate for 60 min at 37 °C in a humid chamber.
8. Remove secondary antibody solution from the wells by placing a tissue paper at the edge of the well. Wash slides 2x in 100 mL of Wash Buffer 'A' in a Coplin slide-staining jar for 5 min, without shaking.
9. During the washes prepare 40 µL of ligation mixture per well, by mixing 8 µL of 5x Ligation Buffer, 31 µL of ultrapure water and 1 µL of ligase.
10. Add 40 µL of ligation mixture to each well. Incubate for 30 min at 37 °C in a humid chamber.
11. Remove ligation mixture from the wells by placing a tissue paper at the edge of the well. Wash slides 2x in 100 mL of Wash Buffer 'A' in a Coplin slide-staining jar for 2 min, without shaking.
12. During the washes prepare 40 µL of amplification mixture per well, by mixing 8 µL of 5x amplification solution, 31.5 µL of ultrapure water, and 0.5 µL of polymerase.

NOTE: The 5x amplification contains fluorescent probes. Protect this mixture from light. Also, protect the slides from light during each of the following steps. If using translucent Coplin slide-staining jars and humid chambers, wrap them in aluminum foil.

13. Add 40 µL of amplification mixture per well. Incubate for 100 min at 37 °C in a humid chamber.
14. Remove amplification mixture from the wells. Wash slides 2x in 100 mL of Wash Buffer 'B' in a Coplin slide-staining jar for 10 min without shaking.
15. Wash slides in 100 mL of Wash Buffer 'B' diluted 1:100 in ultrapure water in a Coplin slide-staining jar for 30 s.
16. Add 20 µL of DAPI containing mounting medium per well to the slides. Close slides with a coverslip and seal slides with nail polish.
17. If imaging, immediately incubate DAPI containing mounting medium for 10–15 min, while protected from light. If not, store the slides at -20 °C for up to 1 week, protected from light.

5. Detection

1. Use confocal microscopy to acquire images of HeLa, *S. cerevisiae* and *E. coli* cells with 20x/0.8 NA, 63x/1.4 NA and 100x/1.4 NA Plan Apochromat objectives, respectively. Excite DNA-stained DAPI with a 405 nm pulsed diode laser. For the PLA signal (for this study) excite with a 561 nm solid-state laser.

Representative Results

Our previous in vitro studies using purified proteins revealed that a subset of human class A and class B JDPs form transient mixed class JDP complexes to efficiently target a broad range of aggregated proteins and possibly facilitate the assembly of Hsp70-based protein disaggregases[7]. We employed PLA to determine whether mixed class (A+B) JDP complexes occur in human cervical cancer cells (HeLa). Human JDPs DNAJA2 (class A) and DNAJB1 (class B) were targeted with highly specific primary antibodies and secondary PLA probes (**Figure 2A-C**). The appearance of red fluorescent punctae indicated the presence of mixed class DNAJA2 and DNAJB1 complexes in HeLa cells (**Figure 2C**) confirming our previous biochemical findings[7]. Each punctum represents an individual protein interaction event in the HeLa cell cytosol/nucleus.

Previous biochemical results obtained from Förster resonance energy transfer (FRET), which detects protein interactions as a readout of the amount of energy transferred from an excited donor fluorophore attached to one protein to a suitable acceptor fluorophore attached to the second protein, indicated that similar mixed class complexes could also occur between yeast JDPs, in vitro[8]. Confirming our biochemical findings, we observed the formation of mixed class complexes between Ydj1 (class A) and Sis1 (class B) in unicellular eukaryote *S. cerevisiae* (baker's yeast) cells with PLA (**Figure 2F**). In yeast, due to their small cell size and the coalescence of multiple punctae, the individual fluorescent dots generated by PLA could often be less distinguishable. A considerable decrease in fluorescent punctae formation was observed after knockout or knockdown of interacting JDPs in both human and yeast cells[8], which demonstrates the high specificity of PLA in capturing these co-chaperone complexes in different cell types. In contrast to their eukaryotic counterparts, the prokaryotic (*E. coli*) JDPs lacked the ability to form mixed class JDP complexes and functionally cooperate to boost protein disaggregation, in vitro[8]. In agreement with the biochemical analysis, we did not observe mixed class JDP complex formation between bacterial DnaJ-YFP (class A) and CbpA-mCherry (class B), the only JDPs in *E. coli* (**Figure 2I**). However, our PLA setup was able to capture other chaperone assemblies involving the two JDPs. For example, we detected chaperone complexes between DnaJ-YFP and DnaK (bacterial Hsp70) (**Figure 2L**) and CbpA-mCherry and DnaK[8] (data not shown). These two bacterial chaperone complexes are extensively characterized both in vitro and in vivo[8,11,12] thus confirming our PLA results. Together, these observations demonstrate the ability of PLA to robustly capture transiently formed chaperone machineries in unicellular/multicellular eukaryotic and prokaryotic cells. The technical controls lacking a primary antibody against one of the interacting JDPs/chaperones, but containing both the mouse and rabbit derived secondary PLA probes, showed little to no background fluorescence signal (**Figure 2A,B,D,E,G,H,J,K**) indicating a lack of false positive signal amplification in our experimental setup.

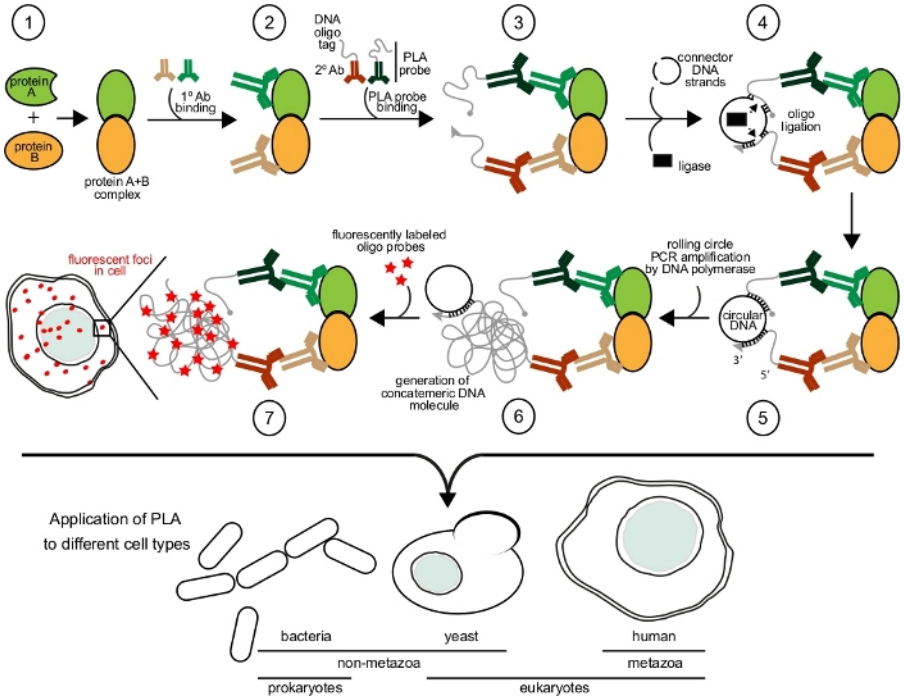

Figure 1: Schematic representation of the chronological steps of the Proximity Ligation Assay. (1) Complex between protein A and protein B. (2) Binding of primary (1°) antibodies (green and light brown) to proteins A and B. The two primary antibodies must be raised in different host species (e.g. mouse, rabbit or goat). (3) Primary antibodies are recognized by species-specific secondary (2°) antibodies covalently attached with DNA oligo tags (PLA probes). (4) When proteins A and B are in complex, the bound PLA probes are in close proximity to facilitate the DNA oligos to hybridize with connector DNA strands, which results in the formation of a circular DNA molecule. Subsequently, a DNA ligase facilitates the joining of DNA strands together by catalyzing the formation of a phosphodiester bond. (5) Initiation of the Rolling Circle Amplification (RCA) of the circular DNA molecule by a bacterial DNA polymerase at 37° C. RCA reaction is primed by one of the antibody-conjugated DNA oligo tags. (6) Generation of a single-stranded concatemeric DNA molecule attached to one of the secondary antibodies. (7) Hybridization of fluorescently-labeled complementary oligonucleotide probes to a unique repetitive sequence in the concatemeric DNA molecule. After the hybridization step, the concatemeric DNA molecule could be visualized as a bright fluorescent dot at the location of the targeted protein complex in fixed cells. Bottom denote the prokaryotic and eukaryotic cell types in which the PLA technique is applicable. Please click here to view a larger version of this figure.

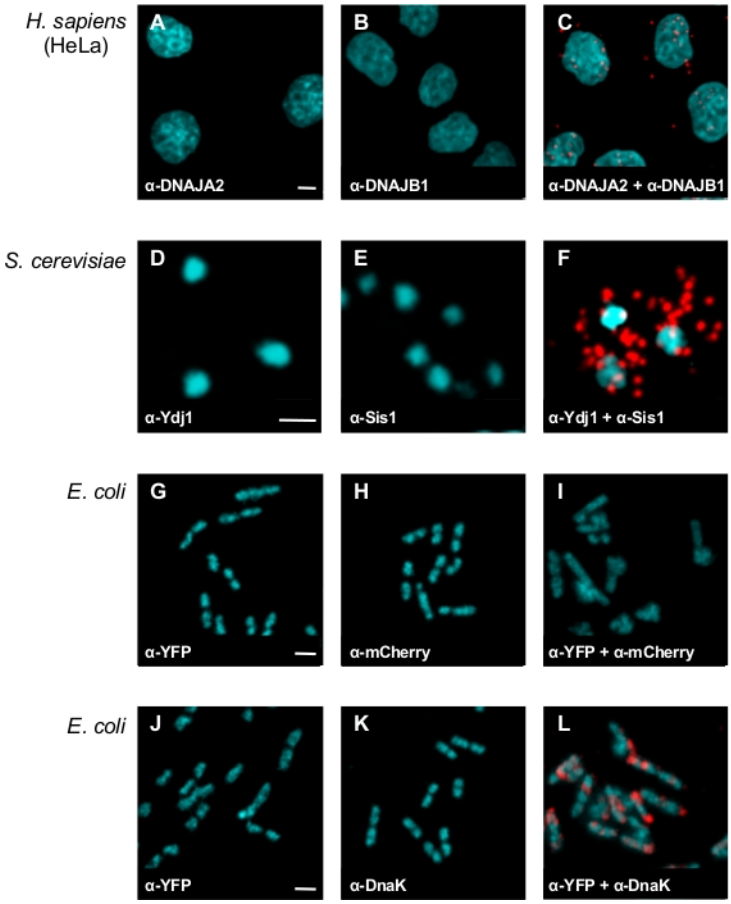

Figure 2. Molecular chaperone assemblies captured by PLA in prokaryotic and eukaryotic cells. (**A-C**) Detection of mixed class JDP complexes formed between DNAJA2 (class A) and DNAJB1 (class B) in human cervical cancer cell line HeLa. PLA performed with (**A**) anti-DNAJA2 antibody alone (negative technical control); (**B**) anti-DNAJB1 antibody alone (negative technical control); (**C**) anti-DNAJB1 and anti-DNAJA2 antibodies together. The appearance of multiple fluorescent dots in panel **C** (positive signal) indicates the presence of protein complexes formed between DNAJA2 and DNAJB1. Each red fluorescent dot/punctum represents a single interaction. Nuclei (DNA) stained with DAPI (cyan). (**D-F**) Detection of mixed class JDP complexes formed between Ydj1 (class A) and Sis1 (class B) in *S. cerevisiae* cells. (**D**) PLA performed with anti-Ydj1 antibody alone (negative technical control); (**E**) PLA performed with anti-Sis1 antibody alone (negative technical control); (**F**) PLA performed with anti-Ydj1 and anti-Sis1 antibodies together. The positive fluorescent signal denotes the presence of Ydj1 and Sis1 complexes in *S. cerevisiae*. Yeast nuclei stained with DAPI (cyan). (**G-L**) Detection of chaperone complexes formed between prokaryotic Hsp70 (DnaK) and JDPs (DnaJ and CbpA) in *E. coli* cells. Due to the lack of specific primary antibodies against prokaryotic JDPs, the *E. coli* DnaJ (class A) and CbpA (class B) were tagged with YFP and mCherry, respectively. (**G, J**) PLA performed with anti-YFP alone (negative technical control); (**H**) PLA performed with anti-mCherry alone (negative technical control). (**I**) PLA performed with anti-YFP and anti-mCherry antibody. The lack of fluorescent dot formation indicates no complex formation between DnaJ and CbpA. (**K**) PLA performed with anti-DnaK antibody alone (negative technical control). (**L**) PLA performed with anti-YFP and anti-DnaK antibodies together. The positive fluorescent signal indicates complex formation between DnaK and DnaJ. Bacterial DNA stained with DAPI (cyan). In addition to containing a single primary antibody, all the negative technical controls were performed in the presence of the respective secondary PLA probes. Scale bars = 10 µm.

Discussion

Co-immunoprecipitation and co-localization based approaches have been used as long-standing methods to characterize protein assembles. The detection of transiently formed specific chaperone complexes is a major challenge with such conventional methods, and as a result, previous findings are largely restricted to qualitative interpretations. The cell lysis-based co-immunoprecipitation techniques often require cross-linking to stabilize protein-protein interactions. Cell lysis increases the risk of disrupting transiently formed chaperone complexes, while cross-linking could introduce non-native interactions, particularly driven by the inherent “stickiness” of most chaperones. When analyzing the Hsp70 chaperone system using co-immunoprecipitation, potential artifacts could arise from (a) high expression levels of the chaperone and (b) solubility issues pertaining to membrane or protein aggregate bound chaperone machineries. Besides, co-immunoprecipitation-based techniques provide no information on cellular localization of the captured protein assemblies, which could be important for delineating the associated functions. The drawbacks of using co-immunoprecipitation techniques are somewhat reduced in co-localization-based approaches that preserve protein localization information. However, the co-localization of two or more proteins could indicate either direct protein-protein association or partitioning of proteins into the same microdomain in cells. Therefore, co-localization analysis is, at best, speculative in predicting protein interactions and lacks any proximity value. Due to the bulk and indiscriminate detection of the targeted proteins, the technique is highly limited in studying specific protein assemblies in cells. This is particularly problematic when the targeted protein(s) could, in parallel, form a wide range of distinct protein assemblies as in the case with the Hsp70 chaperone system. Compared to these conventional methodologies, PLA is a more refined in situ technique developed to robustly detect and quantify native protein associations in cells with preserved protein localization

information. A protein interaction is revealed by this assay based on the proximity (approximately 10-30 nm) between the targeted proteins. PLA is "tunable" in that the range of the proximity distance could be reduced to obtain a more stringent readout by (a) decreasing the length of the oligonucleotide tags attached to the PLA probes and/or (b) conjugating the tags directly to primary antibodies. Care should be taken when interpreting a positive PLA signal. Ideally, a protein interaction should be confirmed with two or more independent methodologies. The intensity of the fluorescent signal in PLA is not as deterministically related to protein cluster size or separation distance between the interacting proteins as is the case with FRET. However, PLA has a high degree of specificity and sensitivity, and could even be used for analyzing very low abundant proteins such as growth factors or cytokines and their interactions in rare cell types or clinical specimens[13].

The Hsp70 chaperone partners with multiple co-chaperones (e.g., JDPs and NEFs) during its cellular lifetime[14] to drive distinct biological functions. The sheer number of probable Hsp70-JDP-NEF machine configurations and their dynamic behavior in cells have largely hampered a detailed understanding of the specific roles of these chaperone machines. Biological tools that allow selective tracing of distinct Hsp70 assemblies are, therefore, required to dissect the overall wiring of this chaperone network in cells. The transiently formed JDP-JDP and JDP-Hsp70 chaperone complexes[7] were effectively captured in cells with PLA, indicating that this technique is suitable for studying molecular interactions with high dissociation constants (e.g., >3 μM K_d). Though, in the current work, the assay is primarily used as a “yes” or “no” type binary indicator for chaperone interaction, the users can employ this technique to obtain semi-quantitative readouts of the degree of interaction by digitally counting the fluorescence signal intensities[15]. However, due to the non-linear amplification of the PLA signal, caution should be exercised when interpreting PLA data in a quantitative manner[16]. Despite the aforementioned advantages, this method has certain limitations. One of the main disadvantages of PLA is that the assay requires cell fixation thus largely limiting its ability to resolve temporal dynamics of protein complexation in live cells. In comparison, in vivo FRET[17] or Bioluminescence Resonance Energy Transfer (BRET)[18] allows the monitoring of similar protein interactions in a spatio-temporal manner in living cells with relatively smaller proximity values (<10 nm). In contrast to PLA, a FRET signal exhibits a strict linear correlation with protein expression levels[16] making FRET the gold standard in quantitative analysis of protein interactions. However, unlike FRET’s dependence on the enigmatic orientation factor κ^2, PLA is not influenced by the orientation of the PLA probes, which increases the probability of capturing a protein complex[19]. In terms of user-friendliness, FRET and BRET require unique expertise thus generally limiting the accessibility of these methodologies to the broader research community. Further, both of these techniques require modification of the interacting proteins by attaching bulky luminescent/fluorescent protein tags that could potentially interfere with protein function and complex formation.

The following steps (1-4) require careful consideration for the successful implementation of PLA in cells. (1) Antibody selection: The commercially available PLA kit is compatible with primary antibodies raised against mouse, rabbit and goat only. PLA requires highly specific primary antibodies that do not bind to off targets. Additionally, it is important to select primary antibodies that are compatible with applications such as immunohistochemistry (IHC), enzyme-linked immunosorbent assay (ELISA), and/or immunoprecipitation (IP) to ensure that the antibodies could recognize antigenic amino acid sequences exposed on the surface of folded proteins. Prior to use, testing of all primary antibodies for their specificity is highly recommended. This can be performed via western blot analysis of whole cell lysates. In cases where the targeted proteins have homologs with small variations in size and sequence similarity (e.g., Hsp70 and JDP paralogs), the specificity of the antibodies could be tested with gene knockdown/knockout approaches or by probing against purified homologs to rule out any potential cross recognition. If suitable antibodies are not available, the targeted proteins could be tagged with epitopes that have antibodies of acceptable quality (**Figure 2F**). (2) Fixation and permeabilization of cells: A treatment of 4% paraformaldehyde and 0.1-0.5% Triton-X100 could be used to fix and permeabilize cells, respectively. The use of 4% paraformaldehyde is a better treatment for preserving protein-protein interactions and cellular ultrastructure compared to fixative conditions employing 99% methanol[20,21,22]. Fixing cells with 99% methanol, however, yields less cytoplasmic background staining, and perhaps is more suitable for specific cases such as detection of cytoskeleton associated protein assemblies[21,22]. Efficient permeabilization of both the plasma membrane as well as organelle membranes to increase antibody accessibility could be achieved with non-ionic surfactant Triton-X100. Triton-X100 can, however, non-specifically remove proteins from the plasma membrane[23,24]. Therefore, for the analysis of protein assemblies associated with cellular membranes, alternative detergents such as saponin or digitonin that targets sterols to permeabilize membranes could be applied[21,22,25]. (3) Disruption of the cell wall: Prior to membrane permeabilization, an additional step involving specific lytic enzymes is needed to increase the penetrability of antibodies in cell types with cell walls (e.g. fungi, plant and bacterial cells). For example, we employed lyticase, which degrades β-glucan to disrupt the cell wall of *S. cerevisiae*[26]. Similarly, the *E. coli* cell wall was disrupted using lysozyme, an enzyme that targets peptidoglycans[27,28]. The cell wall composition and lytic enzyme sensitivity varies with different growth conditions[26] and culture confluence[29,30]. Therefore, the concentration of the lytic enzymes and digestion times may vary and care has to be taken to prevent over- or underdigestion of cells. After the cell wall digestion, the cells are relatively fragile and require crowding agents such as sorbitol for them to remain intact during the washing steps. (4) DNA amplification: The DNA ligation and the rolling circle PCR reaction steps are sensitive to temperature and humidity fluctuations. To ensure robust DNA amplification and reproducibility of the assay, these reactions are required to be performed at 37 °C in a humidity chamber. Importantly, the proccessed cells should be prevented from drying out while performing the assay to avoid any non-specific antibody binding and DNA amplification events that could lead to an increased in background signal.

All things considered, the implementation of this assay does not require unique expertise and sophisticated instrumentation. The successful monitoring of JDP-JDP and JDP-Hsp70 chaperone complexes illustrate the potential application of this technique to trace transiently formed protein assemblies in all cell types. Our implementation of the technique in yeast and bacteria significantly increases the applicability of PLA to study various biological processes mediated by distinct protein assemblies in a broad range of organisms. Further, our work highlights PLA as a promising new protein interaction tool to study evolutionary changes occurring at a molecular level across species[8].

Disclosures

The authors have nothing to disclose.

Acknowledgments

NBN is supported by a special Recruitment Grant from the Monash University Faculty of Medicine Nursing and Health Sciences with funding from the State Government of Victoria and the Australian Government. We thank Bernd Bukau (ZMBH, Heidelberg University, Germany) and Harm H. Kampinga (Department of Biomedical Sciences of Cells & Systems, University of Groningen, The Netherlands) for their invaluable

support and sharing of reagents, Holger Lorenz (ZMBH Imaging Facility, Heidelberg University, Germany) for his support with confocal microscopy and image processing, and Claire Hirst (ARMI, Monash University, Australia) for critical reading of the manuscript.

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