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Javoor Akash
Department of Veterinary Pathology, Veterinary College Shivamogga, Karnataka, India

GM Jayaramu
Department of Veterinary Pathology, Veterinary College Shivamogga, Karnataka, India

B Kavitha Rani
Professor and Head, Department of Veterinary Pathology, Veterinary College Shivamogga, Karnataka, India

SS Manjunatha
Department of Veterinary Pathology, Veterinary College Shivamogga, Karnataka, India

P Ravikumar
Department of Veterinary Pathology, Veterinary College Shivamogga, Karnataka, India

SJ Arun
Department of Veterinary Pathology, Veterinary College Shivamogga, Karnataka, India

P Anupama
Department of Veterinary Pathology, Veterinary College Shivamogga, Karnataka, India

KN Brunda
Department of Veterinary Pathology, Veterinary College Shivamogga, Karnataka, India

CV Santhoshkumar
Department of Veterinary Pathology, Veterinary College Shivamogga, Karnataka, India

Sunkad Meghana
Department of Veterinary Pathology, Veterinary College Shivamogga, Karnataka, India

CS Niranjana Murthy
Department of Veterinary Pathology, Veterinary College Shivamogga, Karnataka, India

Corresponding Author:
Javoor Akash
Department of Veterinary Pathology, Veterinary College Shivamogga, Karnataka, India

Proximity ligation assay and its applications

Javoor Akash, GM Jayaramu, B Kavitha Rani, SS Manjunatha, P Ravikumar, SJ Arun, P Anupama, KN Brunda, CV Santhoshkumar, Sunkad Meghana and CS Niranjana Murthy

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Abstract
Proximity Ligation Assay (PLA) is a powerful tool for studying protein-protein interactions and *in situ* analysis of protein localization in specific cells or tissues. To identify and measure target protein interactions in cells or tissues, PLA combines the specificity of immunofluorescence with the sensitivity of polymerase chain reaction (PCR). The assay is based on the principle that, when two antibodies specific for different epitopes of a target protein are brought into close proximity, they can form a stable protein complex that can be detected by ligation of two oligonucleotides conjugated to the antibodies. The ligation product can then be amplified by PCR and visualized by fluorescence or confocal microscopy. PLA has several advantages over other methods for detecting protein interactions, such as immunofluorescence, co-immunoprecipitation, FRET/BRET, immunoelectron microscopy. Unlike these methods, PLA can detect low-abundance or transient interactions and any *In vitro* protein purification. In addition, PLA can be combined with other techniques like electron microscopy to visualize subcellular localization of proteins in the cell organelles. PLA is a versatile and sensitive tool for studying protein localization in medical diagnostics.

Keywords: Protein, interaction, immunofluorescence, ligation assay, antibodies

Introduction
For an early diagnosis, finding proteins in plasma at low amounts might be critical. Due to reliance on target recognition by pairs of antibodies, current methods used in clinical practice, such as sandwich ELISA, provide for protein detection; nonetheless, detection of low protein concentrations is frequently required. With the aid of target protein binding by antibodies and signal amplification by rolling circle amplification (RCA) in microtiter wells, the proximity ligation assay (PLA) is a novel technique for analytically specific and sensitive protein detection that can be easily adapted to instrumentation used in hospitals (Ebai *et al*., 2007) [1]
Assays for the identification of proteins using antibodies are useful tools for both research and clinical diagnosis. However, the molecular complexity and broad protein concentration ranges of biofluids like plasma make detection more challenging (Clamp *et al*., 2007) [16]. As the concentration of a protein of interest decreases, the difficulty of precise detection increases quickly. Strict requirements are placed on both the analytical specificity and sensitivity when it comes to the early diagnosis of illness using leakage indicators that are particularly coming from afflicted tissues. As a result, high analytical sensitivity, specificity, high accuracy, and extensive dynamic ranges in bioassays are required. Although specialist instruments for measuring very sensitive proteins are now becoming accessible, a method that enhances performance using already-existing instruments may have extensive applications. Specifically targeted proteins can be bound by a single detection reagent in analytically sensitive protein detection experiments to produce observable signals. Although having the potential to identify single molecules, the aforementioned methods need a lot of molecules to be present in a sample in order to effectively distinguish them from background noise. In alternative testing methods, DNA molecules are used to reveal the presence of identified proteins. Immuno-rolling circle amplification (iRCA) and immuno-PCR are instances of such assays. In these techniques, DNA molecules linked to affinity reagents are employed to amplify and detect labeled antibodies with high sensitivity. The analytical sensitivity of these DNA-assisted assays has all been demonstrated in the femtomolar concentration range. The aforementioned immunoassays eliminate background contributions often present in traditional immunoassays, such as nonspecific fluorescence or medium absorbance, because measurable signals may only be evoked by certain detection reagents.

Detecting protein-protein interactions is important for several reasons

1. **Understanding biological processes:** Proteins often work together in complex networks to carry out various biological processes. By identifying the interactions between proteins, researchers can better understand how these processes work and how they can be manipulated.
2. **Identifying drug targets:** Many diseases result from the malfunction of one or more proteins. By identifying the proteins that interact with each other, researchers can identify potential targets for drug development.
3. **Understanding disease mechanisms:** Some diseases result from the disruption of protein- protein interactions or cause the disease via protein-protein interactions. By studying these interactions, researchers can gain insights into the mechanisms underlying these diseases and identify potential therapeutic approaches.
4. **Developing diagnostics:** Some protein-protein interactions can be used as biomarkers for disease. By identifying these interactions, researchers can develop diagnostic tests that can be used to detect diseases at an early stage.

Overall, detecting protein-protein interactions is crucial for advancing our understanding of biological processes, identifying potential drug targets, developing diagnostics, and ultimately improving human health.

What is Proximity Ligation Assay?

A proximity ligation assay (PLA) is a form of analysis utilized to identify and measure the interaction or co-location of particular protein pairs within cells and tissues. This technique relies on antibodies and serves to identify biomolecules and their close physical proximity, extending to any molecules that can be targeted by antibodies. This assay has been modified to detect also cytokines (Gullberg *et al*., 2004) [3] as well as to detect and visualize proteins, protein complexes, and protein modification *in situ* via the in-situ PLA (Soderberg *et al*., 2006) [17]

What is Colocalization?

Colocalization refers to the phenomenon where two or more molecules or structures of interest are found in close proximity to each other within a cell or tissue. It implies that these molecules or structures share the same location or occupy overlapping regions within the sample being studied. (Dunn *et al*., 2016) [18] This colocalization can be physical or functional.

Consider two proteins, Protein A and Protein B (Figure 1). Here both proteins A and B are together, so we can say that they are colocalizing. However, we can't say that they are interacting with each other without signaling or trafficking as such (Dunn *et al*., 2016) [18].

One such example of functional interaction between two proteins can be noticed in Gamma- aminobutyric acid B (GABAB) receptor proteins. GABA stands as the primary inhibitory neurotransmitter within the central nervous system of mammals. Due to its great degree of flexibility, GABA can exist in a variety of low-energy conformations. Three main GABA receptors dubbed GABAA, GABAB, and GABAC receptors have been identified using conformationally constrained analogues of GABA (Chebib *et al*., 1999) [19]. GABAB receptors, categorized as G Protein Coupled Receptors (GPCRs), establish connections with G- proteins, thereby triggering secondary messenger pathways as well as channels for Ca2+ and K+ ions. GPCRs are proteins that have 7 transmembrane domains with N terminal extracellularly and C terminal intracellularly. At N terminal the ligands bind ant via C terminal the signal transduction occurs through cytoplasm. GABAB receptor needs two subunits B1 and B2 to be functional (Robbins *et al*., 2001) [9].

Both B1 and B2 subunits of GABAB receptor are produced in ribosomes of Rough Endoplasmic Reticulum (RER). Then they are packaged in golgi complex and then these two subunits are transported to the plasma membrane of the cell. At N terminal of subunit B1 present on the cell's plasma membrane, the GABA binds and through C terminal present intracellularly, the signal transduction occurs into the cell (Figure 2).

What happens when subunit B2 is deleted?

GABA B1 is blocked at RER and no receptor expressed on the plasma membrane. As there is no N terminal of the B1 subunit for GABA to bind, there is no signal transduction (Figure 3)

What happens when subunit B1 is deleted?

GABA B2 is expressed on plasma membrane but as there is no N terminal of subunit B1 for GABA to bind, so there is no signal transduction (Figure 3)

Therefore, both subunits B1 and B2 are necessary to be expressed on the plasma membrane and colocalize with each other for functioning of GABAB neurotransmitter.

Criterias of protein-protein interaction

There are three criterias of protein-protein interaction:

1. Colocalization and interaction
2. Distinct heteromer specific properties (Properties of interacting proteins)
3. Heteromer selective reagents that alter heteromer specific properties (Loss of protein-protein interactions)

Criteria i: It can be detected by various methods like

- *In situ* hybridization
- Co-Immunoperoxidation (Co-IP)
- Fluorescent Techniques
- Immunohistochemistry
- Immunoelectron microscopy
- Proximity based techniques

The proximity-based techniques include

- Proximity Ligation Assay (PLA)
- Bioluminescence Resonance Energy Transfer (BRET)
- Fluorescence Resonance Energy Transfer (FRET)

Criteria ii: It can be detected by various methods like

- Signalling
- Ligand binding
- Trafficking

Criteria iii: It can be detected by various methods like

- Heteromer selective antibodies
- Transgenic animals
- Small molecule ligands
- Membrane permeable peptides

Criteria i. includes PLA which involves detection of protein-protein colocalization, which is discussed here.

Principle of PLA

To recognize and capture the same target protein, paired proximity probes made of conjugated antibody or aptamer and nucleic acid are utilized. The adjacent nucleic acid sequence then creates a continuous and full sequence, which may be assessed by quantitative real-time Polymerase Chain Reaction (qPCR), under the complementary base pairing of a bridge oligonucleotide and ligation of enzyme (Figure 4).

The basic steps in performing PLA (Figure 5)

Before going for first step of PLA, we need to determine which the two proteins that are colocalizing are or two epitopes on same proteins which are in close proximity (17-40 nm) that we are targeting.

Step 1: Incubation with primary antibodies

Consider two proteins P1 and P2 in the sample of interest interacting with each other. Apply the sample to a solution containing two primary antibodies of the IgG class, which have been produced using distinct species (such as mouse, rabbit, or goat) and are designed to target proteins P1 and P2. One of these primary antibodies, like the mouse anti protein 1, is from one species, while the other, like the rabbit anti protein 1, is from a different species

Step 2: Incubation with secondary antibodies

Secondary antibodies against two primary antibodies are incubated next (anti mouse and anti- rabbit). These two secondary antibodies are tagged with two sequence specific oligonucleotides. The secondary antibodies labeled with oligonucleotides are referred to as PLA probes. These oligonucleotides must possess complementary sequences. The specific assignment of the PLUS or MINUS oligonucleotide sequence to each secondary antibody is inconsequential; what matters is that one secondary antibody is attached to the PLUS sequence and the other to the MINUS sequence.

Step 3: Ligation

When the two proteins are near each other, introducing two oligonucleotides that can pair with the PLA probes will result in the creation of a closed circular template once a ligase enzyme is added.

Step 4: Amplification

The closed circle structure undergoes amplification through a process called rolling-circle amplification, which employs a polymerase enzyme and fluorescently labeled oligonucleotides that match the sequence. This amplification generates a visible signal in the form of a distinct fluorescent spot, detectable and measurable through fluorescence microscopy.

Variations of PLA

Indirect PLA

It involves use of two primary antibodies that recognize the target molecule and a secondary antibodies tagged with oligonucleotide (PLA probes) that binds to the first two antibodies to form a "sandwich" complex around the target molecule.

Direct PLA

It involves use of two primary antibodies that are tagged with oligonucleotides that binds to target molecules. Producing primary antibodies that are tagged with oligonucleotides is difficult and expensive, therefore indirect PLA is more often used rather than direct PLA.

Single recognition PLA (SRPLA)

Instead of using two primary antibodies that recognize different epitopes of the target molecule, SRPLA uses a single primary antibody that recognizes the target molecule. The single recognition primary antibody is labelled to DNA oligonucleotides, which can be amplified and detected using polymerase chain reaction (PCR) or other DNA amplification techniques.

PLA protocol summery: Steps to perform PLA in Laboratory

Samples for PLA can be

- Cells
- Formalin Fixed Paraffin Embedded (FFPE) tissues
- Freezed and Fixed tissues

Steps to perform PLA

Sample Pre-treatment
↓
Blocking (1 hour, @ 37 °C)
↓
Primary Ab. incubation (O/N, @ 4 °C)
↓
PLA Probes incubation (1 hr, @ 37 °C)
↓
Ligation incubation (30 min to 1hr, @ 37 °C)
↓
Amplification incubation (100 min, 37 °C)
↓
Airdry the samples, place coverslips
↓
Observe under fluorescent or confocal microscope

PLA Step by step in cells: Sample Pre-treatment I

- Grow cells in 16 well-chambered slides or chambered cover glasses
- Never grow cells on coverslip, because
- They are very fragile and break very easily
- They have bigger surface than wells, therefore we require more reagents
- The cells grown are easily dried
- Next, Coat the wells using Poly-D-lysine (helps cells to be adhered to well's surface)

Once the cells grow, check for seeding and morphology under microscope. If too much cells and/or artifacts are present, then such wells are discarded. Confluency around 40 to 50% is good, and borders of cells should be clearly visible.

Sample Pre-treatment II

Seeding (200 µL medium + FBS + ATB)
↓
Wash (200 µL PBS)
↓
Fixation (200 µL PAS 4%, 15 min, RT)
↓
Wash (PBS x 2)
↓
Quenching aldehyde groups (Glycine 20mM in PBS, 15 min)
↓
Permeabilization (Triton x-100, 0.05%in glycine 20 mM, 5 min)
↓
Wash (PBS x 2)
↓
Next steps in PLA protocol

The results of PLA in cells, is depicted in Figure 6.

Seeding
Discard the cells grown in all four corner wells because, if you work with inverted confocal microscopy, the cells in these four wells will dispatch and you won't be able to see the cells. 200 µL of medium containing Fetal Bovine Serum (FBS) is taken to grow the cells and antibiotics (ATB) are added (Penicillin-Streptomycin).

Quenching
CHO cells increase unwanted background staining; therefore, they are quenched out by washing cells with Glycine 20mM in PBS for 15 minutes.

Permeabilization
The permeabilization process eliminates additional cellular membrane lipids, creating a pathway for larger molecules like antibodies to enter the cell. This procedure enables the targeting of antigens located within the cell or within organelles. In this procedure, permeabilization is carried out by washing slides in Triton x-100, 0.05% in glycine 20mM for 5 minutes.

PLA Step by step in FFPE tissue sections

Take FFPE tissue sections on slide
↓
Deparaffinization and hydration
↓
Antigen retrieval and permeabilization
↓
Wash and dry the slides
↓
Encircle the sample with hydrophobic pen

Deparaffinization and hydration

Xylene, 20 min
↓
Ethanol 100%, 20 min
↓
Ethanol 95%, 5 min
↓
Ethanol 75%, 5 min
↓
Ethanol 50%, 5 min
↓
Distilled Water, 5 mins

Antigen Retrieval
Chemicals such as formalin which are used for tissue fixing mask the antigenicity of the tissue antigens. So, it is necessary to retrieve that antigenicity so that the antibodies go and bind to those antigens in the tissues. There are two methods of antigen retrieval:

1. Heat-mediated/Heat Induced Epitope Retrieval (HIER)
2. Enzyme-mediated/ Enzyme Induced Epitope Retrieval (PIER)

In PLA using tissues, HIER method is followed where the slide with tissue section is treated with mixture of 10mM Sodium citrate and 0.5% tween 20 at 95-100° C for 10 minutes.

Tween 20: Tween 20 is often used as a detergent or surfactant. It is a type of nonionic detergent used in solutions like wash buffers to reduce nonspecific binding of antibodies and other reagents to the tissue sections or cells. It aids in preventing background staining and enhances the specificity of the antibody- antigen interactions

Sodium citrate: Sodium citrate solution is heated and used to treat tissue sections before applying primary antibodies. The heat and buffer action of sodium citrate help to break cross-links between proteins and reverse some of the effects of formaldehyde fixation. This allows better antibody penetration and binding to the target antigens, resulting in improved staining intensity and specificity

Encircle the sample with hydrophobic pen
Draw a circle around the tissue using a hydrophobic pen. To create a barrier to keep the reagents on the sample, encircle the dry sample using a hydrophobic pen.

PLA Step by step in fixed and frozen tissue sections

Fixed and frozen tissue sections
↓
Thawing and sectioning by Cryotome
↓
Mounting (0.2% 0.05 M gelatin and Tris pH 7.6)
↓
Permeabilization (0.01-0.1% Triton X-100 in PBS for 12 minutes
↓
Wash and dry the slides. Encircle the sample with hydrophobic pen

Results of PLA in tissue samples is depicted in Figure 7.

Combination of PLA and Electron microscopy (EM)
EM has very high resolution therefore, the combination of PLA with EM can be used to detect protein-protein colocalization in subcellular organelles. In this method, instead of using fluorescent labelled oligonucleotides, we use peroxidase labelled oligonucleotides. Then we incubate our sample with gold tagged anti- peroxidase antibodies so that they bind to peroxidase labelled oligonucleotides (Figure 8)
Ultrastructural detection of D1–D2 receptor heteromers using combination of PLA and EM (Figure 8):
In this study, a rat antibody against the D1 receptor and a rabbit antibody against the D2 receptor were employed. The accuracy of these antibodies in recognizing dopamine receptors D1 and D2 had been assessed in a prior study conducted by Lee *et al*. in 2004 [6], and their reliability was further confirmed in mice with deleted D1 and D2 genes, as

demonstrated by Perrreault *et al*. in 2010. To visualize D1-D2 receptor heteromers at the ultrastructural level, the proximity ligation assay (PLA) technique was utilized, followed by immunogold labeling and enhancement through silver, as outlined by Sierra *et al*. in 2015 [10].

Other techniques to detect protein-protein proximity:

1. Immunofluorescence
2. Co-immunoprecipitation
3. Fluorescent Resonance Energy Transfer (FRET)/ Bioluminescence Resonance Energy Transfer (BRET)
4. Immunoelectron microscopy (Transmission Electron Microscopy (TEM))

Immunofluorescence

Immunofluorescence is a method employed in light microscopy with a fluorescence microscope, primarily applied to biological specimens. This technique capitalizes on the selective binding of antibodies to their corresponding antigens, enabling the precise attachment of fluorescent dyes to specific biomolecular targets within a cell. As a result, it becomes possible to observe and analyze the spatial distribution of the target molecule throughout the sample (Zhao *et al*., 2009) [15].

Co-immunoprecipitation (Co-IP)

Also referred to as protein complex immunoprecipitation, involves the use of an antibody that specifically binds to a known protein suspected to be part of a larger protein complex. Through this antibody, the goal is to extract the entire protein complex from a solution, potentially revealing unidentified constituents of the complex. (Lin *et al*., 2017) [7]

FRET/BRET

FRET (Fluorescence Resonance Energy Transfer) and BRET (Bioluminescence Resonance Energy Transfer) are based on nonradiative energy transfer between a donor and an acceptor molecule. In the case of FRET, two fluorophores with appropriately overlapping emission/absorption spectra (the "donor" and the "acceptor") can transfer excited-state energy from donor to acceptor if they are within ~50 Å of each other.

An issue with FRET utilizing fluorophore donors is the need for external illumination to trigger the transfer of fluorescence. This illumination can introduce background noise in outcomes due to the direct activation of the acceptor or the fading of fluorescence (photobleaching). To avoid this drawback, bioluminescence resonance energy transfer (or BRET) has been developed (Bevan and Rees, 2006) [20]. It uses luciferase enzyme (typically the luciferase from Renilla reniformis) to produce bioluminescence (Mo *et al*., 2016) [8].

Immunoelectron microscopy

In this technique, Transmission electron microscopy (TEM) is used to detect protein-protein colocalization. Here, the antibodies are tagged with 5nm gold particles and incubated along with our sample of interest. Then, sample is visualized using TEM (Figure 9)

The comparison between PLA and other techniques to detect protein-protein interaction is given in Table and drawbacks of other protein-protein interaction detection techniques compared to PLA are given in Table 2.

Applications of PLA

Visualization of Borna Disease Virus Protein Interactions with Host Proteins using *in situ* Proximity Ligation Assay

Borna Disease Virus (BDV) causes

- Proventricular Dilatation Disease in psittacine and non-psittacine birds
- Neurological diseases, persistently infecting neurons and glia cells mainly in the central nervous system (CNS) in horse and sheep
- Staggering Disease in Cats

(Kistler *et al*., 2008; Weissenböck *et al*., 2009; Wensman *et al*., 2016) [5, 13, 64]

Visualization of host-virus protein-protein interactions in persistently infected cell cultures of Borna disease virus is depicted in figure 9.

PLA to determine UL37 interactions with gK and UL20 in Herpes simplex virus 1

gK is viral glycoprotein, UL20 is membrane protein and UL37 is a viral protein present in Herpes simplex virus. It is shown that glycoprotein K (gK) and its interacting partner, the UL20 protein, play important roles in virion envelopment. Specifically, virions lacking either gK or UL20 fail to acquire an envelope (Jambunathan *et al*., 2014) [8]. Further investigation revealed that, interaction between viral protein UL37 with both gK and UL20 also plays important role in virion envelopment. Therefore, interactions between these proteins can be targeted and visualized using PLA technique (Figure 10)

Visualization of interaction between two subunits of Platelet Derived Growth Factor-BB (PDGF-BB)

Platelet-derived growth factor (PDGF) holds a pivotal role in orchestrating cell growth and division. Particularly, PDGF assumes a crucial function in shaping blood vessels, prompting their growth from preexisting vascular tissue. Additionally, it propels the proliferation of various mesenchymal cell types, encompassing fibroblasts, osteoblasts, tenocytes, vascular smooth muscle cells, and mesenchymal stem cells. Furthermore, PDGF guides the directed movement, known as chemotaxis, of these mesenchymal cells.

Structured as a dimeric glycoprotein, PDGF comes in three potential configurations: PDGF-AA, composed of two A subunits; PDGF-BB, comprising two B subunits; and PDGF-AB, formed from one A subunit and one B subunit. This intricate molecular framework equips PDGF to exert its multifaceted regulatory effects on cellular processes (Weibrecht *et al*., 2010) [12] (Figure 11).

PLA to visualize c-Myc and Max heterodimer interactions in cultured human fibroblasts

The Myc oncoprotein dimerizes with its partner, Max, to bind DNA, activate transcription, and promote cell proliferation, as well as programmed cell death. Compound 10058 F4 is c-myc inhibitor which causes cell cycle arrest and apoptosis (Figure 12).

Advantages of PLA

- High specificity and Sensitivity
- PLA allows multiple proteins to be analyzed simultaneously, providing a more comprehensive view of protein-protein interactions in a sample
- High resolution
- PLA can be performed on a variety of sample types, including cells, tissues, and even whole organisms,

making it a versatile tool for a wide range of biological studies

PLA is a robust assay that can withstand harsh sample preparation conditions, such as high temperatures, detergents, or harsh fixatives, which makes it well-suited for analyzing proteins in challenging samples.

Limitations of PLA

- The primary constraint of PLA lies in the present scenario where commercially accessible secondary antibodies, which are linked to the PLA probes, exclusively exhibit binding affinity to IgG antibodies derived from rabbits, mice, or goats.
- Consequently, the feasibility of conducting an indirect PLA experiment hinges on the accessibility of primary antibodies originating from any two of the aforementioned sources.
- An alternative approach is to execute a Direct PLA, which involves attaching the primary antibodies to the PLA probes directly; however, this method incurs higher costs compared to carrying out an Indirect PLA.
- It is not a rapid technique and takes more time to perform the assay
- PLA requires multiple steps in sample preparation, this can increase the complexity and time required to perform the assay.

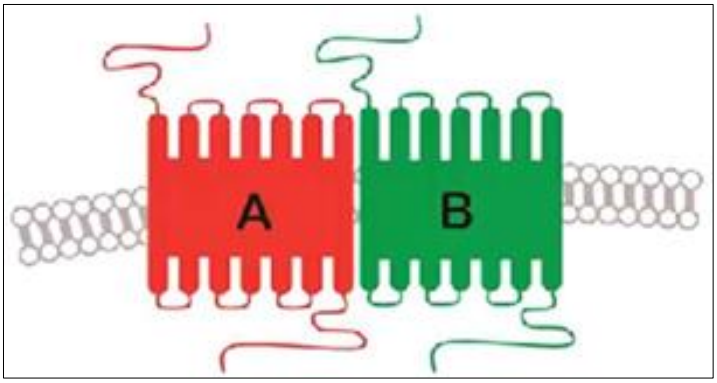

Fig 1: If Protein A and B are colocalizing functionally, there will be Signaling or Trafficking (Dunn *et al*., 2016) [18]

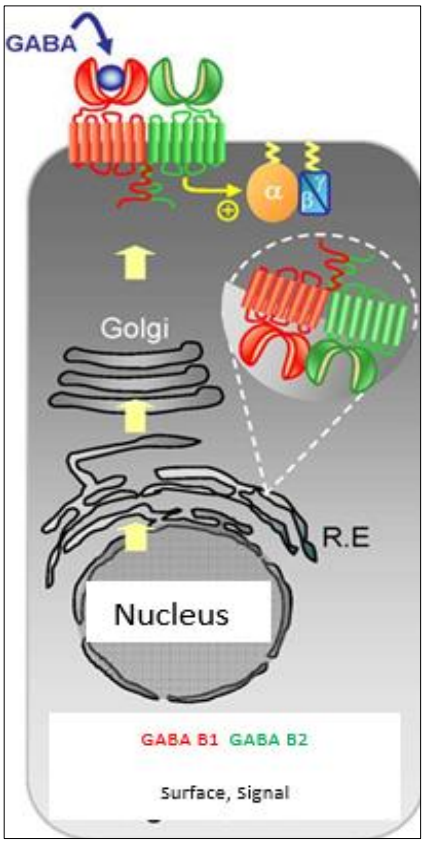

Fig 2: GABAB receptor with subunit B1 (Red) and subunit B2 (Green) colocalizing on the plasma membrane (Robbins *et al*., 2001) [9]

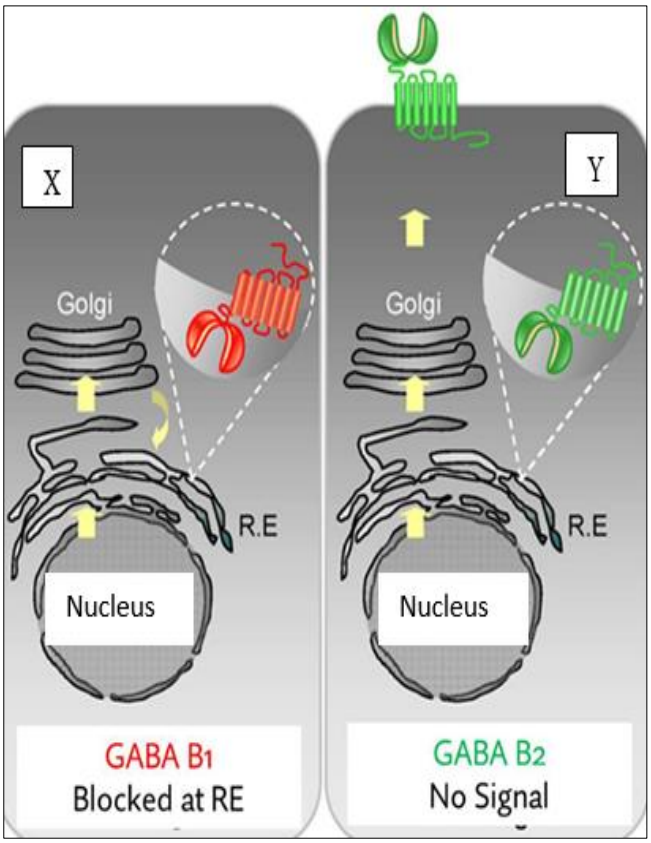

Fig 3: Depicting what happens when subunit B1 is deleted (Y) and subunit B2 is deleted (X) (Robbinson *et al*., 2001) [9]]

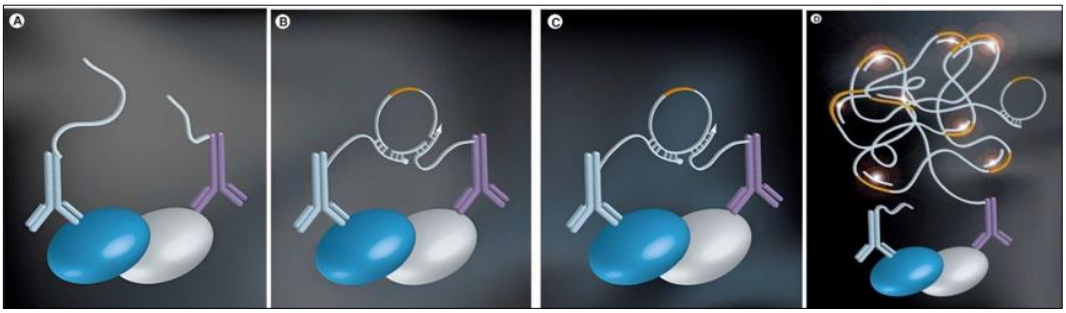

Fig 4: Schematic representation of PLA steps (Sierra *et al*., 2015) [10]

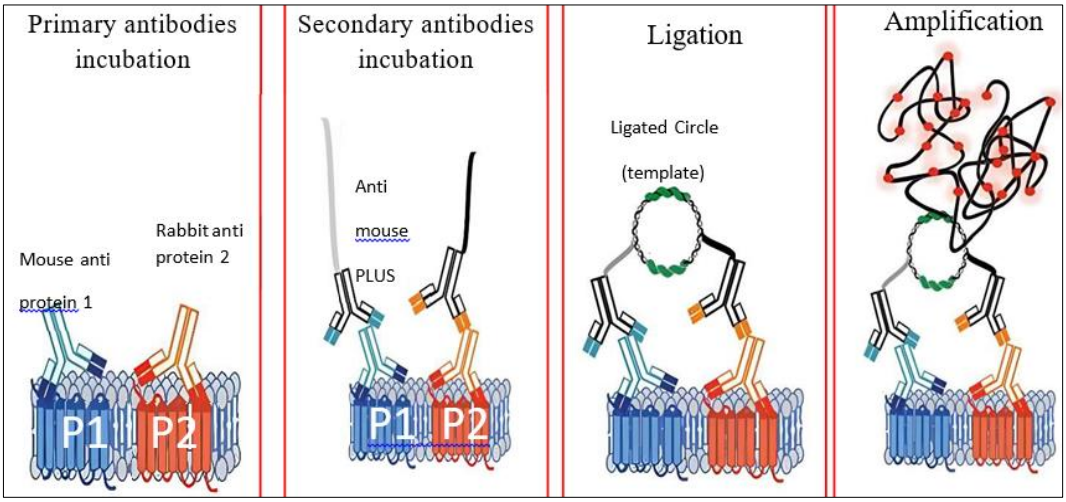

Fig 5: Steps involved in performing PLA (Gomes *et al*., 2016) [2]

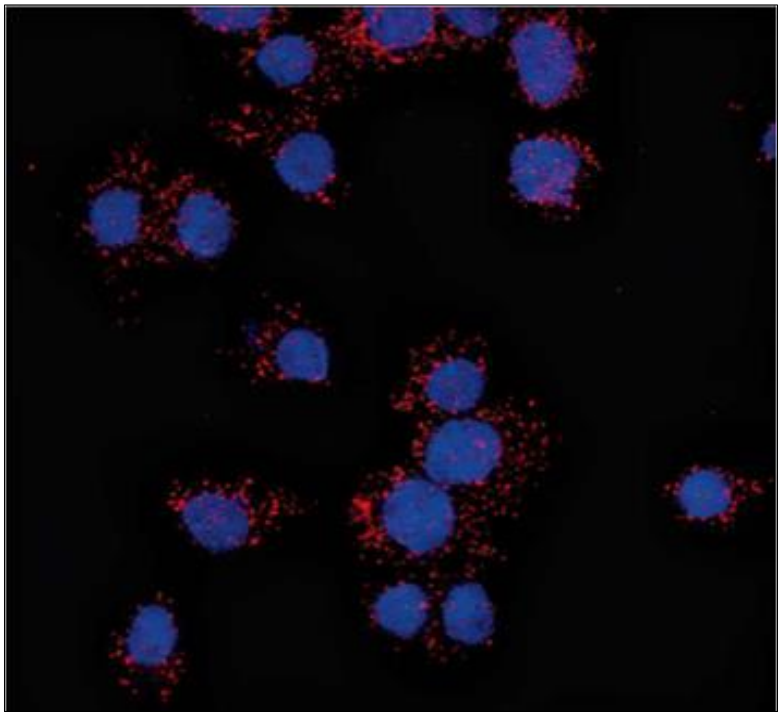

Fig 6: Indirect PLA in cells. Cells co-harboring GPCR protomer A and GPCR protomer B are cultured together and exposed to an anti-mouse antibody that specifically binds to protomer A, as well as an anti- rabbit antibody designed for protomer B. This is followed by treating the cells with PLA probes designated as PLUS and MINUS, tailored to their respective species. The connection between the oligonucleotide sequences triggers the creation of a rolling circle template. Through the action of a polymerase in the presence of fluorescently labeled oligonucleotides, this template is amplified. The resulting amplification generates distinctive red dots, discernible through confocal microscopy, whereas the cellular nuclei are counterstained with DAPI, appearing in blue (Gomes *et al*., 2016) [2]

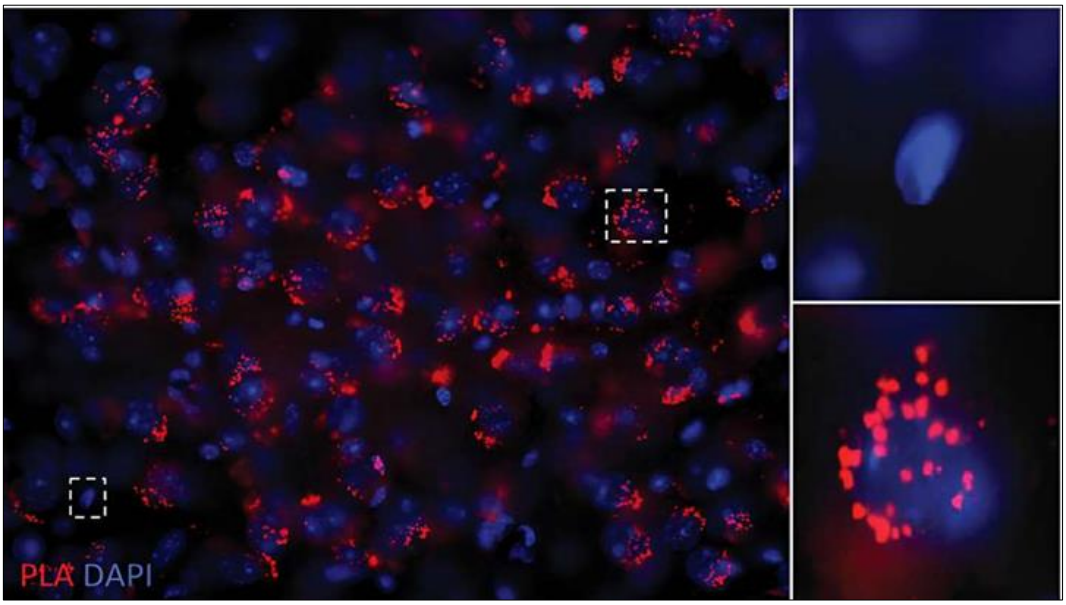

Fig 7: Indirect PLA is performed on a mouse brain slice. The slice is treated with an anti-goat antibody specific for protomer A and an anti-rabbit antibody specific for protomer B. Subsequently, species-specific PLA probes PLUS and MINUS are added. Interaction of oligonucleotide sequences initiates the creation of a rolling circle template. Polymerase amplification with fluorescent oligonucleotides generates red dots visible via confocal microscopy. The left panel shows positive PLA signal (red dots) with blue-stained nuclei (DAPI). The upper right panel offers higher magnification of a PLA-negative cell, while the lower right panel presents higher magnification of a PLA-positive cell. (Gomes *et al*., 2016) [2]

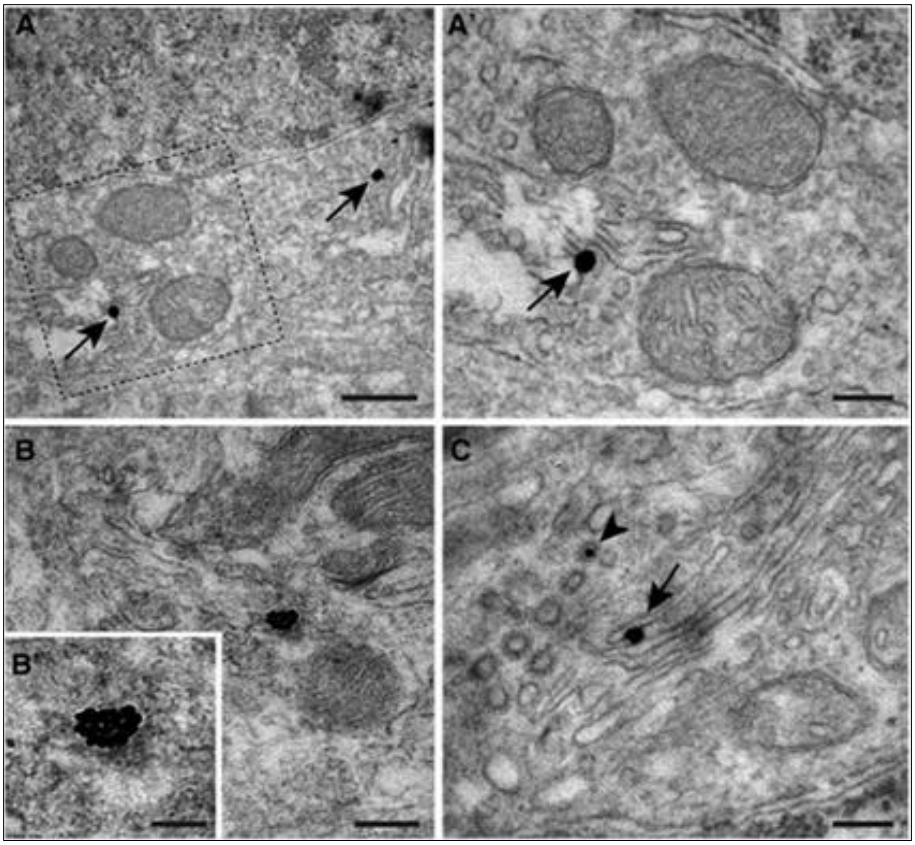

Fig 8: The ultrastructural identification of D1–D2 receptor heteromers was achieved through electron microscopy using the PLA technique, which confirmed their presence in striatal neurons. In panel A and A', two distinct types of D1–D2 receptor heteromers are depicted, each representing individual heteromers. In panel B and B', macromolecular structures are formed by the accumulation of a considerable number of receptor heteromers. In panel C, D1–D2 receptor heteromers are observed within the cisternae of the Golgi apparatus, indicated by an arrow. The scale bars are provided as references for size: 500 nm in panel A, 200 nm in panel A', 500 nm in panel B, 100 nm in panel B', and 200 nm in panel C. (Sierra *et al*., 2015) [10]

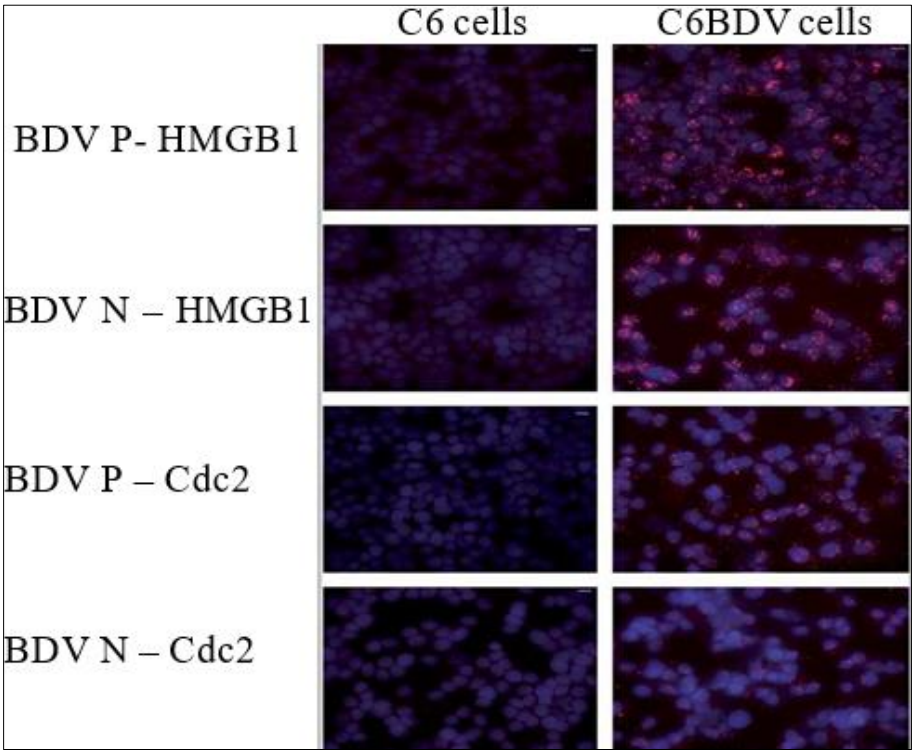

Fig 9: Detection of interactions between host and virus proteins within persistently infected cell cultures was accomplished by visual methods. *In situ* proximity ligation assay (PLA) was conducted to investigate the designated protein-protein interactions in BDV-infected cell cultures (C6BV), with uninfected C6 cells serving as a negative reference. Notably robust positive signals, represented by red dots, were evident in the C6BV cells, contrasting with the C6 cells. Moreover, the quantity of signals per cell can be evaluated using readily available imaging software tools. (Wensman *et al*., 2016) [14]

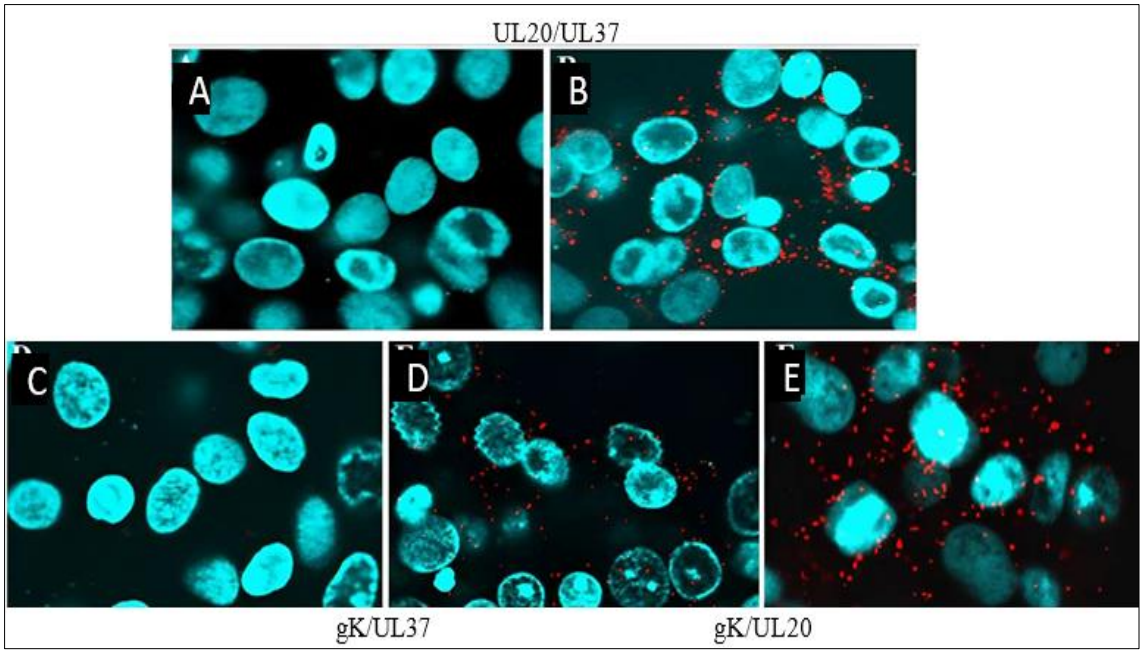

Fig 10: Confocal microscopy images of PLA targeting interactions between gK, UL20, UL37. 'A' and 'C'- healthy uninfected vero cells, therefore there is no PLA signals (Red dots). 'B', 'D' and 'E'- cells infected with herpes simplex virus 1, therefore there is a red PLA signal indicating interaction between proteins (Jambunathan *et al*., 2014) [4]

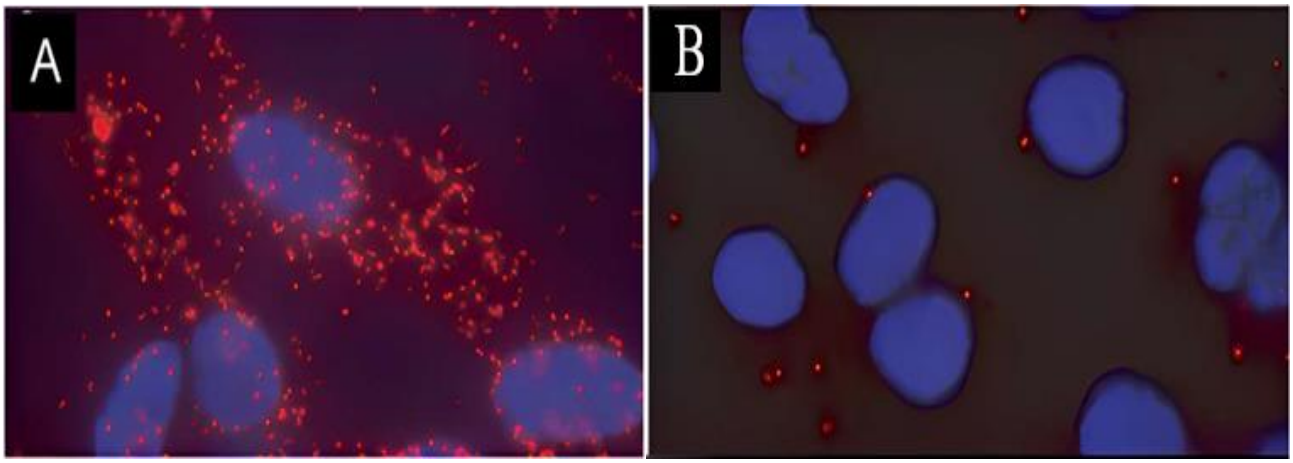

Fig 11: Image 'A' shows distinct phospho-PDGF receptor molecules identified within PDGF-BB stimulated human fibroblasts. Each luminous point corresponds to a single identified and phosphorylated receptor, while the cell nuclei are displayed in a blue hue. Image 'B' displays the recognition of phosphorylated PDGF receptor molecules within human fibroblasts that were not subjected to any stimulation (Weibrecht *et al*., 2010) [12]

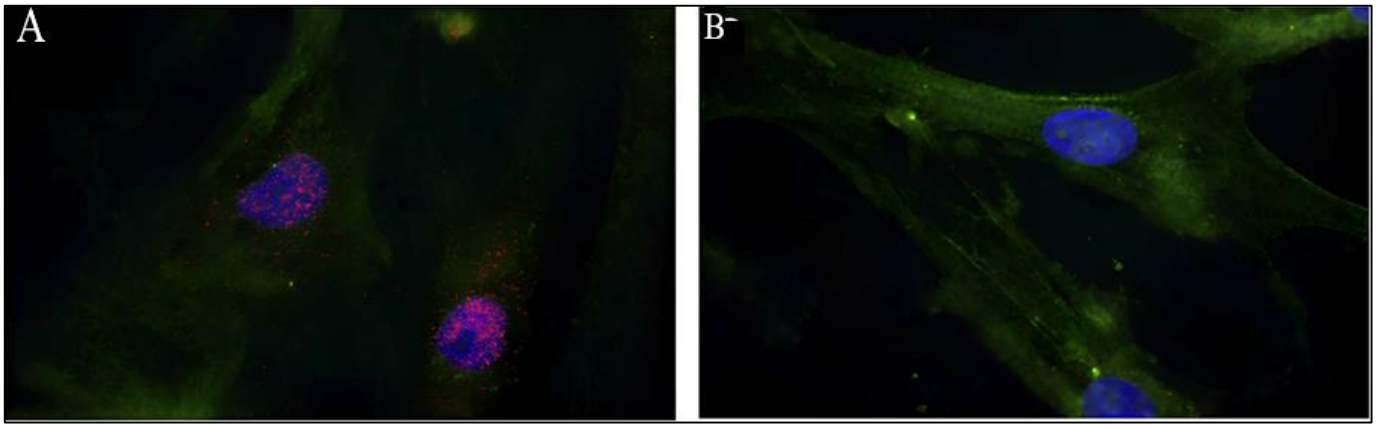

Fig 12: Image 'A': *In situ* PLA with proximity probes reveals c-Myc-Max heterodimers (Red Dots) in human fibroblasts. Image 'B': 6-hour treatment with 10058 F4 disrupts c-Myc-Max interaction, eliminating PLA signals (red dots). Cells stained for actin (green) and nucleus (blue) (Söderberg, 2008) [11]

Table 1: Comparison between PLA and other techniques to detect protein-protein interaction (Gomes *et al*., 2016) [6]

Techniques	Resolution	Signal/ Background ratio	*In vitro* demonstration	*Ex vivo* demonstration	*In situ* localization
Immuno- fluorescence	250 nm	✓	✓	✓	✓
Co-IP	250 nm	✓	✓✓	✓✓	✓✓
Immuno- electron staining	< 10 nm	✓✓✓	✓✓✓	✓✓✓	✓✓✓
FRET/BRET	10nm	✓✓✓	✓✓✓	✓✓	---
PLA	17-40nm	✓✓✓	✓✓✓	✓✓✓	✓✓✓

Table 2: Drawbacks of other protein-protein interaction detection techniques compared to PLA (Gomes *et al*., 2016) [6]

Techniques	Drawbacks
Immuno-fluorescence	Low resolution, no quantification
Co-IP	No direct colocalization
Immuno-electron staining	Technically difficult and expensive
FRET/BRET	Only *In vitro*

Conclusion

Proximity Ligation Assay (PLA) is a powerful and versatile tool for detecting protein- protein interactions in biological samples. With its high specificity, direct detection of protein-protein interactions, and multiplexing capability, it provides valuable insights into cellular processes and biological systems. Efforts are underway to further reduce non-specific binding and false-positive results in PLA, making the assay even more specific and there are works going on for automation of the PLA process which will reduce the time and complexity of the assay. Overall, PLA will advance, bringing more key information on biological systems and cellular processes.

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