Chapter 27

The "In Situ" Proximity Ligation Assay to Probe Protein–Protein Interactions in Intact Tissues

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Abstract

The proximity ligation assay (PLA) is a sensitive and specific technique to visualize proteins, their posttranslational modifications and activation state as well as protein–protein interactions.

The assay is based on the employment of proximity probes, composed by oligonucleotide-conjugated antibodies, to recognize a couple of specific targets. The binding of probes in close proximity allows for their hybridization by connector oligonucleotides, that can form a circular DNA strand. These DNA circles can then be amplified by polymerase chain reaction. Finally, the conjugation of fluorescence-labelled oligonucleotides with the amplification product allows for the localized detection of individual or interacting proteins in cells and tissues.

Here, we describe the use of "in situ" PLA to visualize the localization of protein–protein interactions in intact tissues.

Key words Protein–protein interaction, "In situ" proximity ligation assay (PLA), Intact tissue, Confocal microscopy

1 Introduction

The "in situ" proximity ligation assay (PLA) was developed to provide visualization of proteins and of their posttranslational modifications and activation state. However, PLA also allows for the localized detection of endogenous protein–protein interactions in fixed cells and intact tissues [1–4]. In the latter case, the assay is based on the detection of interacting proteins by a couple of "proximity probes" encompassed by a target-specific antibody, which can either be covalently or non-covalently conjugated with an oligonucleotide sequence. When the probes bind to a couple of ligands located in close proximity (<16 nm) the conjugated-oligonucleotides can be hybridized with two connector oligonucleotides which are then ligated to form a circular DNA molecule [2]. This latter, can be then amplified by polymerase chain reaction (PCR) and hybridized

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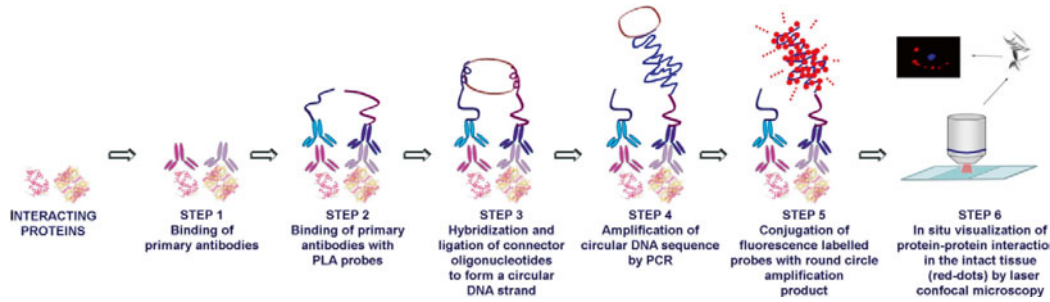

Fig. 1 Schematic representation of detection of protein–protein interactions in fixed tissue or cell cultures by indirect "in situ" PLA. *STEP 1*: the interacting target proteins are recognized by two specific primary antibodies. *STEP 2*: a couple of oligonucleotide-conjugated secondary antibodies (PLA-probes) recognizing the primary antibodies used to bind targeted proteins is added to the sample. *STEP 3*: when the targeted proteins are located in close proximity, the PLA probes can be hybridized to connector oligonucleotides to form a round circle which is then stably ligated to the target template by DNA ligase to form a circular oligonucleotide. *STEP 4*: the circular oligonucleotide is then amplified by PCR during the rolling circle amplification step. *STEP 5*: the resulting product is finally hybridized with fluorescence-labelled oligonucleotides. *STEP 6*: laser confocal microscopy allows for the "in situ" visualization of protein–protein interactions as *red dots* in the slide mounted tissue sections

in turn with fluorescence-labelled complementary oligonucleotides. Hence, at the end of the assay, the interaction of endogenous target proteins can be visualized "in situ" by confocal microscopy [5].

Differently from other standardized methods for detection of protein–protein interactions, such as co-immunoprecipitation (Co-IP) (*see* **Note 1**) or bioluminescence resonance energy transfer (BRET) and fluorescence resonance energy transfer (FRET), which usually fail to reveal the cellular context of molecules, "in situ" PLA allows for the visualization of tissue-, cellular-, and subcellular-specific endogenous antigen interactions. In addition, the assay offers the possibility to investigate the distribution of protein–protein complexes in both physiological and pathological conditions [6, 7] and the way is open toward novel potential applications in the proteomic field.

The "in situ" PLA method can be performed either directly (direct targeting "in situ" PLA), by using primary oligonucleotide-conjugated antibodies, or indirectly (indirect targeting "in situ" PLA) (Fig. 1) by using proximity PLA probes recognizing the primary antibodies bound to the target proteins (*see* **Note 2**).

Recently, we described that indirect targeting "in situ" PLA is an optimal method to visualize localization of endogenous interacting proteins in tissue and in particular, to detect the redistribution of specific protein complexes in the brain [7]. To perform this study we first evaluated the distribution of our target proteins of interest: alpha-synuclein and the dopamine transporter (DAT) by double fluorescence immunohistochemistry in the mouse brain tissue (Fig. 2a).

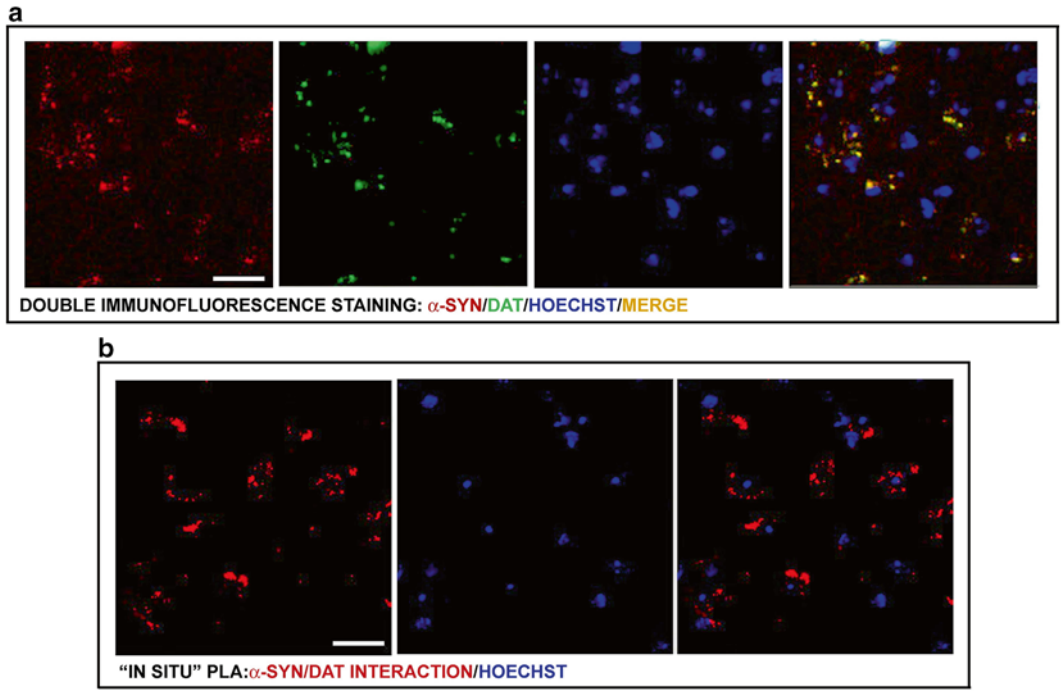

Fig. 2 Double fluorescence immunohistochemical protein labelling and "in situ" PLA-positive signal. (**a**) Visualization of alpha-synuclein (*red*) and DAT (*green*) in the striatum by using 30 μm PFA-fixed cryostat coronal brain sections of C57BL/6 J mice. (**b**) Visualization of DAT/alpha-synuclein complexes by "in situ" PLA in the striatum by using 30 μm PFA-fixed cryostat coronal brain sections of C57BL/6 J mice. *Red dots* are indicative of areas where the two proteins are interacting. Scale bars, 40 μm (panel **a**) and 25 μm (panel **b**)

Then, we optimized the "in situ" PLA protocol to visualize alpha-synuclein/DAT interaction "in situ" on mouse striatal brain sections (Fig. 2b).

Below, we describe an optimized method to perform indirect targeting "in situ" PLA to visualize the localization of interacting proteins in fixed intact tissue and we provide an exhaustive list of advantages and disadvantages linked to the employment of this technique when compared with widely used methods for detection of protein–protein interactions.

2 Materials

Tissue samples must be fixed in ice-cold 4 % paraformaldehyde (PFA) solution (*see* **Note 3**). The experiment can be performed either by using free-floating sections or by open droplet reactions on slide-mounted sections. In the latter case, all the incubations must be performed in a humidity chamber to prevent evaporation. Furthermore, the optimal volume of working solution for the reaction area must be previously determined by the operator.

Before performing "in situ" PLA to visualize protein–protein interactions it is strongly recommended to perform a series of preliminary analysis in the tissue of interest (*see* **Note 4**).

2.1 "In Situ" PLA

Prepare all the solutions in deionized water and store them at 4 °C.

1. Phosphate Buffer Saline (PBS)*:* 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 pH 7.4.
2. 4 % PFA solution pH 7.4: 4 % (w/v) PFA in PBS pH 7.4.
3. 18 % Sucrose solution: 18 % (w/v) sucrose in PBS pH 7.4.
4. Storage Buffer: PBS with 0.1 % BSA.
5. Tris Buffered Saline with Tween-20 (TBS-T): 50 mM Tris–HCl, 138 mM NaCl, 2.7 mM KCl, pH 7.4 containing 0.05 % Tween-20.
6. Saline Sodium Citrate (SSC) buffer 20× stock solution: 3 M Na-Citrate, 0.3 M NaCl, pH 7.0.
7. SSC-T buffer A: 2× SSC buffer containing 0.05 % Tween-20.
8. SSC-T buffer B: 1× SSC buffer containing 0.05 % Tween-20.
9. SSC buffer C: 0.2× SSC buffer.
10. SSC buffer D: 0.02× SSC buffer.
11. PLA probe mix: to prepare oligonucleotide-linked antibodies incubate 100 nM oligonucleotide-streptavidin complexes with 100 nM of biotinylated antibody solution for 1 h at RT. Then directly dilute the deriving PLA probes in PLA solution containing 50 ng/μl RNase A, 2.5 ng/μl poly(A), 2.5 mM cysteine, 5 mM EDTA, and 0.05 % Tween-20 in TBS.
12. Ligation mix: 10 mM Tris-acetate pH 7.5, 10 mM magnesium acetate, 50 mM potassium acetate, 0.05 U/μl T4 DNA ligase, 250 mM NaCl, 250 ng/μl BSA, and 0.05 % Tween-20 in H_2O.
13. Amplification mix: 50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 250 μM dNTPs, 250 ng/μl BSA, and 0.05 % Tween-20.
14. Detection mix: 2× SSC, 7.5 ng/μl poly(A), 250 ng/μl BSA, 0.05 % Tween-20 containing 5 nM of fluorescence-labelled probe (Alexa).

2.2 Microscopy

1. Microscope slides: 1–1.2 mm thick microscope slides and 0.13–0.16 mm thick coverslips.
2. Mounting solution: optimal PLA signal detection in tissue can be achieved by using the VECTASHIELD mounting medium (Vector Laboratories, Burlingame, CA, USA).
3. Laser confocal microscope: Laser confocal microscopes must be equipped with 40× and 60× oil objectives and 543/633 HeNe and 405 Diode excitation lasers plus image acquisition

software. Optimal image acquisitions may be achieved by using a Zeiss LSM 510 Laser Scanning Microscope in combination with LSM Image Browser software (Carl Zeiss, Advanced Imaging Microscopy, Jena, Germany).

4. Image analysis software: Image J (http://rsbweb.nih.gov/ij/) (NIH, Bethesda, MD, USA).

3 Methods

3.1 Indirect Targeting "In Situ" PLA

1. Isolate tissue samples in ice-cold PBS solution and gently wash them with PBS to remove blood. Place tissue in ice-cold freshly made 4 % PFA solution at 4 °C for 24–48 h depending on the size of tissue samples (*see* **Note 5**). Tissue slices (15–30 μm thick) can now be obtained by vibratome sectioning. When choosing to perform cryostat sectioning put tissue samples in 18 % sucrose solution at 4 °C for 24 h for cryoprotection, quickly freeze tissue at −20 °C and cut sections (15–30 μm thick). Use either free-floating or slide-mounted tissue sections (*see* **Note 6**).
2. Set up the PLA experiment in order to have the following samples in duplicate: (a) a sample of interest; (b) a negative control without one of the primary antibody recognizing the target protein of interest; (c) a negative control consisting of a knock-out of one of the targeted proteins; (d) a positive control representing a tissue or cultured cell sample with well-documented interactions between the two targeted proteins.
3. Choose primary antibodies (monoclonal or polyclonal) of IgG-class specific for the targeted proteins (*see* **Note 7**). Mix and dilute the primary antibodies in blocking solution made up by storage buffer containing antibody specific blocking sera and 0.3–0.03 % Triton X-100. Remove the blocking solution and immediately add primary antibodies to samples. Incubate either overnight at 4 °C or for at least 90 min at room temperature (RT).
4. Remove primary antibodies and wash the samples three times for 5–10 min with washing buffer under gentle orbital shaking. Add the PLA probe solution and incubate up to 2 h at either RT or 37 °C. The length of the PLA probe oligonucleotide sequence (*see* **Note 8**) and the concentration of proximity probes must be optimized by the operator in order to avoid unspecific trapping.
5. Prepare the hybridization solution by diluting 125 nM of connector oligonucleotides in the ligation mix. Remove the PLA probe solution, wash the samples at least twice for 5–10 min in TBS-T and incubate them in hybridization solution for 15 min at 37 °C.

6. Wash the samples three times for 5–10 min in TBS-T. Incubate them with the ligation mix supplemented with 0.05 U/μl of T4 DNA ligase for 30 min at 37 °C.
7. Remove the ligation mix and wash the samples three times for 5–10 min in TBS-T. During the last wash prepare the amplification solution by adding 0.125 U/μl phi29 DNA polymerase. Add this solution to samples and incubate them for 90 min at 37 °C.
8. From this stage, the tissue sections must be protected from light to prevent quenching of fluorescence-labelled oligonucleotides. Remove the amplification solution and wash the samples twice for 5–10 min with TBS-T under gentle agitation. Add detection mix containing fluorescence-labelled probes to hybridize the round circle amplification product and incubate for 30 min at 37 °C.
9. Wash the samples twice in TBS-T for 5–10 min and then once for 5–10 min in H_2O. Incubate the sections in 1 μM Hoechst 33342 diluted in H_2O for 1 min at RT.
10. Before performing these washing steps freely floating sections must be mounted on microscope slides. Final washing remove the excess of detection reagents to avoid the formation of unspecific background. The sections must be consequently washed in the following solutions:

    SSC-T A buffer for 5 min

    SSC-T B buffer for 5 min

    SSC C buffer for 5 min

    SSC D buffer for 5 min

    70 % Ethanol for 1 min

    At the end of this step let the sections dry.
11. Slide-mounted sections must be covered with a minimal volume of the appropriate mounting medium (2–3 μl) for laser confocal microscopy and covered with coverslip. Slides should be protected from light and can be stored at 4 °C for several days or at −20 °C for longer periods.
12. Examine the results of PLA by laser confocal microscopy with 40× and 60× oil objectives. Excitation of samples must be obtained either by using 543 or 633 HeNe laser for detection of PLA signal and Diode 405 for detection of Hoechst signal. Optimal height of sections' scanning is about 1 μm. Sequential Z-stuck scanning is useful to appreciate the size of protein complexes. Acquire a minimum of 10 high resolution images from each specimen and analyze them by Image J to calculate the density of PLA puncta. At the first image analysis set threshold manually to discriminate the PLA signal from background fluorescence. Then, apply this threshold setting to all

the following image analysis. Use the built in macro "Analyze particles" to count all the objects in the thresholded image. Discard objects larger than 5 μm to remove nuclei signal, and count the remaining objects as PLA puncta. Density of PLA signal can be estimated by dividing total positive area for the number of positive PLA puncta.

4 Notes

1. Co-immunoprecipitation (Co-IP), a widely used biochemical method to study protein–protein interactions, is based on the use of an antibody to pull down the protein of interest and all interacting proteins. A list of advantages and disadvantages linked to the usage of Co-IP versus "in situ" PLA to detect protein–protein interactions is provided below:

Advantages of Co-IP:

- Low-cost and easy-to-use technique. When appropriate controls are used, Co-IP represents a valid technique for demonstrating protein–protein interaction.
- Allows for detection of native protein complexes in cell lines or tissues in which they are endogenously expressed by using a specific antibody. In these conditions any artificial effects of tags or protein overexpression are avoided.
- The proteins of interest can be expressed in heterologous expression systems with a plasmid encoding a tagged bait protein that circumvents the need for a specific antibody.
- Specificity of this technique can be increased by performing subcellular fractionation step prior to performing Co-IP if the interaction takes place in a specific organelle.
- Analyses of the proteins involved in the complex evaluated by western blot or by mass spectrometry can reveal new interacting partners of a known bait protein.

Disadvantages of Co-IP:

- Low expression levels and the difficulties of obtaining highly specific antibodies recognizing the protein of interest represent the main problem.
- High risk of detecting nonspecific interactions due to the presence of highly abundant contaminant proteins. In addition, the presence of eluted antibody light and heavy chains may interfere with detection of target proteins during western blot analysis.
- Larger molecular complexes might be difficult to analyze.
- Data obtained in transfected cells should be interpreted with caution since they represent artificial systems possible lacking

of appropriate molecular chaperones, escort proteins, or co-receptors. Moreover, artificial protein–protein interactions could be detected due to non-physiological expression levels or interactions with tag.

- The native cellular environment that may affect the integrity of the protein complexes is loss.
- Co-IP of highly hydrophobic membrane proteins or extreme levels of protein expression require solubilization, that itself induces protein–protein aggregation. By contrast, interaction with proteins could occur due to incomplete solubilization.
- Difficulties in identifying less-stable interactions because of the requirement of extensive washing to eliminate nonspecifically bound proteins. However, cross-linking techniques in combination with Co-IP could be applied to stabilize labile protein–protein interactions through covalent bonds.
- It does not allow for the localized visualization of protein–protein interactions "in situ" in intact tissue.

2. When the targeted proteins are localized intracellularly, it is desirable to use cell permeabilization to allow for better penetration of primary antibodies (for instance, 20 % methanol and/or 0.3 % Triton X-100). Please avoid the use of heat-induced or RT antigen retrieval as these techniques may change epitopes' conformations, thus causing detection of false positives or precluding the visualization of protein complexes.
3. When starting from freshly frozen tissue samples, the tissue sections can be cut by a cryostat and then fixed in ice-cold 4 % PFA for 5 min.
4. Perform (a) Co-IP experiments to assay the interaction between the targeted proteins in the tissue of interest; (b) double immunofluorescence staining coupled to confocal microscopy to evaluate the distribution of the targeted proteins in the tissue of interest. This will give the operator an idea about localization and interactions of these proteins in the intact tissue; (c) choose proper negative controls to minimize the possibility of false positive results. For instance, when working with animal tissues, the animals with knockout of one of the targeted proteins would represent an optimal negative control.
5. Tissues with higher amount of blood or fat may require longer fixation time.
6. The use of free-floating sections allows for better penetration and binding of primary antibodies and proximity probes but may lead to false positive results. The use of slide-mounted sections allows for consumption of less reagents. Please note that when working with slide-mounted sections it is recommended to delimit the reaction area with a PAP pen.

7. When using "in situ" PLA to detect interactions between two trans-membrane proteins it is important that the primary antibodies chosen for the assay recognize epitopes of both proteins located on the same face of the plasma membrane.
8. Since the distance between the epitopes is a powerful determinant of PLA efficiency, the length of the PLA probe could be modified to improve the specificity of the assay either by reducing the detection distance or by increasing such a distance (e.g., longer DNA sequences to detect substrates belonging to a multi-protein complex).

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References

1. Fredriksson S, Gullberg M, Jarvius J et al (2002) Protein detection using proximity-dependent DNA ligation assays. Nat Biotechnol 20:473–477
2. Soderberg O, Leuchowius KJ, Gullberg M et al (2008) Characterizing proteins and their interactions in cells and tissues using the in situ proximity ligation assay. Methods 45: 227–232
3. Weibrecht I, Leuchowius KJ, Clausson CM et al (2010) Proximity ligation assays: a recent addition to the proteomics toolbox. Expert Rev Proteomics 7:401–409
4. Soderberg O, Leuchowius KJ, Kamali-Moghaddam M et al (2007) Proximity ligation: a specific and versatile tool for the proteomic era. Genet Eng (N Y) 28:85–93
5. Soderberg O, Gullberg M, Jarvius M et al (2006) Direct observation of individual endogenous protein complexes in situ by proximity ligation. Nat Methods 3:995–1000
6. Trifilieff P, Feng B, Urizar E et al (2011) Increasing dopamine D2 receptor expression in the adult nucleus accumbens enhances motivation. Mol Psychiatry 51:111–118
7. Bellucci A, Navarria L, Falarti E et al (2011) Redistribution of DAT/alpha-synuclein complexes visualized by "in situ" proximity ligation assay in transgenic mice modelling early Parkinson's disease. PLoS One 6:e27959