Visualizing G Protein-Coupled Receptor-Receptor Interactions in Brain Using Proximity Ligation In Situ Assay

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G protein-coupled receptors (GPCRs) constitute the largest family of plasma membrane receptors and the main drug targets in therapeutics. GPCRs can establish direct receptor-receptor interactions (oligomerization), which can also be considered as targets for drug development (GPCR oligomer-based drugs). However, prior to designing any novel GPCR oligomer-based drug development program, demonstrating the existence of a named GPCR oligomer in native tissues is needed as part of its target engagement definition. Here, we discuss the proximity ligation in situ assay (P-LISA), an experimental approach that reveals GPCR oligomerization in native tissues. We provide a detailed step-by-step protocol to perform P-LISA experiments and visualize GPCR oligomers in brain slices. We also provide instructions for slide observation, data acquisition, and quantification. Finally, we discuss the critical aspects determining the success of the technique, namely the fixation process and the validation of the primary antibodies used. Overall, this protocol may be used to straightforwardly visualize GPCR oligomers in the brain. © 2023 The Authors. Current Protocols published by Wiley Periodicals LLC.

Basic Protocol: Visualization of GPCR oligomers by proximity ligation in situ assay (P-LISA)
Support Protocol: Slide observation, image acquisition, and quantification

Keywords: brain • GPCR • oligomerization • proximity ligation assay

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INTRODUCTION

G protein-coupled receptors (GPCRs) constitute the largest family of plasma membrane receptors in eukaryotes (∼800 GPCRs in humans). GPCRs are grouped into six different classes, from which only class A, B, C, and F are found in humans. The expression

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pattern of each class of GPCRs can vary widely across different tissues, depending on their specific physiological functions. Generally, class A, the most abundant with 719 members (Congreve et al., 2020), is the most widely expressed of the four classes, while class B and C GPCRs tend to be more tissue specific. GPCRs have a distinctive structure composed of 7 transmembrane helices joined by three intracellular and three extracellular loops, plus an intracellular C-terminus and an extracellular N-terminus. GPCRs bind to extracellular ligands (e.g., neurotransmitters, hormones) and transduce the signal into the cell. The nature of GPCR transduction depends on the specific effector protein that the receptor couples with (e.g., protein G, β-arrestin) (Hilger et al., 2018), thus presenting an extensive variety of signaling processes. The diverse signaling pathways activated by GPCRs allow them to regulate a wide range of cellular functions, including sensory perception, hormone secretion, neurotransmitter release, and immune response. Dysregulation of GPCR signaling has been associated with many diseases, including cancer, cardiovascular disease, metabolic disorders, and neurological disorders. Therefore, GPCRs are important drug targets in pharmacotherapy; more than a third of FDA-approved drugs act on GPCR (Hauser et al., 2017).

GPCRs can establish direct protein-protein interactions, not only with their effector proteins but also with other GPCRs, allowing fine-tuning of GPCR functioning (Ciruela et al., 2010; Møller et al., 2018; Rukavina Mikusic et al., 2020). Upon GPCR oligomerization, ligand-receptor interactions can be altered, resulting in novel pharmacological properties (Ferré et al., 2022). In fact, changes in ligand binding (i.e., orthosteric and allosteric properties) and/or efficacy (i.e., intrinsic activity diversity) have been associated with GPCR oligomerization (Fernández-Dueñas et al., 2012; Ferré et al., 2022; Sarasola et al., 2022). Consequently, the study of GPCR oligomerization may offer new opportunities toward the discovery of new pharmacological tools.

The existence of protein-protein interactions (PPIs) can be assessed by several methodologies. Some are based on a protein complementation assay (PCA) that relies on the reconstitution of a functional protein (e.g., YFP, *R*Luc, transcription factor) from two physically separated inactive halves fused to each partner protein (Gandía et al., 2008). Partner oligomerization enables reporter protein complementation. A good example is the yeast two hybrid system (Y2H), where the PPI drives the reconstitution of a functional transcription factor, thus promoting the expression of a reporter gene. Other PCAs rely on the complementation of fluorescent (Bimolecular Fluorescence Complementation, BiFC) or bioluminescence (Bimolecular Luminescence Complementation, BiLC) (Ciruela et al., 2010). Alternatively, fluorescent resonance energy transfer (FRET) approaches have been commonly used to assess PPIs. These approaches also use genetically modified GPCRs (i.e., tagged with fluorescent proteins) overexpressed in heterologous expressing systems. In general, these technologies do not definitely prove the existence of direct PPIs under native conditions; therefore, several techniques based on antibody-based fluorescent probes have emerged to overcome this limitation. The proximity ligation in situ assay (P-LISA) uses primary antibodies to detect receptors, which are then recognized by secondary antibodies conjugated to a single-stranded oligonucleotide (referred to as the PLA probe). Then, if the two receptors are in close proximity (closer than 40 nm), the PLA probes will hybridize with two connector oligonucleotides and a ligase will generate a closed-circle DNA template that can be amplified through a rolling-circle amplification step. Finally, by using complementary fluorescent-labeled oligonucleotides, it is possible to detect the receptor-receptor interaction (Söderberg et al., 2006). Interestingly, P-LISA has been successfully used to demonstrate the existence of several GPCR oligomers in native tissue (Fernández-Dueñas et al., 2015; López-Cano et al., 2019; Romero-Fernandez et al., 2022; Taura et al., 2015; Trifilieff et al., 2011).

Below, we describe in detail the whole process of performing P-LISA in the brain, a tissue that expresses many GPCRs that regulate important aspects of brain function (Huang et al., 2017). Therefore, a step-by-step protocol is provided to perform P-LISA in brain slices (Basic Protocol). Additionally, a support protocol will guide the reader through slide observation, image acquisition, and quantification (Support Protocol).

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

VISUALIZATION OF GPCR OLIGOMERS BY PROXIMITY LIGATION IN SITU ASSAY (P-LISA)

BASIC PROTOCOL

P-LISA is a technology that overcomes the capacity of classical immunohistochemical assays to allow the direct visualization of endogenous PPIs in situ (Söderberg et al., 2006). The P-LISA, as a proximity ligation assay approach (Fredriksson et al., 2002), depends on dual proximal binding by pairs of detection reagents, for example, two primary antibodies that recognize target antigens (e.g., GPCR) under study (Fig. 1A). Primary antibodies, which are raised in different species, are readily detected by specific secondary antibodies containing a single complementary short DNA strand, also called a PLA probe (Fig. 1B). When PLA probes from two secondary antibodies are close to each other (≤40 nm), they can interact, and aided by two subsequently added linear connector oligonucleotides, they generate an amplifiable circular DNA strand (Fig. 1C) that serves as template for a local rolling-circle amplification (RCA) reaction (Fig. 1C). The RCA reaction generates a single-stranded product covalently linked to the antibody-antigen complex, which can be easily detected by complementary hybridization of fluorescent-labeled oligonucleotides (Fig. 1C). Overall, protein targets can be readily detected and localized with single-molecule resolution by means of fluorescence microscopy, thus allowing unbiased quantification of PPIs in unmodified cells and tissues. Hence, although other technologies can be implemented to unequivocally demonstrate the occurrence of GPCR oligomerization in native tissue (i.e., TR-FRET using fluorescent ligands), the

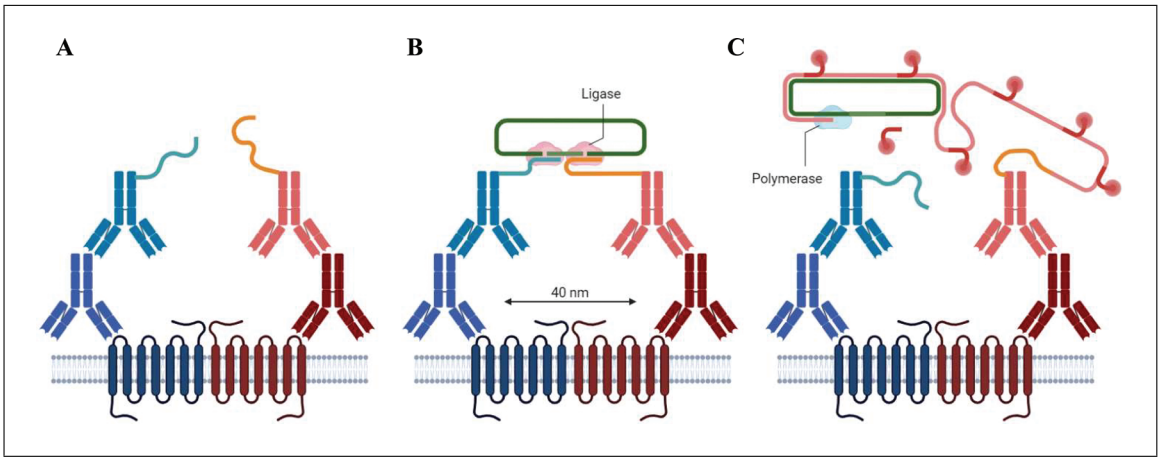

Figure 1 Schematic representation of the proximity ligation in situ assay (P-LISA). **(A)** Specific primary antibodies against GPCRs are used to detect each receptor, and secondary specie-specific antibodies conjugated with oligonucleotides (PLA probe MINUS and PLA probe PLUS, illustrated as blue and orange bands, respectively) recognize the primary antibody. **(B)** Two oligonucleotides (illustrated as green and clear-green bands) hybridize with the PLA probes. When the PLA probes are in close proximity (≤40 nm) a circular ssDNA might be formed (dark green rectangle). The open circular ssDNA could be closed by the action of T4 DNA-Ligase (illustrated as a pink oval). **(C)** Closed circular ssDNA can serve as a template for phi29 DNA polymerase (illustrated as a sky-blue oval) that extends the 3'-OH ends of one of the PLA probes, acting as a primer for rolling circle amplification (RCA). Finally, the generated concatemeric product is hybridized with fluorescent oligonucleotide probes (red spots).

P-LISA assay is a powerful and straightforward technology to uncover these types of subcellular events, either transient or weak interactions, within specific subpopulations of cells in situ.

Materials

CD1 mouse (in-house colony)
Ketamine (50 mg/ml; Ketalar, Panpharma)
Xylazine (20 mg/ml; Rompun, Bayer)
70% ethanol
4% formaldehyde solution (Sigma-Aldrich, cat. no. 1040021000)
1× PBS (see recipe)
Sodium azide (Sigma-Aldrich, cat. no. 769320-100G)
Walter's antifreezing solution (see recipe)
Triton X-100 (Sigma-Aldrich, cat. no. X100-1 L)
Blocking solution (see recipe)
Normal donkey serum (NDS; Jackson ImmunoResearch Laboratories, cat. no. 017-000-121)
Duolink In Situ PLA Probe Anti-Goat PLUS (Sigma-Aldrich, cat. no. DUO92003-30RXN)
Duolink In Situ PLA Probe Anti-Rabbit MINUS (Sigma-Aldrich, cat. no. DUO92005-30RXN)
P-LISA wash buffer A (see recipe)
5× ligation solution (see recipe)
T4 DNA ligase (1 U/μl; Promega, cat. no. M1801)
5× rolling-circle amplification (RCA) solution (see recipe)
phi29 DNA polymerase (Life Technologies, cat. no. EP0091)
P-LISA wash buffer B (see recipe)
Duolink in situ mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, cat. no. DUO82040-5ML)
Transparent nail polish

27-gauge needle
1-cc syringe
Rack (for dissection)
Tape or pins
Surgical scissors
Forceps
Perfusion valve
25-gauge, 25-mm perfusion needle
50-ml syringe reservoir
Vibratome (VT1200, Leica Lasertechnik GmbH)
Round brush, sizes 10/0 to 2/10
Incubator, 37°C
Opaque Eppendorf tubes or foil-covered tubes
Microscope slides and coverslips

Fixed tissue preparation

The recommended dose for anesthetizing the animal (i.e., mouse) with the combination of ketamine and xylazine is: 100 mg/kg body weight for ketamine and 10 mg/kg body weight for xylazine (Flecknell, 1993). This guideline provides an onset of 3-5 min with 30-40 min of anesthesia duration for surgical purposes.

Of note, in our protocol, animals are housed and tested in compliance with the guidelines described in the Guide for the Care and Use of Laboratory Animals (Clark et al.,

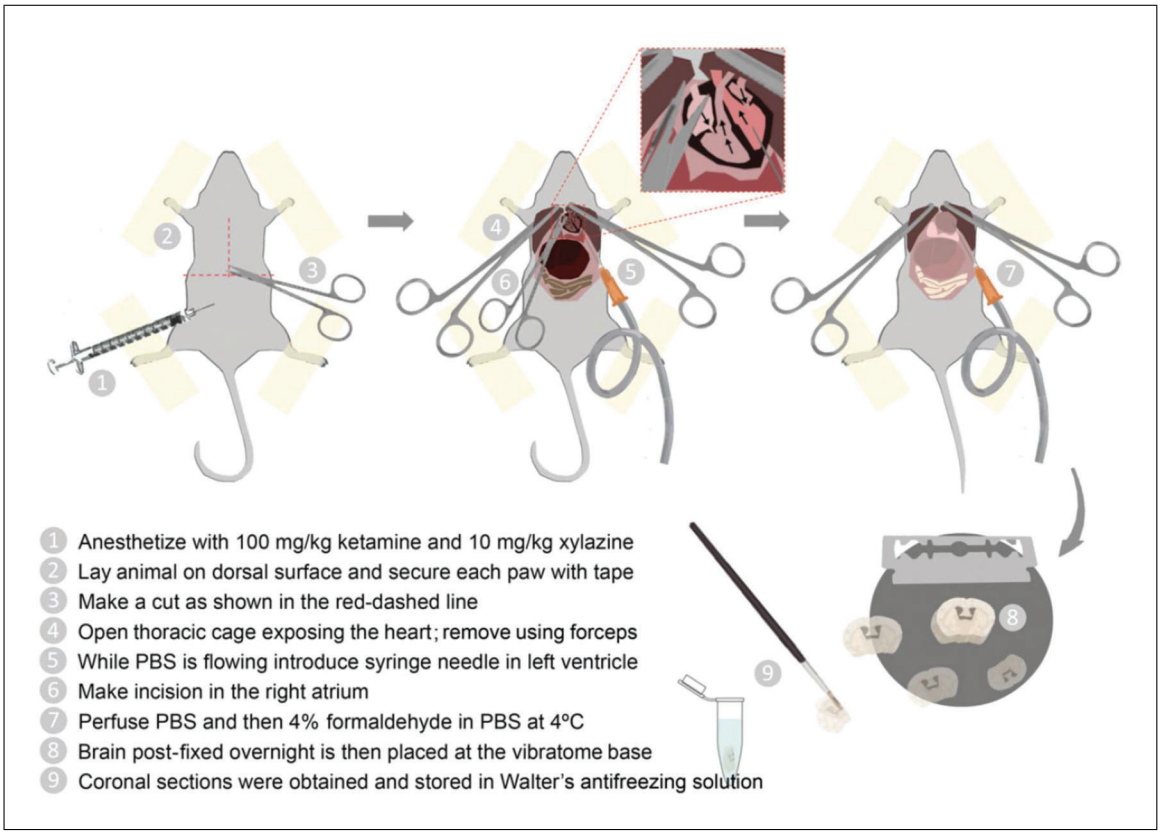

Figure 2 Schematic representation of tissue fixation and brain slice generation. The steps show the standard procedure for obtaining brain slices from a whole fixed animal. The anesthetized animal is perfused intracardially with a solution containing 4% formaldehyde. The brain is then extracted and post-fixed overnight. Finally, brain slices are obtained using a vibratome and kept at −20°C.

1997) and following the European Community, law 86/609/CCE, FELASA and ARRIVE guidelines. The protocol is approved by our Committee on Animal Use and Care. Thus, the animals are housed in standard cages with ad libitum access to food and water and maintained under controlled standard conditions (12 hr dark/light cycle starting at 7:30 a.m., 22°C temperature and 66% humidity). Regarding anesthesia, we recommend a barbiturate type (i.e., ketamine/xylazine combination), since gaseous types (i.e., fluothane) tend to constrict vessels, resulting in poor fixative perfusion.

1. Add 2 ml 50 mg/ml ketamine (100 mg) and 0.5 ml 20 mg/ml xylazine (10 mg) to 7.5 ml sterile water to generate 10 ml ketamine/xylazine anesthetic solution. Mix very well by gentle inversion.

   This solution can be stored at 4°C for a maximum of 2 weeks.

2. Weigh the animal and inject intraperitoneally 0.1 ml of the ketamine/xylazine anesthetic solution per 10 g body weight (100 mg/kg ketamine, 10 mg/kg xylazine) using a 27-gauge needle and 1-cc syringe (Fig. 2). Wait 3-5 min until the animal is deeply anesthetized.

   To increase the duration of the anesthetic effect, the dose of xylazine can be increased (i.e., 15 mg/kg).

3. Once the animal is unconscious, check that the withdrawal reflexes (toe pinch) are absent. Lay the animal dorsally on a rack above a container and secure each paw to the rack with tape or a pin (Fig. 2).

4. Clean the ventral chest area with 70% ethanol and make an inverted T-cut through the skin and the abdominal wall caudal to the last rib. Open the thoracic cavity by cutting with scissors through the base of the sternum, perpendicular to the first cut.

   Be careful not to damage the heart or lungs (Fig. 2).

5. Expose the heart by holding the rips out using forceps. Tear the pericardium with the help of blunt forceps (Fig. 2).

6. Switch on the perfusion valve to start the flow. Insert the perfusion needle (25-gauge, 25-mm or wider if the flow rate is not fast enough) into the left ventricle and make an incision in the right atrium to quickly drain fluids (a great deal of blood will be released; Fig. 2).

7. Perfuse with 100 ml cold 1× PBS.

   The reservoir (i.e., 50-ml syringe) containing the PBS solution should be maintained (and continuously refilled) at 100 cm above the animal to ensure sufficient perfusion pressure through the circulatory system. The blood from the circulatory system will exit through the incision performed in the right atrium.

   Increase the flow using larger gauge needles or increasing the height of the reservoir. This will provide better results. Typically, it will take 1-10 min to wash the circulatory system with the PBS solution, following which the eyes and liver will become clear. For rat, use 200 ml 1× PBS solution.

8. Perfuse with ∼100 ml of ice-cold 4% formaldehyde in 1× PBS.

   Tremors should be observed within seconds and at the end of the fixation process and the animal should be stiff.

   Due to the toxic nature of formaldehyde, all perfusion procedures should be performed under the fume hood, and gloves and a lab coat should be used. The formaldehyde should be collected and stored for disposal according to the regulations of your institution. For rat, use 200 ml of fixative solution. The organs and the whole animal should be rigid (including the tail) to the touch once the fixative solution has passed through the body.

9. Dissect and post-fix the brain by incubation (24 hr) in the same fixative solution (4% formaldehyde) at 4°C. Swirl occasionally. Wash the brain with 1× PBS by exchanging the medium three times, swirling each time.

   The brain can then be stored in 1× PBS containing 0.1% (w/v) sodium azide at 4°C.

10. Obtain brain coronal sections (50-70 μm) using a vibratome (Fig. 2).

11. Keep the slices in Walter's antifreezing solution at −20°C until use.

    Slices should be manipulated with a round brush (size 10/0) to avoid tissue damage (Fig. 2).

Free-floating P-LISA protocol

12. Rinse the slices three times for 10 minutes each with 1 ml 1× PBS to remove Walter's antifreezing solution (Fig. 3, step 1).

    Use the round brush to manipulate the slices during the washing steps.

13. Wash slices with 1 ml 1× PBS containing 0.3% Triton X-100 for 2 hr at room temperature (Fig. 3, step 2).

14. Wash the slices three times for 10 min each with 1 ml of 1× PBS containing 0.05% Triton X-100 (Fig. 3, step 2).

15. Incubate slices with 250 μl blocking solution for 2 hr at room temperature (Fig. 3, step 3).

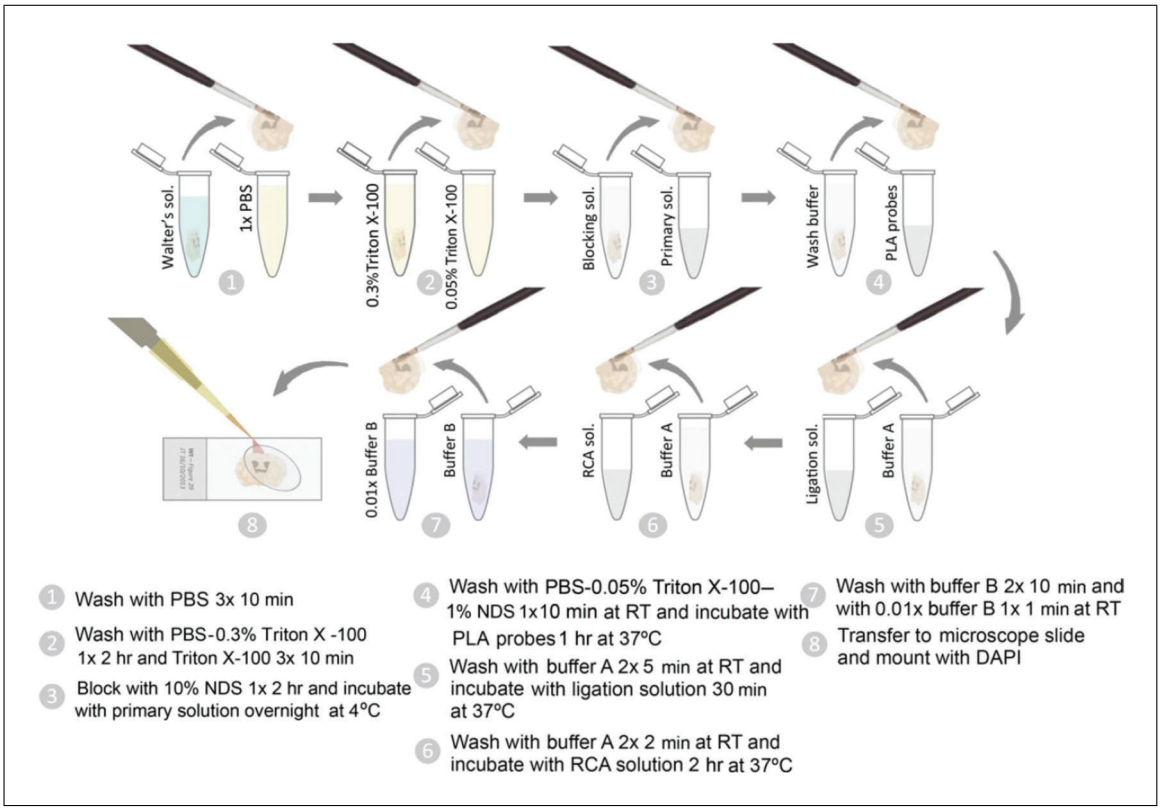

Figure 3 Flow chart showing all the incubation steps of the P-LISA. Several free-floating steps are performed: permeabilization, blocking, primary antibody incubation, PLA probe recognition, ligation, RCA (including detection) and mounting.

16. Incubate the slices with 100 µl blocking solution containing the primary antibodies overnight at 4°C (Fig. 3, step 3).

    Primary antibodies against the antigens under study (i.e., $A_{2A}R$ and D_2R) should be raised in different animal species to avoid cross-reactivity of the PLA probes. For example, in the example provided here, goat anti-$A_{2A}R$ and rabbit anti-D_2R polyclonal antibodies (Frontier Institute Co. Ltd., Shinko-nishi, Ishikari, Hokkaido, Japan) are used, as previously described (Fernández-Dueñas et al., 2015). In addition, primary antibody specificity should previously be demonstrated by conventional immunofluorescence staining under the same experimental conditions described here. In addition, each antibody pair used should be specifically titrated for the P-LISA experiment.

17. Dilute 1:5 the two PLA probes provided by the manufacturer in a blocking solution and allow the mixture to stabilize for 20 min at room temperature.

    PLA probes are species-specific secondary antibodies that contain a unique short DNA strand attached to them. Hence, PLA probes are verified both for specificity (e.g., for a goat primary antibody use a PLA-probe anti-goat) and for complementarities (e.g., one PLUS and another MINUS).

18. Wash slices for 10 min with 1 ml of 1× PBS containing 0.05% Triton X-100 and 1% NDS at room temperature (Fig. 3, step 4).

19. Incubate slices with 100 µl blocking solution containing the PLA probes for 1 hr at 37°C (Fig. 3, step 4).

    Vortex the PLA probe mixture before adding to the slices. Note that steps 6 to 8 of Fig. 3 are performed in a similar manufacturer protocol manner.

20. Wash slices twice for 5 min each with 1 ml P-LISA wash buffer A at room temperature (Fig. 3, step 5).

21. Prepare 1× ligation solution by diluting 1:5 the 5× ligation stock solution (see recipe) with high grade pure water.

22. Incubate slices with 100 µl of 1× ligation solution containing 0.025 U/µl of T4 DNA ligase (prepared by diluting 1:40 the 1 U/µl T4 DNA ligase into the 1× ligation solution) for 30 min at 37°C (Fig. 3, step 5).

    The T4 DNA ligase should be maintained at −20°C until diluted; use a freezing block (−20°C) to transport. The ligation solution containing the diluted T4 DNA ligase should be used immediately upon preparation.

23. Wash slices twice for 2 min each with 1 ml P-LISA wash buffer A at room temperature (Fig. 3, step 6).

24. Prepare the RCA solution (1×) by diluting 1:5 the 5× RCA solution (see recipe) with high grade pure water.

    The RCA solution (1×) contains 1 mM dNTPs, 5% glycerol, 0.25 µg/µl BSA and 0.05% Tween 20 according to previously reported information (Jarvius et al., 2007). In addition, this solution contains the fluorescently labeled red probe that hybridizes with the single-stranded RCA product and that can be visualized using a red-based laser system (see below).

25. Dilute 1:40 phi29 DNA polymerase (10 U/µl) into the RCA solution (1×). Incubate slices with 100 µl RCA solution containing 0.25 U/µl of phi29 DNA polymerase for 2 hr at 37 ° C (Fig. 3, step 6).

    This step must be performed using opaque Eppendorf tubes (or tubes covered with aluminum foil), since the RCA solution is light sensitive. The phi29 DNA polymerase should be maintained at −20°C until diluted; use a freezing block (−20°C) for transport. The RCA solution containing the diluted phi29 DNA polymerase should be used immediately upon preparation.

26. Wash slices twice for 10 min each with 1 ml P-LISA wash buffer B at room temperature (Fig. 3, Step 7).

27. Wash slices for 1 min with 1 ml of 0.01× P-LISA wash buffer B at room temperature (Fig. 3, Step 7).

    This wash step aims to remove salts from the slice prior to mounting for observation. Thus, we recommend keeping slices in wash buffer B for 5-10 min until ready for mounting (slices may collapse and deform if they stand for a long time in 0.01× wash buffer B).

28. Place and extend the slices on a microscope slide containing a drop of 0.01× P-LISA wash buffer B (Fig. 3, step 8).

29. Add 10 µl of Duolink in situ mounting medium (or an equivalent) containing DAPI to each slice. Place a coverslip on top of the slice and avoid the formation of air bubbles. Seal the cover slip with transparent nail polish (Fig. 3, step 8).

    The slides are kept in the dark for at least 1 hr before analysis in a confocal microscope. The slides can be stored at 4°C for a maximum of 2 weeks, and for longer periods at -20^0C.

SUPPORT PROTOCOL

SLIDE OBSERVATION, IMAGE ACQUISITION, AND QUANTIFICATION

Slide observation, image acquisition, and quantification constitute a key step for the interpretation of the P-LISA results. Indeed, obtaining good P-LISA images depends on various optical factors that are far beyond the scope of the present protocol and have been discussed elsewhere (Smith, 2011). Consequently, the use of a laser scanning confocal microscope (LSCM) system allowing a specimen's depth discrimination and contrast is

highly recommended. Therefore, the P-LISA-stained brain slice mounted on a confocal fluorescence microscope slide is observed as a static image in the LSCM using the appropriate excitation/emission wavelengths. Once the whole brain slice is observed, a region of interest (ROI) is selected and the thickness within the z-plane is determined. Next, serial optical thin sections are collected through the fluorescent ROI in steps of 0.2-0.5 μm perpendicular to the z-axis (microscope optical axis) using proper illumination of the laser system. The image series are collected by coordinating incremental changes in the microscope fine focus mechanism—by using a stepper motor—with sequential image acquisition at each step. Therefore, the information from each collected image is limited to a definite specimen plane. Since confocal microscopy allows for a reduction in background fluorescence and improves the signal-to-noise ratio, the contrast and definition of the collected images are dramatically improved over wide-field microscopy (Smith, 2011). On the other hand, confocal optical sectioning eliminates artifacts that occur during physical manipulation (i.e., sectioning and P-LISA) of tissue specimens. Finally, the confocal optical sections in the x-y plane and collected on the z-axis are combined along the z-axis with the confocal microscopy software package, projecting a view of fluorescence intensity. Alternatively, image stacks can be composed and analyzed using an open-source Java-based image processing program allowing Java plugins and recordable macros (i.e., ImageJ or Fiji; Schneider et al., 2012).

Materials

Laser scanning confocal microscope (LSCM; TCS 4D, Leica, or equivalent)
ImageJ (see Internet Resources)
Example of DAPI-stained nuclei and PLA probe stacks of images
Program that automates a series of ImageJ commands (recorded macro)

Slide observation

1. Visualize the region of interest (ROI) of each slice (i.e., striatum) using LSCM (i.e., Leica TCS 4D) with the aid of a 60× N.A. = 1.42 oil objective.

   The use of an LSCM is essential to allow the precise location of the RCPs within a slice of 50-70 μm thickness. The DAPI stained nuclei within the ROI are visualized by exciting the slide at 364 nm using an UV laser and the fluorescence emission is collected at 460 nm. Similarly, the PLA probe detection is visualized throughout the oligonucleotide probes typically labeled with a red fluorophore (λ_{ex}=594 nm and λ_{em}=624 nm) using a 561-nm laser system.

Image acquisition

2. Acquire independently the DAPI and PLA probe high-resolution images from a 5-μm thick optical section of each ROI. Establish a z-interval of 0.2 μm, to obtain a z-stack of 25 sections.

   The images should ideally be saved as an 8-bit RGB .tiff-format image and the dimensions can be either 1024 × 1024 or 512 × 512.

Quantification

Image processing

3. Open ImageJ and load the stacks "DAPI.tif" and "PLA.tif."

   This can be achieved by selecting [File > Open…], or simply by dragging and dropping the files into the ImageJ menu bar.

4. Create a maximum intensity projection image for both stacks by selecting [Image > Stack > Z project… > Max intensity].

   This will generate a new image that displays the maximum pixel intensity across all the slices in the stack. (Fig. 4).

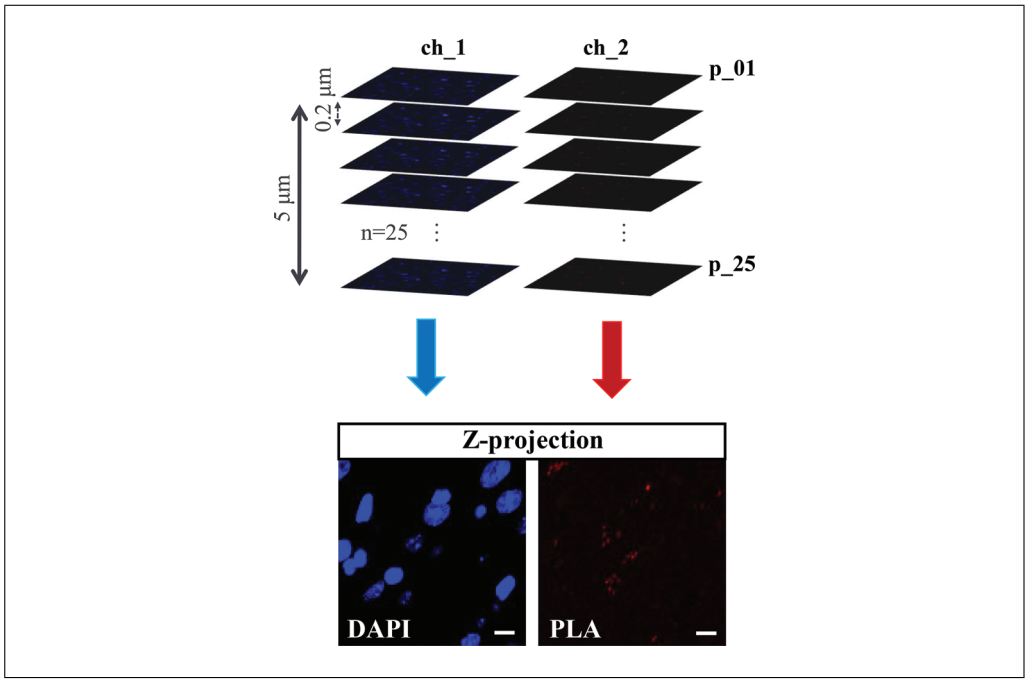

Figure 4 Schematic representation of the confocal image stacks (n = 25) acquired for each fluorescence channel (i.e., ch_1 and ch_2). Stacks are projected through the Z axis to generate the Z-projected image of each staining. Scale bar: 10 µm.

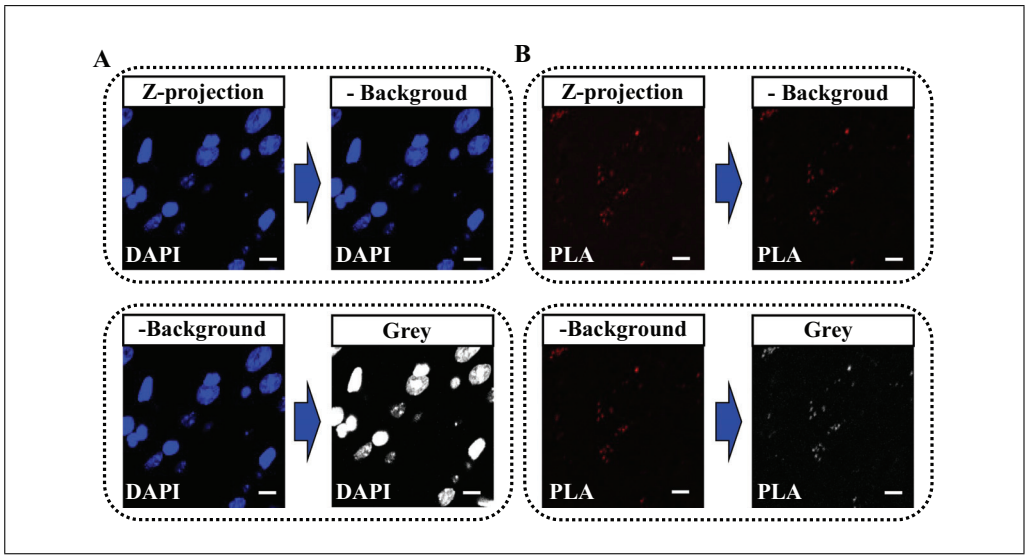

Figure 5 Scheme of the specific sequence of ImageJ commands necessary to perform a Z projection from a stack (n = 25) of DAPI **(A)** and PLA **(B)**. The background is subtracted using the rolling ball radius approach of 50.0 pixels (– Background; upper panels). Next, the image is transformed into a gray image (Grey; lower panels). Scale bar: 10 µm.

5. Perform a background subtraction on both z-projection images using the built-in function in ImageJ by selecting [Process > Subtract background…].

Although the nuclei and PLA spots differ in size, we found that the default rolling ball radius of 50.0 pixels produced good results for both sample images (Fig. 5). To ensure accurate background subtraction, set the radius to at least the size of the largest object in the image that is not part of the background (i.e., nuclei). To determine the radius of the largest objects in your image, you can use the line tool to draw a line from one edge of the object to the other, and the length will be reported in the ImageJ toolbar. The default rolling ball radius value in ImageJ is 50.0 pixels, which may be suitable for some images, but might need to be adjusted for others depending on the size of the objects in question.

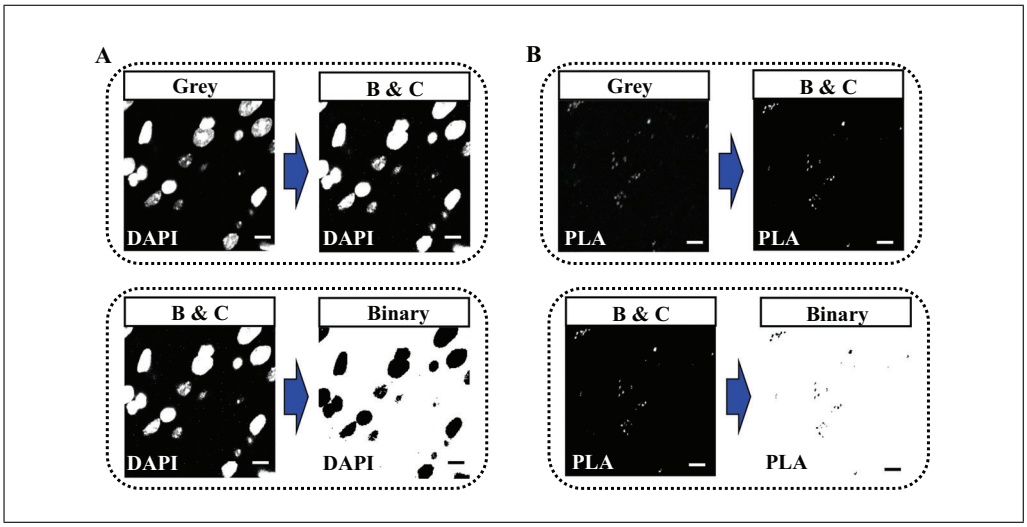

Figure 6 Digital transformations scheme for image analysis in ImageJ. Example images of transformations of each channel transformations of DAPI-stained nuclei (**A**) and PLA dots (**B**). Brightness and contrast adjustments (B & C; upper panels) of the grayscale images from Figure 5 are subtracted. Next, a binary image transformation is performed (lower panels). Scale bar: 10 µm.

6. Enhance the visual contrast of the images by displaying both z-projections in grayscale (as shown in Fig. 5), by selecting [Image > Lookup Tables > Grays] and selecting the grayscale option.

   This will help bring out the details and make it easier to distinguish between different structures in the image.

7. Adjust the brightness and contrast by selecting [Image > Adjust > Brightness/Contrast…] and adjust the settings until you reach the desired level of contrast.

   This step can help enhance the image if you find that the spots in your z-projections are too weak or difficult to see. However, be careful not to change the threshold values from one image to the next, as this can affect the comparability of your results. Figure 6 provides an example of how adjusting brightness and contrast can improve the visibility of spots.

   When adjusting the brightness and contrast of your z-projections, aim to make the background as dark as possible and the signal as uniform and bright as possible. Keep a record of the settings used so that you can apply the same parameters to the rest of the images in your experimental set. Note that the settings you use may differ between channels, but they must remain consistent for the corresponding channels across all the images analyzed. It is important to remember that changing the background and contrast of the images will alter the original intensity values. Although this may not be critical for particle analysis, it could potentially impact other parameters and subsequent image analysis. Therefore, it is important to exercise caution and ensure that any adjustments made are necessary and well-documented.

8. Apply a smoothing filter to improve the image quality by selecting [Process > Smooth] and select the desired smoothing filter.

   If your resulting images exhibit excess noise or have particles with holes or gaps, this can help reduce noise and improve the appearance of particles in the image, making them easier to analyze.

   If individual particles show gaps that could affect counting (particle analysis; nuclei analysis and PLA signal analysis, steps 11 and 13, respectively), then use the smooth filter to blur the active image or selection. This filter replaces each pixel with the average of its 3 × 3 surroundings.

9. Segment z-projections and convert them into binary images (black-and-white representations of the original image; as shown in Fig. 6) by selecting [Process > Binary > Make Binary] and select the desired thresholding method.

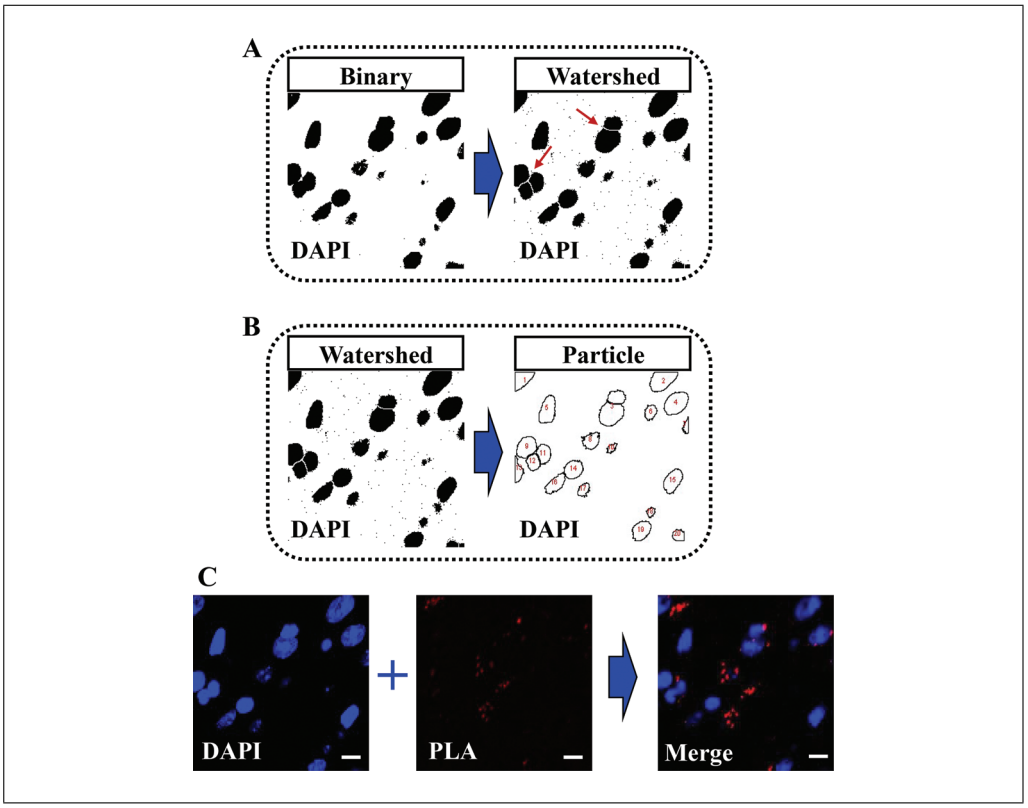

Figure 7 Particle signal analysis scheme in ImageJ. Example of DAPI-stained nuclei and PLA probe images with specific instructions for: **(A)** optional watershed filter application; **(B)** particle analysis and counting of the total number of nuclei; **(C)** composite transformation of both channels. Red arrows in (A) indicate examples of object segmentation and separation achieved by applying the watershed filter. Scale bar: 10 µm.

This will help separate the foreground (signal) from the background, making it easier to identify and analyze individual particles and facilitate the analysis.

ImageJ provides various global thresholding methods that can be accessed through [Image > Adjust > AutoThreshold]. This feature allows you to test different methods and select the one that best fits your specific images. For the provided sample images, the default automatic thresholding method is sufficient. This method works by taking a test threshold and calculating the averages of the pixels below and above it. Then it computes the average of these two values, increments the threshold, and repeats the process iteratively until the threshold is greater than the composite average. This approach can effectively distinguish between the foreground (signal) and the background, producing binary images that are suitable for further analysis.

Nuclei analysis

In this section, the cells of interest are counted using the binary mask that results from nuclei segmentation.

10. Perform a watershed transformation (Fig. 7A) to separate objects that appear contiguous in the binary mask by selecting [Process > Binary > Watershed].

    This step is useful when the nuclei overlap or are very close to each other and are therefore difficult to resolve spatially.

11. Count nuclei >30 μm^2 in the resulting binary image by selecting [Analyze > Analyze particles …] (Fig. 7B) and setting the following parameters: Size (μm^2), 30-Infinity; Show, Outlines; and Display results.

    It is well established that within the brain (i.e., striatum), neurons and glial cells can be discriminated by the size of their respective nucleus (Matamales et al., 2009). Therefore,

if the interaction upon study is expected to take place in neurons, as the example provided here, the glial nuclei (<30 μm^2) can be excluded from the quantification.

PLA signal analysis

In this section, the number of PLA probe particles is counted on its corresponding binary mask. Then, a composite image containing both nuclei PLA spots is created, and the ratio between the PLA probe particles and the number of nuclei calculated.

12. Apply a watershed transformation by selecting [Process > Binary > Watershed].

    This step can be performed if the PLA signal overlaps, and thus the spots are difficult to resolve spatially (Fig. 7A).

13. Count PLA probe particles >0.3 μm^2 in the resulting PLA watershed image by selecting [Analyze > Analyze particles..] and setting the following parameters: Size (μm^2), 0.3–10; Show, Outlines; and Display results.

    Studies have reported that PLA probe particles may exceed the size of 0.3 μm^2 particles (Taura et al., 2015).

14. For visualization purposes, create a composite image that combines the projected DAPI and PLA channels by selecting [Image > Color > Merge channels…] and follow these steps:

    a. Select the MAX_PLA image as C1 (red)
    b. Select the MAX_DAPI image as C3 (blue).
    c. Select the Create composite option to generate the merged image (Fig. 7C).

    This approach can be useful for presenting your results in a clear and concise manner.

15. Obtain a quantitative measure of the PLA signal by calculating the ratio between the number of PLA probe particles and the number of nuclei present in the sample.

    Divide the number of PLA probe particles by the number of nuclei in the same image to obtain the desired ratio.

16. Graph data that contain appropriate negative controls (Fig. 8).

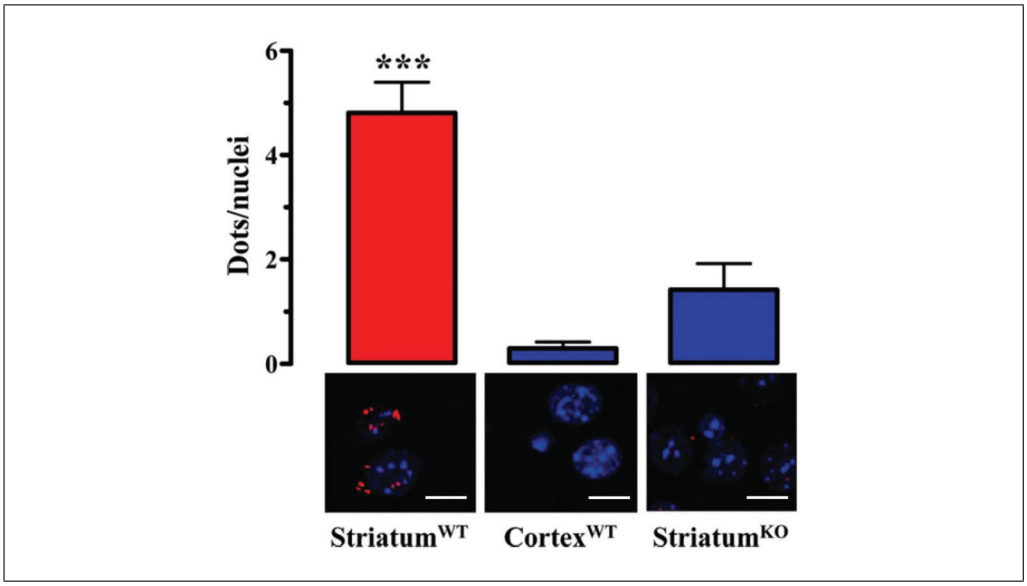

Figure 8 Representative P-LISA result. Illustrative images of dual recognition of D_2R and $A_{2A}R$ P-LISA in the striatum and cortex of a wild-type animal ($Striatum^{WT}$ and $Cortex^{WT}$, respectively) and the striatum of a $A_{2A}R$ knockout animal ($Striatum^{KO}$). The PLA signal in the striatum was significantly (***$p < 0.001$) higher than in the cortex or striatum of $A_{2A}R$-KO mice. The values correspond to the mean ± s.e.m. (dots/nuclei) of at least 6 animals for condition. Adapted from (Fernández-Dueñas et al., 2015). Scale bar: 10 µm.

To establish several negative controls, it is essential to perform the same procedure under different conditions. Specifically, the procedure should be performed in the absence of primary antibodies as a negative control. Additionally, brain slices of the knockout animal should be used to verify the specificity of the staining for at least one of the targeted receptors. Lastly, defined negative brain regions that are expected to be devoid of targeted receptors should be used as negative controls as well.

17. Perform a suitable statistical analysis of the results (Fig. 8).

REAGENTS AND SOLUTIONS

Use distilled deionized water in all recipes and protocol steps.

Blocking solution

Mix 10% normal donkey serum (NDS; Jackson ImmunoResearch Laboratories, cat. no. 017-000-121) and 0.05% Triton X-100 (Sigma-Aldrich) in 1× PBS (see recipe)

Formaldehyde solution, 4% in 1× PBS

Mix 107.4 ml of 36.5%-38% formaldehyde solution (Sigma-Aldrich) with 100 ml 10× PBS (see recipe) and bring up to 1 L with H_2O
Prepare fresh

Ligation solution, 5×

1.25 M NaCl (73.05 mg/ml)
5 mM ATP (2.75 mg/ml)
0.125% BSA (1.25 mg/ml; Sigma-Aldrich)
0.25% Tween-20
Store at −20°C up to 1 year

Phosphate buffered saline (PBS), 10×

80.7 mM Na_2HPO_4 (28.9 g/L)
14.7 mM KH_2PO_4 (2 g/L)
1.4 mM NaCl (80 g/L)
27 mM KCl (2 g/L)
Adjust pH to 7.2 using HCl or NaOH
Store at room temperature up to 6 months

Phosphate buffered saline (PBS), 1×

Dilute 1:10 the 10× PBS (see recipe) in H_2O
Store at 4°C up to 1 month

P-LISA wash buffer A

150 mM NaCl (8.8 g/L)
10 mM Tris base (1.2 g/L)
0.05% Tween-20 (0.5 ml/L)
Adjust pH to 7.4 using HCl
Filter solution through a 0.22-μm filter
Store at 4°C up to 1 month

P-LISA wash buffer B

100 mM NaCl (5.84 g/L)
200 mM Tris (4.24 g/L Tris base and 26 g/L Tris·HCl)
0.05% Tween-20 (0.5 ml/L)
Adjust pH to 7.5 using either HCl or NaOH

Filter solution through a 0.22-μm filter
Store at 4°C up to 1 month

Rolling-circle amplification (RCA) solution, 5×

250 mM Tris·HCl (39.4 g/L)
50 mM $MgCl_2$ (4.76 g/L)
50 mM $(NH_4)_2SO_4$ (6.6 g/L)
Adjust pH to 7.5 using NaOH
5 mM dNTPs (1 mM of each)
25% glycerol
0.125% BSA (1.25 mg/ml; Sigma-Aldrich)
0.25% Tween-20
Store at −20°C up to 1 year

Walter's antifreezing solution

30% glycerol
30% ethylene glycol (Sigma-Aldrich)
in 1× PBS (see recipe), pH 7.2
Store at −20°C up to 1 year

COMMENTARY

Background Information

Detection of low-abundance and/or transient receptor-receptor interactions requires both high selectivity and sensitivity. The former may be achieved by using antibodies and the latter by means of enzymatic activities. Hence, the use of antibodies is a suitable and resourceful approach for the detection of endogenously expressed receptors and their interactions. On the other hand, the sensitivity may be boosted by enzyme-coupled activity, prompting potent signal amplification. Accordingly, the PLA combines the dual recognition of an antibody-targeted assay with a split-reporter enzymatic approach, thus generating a selective and sensitive method for the specific detection of receptor-receptor interactions (Fernández-Dueñas et al., 2021).

The notion of using DNA ligation for analytical purposes was initially proposed in the early 1970's after the discovery of ligases (Besmer et al., 1972). Subsequently, the use of this concept ended with the description of proximity-dependent DNA ligation assays, or simply PLA, allowing the detection of zeptomole (40×10^{-21} mol) amounts of the cytokine platelet-derived growth factor (PDGF) without washes or separations (Fredriksson et al., 2002). More recently, PLA or P-LISA was developed to enable the detection of individual proteins and protein-protein interactions in cultured cells and in native tissue (Söderberg et al., 2006, 2008). Interestingly, the P-LISA has both single-molecule resolution and superior dynamic range compared to those of other current antibody-based protocols, thus allowing relative signal quantification and localization.

Critical Parameters

Tissue fixation

Tissue fixation is one of the most critical steps for the success of P-LISA. Indeed, since P-LISA depends on specific epitope-antibody interactions, the preservation of tissue morphology and antigenicity of target molecules (i.e., GPCR) is extremely important, as happens for all immunohistochemical procedures (Ramos-Vara & Miller, 2014). Accordingly, incomplete fixation (underfixation) of tissue (e.g., brain) may lead to rapid proteolytic degradation of target proteins (i.e., GPCRs), thus reducing specific immunoreactivity. Importantly, within the brain there are areas that easily fix (e.g., cortex) while others (e.g., striatum) are more resilient to perfuse with the fixative solution and thus are prone to get underfixed. An underfixed tissue (i.e., with pink-colored appearance) will render a low number of P-LISA puncta, thus underestimating the amount of P-LISA signal (i.e., GPCR interaction) and minimally reducing the differences from the background. Hence, a poor fixation cannot be corrected at a later stage and is a common source of problems for the detection of P-LISA. On the other hand, an excess of fixation (overfixation) may end with epitope masking or with an intense

nonspecific P-LISA background, which may blur specific labeling. Generally speaking, underfixation is a much greater problem than overfixation and consequently, special attention should be paid to all key steps within the tissue fixation process (i.e., fixative, duration, temperature, and pH; Fig. 2), which will in fact affect the degree of tissue fixation and thus the P-LISA detection.

Whole animal perfusion fixation, as presented here, may not be sufficient to fix the tissues of interest. In such cases, dissected tissue (e.g., brain) can be immersed in fixative solution overnight (i.e., post-fixation). To enhance the penetration of the fixative during post-fixation, it is recommended that tissues be no thicker than 10 mm. On the other hand, the volume of the fixative should be 50-100 times larger than the volume of the tissue. If the tissue floats, it should be submerged. Again, it is important to optimize both fixation and post-fixation conditions, since underfixation or overfixation may reduce or preclude the P-LISA signal.

Finally, it is important to mention here that while sample preparation in fixed tissue-based PLA methods (i.e., P-LISA) is very laborious and therefore time-consuming, the implementation of PLA techniques in isolated cultured cells could be a suitable and easy alternative to visualize GPCR interactions. Indeed, since the fixation procedure for cultured cells is quite straightforward, most users will not have difficulty obtaining appropriate results in adherent or suspended cell lines (Leuchowius et al., 2011).

Antibodies

The most important aspect when designing a P-LISA experiment is primary antibody pair selection. Indeed, the critical feature of any primary antibody is specificity for the epitope (i.e., GPCR), therefore all primary antibodies used in P-LISA should be first tested for specificity in immunohistochemistry experiments using proper controls (see below). Subsequently, all P-LISA steps must be optimized to visualize specific staining and minimize nonspecific background signals. This initial antibody characterization of the incubation conditions includes: (i) working dilution (i.e., primary antibody concentration), (ii) temperature, (iii) diluent solution, and (iv) incubation time. All these conditions should be empirically optimized by means of immunohistochemistry experiments for each primary antibody, either monoclonal or polyclonal, and also for the specimen used.

The binding of the antibody to its recognizing protein depends on the preservation of the native conformational state of the target protein. Importantly, access of the antibody to its epitope could be conditioned by post-translational modifications, protein-protein interactions, diluent salt concentration, temperature, pH, and fixation (see above). Since polyclonal antibodies can recognize multiple epitopes within the target protein, they are less prone to being influenced by conformational changes. On the contrary, because monoclonal antibodies bind with high affinity and specificity to a single epitope, disruption of the target protein conformation may preclude recognition and thus antigen detection. Additionally, polyclonal antibodies are more stable over a range of pH and salt concentrations when compared to monoclonal antibodies. Overall, polyclonal antibodies are used more frequently in P-LISA experiments, but when the detection of close related proteins (i.e., homologous receptors) is compromised then the use of monoclonal antibodies is recommended.

Optimization of P-LISA detection is initially assessed by maintaining the temperature and incubation time constant, while varying the concentration of the primary antibody to determine the optimal P-LISA signal. Therefore, a high affinity primary antibody may be used at high concentrations for shorter incubation times or at lower concentrations but with a longer time. However, longer incubation periods (i.e., overnight) at lower temperatures (i.e., 4°C) are often preferred to promote specific staining and guarantee antibody penetration throughout the tissue section. To increase antibody penetration, incubations could be performed with free floating tissue sections.

Controls

As a general rule in research—and P-LISA is not an exception—proper controls are key elements to consider any experimental approach as a trustworthy technique. Accordingly, in addition to the above-mentioned considerations, some key controls should be considered when P-LISA experiments are performed. First, the nonspecific binding of PLA probes to the sample tissue should be checked. Hence, the whole protocol should be performed in the absence of the primary antibodies. Because PLA probes are not expected to recognize any sample epitope apart from the primary antibodies used in the P-LISA experiment, the resulting stain should be low. Second, to confirm the specificity of

Table 1 Frequent P-LISA Problems: Possible Causes and Solutions

Problem	Causes	Solutions
Low Signal	Underfixation	Revise concentration, temperature, and pH of the fixative solution; increase duration of the fixation process
	Inappropriate primary antibody	Select an antigen-specific antibody and validate its antigen detection properties by alternative methods (e.g., Immunoblot, etc.)
	Wrong concentration (i.e., too low) of the primary or PLA probes	Optimize the concentration
	The activity of primary antibody or PLA probes is lost	Check the antibody storage condition and perform an experiment using a positive control
	Wrong pH of the primary antibody solution	Ensure that the primary antibody works properly
	Deficient permeabilization	Increase the concentration of the permeabilizing agent (i.e., Triton X-100)
	Low or no fluorescence signal	The fluorescently-labeled probe should be stored in the dark
High background signal	The concentration of the primary antibody was too high	Dilute the primary antibody
	The incubation time of the primary antibody was too long	Reduce the incubation time of primary antibodies
	The temperature of primary antibody incubation was high	Perform the primary antibody incubation at 4°C to allow an increased incubation time and thus antibody penetration
	Serum was absent or insufficient in the blocking solution	Revision of serum concentration and/or increase in blocking time
	Inadequate buffer A/B washing	Wash with buffer A/B for an adequate amount of time and several times to eliminate fixatives
	Polyclonal primary antibody	Try a monoclonal primary antibody
Nonspecific staining	Overfixation	Reduce the duration of the fixation process; revise concentration, temperature, and pH of the fixative solution
	Deficient washing after incubation with primary antibodies and PLA probe	Wash with buffer A/B appropriately
	The concentration of the primary antibody or PLA probe was too high	Decrease the concentration of primary antibody or PLA probe
	The PLA probe is the same species as the sample origin	Change the PLA probe

the primary antibodies in a P-LISA experiment, tissue samples where either one or both receptors are not expressed are required. Therefore, tissues obtained from knockout animals should be used in the detection of P-LISA. Finally, another interesting control may consist of performing the P-LISA detection in a tissue or a region of the tissue where the interaction is expected not to occur. Overall, three types of controls are recommended in P-LISA experiments: (i) PLA probe specificity, (ii) primary antibody specificity, and (iii) sample/tissue selectivity.

Even though the output of the P-LISA technique is an image, proper computational processing allows us to quantify data. This can be achieved by using the ImageJ program "Analyze particle" function located in the Analyze menu toolbar. To be able to have reliable values of the PLA probes dots, it is important to process all images using the same settings for background subtraction and contrast

enhancements. It is also crucial to determine which size dots can be considered as background, since the function will only count particles that fit in a given size range.

Troubleshooting

The success of the P-LISA method depends primarily on the quality of the antibodies used and the sample preparation mode. The main problem often found when using antibodies is the lack of antigen recognition, cross-reactivity (i.e., the antibody binds to other proteins), and nonspecific absorption (i.e., the antibody is trapped within the sample in an unspecific manner). These facts might limit the specificity of the P-LISA method. On the other hand, the use of an enzymatic activity for signal amplification might also prompt false P-LISA detection. In general, the difficulties often found to cause a lack of detection, nonspecific staining, or a high background are shown in Table 1.

Understanding Results

We took advantage of P-LISA to detect D_2R-$A_{2A}R$ oligomers in the striatum of control and parkinsonian rats (Fernández-Dueñas et al., 2015). In our hands, the P-LISA signal observed for this GPCR oligomer in the rat or mouse striatum was consistent and within the same range (5-6 dots/nuclei) to that described previously (Trifilieff et al., 2011). Interestingly, our negative controls (i.e., $A_{2A}R$-KO and brain cortex) provided 0.5-1.5 dots/nuclei. Therefore, the specific P-LISA signal for D_2R-$A_{2A}R$ complexes in the rat/mouse striatum is typically about 3-10 times higher than the background signal. In fact, the obtained P-LISA results were validated using two alternative techniques (i.e., immunogold staining and electron microscopy detection, and RET experiments using fluorescent ligands; Fernández-Dueñas et al., 2015). In general, P-LISA is a powerful and straightforward technique for visualizing receptor-receptor interactions in native tissue provided it is performed under optimal conditions and also includes proper appropriate controls.

Time Considerations

Typically, the entire protocol can be performed in o1 week. On the first day, the animal is anesthetized and fixed, and the brain is post-fixed overnight. The second day, the brain is sliced and stored at −20°C in Walter's antifreezing solution. The third day, the slices are washed, blocked and incubated overnight with primary antibody solution. On the fourth day, the slices are incubated with the PLA probes, then the ligation and the RCA is performed; finally the slices are mounted on a slide and left overnight to make sure the samples are ready. Finally, the last day, the acquisition and the image processing can be performed.

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Author Contributions

Jaume Taura: Conceptualization, data curation, investigation, methodology, writing - original draft, writing - review and editing; **Marc López-Cano:** Conceptualization, data curation, investigation, methodology, writing - original draft, writing - review and editing; **Víctor Fernández-Dueñas:** Formal analysis, supervision, writing - original draft, writing - review and editing; **Francisco Ciruela:** Conceptualization, data curation, funding acquisition, methodology, project administration, resources, supervision, writing - original draft, writing - review and editing.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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Internet Resources

https://imagej.net/ij/index.html

Image J program version 1.53u and installation instructions. To ensure accurate quantification, we recommend using BMP, TIFF, PNG, or GIF formats as they preserve all the information in the image.