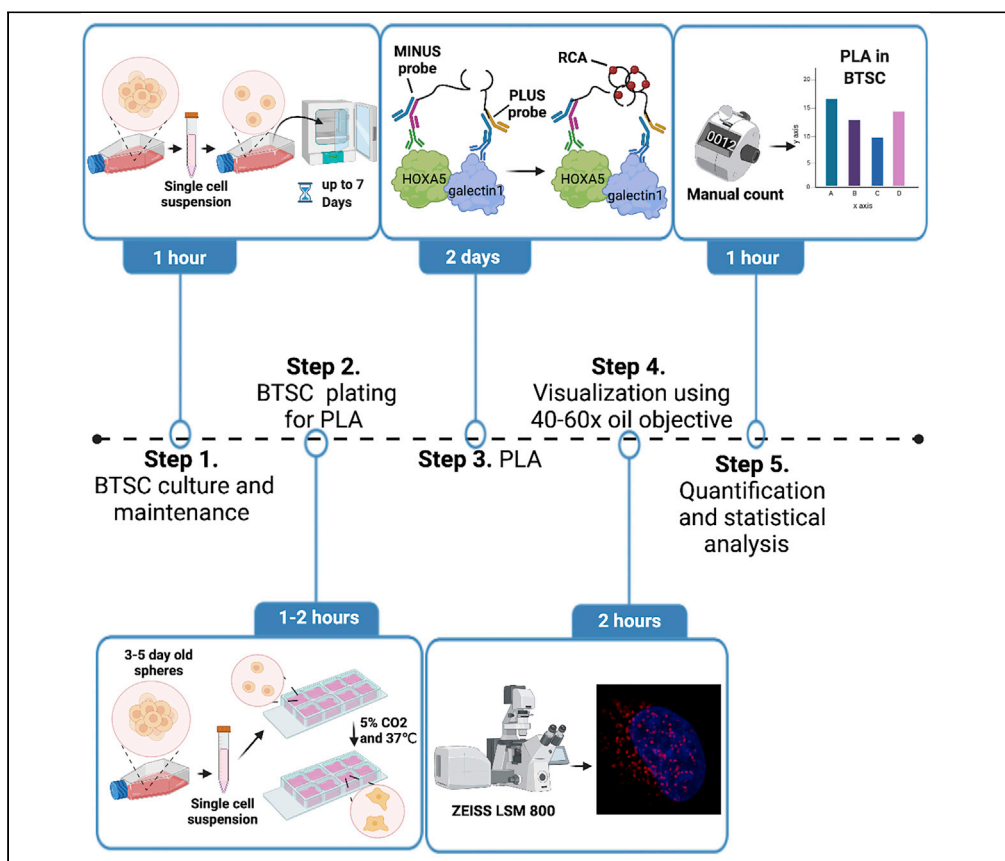


Protocol

In situ detection of protein-protein interaction by proximity ligation assay in patient derived brain tumor stem cells



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Highlights

Proximity ligation assay to detect protein-protein interaction in BTSCs

Optimized plating conditions for BTSC preparation using minute starting material

Inclusion of appropriate controls for the PLA to ensure specificity and precision

Alternative reagents and buffers to allow user to adapt the protocol to their needs

Improper or aberrant protein-protein interactions can lead to severe human diseases including cancer. Here, we describe an adapted proximity ligation assay (PLA) protocol for the assessment of galectin-HOXA5 in brain tumor stem cells (BTSCs). We detail the steps for culturing and preparation of BTSCs followed by PLA and detection of protein interactions *in situ* using fluorescent microscopy. This PLA protocol is optimized specifically for BTSCs and includes key controls for effective result analysis.

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

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Protocol

In situ detection of protein-protein interaction by proximity ligation assay in patient derived brain tumor stem cells

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SUMMARY

Improper or aberrant protein-protein interactions can lead to severe human diseases including cancer. Here, we describe an adapted proximity ligation assay (PLA) protocol for the assessment of galectin-1-HOXA5 interaction in brain tumor stem cells (BTSCs). We detail the steps for culturing and preparation of BTSCs followed by PLA and detection of protein interactions *in situ* using fluorescent microscopy. This PLA protocol is optimized specifically for BTSCs and includes key controls for effective result analysis.

For complete details on the use and execution of this protocol, please refer to Sharanek et al. (2021).

BEFORE YOU BEGIN

There are a variety of methods by which protein-protein interaction can be assessed which include but are not limited to immunohistochemical staining, co-immunoprecipitation, affinity chromatography, fluorescence resonance energy transfer (FRET) and proximity ligation assay (PLA) (Edidin, 2003; Rao et al., 2014). In contrast to classical methods such as co-immunoprecipitations, PLA allows the scientist to conduct the experiment with a small amount of starting material and to visually recognize an interaction at single cell resolution in cells or tissue (Leuchowius et al., 2013). First described for protein-protein interaction in 2002 by Fredriksson et al., this assay outlines a procedure in which there is simultaneous recognition of target sequences by two distinct signal probes. If the proteins of interest are in proximity, ligation will occur between the oligonucleotides of the signal probes and form a closed DNA circle. Through rolling circle amplification (RCA), a detection signal will be generated (Fredriksson et al., 2002). This simple and cost-effective method for the detection of protein-protein interactions allows for the highly precise visual confirmation that the proteins in questions are less than 40 nm from each other, indicative of interaction (Bagchi et al., 2015).

The adapted Duolink PLA protocol that we describe here includes culturing of BTSCs as well as a detailed method in which BTSCs are specifically processed prior to beginning the experiment in order to obtain optimal results (Figure 1). We also describe key controls that are required for effective



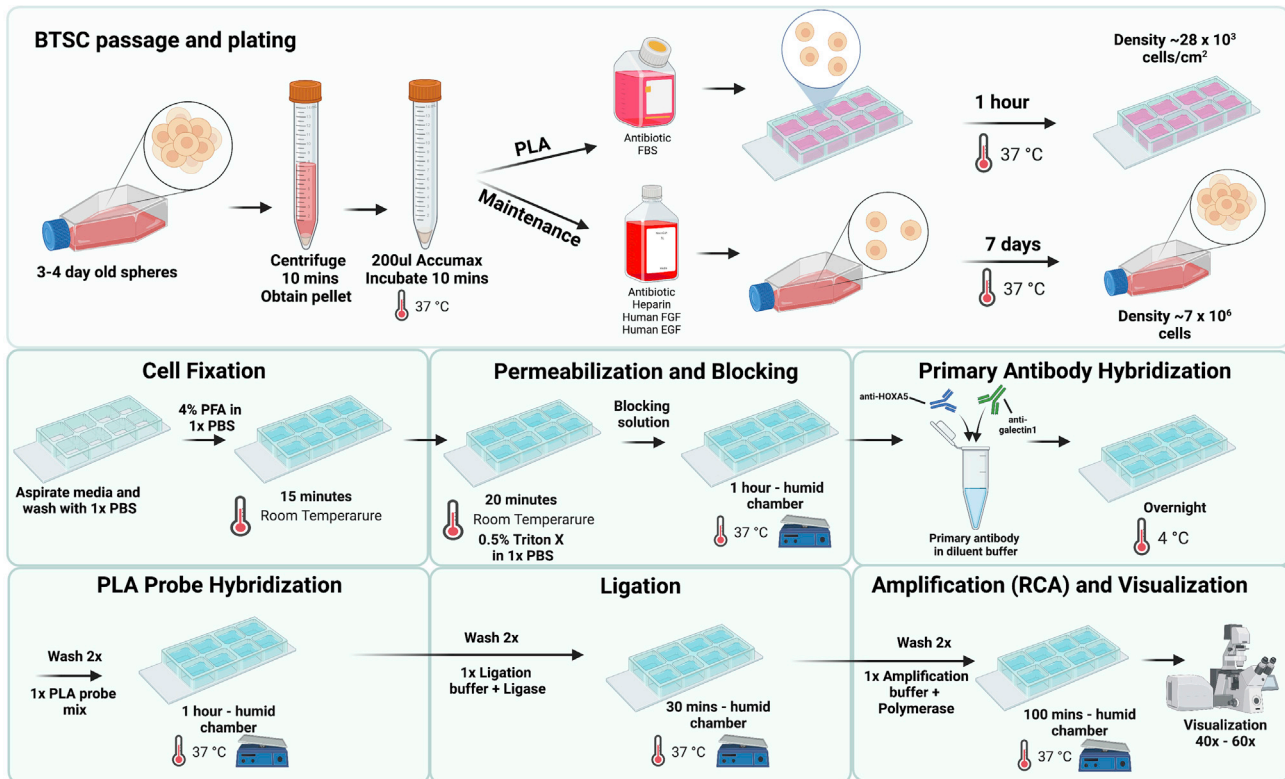


Figure 1. Workflow diagram

Schematic representation of BTSC maintenance and plating for PLA followed by sequential steps to for cell fixation, permeabilization and blocking, primary antibody hybridization, PLA probe hybridization, ligation, amplification and finally visualization of PLA signal. Created using [Biorender.com](https://biorender.com).

result analysis and highlight steps that are essential when conducting this type of fluorescence based experiments in brain tumor stem cells.

Similar protocols for PLA experiments may be found in other journals ([Alam, 2018](#); [Bagchi et al., 2015](#)), however, this protocol specifically describes optimization of PLA in BTSCs.

Set up of reagents

⌚ Timing: ~ 2 days

In this section, we describe the critical aspects that should be considered when choosing the reagents to perform the PLA experiments.

1. Primary antibodies.
 - a. Choice of primary antibodies: PLA experiments rely on the selection of two primary antibodies that are preferably validated for immunostaining in cells and/or tissues.

⚠ **CRITICAL:** The two primary antibodies must be raised in two different species and must bind with high specificity and affinity to the target proteins under the same fixation-permeabilization conditions. Most PLA probes that are commercially available are IgG class. Thus, primary antibodies of IgG-class that are either monoclonal or polyclonal should be selected.

Note: To assess galectin-1-HOXA5 interaction, we have tested several antibodies. The combination of the monoclonal mouse anti-galectin-1 (Cell Signaling Technology, #40103) and the polyclonal rabbit anti-HOXA5 (Abcam, #ab82645) showed optimal specificity for recognizing the galectin1 and HOXA5 proteins, respectively.

- b. **Determination of primary antibody dilution:** The recommended manufacturer dilution used for immunocytochemistry or immunofluorescence can be used for PLA experiments. However, to obtain a quantifiable PLA signal and minimal background, the concentration of the primary antibodies must be titrated. To titrate the antibody, first start with a concentration of each antibody that results in a saturated signal and then perform a serial dilution of the primary antibodies until distinct PLA signals are detected in puncta. For our PLA studies, both antibodies against galectin-1 and HOXA5 generated optimal and quantifiable signal when used at a dilution of 1:400.
- c. **Determination of the fixation-permeabilization method:** The fixation and permeabilization method should be optimized. The recommendations provided in the data sheet for each of the primary antibodies are used.

△ **CRITICAL:** The fixation and permeabilization condition should be the same and optimal for each of the two primary antibodies.

Note: In our experiments, 4% Paraformaldehyde (PFA) was used for fixation and 0.5% Triton X-100 in 1 × PBS was the optimal permeabilization method for each of the primary antibodies.

2. Choice of the PLA reagents.

Note: The PLA reagents are available to be purchased in a kit from Sigma-Aldrich (e.g., Duolink In Situ Red Starter Kit Mouse/Rabbit, Sigma-Aldrich, # DUO92101). The starter kits include all the reagents required for a PLA reaction including a pair of PLA probes, detection reagents, wash buffers, and a mounting solution. However, to be more cost-effective, the necessary *in situ* PLA reagents can be purchased separately and the remaining reagents can be prepared in the laboratory. In the “materials and equipment” sections we provide the protocols to prepare alternative buffers and solutions.

When choosing the *In Situ* PLA reagents, two critical elements should be considered:

- a. Both a Minus and a Plus PLA probe (complementary oligonucleotide conjugated secondary antibodies) are required.

△ **CRITICAL:** The choice of PLA probes depends on the species of the chosen primary antibodies. For instance, if the primary antibodies are raised in rabbit and mouse, one can choose an anti-rabbit Plus probe (Duolink In Situ PLA Probe Anti-Rabbit PLUS, Sigma-Aldrich, #DUO92002) and an anti-mouse Minus probe (Duolink In Situ PLA Probe Anti-Mouse MINUS, Sigma-Aldrich, #DUO92004).

Alternatively, one may choose an anti-mouse Plus probe (Duolink In Situ PLA Probe Anti-Mouse PLUS, Sigma-Aldrich, # DUO92001) and an anti-rabbit Minus probe (Duolink In Situ PLA Probe Anti-Rabbit MINUS, Sigma-Aldrich, # DUO92005).

- b. **PLA detection reagents:** Multiple fluorescent Duolink detection reagents are available (Red, green, orange and far red). Before beginning the experiment, ensure your microscope is equipped with the appropriate filter for the chosen fluorescence. Alternatively, Duolink bright field PLA systems are available for purchase.

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|--|---|
| Antibodies | | |
| Mouse monoclonal anti-galectin 1 (1:400) | Cell Signaling Technology | 40103 |
| Rabbit polyclonal anti-HOXA5 (1:400) | Abcam | ab82645 |
| Chemicals, peptides, and recombinant proteins | | |
| NeuroCult NS-A medium | STEMCELL Technologies | 05751 |
| Penicillin (100 U/mL)/Streptomycin (100 µg/mL) | Sigma-Aldrich | P4333 |
| Heparin solution (2 µg/mL) | STEMCELL Technologies | 07980 |
| Human EGF (20 ng/mL) | Miltenyi Biotec | 130-093-825 |
| Human FGF (10 ng/mL) | Miltenyi Biotec | 130-093-838 |
| Accumax solution | Innovative Cell Technologies | AM105 |
| Dulbecco's Modified Eagle's Medium (DMEM) | WISENT | 319-005-CL |
| Fetal Bovine Serum (FBS), Premium quality | WISENT | 080-450 |
| D-PBS | WISENT | 311-425-CL |
| Critical commercial assays | | |
| Duolink® <i>In Situ</i> Red Starter Kit Mouse/Rabbit (Includes Blocking solution, PLA Plus and Minus Probes, 5× Ligation Buffer, Ligase, 5× Amplification Buffer, Polymerase, Wash Buffers and Mounting solution with DAPI) | Sigma-Aldrich | DUO92101 |
| Experimental models: Cell lines | | |
| Human: Brain Tumor Stem cell (BTSC) 73, Passage 20–25 | Dr. Samuel Weiss - University of Calgary | N/A |
| LGALS1 CRISPR-CAS9 KO, Passage 20–25 | This study | N/A |
| HOXA5 CRISPR-CAS9 KO, Passage 20–25 | This study | N/A |
| Software and algorithms | | |
| Prism 8 | GraphPad | http://graphpad.com/ |
| Other | | |
| Triton-X 100 | Sigma-Aldrich | 9002-93-1 |
| Paraformaldehyde 16% (w/v) in aqueous solution methanol-free | VWR | ICNA0219998380 |
| Nunc Lab-Tek, II CC2 Chamber Slide System | Thermo Scientific | 154941 |
| T-75 culture flask for cells in suspension | Sarstedt | 833911502 |
| 37°C, 5% CO2 incubator for cell culture | VWR | VWR51014995 |
| Parafilm | Sigma-Aldrich | P7543 |
| Scale | Sartorius | BL1500 |
| PH meter | Thermo Fisher Scientific | OrionSTAR A111 |
| Water Bath | Thermo Fisher Scientific | TSGP10 |
| Sorvall ST8 Centrifuge | Thermo Fisher Scientific | 75007200 |
| Vortex 2 Geneie | Scientific Industries | G560 |
| Glass cover slips compatible with fluorescence microscopy | VWR | 48393-070 |
| Rocking Platform Shaker | VWR | 200 |
| Freeze block/Benchtop Coolers | Thermo Scientific | 355501 |
| Polyoxyethylene-20 (TWEEN 20) | Bio Basic | 12161902 |
| TRIS | BioShop | TRS003.5 |
| Humid Chamber | Jahani-Asl Lab | N/A |
| Fluorescence confocal microscope equipped with: a) excitation/emission filters compatible with red fluorophore and the nuclear stain, DAPI, b) Camera and software for image acquisition | ZEISS | LSM 800 |

MATERIALS AND EQUIPMENT

⌚ **Timing:** ~3 h

In this section, we describe the reagents that can be prepared and stored for later use. The reagents that are required to be freshly prepared are described in the [step-by-step method details](#) section.

△ **CRITICAL:** Many Duolink PLA reagents are supplied as concentrated stocks and must be diluted prior to use. Do not store diluted Duolink PLA reagents.

- **BTSC growth medium:** Prepare BTSC growth medium by supplementing NeuroCult NS-A medium with 100 U/mL penicillin, 100 µg/mL streptomycin, heparin, human EGF and human FGF as follows:

| Reagent | Final concentration | Amount |
|---|---------------------|---------|
| Streptomycin (50 mg/mL)- penicillin 50,000 U/mL | 0.5 mg/mL:500 U/mL | 500 µL |
| Heparin (2 mg/mL; 1000 IU/mL) | 2 µg/mL:1 IU/mL | 50 µL |
| Human EGF (40 mg/mL) | 20 µg/mL | 25 µL |
| Human FGF (20 mg/mL) | 10 µg/mL | 25 µL |
| NeuroCult NS-A medium | N/A | 49.4 mL |
| Total | N/A | 50 mL |

Note: BTSC growth medium supplemented with growth factors (EGF and FGF) can be stored at 4°C and used within 7–10 days. Bring the growth medium to room temperature for ~1 h before use, or warm it up in a water bath at 37°C for 10 min prior to use.

- **BTSC plating medium:** Prepare BTSC plating medium by supplementing DMEM medium with 100 U/mL penicillin, 100 µg/mL streptomycin and 10% Fetal Bovine Serum (FBS) as follows:

| Reagent | Final concentration | Amount |
|---|---------------------|---------|
| Streptomycin (50 mg/mL)- penicillin 50,000 U/mL | 0.5 mg/mL:500 U/mL | 500 µL |
| FBS | 10% | 5 mL |
| DMEM media | N/A | 44.5 mL |
| Total | N/A | 50 mL |

Note: BTSC plating medium can be stored at 4°C for up to 1 month. Bring the plating medium to room temperature ~1 h before use. Alternatively, warm it up in a water bath at 37°C for 10 min.

Alternatives: BTSC growth medium that is serum free (prepared in the previous step) can be used. In this case, the culture slides should be coated with laminin for 3 h prior to use at a concentration of 10 µg/mL.

- **Fixative solution (Paraformaldehyde (PFA) 4%):** Make a 4 times dilution of PFA (16%) in 1 × PBS. The 4% solution can be store at 4°C for one month.

Note: For this experiment purchased 1 × PBS (WISENT, # 311-425-CL) was used. Nucleases may impact amplification, if this occurs trouble shooting using RNase and DNase free PBS (Thermo Fisher Scientific, # 70011044) may be required.

Note: The 4% PFA must be at room temperature (22°C–25°C) before use.

- **Permeabilization solution (0.5% Triton X-100 in 1 × PBS):** Add 100 µL of Triton X-100 to 20 mL of room temperature (22°C–25°C) 1 × PBS. Store the solution at 4°C for up to one month.
- **Blocking solution:** A blocking solution is included in the Duolink *In Situ* Starter kits purchased from Sigma. Alternatively, a blocking solution can be prepared by dissolving 3 mg of Bovine serum

albumin (BSA) in 100 mL of 1× PBS to make 3% BSA-PBS. Aliquot and store at −20°C for up to 12 months.

- **Antibody diluent:** The antibody diluent solution is used to dilute the primary antibodies and the Minus and Plus PLA probes. This is provided in the Duolink Starter kit. Alternatively, a homemade antibody diluent solution can be prepared in the laboratory as follows: dissolve 1 mg of BSA in 100 mL 1× PBS. Aliquot the prepared solution and store at −20°C for up to 12 months. Note that the blocking solution can be used as antibody diluent if the background is high.
- **1× wash buffer A:** Wash buffer A is provided as a powder in the Duolink Starter kit. However, it can also be prepared in the lab as follows:

| Reagent | Final concentration | Amount |
|-------------------------|---------------------|--------|
| NaCl | 0.15 M | 8.8 g |
| Tris base | 0.01 M | 1.2 g |
| Tween 20 | 0.05% | 0.5 mL |
| Milli-Q Ultrapure water | N/A | 1 L |

Adjust pH to 7.4 using HCl. Filter the solution through a 0.22 µm filter and store at 4°C for up to 12 months and protected from light.

- **1× wash buffer B:** Wash buffer B is provided as a powder in the Duolink Starter kit. However, it can also be prepared in the lab as follows:

| Reagent | Final concentration | Amount |
|-------------------------|---------------------|--------|
| NaCl | 0.10 M | 5.84 g |
| Tris base | 0.04 M | 4.24 g |
| Tris-HCl | 0.16 M | 26.0 g |
| Milli-Q Ultrapure water | N/A | 1 L |

Adjust pH to 7.5 using HCl. Filter the solution through a 0.22 µm filter and store at 4°C for up to 12 months and protected from light.

Note: Bring the solutions to room temperature (22°C–25°C) before use.

Alternatives: If a Duolink PLA Starter kit is chosen, the wash buffer A and B will be provided. Dissolve one pouch of each buffer in Milli-Q Ultrapure water to a final volume of 1 L and store at 4°C.

- **0.01× wash buffer B:** Dilute wash buffer B (1:100) in Milli-Q Ultrapure water.

STEP-BY-STEP METHOD DETAILS

Brain tumor stem cell (BTSC) culture and maintenance

⌚ **Timing:** ~1 h

This section describes the passaging and maintenance of BTSC spheres. Incubation and growth time may vary depending on the number of cells required for a specific assay or experiment.

1. Maintain patient derived BTSCs in 5% CO₂ at 37°C in BTSC growth medium and in T-75 culture flasks for cells in suspension as spheres.
2. Collect BTSC spheres by gently mixing the flask to ensure collection of as many spheres as possible, and transfer the content to a 15 mL falcon tube using a 10 mL pipette.
3. Centrifuge for 10 min at 200 × g at room temperature (22°C–25°C).

4. Using a vacuum pump, carefully aspirate media as to not disturb the pellet.
5. Add 200 μL Accumax cell dissociation solution to the pellet. Using a P200 pipette gently triturate the sample.
6. Incubate at 37°C for a total of 10 min. After 5 min of incubation, gently flick the tube 3–5 times to prevent the settling of the BTSC spheres at the bottom of the tube.
7. Following the incubation period, add 800 μL of BTSC growth media.
8. Triturate the cell suspension using a 1000 μL pipette followed by a 200 μL pipette to complete dissociation if required.
9. Count the number of viable cells using an exclusion dye such as trypan blue.

Note: If extra cells are obtained, BTSCs can be cryopreserved. Following Accumax dissociation, centrifuge the cells to discard the media. Resuspend the cell pellet in 500 μL of BTSC growth media containing 10% DMSO, transfer to a cryogenic tube and store at -80°C overnight (12–16 h). Transfer to a liquid nitrogen storage box for long term storage.

10. Seed cells at a density of $\sim 0.5 \times 10^6$ cells in a T-75 flask in 10 mL BTSC growth medium.

Note: BTSC lines can be seeded at variable densities based on their growth rate. The growth rate is variable from one BTSC line to another depending on the co-occurrence of oncogenic mutations that each cell line harbors. For example, BTSC lines that harbor the EGFRvIII mutation have a higher proliferation rate than those that lack the mutation. Typically, BTSC lines must be passaged every week.

11. Maintain cells up to 7 days in culture. This will yield around $\sim 7 \times 10^6$ cells. If BTSC spheres are less than 150 μm in diameter and the media has become acidic, recognized by a yellow color, then add 3 mL of fresh BTSC media to the flask until next passage.

Preparation of BTSCs

⌚ **Timing:** $\sim 1\text{--}2$ h

This section describes the procedure required to prepare BTSCs for proximity ligation assay (PLA). The following details are described for a Lab-Tek II, CC2-treated chamber slide 0.7 cm^2 sample well. However, cell density can be adjusted according to the chamber slide in use.

Note: The quality of chamber slides is very important. Various brands were tested but Nunc Lab-Tek II CC2 Chamber Slide System has given us the best results. Chamber slides with a synthetic base should be avoided. In general, an optically clear glass base works optimally.

△ CRITICAL: If you are using other chamber slides with low adhesive properties, a 10 $\mu\text{g}/\text{mL}$ poly-D-lysine-coating (PDL) solution may be required prior to use. The working PDL solution can be made from a 2 mg/mL stock (Fisher Scientific, # CB-40210) using sterile water. PDL coating solution should be left on the slide overnight (12–16 h) in an incubator at 37°C and washed thoroughly (3 times) with sterile water prior to addition of cell suspension. If you are using serum free media, the chamber slides should be coated first with a 10 $\mu\text{g}/\text{mL}$ PDL solution overnight (12–16 h) at 37°C followed by a 10 $\mu\text{g}/\text{mL}$ laminin solution for 3 h at 37°C and washed once with PBS prior to use. Laminin solution can be made from a 1–2 mg/mL stock (Sigma-Aldrich, #L2020) with sterile water.

12. Warm BTSC plating medium in a water bath at 37°C for ~ 10 min.
13. Incubate wells with 300 μL of warm BTSC plating media in a biological safety cabinet.
14. Take 3–4 days old BTSCs from step 11. BTSCs spheres will be around 250 μm in diameter.

15. Using a 10 mL serological pipette, collect media and cells by gently mixing and pipetting all the BTSC spheres and transfer them to a 15 mL polypropylene conical tube.
16. Centrifuge for 10 min at $200 \times g$ at room temperature (22°C–25°C) and discard the media by aspiration.
17. Add 200 μL of Accumax solution and mix gently using a P200 micropipette and incubate at 37°C for 10 min in the incubator or in a water bath.

Note: During the incubation, the large spheres have the tendency to settle at the bottom of the tube. After 5 min of incubation, gently flick the tubes 3–5 times to prevent the spheres from settling down and allow an efficient cell dissociation by Accumax.

18. Remove the cell-Accumax containing tubes from the incubator and triturate gently 10 times using a P200 micropipette and low retention filtered long pipette tips to homogenize.

Note: Certain BTSC lines require a shorter Accumax incubation time to be dissociated into single cell suspensions. The incubation time is the amount of time needed to effectively dissociate the BTSC spheres.

△ CRITICAL: It is important to obtain a single cell suspension. Cell clumps will result in a non-homogenous culture leading to overlapping PLA puncta and preventing the quantification of PLA signals at single cell resolution.

19. Add 800 μL of BTSC plating media to the 200 μL Accumax-cell suspension.

△ CRITICAL: Maintain BTSCs in serum-free medium. Following cell counting, if the remaining cell-suspension is being passaged for future use, take the cells needed for maintenance before adding the serum-containing plating medium for PLA experiments. In this case adjust the volume of the plating medium being added to obtain a total volume of 1 mL.

20. Count the number of viable cells using an exclusion dye such as trypan blue.
21. Prepare a cell suspension of 10^5 cells/mL in BTSC plating medium and homogenize by pipetting up and down using a P1000 micropipette set at 800.
22. Remove the media from the wells from step 13.
23. Plate BTSCs at a density of $\sim 28 \times 10^3 / \text{cm}^2$ by distributing 200 μL of the cell suspension to each 0.7 cm^2 well.

△ CRITICAL: Cell density is an important parameter that impacts the quantification of PLA signal. The confluency of cells for PLA assay should be within 50%–70% to allow quantification of PLA signal at a single cell resolution. The optimal number of cells to be plated should be tested. When using different cell lines, perform a serial dilution of cell numbers. For most of BTSC lines 28×10^3 in 0.7 cm^2 well is an optimal cell density.

24. Incubate the newly plated cells under the biological hood for 5 min with minimum vibrations to allow the cells to attach to the bottom of the wells. Incubate cells for 1 h at 37°C with 5% CO_2 in an incubator.

△ CRITICAL: Ensure that the BTSC spheres are well dissociated and there are no apparent cell aggregates in the cell suspension. It is important to check the wells before incubating at 37°C to ensure a homogeneous distribution of the cells in each well. When this is confirmed, avoid shaking the plate until the cells are attached to the wells. Cell aggregates will lead to overlapping PLA signals and high background.

25. After 1 h of incubation, move to the fixation and permeabilization step.

△ **CRITICAL:** Following the incubation period, it is highly recommended to check the cells under the microscope before continuing the assay. This is to make sure that the cells have adhered to the bottom of the well and to verify that cell distribution is homogeneous and that there are no cell aggregates.

Cell fixation

⌚ **Timing:** ~20 min

This section describes the method used to fix BTSCs for subsequent processing. Other cell lines may require optimization of fixation parameters to obtain optimal results.

26. Aspirate media and wash cells by gently distributing 200 μ L 1 \times PBS to each chamber. Immediately remove the PBS and repeat the wash one more time.

Note: A low speed aspirator is used, but pipette aspiration also works to aspirate the solution.

27. Aspirate PBS and fix the cells by adding 100 μ L of room temperature (22°C–25°C) 4% PFA in 1 \times PBS to each well.

△ **CRITICAL:** Protein-protein interaction could be modulated by different stress signals. Therefore, rapid fixation of the cells is recommended to avoid any impact of experimental procedures on the protein-protein complexes.

28. Incubate for 15 min at room temperature (22°C–25°C).

29. Aspirate PFA and wash the cells twice with room temperature (22°C–25°C) 1 \times PBS.

Note: PFA should be handled under a certified chemical fume hood. Perform the preparation of PFA, fixation of cells and washing steps after PFA incubation under the hood.

△ **CRITICAL:** Choosing the right fixative is very important because it can impact the efficiency of the antibody binding to its substrate thereby reducing the expected signal.

⏸ **Pause point:** The experiment can be stopped at this step and the fixed cells can be stored at 4°C for later processing. Fixed cells can be stored up to ~ 1 month at 4°C. If storing fixed cells for a short period (less than 1 week), ensure that the well is filled with 1 \times PBS and wrapped with parafilm to avoid drying of the wells. If storage for more than 1 week is required, store sample at 4°C with 0.01% sodium azide in 1 \times PBS.

Permeabilization and blocking of cells

⌚ **Timing:** ~40 min

This section describes the method in which cells are permeabilized at the level of the cellular membrane and the nuclear membrane. Once permeabilization is completed, subsequent reagents can move freely between compartments. For this reason, blocking of non-specific binding sites is essential to achieve a quantifiable and accurate signal.

30. Gently remove the PBS solution with a vacuum pump.

△ **CRITICAL:** PFA fixation will not permeabilize the samples, thus a separate permeabilization step is needed. Permeabilization allows antibodies to access intracellular structures while leaving the morphological characteristics of the cells intact. Triton, and saponin

are examples of permeabilization reagents which act by disrupting the cellular membrane (Jamur and Oliver, 2010). If alcohol fixative is used (e.g., 70% ethanol, methanol) no permeabilization reagent is needed (Jamur and Oliver, 2010).

31. To permeabilize fixed cells, add 100 μ L of permeabilization solution (0.5% Triton X in 1 \times PBS) and incubate for 20 min at room temperature (22°C–25°C).

△ CRITICAL: Protein-protein interaction may occur in the various cell compartments (e.g., cytoplasmic, nuclear, mitochondrial). Epitope access in the different cell compartments may require different levels of permeabilization. In our experiments, 0.5% Triton X-100 in 1 \times PBS was efficient to permeabilize the nuclear membrane to assess HOXA5-galectin1 interaction in the nucleus.

32. Remove the permeabilization solution with a vacuum pump.
33. Wash twice with room temperature (22°C–25°C) 1 \times PBS.
34. Mix the blocking solution and add 100 μ L of the blocking solution to each well.

Alternatives: The blocking buffer is commercially available in the Duolink PLA kit however, a homemade solution can be prepared (See section – Materials and equipment).

35. Incubate the chamber slides in a heated humid chamber for 1 h at 37°C under gentle agitation on a up/down rocking shaker (2 rpm).

△ CRITICAL: Ensure that the entire well/sample is covered with the blocking solution. Avoid drying of the samples as this will increase the background.

⏸ Pause point: The wells with sample containing blocking solution can be stored overnight at 4°C in a humid chamber.

Primary antibody hybridization

⌚ **Timing:** overnight (12–16 h)

This section describes the method in which permeabilized and blocked cells are incubated with diluted primary antibodies against the desired proteins to achieve an antibody-antigen reaction. This reaction is critical as it will determine the sensitivity of the assay.

36. In one Eppendorf tube, dilute the primary antibodies (specific to the proteins of interest) to the optimal dilution in the antibody diluent buffer, as described above. In the current protocol, mouse galectin-1 and rabbit HOXA5 antibodies are used at a dilution of 1:400 in the diluent buffer.

Alternatives: The antibody diluent buffer is commercially available in the Duolink PLA kit, however, it can be prepared in-house (See section - Materials and equipment).

△ CRITICAL: Multiple negative controls can be used. Omitting the primary antibodies provides a negative control for the PLA signal. An ideal negative control, however, is to generate knockout (KO) cells for the protein of interest and run the procedures in parallel. In our experiment, we probed the LGALS1 CRISPR KO BTSCs (negative control 1) and HOXA5 CRISPR KO BTSCs (negative control 2) with the pair of galectin-1 and HOXA5 primary antibodies. Additionally, a combination of a primary antibody with an anti-IgG control can be used as a negative control. For example, a combination of mouse

anti-galectin-1 and rabbit anti-IgG or a combination of rabbit anti-HOXA5 and mouse anti-IgG can be used as negative controls.

37. Following blocking, add 100 μ L of 1 \times PBS to each well without removing the blocking solution and rapidly aspirate using a vacuum pump.

Alternatives: Aspiration can be done using a micropipette. However, when the assay is performed on multiple samples, it is recommended to use a vacuum pump to move quickly to the next step and avoid drying of the samples.

38. Add 40 μ L of primary antibody-diluent solution and incubate overnight (12–16 h) at 4°C.

△ CRITICAL: Incubate samples overnight (12–16 h) at 4°C in a humid chamber to avoid sample drying and on an up/down rocking shaker to ensure an even distribution of the solution. Fill unused wells with 1 \times PBS.

Primary antibody washing

⌚ Timing: ~ 20 min

This section describes the method used to wash samples following the antibody hybridization step. Efficient washing is essential as the PLA secondary antibody probes will hybridize only with antibodies that remain after washing.

39. Remove buffer A from the fridge (4°C) and bring it to room temperature (22°C–25°C) before use (~1 h).

Alternatives: Although the wash buffer A is commercially available (Duolink In Situ Wash Buffer A in starter kit), a homemade wash buffer A can be prepared in the laboratory (See section - Materials and equipment).

40. Without removing the primary antibody-diluent buffer solution, add 300 μ L of wash buffer A in each well and rapidly aspirate using a vacuum pump.
41. Add 300 μ L of wash buffer A to each well and incubate for 5 min at room temperature (22°C–25°C) in a humid chamber on an up/down rocking shaker at low speed (2 rpm).
42. Aspirate the wash buffer A and repeat washing twice.

Note: During the washing step, prepare PLA Probes for the next step (PLA probe incubation).

PLA probe incubation

⌚ Timing: ~1 h

This section describes the method of how PLA probes are prepared and incubated with the sample. The PLA secondary antibody probes are conjugated to oligonucleotides that will give rise to a circular product if in proximity, indicative of a protein-protein interaction.

43. While the samples are being washed, take the PLA Plus and Minus probes out of the fridge (4°C) and vortex briefly.
44. Dilute the 5 \times PLA probes in antibody diluent buffer at a dilution of 1:5. For each sample, prepare 40 μ L of 1 \times PLA probes as follows:

| Reagent | Amount |
|-------------------------|------------|
| PLA probe MINUS stock | 8 μ L |
| PLA probe PLUS stock | 8 μ L |
| Antibody diluent buffer | 24 μ L |

Note: To ensure efficiency, prepare a master mixture for multiple samples and mix well before applying to the wells. Also prepare a master mixture for the negative controls.

45. Aspirate the wash buffer A from last washing using a vacuum pump.
46. Add 40 μ L of 1 \times PLA probe mix to each sample, including the negative controls and incubate in a preheated humid chamber for 1 h at 37°C on an up/down rocking shaker at low speed (2 rpm).

PLA probe washing

⌚ Timing: ~ 20 min

This section describes the method used for washing sample following PLA probe incubation. Efficient washing is essential as the ligase will ligate secondary antibody conjugated oligonucleotides that remain bound to the primary antibody.

47. Without removing the PLA probe, add 300 μ L of wash buffer A to each well and rapidly aspirate using a vacuum pump.

Note: The commercial or the homemade wash buffer A can be used.

48. Add 300 μ L of wash buffer A to each well and incubate for 5 min at room temperature (22°C–25°C) in a humid chamber on an up/down rocking shaker at low speed (2 rpm).
49. Aspirate the wash buffer A and repeat washing twice.

Note: During the washing step, prepare the ligation solution for the next step (Ligation).

Ligation

⌚ Timing: ~45 min

This section describes the method in which the ligation mixture is prepared and how the incubation is conducted to allow for maximal ligation of the oligonucleotide probes. The ligase enzyme will hybridize the two oligonucleotides of the two PLA probes and form a closed DNA circle if the two proteins of interest are in proximity (less than 40 nm).

50. During the last wash, thaw the 5 \times ligation buffer (stored at –20°C) before use.
51. Prepare 1 \times ligation buffer by diluting the 5 \times ligation buffer in Milli-Q water. For each sample, prepare 40 μ L of 1 \times ligation buffer as follows:

| Reagent | Amount |
|----------------------------|------------|
| 5 \times Ligation buffer | 8 μ L |
| Milli-Q Ultrapure water | 32 μ L |

⚠ CRITICAL: Make sure the ligation buffer is completely thawed and well homogenized prior to use. Keep the prepared 1 \times ligation buffer at room temperature.

52. Retrieve the ligase (1 U/ μ L) from the freezer using a freezer block (-20°C). Prepare 40 μ L of 1 \times ligase solution per reaction as follows:

| Reagent | Amount |
|---|------------|
| Ligase (1 U/ μ L) | 1 μ L |
| 1 \times ligation buffer (from step 35) | 39 μ L |

△ CRITICAL: Add the ligase to the 1 \times ligation buffer immediately prior to addition to the sample. Ligase mix should remain on the freezer block.

Note: For maximum efficiency, prepare a master mixture for multiple samples and mix well before applying to the wells, do the same for the negative controls.

53. Gently remove the wash buffer A from last wash using a vacuum pump.
 54. Add 40 μ L of the ligation reaction mixture into each well including negative controls.
 55. Incubate for 30 min at 37°C in a humid chamber under gentle agitation on a up/down rocking shaker (2 rpm).

Ligation washing

⌚ Timing: \sim 20 min

This section describes the method used for washing sample following ligation. Efficient washing is essential as amplification will take place based on the DNA circle formed in the ligation step.

56. Without removing the ligation-ligase solution, add 300 μ L of wash buffer A to each well and rapidly aspirate using a vacuum pump.
 57. Add 300 μ L of wash buffer A to each well and incubate for 5 min at room temperature (22°C – 25°C) in a humid chamber on an up/down rocking shaker at low speed (2 rpm).
 58. Aspirate wash buffer A and repeat washing twice.

Note: During this washing step, prepare the rolling circle amplification (RCA) solution for the next step.

Rolling circle amplification (RCA)

⌚ Timing: \sim 100 min

This section describes the method in which the amplification mixture is prepared and incubated with sample. The circular DNA probe will be amplified via rolling circle amplification. The rolling circle amplification reaction occurs in the presence of DNA polymerase and the nucleotide mixture present in the amplification buffer. The amplification buffer contains fluorescently labeled oligonucleotides which will hybridize to a specific sequence in the rolling circle amplification product and the signal will be easily visible as a distinct fluorescent spot by a fluorescence microscope.

59. During the last wash, thaw the 5 \times amplification buffer (stored at -20°C).
 60. Prepare 40 μ L of amplification buffer per reaction as follows:

| Reagent | Amount |
|-------------------------|--------|
| 5× amplification buffer | 8 μL |
| Milli-Q Ultrapure water | 32 μL |

△ **CRITICAL:** The amplification buffer contains fluorescent dyes and should be protected from the light. Ensure the amplification buffer is completely thawed and mixed prior to using.

- Keep the prepared 1× amplification buffer at room temperature (22°C–25°C) and protected from light.
- Retrieve the polymerase enzyme (10 U/μL) from the freezer using a freeze block (–20°C). Prepare 40 μL of polymerase solution per reaction as follows:

| Reagent | Amount |
|--|---------|
| Polymerase (10 U/μL) | 0.5 μL |
| 1× amplification buffer (from step 45) | 39.5 μL |

△ **CRITICAL:** Add the polymerase to the 1× amplification buffer immediately prior to addition to the sample. Polymerase should stay on the freezer block while the prepared mix stays at room temperature (22°C–25°C) protected from light.

Note: To maximize efficiency, prepare a master mixture for multiple samples and mix well before applying to the wells. Also prepare a master mixture for the negative controls.

- Gently remove the wash buffer A from last wash with a vacuum pump.
- Add 40 μL of the amplification mix into each well including negative controls.
- Incubate for 100 min at 37°C and protected from light in a humid chamber under gentle agitation on an up/down rocking shaker (2 rpm).

△ **CRITICAL:** Follow the recommended amplification time as extended amplification duration can cause signal coalescence.

Rolling circle amplification washing

⌚ **Timing:** ~ 20 min

This section describes the method used for washing sample following amplification.

- Bring the wash buffer B (stored at 4°C) to room temperature (22°C–25°C) before use (~1 h).

Alternatives: Although the wash buffer B is commercially available (Duolink In Situ Wash Buffer B – available in the starter kit), a homemade wash buffer B can be prepared in the laboratory (See section - Materials and equipment).

- Without removing the amplification-polymerase solution, add 300 μL of wash buffer B to each well and rapidly aspirate using a vacuum pump.
- Add 300 μL of wash buffer B to each well and incubate for 10 min at room temperature (22°C–25°C) in a humid chamber on an up/down rocking shaker at low speed (2 rpm).
- Aspirate the wash buffer B and repeat the washing for another 10 min.
- Aspirate the wash buffer B and wash with 300 μL of the 0.01× wash buffer B for 1 min.

Note: At this step, the PLA-stained samples can be further immunostained using classical immunofluorescence or probe-based methods. In this case, avoid to use primary antibodies raised in the same species as those used for the PLA assay. Ensure that the probes or secondary antibodies are conjugated to different fluorophores than those of the PLA to be able to identify each signal. It is possible to co-stain the PLA signals with dyes that stain live cells such as labeling the mitochondria with MitoTracker. In this case, the mitochondria should be labeled with the MitoTracker dye in live cells before fixing the cells for the PLA assay (Sharanek et al., 2020).

Mounting the slides for imaging

⌚ Timing: ~ 30 min

This section describes the method used to prepare slides and mount slides using a mounting solution containing DAPI to subsequently visualize the nucleus and PLA puncta using fluorescent microscopy. Effective mounting of slides is important for the quality visualization and to ensure samples can be stored if needed.

71. Aspirate the 0.01 × wash buffer B using the vacuum pump.
72. Using the removal tool provided with the Lab-Tek chamber slides, carefully separate the chamber from the slide.
73. Remove any additional liquid remaining on the slide by holding the slide horizontally on a filter paper or a paper towel.
74. Air-dry the slide for 3 min at room temperature (22°C–25°C) and protect from light.
75. Add 3 μL of mounting medium with DAPI (Duolink *In Situ* Mounting Medium with DAPI- available in the starter kit) on each sample slide section.

Alternatives: Duolink *In Situ* Mounting Medium with DAPI can be replaced by ProLong Gold antifade reagent with DAPI (Life Technologies, # P-36931), or other mounting media with DAPI.

76. Place a rectangular cover glass on the slide and make sure that the mounting medium is spread evenly over the samples.

Note: Avoid forming air bubbles under the coverslip.

77. Seal the edges of the coverslip to the slide with clear nail polish.
78. Keep the slides under the fume hood for 30 min and protected from light to allow the nail polish sealing to dry.

⏸ **Pause point:** One can store the slides at –20°C for later acquisition of the microscopic images. Mounted slides can be stored at –20°C and protected from light for up to 6 months.

Image acquisition and quantification

⌚ Timing: ~ 3 h

This section describes the parameters used to visualize the PLA signal and to compare this signal with other conditions (ex. wild type vs. knockout cells). Following visualization, PLA puncta can be quantified as described in this section and statistical analysis can be carried out to determine the presence of the signal and in which compartment the signal is originating from.

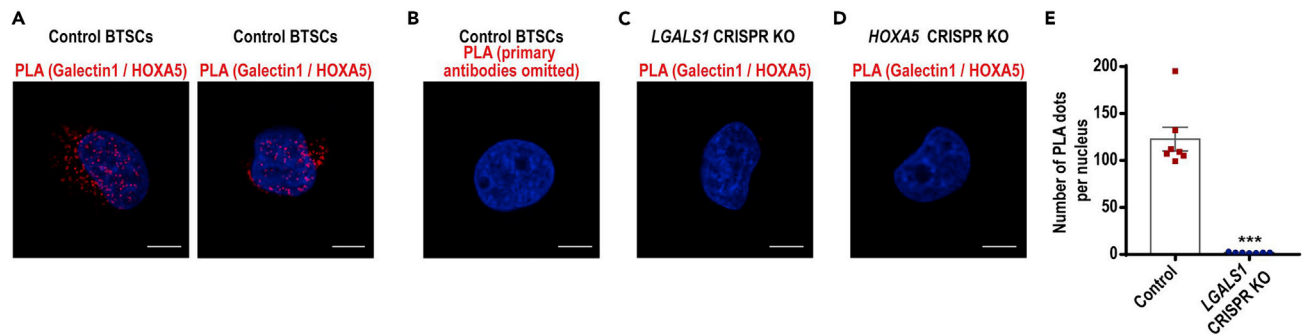


Figure 2. Detection of galectin1-HOXA5 protein interaction by proximity ligation assay in brain tumor stem cells (BTSCs)

Control BTSCs, *LGALS1* CRISPR KO and *HOXA5* CRISPR KO BTSCs were subjected to PLA, as described above.

(A) Each red spot represents a single interaction between *HOXA5* and galectin1 molecules.

(B–D) Controls include the omission of primary antibodies (B), PLA in *LGALS1* CRISPR KO (C) and PLA in *HOXA5* CRISPR KO (D). Nuclei were stained with DAPI. Scale bar, 10 μ m.

(E) The number of PLA dots per nucleus was quantified in control BTSCs and *LGALS1* CRISPR KO. Data are presented as the means \pm SEM, $n = 7$ images. Each dot represents the number of PLA dots per nucleus in each image. Unpaired two-tailed t test, $p < 0.0001$.

79. Visualize and capture images using a fluorescence microscope with the appropriate filters for the detection of DAPI and the used fluorophore with 40–60 \times objective. In our experiment, PLA signals were captured using a 63 \times oil objective lens (Figure 2) on a laser scanning confocal microscope (ZEISS LSM 800). It is critical that the acquisition parameters for the images in the experimental and control conditions be kept the same in order to ensure proper comparison.
80. Count the number of PLA dots and nuclei in each image. Divide the number of PLA dots by the number of nuclei in each image to obtain the number of PLA dots per nucleus. To enable statistical analysis a minimum of $n=3$ biological replicate images should be assessed. Plot data as shown in Figure 2E.

Note: In this protocol we performed manual quantification of the PLA dots. However, automated analysis can be performed using the ImageJ software (<https://imagej.nih.gov/ij>).

81. Perform statistical analysis by means of a student t-test to determine the significance between the mean values of the two conditions that are being compared (number of PLA dots per nucleus in control versus *LGALS1* CRISPR KO).

Note: A student t-test can be used when comparing the means of two different conditions, however one way ANOVA for multiple comparisons may be employed to compare three or more conditions.

EXPECTED OUTCOMES

Successful execution of this assay should yield a PLA signal that is recognized as discrete fluorescent spots (Figure 2A) if the proteins are less than 40 nm from each other (Bagchi et al., 2015; Fredriksson et al., 2002). In our experiments control BTSCs were used as well as CRISPR KO lines to determine the specificity of the signal (Figures 2C and 2D). PLA signals should not be detected in the sample in which the primary antibodies were omitted (negative control 1) (Figure 2B), nor in the *LGALS1* or *HOXA5* CRISPR KO (negative control 2–3, respectively) (Figures 2C–2E). Finally, if a PLA signal is identified when there is no primary antibody, this may suggest that the PLA kit is no longer functioning as it may be self-ligating. Furthermore, if a PLA signal is detected in the KO, troubleshooting steps should be considered such as blocking time, primary antibody dilution and duration of RCA.

LIMITATIONS

This protocol outlines the use of PLA which requires two antibody recognition events; the primary antibodies binding to the two proteins of interest, and the secondary antibodies binding to the primary

antibodies (Weibrecht et al., 2010). Although this method is useful in generating highly precise and quantifiable results, it is limited to the availability of primary antibodies from specific sources if commercially available secondary antibody probes are used. Alternatively, a direct method can be used in which the primary antibodies are directly conjugated to the PLA probes (Weibrecht et al., 2010).

TROUBLESHOOTING

Problem 1

There is a high background signal which may be the result of improper primary antibody concentration, inefficient blocking or the samples being dried out.

Potential solution

Titrate the primary antibodies individually to assess at which concentration the puncta are clearly identifiable. This is accomplished by starting with a concentration at which the signal is saturated followed by serial dilution. Furthermore, increasing the duration of incubation with blocking solution (step 35) can help in reducing the non-specific binding and the background signal. In addition, ensuring that the sample remains moist during the entire experiment is essential to avoid any additional background, this can be achieved by placing the samples in a humid chamber during each incubation period as well as filling all unused wells with PBS.

Problem 2

There is no PLA signal in the nucleus although the proteins of interest are in the nucleus, but there is signal throughout the rest of the cell. This may be due to the duration of permeabilization.

Potential solution

Increase the duration of incubation with permeabilization solution (0.5% Triton X-100 1 × PBS) (step 31). Incubation time may be increased as long as 30 min, although increasing the duration of incubation any further may impact the structure of the cell.

Problem 3

Lack of a PLA signal even though other methods of protein-protein interaction have consistently shown that the proteins in question are interacting. Although there may be multiple possible explanations for this, some include inefficient binding or no binding of the primary antibody to the target protein, inefficient ligation and amplification. In addition, there could be a possibility that the reagents are expired.

Potential solution

Ensure that the primary antibodies are binding the target protein efficiently. This can be done by testing the antibody using another antibody-protein based assay such as immunofluorescence (IF). Furthermore, the ability of the primary antibody to bind the target protein is impacted by appropriate fixation (steps 26–29) and permeabilization (steps 30 and 31) of the cells. In addition, proper ligation conditions are essential when conducting PLA. For instance, ligation mixture may not be as efficient if excess wash buffer is left on the sample as this will dilute the ligation solution (steps 47–49). In addition, ensure that the ligase is added immediately prior to use to ensure the enzyme is not denatured (step 52). Finally, when working with low abundance binding proteins, amplification duration can be extended to allow a more robust signal to be visualized (step 65).

Problem 4

There are few to no cells that can be identified upon visualization. This may be due to inefficient adhesion of the BTSCs to the slide surface.

Potential solution

Incubate single BTSC suspension on the chamber slide for up to 6 h to ensure efficient attachment (step 24) of the cells to the surface of the slide, however different cell lines may require variable

incubation times. It is critical that the cells properly adhere to the slide for all subsequent PLA steps. If after 6 h cell attachment is still poor, coating with PDL and/or laminin solution may be required prior to plating the single cell suspension. These agents will promote further attachment to the slide surface. PLA can also be performed on BTSCs under floating conditions in an Eppendorf tube (Jahani-Asl et al., 2016), however, the method described here requires less reagents and is more efficient.

Problem 5

The PLA signal is very faint although the specificity of the antibody has been verified, and the duration of ligation and amplification has been optimized. This may be due to the sample being exposed to light for a prolonged period of time.

Potential solution

Once the amplification solution is added to the sample, it is imperative that the sample be shielded from light during amplification incubation (step 65), subsequent washing steps (steps 66–70) and during mounting as much as possible (steps 71–78). This can be achieved by either using a light omitting humid chamber or by wrapping the humid chamber in a light resistant material such as aluminum foil.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Arezu Jahani-Asl (arezu.jahani@uottawa.ca).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate any unique datasets.

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AUTHOR CONTRIBUTIONS

A.S. optimized the conditions for PLA of BTSCs and performed experiments. A.S. and L.R. have performed PLA experiments, jointly wrote the protocol, and generated the figures. V.S.D. contributed to reagents and supervision of L.R. A.J.-A. conceived the research program, contributed to writing and editing of the paper, and provided supervision and funding.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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