Protocol
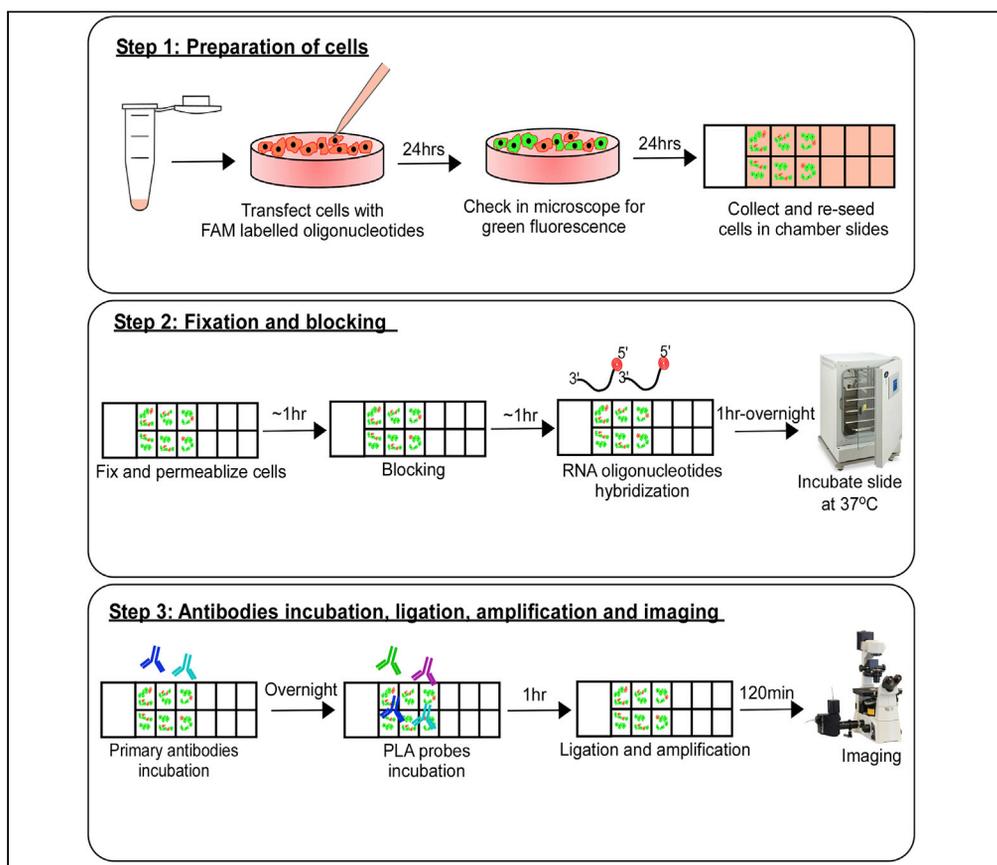
Optimized proximity ligation assay (PLA) for detection of RNA-protein complex interactions in cell lines

Step 1: Preparation of cells

Transfect cells with FAM labelled oligonucleotides — 24hrs — Check in microscope for green fluorescence — 24hrs — Collect and re-seed cells in chamber slides

Step 2: Fixation and blocking

Fix and permeablize cells — ~1hr — Blocking — ~1hr — RNA oligonucleotides hybridization — 1hr-overnight — Incubate slide at 37°C

5′ 5′
3′ 3′

Step 3: Antibodies incubation, ligation, amplification and imaging

Primary antibodies incubation — Overnight — PLA probes incubation — 1hr — Ligation and amplification — 120min — Imaging

Jasmine George, Sonam Mittal, Ishaque P. Kadamberi, Sunila Pradeep, Pradeep Chaluvally-Raghavan

pchaluvally@mcw.edu

Highlights

FXR1 binds to the AU-rich elements (ARE) within cMYC 3′UTR

Use of fluorescence-labeled mRNA improves the specificity of PLA reaction

Linear connectors linked to the probes produce high levels of PLA signals

Conventional proximity ligation assay (PLA) suffers from target specificity issues that curtail their accuracy on interpreting proximal interactions in cell biology. Here, we present a reliable and sensitive approach by including a fluorochrome-labeled mRNA fragment along with biotin-labeled RNA probe and a target-specific antibody, which were used to generate proximal ligation signals through linear connectors in intact cells. This protocol will be particularly useful for studying the proximal interactions between RNA binding proteins (RBPs) and their target mRNAs in cells.

Protocol

Optimized proximity ligation assay (PLA) for detection of RNA-protein complex interactions in cell lines

Jasmine George,[1,4] Sonam Mittal,[1] Ishaque P. Kadamberi,[1] Sunila Pradeep,[1,2,3] and Pradeep Chaluvally-Raghavan[1,2,3,5,]*

[1]Department of Obstetrics and Gynecology, Milwaukee, WI 53226, USA

[2]Department of Physiology, Milwaukee, WI 53226, USA

[3]Medical College of Wisconsin Cancer Center, Milwaukee, WI 53226, USA

[4]Technical contact

[5]Lead contact

*Correspondence: pchaluvally@mcw.edu
https://doi.org/10.1016/j.xpro.2022.101340

SUMMARY

Conventional proximity ligation assay (PLA) suffers from target specificity issues that curtail their accuracy on interpreting proximal interactions in cell biology. Here, we present a reliable and sensitive approach by including a fluoro-chrome-labeled mRNA fragment along with biotin-labeled RNA probe and a target-specific antibody, which were used to generate proximal ligation signals through linear connectors in intact cells. This protocol will be particularly useful for studying the proximal interactions between RNA binding proteins (RBPs) and their target mRNAs in cells.

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

BEFORE YOU BEGIN

Order RNA oligonucleotides, prepare buffers, and make the cells ready. The optimal number of cells to be plated should be tested. We initially performed a serial dilution of cell for calibration. For example, for a 24-well plate, we plated 5×10^3, 1×10^4 and 1×10^5 HeyA8 cells and cultured overnight.

The study of RNA–protein interactions has primarily relied on the use of biochemical technique, such as immunoprecipitation or pull-down assays which often gives off target effects (Niranjanakumari et al., 2002). Target specificity issues are also associated with fluorescence *in situ* hybridization (FISH) assay that have been developed to visualize nucleic acid targets in cells. PLA protocols have been developed and refined for advancement to study RNA–protein interactions in cells (Jung et al., 2013a; Jung et al., 2013b; Weibrecht et al., 2013). However, these methods are relatively complex with involvement of local-ized cDNA synthesis using a primer containing locked nucleic acid-modified bases followed by visualiza-tion with padlocked probes and target-primed amplification.

Here, we describe a modified PLA approach (Figure 1) by including a fluorochrome-labeled mRNA oligonucleotide along with a biotin-labeled RNA probe to study defined RNA-protein complexes in cells step-by-step for proximity detection of the RNA-protein complex in - cancer cells. Our enhanced optimized PLA protocol can also be used to detect RNA-protein complexes in different cellular compartments.

Preparation of reagents
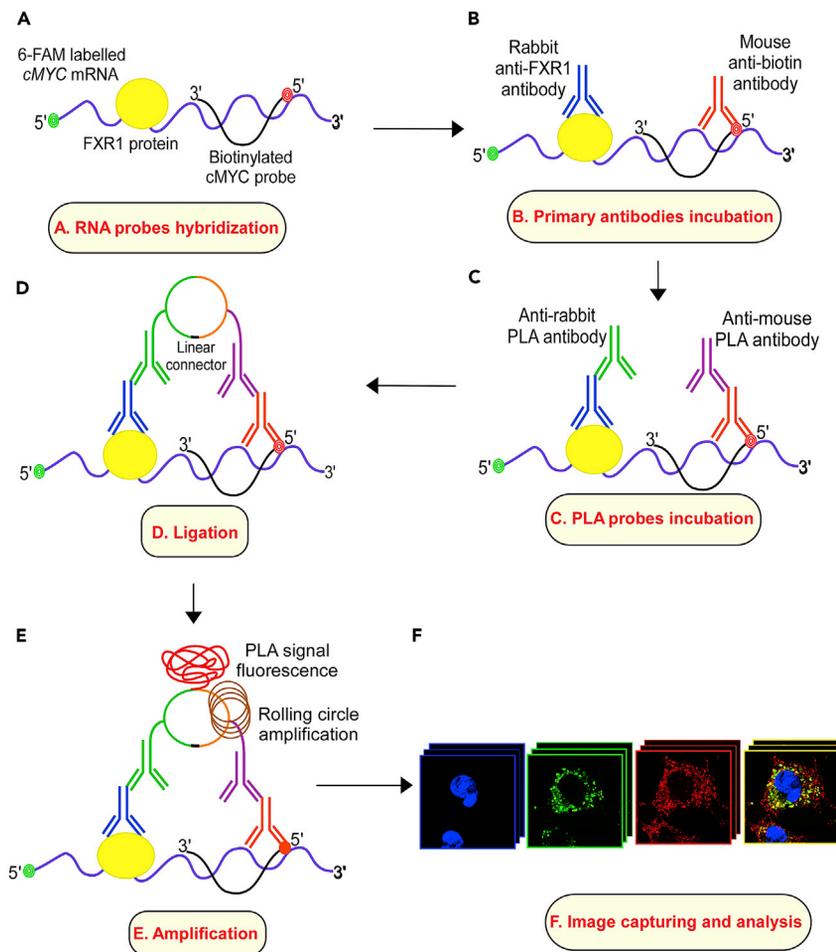
⏱ Timing: approximately 2 h

Figure 1. Schema of workflow of PLA developed for detecting target-specific interactions between RNA sequences and RBP

(A) RNA probe hybridization was performed using a biotinylated PLA probe (black with biotin indicated in red), which targets a specific mRNA sequence in MYC gene close to the binding site of FXR1 protein (yellow) within *MYC* mRNA (purple line with FAM indicated in green at the 5'end) is performed.

(B) Two primary antibodies raised in different species are added. In this case a rabbit antibody (blue) targets the FXR1, and a mouse antibody (red) targets the biotin label in the PLA probe are used.

(C) Two secondary PLA species-specific antibodies (green and pink) conjugated with oligonucleotides are then added.

(D) The conjugated oligonucleotides are joined and circularized by ligation upon introduction of linear connector oligonucleotide (black).

(E) The PLA probe acts as a primer for target mRNA generated concatemeric sequences during rolling circle replication (the amplified circular molecule is annotated in brown).

(F) In the last step, labeled oligos hybridized to the complementary sequences within the amplicon are visualized and quantified as discrete spots of PLA signals using microscopy.

⚠ CRITICAL: Always keep reagents on ice.

1. **Selection of RNA oligonucleotides:** For transfection of cells, FAM fluorophore or any other fluorophore of choice should be used for labeling of oligonucleotides and can be of 100 bp selected from 3′ untranslated region (3′UTR), coding sequence (CDS) or 5′UTR region. Typically, Cy3b, FITC or FAM can be used, which are characterized by a long fluorescent lifetime and have good resistance to photobleaching. The PLA probes targeting the RNA of interest are RNA oligonucleotides consists of 40–50 nucleotide (nt) sequence

Table 1. FAM labeled oligonucleotides transfected in cells for PLA

Oligonucleotide	Sequence (5′-3′)
1	Caaaugcaaccucacaaccuuggcugagucuugagacugaaagauuua gccauaauguaaacugccucaaauuggacuuugggcauaaaagaacuuuuuu
2	Cuuuaacagauuuguauuuaagaauuguuuuuaaaaaauuuuaagauuu acacaauguuucucuguaaauauugccauuaaaauguaaauaacuuuaauaa

Sequences of oligonucleotides were designed as probes for *MYC* mRNA 3′UTR containing ARE sequences.

complementary to (or sense to) the RNA of interest. They should be 7 nucleotides far from the predicted site of binding, labeled with biotin at 5′end and 30 bp in length. In our setting, we have used probes that target 7 nucleotides far from the AU-Rich Element (ARE) located in the 3′UTR of target mRNA. Sequences of oligonucleotides used in this study are shown in Tables 1 and 2.

2. **Optimization of primary antibody treatment:** For PLA, the two primary IgG antibodies with high specificity for the protein of interest can be used for detecting the desired protein-protein or RNA-protein interaction. Both monoclonal and polyclonal antibodies are applicable in this step. To enable detection, the two primary antibodies must be raised in two different species because these antibodies will be detected with species specific primer-tagged secondary antibodies. Both primary antibodies bind to the proteins when the maximum distance between two target proteins is 40 nm to be able to create signals. The fixation and permeabilization conditions also should be same for both antibodies. One can start with a saturated or recommended concentration of primary antibody (1:100 dilution in most cases) and then titrate each primary antibody in different dilution to determine optimum binding without background, until the number of quantified PLA complex start decreasing. In our settings, we observed that FXR1 and biotin antibodies can be used at 1:100 dilution.

Note: Confirm that primary antibodies have been validated for immunofluorescence applications before PLA. Primary antibodies should be titrated for their optimum concentration for the best staining intensity without nonspecific background staining.

3. **Optimization of fixation and permeabilization step:** The fixation and permeabilization conditions must be optimized depending upon the cell type and antibodies used. We started using the vendor recommended concentration when calibrating the assays, then decreased or increased based on signals. Cells were fixed using 4% PFA and permeabilization was performed using ice-cold methanol.

4. **4% Paraformaldehyde (PFA) (vol/vol):** For making 4% PFA solution, add 4 g of EM grade PFA to 80 mL of 1× PBS. The powder will not immediately dissolve into solution. Slowly raise the pH by adding 1 N NaOH dropwise from a pipette until the solution clears and stir gently on a heating block at ∼60°C. Once the PFA is dissolved, adjust the pH to 7.0–7.4 and make up the volume to 100 mL.

△ CRITICAL: 4% PFA should be prepared under a chemical ventilated hood as PFA is toxic and can emit formaldehyde gas, which is a known human carcinogen, and can irritate the

Table 2. Oligonucleotides used in PLA experiment

Oligonucleotide	Sequence (5′-3′)
PLA probe 1	ugacggaguuuaaccugaaacccguauuuu
PLA probe 2	gacauuuauaacgguaauuuacauuuauug

Oligonucleotides were designed complementary sequences to target 7 nucleotides far from ARE in human *MYC* mRNA 3′UTR.

eyes and skin. Keep the bottle containing 4% PFA container tightly closed when not in use at 4°C.

5. **Probe solution:** Dilute each PLA probe (1:5) in 1✕ antibody diluent.
6. **Ligation reaction buffer:** Dilute the ligase (40✕) in the ligation solution (5✕) with milliQ water immediately before use.
7. **Polymerase:** Dilute the polymerase (80✕) in the amplification solution (5✕) with milliQ water immediately before use.
8. **Wash buffer A and B:** Wash Buffers A and B should be made prior to beginning the assay by dissolving the contents of one pouch in high purity water to a final volume of 1 L.

> *Note:* Solutions may be stored at 20°C–25°C for short term storage (less than two weeks) or at 4°C for long term storage.

> *Alternatives:* Alternatively, recipe for wash buffer A and B is as follows:

Buffer A		
Reagent	Final concentration	Amount
NaCl	0.01 M	8.8 g
Tris base pH 7.5	0.15 M	1.2 g
High purity water	–	800 mL
Tween-20	0.05%	0.5 mL

Buffer B		
Reagent	Final concentration	Amount
NaCl	0.2 M	5.84 g
Tris base pH 7.5	0.1 M	4.24 g
High purity water	–	500 mL

> *Note:* Filter the solutions using 0.22 μm filters and store at 4°C. Bring the solutions to 20°C–25°C before use.

> △ CRITICAL: Hydrochloric acid is toxic if inhaled and should be handled under a fume hood. Handler should wear appropriate personal protective equipment (PPE) to cover skin and eyes.

9. **0.01✕ Wash buffer B:** Dilute 1:100 buffer B 1✕ in distilled water.
10. **Reaction volume:** Use 20 μL–30 μL of total reaction volume for one cover slip (12 mm diameter) or for each well in 12 well chamber slide.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Biotin (1:100)	Rockland	Cat# 200-301-098, RRID: AB_2611059
FXR1 (1:100)	Cell Signaling Technology	Cat# 12295S; RRID: AB_2797875
Chemicals, peptides, and recombinant proteins		
Antibiotic (Penicillin/Streptomycin)	Thermo Fisher Scientific	Cat# 15140122

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Ethyl alcohol	Sigma-Aldrich	Cat# E7023
FBS	Atlanta Biologicals	Cat# H17112
Lipofectamine 2000	Thermo Fisher Scientific	Cat# 11668027
Methanol	Sigma-Aldrich	Cat# 34860
Paraformaldehyde	Sigma-Aldrich	Cat# 158127
DPBS	Thermo Fisher Scientific	Cat# 14190144
RPMI-1640	Thermo Fisher Scientific	Cat#11875119
Trypsin	Thermo Fisher Scientific	Cat# 15400054
ProLong Glass Antifade Mountant	Thermo Fisher Scientific	Cat# P36931
Critical commercial assays		
PLA Kit anti-rabbit	Sigma-Aldrich	Cat# DUO92002
PLA Kit anti-mouse	Sigma-Aldrich	Cat# DUO92004
PLA Kit in situ detection reagent	Sigma-Aldrich	Cat# DUO92008
Experimental models: Cell lines		
HeyA8	MD Anderson Cancer Center	N/A
Oligonucleotides and recombinant proteins		
FAM labeled cMYC Oligonucleotides (Please see Table 1)	GenScript	N/A
PLA probes (Please see Table 2)	Sigma-Aldrich	N/A
Software and algorithms		
Image J: J Coloc 2 image analysis plug-in	National Institutes of Health	https://imagej.net/Coloc_2
ImageJ: JACoP plugin	National Institutes of Health	http://rsb.info.nih.gov/ij/plugins/track/jacop.html
Microscope imaging software	LSM 510	N/A
Other		
12 Well Chamber, removable: microscopy glass slides, sterilized	ibidi USA, Inc.	Cat# 81201
Microscope cover glasses	Thermo Fisher Scientific	Cat# 12-544-14
CO_2 incubator, Model 3010	Thermo Fisher Scientific	N/A
Tabletop centrifuge	Eppendorf	Cat# 5427 R
Vortex Genie	LMS Co.,	Cat# VTX-3000L
Rocking shaker, Model no. 5	Reliable Scientific Inc.	N/A
Fluorescence microscope equipment with excitation/emission filters compatible with green and red fluorophores and nuclear stain	Zeiss	N/A

MATERIALS AND EQUIPMENT

Here we have not included basic laboratory materials like cell culture materials, microcentrifuge tubes, glassware, pipette tips, as these materials must be available in the laboratory. All the following materials can be substituted by alternative equivalent materials or with suitable equipment.

Microscope and imaging

We used LSM510 confocal laser scanning microscope (Zeiss, Oberkochen, Germany) for imaging in 3 separate fluorochrome channels, including DAPI, FAM, and Texas Red for PLA signal. Microscopy and imaging setup was controlled by LSM 510 software (Zeiss, Oberkochen, Germany). For capturing each image, we use magnification 40×, channel 1 (DAPI) of wavelength 405 nm with exposure time of 3 ms, channel 2 (FAM) of wavelength 488 nm with exposure time of 300 ms, and channel 3 (PLA) of wavelength 545 nm with exposure time of 200 ms. The image analysis method described in this protocol can be used for multiple analysis including z-stack images.

Note: At least, three to four different locations of interest on each slide from three independent experiments per sample should be acquired for statistical analysis.

Alternatives: Alternative microscopic systems are also applicable for imaging. An upright fluorescent microscopy is preferred with a minimum of 40× magnification objective for capturing images.

> △ CRITICAL: At least two fluorescent channels are needed for detecting protein-protein co-localization; a 455 nm UV channel for the acquisition of DAPI or similar staining probes for nuclear, and a 488 nm green filter or a 545 nm red filter is required for the acquisition of PLA complexes.

Image analysis

The images, which are acquired by Zeiss confocal laser scanning microscope are processed using ImageJ (Version 1.0) together with Java image acquisition plugins (Schindelin et al., 2012) for the quantification of the mRNA-protein interactions were described in this protocol.

STEP-BY-STEP METHOD DETAILS
Preparation of cells and oligonucleotides transfection

⏱ Timing: variable 24–48 h

The following steps describes the procedure of how to deliver 6-FAM labeled oligonucleotides to the cells using the Lipofectamine® 2000 transfection reagent (Thermo Fisher Scientific). We have utilized this procedure for the delivery of 6-FAM labeled oligonucleotides successfully with several cell lines including HeyA8, OVCAR8, OVCAR5. Standard working concentrations of oligonucleotides are 2 nM–5 nM. The optimal oligonucleotides concentration should be determined in preliminary experiments for each cell type.

1. Seed 1×10^4 HeyA8 cells per well of 24-well plate in 1 mL of culture medium per well for transfection.
2. Next day, transfect the cells using Lipofectamine 2000 according to manufacturer instructions (Thermo Fisher Scientific) with 5′ 6-FAM-labeled oligonucleotides (n=2, Table 1) synthesized by GenScript (Piscataway, NJ, USA). In brief, 2 nM oligonucleotides were added to 100 μL Opti-MEM medium and 2.5 μL lipofectamine 2000 was added to 100 μL Opti-MEM medium. Both solutions were incubated at 20°C–25°C for 5 min, before, the oligonucleotide solution was added to the lipofectamine solution. The mixture was then incubated at 20°C–25°C for 20 min and then transfection mixture was added to the cells. After 4 h, the transfection reagent containing medium was changed to regular culture medium.
3. After 24 h of transfection, cells were trypsinized and counted for cell density using an automated cell counter or a hemocytometer.
4. Cells were resuspended to a density of 1×10^3 cells in 1,200 μL of growth media and then added 100 μL equally into the wells of 12-well glass bottom chamber slides.
5. Cells were allowed to attach on glass bottom for 5 min under the hood, then incubated at 37°C with 5% CO_2 for 24 h.
6. For MYC knockdown, 5 nM pre-designed siRNAs for human MYC (Thermo Fisher Scientific) were transfected using the Lipofectamine RNAiMAX transfection reagent according to manufacturer instructions (Thermo Fisher Scientific) in HeyA8 cells. After 48 h, cells were again transfected with 5′FAM-labeled oligonucleotides.

> △ CRITICAL: The confluency of cells for PLA assay, should be at 40%–60% confluency. Too few cells will cause the culture to grow poorly without cell-to-cell contact. High confluency of cells results in contact inhibition, making cells resistant to the uptake of foreign DNA. Actively dividing cells take up introduced DNA better than quiescent cells. Therefore, cell density is an important parameter that impacts transfection efficiency.

⚠ CRITICAL: One crucial factor is to ensure that the cells are in a healthy state, properly mixed and evenly distributed into the wells. Cells should be checked before the incubation at 37°C to make sure that the suspended cells are homogeneously distributed in each well without cellular aggregation. When this is confirmed, do not further shake the slides until the cells are attached. After 24 h growth, the cells should be checked for green color (FAM fluorophore) in a microscope before doing PLA.

Fixation, permeabilization, and blocking of cells

⏱ Timing: 50 min

7. Aspirate off media, wash with 1× DPBS and fix the cells with 4% PFA in PBS (fixative solution) for 20 min at 25°C.
8. Gently discard the fixative solution.
9. Wash with 1× DPBS and add 100 μL of ice-cold methanol or acetone to cells.
10. Incubate at −20°C for 15–30 min to permeabilize.
11. Gently discard the methanol solution.
12. Wash the cells two times with 1× DPBS gently.
13. Add 50 μL of the blocking solution on chambers glass slide and incubate for 1 h at 4°C in a humidified chamber.

Oligonucleotide's hybridization

⏱ Timing: 1 h process overnight

14. Add 100 nM specific biotin labeled oligonucleotide probes to fresh blocking buffer, heat at 70°C for 3 min, put on ice immediately and add 30 μL in each well of a chamber glass slide. Incubate for 1 h at 37°C or alternatively at 4°C overnight in a humidified chamber.

⚠ CRITICAL: Heating probes at 70°C for 3 min, then immediately cool in an ice-water bath increases the efficiency of probe binding to the target site.

Primary antibody incubation

⏱ Timing: Overnight

15. To detect the RNA-protein proximal interaction, dilute both primary antibodies biotin (anti-mouse) and FXR1 (anti-rabbit) in 1:100 ratio as the final dilution in the 1× antibody diluent.

⚠ CRITICAL: The concentration and species source of the primary antibodies are essential for the detection of protein-protein or RNA-protein interactions, as discussed previously. Therefore, to choose the appropriate antibodies for the PLA assay, one should perform in advance both a positive and a negative control. For example, we used the same dilution condition for biotin antibody alone or FXR1 antibody alone for primary antibody incubation followed by proximity ligation probe hybridization with PLA probes. Similarly, we used PLA probe Anti-Rabbit Plus and PLA probe Anti-Mouse Plus for FXR1 detection. This allowed us to determine if the primary antibody is suitable for PLA assay. For a negative control, we used two PLA probes Anti-Mouse Plus for biotin and Anti-Rabbit Plus for FXR1. The same coupled oligonucleotides will not allow the ligation between them, therefore, any signals detected under this condition should be considered as non-specific background. We also used no primary antibodies and no PLA probes controls, which further confirms specificity of your antibodies and PLA probes (Figure 2).
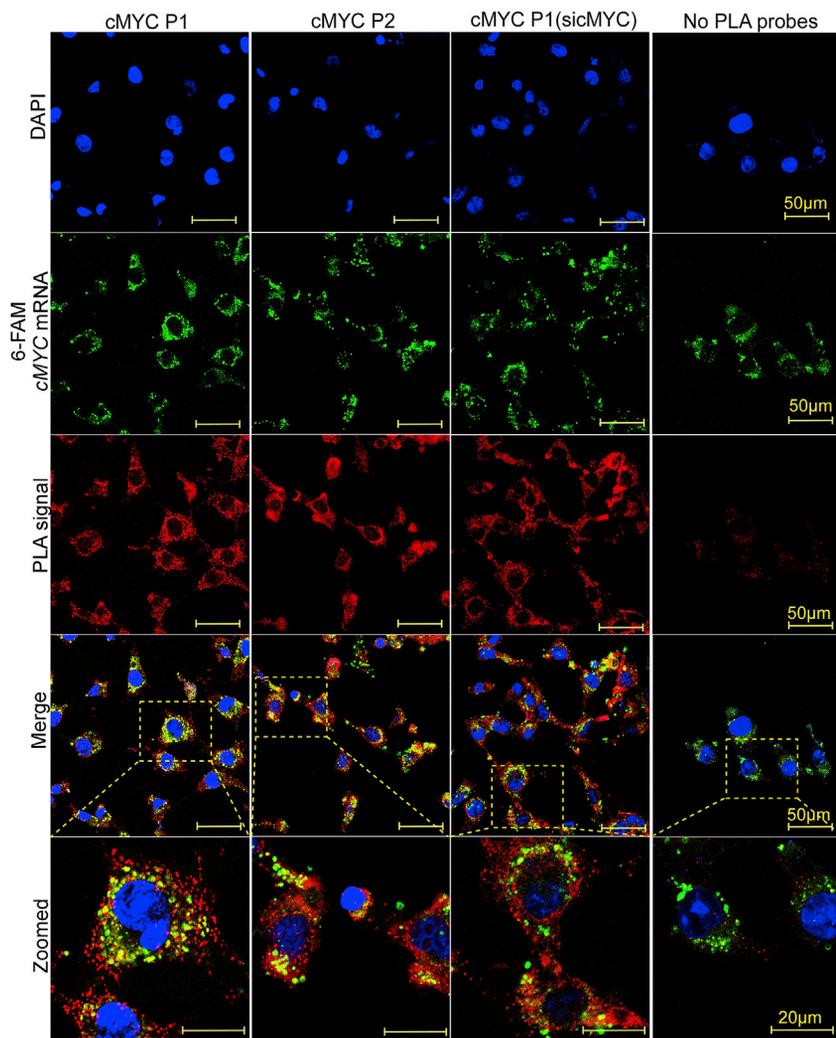
Figure 2. FXR1 binds to the AU-rich elements (ARE) within MYC 3′UTR

Representative fluorescence images of HeyA8 cells, prior transfected with FAM-labeled MYC oligonucleotides (Probe1 and 2), and then, replated, fixed, permeabilized, and incubated with anti-FXR1 antibody and oligonucleotide PLA probes anti-sense to MYC 3′UTR. MYC was transiently knockdown using MYC siRNA followed by FAM-labeled cMYC oligonucleotides (Probe 1) treatment. PLA was performed to detect the interaction of FXR1 protein and the MYC mRNA (yellow merge color); nuclei were stained with DAPI (blue). Images were zoomed in by x4 times to show FXR1 protein and *MYC* mRNA colocalization in cells. Scale bars: 50 and 20 μm, respectively.

16. Add a solution of 1× PBS in each well without removing the blocking solution and gently aspirate with vacuum pump immediately.
17. Add 30 μL of the corresponding primary antibodies solution in the chamber slide and incubate for overnight at 4°C in a humidified chamber.

Note: Do not allow the sample to dry. Fill with 1× PBS in non-used wells of the chamber slides.

18. Add Wash Buffer A in each well. Incubate on a shaker at slow speed (10–30 × *g*) for 5 min. Remove the wash buffer with vacuum pump and repeat this step twice.

Note: The Wash buffer is conserved at 4°C. Before using, it should be recovered at 20°C–25°C during ∼30 min to reach the 20°C–25°C.

Proximity ligation probe hybridization

⊙ Timing: 60 min

19. Dilute the two PLA probes Anti-Mouse Plus and Anti-Rabbit Plus stock solutions in antibody diluent to 1:5 dilution.
20. Add 30 μL of the PLA probes mixed solution on the chamber slide. Incubate for 1 h at 37°C.
21. Add Wash Buffer A in each well. Incubate on a shaker at low speed. Remove the wash buffer with vacuum pump and repeat this step twice.

    Note: During the washing step, one should start to prepare the ligation solution at 20°C–25°C for the next step.

Oligonucleotide ligation

⊙ Timing: 45 min

22. At the second time of the washing step, prepare the ligation reaction solution as follows:

Component	Volume to add per reaction (μL)	Final concentration
5× Ligation buffer	6 μL	1×
Ligase (1:40)	0.75 μL	1 U/mL
Milli-Q water	23.25 μL	n/a

    Note: Ligase should stay on ice block. However, the prepared mix should stay at 20°C–25°C.

23. Gently remove the wash buffer A with a vacuum pump after the second wash of ligation solution.
24. Add 30 μL of the ligation reaction mix on the cover glass. Incubate for 30 min at 37°C.
25. Add Wash Buffer A in each well. Incubate on a shaker at low speed. Remove the wash buffer with a vacuum pump and repeat this step twice.

    Note: During the washing step, one should start to prepare the rolling cycle amplification solution at 20°C–25°C for the next step, and make sure that the amplification solution is protected from light.

Rolling cycle amplification

⊙ Timing: 90 min

26. At the second time of the previous step, prepare the amplification reaction mix as follows:

Component	Volume to add per reaction (μL)	Final concentration
5× Amplification buffer	6 μL	1×
Polymerase (1:80)	0.375 μL	1 U/mL
Milli-Q water	23.625 μL	n/a

    Note: The amplification buffer contains fluorescent dyes and should be protected from light. This mix should stay at 20°C–25°C.

27. Gently remove the wash buffer A with a vacuum pump after the second time of previous step.

28. Add 30 μL of the amplification reaction mix in the chamber slide. Incubate for 1.5 h at 37°C in the dark.
29. Add wash Buffer B in each well. Incubate on a shaker at low speed. Remove the wash buffer with a vacuum pump and repeat this step twice.

> *Note:* The slide should be protected from light during this step.

Rolling cycle amplification washing

> ⏱ Timing: 20 min

30. Gently remove the wash buffer B with a vacuum pump.
31. Add 100 μL of 0.01× wash buffer B diluted in high purity water.

> *Note:* Users can process the samples at this step for further immunostaining by using antibody of their choice and routine immunofluorescence assay. Please avoid performing further immunostaining without washing at least once with 1:100 diluted wash buffer B since high concentration of buffer B may diminish the antibody staining efficacy.

32. Remove the chambers with the help of clean forceps.
33. Air-dry for 2 min.

> *Note:* Protect from light. Alternatively, the slide can be further processed for cytoplasmic staining or co-staining with immunofluorescence. Please avoid overly drying which will decrease the PLA signal intensity.

Slide mounting

> ⏱ Timing: 15–30 min

34. Add 50 μL of PLA mounting medium on glass slide.

Alternatively: Add 50 μL of mounting medium with DAPI on glass slide.

Carefully with the help of forceps put the cover slip over slide slowly to make sure mounting medium spread all over the slide.

> *Note:* Avoid producing air bubbles in the process.

Seal with nail polish. Alternatively, if one uses ProLong Glass Antifade Mountant, (Thermo Fisher Scientific, P36931), sealing with nail polish is not necessary.

> ⏸ Pause Point: The slide can be stored at 4°C for long time before processing to microscopy image acquisition. In our laboratory, we have tested to store the slides for maximum 3 months at 4°C and we do not see obvious decrease of the fluorescence intensity.

> ⚠ CRITICAL: Do not freeze the slide. Freezing at −20°C or −80°C will create large crystal particles in the mounting medium which will influence the image acquisition quality.

Confocal microscope setup and imaging

> ⏱ Timing: Variable, it may take multiple sessions of several hours (4–24 h) to obtain sufficient data. The pixel resolution for the different objective lens used will depend on the specific

parameters and the confocal microscope being used. We list the key parameters required for the image acquisition in the following table:

Parameter name	Parameter value	Note
Objective	20×	Required for high throughput image acquisition
Objective	40× with water	Required for high quality image acquisition
Objective	400×	Required for the zoomed images
Filter	405 nm/450 nm	Required for DAPI nuclear staining visualization
Filter	488 nm	Required for FAM fluorophore staining visualization
Filter	545 nm	Required for Cy3 red PLA staining

35. Scan images at 20× lens first to locate the cells on slide followed by scanning with 40× lens. At least 3 images for each group should be captured and saved for further analysis.

    Note: Scanning images using 20× lens first is helpful to have a general view of the distribution of signals and to identify the cells of interest, which is a preliminary step before locating the scanning position using 40× or 63× lens. Process image acquisition with 20× objective, starting with DAPI channel (450 nm), then FAM channel (488 nm), then Cy3 channel (545 nm).

    Note: If you are using different fluorescent tag for the detection of proximity ligation assay, please optimize the corresponding filter. The PLA fluorescence are available for four different colors. Apart from the color used in this protocol, three additional fluorophores are also applicable. For green fluorescence, one should use a 488 nm/527 nm filter; For Cy3 orange fluorescence, one should use a 554 nm/576 nm filter; and for Cy5 far red fluorescence, one should use a 644 nm/670 nm filter.

    ⚠ CRITICAL: All slides from the same experiment should be imaged using the same conditions. At 40× don't forget to use water on lens and keeping the slides facing glass cover on the bottom. Check to see if there is any fluorescence in an unstained well of the slide. If there is, then this is autofluorescence in the cells.

PLA signal analysis and quantification

Images acquired by Zeiss confocal laser scanning microscope are saved as TIFF format. Zeiss software (LSM 510) allows the user to access images with TIFF format. The relative number of PLA dots were then quantified using ImageJ software as below.

36. Open a TIFF image in image J software, in the main menu of software and go to Process/Binary/ Make Binary to convert the TIFF images into Binary format.
37. After converting images to binary format, select region of interest that represent only one cell by using selection tool from main menu. Then, select Process/Filter/Median 2.0. Click main menu again and select Process/Binary/Watershed to segment spots (Figures 3A and 3B).
38. In the main menu of image J, select Process/Find Maxima to exclude edge maxima and light background. Select "Count" in the output type. This gives automatic calculation of PLA spots which are presented as medium ± standard deviation for 3 different images (Figure 3C). Select "Point section" in the output type. This marks each PLA spots with yellow crosses from single cell.

Analysis and quantification of co-localization coefficients

39. In this protocol we determined the co-localization between *MYC* mRNA and FXR1 protein and, co-localization coefficients were calculated using the JACoP plugin in ImageJ.
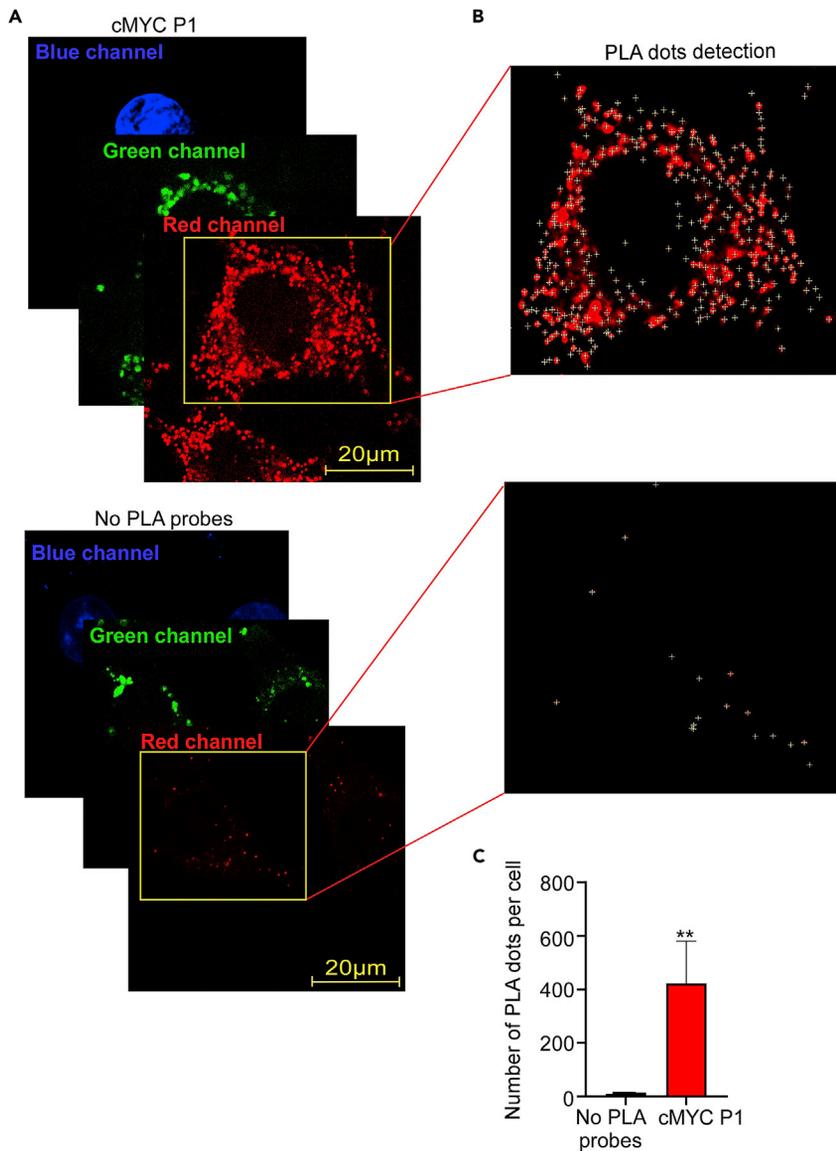
Figure 3. Experimental workflow employed to quantitate PLA signals per cell

(A) Images of in situ PLA were captured using mouse anti-Biotin targets *MYC* mRNA and rabbit anti-FXR1 antibody in HeyA8 cells that were transfected with 6-FAM *MYC* mRNA (Green) and *MYC* probe P1. Scale bar: 20 μm.

(B) PLA signals generated were labeled individually and quantified using Image J software, where cross marks indicate PLA signals.

(C) Quantitative graph of PLA dots was identified per cell. Error bars represents mean $\pm$ SEM; **p<0.01 and n=4.

40. Open a high magnification (400×) TIFF image in image J software and identify the cells that shows good interaction between *MYC* mRNA and FXR1 protein. Go to main menu of image J and select Image/Color/Split image. Split the image into individual images displaying MYC (green), PLA dots (red), and DAPI. Save the individual images (Figure 4B).

41. Select Plugin/JAcoP in the main menu of image J and choose the two channels for analysis in JACoP, e.g., Red (PLA dot) for Image A and Green (MYC) for Image B. Save these images.

42. Go to analysis tab in the main menu and select the parameter appropriate to you. We used Overlap and Manders' coefficients in our analysis.
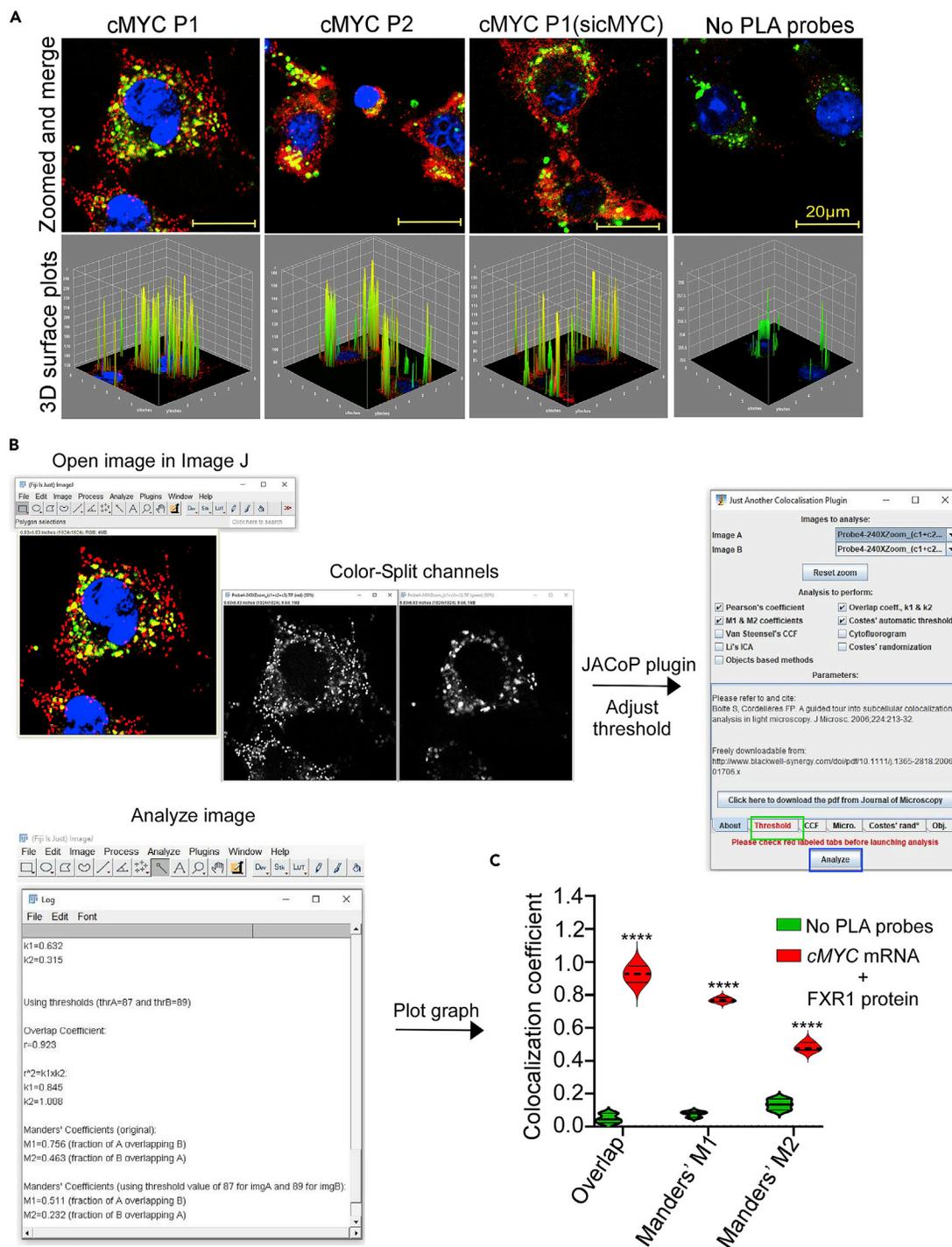
Figure 4. Experimental workflow of how colocalization coefficient was determined for RNA-protein proximal interactions

(A) Representative images show colocalization of *MYC* mRNA and FXR1 protein were captured and presented as 3D surface intensity plots. Scale bar: 20 μm.

(B) Workflow employed for determining colocalization signals using JACoP plugin for ImageJ and the signal overlap using Manders' coefficient value for both A (Green) overlapping B (Red) i.e., M1 and B (Red) overlapping A (Green) i.e., M2.

(C) Quantitative violin plot represents colocalization coefficient of *MYC* mRNA and FXR1 protein. Data in the graphs represent mean ± SEM; ****p<0.0001, n=8.

Note: Overlap coefficient is based on Pearson's coefficient signal that is dependent on linear signals while Manders' coefficient is based on ratios of two signals on top of the compartment and vice versa. Two independent correlation coefficient analysis will provide more accurate degree of relationship between co-localization signals.

43. Select Analysis or Threshold commands in the window, then adjust the signal for channel A and B to the level that can clearly differentiate between background without signal saturation. Click analyze (Figure 4B).
44. After clicking analyze, a data window will open in image J. Record the Manders' coefficients and Overlap coefficient (using threshold value for both, A overlapping B, and B overlapping A).
45. Plot the data in the form of table or graph, then perform statistical analysis using quantitated values (Figure 4C). All statistical analyses were done using GraphPad software.

EXPECTED OUTCOMES

Over the past few years, great progress have been made on uncovering the functions of RBPs by identifying the targets of RNA Binding Proteins (RBPs) in various cellular lineages (Kelaini et al., 2021). RBPs are known for influencing the stability of mRNAs and protein translation (Glisovic et al., 2008). There are several advantages of our fluorescence labeled enhanced-PLA technique over traditional RNA immunoprecipitation assays used for studying RNA-protein interactions such as RNA-immunoprecipitation (RIP) or crosslinked immunoprecipitation (CLIP) approaches.

In this approach, we describe a simple, reproducible, and easily executable proximity ligation-based method called enhanced-PLA for visualizing and quantifying *MYC* mRNA as the target of FXR1 in single-cell resolution, which can be visualized as dot-like structures (Figure 1). Enhanced-PLA method we established can identify RNA-protein complexes at subcellular level in single cell resolution with improved specificity (Figures 1–4). The chances of false-positive results are limited when we used our enhanced PLA protocol. Cautionary steps included in our approach eliminate conventional issues associated with quantifications of localized signals in heterogeneous cell populations. Moreover, easy to use approach of this protocol, could make this method applicable to many researchers conveniently.

Along with this assay, we also included the description and tips for both image analysis and quantification of proximal binding sites of FXR1 and its target mRNA by counting of dots representing PLA signal by using image J software. Recently, we have published our enhanced PLA procedure to detect interaction between AU-rich elements present within the 3′UTR region of *MYC* mRNA and FXR1 protein at the single-cell level in ovarian cancer cell lines (George et al., 2021). In principle, this protocol can be extended to implement the detection of the mRNA-protein as well as protein-protein interaction in a variety of cells.

Background noise due to lack of specificity is also very minimal in our enhanced PLA protocol. Therefore, quantification of localized signals is highly accurate compared to conventional imaging approaches. To overcome the limitation of any non-specific background fluorescence, exist, we employed fluorophore labeled oligonucleotides in our enhanced PLA assay. Specifically, for *in vitro* transfection, we used 5′-FAM labeled oligonucleotides spanning the 3′UTR region of *MYC* mRNA (n=2, Table 1) and for hybridization, we used oligonucleotides sequences that comes in the 5′-FAM labeled oligonucleotides (n=2, Table 2). Therefore, our modified PLA gives more specificity in terms of detecting FXR1 protein and its target protein at single-cell resolution (Figure 2). To confirm PLA signals are derived from MYC mRNA upon FXR1 binding, we knocked down MYC using target-specific siRNAs and performed PLA assay. As expected, MYC knockdown did not result in any loss of proximity signal demonstrated the specificity of our assay (Figure 2).

At the end of this protocol, RNA-protein interaction and co-localization signals were analyzed using Fiji image J software. PLA signals were formed were counted as individual spots per cell from 4 different images (Figures 3A and 3B). For this, we quantified the absolute number of *MYC* mRNA-FXR1 complex formed as PLA spots in HeyA8 cell line, which was transfected with *MYC* mRNA (Figure 3). The colocalization coefficient between *MYC* mRNA and FXR1 protein was determined using JacoP plugin of image J software. To visualize the co-localization signals better and convincingly, we generated 3D surface plot in image J using 3D surface plot plugin (Figure 4A), then calculated the co-localization coefficient between *MYC* mRNA and FXR1 using Manders' algorithm for quantification with appropriate statistical power after adjusting threshold in each image. For example, Figure 4B represents the co-localization data from at least 8 individual images.

LIMITATIONS

PLA probes or oligonucleotides

It has been reported that FXR1 binds to AU-rich elements (AREs) within the 3'untranslated region (3'UTR) and enhances the stability of other mRNAs (Li et al., 2018; Vasudevan and Steitz, 2007). Therefore, we designed the PLA probes specifically for AREs within the 3'UTR region of *MYC* mRNA. Any probe prediction tool such as (http://bclab.inha.ac.kr/pridictor/result.aspx) could be used to design target specific probes that bind to the given mRNA for RBPs which are poorly studied for their targets and sequence specificity (Tuvshinjargal et al., 2016). This approach will identify several potential binding sites within mRNA. It is highly recommended to perform an RNA electrophoretic mobility shift assay (REMSA) to confirm if the identified sequences exhibit binding affinity with a specific RBP (George et al., 2021).

RNA oligonucleotides or fluorescently labeled oligonucleotides are easily degraded by RNases. Thus, the major limitation of this protocol is the degradation of oligonucleotide probe due to stability associated factors during freeze-thaw and RNase activity when handling at 20°C–25°C. To overcome this, before proceeding clean the workbench with agents that destroy RNases and always use chemicals, reagents, and water that are certified as RNase free. Furthermore, fluorescently labeled RNA oligonucleotides should be stored in TE (Tris-EDTA) buffer at −80°C in amber tubes.

Moreover, for a successful PLA, the quality and purity of oligonucleotide probes are highly essential. Therefore, it is important to include appropriate controls for validating expected levels of any gene of interest. To test for probe specificity, we recommend performing independent evaluation of probes in cells or conditions, where the target genes are deleted or not expressed as a negative control. In addition, some target genes may not work due to the secondary structures formed in target mRNA, due to the chances of degradation of probes or their affinity to other cellular components. In addition, it is always recommended to perform PLA with a control probe for target RNAs that are widely expressed in the desired cells or cell line.

Antibodies selection

Another concern is the high background fluorescence. To avoid the background noise, it is highly recommended to use target specific and pre-validated primary antibodies with seldom off-target reactions.

TROUBLESHOOTING

One of the challenges of this protocol is that the consequences of any technical mistakes happened on either Day 1 or 2 will be realized, when visualize the cells upon microscopy on Day 3. In addition, there could be technical errors, such as over confluency of the cells, transfection issues, over fixation issues, drying of cells, high background levels, bubbles, etc. Possible solutions for some of these issues have already been described within the protocol. Here are some additional issues one may encounter, along with possible solutions that may help with optimization.

Problem 1
Related to step: preparation of cells.

No cells or cells are not adherent to glass bottom chambered slide.

Potential solution
Pre-treat slide with 0.01% Poly-L-lysine.

Problem 2
Related to step: RNA probe transfection.

No signal or low signal.

Potential solution
For first experiments with any new probe sets, appropriate control probe, which target any widely expressing gene in the target cell should be used to assess if there is any technical errors during the setup of the assay or during imaging as well as to avoid any misidentifications due to artifacts. The control sample should display signals in most cells.

Problem 3
Related to step: PLA probes incubation.

No PLA spots.

Potential solution
Hybridization solution was not properly mixed, or a higher concentration of PLA probes is required.

Problem 4
Related to step: fixation and permeabilization of cells.

High signal-to-noise as background.

Potential solution
This may be due to drying of samples during incubation. Add PBS 1× in empty wells in the same incubation chamber slides-and place wet Kimwipes in the box where slides keep.

Problem 5
Related to step: rolling cycle amplification washing.

High background or non-specific staining in nuclei.

Potential solution
This may be due to wash buffer B which could be expired. Wash buffer B can be stored at 4°C only for 1 month.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Pradeep Chaluvally-Raghavan (pchaluvally@mcw.edu)

Materials availability
This study did not generate new unique reagents.

OPEN ACCESS

Data and code availability

This study did not generate any codes.

AUTHOR CONTRIBUTIONS

P.C.R., S.P., and J.G. conceived the study, generated hypotheses, and designed the experiments. J.G. performed most of the experiments. J.G., S.M., and I.P.K. did analysis for images, prepared figures, and the draft of the manuscript. S.P. and P.C.R. edited the manuscript. P.C.R. provided scientific direction, allocated funding, and finalized the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

George, J., Li, Y., Kadamberi, I.P., Parashar, D., Tsaih, S.W., Gupta, P., Geethadevi, A., Chen, C., Ghosh, C., Sun, Y., et al. (2021). RNA-binding protein FXR1 drives cMYC translation by recruiting eIF4F complex to the translation start site. Cell Rep. *37*, 109934. https://doi.org/10.1016/j.celrep.2021.109934.

Glisovic, T., Bachorik, J.L., Yong, J., and Dreyfuss, G. (2008). RNA-binding proteins and post-transcriptional gene regulation. FEBS Lett. *582*, 1977–1986. https://doi.org/10.1016/j.febslet.2008.03.004.

Jung, J., Lifland, A.W., Alonas, E.J., Zurla, C., and Santangelo, P.J. (2013a). Characterization of mRNA-cytoskeleton interactions in situ using FMTRIP and proximity ligation. PLoS One *8*, e74598. https://doi.org/10.1371/journal.pone.0074598.

Jung, J., Lifland, A.W., Zurla, C., Alonas, E.J., and Santangelo, P.J. (2013b). Quantifying RNA-protein interactions in situ using modified-MTRIPs and proximity ligation. Nucleic Acids Res. *41*, e12. https://doi.org/10.1093/nar/gks837.

Kelaini, S., Chan, C., Cornelius, V.A., and Margariti, A. (2021). RNA-binding proteins hold key roles in function, dysfunction, and disease. Biology *10*. https://doi.org/10.3390/biology10050366.

Niranjanakumari, S., Lasda, E., Brazas, R., and Garcia-Blanco, M.A. (2002). Reversible cross-linking combined with immunoprecipitation to study RNA-protein interactions in vivo. Methods *26*, 182–190. https://doi.org/10.1016/S1046-2023(02)00021-X.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. Nat. Methods *9*, 676–682. https://doi.org/10.1038/nmeth.2019.

Tuvshinjargal, N., Lee, W., Park, B., and Han, K. (2016). PRIdictor: Protein-RNA Interaction predictor. Biosystems *139*, 17–22. https://doi.org/10.1016/j.biosystems.2015.10.004.

Vasudevan, S., and Steitz, J.A. (2007). AU-rich-element-mediated upregulation of translation by FXR1 and Argonaute 2. Cell *128*, 1105–1118. https://doi.org/10.1016/j.cell.2007.01.038.

Weibrecht, I., Lundin, E., Kiflemariam, S., Mignardi, M., Grundberg, I., Koos, B., Nilsson, M., and Soderberg, O. (2013). In situ detection of individual mRNA molecules and protein complexes or post-translational modifications using padlock probes combined with the in situ proximity ligation assay. Nat Protoc. *8*, 355–372. https://doi.org/10.1038/nprot.2013.006.