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A Novel Method to Quantify RNA–Protein Interactions In Situ Using FMTRIP and Proximity Ligation

C. Zurla, J. Jung, E.L. Blanchard, and P.J. Santangelo

Abstract

RNA binding proteins (RBP) and small RNAs regulate the editing, localization, stabilization, translation, and degradation of ribonucleic acids (RNAs) through their interactions with specific cis-acting elements within target RNAs. Here, we describe a novel method to detect protein–mRNA interactions, which combines FLAG-peptide modified, multiply-labeled tetravalent RNA imaging probes (FMTRIPs) with proximity ligation (PLA), and rolling circle amplification (RCA). This assay detects native RNA in a sequence specific and single RNA sensitive manner, and PLA allows for the quantification and localization of protein–mRNA interactions with single-interaction sensitivity.

Keywords

FMTRIPS; PLA; RCA; Posttranscriptional regulation; mRNA binding proteins

1 Introduction

As a gene is being transcribed, posttranscriptional events take place, including cotranscriptional mRNA processing (capping, splicing, 3' end processing), nucleocytoplasmic export, and mRNA localization, prior to translation, mRNA stabilization, translational regulation, and decay. Different sets of RNA binding proteins (RBPs) are associated with mRNAs during each stage of their maturation [1 – 3]. These interactions depend on the RBPs availability in response to cellular or extracellular cues, as well as mRNA localization within different cellular compartments, which determines the assembly of temporally and spatially dynamic RNP complexes called messenger ribonucleoproteins (mRNPs) [4, 5]. Their specific molecular composition determines “the mRNA state,” which is whether a transcript is immediately translated, transported to specific sites prior to translation, or transported to specific sites for storage and translation or tagged for degradation through quality control mechanisms.

Protein–mRNA interactions are characterized by an inhomogeneous spatiotemporal distribution within cells and across individual cells. Therefore, their investigation requires the development of ultrasensitive techniques. Single molecule approaches have emerged as powerful tools to resolve complex cellular processes [6 – 8], which are otherwise masked by ensemble averaging provided by most biochemical methods [9 – 11]. The main goal in the study of protein–RNA interactions at the single molecule level is to permit the simultaneous

detection and quantification of both the mRNA and its interactions with specific trans-acting factors with single molecule and single-interaction sensitivity. In the present review we provide a detailed protocol of a novel method with the potential to achieve these objectives.

The RNA imaging strategy that we utilize is based on the use of MTRIPs, previously described in Santangelo et al. [12, 13]. MTRIPs are fluorescently labeled tetravalent single RNA sensitive probes consisting of a neutravidin core and four biotinylated fluorescently labeled oligonucleotides for specific mRNA targeting. MTRIPs are delivered to the cytoplasm of cultured cells via reversible membrane permeabilization using Streptolysin-O. Their chimeric 2'OMe-RNA nature ensures an optimal level of affinity to bind target mRNAs without inhibiting their function and metabolism, and without the toxicity observed for other chemical modifications. To date, MTRIPs have been successfully utilized for the quantification of endogenous mRNAs such as β -actin, c-myc, and polyA+ transcripts, and the colocalization with regulatory proteins and RNA granules in both fixed and living cells [14 – 16]. MTRIPs were also utilized to study the RSV genome, its organization in viral particles, its localization in infected cells and colocalization with viral and host proteins [17, 18]. In the protocol we describe here, mRNAs are identified using FMTRIPs, a peptide-modified version of MTRIPs, where a flag-tagged neutravidin constitutes the scaffold for the tetravalent probes (Fig. 1).

Typically, proteins are imaged using immunofluorescence (IF) or overexpression with fusion Fluorescent proteins. Colocalization and correlation functions are often utilized to observe the cellular localization of mRNAs and related proteins and infer their association. However, these methods of analysis are poor indicators of physical interactions, because of limits in the optical resolution of the microscopes commonly utilized for these measurements. Here, we described a different approach based on a proximity ligation assay (PLA) that permits to quantify specific mRNA–protein interactions in situ.

PLA is a commercially available kit, whose overall approach was described in Soderberg et al. [19, 20]. It is generally utilized to study protein–protein interactions in fixed cells and tissues [21, 22]. The sample is initially incubated with primary antibodies specific for the proteins of interest and then the oligonucleotide-labeled proximity probes are added. If <40 nm apart, the oligonucleotides on the proximity probes come together to form a template for a circularized DNA strand by ligation. One of the proximity probes oligonucleotides then serves as template for the rolling circle amplification (RCA), which results in a coiled single-stranded DNA. The PLA product is detected by hybridizing complementary fluorescently labeled oligonucleotides. In PLA experiments using FMTRIPs one antibody targets the protein of interest, and the other one the flag tag on neutravidin (Fig. 2).

Using this method, we visualized and quantified interactions the genomic RSV RNA (labeled with FMTRIPs) and the viral N protein [23]. The results validated previous evidence obtained both via colocalization analysis and super resolution microscopy [17, 18]. We also interrogated the localization and frequency of interactions of native mRNAs with RBPs involved in posttranscriptional regulation such as HuR and TIA1, at both native and modulated protein levels [23, 24]. The results of our studies allowed to introduce a novel mechanism for fine-tuning of programmed cell death 4 (PDCD4), a tumor suppressor gene,

in the regulation of protein levels to prevent neoplastic transformation [24]. Last, this method was also used to quantify the interactions between native mRNAs and microtubules, microfilaments and intermediate filaments, which are correlated with mRNA translation, during oxidative stress conditions [25]. The results we obtained were consistent with evidence obtained by our lab and other groups [15, 26 – 28].

2 Materials

2.1 Cell Culture and Reagents

1. Cells of choice.
2. High glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % FBS, 100 μ /ml Penicillin and 100 μ g/ml streptomycin or other complete media.
3. 24-Well plates.
4. No 1.5 glass coverslips 10 mm.
5. Phosphate buffered saline without Calcium and Magnesium (PBS- Ca^{2+} - Mg^{2+}).
6. Optimem.
7. 1 % Paraformaldehyde in 1 $\times$ PBS (PFA).

2.2 FMTRIPs Assembly and Delivery

1. Biotinylated oligonucleotides with internal amino groups.
2. cy3b NHS ester or, alternatively, Dylight650 NHS ester.
3. Dimethyl Sulfoxide (DMSO).
4. 0.1 M Sodium Bicarbonate.
5. Neutravidin.
6. Flag tag-hyNic (Solulink).
7. S-4FB (Solulink).
8. 10 $\times$ Modification and 10 $\times$ Conjugation buffers (Solulink).
9. 2 μ /ml Streptolysin-O.
10. Tris (2-carboxyethyl)phosphine.
11. 1 $\times$ Phosphate buffered saline (PBS).
12. 3 and 30 kDa centrifugal filters.

2.3 Proximity Ligation Assay

1. Primary antibodies of choice.
2. Primary antibody solution: 0.25 % gelatin, 0.5 % Triton X-100, 0.5 % donkey serum and 1 % BSA in PBS.

3. PLA probes solution: 0.05 % Tween-20 in PBS.
4. PLA kit (Olink Bioscience).
5. 0.2 % Triton X-100.
6. Modified blocking solution: 0.5 % Tween-20, 0.1 % Triton X-100, 0.1 % gelatin, 2 % donkey serum and 1 % bovine serum albumin (BSA) in PBS.
7. PLA mounting media with DAPI.
8. Slides holders.
9. Beaker.
10. Humidity chamber.
11. Shaker.

2.4 Microscope Operation and Processing

1. Zeiss Axiovert 200 M with an UltraVIEW Spinning disk Confocal microscope (PerkinElmer).
2. 60× numerical aperture (NA) 1.4 Plan-Apochromat objective (Zeiss).
3. Flash 4.0v2CMOS camera (Hamamatsu).
4. Imaging and processing via the Volocity software (PerkinElmer).

3 Methods

3.1 Neutravidin Labeling with Flag Peptides

Neutravidin represents the core of MTRIPs, since it allows for the tetramerization of the biotinylated mRNA-targeting oligonucleotides. In PLA studies, ideally, the neutravidin should also provide for the epitopes recognized by the primary antibody. The primary antibodies for PLA are crucial reagents, which must be carefully selected for purity and specificity. Unfortunately, we could not identify any anti-neutravidin (or streptavidin) antibodies suitable for our studies. As an alternative strategy, we opted for a flag-peptide-based system, since several highly specific anti-flag antibodies are commercially available. FMTRIPs are a Flag peptide-modified version of MTRIPs. The following protocol, schematically illustrated in Fig. 3a, is optimized to produce neutravidin molecules labeled, on average, with 2 Flag-peptides, which ensures maximum PLA efficiency. Indeed, we observed that three Flags per neutravidin caused reduced PLA efficiency, probably due to steric hindrance or self-quenching of the fluorophores used for detection [23]. The use of HyNic-4FB system permitted to perform a controlled reaction, which results in a stable, covalent bond between the peptide and the neutravidin. The degree of labeling can be quantified after neutravidin conjugation to both 4FB and Flag-peptide, for quality control.

1. Dilute 80 µl of 10 mg/ml neutravidin in 1× Modification buffer to have a 2 mg/ml solution.
2. Add 4.9 µl of 20 mg/ml S-4FB in DMSO (corresponding to 30× molar excess).

3. Incubate the reaction for 2 h at room temperature.
4. Add 400 μ l of 1 $\times$ conjugation buffer and remove unincorporated 4FB using a 30 kDa filter (3 times at 10,000 $\times g$ for 5 min).
5. Recover the neutravidin-S4FB by reverse spinning (2 min at 1000 $\times g$) and adjust the concentration of the solution to 2 mg/ml using 1 $\times$ conjugation buffer (*see Note*¹).
6. Add 5 μ l of Flag-HyNic 10 mg/ml in DMSO (corresponding to 3 $\times$ molar excess).
7. Incubate overnight at room temperature.
8. Add 350 μ l of PBS and clean up using a 30 kDa filter (3 times at 10,000 $\times g$ for 5 min).
9. Recover the neutravidin-Flag by reverse spinning (2 min at 1000 $\times g$).
10. Determine the molar substitution ratio by measuring the ratio of the concentration of the S4-FB-HyNic bond and the concentration of neutravidin. The reaction between 4-FB reagent and HyNic-modified proteins leads to the formation of a traceable absorbance signal at 350 nm with a molar extinction coefficient of 24,000 mol⁻¹ cm⁻¹ (*see Note*²).
11. Dilute the Flag-tagged neutravidin to 6 μ M in 1 $\times$ PBS and store at 4 °C.

3.2 Oligonucleotide Labeling with Fluorophores

The oligonucleotides utilized for FMTRIPs consist of a 5' biotin modification, a linker sequence, composed typically by 6 thymidine nucleotides, and a ~20 bases-long antisense hybridization sequence, composed by 2'-O-Methyl RNA nucleotides, and three to four C6-amino modified thymines. The linker sequence is included to extend the hybridization region from the neutravidin, facilitating the binding to target mRNAs. The hybridization sequence should have a GC content of about 50 %, should not form secondary structures or primer dimers (which can be verified using software like MFOLD), and should be aligned against known transcripts, to ensure specificity for the mRNA of interest. The antisense sequence should not overlap known binding sites for regulatory proteins or miRNAs. The C-6 amino modified thymines are used as sites of conjugation of fluorophores, typically Cy3b or Dylight 650, which are characterized by a long fluorescent lifetime and good resistance to photobleaching. We typically utilize 3 FMRTIPS per mRNA, preferentially targeting the 3'UTR. The following protocol describes the oligonucleotide-labeling procedure we currently utilize, which yields a DOL of about 2–3 fluorophores per oligonucleotide (Fig. 3b).

1. Resuspend cy3b in DMSO at 25 mM concentration. The resuspended cy3b can be subsequently stored at –20 °C in ready-to-use aliquots.

¹The molar substitution ratio (number of S4-FB per neutravidin) can be measured using the 2-HP reagent according to the manufacturer protocol. The reaction between the 2-HP reagent and 4FB-modified proteins leads to the formation of a traceable absorbance signal at 350 nm with a molar extinction coefficient at 18,000 l/mol cm.

²The concentration of neutravidin should be determined using a BCA assay or a Bradford assay. The concentration of neutravidin cannot be measured via UV–Vis at 280 nm in a reliable way because of the S4-FB-HyNic bond.

2. Resuspend the biotinylated oligonucleotide in nuclease free water at 450 μM
3. Combine 6 μl of oligonucleotide, 3.5 μl of fluorophore and 37.5 μl of 0.1 M Bicarbonate buffer
4. Vortex gently for 4 h protected from light at room temperature
5. Remove unincorporated dyes by centrifugation three times in 1 $\times$ PBS using 3 kDa filters (20 min at 10,000 $\times g$).
6. Reverse spin in a new collection tube to retrieve the labeled oligonucleotide (2 min at 1000 $\times g$).
7. Determine oligonucleotide molar concentration and degree of labeling using UV-Vis. Measure the absorbance at 260 nm and at 565 nm. The molar concentration of nucleic acids and dye is given by [Concentration] = Abs $\times$ dilution factor $\times \epsilon$ ($\epsilon_{\text{cy3b}} = 150,000 \text{ M}^{-1} \text{ cm}^{-1}$; ϵ_{oligo} is provided by the manufacturer). The degree of labeling (DOL) is given by the ratio of [oligo]/[dye] (see **Notes** ³ and ⁴).
8. Dilute the labeled oligonucleotide at 30 μM in 1 $\times$ PBS and store at -20°C in ready-to-use aliquots.

3.3 FMTRIPs Assembly

The following protocol describes the final step to obtain functional FMTRIP molecules (Fig. 3b). FMTRIPs should be freshly prepared and purified before delivery to live cells. If different hybridization anti-sense sequences are utilized, each FMTRIP should be assembled and purified separately. At the end of this section we will discuss two assays that can be utilized to verify the successful assembly of the probes.

1. Combine 5 μl of 30 μM cy3b-labeled oligonucleotide and 5 μl of 6 μM neutravidin-flag.
2. Incubate for 1 h at room temperature protected from light.
3. Remove excess of oligonucleotide by centrifugation three times in 1 $\times$ PBS using 30 kDa (5 min at 10,000 $\times g$).
4. Recover the FMTRIPs by reverse spinning (2 min at 1000 $\times g$). The concentration of the FMTRIPs is about 1 μM .
5. Using the same procedure, assemble MTRIPs (containing neutravidin without Flag-peptide that will be utilized as a control experiment). See **Note** ⁵.

³High DOL often imply over-labeling, which causes quenching of fluorescence and/or inefficient binding of the oligonucleotide to its target mRNA.

⁴Another dye we routinely use for oligonucleotide labeling is Dylight 650 NHS ester. After oligonucleotide labeling with this dye, unincorporated fluorophores are not efficiently removed using 3 kDa filters, due to the larger molecular weight ($>1000 \text{ g/mol}$). We recommend using Zeba desalting columns (Thermo Fisher scientific) according to manufacturer instructions at least three times. $\epsilon_{650} = 250,000 \text{ M}^{-1} \text{ cm}^{-1}$.

⁵We routinely use FMTRIPs designed to target at least three sequences in the 3' UTR of target mRNAs, near the RPB binding site, since PLA detects interaction between elements $<40 \text{ nm}$ apart. We observed that increasing the number of FMTRIPs did not significantly affect PLA frequency.

Successful assembly of FMTRIPs should be verified in preliminary measurements, to ensure that the conjugation of flag-peptides does not compromise the ability of neutravidin to bind to the biotinylated oligonucleotides. This can be tested using, first of all, a gel retardation assay where the migration of biotinylated and fluorescently labeled oligonucleotides is compared to that of FMTRIPs in a native 20 % TBE polyacrylamide gel. The latter, will result in a band shifted toward higher molecular weights than the former. The effect of the flag-peptide modification can be easily observed by comparing the bands obtained after assembly of MTRIPs (with unmodified neutravidin) and FMTRIPs (Fig. 4a). Successful assembly of FMTRIPs can also be verified after deposition of the probes on glass coverslip. The probes are incubated for 30 min in growth media at low concentration (1 nM) in order to deposit single molecules. The media is removed, and the probes are fixed in 1 % PFA and mounted. This is a procedure we routinely utilize to test new imaging molecules, and the samples can be easily analyzed using wide field deconvolution microscopy or confocal microscopy. Experiments performed using a mixture of probes labeled with two different dyes (such as cy3b and Dylight 650) demonstrated that they do not form aggregates because they displayed similar mean fluorescence intensities and no colocalization between the two dyes was observed (Fig. 4b). FMTRIPs can be additionally visualized using immunofluorescence (IF) after fixation and blocking with a flag-tag antibody and a suitable secondary antibody. Experiments performed using cy3b labeled FMTRIPs and Alexafluor 488 labeled anti-flag antibody yielded high colocalization between the two fluorescent signals, demonstrating that the modified neutravidin retain their functionality (Fig. 4c).

3.4 Delivery to Cells

The following protocol describes the procedure to deliver FMTRIPs (and MTRIPs for control experiments) to cells using the pore-forming toxin Streptolysin-O (SLO). We utilized this delivery method successfully with several cell lines and primary cells including A549, HeLa, U2OS, Vero, VERO C1008, Hep, LnCaP, RAW 286.7, DU145, MDBK, MDCK, HDF, CEF, MCF-7. Standard working concentrations of SLO are 0.15 μml up to 0.8 μml . The optimal SLO concentration should be determined in preliminary experiments for each cell type, as described at the end of this paragraph.

1. Cells are plated the day prior to the experiment on 10 mm coverslips in 24 well plates (*see Note 6*).
2. Activate SLO by adding 1.5 μl TCEP to 100 μl of 2 μml SLO in H_2O and incubate 1 h at 37 °C (*see Note 7*).
3. Wash cells once with PBS without Calcium and magnesium
4. Dilute the 100 μl of activated SLO 1:10 in Optimem to have a 0.2 μml solution.
5. Add the FMTRIPs to the activated SLO. The final concentration of the FMTRIPs for delivery is about 30 nM.

⁶Prior to plating, the coverslips are cleaned with ethanol, dried with a kimwipe, and incubated at 37 °C submerged in complete media for at least 30 min to promote cell adhesion.

⁷SLO is dissolved in molecular biology grade water to a concentration of 25,000 μml , then aliquot into 2 μml (100 μl each) and stored at -20 °C. Do not use DEPC-treated water and do not freeze/thaw the aliquots, as this will decrease SLO activity.

6. Add to each well 250 μ l probes and incubate 10 min at 37 °C
7. Remove probes and add complete media. Incubate for 15 min at 37 °C for recovery.
8. Fix cells in 1 % PFA for 10 min at room temperature (*see Note* ⁸).
9. Wash once with PBS.
10. Permeabilize cells using 0.2 % Triton X for 5 min at room temperature.
11. Wash once with PBS.
12. Block using modified blocking buffer for 1 h at 37 °C (*see Note* ⁹).
13. Wash once with PBS for 5 min.

The optimal SLO concentration for efficient delivery can be tested using a given amount (30 nM) of a probe targeting, for example, β actin mRNAs and a scrambled probe (Fig. 5). The former will appear homogeneously distributed in the cell cytoplasm, near the nucleus and around the edges of the cell. RNA granules display heterogeneous intensity profiles, depending on the RNA content of different RNPs. The latter, instead, will appear prevalently distributed around the cell nucleus and not around the edges of the cell. Untargeted probes display uniform intensities, comparable to those of single probes. Low SLO concentrations result in poor probe delivery, with most of the fluorescent signal observed on glass, outside the cell. High SLO concentrations results in sequestration of probes within hollow vesicles in the cell cytoplasm.

3.5 Proximity Ligation Assay

All incubations are performed with coverslips face-down on parafilm in a humidity chamber to keep the reaction volume $\sim$ 40 μ l as recommended by the manufacturer. All washings are done placing the coverslips in a coverslip holder submerged in a beaker containing the wash buffer. For gentle shaking we use a bench rocker set at low speed. Experiments should be designed including two controls: (a) omitting one of the primary antibodies and (b) Using MTRIPs instead of FMTRIPs (Fig. 6).

1. Incubate samples with the primary antibody diluted in the primary antibody solution for 30 min at 37 °C (*see Note* ¹⁰).
2. Wash 10 min in buffer A (provided by manufacturer).
3. Incubate with PLA plus and minus probes for 30 min at 37 °C.
4. Wash 10 min in buffer A.
5. Incubate with the ligase diluted as recommended for 30 min at 37 °C.

⁸Different fixatives have been tested and were successfully utilized for PLA with FMTRIPs, such as 100 % Methanol, 50 % Methanol: 50 % Acetone or PFA in BRB 80 buffer (ideal to preserve microtubules).

⁹In preliminary experiments we tested both the blocking solution provided by the manufacturer and the modified blocking solution described here. While the former resulted in nonspecific signal, the latter efficiently eliminated the background signal.

¹⁰The concentration of the primary antibodies must be determined accurately for PLA (usually dilution 1:500 or higher, depending on the antibodies).

6. Wash 10 min in buffer A.
7. Incubate with the Amplification solution diluted as recommended for 100 min at 37 °C (*see Notes* ¹¹ and ¹²).
8. Wash with 1× buffer B (provided by manufacturer) for 20 min.
9. Wash with 0.01× buffer B for 1 min.
10. If immunostaining is required, wash once with 1× PBS before adding the primary antibody. For example, phalloidin staining (which binds to F-actin) can be used to identify the contour of single cells in the sample for subsequent analysis (*see Note* ¹³).
11. Mount with Duolink mounting medium with DAPI and wait for 15 min with samples protected from light.
12. Seal with nailpolish before imaging. Slides should be stored at −20 °C.

3.6 Detection and Analysis

In the current paragraph we will describe the detection and analysis procedure we perform utilizing the Volocity software (PerkinElmer) using a spinning disk confocal microscope. We also used laser scanning confocals as well as wide field deconvolution microscopes successfully. Analysis is performed on single cells identified by the mRNA signal or immunofluorescence.

1. Record image stacks at 300 nm intervals to adequately sample volumes.
2. Import the acquired files in the Volocity analysis software and linearly contrast enhance for display
3. In a single cell, determine the mRNA volume based on standard deviation intensity of the probes.
4. Identify the PLA puncta as “Objects” by their standard deviation intensity then separate into individual punctae using the “separate touching object” tool
5. The PLA punctae can be further filtered based on their size and maximum intensity (*see Note* ¹⁴).
6. The PLA frequency can be measured as the ratio of the number of PLA punctae and the fluorescent FMTRIP volume. In this way, interactions are normalized to the mRNA signal, allowing comparison of the quantification between cells (*see Note* ¹⁵)

¹¹The amplification solution contains the “detection reagent,” which is light sensitive. In this step and all the subsequent passages, samples should be protected from light.

¹²The detection reagent is available green, red, orange, and far-red and is therefore amenable for different filter sets and potentially ideal for multiplexing. We routinely utilize the far-red one, because it is the brightest and most photostable.

¹³We noted that performing immunofluorescence before the PLA protocol yields generally poor results probably as result of the washings with the required buffers.

¹⁴A PLA product is a micron-sized fluorescent puncta with consistent size and intensity for a variety of analytes, antibodies, and cell types.

¹⁵It is important to notice that not every FMTRIP participates in PLA productively for at least two reasons: (a) PLA detects interactions present at the time of fixation and (b) the distance between the FMTRIPs and the antibody against the protein of interest

7. Perform statistical analysis on the results using a software like Sigma plot.

3.7 Conclusions and Future Perspectives

The use of FMTRIP and PLA offers a relatively simple approach to the investigation of protein–mRNA interactions in single cells with single-interaction sensitivity. Protein–mRNA interactions were quantified by measuring the PLA frequency, defined as the ratio of the number of PLA punctae and the mRNA volume (identified by the fluorescent probes labeling the mRNAs). The limits of traditional colocalization measurements can be overcome using PLA because the antibodies are not utilized for direct observation of a protein but their specific binding to target proteins is necessary to start a reaction which amplifies the signal rendering the interaction visible. The result of the assay can be observed using a deconvolution or a confocal microscope. Various fixatives can be utilized to preserve specific cellular components without affecting the specificity and quality of the results. Crucial reagents in the assay are the primary antibodies specific for the Flag tag on FMTRIPs and the protein of interest, which have to be carefully tested in initial assays. Potentially all proteins involved in translational regulation can be examined, provided “a good” antibody, such as TTP, TIAR, or CUG-BP, but also proteins involved in mRNA decay like DCP1a or the proteasome components. The effect of RBPs on miRNA binding could additionally be addressed analyzing the interactions between the RNA-induced silencing complex (RISC) or Ago2 and RBPs. Identifying the localization of these interactions and whether RBPs bind cooperatively or competitively with other RBPs can help establish a model system for examining how changes in RBPs modulate mRNAs and their translation.

Critical aspects of the presently described method can be improved. PLA relies on two enzymatic reactions catalyzed by the ligase and phi29 DNA polymerase. Although these enzymatic reactions are generally predictable, they do not allow for optimization. Ensuring uniform enzymatic reactions across all the samples is virtually impossible. Potentially, this could be overcome by using a method that does not rely on enzymatic reactions, but yet ensures signal amplification. The proximity dependent Hybridization chain reaction method (proxHCR) recently described by Koos et al. [29] might represent a useful implementation to FMTRIP-based mRNA imaging for the detection of protein–mRNA interactions. In proxHCR when two oligonucleotide hairpins conjugated to antibodies bind in close proximity, they can be activated to reveal an initiator sequence. This starts a chain reaction of hybridization events between a pair of fluorophore-labeled oligonucleotide hairpins, generating a fluorescent product. This method would allow for detection of protein–protein or RNA–protein interactions without the need of enzymes. In conclusion, the quantitative characterization of the distribution of interactions for various mRNAs and RBPs provided by our method would be helpful in establishing the cell-to-cell variability for posttranscriptional regulatory events and how they contribute to mRNA “state.”

may exceed the distance for proximity ligation. For this reason, PLA represents a powerful tool for quantifying and comparing the relative change of interactions between different experimental conditions, rather than obtaining absolute numbers. Moreover, when performing PLA experiments, mRNAs and proteins are typically under-sampled in order to detect their interactions randomly for comparative analyses. Usually, the FMTRIPs and primary antibodies are used in concentrations that provide minimal nonspecific binding. While this allows for detection of relative differences between samples, it may cause under-sampling of the interactions especially for less abundant mRNAs.

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Fig. 1.

Delivery of Flag-tagged MTRIPs (FMTRIPs): **(a)** FMTRIPs are composed by a flag-tagged (green) neutravidin core (yellow) and four fluorescently labeled (red) oligonucleotides. They are delivered into live cells via SLO-mediated reversible membrane permeabilization. **(b)** FMTRIPs rapidly bind to target mRNAs while cells recover after SLO treatment. **(c)** Single mRNAs bind multiple probes, and can be recognized by the enhanced signal to noise ratio (T = FMTRIPs bound to mRNA, UT = unbound FMTRIPs)

Fig. 2.
PLA to detect protein-mRNA interactions using FMTRIPs: **(a)** Antibodies (*light blue* and *magenta*) bind to the Flag tag on FMTRIP and to the RNA binding protein (RBP) of interest. **(b)** Proximity probes (*dark blue* and *magenta*) bind to the primary antibodies and ligation occurs **(c)** A cy5-equivalent hybridized product (*green*) is synthesized via RCA

Fig. 3.

Details of FMTRIP composition and assembly: **(a)** neutravidin (*yellow*) is modified with 2 HyNic-Flag-tags (*green*) via the 4FB Linker (*blue*). **(b)** Biotinylated oligonucleotides are fluorescently labeled with cy3b (*red*) and subsequently incubated with Flag-tagged neutravidins to obtain functional FMTRIPs. *Red* asterisks represent “checkpoints” where reaction products can be quantified

Fig. 4.

Flag-tagged neutravidin as a core for functional mRNA imaging probes: **(a)** Migration of oligonucleotides, MTRIPs, and FMTRIPs in a 20 % TBE gel demonstrating the band shift due to the assembly of functional FMTRIPs. **(b)** Single cy3b (*red*) and 650 (*green*) labeled FMTRIPs do not aggregate or colocalize when deposited on glass. **(c)** Colocalization between cy3b-labeled FMTRIPs (*red*) and Flag immunofluorescence (*green*). Scale bars are 2.5 μm

Fig. 5.

Observation of efficient SLO delivery of probes: **(a)** Comparison of the distribution of 60 and 30 nM probes targeting β actin mRNA and 30 nM of untargeted probes. Phalloidin was used to identify the contour of the cell, and to demonstrate localization of targeted MTRIPs to the edges of the cell. Nuclei are stained with DAPI. **(b)** Mean MTRIP intensity observed in the experiments in **(a)**

Fig. 6.

Visualization of interactions between β actin mRNA and stress F-actin: Three cy3b labeled FMTRIPs (*red*) targeting the 3'UTR of β actin mRNA were delivered to cells using SLO. F-actin was visualized using Phalloidin Alexafluor488 (*green*). For PLA, we used an α Flag primary antibody, an α 488 primary antibody, and far-red detection reagent (*gray*). We routinely perform two control measurements, one using MTRIPs and one omitting one of the primary antibodies. Nuclei are stained with DAPI (*blue*). Scale bar is 10 μ m