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Quantification and localization of protein-RNA interactions in patient-derived archival tumor tissue

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Abstract

Abnormal post-transcriptional regulation induced by alterations of mRNA-protein interactions is critical during tumorigenesis and cancer progression and is a hallmark of cancer cells. A more thorough understanding is needed to develop treatments and foresee outcomes. Cellular and mouse tumor models are insufficient for vigorous investigation as they lack consistency and translatability to humans. Moreover, to date, studies in human tumor tissue are predominately limited to expression analysis of proteins and mRNA, which do not necessarily provide information about the frequency of mRNA-protein interactions. Here, we demonstrate novel optimization of a method that is based on fluorescent *in situ* hybridization and proximity ligation techniques, to quantify mRNA interactions with RNA binding proteins relevant for tumorigenesis and cancer progression in archival patient-derived tumor tissue. This method was validated for multiple mRNA-protein pairs in several cellular models and in multiple types of archival human tumor samples. Furthermore, this approach allowed high-throughput analysis of mRNA-protein interactions across a wide range of tumor types and stages through tumor microarrays. This method is quantitative, specific, and sensitive for detecting interactions and their localization at both the individual cell and whole tissue scales with single interaction sensitivity. This work presents an important tool in investigating post-transcriptional regulation in cancer on a high-throughput scale, with great potential for translatability into any applications where mRNA-protein interactions are of interest.

Keywords

archival tumor samples; post-transcriptional regulation; proximity ligation assay; fluorescent *in situ* hybridization; cancer

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Contributions

E.L.B and P.J.S. planned the experiments and interpreted the data. E.L.B., D.A., and S.M.B. performed experiments. E.L.B., C.Z., D.V., and P.J.S. wrote and edited the paper.

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Introduction

After transcription, processing, and transport, mRNA translation and stability is tightly regulated by miRNAs and RNA-binding proteins (RBPs) in a time- and space-dependent manner (1–3). Among these, human antigen R, HuR, promotes mRNA stability, while T-cell intracellular antigen-1 related protein, TIAR, represses translation (3). Abnormal post-transcriptional regulation induced by altered RBP interactions plays a critical role in deregulating mRNA function, which can result in altered cellular states and development of cellular cancer hallmarks, such as suppression of apoptosis (3). For example, cytoplasmic levels of HuR, which is predominantly nuclear in healthy cells, correlate with cancer pathogenesis and malignancy (3). In addition, HuR overexpression is associated with COX-2 overexpression, which is directly involved in tumor growth (3–6). Overexpression of both HuR and COX-2 has been observed in many cancer types, including colon and lung cancers (3,7). Understanding these aberrant RBP interactions is fundamental to improve biological understanding of tumorigenesis, to predict outcome, and to screen new treatments. Indeed, targeting RBPs and their interactions with mRNA is a strong cancer therapeutic interest (8,9).

Current techniques to detect RNA-protein interactions, such as immunofluorescence colocalization, immunoprecipitation, or fluorescence resonance energy transfer, either lack sensitivity, the ability to localize interactions, or the ability to characterize cell-to-cell heterogeneity. Additionally, these methods often require the analysis of a large number of cells, and cannot be used in fixed, archival, human tissues (10–13). Recent work by Roussis et al. and Zhang et al. attempted to alleviate these limitations but did not allow for simultaneous detection of mRNA or interactions with RBPs in tissue (14,15). Characterizing interactions in archival, patient-derived tissue is critical for a vigorous investigation of abnormal RBP interactions during tumorigenesis. While cellular models can provide insight, it has been shown that cancer cellular models and mouse tumor models can generate significantly different results (16). Cellular models alone, therefore, are not sufficient for a robust investigation. Mouse tumor models have been extensively used; however, the discrepancies between mouse and human anatomy and physiology limit any conclusion translatability, as evidenced by the low FDA approval rate for cancer drugs developed in mice (17). Therefore, vigorous investigations into post-transcriptional regulation in cancer necessitate human study, particularly in archival samples. Significant work in human samples has focused on the analysis of protein and mRNA expression with immunohistochemistry, but not on their interactions (18–22). Since simultaneous overexpression of a protein and mRNA does not necessarily stipulate interaction, this may misconstrue their importance as therapeutic targets. Furthermore, immunohistochemistry is typically semi-quantitative (23). Therefore, our objective was to develop a method to quantify mRNA-RBP interactions applicable to archival, patient-derived tissue.

We have previously demonstrated the use of a proximity ligation assay (PLA) to visualize RNA and quantify its RBP interactions, upon delivery of FLAG-tagged and fluorescently labeled 2'-O-methyl (2'OM) based multiply-labeled tetravalent RNA imaging probes (MTRIPs) to live cells, followed by fixation (24,25). The epitope tag on the MTRIP and the protein of interest are recognized by primary antibodies and subsequently by secondary

antibodies conjugated to specific oligonucleotides. If the oligonucleotides, and consequently the mRNA and protein, are within ~40 nm of distance, the oligonucleotides are ligated and are amplified by rolling circle amplification. The amplification is detected with fluorescent complementary oligonucleotides, creating a puncta per interaction.

Since live cell delivery is incompatible with patient-derived, archival tissue sections, here we optimized V5-tagged and peptide nucleic acid (PNA)-based MTRIPs (PMTRIPs) to visualize mRNA and quantify mRNA-protein interactions by fluorescent *in situ* hybridization (FISH) and PLA. (Figure 1A–B) (24,25). This PNA-based approach allowed us for the first time to *sensitively, specifically, and quantitatively detect native mRNA and its interactions with target proteins, via FISH-PLA, with single-interaction sensitivity, while providing localization information across single cells at tissue level in both frozen and paraffin-embedded (PFFE) archival samples.*

Materials and Methods

Cell culture

A549, HeLa, and Veros (ATCC) were maintained in DMEM (Lonza) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and 1% penicillin-streptomycin (Life Technologies). HT-29s and DLD-1s (ATCC) were maintained in RPMI-1640 (Lonza) supplemented with 10% FBS and 1% penicillin-streptomycin. All cells were sustained at 37°C and 5% CO₂. Cells were authenticated by ATCC, regularly checked for Mycoplasma contamination, and used for up to 18 passages from thawing.

Drugs and transfections

Where indicated, cells were treated with 5 µg/mL of actinomycin-D (ActD) for 1 hour at 37°C. 200 ng of c-Myc-GFP-AB or c-Myc-GFP-A plasmids were transfected via the Neon Transfection System (Life Technologies) to Veros in a 96 well plate. To knockdown HuR, HeLas were transfected with 200 nM of On-TARGET SMARTpool HuR siRNA (Thermo Fisher) or 200 nM of On-TARGETplus Non-targeting siRNA #1 (Thermo Fisher) with Lipofectamine 2000 (L2K) for 48 hours. To knockdown COX-2, HT-29s were transfected with 100 nM oCOX-2 siRNA (Thermo Fisher) or 100 nM of On-TARGETplus Non-targeting siRNA #1 with L2K for 24 hours.

PNA oligonucleotide design and fluorescent labeling

PNA oligonucleotides (PNA Bio) (Supplementary Table 1) were designed with a 5' biotin and an O-K-O-K spacer according to recommendations from the manufacturer. Sequences were chosen to be complimentary to mRNA sequences as specified from the NCBI GenBank. Several COX-2 PNA oligonucleotides were designed with gamma lysines as indicated with * for fluorescent labeling. Gamma lysines in COX-2 PNA were fluorescently conjugated to cy3B-NHS ester fluorophores (GE Healthcare) according to manufacturer's protocol.

PMTRIP preparation and assembly

Trilink's Solulink technology was used to conjugate a V5 epitope tag to NeutrAvidin, using the maleimide hynic and S-4FB crosslinkers according to manufacturer instructions. V5-NeutrAvidin was then purified via a 30KD MWCO centrifugal filter (Millipore Sigma). Last, V5-NeutrAvidin and 5' biotinylated PNA oligonucleotides (PNA Bio) were incubated at a 1:5 mol ratio for 1 hour at RT followed by filtration with a 30 KD MWCO centrifugal filter.

Immunofluorescence

A549s were infected with respiratory syncytial virus at a multiplicity of infection of 3 for 24 hours. Cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) for 10 minutes at RT and permeabilized with 0.2% Triton X-100 (Sigma Aldrich) for 5 minutes at RT. Cells were incubated overnight in 2x saline sodium chloride (SSC) (Sigma Aldrich) with either 10% or 0% formamide. Next, cells were blocked with 5% bovine serum albumin (BSA) (Sigma Aldrich) for 30 minutes at 37°C and stained with antibodies for the fusion protein, F, (Palivizumab, Medimmune, 1 µg/mL) and the matrix protein, M, (C781, 1 µg/mL, gift from James Crowe) for 30 minutes at 37°C. Cells were washed with 1xPBS and stained with the respective secondary antibodies (Jackson ImmunoResearch, 1:250 dilution) for 30 minutes at 37°C. Finally, cells were stained with DAPI and mounted in prolong gold (Thermo Fisher).

Live cell mRNA-protein PLA

PLA was performed as previously discussed using V5-tagged MTRIPS with either 2'OM or PNA (24). A549s plated in a 96 well plate were treated with ActD. MTRIPs were assembled with either PNA oligonucleotides or 2'OM oligonucleotides (Biosearch) and were delivered to cells with .4 U streptolysin-O (Sigma Aldrich) in OPTIMEM (Invitrogen) for 15 minutes at 37°C. Streptolysin-O/OPTIMEM was then removed and the cells were incubated for 10 minutes at 37°C in DMEM before fixing.

PLA in cells

Cells were fixed in 1% PFA and PLA was performed according to manufacturer instructions (Sigma Aldrich). Blocking buffer was 1% BSA, 2% donkey serum, and 0.2% gelatin in 1xPBS. Primary antibodies were V5 (Abcam, Cat.# ab9116, 1:1000 dilution) and either HuR (Santa Cruz, Cat.# sc-5261, 1:1000 dilution) or TIAR (Santa Cruz, Cat.# sc-398372, 1:10 dilution) diluted in a buffer of 1% BSA, 0.2% donkey serum, and 0.25% gelatin in 1xPBS. Controls include PLA with no primary antibodies, PLA with FISH with a scrambled oligonucleotide, and PLA with MTRIPs lacking a V5 epitope tag.

FISH for IVT mRNA colocalization

HeLas plated in a 96 well plate (Cellvis) were transfected with 200 ng of luciferase mRNA via L2K. The mRNA was labeled prior to delivery with Dylight-650 MTRIPs, as previously described (26). After 5 hours, cells were fixed with 1% PFA and permeabilized overnight with 70% ethanol at 4°C. Cells were then blocked for endogenous biotin with an endogenous biotin blocking kit (Thermo Fisher). For DNA oligonucleotide FISH (Stellaris Biosearch Technology), cells were incubated with 200 nM of Quasar-570 labeled

oligonucleotides specific for the luciferase mRNA overnight at 37°C in a hybridization buffer of 2xSSC with 10% formamide (Sigma Aldrich), 10% dextran sulfate (Sigma Aldrich), 0.5% tRNA (Thermo Fisher), 0.5% ssDNA (Thermo Fisher) and 0.2% BSA (New England Biolabs). Next, cells were washed for 30 minutes in 2xSSC with 10% formamide and 10% dextran sulfate at 37°C before mounting in DAPI mounting medium. For PNA-based FISH, NeutrAvidin was first conjugated to cy3b NHS-ester according to manufacturer instructions and assembled into PMTRIPs. 20 nM of the PMTRIPs were incubated with cells overnight 37°C in a hybridization buffer of 2x SSC with 0.5% tRNA, 0.5% ssDNA and 0.2% BSA in 1xPBS. Last, cells were washed for 30 minutes in 2xSSC at 37°C before mounting in DAPI mounting medium.

For tissue studies, luciferase mRNA was pre-labeled with Dylight-650 MTRIPs as previously described (26). 10 µg of mRNA in Ringer's lactate buffer (Bioo Scientific) either naked or complexed with Viomer Red (Origene) was injected into the anterior tibialis muscle of BALB/C mice. After 2 hours, mice were sacrificed and the muscle was removed and fixed in 4% PFA overnight. Tissue was fixed in sucrose overnight, embedded in OCT and snap-frozen in isopentane. Tissue sections were sliced to 10 µm thick sections using a cryostat. Tissue was permeabilized in 50:50 methanol acetone at -20°C for 20 minutes and FISH was performed as described above.

Mice were housed and manipulated under specific-pathogen-free conditions in the animal care facilities and experiments were performed in accordance with IACUC protocols approved by the Georgia Institute of Technology.

FISH-PLA

Indicated cells in a 96 well plate were treated with ActD. Cells were fixed with 1% PFA and permeabilized with 70% ethanol overnight at 4°C. V5-tagged PMTRIPs at specified concentrations were delivered overnight at 37°C in a hybridization buffer of 2xSSC with 0.5% tRNA, 0.5% ssDNA and 0.2% BSA in 1x PBS. PLA was then performed between the V5 tag and HuR or TIAR. To test dextran sulfide's effect, FISH hybridization buffer contained either 0%, 5%, or 10% dextran sulfate.

RNA immunoprecipitation

HT-29s were treated with siRNA as previously described. An Imprint RNA immunoprecipitation (RIP) kit (Sigma Aldrich) was used with either a HuR or nonspecific antibody. qRT-PCR was performed for COX-2 or GAPDH using Taqman primers (Thermo Fisher).

FISH-PLA *in situ* in tissue

Formalin fixed and paraffin embedded (PFFE) tissue sections (Origene) or microarrays (US Biomax) were deparaffinized in xylene, followed by decreasing percentages of ethanol (100%, 95%, 70%, and 50%) before rinsing in water. The tissue was then immersed in 1x citrate buffer (Wako) for antigen retrieval via steaming for 20 min. Next, the tissue was permeabilized by incubation in PBST (1xPBS, tween-20) for 10 min at RT and then was equilibrated in FISH wash buffer (2xSSC in H₂O) for 5 min at RT before FISH-PLA was

performed as previously described. The blocking buffer was composed of 1.25% BSA and 5% donkey serum in 1xPBS, while primary antibodies (HuR, 1:200; TIAR, 1:10; V5 1:750) were diluted in 1% BSA, 1% donkey serum, and 0.2% Triton X-100 in 1xPBS.

Tissue sections flash frozen in OCT in liquid nitrogen (Origene) were stored at -80°C . After thawing, the tissue was fixed in 50:50 methanol-acetone for 10 min at -20°C . Tissue was then equilibrated in FISH wash buffer for 5 min at RT before FISH-PLA as described above.

IRB approval was not required for this work under IRB exemption 4, as tissue was purchased fully deidentified.

Immunohistochemistry

PFFE tissue was deparaffined and processed for antigen retrieval. The tissue was permeabilized by incubation in PBST for 10 min at RT, before incubation with specified primary antibodies overnight at 4°C . Endogenous peroxidase was blocked with 3% H_2O_2 for 15 minutes at RT, followed by incubation with a horseradish peroxidase conjugated secondary antibody (Jackson ImmunoResearch, 1:1000 dilution). Finally, tissue was incubated with 3,3'-diaminobenzidine (Sigma Aldrich) for 5–10 minutes at RT, followed by hematoxylin staining.

Tissue FISH

FISH was performed as described above for FISH-PLA. 100 nM of V5-tagged PMTRIPs were delivered, and targeted with an anti-V5 antibody overnight at 4°C , followed by a fluorescent secondary antibody for 30 minutes at 37°C , then DAPI staining for 5 minutes at RT.

Microscopy

Images for *in vitro*, frozen tissue, and microarray experiments were acquired using a Hamamatsu Flash 4.0 v2 sCMOS camera on a PerkinElmer UltraView spinning disk confocal microscope on a Zeiss Axiovert 200M body. A 63x, NA 1.4 Zeiss Plan-Apochromat oil objective, a 20x, NA 0.8 Zeiss Plan-Apochromat air objective, or a 10x, NA 0.3 Zeiss EC-Plan-NEOFLUAR air objective were used for images. Imaging was controlled by Volocity acquisition software (PerkinElmer). Image stacks were recorded at 200 nm intervals. Tiled images were stitched using Volocity acquisition software with 25% overlap. Images for PFFE tissue experiments were acquired using a Zeiss laser scanning confocal microscope with a Zeiss LSM 710 confocal scan head on an AxioObserver Z1 inverted microscope stage using a 20x, NA 0.8 Zeiss Plan-Apochromat air objective. Image stacks were recorded at 3 or 4 μm intervals. Tiled images were stitched using Zeiss acquisition software with 10–25% overlap. Brightfield images were taken with a Zeiss AxioCam color camera on an AxioObserver Z1 inverted microscope body using a 20x, NA 0.8 Zeiss Plan-Apochromat air objective. Images were linearly contrast enhanced for visual clarity.

Quantification and Statistical Analysis

All quantification and analysis were performed on unenhanced data. For *in vitro* data, Volocity acquisition software was used to draw regions of interest around individual cells

and quantify the number of PLA puncta per cell above a specified signal threshold. 30 cells were counted per condition. Either a one-way ANOVA/Kruskal-Wallis test with a Dunn's multiple comparisons test, or a two-way ANOVA with a Tukey's multiple comparisons test were used to determine statistical significance, as indicated in the figure captions. For *in vivo* data, Volocity acquisition software was used to measure the area and sum of signal intensities of PLA puncta for each tissue section. To remove autofluorescence signal, fluorescent signal in an unused channel was recorded. Volocity acquisition software subtracted any measured PLA signal that colocalized with the autofluorescence signal. Immunohistochemistry data was analyzed with ImageJ, where the area of signal above a set threshold was measured. A one-way ANOVA with a Tukey's multiple comparisons test or t-test were used to determine significance. Statistics were performed in GraphPad Prism 7.0, as indicated in captions. n values are the result of biological replicates.

Results

PMTRIPs' high specificity as RNA imaging probes

FISH is the prevalent method for nucleic acids detection in cells and tissues (27). In order to use MTRIPs as FISH probes, we replaced 2'OM bases with PNA, hypothesizing this would improve the specificity and binding efficiency to target mRNAs in fixed samples. While PNA possesses the same nucleotide bases as DNA, its backbone consists of N-(2-aminoethyl) glycines, which confers a neutral charge (28–31). Consequently, PNA binds more strongly to target molecules, as there is no electrostatic repulsion (28–31). Furthermore, non-specific interactions with charged molecules are reduced (28–31). The resulting high binding affinity and specificity renders PNA an ideal RNA imaging tool. Additional advantages of this chemistry are its stability in the cellular environment, in a wider range of pH and temperature, as well as its resistance to nucleases and proteases (28–31).

First, we compared the use of PNA and 2'OM probes upon live cell delivery, by quantifying the interactions between polyadenylated mRNA and HuR with the previously described PLA assay (24,25). Incorporation of PNA increased the binding efficiency of MTRIPs to target mRNA (28). Accordingly, we measured more mRNA-HuR interactions (puncta) using PMTRIPs compared to 2'OM-based MTRIPs (Supplementary Figure 1A).

Next, we optimized a protocol to use PMTRIPs post-fixation as FISH probes (Figure 1C). In standard FISH protocols, buffers containing formamide and dextran sulfate are used to promote hybridization. However, formamide is known to denature proteins (32) and can prevent antibody binding to target epitopes (Supplementary Figure 1B). Furthermore, dextran sulfate inhibits polymerase function (33), an essential step for PLA.

We developed a hybridization buffer devoid of these reagents, and confirmed the sensitivity and specificity of PMTRIPs to detect target mRNA in cells and tissue, upon delivery of synthetic mRNA and colocalization analysis. We pre-labeled a luciferase-encoding *in vitro* transcribed (IVT) mRNA with DyLight 650 MTRIPs targeting its 3'UTR (26). The mRNA was then delivered to cells via L2K or to mice by intramuscular (IM) injection, either naked or using a modified form of PEI. After fixation, FISH was performed using either the Quasar

570 DNA-based probes or cy3B-labeled PMTRIPs (Supplementary Figure 1C–E). Both DNA and PMTRIPs colocalized with the luciferase mRNA in cells and in tissue, upon vehicle-mediated delivery. However, when the pre-labeled mRNA was delivered naked to muscle, a less efficient method, colocalization was only observed using PMTRIPs, demonstrating their high binding efficiency.

PMTRIPs-based FISH was also demonstrated in different cell lines, where various target endogenous mRNAs were successfully visualized, including polyadenylated transcripts, Actin- β and COX-2 mRNAs (Supplementary Figure 1F–H).

Quantification of mRNA-protein interactions by PMTRIP FISH-PLA in cells

We next validated the use of PMTRIPs for FISH-PLA in cells. A V5-epitope tag was conjugated to the NeutrAvidin core of PMTRIPs, which were hybridized via FISH to target mRNAs.

PLA was performed using primary antibodies against the V5 tag and the protein of interest. We first quantified interactions between the polyadenylated mRNA and HuR or TIAR in A549s (Figure 1D). Cells were treated with ActD prior to fixation to promote HuR and TIAR cytoplasmic localization and mRNA binding (34,35). A significant number of mRNA interactions per cell was measured with both HuR and TIAR compared to controls. As expected, no PLA puncta between HuR and polyadenylated mRNA were detected in cells exposed to dextran sulfate (Supplementary Figure 2 A–B). Accordingly, a significant number of interactions over controls were observed between COX-2 mRNA and either HuR or TIAR in HT-29s, which overexpress COX-2 mRNA (Figure 1E). Similar results were observed measuring RBP interactions with Actin- β mRNA (Figure 1F) compared to their controls. By measuring PNA FISH-PLA in multiple cell types, with two different proteins, two specific mRNAs and polyadenylated mRNAs, we demonstrated that this method is easily translatable to any mRNA-protein pair of interest.

To confirm that the incorporation of PNA bases was necessary to achieve increased specificity, a similar FISH-PLA assay was performed using 2'OM-based MTRIPs to quantify HuR interactions with polyadenylated mRNA. More interactions were detected with 2'OM-based MTRIPs than with PMTRIPs, including a higher number of nonspecific interactions in the scrambled control (Supplementary Figure 2C).

Sensitivity of PMTRIP FISH-PLA to variations in mRNA and protein levels

We next demonstrated that PMTRIP FISH-PLA sensitively quantifies changes in interaction frequency as a result of altered mRNA or protein levels. First, we compared the frequency of HuR interactions with polyadenylated mRNA in control conditions, when HuR remained predominantly nuclear, or upon ActD treatment, which induces HuR cytosol translocation (34). A significant number of interactions compared to controls were measured in untreated cells. As expected, the number of interactions significantly increased in cells treated with ActD, as a result of the higher HuR cytoplasmic concentration (Figure 2A, Supplementary Figure 2D). Next, cells were transfected with a plasmid encoding for c-Myc-GFP designed either with (AB) or without (A) a HuR binding site (Figure 2B, Supplementary Figure 2E). Only cells transfected with c-Myc-GFP containing a HuR binding site displayed significant

protein-mRNA interactions. Finally, we knocked down HuR expression in cells by HuR siRNA transfection (Figure 2C, Supplementary Figure 2F). Cells with reduced HuR levels displayed significantly fewer interactions with polyadenylated mRNA than those transfected with a non-targeted siRNA.

We also demonstrated PMTRIP FISH-PLA sensitivity to variations of mRNA expression. First, we knocked down COX-2 mRNA in HT-29s by COX-2 siRNA transfection (Figure 2D, Supplementary Figure 2G). Cells with knocked down COX-2 expression produced significantly fewer interactions between HuR and COX-2 mRNA than those with a non-targeted siRNA. We next compared these results to those obtained with RIP. We transfected cells with COX-2 or non-targeted siRNA, pulled down RNA-protein complexes with either a HuR or a nonspecific antibody and performed qRT-PCR for COX-2 or GAPDH (Figure 2E). Our pulldown results were comparable to our prior results with FISH-PLA; COX-2 RNA was detected in significant levels over the nonspecific antibody pull-down in cells treated with the non-target siRNA, but not after the COX-2 siRNA. No significant levels of GAPDH were found, as HuR does not interact with GAPDH (36). Finally, we compared HuR and TIAR interactions with COX-2 mRNA in DLD-1s, characterized by low COX-2 mRNA expression, and HT-29s, with high COX-2 mRNA expression (Figure 1E, Figure 2F). As expected, fewer interactions were observed in DLD-1s compared to HT-29s. Collectively, these results demonstrated that PMTRIP FISH-PLA is highly specific, sensitive, and comparable to RIP and therefore is a valid assay to assess variations of mRNA-protein interactions.

PMTRIP FISH-PLA in patient-derived, archival tissue

Finally, we quantified mRNA-protein interactions in fixed, archival patient-derived tissue sections. First, we measured interactions between polyadenylated mRNA and either HuR or TIAR in PFFE-embedded metastatic, stage IV, colon cancer tissue samples, both at the level of whole tissue and single cells. Individual PLA puncta were not resolvable in whole tissues when imaged at low magnification. Therefore, total PLA signal area and the sum of intensities of PLA signal were quantified, since these analysis methods produced similar results in cells (Supplementary Figure 3A–C). A significant increase in interactions between polyadenylated mRNA and HuR or TIAR were observed in metastatic colon tumor samples compared to healthy samples (Figure 3A–F, Supplementary Figure 3D–I). Similarly, in colon tumor samples, both HuR and TIAR displayed significant interactions with COX-2 mRNA over healthy tissue and controls (Figure 4A–F, Supplementary Figure 4A–F). Interestingly, interactions with HuR were detected in the villi in healthy colon sections. These interactions were likely due to the high HuR expression previously observed in healthy colon villi (6). FISH-PLA results were correlated by the high expression of HuR, TIAR, and COX-2 mRNA in colon cancer (Figure 4G–H, Supplementary Figure 4G). We further demonstrated the versatility of the assay by measuring mRNA-RBP interactions in PFFE-embedded metastatic, stage IV, lung cancer tissue. We found significant interactions for all measured mRNA-protein pairs in tumor samples compared to healthy tissues or negative controls (Figures 5A–F, 6A–F, Supplementary Figures 5A–F, 6A–F). Critically, we observed that RBP interactions were heterogeneously distributed across the cancer tissue sections, demonstrating that our assay allows to preserve and acquire spatial information at

both the tissue and single cell scale. To fully demonstrate the versatility, we measured interactions between polyadenylated mRNA and HuR in frozen, archival, patient derived, colon cancer tissues, where we found similar results to those observed in PFFE tissue (Supplementary Figure 7A–C).

Last, we demonstrated the potential of this assay for an in-depth study of the role of RBPs in tumorigenesis by investigating polyadenylated mRNA-TIAR and COX-2 mRNA-HuR interactions in a tissue microarray of 60 lung tumor cores of different stages and types, alongside their matching healthy counterparts (Figure 7A–B). While some tumor cores demonstrated significantly more interactions than their healthy counterparts, we could not identify a universal trend across all stages and types. For example, stage II tumors produced a higher-fold change of polyadenylated mRNA-TIAR interactions than stage III tumors. Similarly, only adenocarcinoma tumors produced increased polyadenylated mRNA-TIAR interactions. COX-2-HuR interactions did not display a change in stage II tumors, decreased in stage III tumors and large cell, small cell, and squamous cell carcinomas, and increased in adenocarcinoma tumors. To fully investigate tumor complexity, further work is required to measure a larger sample size for a strong statistical analysis. Future studies will be aimed at developing this assay to investigate multiple mRNA/RBP interactions simultaneously.

Discussion

Here, we demonstrated the use of PMTRIPs in a mRNA-protein interaction assay in both fixed cells and fixed, archival, patient-derived tissue. While FISH is a common technique for detecting RNA post-fixation, the use of formamide and dextran sulfate to promote hybridization limits its universality. Though many antibodies tolerate formamide exposure, formamide exclusion was required to render our method suitable for any protein/mRNA pair. Furthermore, the use of dextran sulfate precludes the use of PLA post-FISH. As a result of their high specificity, PMTRIPs eliminated the need for formamide and dextran sulfate, creating an assay suitable to quantify any pair of interest with high specificity. We established the high specificity and affinity of PMTRIPs for both live and FISH-PLA post-fixation, compared to DNA oligonucleotides or 2'OM-based MTRIPs. This approach was successfully applied to samples treated with multiple tissue fixation methods and without the need for protease treatment. Critically, we quantified variations of interactions frequency in multiple cell types, between multiple mRNAs and protein pairs, with both wild-type and modulated levels of mRNA and protein, with results analogous to those obtained by RIP, while maintaining cell-to-cell heterogeneity information.

In fixed, archival tissue, we detected significant interactions between both HuR and TIAR with either polyadenylated mRNA or COX-2 mRNA in colon or lung tumor tissue compared to controls. It is important to note that tissue sections are characterized by detectable natural autofluorescence, particularly in colon cancer and healthy lung tissue samples, which can be easily removed during image processing. Significant mRNA-HuR interactions were expected, due to the known role of HuR in cancer pathogenesis (3–5). The detected COX-2-HuR interactions support the role of HuR in promoting COX-2 overexpression in cancer previously suggested in the literature by simultaneous overexpression (5). The increase of interactions between TIAR and polyadenylated or COX-2 mRNA in both colon and lung

cancer was unexpected. TIAR represses translation and is thought to be a tumor suppressor, so we anticipated to measure fewer interactions with mRNA in tumors (18). However, the observed interactions correlated with the increased expression of TIAR and COX-2 mRNA observed in colon cancer samples. Strong TIAR signal has previously been observed in infiltrating inflammatory cells in tumors (18), which may explain the significant interactions detected by our assay, which relies on the ability to observe interactions on a whole tumor level. These interactions require further investigation, including the characterization by cell type and their heterogeneous spatial distribution across the tumor via immunofluorescence staining post-FISH-PLA.

While the FISH-PLA results correlated with protein and mRNA expression levels in colon cancer, FISH-PLA quantifies specific mRNA-protein interactions. Additionally, IHC expression analysis is typically subjective and only semi-quantitative (23). Moreover, simultaneous upregulation does not necessarily indicate interaction; multiple RBPs promote mRNA stability but many mRNAs share the same binding sites, making it difficult to conclude which interactions are occurring simply by quantifying protein or mRNA expression levels (1,3,4,34,37). Proteins binding can be competitive or cooperative and this ultimately determines mRNA fate (37). FISH-PLA specifically quantified the increase of protein-mRNA interactions in colon cancer, making it an ideal tool to investigate the relevance of post transcriptional regulation in cancer.

Interactions between mRNA and proteins were detectable on both the whole tissue and individual cell levels. While the COX-2 mRNA alone was not visible at the lower magnification, it could be observed in the individual cells at higher magnifications. Co-localizing the mRNA and mRNA-protein interactions is critical, as this provides information about the contribution of altered mRNA expression to tumorigenesis.

Preliminary PMTRIP FISH-PLA in a lung tumor tissue microarray revealed a lack of an obvious trend in protein-mRNA interactions, which was not unexpected. Tumors are complex, heterogeneous microenvironments, and further studies/methodologies are required for a full statistically significant analysis of tumor metrics and their correlation with abnormal interactions. The observed increase in interactions for TIAR with polyadenylated mRNA in adenocarcinomas compared to squamous cell carcinomas was consistent with previously discussed evidence obtained of expression levels (18). Additionally, increased cytoplasmic HuR levels have previously been demonstrated to correlate with high COX-2 expression in lung adenocarcinomas (19), which is supported by the increase in interactions observed here. However, the decrease in COX-2 and HuR interactions observed in lung squamous cell carcinomas was contradictory to the conclusions drawn from the previously reported concurrent increase in COX-2 and HuR expression (19,20). This provides further strong evidence for the need of PMTRIP FISH-PLA to fully investigate abnormal RBP interactions, rather than relying only on expression levels. This discrepancy may explain the conflicting reports in the literature about the prognostic value of HuR expression (19,20), and will be investigated further in future work. Advancing this methodology to multiplexing capability will increase ease of use and expand the study of tumor metrics. A comprehensive study of RBP interactions across multiple tumor types and stages will be easily achievable

with FISH-PLA, providing knowledge about tumor progression and presenting possible targets for future treatments.

In conclusion, we demonstrated a PNA-based FISH-PLA method to simultaneously detect mRNA and its interactions with RBPs, with single-interaction sensitivity, in fixed, archival patient-derived tissue samples. This method is specific, quantifiable, and versatile. We demonstrated that abnormal mRNA-proteins interactions can be spatially resolved and quantified in various types of tumors in patient-derived samples. We believe PMTRIP FISH-PLA will be a valuable tool in investigating cancer biology on a high-throughput scale, with great potential for translatability into any disease where abnormal RNA-protein interactions occur.

Data Availability

All data from this study are available from the corresponding author upon reasonable request.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Chen C-YA, Shyu A-B. AU-rich elements: characterization and importance in mRNA degradation. *Trends Biochem Sci.* 1995;20:465–70. [PubMed: 8578590]
2. Dreyfuss G, Kim VN, Kataoka N. Messenger-RNA-binding proteins and the messages they carry. *Nat Rev Mol Cell Biol.* 2002;3:195–205. [PubMed: 11994740]
3. Wurth L Versatility of RNA-Binding Proteins in Cancer. *Comp Funct Genomics* [Internet]. 2012;2012 Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3359819/>
4. Kouwenhove, Kedde M, Agami R MicroRNA regulation by RNA-binding proteins and its implications for cancer. *Nat Rev Cancer.* 2011;11:644–56. [PubMed: 21822212]
5. Dixon DA, Tolley ND, King PH, Nabors LB, McIntyre TM, Zimmerman GA, et al. Altered expression of the mRNA stability factor HuR promotes cyclooxygenase-2 expression in colon cancer cells. *J Clin Invest.* 2001;108:1657–65. [PubMed: 11733561]

6. Giammanco A, Blanc V, Montenegro G, Klos C, Xie Y, Kennedy S, et al. Intestinal Epithelial HuR Modulates Distinct Pathways of Proliferation and Apoptosis and Attenuates Small Intestinal and Colonic Tumor Development. *Cancer Res.* 2014;74:5322–35. [PubMed: 25085247]
7. Soslow RA, Dannenberg AJ, Rush D, Woerner BM, Khan KN, Masferrer J, et al. COX-2 is expressed in human pulmonary, colonic, and mammary tumors. *Cancer.* 2000;89:2637–45. [PubMed: 11135226]
8. Blanco FF, Preet R, Aguado A, Vishwakarma V, Stevens LE, Vyas A, et al. Impact of HuR inhibition by the small molecule MS-444 on colorectal cancer cell tumorigenesis. *Oncotarget.* 2016;7:74043–58. [PubMed: 27677075]
9. Lin G-L, Ting H-J, Tseng T-C, Juang V, Lo Y-L. Modulation of the mRNA-binding protein HuR as a novel reversal mechanism of epirubicin-triggered multidrug resistance in colorectal cancer cells. *PLOS ONE.* 2017;12:e0185625. [PubMed: 28968471]
10. Taneja KL, Lifshitz LM, Fay FS, Singer RH. Poly(A) RNA codistribution with microfilaments: evaluation by in situ hybridization and quantitative digital imaging microscopy. *J Cell Biol.* 1992;119:1245–60. [PubMed: 1360014]
11. Dreyfuss G, Choi YD, Adam SA. Characterization of heterogeneous nuclear RNA-protein complexes in vivo with monoclonal antibodies. *Mol Cell Biol.* 1984;4:1104–14. [PubMed: 6204191]
12. Turner B, Melcher SE, Wilson TJ, Norman DG, Lilley DMJ. Induced fit of RNA on binding the L7Ae protein to the kink-turn motif. *RNA.* 2005;11:1192–200. [PubMed: 15987806]
13. Rackham O, Brown CM. Visualization of RNA–protein interactions in living cells: FMRP and IMP1 interact on mRNAs. *EMBO J.* 2004;23:3346–55. [PubMed: 15282548]
14. Roussis IM, Guille M, Myers FA, Scarlett GP. RNA Whole-Mount In situ Hybridisation Proximity Ligation Assay (rISH-PLA), an Assay for Detecting RNA-Protein Complexes in Intact Cells. *PLoS ONE* [Internet]. 2016 [cited 2017 Apr 6];11 Available from: <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4732756/>
15. Zhang W, Xie M, Shu M-D, Steitz JA, DiMaio D. A proximity-dependent assay for specific RNA–protein interactions in intact cells. *RNA.* 2016;22:1785–92. [PubMed: 27659050]
16. López de Silanes I, Fan J, Galbán CJ, Spencer RG, Becker KG, Gorospe M. Global Analysis of HuR-Regulated Gene Expression in Colon Cancer Systems of Reducing Complexity. *Gene Expr.* 2018;12:49–59.
17. Day C-P, Merlino G, Van Dyke T. Preclinical Mouse Cancer Models: A Maze of Opportunities and Challenges. *Cell.* 2015;163:39–53. [PubMed: 26406370]
18. Sánchez-Jiménez C, Ludeña MD, Izquierdo JM. T-cell intracellular antigens function as tumor suppressor genes. *Cell Death Dis.* 2015;6:e1669. [PubMed: 25741594]
19. Giaginis C, Alexandrou P, Tsoukalas N, Sfiniadakis I, Kavantzis N, Agapitos E, et al. Hu-antigen receptor (HuR) and cyclooxygenase-2 (COX-2) expression in human non-small-cell lung carcinoma: associations with clinicopathological parameters, tumor proliferative capacity and patients' survival. *Tumor Biol.* 2015;36:315–27.
20. Kim GY, Lim S-J, Kim YW. Expression of HuR, COX-2, and survivin in lung cancers; cytoplasmic HuR stabilizes cyclooxygenase-2 in squamous cell carcinomas. *Mod Pathol.* 2011;24:1336–47. [PubMed: 21572400]
21. Hopkins TG, Mura M, Al-Ashtal HA, Lahr RM, Abd-Latip N, Sweeney K, et al. The RNA-binding protein LARP1 is a post-transcriptional regulator of survival and tumorigenesis in ovarian cancer. *Nucleic Acids Res.* 2016;44:1227–46. [PubMed: 26717985]
22. Mura M, Hopkins TG, Michael T, Abd-Latip N, Weir J, Aboagye E, et al. LARP1 post-transcriptionally regulates mTOR and contributes to cancer progression. *Oncogene.* 2015;34:5025–36. [PubMed: 25531318]
23. de Matos LL, Trufelli D, Luongo de Matos M, Aparecida da Silva Pinhal M. Immunohistochemistry as an Important Tool in Biomarkers Detection and Clinical Practice. *Biomark Insights.* 2010;5:9–20. [PubMed: 20212918]
24. Jung J, Lifland AW, Zurla C, Alonas EJ, Santangelo PJ. Quantifying RNA–protein interactions in situ using modified-MTRIPs and proximity ligation. *Nucleic Acids Res.* 2013;41:e12–e12. [PubMed: 22952158]

25. Jung J, Lifland AW, Alonas EJ, Zurla C, Santangelo PJ. Characterization of mRNA-Cytoskeleton Interactions In Situ Using FMTRIP and Proximity Ligation. *PLOS ONE*. 2013;8:e74598. [PubMed: 24040294]
26. Kirschman JL, Bhosle S, Vanover D, Blanchard EL, Loomis KH, Zurla C, et al. Characterizing exogenous mRNA delivery, trafficking, cytoplasmic release and RNA–protein correlations at the level of single cells. *Nucleic Acids Res*. 2017;45:e113–e113. [PubMed: 28449134]
27. Levsky JM, Singer RH. Fluorescence in situ hybridization: past, present and future. *J Cell Sci*. 2003;116:2833–8. [PubMed: 12808017]
28. Hyrup B, Nielsen PE. Peptide Nucleic Acids (PNA): Synthesis, properties and potential applications. *Bioorg Med Chem*. 1996;4:5–23. [PubMed: 8689239]
29. Shakeel S, Karim S, Ali A. Peptide nucleic acid (PNA) — a review. *J Chem Technol Biotechnol*. 2006;81:892–9.
30. Nielsen PE. PNA technology. *Mol Biotechnol*. 2004;26:233–48. [PubMed: 15004293]
31. Dean DA. Peptide nucleic acids: versatile tools for gene therapy strategies. *Adv Drug Deliv Rev*. 2000;44:81–95. [PubMed: 11072107]
32. Matthiesen SH, Hansen CM. Fast and Non-Toxic In Situ Hybridization without Blocking of Repetitive Sequences. *PLOS ONE*. 2012;7:e40675. [PubMed: 22911704]
33. Viennois E, Chen F, Laroui H, Baker MT, Merlin D. Dextran sodium sulfate inhibits the activities of both polymerase and reverse transcriptase: lithium chloride purification, a rapid and efficient technique to purify RNA. *BMC Res Notes*. 2013;6:360. [PubMed: 24010775]
34. Peng SS, Chen CY, Xu N, Shyu AB. RNA stabilization by the AU-rich element binding protein, HuR, an ELAV protein. *EMBO J*. 1998;17:3461–70. [PubMed: 9628881]
35. Zhang T, Delestienne N, Huez G, Kruys V, Gueydan C. Identification of the sequence determinants mediating the nucleo-cytoplasmic shuttling of TIAR and TIA-1 RNA-binding proteins. *J Cell Sci*. 2005;118:5453–63. [PubMed: 16278295]
36. López de Silanes I, Gorospe M, Taniguchi H, Abdelmohsen K, Srikantan S, Alaminos M, et al. The RNA-binding protein HuR regulates DNA methylation through stabilization of DNMT3b mRNA. *Nucleic Acids Res*. 2009;37:2658–71. [PubMed: 19270063]
37. Wigington CP, Jung J, Rye EA, Belauret SL, Philpot AM, Feng Y, et al. Post-transcriptional Regulation of Programmed Cell Death 4 (PDCD4) mRNA by the RNA-binding Proteins Human Antigen R (HuR) and T-cell Intracellular Antigen 1 (TIA1). *J Biol Chem*. 2015;290:3468–87. [PubMed: 25519906]

Figure 1: PMTRIP FISH-PLA quantifies and localizes mRNA-protein interactions in fixed cells. Schematic representation of **A**) V5 conjugation to NeutrAvidin **B**) PMTRIP assembly and **C**) FISH-PLA workflow **D**) 5 nM of V5 tagged PMTRIPs targeting polyadenylated mRNA were delivered as FISH probes in A549s. PLA (white) was performed between the V5 tag and indicated proteins. Extended focus images shown. Scale bars indicate 10 μ m. Quantification of PLA is shown. Statistics were performed with a one-way ANOVA with a Dunn's multiple comparisons test, where $n=30$ and ** $p < 0.002$, *** $p < 0.0009$, and **** $p < 0.0001$. 95% confidence intervals are shown in red. **E**) 20 nM of V5 tagged PMTRIPs

targeting COX-2 mRNA were delivered to HT-29s as FISH probes. PLA (white) was performed between the V5 tag and indicated proteins. Extended focus images are shown. Scale bars indicate 10 μ m. Quantification of PLA is shown. Statistics were performed with a one-way ANOVA with a Dunn's multiple comparisons test, where $n=30$ and ** $p < 0.008$, *** $p < 0.002$, and **** $p < 0.0001$. 95% confidence intervals are shown in red. **F)** 5 nM of V5-tagged PMTRIPs targeting β -actin mRNA were delivered as FISH probes to HT-29s. PLA (white) was performed between the V5 tag and indicated proteins. Extended focus images are shown. Scale bars indicate 10 μ m. Quantification of PLA is shown. Statistics were performed with a two-way ANOVA with a Tukey's multiple comparisons test, where $n=30$ and * $p < 0.031$, ** $p < 0.0035$, *** $p < 0.0003$, and **** $p < 0.0001$. 95% confidence intervals are shown in red.

Figure 2: PMTRIP FISH-PLA can sensitively measure variations of mRNA-protein interactions at modulated protein and mRNA expression levels.

A) A549s were treated either with 5 μ g/ml of ActD or without ActD for 1 h prior to fixation. 5 nM of V5 tagged PMTRIPs targeting polyadenylated mRNA were delivered as FISH probes. PLA (white) was performed between the V5 tag and HuR. Representative extended focus images are shown, with representative control images in supplementary figure 2. Scale bars indicate 10 μ m. Quantification of PLA is shown. Statistics were performed with a two-way ANOVA with a Tukeys's multiple comparisons test, where $n=30$ and **** $p < 0.0001$. 95% confidence intervals are shown in black. **B)** 200 ng of c-Myc-GFP plasmid either with (AB) or without (A) a HuR binding site was delivered to Veros via L2K transfection. 5 nM of V5 tagged PMTRIPs targeting c-Myc mRNA were delivered as FISH probes and PLA was performed between the V5 tag and HuR. Representative extended focus images are shown, with representative control images in supplementary figure 2. Scale bars indicate 10 μ m. Quantification of PLA is shown. Statistics were performed with a two-way ANOVA with a Tukeys's multiple comparisons test, where $n=30$ and **** $p < 0.0001$. 95% confidence intervals are shown in black. **C)** HeLas were treated with either 200 nM of HuR

siRNA or Non-targeting siRNA for 48 h before fixation. 5 nM of V5 tagged PMTRIPs targeting polyadenylated mRNA were delivered as FISH probes. PLA (white) was performed between PMTRIPs and HuR. Representative extended focus images are shown, with representative control images in supplementary figure 2. Scale bars indicate 10 μ m. Quantification of PLA is shown. Statistics were performed with a two-way ANOVA with a Tukeys's multiple comparisons test, where $n=30$ and **** $p < 0.0001$. 95% confidence intervals are shown in black. **D)** HT-29 cells were treated with either 100 nM of COX-2 siRNA or Non-targeting siRNA for 24 h before fixation. 20 nM of V5 tagged PMTRIPs targeting COX-2 mRNA were delivered as FISH probes. PLA (white) was performed between PMTRIPs and HuR. Representative extended focus images are shown, with representative control images in supplementary figure 2. Scale bars indicate 10 μ m. Quantification of PLA is shown. Statistics were performed with a two-way ANOVA with a Tukeys's multiple comparisons test, where $n=30$, ** $p < 0.007$, and **** $p < 0.0001$. 95% confidence intervals are shown in black. **E)** HT-29 cells were treated with either 100 nM of COX-2 siRNA or Non-targeting siRNA for 24 h before lysing. RNA was pulled down with either a HuR or a nonspecific antibody. qRT-PCR was performed for either COX-2 or GAPDH RNA. Statistics were performed with a two-way ANOVA with a Tukey's multiple comparison test where $n=3$, * $p < 0.031$, and ** $p < 0.0032$. Standard deviations are shown. **F)** 20 nM of V5-tagged PMTRIPs targeting COX-2 mRNA were delivered as FISH probes to cells with high COX-2 expression (HT-29) or low COX-2 expression (DLD-1). PLA (white) was performed between the V5 tag and indicated proteins. Representative extended focus images of DLD-1 cells are shown. HT-29 images are shown in figure 1. Scale bars indicate 10 μ m. Quantification of PLA is shown for all experimental conditions. Statistics were performed with a two-way ANOVA with a Tukey's multiple comparisons test, where $n=30$ and *** $p < 0.0005$, and **** $p < 0.0001$. 95% confidence intervals are shown in black.

Figure 3: PMTRIP FISH-PLA can quantify and localize polyA-HuR interactions in colon tissue. 5 nM of V5 tagged PMTRIPs targeting polyadenylated mRNA were delivered as FISH probes to tissue samples. PLA (green) was performed between the V5 tag and HuR. **A**) Extended focus images of cancer tissue are shown, with a 1 mm scale bar. **B**) Extended focus images of control tissue are shown, with a 1 mm scale bar. **C**) Quantification of PLA signal in (a,b) are shown. Statistics were performed with a one-way ANOVA with a Tukey's multiple comparisons test, where $n=3$ and $* p < 0.03$, and $** p < 0.009$. Standard deviations are shown in red. **D**) Extended focus cropped images of full tissues in (a) as indicated by white boxes. Scale bars are 100 μm . **E**) Extended focus images of tissue FISH-PLA at 63x are shown with a 10 μm scale bar. **F**) Extended focus cropped images of control full tissue in (b) as indicated by white boxes. Extended focus images of tissue FISH-PLA at 63x are also shown. Scale bars are 100 μm for 20x and 10 μm for 63x images.

Figure 4: PMTRIP FISH-PLA can quantify COX-2-HuR interactions in colon tissue. 20 nM of V5 tagged PMTRIPs targeting COX-2 mRNA were delivered as FISH probes to colon cancer samples. PLA (green) was performed between the V5 tag and HuR. **A)** Extended focus images of cancer tissue are shown, with a 1 mm scale bar. **B)** Extended focus images of control tissue are shown, with a 1 mm scale bar. **C)** Quantification of PLA signal in (a,b) are shown. Statistics were performed with a one-way ANOVA with a Tukey's multiple comparisons test, where n=3 and * p < 0.034. Standard deviations are shown in red. **D)** Extended focus cropped images of full tissues in (a) as indicated by white boxes. Scale

bars are 100 μm . **E)** Extended focus images of tissue FISH-PLA at 63x are shown with a 10 μm scale bar. COX-2 FISH shown in red. **F)** Extended focus cropped images of control full tissue in (b) as indicated by white boxes. Extended focus images of tissue FISH-PLA at 63x are also shown. Scale bars are 100 μm for 20x and 10 μm for 63x images. COX-2 FISH shown in red. **G)** Immunohistochemistry for HuR was performed on colon cancer and healthy colon tissue. Single plane representative images are shown. Scale bars are 50 μm . Quantification of the area of HuR expression normalized by the area of nuclei is shown. Statistics were a Mann-Whitney t-test, where $n=5$ and $** p < 0.008$. **H)** 100 nM of V5 tagged PMTRIPs targeting COX-2 mRNA were delivered as FISH probes to colon samples. FISH (green) was visualized via V5 staining. Extended focus images are shown, with 50 μm scale bars. Quantification of the area of COX-2 mRNA expression normalized by the area of nuclei is shown. Statistics were a one-way ANOVA, where $n=10$, $* p < 0.02$, and $**** p < 0.0001$.

Figure 5: PMTRIP FISH-PLA can quantify polyA-HuR interactions in lung tissue.

5 nM of V5 tagged PMTRIPs targeting polyadenylated mRNA were delivered as FISH probes to colon cancer samples. PLA (green) was performed between the V5 tag and HuR. **A)** Extended focus image of cancer tissue is shown, with a 1 mm scale bar for full tissue. **B)** Extended focus images of FISH-PLA (green) controls are shown. Scale bar is 1 mm. **C)** Extended focus cropped image of full tissues in (a) are indicated by white boxes. Scale bar is 100 μm . **D)** Extended focus images of tissue FISH-PLA at 63x is shown with a 10 μm scale bar. **E)** Extended focus cropped images of full tissues in (b) are indicated by white boxes.

Extended focus images of tissue FISH-PLA at 63x are also shown. Scale bars for (e) are 100 μm for 20x images and 10 μm for 63x images. **F)** Quantification of PLA signal in (a, b). Statistics were performed with a one-way ANOVA with a Tukey's multiple comparisons test, where $n=3$ and ** $p < 0.0082$ and **** $p < 0.0001$. Standard deviations are shown in red.

Figure 6: PMTRIP FISH-PLA can quantify COX-2-HuR interactions in lung tissue.

20 nM of V5 tagged PMTRIPs targeting COX-2 mRNA were delivered as FISH probes to colon cancer samples. PLA (green) was performed between the V5 tag and HuR. **A)** Extended focus image of cancer tissue is shown, with a 1 mm scale bar for full tissue. **B)** Extended focus images of FISH-PLA (green) controls are shown. Scale bar is 1 mm. **C)** Extended focus cropped image of full tissues in (a) are indicated by white boxes. Scale bar is 100 μm . **D)** Extended focus images of tissue FISH-PLA at 63x is shown with a 10 μm scale bar. COX-2 FISH is shown in red. **E)** Extended focus cropped images of full tissues in (b)

are indicated by white boxes. Extended focus images of tissue FISH-PLA at 63x are also shown. Scale bars for (e) are 100 μm for 20x images and 10 μm for 63x images. COX-2 FISH is shown in red. **F)** Quantification of PLA signal in (a, b). Statistics were performed with a one-way ANOVA with a Tukey's multiple comparisons test, where $n=3$ and $* p < 0.049$. Standard deviations are shown in red.

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Figure 7: PMTRIP FISH-PLA can quantify mRNA-protein interactions in fixed, archival lung microarrays.

A) 5 nM of V5 tagged PMTRIPs targeting polyadenylated mRNA were delivered as FISH probes to a lung microarray. PLA (green) was performed between the V5 tag and TIAR. Cropped image is indicated by the white box. Scale bars are 1 mm. Quantification of PLA signal is shown. SEM is indicated in red. **B)** 20 nM of V5 tagged PMTRIPs targeting COX-2 mRNA were delivered as FISH probes to lung microarray. PLA (green) was performed

between the V5 tag and HuR. Cropped image shown is indicated by the white box. Scale bars are 1 mm. Quantification of PLA signal is shown. SEM is indicated in red.

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