Single mRNA Detection Using MTRIPS-FISH

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Abstract:

RNA localization and interactions within a cell can give valuable insight into the cell's actions and reactions, especially during a disease state. Assays that give spatial information about RNA localization in cells are extremely useful as controls in interaction studies and also in providing functional understanding of cellular processes. For this purpose, Fluorescent *In-Situ* Hybridization (FISH) assays use fluorescently labeled strands of nucleic acids that are complementary to the RNA of interest to illuminate where the RNA is at that point in time. While much success has been achieved in visualizing single RNA molecules in live cells by the Santangelo Laboratory, FISH in fixed tissues with single-molecule specificity is more difficult to achieve. Here, we explore different methods of obtaining single-molecule specificity with previously validated MTRIPs molecules. We discovered that using prelabeled PNAs bound to the neutravidin protein lead to highly specific detection of single-molecule RNA, while allowing for other assays, such as Proximity Ligation, to occur simultaneously.

Introduction:

The closer one looks into cellular processes, the more complicated these microscopic interactions become. Biomolecules like nucleic acids, proteins, and fats, are responsible for almost all functions of the body. RiboNucleic Acids (RNA) are nucleic acids that serve a variety of functions in the cell. The three main types of RNA are messenger RNA (mRNA), transfer RNA (tRNA) and ribosomal RNA (rRNA). mRNA makes functional 'blueprints' of DNA that it carries to ribosomes, where tRNA uses the blueprint to assemble proteins[1]. These proteins can have a variety of functions in the cell – from preserving the cell's structural integrity, to catalyzing metabolic reactions, and even helping to regulate the translation and folding of other proteins.

RNA and proteins interact at many points in the cell cycle, notably during the translation, moving, and preparation of RNA[2]. These interactions are important, not just in the healthy cell cycle, but also in altered cellular environments, such as viral infections or tumorigenesis. An understanding of protein interactions and their cellular localization in the diseased state can give valuable insights into how to stop or treat infection.

This understanding is currently based on many different biomolecular assays designed to identify and quantify RNA and proteins in the cell. Fluorescent *In-Situ* Hybridization (FISH) is one such assay that uses fluorescent probes complementary to RNA or DNA sequences of interest to determine their location and quantity in the cell[3]. Another assay that is used to understand the internal mechanisms of RNA and proteins is the Proximity Ligation Assay (PLA). PLA involves two probes that bind to the two molecules of interest. If the probes are within 40 nm, they produce a detectable, punctate signal where each puncta represents a single interaction[4].

Review of Related Literature:

FISH is a molecular technique that built upon the earlier work of immunofluorescence pioneered by Coons et al in 1941[5], and *In-Situ* Hybridization first described by Pardue and Gall in 1969[6]. These two techniques were combined in the first non-radiolabelled ISH in 1975[7], representing the first instance of FISH. Since then, much work has been done to improve FISH, specifically to allow for single molecule specificity. Each solution has its benefits in certain situations. For example, Huang et. al. used a fluorescent resonance energy transfer (FRET) based hybridization chain reaction (HCR) to visualize mRNA in cells. HCR features hairpins of RNA, which are highly stable and programmable[8]. Another method uses DIG-UTP-labeled RNA, whose signal are amplified with a tyramide amplification buffer[9]. A study by Xie et. al. showed that using commercially available Z shaped RNA probes, signal could be significantly amplified using the company's kits in order to visualize even for mRNAs that were not plentiful in their cells[10]. These methods are complex and are dependent on purchased products that cannot be modified to be used for FISH and PLA simultaneously.

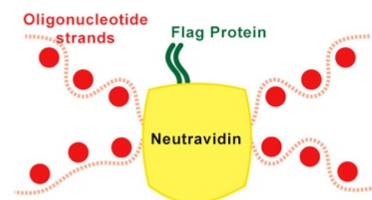

Figure 1: Illustration of the MTRIP molecule. The central neutravidin protein (yellow) is labeled with a flag protein (green) for PLA and attached to four oligonucleotide strands (yellow) that are specific to a certain RNA. Image adapted from Jung (2013)

The Santangelo Laboratory has created an assay using a new probe called Multiply-labeled Tetravalent RNA Imaging Probe (MTRIP), which can be used to fluorescently visualize individual RNA molecules and conduct PLA. A neutravidin protein is bound via biotin to four oligonucleotide strands; each of these strands is complementary to the RNA of interest, and also can be modified to have three fluorophores attached. Therefore, each MTRIP molecule colocalizes many fluorophores, yielding a bright signal[11]. MTRIPs are easily modified to facilitate PLA by adding an epitope tag, such as FLAG or V5, to the neutravidin. If the antibody targeting

this tag is close enough to the antibody targeting the protein of interest, the signal can be amplified via Rolling Circle Amplification[2, 12]. When viewing the interactions between RNA and proteins of interest, it is helpful to concurrently see the total RNA localization itself. Comparison across multiple cells is more significant when interactions are normalized by the total amount of RNA, and this is found via MTRIPs intensity.

Previously, the MTRIPs were delivered in live cells prior to fixation, both for RNA imaging and for interaction studies. However, there is also a need to visualize RNA and perform these studies in *fixed* tissue, such as patient samples, which are more than just models – they are the diseased state. Therefore, the method is being modified to be effective in a FISH assay.

FISH typically is performed with additives to the buffer such as formamide to promote probe affinity. However, these substances can have a detrimental effect on antibody use post-FISH, preventing the use of FISH before interaction studies with proteins. To alleviate the need for these buffers, the MTRIPs were modified so that the oligonucleotides used are peptide nucleic acids (PNAs), which have a high affinity for the RNA[13, 14]. However, previous work in the laboratory has found modifying PNA oligonucleotides with fluorophores to be difficult. To accommodate this, the fluorophore is instead attached directly to the neutravidin, occluding the epitope tag. Using this method, MTRIPs FISH probes are not currently RNA single molecule sensitive.

Hypothesis:

An increased concentration of MTRIPs delivered to fixed cells will produce strong fluorescent signal in clear puncta. Similarly, using pre-labeled PNA in the MTRIPs will colocalize many fluorophores in one area, yielding a bright and punctate signal.

MTRIPs in Fixed Cells:

In order to achieve strong, punctate signal in MTRIP FISH, a number of methods were examined. For consistency throughout all the experiments, FISH for human β-actin in A549 cells was explored initially, with the intent of applying the developed methods to other cell lines and genes of interest. The first method investigated involved increasing the concentration of the FISH probes that are delivered to the cells; both the number of probes (1, 3, 5, or 7) and the concentrations (1nM, 5nM, and 10nM) were varied to determine optimal concentration. The second method utilized PNA molecules that were purchased from PNA-Bio pre-labeled with Cy3b fluorophores. The makeup of the neutravidin (unlabeled, versus Cy3b-labeled), the concentration of the probes (1nM, 5nM, and 10nM), and the number of probes (1, or all 4) were all varied.

Negative controls for both experiments were MTRIPs made with a fluorescently labeled neutravidin and Luciferase or RSV PNAs – nucleic acids that are not found naturally in A549. Wells treated with these MTRIPs should have no fluorescent signal. A second negative control used was MTRIPs made with fluorescent neutravidin with biotin instead of PNAs; this control should not have signal, and confirms that the neutravidin is attaching to RNA, not endogenous biotin in the cells. Additionally, a negative control that has no probes was used to confirm that there was no contamination of the cells with fluorescent materials.

The positive control for the second were made using labeled neutravidin and unlabeled PNA for human β-actin simultaneously. Signal from these probes should perfectly coincide with the experimental FISH.

Materials and Methods:

a. Creation of Labeled Neutravidin Protein

3nmol of 167µM neutravidin (ThermoFisher) was mixed with 7.5µL of 19 mg/mL dye: Cy3b (GE) or Alexa-Fluor 647 (ThermoFisher). 30µL of 0.1M bicarbonate buffer was added, and the mixture was vortexed for 2 hours. Mixture was filtered with 10kDa filter at 14k rcf for10-15 mins until run-through was clear (approximately 5 times).

b. Cell Preparation

100µL of A549 cells (3x10^6 cell/mL) were plated in a glass-bottom 96-well plate and allowed to grow for 24 hours in DMEM (ThermoFisher). Cells were washed in DPBS without calcium or magnesium (ThermoFisher) and fixed in 100µL of 4% PFA (EMS) for 10 minutes at room temperature. Cells were washed in 1x PBS, and 100µL of 70% EtOH was added. Cells were refrigerated at 4˚C overnight. Cells were washed in 1x PBS. Biotin Blocking was performed via manufacturers protocol (ThermoFisher).

c. MTRIPs Preparation

0.625µL of 6µM neutravidin was added to 0.625µL of a single PNA probe (PNA Bio) in a 1.5mL centrifuge tube, and 1.25µL of 1x PBS was added for volume. This mixture was covered and allowed to sit for an hour at room temperature. 400µL 1x PBS was added to the tubes, and the mixed contents were added to a 30kDa filter tube. If needed to combine MTRIPs molecules, all probes were combined in the same 400µL. The filters were spun at 14k rcf for 5 minutes. Filters were flipped into a new tube and spun at 1k rcf for 1 minute. The final centrifuge tube contained approximately 30µL of 81.25nM MTRIPs.

d. Experimentation

Hybridization buffer was made by diluting 5µL of Salmon Sperm DNA (Invitrogen), 5µL of yeast tRNA (Ambion), and 2µL BSA (Ambion) in 1 mL of 2x SSC (Thermo). Prepared MTRIPs was diluted to the desired concentrations in hybridization buffer, and 40 mL was added to the cells. Cells were incubated at 37˚C overnight in a humid chamber. Cells were washed in 40µL of 2xSSC for 30 minutes at 37˚C. DAPI was diluted to 300nM, and 40µL was added to the cells for 5 minutes at room temperature. Cells were washed in 1x PBS and treated with 18µL of ProLong reagent (Cell Signaling Technology).

e. Imaging

Wells were imaged with a 63×, NA 1.4 Zeiss Plan-Apochromat oil objective on a Hamamatsu Flash 4.0 v2 sCMOS camera on a PerkinElmer UltraView spinning disk confocal microscope on a Zeiss Axiovert 200M body. Z-stacks of 0.2µm were taken in the DAPI, Cy3 and Cy5 channels. Laser intensities and exposure varied in iterations of the experiment but remained constant for all conditions in a single experiment.

f. Image Analysis

Images were analyzed in the Volocity acquisition software (PerkinElmer). First, all images were linearly contrast enhanced for clarity. Individual cells were selected with the freeform selection tool, and the measure tool was used to calculate the intensity of each channel and the Manders' Colocalization Coefficients. ImageJ (NIH) software was used to generate RGB plots.

g. Data Analysis

Sum of intensities was normalized to cell volume calculated by Volocity analysis software. Data analysis was done in GraphPad Prism. Analysis was begun by testing if the data set followed a

Gaussian distribution using the D'Agostino-Pearson and the Shapiro-Wilk normality test. Statistical analysis between groups was done via two-sided t-test for normally distributed data, and by Mann-Whitney tests for non-Gaussian data. Significance was determined at a 0.05 level.

Results:

Several methods were employed in order to obtain bright, punctate signal corresponding to human β-actin RNA by MTRIPs FISH. The first was to increase the concentration of the FISH probes to obtain both brighter and more punctate signal. In order to determine whether increasing probe concentration allows for bright, single-molecule detection of RNA, the concentration of MTRIPs with unlabeled human β-actin was varied from 1 to 10nM. Both concentrations led to clear delineations of the cells, with little overlap between the β-actin PNA signal and the scrambled control PNA signal as illustrated in Figure 2. The fluorescent intensity of the 10nM signal (n=21) over the 1nM signal (n=21) was significantly different with $p < 0.0001$.

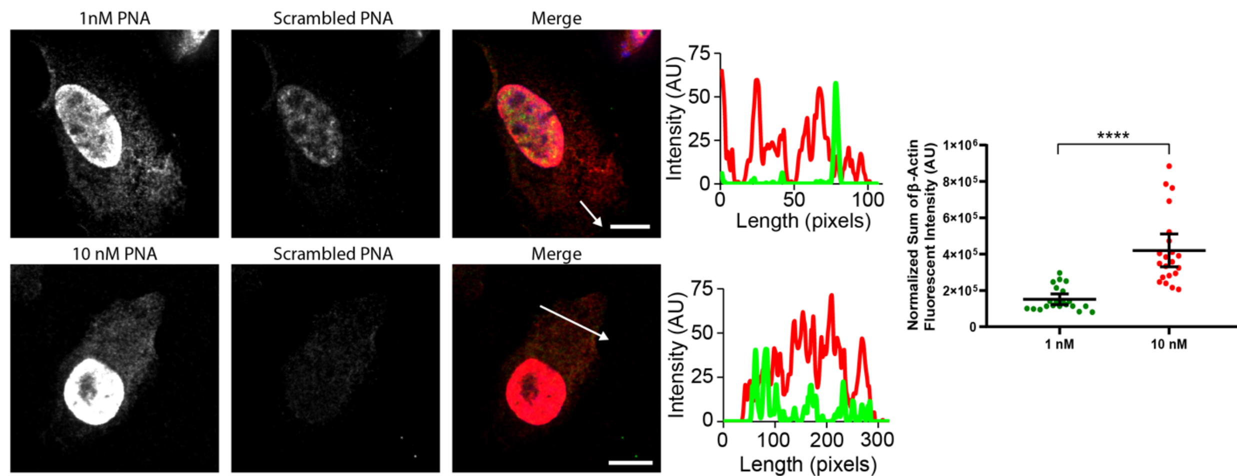

Figure 2: Comparison of 1nM and 10nM human β-actin FISH. Cell images show the lack of overlap between the β-actin and the 10nM scrambled control. RGB plots of the merged image supports this idea. Contrasting intensities of the two concentrations across 21 cells for each group yields a p value < 0.0001 (****). Scale bar represents 10μm.

Next, pre-labeled human β-actin was integrated into the MTRIPs molecule and used as the FISH probe. The success of labeled human β-actin PNA (labeled PNA) was determined by comparing to a positive control (unlabeled PNA) and a negative control (RSV or Luciferase PNA). As shown in Figure 3a, the labeled and unlabeled PNAs coincide in both the cell images and the RGB plot, while the labeled and scrambled PNA show little overlap. To determine colocalization, the two Manders Coefficients (Figure 3b) were computed for 30 cells with labeled PNA and various scrambled controls and 26 cells with labeled PNA and unlabeled PNA. M1, which

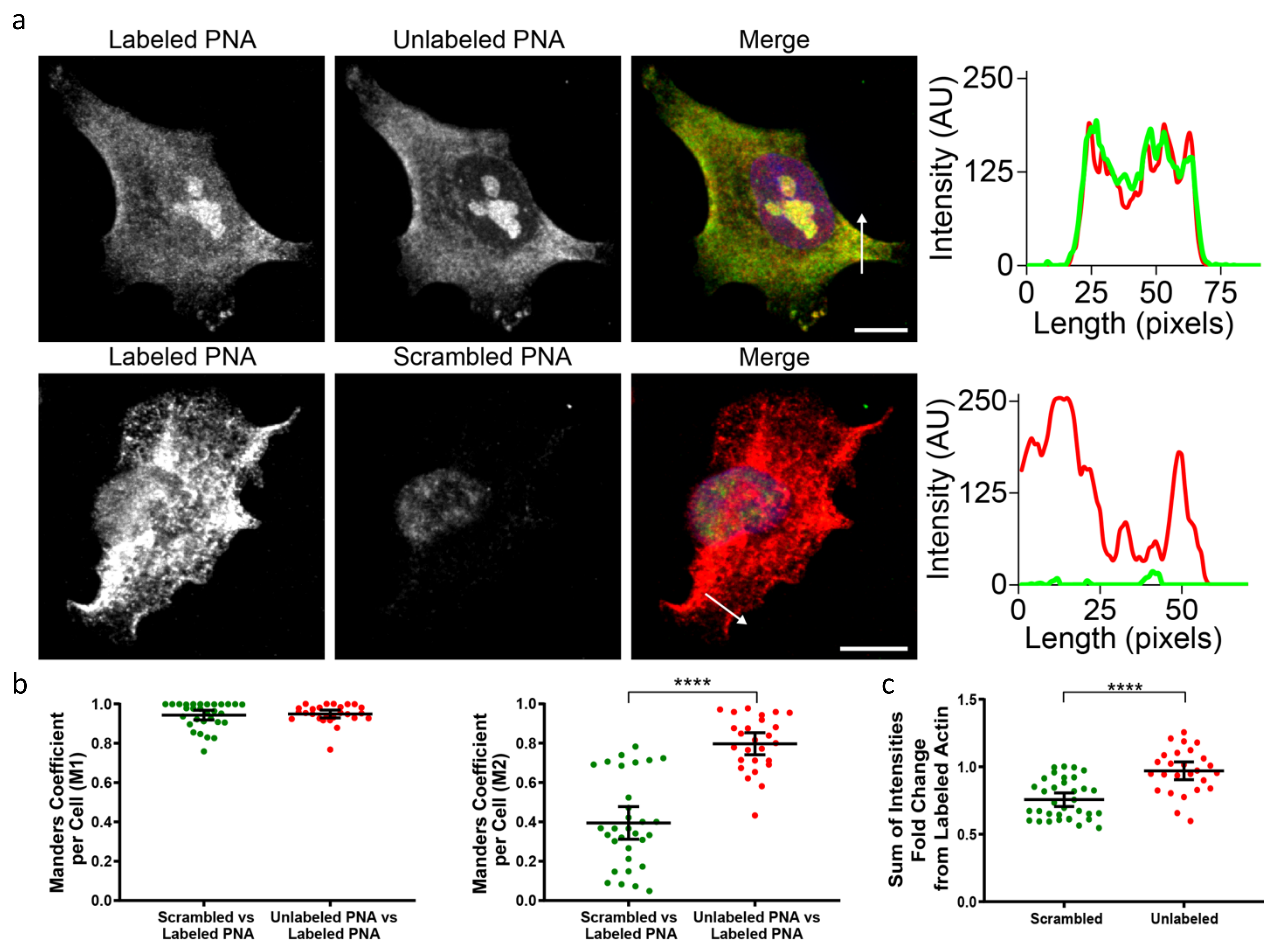

*Figure 3: Analysis of labeled PNA FISH based on colocalization and fluorescent intensity fold change over positive and negative controls. a) Representative cell images of FISH using the labeled PNA (left), the control (center), and a merged view (right) on which the RGB plot was established. Scale bar indicates 10μm. b) Manders Coefficients – M1 and M2 – between the control and the labeled PNA. Scrambled control (n = 30) and unlabeled PNA (n = 26) did not differ significantly in M1 (p = 0.8993), but the positive control had significantly higher M2 (**** p < 0.0001). c) Normalized intensities of the two controls were significantly different at p < 0.0001 (****).*

represents the amount of the second probe (unlabeled or scrambled PNA) that is found in areas with labeled PNA, was not significantly different between the two groups ($p = 0.8993$). This is likely due to the fact that the control PNA is always found in conjunction with the labeled PNA by virtue of the large amount of the labeled PNA in the cells. M2, which represents the amount of labeled PNA found in areas with the second probe (unlabeled or scrambled PNA), is therefore a better measure of colocalization. The M2 of the labeled vs unlabeled group was significantly higher ($p < 0.0001$) than the M2 of the labeled vs scrambled group. Additionally, the ratio of labeled to unlabeled fluorescent intensity was significantly higher ($p < 0.0001$) than the ratio of labeled to scrambled fluorescent intensity (Figure 3c).

The two FISH methods were directly compared (Figure 4) by visualizing the labeled PNA groups and the 10nM unlabeled PNA experiment groups. Both of these concentrations were chosen based on the strong, punctate signal they yielded in the previous experiments. The signal is strong and covers the full cell in both groups, including a visually brighter signal around the edges of the cell, typical of β-actin mRNA[15]. The fluorescent intensities were calculated to further compare the two. The unlabeled PNA always comprised extremely bright signal at the nuclei, and therefore signal in

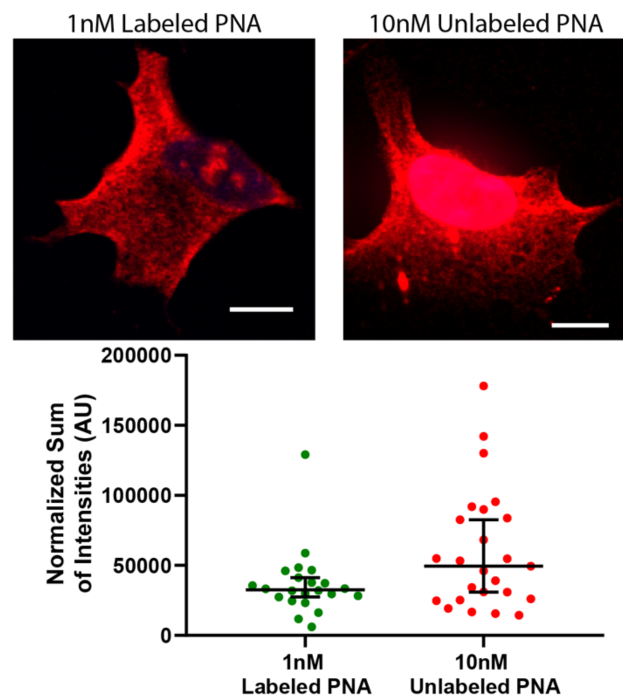

Figure 4: Comparison of labeled PNA to 10nM unlabeled PNA. Representative images of the labeled PNA and the unlabeled PNA are shown with scale bar of 10μm. The sum of fluorescent intensities normalized by cell volume were not found to be statistically different (p = 0.0860) by Mann-Whitney Test.

the nuclei was excluded in Volocity. The labelled PNA and 10nM unlabeled PNA were not significantly different (p = 0.0860).

Discussion:

Both mechanisms of FISH evaluated in this paper succeeded at producing strong punctate signal that was different from scrambled controls, and similar to positive controls. Save the nuclear brightness in the unlabeled PNA experiments, the cellular distribution of the mRNA was consistent with literature. Using larger concentrations of unlabeled PNA produces comparable signal to using a smaller concentration of labeled PNA; using labeled PNA is preferred however, since this leaves the epitope tag available for future analysis. A specific assay that can be performed using this protein is the Proximity Ligation Assay. PLA is a powerful molecular tool that scientists use to deduce – initially – protein interactions in cells. Using the MTRIP probe, the assay can be conducted to determine proximity of RNA and proteins or RNA and other RNA, which can illuminate patterns of expression and post-translational modifications of genes. A preliminary validation experiment of this application was conducted to determine proximity of β-Actin mRNA and HuR, which is overexpressed in the cytoplasm when the cells are stressed with Actinomycin D (ActD). Negative controls for PLA include a control with no primary (I˚) antibody, a control with MTRIPs for RNA not found in A549 cells (RSV mRNA specifically), and a control with MTRIPs without the V5 flag protein. Results of this experiment are summarized in Figure 5.

Negative controls were clean of PLA puncta, indicating good specificity of the assay. Additionally, the -ActD control showed fewer interactions between RBP and the mRNA, which is consistent with literature[16].

Conclusion:

Both the methods discussed in this paper presented strong punctate mRNA signal which visually accorded with current literature. The laboratory proposes the labeled PNA method to be used for future work, due to its capability for further analysis such as PLA and the added benefit of using a lower concentration of probe.

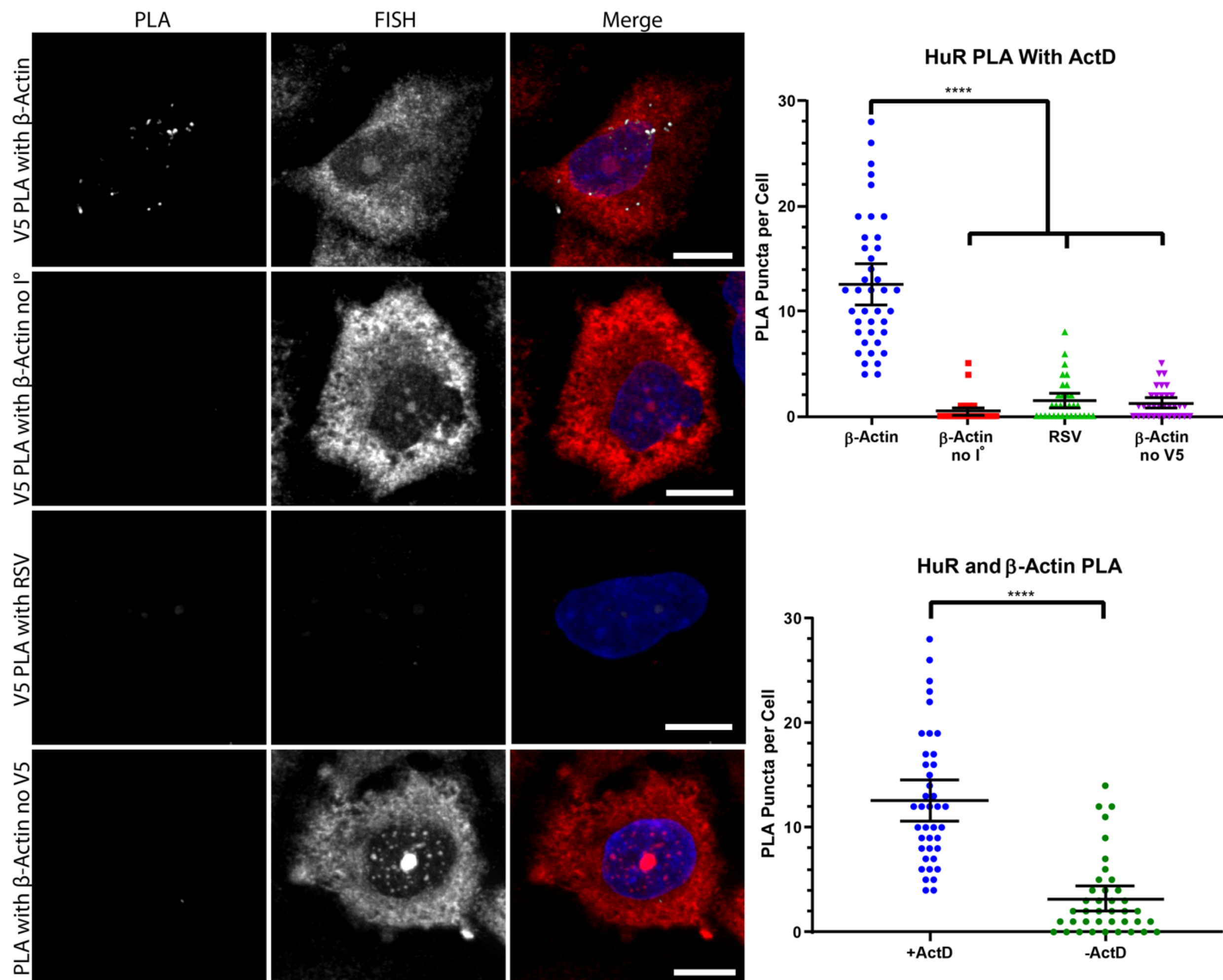

Figure 5: Analysis of β-Actin interactions with RBP with MTRIPs PLA. Representative images of PLA with RBP and mRNA (left), the MTRIPs labeled mRNA (center) and the merged image (right) at different PLA conditions. Scale bar indicates 10µm. PLA puncta per cell was significantly different (p<0.0001 via Mann-Whitney test) in the experimental group over the three controls. PLA puncta per cell as also significantly different when the cells were stressed with ActD versus when there was no ActD treatment (p<0.0001 via Mann-Whitney test).

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