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Can we observe changes in mRNA “state”? Overview of methods to study mRNA interactions with regulatory proteins relevant in cancer related processes

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Abstract

RNA binding proteins (RBP) regulate the editing, localization, stabilization, translation, and degradation of ribonucleic acids (RNA) through their interactions with specific *cis*-acting elements within target RNAs. Post-transcriptional regulatory mechanisms are directly involved in the control of the immune response and stress response and their alterations play a crucial role in cancer related processes. In this review, we discuss mRNAs and RNA binding proteins relevant to tumorigenesis, current methodologies for detecting RNA interactions, and last, we describe a novel method to detect such interactions, which combines peptide modified, 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 *in situ*.

1 Introduction

As a gene is being transcribed, post-transcriptional events take place, including co-transcriptional mRNA processing (capping, splicing, 3' end processing), nucleocytoplasmic export, mRNA localization prior to translation, mRNA stabilization, translational regulation and decay. From the moment of transcription, mRNAs are frequently associated with regulatory factors, including RNA binding proteins (RBPs)¹ and miRNA.^{2–4} The molecular composition of these so called messenger ribonucleoproteins (mRNPs) determines the “state” of a transcript, which means if it is immediately translated, transported to specific sites prior to translation, transported to specific sites for storage and translation, or tagged for degradation through quality control mechanisms (Fig. 1).⁵ mRNA–RBP interactions depend on the availability of RBPs 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.⁶ The complicated network of regulatory factors and target RNAs ultimately establishes the gene expression profile of a cell.

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Regulatory sequences target of RBPs are found in all parts of the mRNA molecule, in the 5' and 3' untranslated regions (UTRs), in the open reading frame (ORF) and in the poly(A) tail. In particular, the fate of many mRNAs encoding tumor suppressors, cytokines and inflammatory mediators are controlled by AU-rich elements (ARE), RNA sequences located within the 3'-untranslated region (3'-UTR) of transcripts, and their respective binding proteins (ARE-BPs).⁷⁻¹⁰

mRNA targets of ARE-BPs have been found to contribute to cell proliferation (cyclins A1, B1, D1, E1, CDK2, c-myc, c-fos, and eIF4E), evasion of apoptosis (akt1, bcl-2, p21, p53, c-myc), angiogenesis (HIF-1 α , VEGF, COX-2, IL-8) and invasion and metastasis (MMP-9).¹¹ Numerous pro- and anti-inflammatory factors undergo ARE mediated regulation including TGF- β , TNF- α , IL-2, 3, 6, 8, 10, 12, GM-CSF, and iNOS.¹²

Among the best characterized ARE-BPs, tristetraprolin (TTP) has been shown to regulate target mRNA stability *via* deadenylation.¹³ TTP plays a major role in pro-inflammatory cytokine production in response to endotoxins and prevents the development of septic shock. Mice lacking TTP develop arthritis, cachexia, dermatitis and formation of auto-antibodies due to excessive release of TNF- α and GM-CSF by macrophages and neutrophils.¹⁴ TTP also regulates IL-17, a cytokine involved in the innate immune response and inflammatory diseases. TTP is often downregulated in many tumors, most prominently in breast, colon, and prostate.^{15,16} Suppression of TTP promotes the epithelial-mesenchymal transition and metastasis.¹⁷ Restoring TTP expression was shown to suppress three key tumorigenic phenotypes: cell proliferation, resistance to proapoptotic stimuli, and expression of VEGF.¹⁸⁻²⁰

AU-rich element RNA-binding protein 1 (AUF1) determines the fate of ARE containing mRNAs by altering their stability.²¹ AUF1 does not directly target degradation of mRNAs but, rather, interacts with different co-factors such as heat-shock proteins, translation initiation factors and poly(A) binding proteins to prevent translation.²² AUF1 KO mice show severe endotoxic shock as a result of excessive production of TNF- α , IL-2 and IL-1B.²³ AUF1 deregulation has been linked to tumorigenesis. Transgenic mice highly expressing the p37 isoform of the AUF1 protein, characterized by the highest affinity for AREs *in vitro* and the highest destabilizing effect, develop sarcomas, which express cyclin D1.²⁴

HuR (Hu-antigen R) interacts with more than 7000 mRNAs and it frequently protects target mRNAs from degradation. It regulates several mRNAs encoding pro-inflammatory proteins including TNF- α , GM-CSF, IL-6, IL-8 and COX-2.^{25,26} COX-2 and the angiogenic and proliferative factors VEGF and IL-8 are overexpressed in colon cancer cells, presumably due to the binding and stabilization by overexpressed HuR. These factors are also up regulated in malignant brain tumors that overexpress HuR.²⁷ Wang and colleagues showed that the expression and half-lives of cyclins A and B1 mRNAs could be reduced in colorectal carcinoma (RKO) cells by knocking down HuR expression with antisense oligonucleotides.²⁸ Cells with decreased HuR have reduced growth, indicating a role for this RNA-binding protein in regulating cell proliferation *via* cyclin mRNA stabilization. Additionally, mice expressing TNF- α mRNA containing mutations in the HuR binding site

display autoimmune diseases resembling human systemic lupus erythematosus.²⁹ Mice with conditional KO in thymocytes have defects in T cell migration and proliferation, highlighting HuR importance in adaptive immunity.³⁰ Increase in both nuclear expression and cytoplasmic localization of HuR, is associated with a poor cancer prognosis.^{31–34} HuR not only stabilizes mRNAs, but it also suppresses their translation by cooperation with T-cell intracellular antigen-1 (TIA1) and TIA1-related protein (TIAR), which are translational silencers.³⁵

Butyrate response factors 1 and 2 (BRF1/2) induce nonsense mediated decay (NMD) of various cytokines including TNF- α and GM-CSF.^{36–38} Deficient mice are embryonic lethal or die soon after birth.³⁹ BRF1/2 have been also known to be involved in the development of fertility and haematopoiesis.^{40,41}

These examples clearly demonstrate that alterations in post-transcriptional regulatory mechanisms influence initiation and progression of cancer as well as the innate and adaptive immune response, affecting cell phenotype, proliferation, differentiation, invasion, metastasis, apoptosis, and angiogenesis. Aberrant localization, expression, and interactions of RBPs with mRNAs occur within tumors^{42–44} and these interactions correlate with treatment resistance.^{45–47}

Most of the ARE-BPs that regulate the translation of inflammatory cytokines, growth factors, angiogenesis, apoptosis and differentiation factors participate in the response to stresses of various nature, including oxidative stress, starvation or heat shock. These stimuli cause translation to be reprogrammed, so that proteins involved in cell survival are synthesized. The stress response is characterized by the formation of cytoplasmic processing bodies (PBs) and stress granules (SGs).⁴⁸ PBs contain factors involved in mRNA degradation/decay machinery, including non-sense and ARE-mediated, the RNA interference (RNAi) machinery and surveillance, translational repression and gene silencing factors.^{49–51} PBs are typically considered sites of mRNA decay, but they may also represent sites of protein storage/modification.⁵² SGs are non-membranous structures that form in response of several different stimuli that cause polysome disassembly. SGs contain translationally-stalled complexes (48S-complexes), TIAR/1 and HuR, kinases and phosphatases, scaffolding and adaptor proteins, ubiquitin modifying enzymes, helicases, ribonucleases, transferases.^{48,53} SGs are typically considered sites of mRNA stabilization/storage, and may facilitate rapid reactivation of translation upon stress recovery, since the ribosome preinitiation complexes are retained in an assembled state.⁵⁴ SGs are dynamic structures in equilibrium with polysomes and with PBs, with whom they share several proteins, including TTP, BRF1 and HuR.^{55,56}

Recent studies indicate that perturbation in SG and PB formation is associated to cancer initiation and progression.⁵⁷ RNA granules may act as important signaling/regulatory centers exploited by cancer cells to survive in hostile tumor environments, such as hypoxia, hyperosmolarity, oxidative or ER stress and nutrient starvation.⁵⁸ Sodium arsenite, the most effective agent used to induce SGs and PBs in cultured cells, can promote resistance to apoptosis, possibly by sequestering or inactivating pro-apoptotic factors.⁵⁹ This mechanism

is still poorly understood and yet complicated by the fact that stresses of various natures induce SGs with different molecular composition, triggered by different cellular pathways.⁵⁸

Formation of SGs and/or PBs has also been associated with chemotherapy or radiotherapy resistance. Several drugs with established anti-tumor activity (Bortezomib, 5-fluorouracil, MG-123) induce SG formation, and this correlates, in certain cancer cell lines, with resistance to treatment.^{60–63} Indeed, depletion of specific proteins involved in the stress response has been shown to restore the pro-apoptotic function of the drug.⁶⁴ Sodium selenite induces formation of SGs that are functionally distinct from the canonical ones, since they promote cell death, rather than cell survival.⁶⁵ DNA-incorporating chemotherapeutic metabolites do not trigger SG formation but rather potentially increase the number of PBs in cancer cells.⁶⁶

Given the complexity of the network of protein–mRNA interactions responsible for the regulation of mRNA metabolism during tumorigenesis, the stress and immune responses, it has become more and more relevant to identify, within a cell population, whether specific mRNAs are translated, degraded, stalled in translation and/or stabilized or destabilized by means of interactions with specific RBPs. The variation of these mRNA “states”, which largely account for the heterogeneity observed in cancer cells, and their changes in distribution during pathogenesis are not easily quantifiable, especially with single cell sensitivity.

Current analytical techniques cannot provide information on the localization or variability of these interactions on a per cell basis, critical to characterizing tumors, which exhibit high levels of heterogeneity. In addition, analytical techniques often suffer from low sensitivity, precluding their use with small numbers of cells. Current imaging techniques for studying RNA interactions often use fluorescent fusion-proteins or transgenic methods to visualize modified RNAs, but these methods are often not quantitative and lack the ability to detect interactions at native levels and stoichiometry, precluding their use in tissue samples. Studies based on genome-wide approaches and the development of complementary methods that provide a detailed subcellular localization of selected mRNA targets and interacting proteins are required to further dissect the elusive mechanisms underlying mRNA–protein interactions during post-transcriptional regulation and their role in determining the mRNA “state” during tumorigenesis, stress response and immune response.

In the present review we will discuss some of the main approaches utilized for the investigation of the interactions of mRNAs with *trans*-acting factors. We will also introduce a novel approach with the potential to overcome the discussed limitations. This method allows for the simultaneous detection and quantification of both the mRNA and its interactions with specific *trans*-acting factors with single molecule and single interaction sensitivity, simultaneously, *in situ* by combining two established techniques: (1) single RNA sensitive, multiply-labeled, tetravalent RNA imaging probes (MTRIPS) modified with Flag tags (FMTRIPS)⁶⁷ and (2) and the commercially available proximity ligation assay (PLA).⁶⁸ We will summarize the main results of recent publications where, using this assay, we interrogated the localization and frequency of interactions of RBPs with native mRNAs at both native and modulated protein levels. The results of our studies allowed to introduce a

novel mechanism for fine-tuning the expression of programmed cell death 4 (PDCD4), a tumor suppressor gene, to prevent neoplastic transformation.⁶⁹

2 Current methodologies for the study of protein–RNA interactions

To capture the physiological role of RBPs in mRNA regulation during cancer related processes, we need, first of all, to unravel the network of mRNA–RBP interactions in various cellular contexts. Second, we need to integrate this knowledge using computational approaches in order to review quantitatively the regulatory effects of RBPs. This includes measuring protein and target RNA levels in different cell types and tissues, addressing cooperation or competition among RBPs and accounting for redundancies in protein families or regulatory pathways. In the following paragraphs we will describe some of the main techniques that are currently utilized to address these needs, focusing on genome-wide high-throughput methods to discover protein–RNA interactions as well as fluorescently based single molecule approaches developed to investigate such interactions at the single cell level.

2.1 Analytical approaches

In order to define which RNAs are bound by a specific protein and define the specific protein binding sites, SELEX (Systematic Evolution of Ligands by Exponential enrichment) and RNA-compete are the main techniques utilized *in vitro* (Fig. 2). In both methods, a RBP of interest is incubated with a pool of RNA oligonucleotides. The selected RNAs are then isolated and sequenced or hybridized to a microarray for detection. In particular, SELEX consists of several rounds of binding and amplification of RNA molecules (Fig. 2A).⁷⁰ RNAcompete queries a designed RNA pool under competitive conditions and assays the bound RNAs using a microarray (Fig. 2B).⁷¹ Since an excess of RNA is utilized, RNA molecules compete for binding to the protein and the relative abundance can be used to assess relative affinities. These approaches have generated catalogs of RNA binding motifs for numerous RBPs in many organisms and are undoubtedly a valuable resource. However, due to their *in vitro* nature, they may not reflect true biological interactions and do not determine the structural specificity of RBPs. SELEX, coupled with high throughput sequencing, has been also recently used to determine the binding preferences of RBPs in cells. However, the RNA binding analysis is generally biased toward the highest affinity targets.⁷²

To overcome this limitation, *in vivo* genome wide analysis approaches were developed (Fig. 3). RNA immunoprecipitation assays (RIP), followed by microarray analysis or sequencing (RIP-chip and RIP-seq) consist of using an antibody against the endogenous protein of interest or an epitope tagged expressed protein in order to immunoprecipitate target RNAs (Fig. 3A).⁷³ The RNAs are transcribed into cDNA, amplified and identified. Bioinformatics tools are then utilized to identify the protein binding site. RIP can be performed using native purification conditions, with the advantage to preserve the native complexes present in the cell, and it has been successfully utilized in various tissues and species, from yeast to mammals. The main limitation of this approach is that results are naturally biased toward abundant transcripts, so that abundant non-specific interactions may mask less abundant specific ones.^{74,75} Non physiological interactions may occur in solution, depending on the

preparation of cell lysates.⁷⁶ Therefore, results necessarily require validation *via* other approaches. If an affinity tag is used for the immunoprecipitation, this approach also requires the ability to express a tagged version of the RBP of interest in the cell.

The primary deficiency of RIP-chip is the inability to precisely identify the location of the binding site of the RNA-interacting component. To overcome this limit and validate native assays, cross-linking and immunoprecipitation (CLIP) was developed (Fig. 3B).⁷³ Here, short wavelength UV light is utilized to create covalent bonds between RNA and proteins in cells within a range of few Angstroms.⁷⁷ The complexes are then purified using stringent wash conditions followed by denaturation of all complexes by analysis with microarray or high throughput sequencing. The limits imposed by the low efficiency of UV crosslinking, can be overcome by a PAR-CLIP (photoactivatable ribonucleoside enhanced) approach, which requires the incorporation of a nucleotide analog into cells, followed by treatment with long wavelength UV light (Fig. 3C).⁷⁸

The latter approach is valuable, but it can be applied only to cultured cells and not to primary tissues. A major limit of UV crosslinking is that not all protein–RNA interactions are efficiently captured; for example, proteins that interact with the RNA backbone are crosslinked less efficiently than those that interact with the nucleic acids bases. Moreover, interactions involving protein complexes are not captured.⁷⁹

RIP-chip studies from diverse organisms and biological conditions have uncovered putative regulatory elements, regulatory modules, and RNP remodeling in response to stimuli.^{80,81} These methods have provided data in support of the post-transcriptional operon/regulon model, in which RNPs coordinate the expression of transcripts encoding functionally related proteins through dynamic interactions.⁸²

Tennenbaum *et al.* were the first to apply RIP-chip to study the RNA fraction bound to HuB, HuA/HuR, eIF4E and PABP in P19 embryonic carcinoma cell lysates and identified a subset of ARE-containing mRNAs encoding cell-cycle regulators and transcription factors. HuB, PABP and eIF-4E mRNP complexes were found to contain pools of mRNAs different from one another and from the total cellular transcriptome. Moreover, the population of mRNAs detected in the HuB–mRNP complexes changed upon treatment of HuB-expressing P19 cells with retinoic acid (RA) to induce neuronal differentiation, and were found to include additional ARE-containing mRNAs known to be up-regulated in neurons.⁷³ De Silanes and colleagues used this approach to identify HuR and TIA-1 target mRNAs in cells. In both cases, computational analysis led to the elucidation of U-rich motifs present on target mRNAs.^{83,84} A more recent application of these methods allowed Mukerjee *et al.* to examine HuR-dependent regulation on target gene expression. PAR-CLIP was used to identify high-resolution HuR binding sites while RIP-chip analysis was used as a complementary biochemical method to quantify HuR–mRNA association. The authors observed that the cumulative number of HuR binding sites per transcript was predictive of both the overall association of HuR with the transcript and the HuR-dependent stabilization.⁸⁵

In contrast to the described “protein-centric” methods, proteomic approaches focus on the identification of RBPs binding to a given RNA by RNA affinity capture methods (Fig. 4A). This is achieved by isolating *via* pull down the RNA of interest, after crosslinking to preserve protein–RNA interactions. *In vitro* approaches generally employ a synthetic RNA bait to capture and identify proteins from cellular extracts, for example tethering the target RNA molecule to a solid support or using an RNA aptamer to a protein, which can be either immunoprecipitated or attached to a solid support.^{86,87} *In vivo* approaches capture the RNA–protein complexes present in cells. This has been performed using oligonucleotides complementary to the native RNA sequence,⁸⁸ *via* coexpression of the MS2 coat protein and the RNA sequence encoded with the MS2 aptamer⁸⁹ and through the delivery of a peptide nucleic acid (PNA) complementary to the target RNA and linked to a photoactivatable reagent that will cross-link the PNA to the nearby proteins.⁹⁰ After purification using either native or denaturing conditions, the total proteome can be analyzed by western blot or by mass spectrometry (MS). The advantage of the latter approach is that all proteins can be identified in the sample, including those that are not visible *via* gel electrophoresis. MS can be quantitative or non-quantitative (Fig. 4B). In the latter case, purified proteins from the RNA sample of interest and a control are separated by gel electrophoresis and stained for total protein. Protein bands present only in the sample of interest are extracted and identified *via* MS. Alternatively, all proteins in the sample can be analyzed.^{91,92} Quantitative MS can be performed, for example, using the SILAC approach, where cells are metabolically labelled to generate tagged protein pools for MS analysis, and the isotopes content is compared to provide direct quantification.⁹³ This approach has been used recently to investigate and validate binding sites determined using PAR-CLIP.⁸⁶

More recently, efforts have been made to combine the results of these complementary approaches to define the role of RBPs in the regulation of the functions of various RNAs. Castello *et al.* and Baltz *et al.* developed an *in vivo* interactome capture assay to screen HeLa and HEK 293 RBPs, respectively.^{94,95} Protein mRNA interactions were stabilized by covalent UV crosslinking and the poly(A) mRNAs were subsequently captured on oligo(dT) magnetic beads following cell lysis. The RNAs were analyzed by next generation sequencing and the proteins by MS. The results permitted the identification of hundreds of previously unknown proteins and established this method as a useful tool to study the mRNA interactome composition and dynamics in different biological conditions.

In summary, the described methods provide excellent tools for the discovery and validation of protein–RNA interactions and represent promising approaches to investigate changes in the mRNA state resulting from metabolic changes, difference in cell growth, various stresses or responses to drugs. A general limit of both protein-centric and RNA-centric methods is low sensitivity, so that sufficient material needs to be generated for the analysis. Typically, $1\text{--}5 \times 10^6$ cells are used for a single RIP. Sufficient material also needs to be purified for MS analysis, particularly for the investigation of low abundance RNA–protein complexes. Moreover, these techniques cannot provide information on the localization of mRNAs and RBP and their interactions on a per cell basis, nor on the cell to cell variability in response to cellular or extracellular cues. Various fluorescence microscopy techniques have been

adapted to cell imaging to overcome such limitations, and they will be discussed in the following paragraphs.

2.2 Fluorescence imaging methods

Biology is stochastic in nature leading to diverse, spatio-temporally inhomogeneous distribution of molecules within cells and across individual cells. Therefore, its investigation requires the development of ultrasensitive techniques. Single molecule approaches have emerged as powerful tools to resolve complex cellular processes that are otherwise masked by ensemble averaging provided by most analytical methods.⁹⁶ For example, the latter approaches cannot discriminate if functionally important RNAs are expressed in low quantity across all cells of a sample, or are only selectively expressed in few cells within a sample. The main goal in the study of protein–RNA interactions at the single molecule level resides in the ability to target and visualize single RNAs, as well as observe and quantify interactions with regulatory proteins. Proteins are typically identified in fixed cells using immunofluorescence (IF), or in living cells by exogenous expression of a fusion-version of the protein of interest (such as GFP) *via* plasmid transfection or lentivirus infection.

2.2.1 Visualization of RNAs at the single molecule level in cells—Recent years have seen a great effort in the development of a set of tools to image RNAs with single molecule sensitivity.⁹⁷ Among them, single molecule sensitive fluorescence *in situ* hybridization (smFISH) consists of labeling target RNAs with several (at least 30) complementary fluorescently labeled oligonucleotides (typically DNA) *in situ*, upon fixing and permeabilizing the cells/tissues.⁹⁸ The single-molecule sensitivity of this method has been validated in several subsequent experiments and its relative simplicity has led to its commercialization (Stellaris probes by Biosearch technology).^{99,100} This method permitted to observe and quantify in HCC1954 breast cancer cells the ERBB2, AKT1, and AKT3 mRNAs, using a different fluorophore per RNA. The results were found to correlate with RNA-Seq data.¹⁰¹ Single mRNA molecules were also observed in single cells within intact histological tissue sections.¹⁰² However, this method cannot be used to study short transcripts or small RNAs, it requires harsh hybridization conditions, which may affect subsequent manipulation of the sample, such as performing immunofluorescence, and, last, it can only be applied to fixed samples, preventing live-cell studies.

To visualize RNAs in living cells, single-label, linear, nucleic acid probes and molecular beacons (MB) have been used.^{103–105} These probes are typically composed of 2′O-methyl RNA bases to alleviate RNase H mediated cleavage of RNA/DNA duplexes and probe degradation. These probes lack of single molecule sensitivity, given the types of microscopes typically utilized, such as widefield, widefield plus deconvolution, and laser scanning confocals. As a consequence, a large number of probes needs to be utilized to enhance the fluorescent signal, and this may interfere with the binding of regulatory factors vital to the RNA function, like binding proteins or miRNA. Third, the delivery strategies applied are usually not ideal. Microinjection, tends to lead to passive transport of probes into the nucleus, and consequently hampers cytoplasmic RNA labeling.^{104–107} Electroporation, typically, requires the cells to be trypsinized and this may cause alteration in gene expression, and cause significant cytoskeletal changes.^{108,109}

Currently, the prevalent technique to visualize RNA in living cells consists in utilizing a GFP-fusion of the phage coat protein MS2 which binds specifically to a 19 nt RNA stem-loop sequence.^{110,111} Both the mRNA of interest, carrying the MS2 target sequence into its 3'-UTR, and MS2-GFP are expressed within a living cell. The MS2-GFP binds to the expressed mRNA and GFP fluorescence indicates the mRNA position. In order to increase the signal above the background of unbound fusion proteins and achieve single molecule sensitivity, multiple MS2-GFP binding domains are inserted into the target RNA.^{111,112}

Two similar strategies have been used to study RNAs in mammalian cells. The first utilizes GFP-RNA binding peptide fusion probes, which bind to a 15 nt RNA hairpin encoded in the expressed target RNA.¹¹³ The second relies on probes containing the Pumilio homology domains (PUM-HD), fused to sections of split EGFP, which target two closely spaced 8 nt native sequences.^{114,115}

These methods can only be used in cell types that allow for efficient transfection. Moreover, plasmid-derived mRNA often have heavily modified 5'- and 3'-UTR sequences, carrying “bulky” molecules, which can strongly influence mRNA translational efficiency, decay, and stability.^{116–118} To reduce the size of the reporter protein a recent approach utilized a GFP-derived group of fluorophores bound by an RNA aptamer called “Spinach”. The Jaffrey lab reported the ability of Spinach to readily permit the visualization of RNAs in live cell experiments and demonstrated its improved resistance to photobleaching with respect to GFP.^{119,120}

2.2.2 Advances in the visualization of RNA–protein interactions—The techniques just described have been implemented for the study of protein–RNA interactions in cells. smFISH is typically combined with IF staining of endogenous proteins, and colocalization analysis is utilized. This method was used by Shih *et al.* to study interactions between RISC (RNA-induced silencing complex), SG and PB components with miRNA-regulated mRNAs.¹²¹ However, colocalization analysis cannot guarantee that interactions occur, due to the resolution limitations of most optical microscopy techniques. To directly visualize protein–RNA interactions, Fluorescence Resonance Energy Transfer (FRET) and Fluorescence Complementation (FC) were recently utilized (Fig. 5). FRET is a photophysical phenomenon in which energy is transferred between two appropriate fluorophores, donor and acceptor, that are in proper orientation and distance (usually, <10 nm). The RNA is typically labeled with either SytoxOrange or the MS2 system.^{122,123} FRET was successfully utilized to visualize interactions of the RNA binding proteins hnRNP H, PTB, Raver1 and their RNA targets with both temporal and spatial resolution (Fig. 5A). The MS2 system has also been used in FC experiments.¹²⁴ Here, the mRNA of interest is tagged with the MS2 cassette, the MS2 coat protein is fused to a split fragment of the yellow fluorescent protein (YFP) variant, Venus, while the complementary portion of Venus is fused to the RNA-binding protein of interest (Fig. 5B). If the RNA-binding protein interacts with the RNA sequence of interest, the two separate Venus fragments unite, emitting a YFP signal. This strategy was used to visualize FMRP and IMP1 interactions with mRNAs *in situ* in living cells. A similar system consisting of the split mCherry reporter and HIV Rev-*RRE* and Tat-*TAR* peptide-RNA pairs was used to image influenza A viral mRNA interactions with key adapter proteins for mRNA nuclear export in the TAP cellular pathway.¹²⁵ It has been

observed that the maturation of the reporter protein after formation of the protein–protein complex takes a considerable amount of time. Therefore, temporal and spatial regulation in response to physiological events cannot be reliably observed using FC.¹²⁶

Recently, single molecule techniques like fluorescence (cross-) correlation spectroscopy (FC(C)S) have been used to quantify protein–RNA interactions in living cells. FCS analyzes the fluctuations of the concentration of single fluorescently-labeled molecules and their diffusion properties. Differentially labeled molecules can be used in FC(C)S to study multi-molecular interactions by confocal microscopy.¹²⁷ According to Wachsmuth *et al.*, FC(C)S experiments in live cells “are based on a manual, labor intensive workflow of image optimization and acquisition” and are prone to bias. In their recently published paper, they described a fully automated approach to overcome this challenge and illustrate the potential of their system as a tool for a systematic characterization of protein complexes in a cell-based environment.¹²⁷ Lastly, fluorescence fluctuation spectroscopy (FFC) was recently used by Wu *et al.* to study protein–mRNA interactions in single live cells.¹²⁸ This technique, closely related to FCS, utilizes brightness analysis to detect interactions between molecules, by characterizing the average fluorescence intensity of a single particle. The authors used the MS2 technology to observe interactions of β actin mRNA and ZBP1 in mouse embryonic fibroblasts (MEFs) and hippocampal neurons and to measure the association of mRNAs with mCherry-labeled ribosomes in different cellular compartments.¹²⁸ Although these methods are very elegant and powerful, they require precise validation and often involve laborious and complex analysis. Moreover, all the described techniques have been applied, at present, to only a very limited number of proteins and mRNAs, so that further experiments are necessary to fully establish their potential.

In the following paragraphs we describe the setup and application of a novel system for the study of native protein–mRNA interactions *in situ* in single cells, with single-interaction sensitivity. This assay can be performed in one working day and the results can be readily observed using a deconvolution or confocal microscope. This method does not require complex validation or analysis and is yet applicable to the study of plasmid derived systems. Here, we discuss the imaging and quantification of native mRNA–RBPs interactions, focusing on proteins modulating mRNA stability.

3 FMTRIP-PLA: design and overview

3.1 FMTRIPS characterization and ability to bind to specific mRNAs in live cells

The RNA imaging strategy, composition and delivery of MTRIPS (Fig. 6) has been comprehensively described in Santangelo *et al.*^{129,130} MTRIPS are fluorescently labeled tetravalent single RNA sensitive probes consisting of a neutravidin core and four biotinylated fluorescently labeled oligonucleotides for specific mRNA targeting (Fig. 6A). MTRIPS are delivered to the cytoplasm of cultured cells *via* reversible membrane permeabilization using Streptolysin-O (Fig. 6B). 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 poly(A) transcripts, and the colocalization with regulatory proteins and

RNA granules in both fixed and living cells.^{131–133} MTRIPS were also utilized to study the RSV genome, its organization in viral particles, its localization in infected cells and interactions with viral and host proteins.^{134,135} As previously mentioned, colocalization is a poor indicator of physical interactions, due to limitation in optical resolution. Therefore, a proximity ligation assay (PLA) and rolling circle amplification (RCA) was utilized to detect specific mRNA protein interactions in cells. mRNAs were identified using FMTRIPS, a peptide-modified version of MTRIPS, where a flag-tagged neutravidin constitutes the scaffold for the tetravalent probes (Fig. 6C).

3.2 Proximity ligation assay and rolling circle amplification using FMTRIPS delivered to live cells

PLA is a commercially available kit, whose overall approach was described in Soderberg *et al.*⁶⁸ It is generally utilized to study protein–protein interactions in fixed cells and tissues.^{136,137} 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 RCA, which results in a coiled single-stranded DNA. The PLA product is detected by hybridizing complementary fluorescently labeled oligonucleotides, and it appears as puncta of consistent size and intensity for a variety of analytes, antibodies and cell lines. The “detection reagent” is available in green, red, orange and far-red, and is therefore amenable for different filter sets and potentially ideal for multiplexing.

In PLA using FMTRIPS one antibody targets the protein of interest, and the other one the flag tag on neutravidin (Fig. 6D).^{67,138} The antibody selection and blocking conditions must be accurately determined in control experiments (using for example neutravidins without flags) to ensure specificity. 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. In RSV infected cells, PLA was used to observe interactions between the genomic RNA (labeled with FMTRIPS) and the viral N protein while no signal was observed using MTRIPS⁶⁷ (Fig. 6E). The results validated previous evidence obtained *via* both colocalization analysis and super resolution microscopy.^{134,135}

In the following paragraphs we will summarize the main results of recent publications demonstrating the potential for PLA to be used to study the “mRNA state” *via* detection and quantification of interactions with regulatory proteins and the interplay between them.

4 Quantification of changes of RBP–mRNAs interactions using FMTRIPS and PLA

4.1 Quantification of mRNA interactions with HuR

HuR is a ubiquitously expressed member of the ELAV family of RNA binding proteins, and it binds to the poly(A) tail and 3'UTR of several transcripts.^{139–141} As previously described, HuR regulation is crucial for the stability and translation of many ARE-containing mRNAs

which, often, encode for proteins involved in the cell cycle, carcinogenesis, immune and stress responses.^{142–144} However, HuR also regulates the stability of mRNAs of housekeeping genes, like β -actin.¹⁴⁵ In cells, it is mainly localized in the nucleus, but it shuttles between nucleus and cytoplasm when cells are exposed to certain stimuli, such as oxidative stress or transcription inhibition, to stabilize mRNAs in the cytoplasm.¹⁴⁶

In Jung *et al.* we first quantified poly(A) mRNA interactions with HuR in HeLa cells (Fig. 7A).⁶⁷ Cells were also treated with Actinomycin D (ActD), which caused transcriptional repression and shuttling of HuR from the nucleus to the cytoplasm, and/or transfected with a plasmid encoding GFP-HuR. Treating cells with ActD resulted in a progressive decrease of the mRNA volume over time and an increase of the frequency of PLA between mRNA and HuR in the cytoplasm (Fig. 7B). Similar results were obtained using FMTRIPS specific to β -actin mRNA (Fig. 7C).

The effect of HuR overexpression was also observed in untreated cells, or treated with ActD. The changes in PLA frequency were measured. As expected, PLA frequency increased when HuR was overexpressed, and even more so in cells treated with ActD (Fig. 7B–C). To conclusively demonstrate the specificity of the assay, cells were also transfected using a HuR siRNA, which reduced dramatically the PLA frequency (Fig. 7D). Ultimately, these experiments permitted to observe the effect of cell-to-cell-variation in time within a cell population as a consequence of (a) plasmid or siRNA transfection (b) FMTRIP number and (c) ActD exposure. Importantly, since the cytosolic HuR could not be observed *via* IF, because of the saturating signal in the nucleus, colocalization measurements were precluded.⁶⁷ Our assay proved to be ideally sensitive to locate proteins in the cytoplasm, because of the productive PLA signal resulting from interactions with mRNAs. Last, during these studies, control experiments were performed transfecting HeLa cells with different plasmids encoding for various truncations of the cmc mRNA 3'UTR, which contains a HuR binding site.¹⁴⁴ PLA was observed only upon transfection of plasmids containing the whole protein binding site, confirming that FMTRIP and PLA can successfully be utilized for the study of both native and non-native interactions.

4.2 Interplay between HuR and TIA1 in the regulation of the onco-suppressor PDCD4

In addition to the conventional role of HuR in stabilizing mRNAs, recent work has revealed that HuR can positively regulate the translation of target transcripts without affecting their stability, or even repress the expression of target transcripts *via* cooperative interactions with the miRNA machinery.^{147–149} Moreover, HuR interacts with other AU- or U-rich element RNA binding proteins, both cooperatively and competitively, to fine tune the expression of target transcripts.^{150,151} Recent transcriptome-wide studies showed that both HuR and TIA1 bind to the 3'UTR in the AU-rich region of target transcripts. TIA1 is distributed in both the cytoplasm and the nucleus of cells, and, as HuR, shuttles between the two cell compartments as a consequence of external stimuli, such as oxidative stress.^{152,153} This evidence suggested that these RBPs may coordinately regulate a number of mRNAs, including the tumor suppressor PDCD4, whose regulation is altered in a number of cancer types.¹⁵⁴ PDCD4 encodes for a protein that inhibits the activity of the eukaryotic translation factor 4A (EIF4A).¹⁵⁵ Its expression is regulated at the transcriptional level by the transcription factor

v-myb as well as through DNA methylation.^{156,157} At the translational level, PDCD4 mRNA is known to be regulated by miR-21 and by the splicing factor SRSF3.^{158,159} In Wigington *et al.*, PDCD4 was first identified as a novel target for HuR and TIAR, whose binding sites were shown to overlap in the 3'UTR.⁶⁹ Knockdown of HuR and/or TIA1 in MCF-7 cells, a breast cancer cell line, resulted in significant reduction of PDCD4 mRNA, supporting a role for these factors in positively regulating mRNA levels. FMTRIPS targeting the 3'UTR of the PDCD4 mRNA were designed, avoiding the HuR binding site. Using FMTRIPS and PLA, individual interactions between native transcripts and either HuR or TIA1 were quantified in single cells *in situ* (Fig. 8A–C). Overexpression of either HuR or TIA1 increased, as expected, PLA frequency (Fig. 8B–D). To determine if the two proteins compete or cooperate in the translational regulation of PDCD4 mRNA, TIA1 was overexpressed and the PLA frequency between HuR and PDCD4 mRNA was measured. TIA1 overexpression caused a reduction of the interactions between HuR and the transcript; knocking down TIA1 increased such interactions (Fig. 8B). Similarly, knock down of HuR increased interactions of TIA1 with PDCD4 mRNA, while overexpression of HuR reduced them (Fig. 8D). These results suggested that the PDCD4 transcript is competitively bound by HuR and TIA1. As previously mentioned, the PDCD4 mRNA undergoes regulation by several different factors at each level of maturation. This study provided evidence for the interplay of two additional RBPs, TIA1 and HuR, and established this approach as a useful tool, complementary to genome wide approaches, to investigate the complex regulation of a tumor suppressor function (Fig. 8E).

5 Conclusions

In the current review we described various techniques currently utilized for the study of protein–RNA interactions, with an emphasis on regulation factors involved in post-transcriptional regulation crucial in cancer related processes. Genome wide approaches offer the unique means to discover new potential networks and identify and characterize the main players. Single molecule based approaches utilize these information to provide spatio-temporal details at the single cell level, accounting for cell to cell variability and sample inhomogeneity.

We also introduced a novel, relatively simple approach to the investigation of protein–mRNA interactions in single cells with single-interaction sensitivity, and its applications. Well characterized regulatory proteins were utilized to validate our assay and the effects of changes of their concentration and localization were analyzed *via* overexpression/silencing. Drugs that induce transcriptional inhibition, cytoskeleton disruption and oxidative stress were additionally used to treat cells, to mimic potential mechanisms involved in the development of an aberrant phenotype. The limits of traditional colocalization measurements were overcome using PLA, as exemplified in our studies of interactions between HuR and mRNAs. Despite overexpression, HuR is not detectable *via* immunostaining in the cell cytoplasm, because of dynamic range limits. In PLA, 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. The crucial reagents in the assay are the primary antibodies specific for the Flag peptide 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 and TIAR, but also proteins involved in mRNA decay 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.

It is important to note that not every FMTRIP participates in PLA productively for at least two reasons: (1) PLA detects interactions present at the time of fixation and (2) the distance between the FMTRIPs and the antibody against the protein of interest 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 non-specific binding. While this allows for detection of relative differences between samples, it may cause under-sampling of the interactions especially for less abundant mRNAs. 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 post-transcriptional regulatory events and how they contribute to mRNA “state”, overall protein production, and aberrant tissue phenotypes within a cellular or tissue context.

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Fig 1.

Schematic representation of the role of RBPs in various post-transcriptional processes in eukaryotic cells. RBPs (represented by circles of different colors) mediate splicing of pre-mRNAs into mature mRNAs in the nucleus, which are then exported into the cytoplasm by other RBPs. Depending on whether RBPs are bound to pre-mRNAs or mRNAs to form a RBP complex, RNPs are classified as hnRNP or mRNPs, respectively. In addition, RBPs are responsible for the localization of mRNAs to specific cellular compartments. In the cytoplasm, RBPs also govern the stability of transcripts and control the translation into proteins.

Fig 2.

In vitro methods for determining RBP targets: (A) SELEX consists of several rounds of binding and amplification of RNA molecules (B) RNA-competite queries a designed RNA pool under competitive conditions and assays the bound RNAs using a microarray.

Fig 3.

In vivo methods for determining RBP targets: (A) RIP-chip and RIP-seq determine bound RNAs by analyzing immunoprecipitated RNPs by microarrays or high throughput sequencing. (B) UV cross-linking and immunoprecipitation allows stringent washing and RNAase treatment of bound RNAs. (C) In PAR-CLIP cells are treated with a modified nucleoside (4SU or 6SG), which is incorporated into transcribed RNAs. The modified nucleoside can be crosslinked using longer wavelength UV radiation.

Fig 4. RNA-centric methods for the purification and identification of RNA binding proteins. (A) Examples of purification schemes for RNA binding proteins using *in vivo* and *in vitro* approaches. In this example, the MS2 system is used as tagging method. (B) MS is commonly used to identify the samples via non quantitative or quantitative approaches, such as SILAC.

Fig 5.

Schematic representation of FRET and FC approaches: (A) schematic representation of the strategy utilized by Huranova *et al.* to visualize RNA–protein interactions inside cells. (B) Schematic representation of the fluorescence complementation method utilized by Rackham *et al.* to study protein RNA interactions in living cells.

Fig 6.

Design and validation of FMTRIPS: (A) schematic representation of MTRIPS, consisting of a neutravidin core (yellow) and four fluorescently labeled oligonucleotides (typically with Cy3b, red), complementary to the mRNA of interest. (B) MTRIPS are delivered to the cytoplasm of cells using Streptolysin-O mediated reversible membrane permeabilization to target endogenous or plasmid derived mRNAs. (C) FMTRIPS are a Flag-tagged version of MTRIPS labeled with one or more Flag peptides *via* a covalent bond (D) schematic representation of the PLA reaction: antibodies (light blue and magenta) and proximity probes (dark blue and magenta) bind to FMTRIPS and RBPs (brown) scaffolded on mRNAs. The probes are ligated *via* enzymatic reaction to synthesize a fluorescently labelled hybridized product *via* RCA (typically using a far red dye, green (E) visualization of PLA (green) between FMTRIPS delivered to RSV infected A549 cells 12 h post infection and RSV N *via* widefield deconvolution microscopy. Nuclei are stained with DAPI (Blue). Scale bar is 5 μ m.

Fig 7.

Scheme and quantification of PLA between HuR and mRNAs: (A) schematic representation of PLA between FMTRIPS targeting the poly(A) tail of mRNAs and HuR. (B) Mean PLA frequency between HuR and FMTRIPS targeting poly(A) mRNAs in untransfected HeLa cells or transfected with a HuR-GFP plasmid in control conditions or after treatment with ActD. (C) Mean PLA frequency between HuR and FMTRIPS targeting β-actin mRNAs in untransfected HeLa cells or transfected with a HuR-GFP plasmid in control conditions or after treatment with ActD. (D) Mean PLA frequency between HuR and FMTRIPS targeting poly(A) mRNAs (PA) or untargeted probes (UT) in untransfected HeLa cells or transfected with either a HuR-GFP plasmid a control siRNA or a HuR siRNA. Error bars are standard deviation.

Fig 8.

Scheme and quantification of PLA between HuR or TIA1 and PDCD4 mRNA: (A) schematic representation of PLA between FMTRIPS targeting the 3'UTR of PDCD4 mRNA and HuR. (B) Mean PLA frequency between HuR and FMTRIPS targeting PDCD4 mRNA in untransfected MCF-7 cells or transfected with either a TIA1-GFP plasmid, a control siRNA or a HuR siRNA. (C) Schematic representation of PLA between FMTRIPS targeting the 3'UTR of PDCD4 mRNA and TIA1. (D) Mean PLA frequency between TIA1 and FMTRIPS targeting PDCD4 mRNA in untransfected MCF-7 cells or transfected with either a HuR-GFP plasmid, a control siRNA or a TIA1 siRNA. Error bars are standard deviation. *** indicates statistically different data. (E) Model for the post-transcriptional regulation of PDCD4 mRNA in the cytoplasm. Both HuR (magenta) and TIA1 (green) are predominantly nuclear but shuttle in and out of the cytoplasm (bi-directional red arrow) and competitively bind to the 3'UTR of PDCD4 mRNA.