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Probes for Intracellular RNA Imaging in Live Cells

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Abstract

RNA localization, dynamics, and regulation are becoming increasingly important to our basic understanding of gene expression and RNA virus pathogenesis. An improved understanding of these processes will be necessary in order to identify new drug targets, as well as to create models of gene expression networks. Much of this new understanding will likely come from imaging studies of RNA, which can generate the spatiotemporal information necessary to characterize RNA within the cellular milieu. Ideally, this would be performed imaging native, nonengineered RNAs, but the approaches for performing these experiments are still evolving. In order for them to reach their potential, it is critical that they have characteristics that allow for the tracking of RNA throughout their life cycle. This chapter presents an overview of RNA imaging methodologies, and focuses on a single RNA sensitive method, employing exogenous probes, for imaging, native, nonengineered RNA in live cells.

1. Introduction

Over the past decade, there is increasing data to suggest that RNA molecules have a wide range of functions in living cells, from physically conveying and interpreting genetic information, to essential catalytic roles, to providing structural support for molecular machines, to gene silencing. These functions are realized through control of their expression level, via transcription factors, stability, and degradation rates, by RNA binding proteins and miRNA, and through their spatial distribution. *In vitro* methods that use purified DNA or RNA obtained from cell lysates can provide a measure of RNA expression level within a cell population; however, they cannot reveal the spatial and temporal variation of RNA and their interactions with regulatory factors within a single cell.

In addition, there has been substantial evidence that the spatial regulation of mRNA is mediated by processing bodies (p-bodies or PB), and stress granules (SG), when exposed to certain environmental stimuli (Anderson and Kedersha, 2009a,b; Buchan and Parker, 2009), and the RNA exosome (Lin *et al*., 2007). The core constituents of SGs are components of a noncanonical, translationally silent 48S preinitiation complex that includes the small ribosomal subunit, and early initiation factors eIF4E, eIF3, eIF4A, eIFG, and PABP. SGs also contain mRNAs and a set of mRNA binding proteins that regulate mRNA translation and decay, as well as proteins that regulate various aspects of mRNA metabolism. PBs consist of a core of proteins involved in mRNA repression and degradation. They include the mRNA decapping machinery, as well as key effectors of microRNA (miRNA)-mediated RNA interference (RNAi), such as Argonaute-2 (Ago2), miRNAs, and their cognate mRNAs (Anderson and Kedersha, 2006, 2008, 2009a,b; Kedersha and Anderson, 2007; Kedersha *et al*., 2005, 2008). The RNA exosome is a multisubunit 3′–5′ exoribonuclease complex that participates in degradation and processing of cellular RNA (Tomecki and Dziembowski, 2010; Tomecki *et al*., 2010) and has been shown to localize near the nucleus during stress(Lin *et al.*, 2007). In general, how specific mRNAs interact with these localized structures in time and space under various conditions will likely be elucidated through RNA

imaging. Therefore, the ability to image specific RNAs in living cells in real-time promises to provide information on RNA synthesis, processing, transport, localization, and degradation. This information should offer new opportunities for advancement in molecular biology, disease pathogenesis, drug discovery, and medical diagnostics.

2. Imaging RNA in Live Cells

Currently, expressing both the RNA and a fluorescent tag using plasmid-based systems is the state-of-the-art for imaging RNA. The enabling technology is the fusion of GFP (or any fluorescent protein) with a sequence-specific RNA binding protein or peptide. The initial system utilized the phage coat protein MS2 fused to GFP (Brodsky and Silver, 2002; Fusco *et al*., 2003) as the probe, which will bind specifically to a 19 nt RNA stem-loop forming sequence. When a target mRNA, with this target sequence inserted into its 3′-UTR, and MS2-GFP, were expressed within a living cell, MS2-GFP bound to the expressed mRNA containing the “tag” sequence and GFP fluorescence constituted the indicator of mRNA position. In order to increase the signal from valid RNAs above the background of unbound fusion proteins, multiple MS2-GFP binding domains were inserted into the target RNA. When 24 binding sites (capable of binding 48 MS2-GFP molecules) were inserted, single molecule sensitivity was achieved (Fusco *et al.*, 2003; Shav-Tal *et al*., 2004). Since the initial use of this system, two additional strategies using plasmid-expressed probes have been demonstrated in mammalian cells: GFP-RNA binding peptide fusion probes, which bind to a 15 nt RNA hairpin encoded in the expressed target RNA (Daigle and Ellenberg, 2007) and probes composed of Pumilio homology domainss (PUM-HD) fused to sections of split EGFP, which target two closely spaced 8 nt native sequences (Ozawa *et al*., 2007; Yamada *et al*., 2011). These systems have been applied to the study of cytoplasmic mRNA, nuclear mRNA, and mitochondrial RNA.

Employing plasmid-derived probes and RNA gives these methods tremendous flexibility, but they do have limitations. First, they can only be used in cell types that allow for efficient transfection. Second, plasmid-derived mRNA often lack the correct number and position of introns and the exact 5′- and 3′-UTR sequences, which can strongly influence mRNA translational efficiency, decay, and stability (de Silanes *et al*., 2007; Giorgi and Moore, 2007; Jambhekar and Derisi, 2007). In addition, plasmid-derived RNAs are often overexpressed, possibly changing the fundamental stoichiometry underlying RNA expression. Of the techniques mentioned above, only the PUM-HD fusions have the ability to study native, nonengineered RNAs. However, they do require the ability to transfect and express the PUM-HD fusions efficiently, and the user must optimize their amino acid sequence for a given RNA.

A new extension of this methodology, which still utilizes the fluorescent protein-MS2 fusion protein as a probe, consists of transgenic mice with the 24× MS2 repeats incorporated into the 3′-UTR of the native β-actin mRNA (Grunwald and Singer, 2010; Lionnet *et al*., 2011). This system resolves the issue of overexpression but requires that cells are removed from the animal and then transfected with GFP-MS2 in order to image the RNAs. This is a powerful system, but it does have limitations: it is uncertain whether this can be repeated with other genes, the expense associated with creating transgenic animals for each gene is not insignificant, the method cannot be used with other species, and this method does not allow for multiplexing.

Another approach to RNA imaging has been to use fluorescently labeled exogenous antisense probes that target RNA via Watson-Crick pairing. These methods seek to target and image, native, nonengineered RNA, and there is no requirement of protein expression. The specific approaches used, though, can greatly affect the observations. As a result, the

variations reported have limited the usage of this approach for studying RNA biology. The types of probes that have been published include single-label, linear, nucleic acid probes (Molenaar *et al*., 2001, 2004) with and without the application of fluorescence resonance energy transfer (FRET), molecular beacons (MB) (Bao *et al*., 2004; Bratu *et al*., 2003; Nitin *et al*., 2004; Santangelo, 2010; Tyagi and Alsmadi, 2004), also with and without FRET, ratiometric approaches with MBs (Chen *et al*., 2007), and more recently, single RNA sensitive, multiply labeled polyvalent RNA imaging probes (Lifland *et al*., 2010; Santangelo *et al*., 2009).

Exogenous probes, such as those mentioned above, have been delivered to living cells using endocytosis, microinjection (Bratu *et al*., 2003; Chen *et al*., 2007; Mhlanga *et al*., 2005; Tyagi and Alsmadi, 2004; Vargas *et al*., 2005), electroporation (Chen *et al*., 2008; Spiller *et al*., 1998), peptide-mediated delivery (Lifland *et al*., 2010; Nitin *et al*., 2004), and streptolysin O (SLO) (Bao *et al*., 2004; Lifland *et al*., 2010; Paillasson *et al*., 1997; Rhee *et al*., 2008; Santangelo and Bao, 2007; Santangelo *et al*., 2005, 2009; Utley *et al*., 2008). The types of microscopy utilized for interrogation have included widefield, widefield plus deconvolution, and laser scanning confocal.

3. Limitations of Single-Label Probes and Molecular Beacons

To date, fluorescent protein-based systems are still the state-of-the-art in RNA imaging. The reason for this is likely twofold, the need for probe delivery versus probe and target expression, and the lack of single molecule sensitivity. Probe delivery has been achieved using multiple methods and agents, such as microinjection, electroporation, cationic molecules, and SLO. Microinjection requires costly equipment, specific expertise and has limited throughput. It also suffers from the problem of probe sequestration within the nucleus, unless probes are tetramerized via the use of streptavidin and neutravidin (Bratu *et al*., 2003; Mhlanga *et al*., 2005; Tyagi and Alsmadi, 2004; Vargas *et al*., 2005). These barriers will likely always limit its general use. Cationic molecules also tend to deliver probes to the nucleus, often utilize the endocytic pathway which contain high levels of acid hydrolases, and restrict the release of probe into the cytoplasm, also limiting their use. Electroporation, typically, requires the cells to be nonadherent or trypsinized in order for it to function and also requires specific equipment (Spiller *et al*., 1998). Trypsinization may specifically be problematic in that it may alter gene expression, as it changes the cytoskeleton significantly. SLO, though, has been used for the past 18 years intermittently by researchers in multiple cell types including primary cells and has many positive characteristics for probe delivery, such as low cost, delivery to all cells within a dish, very low mortality, no observable changes in cell morphology, does not induce stress granules, does not change p-body or stress granule number when induced, and does not change RNA localization to lamellae. SLO delivery does have drawbacks, such as it cannot deliver probes much larger than 30 or 40 nm, and at the concentrations typically utilized, it is difficult to deliver large numbers of probe (>10,000). The limits in delivery quantity though, are not a problem for RNA imaging, as few messenger RNAs are in quantities <1000. In general, we have found that SLO delivery is a convenient, nonperturbing method for delivering exogenous probes and nanoparticles (Barry *et al*., 1993; Clark *et al*., 1999; Giles *et al*., 1998; Lifland *et al*., 2010, 2011; Lindquist *et al*., 2010; Santangelo and Bao, 2007; Santangelo *et al*., 2006, 2009; Spiller *et al*., 1998; Utley *et al*., 2008; Walev *et al*., 2001; Zurla *et al*., 2011).

The second reason, we postulate, for the limited use of exogenous probes, compared with the MS2-GFP system, has been the lack of single molecule sensitivity. The MS2-GFP system, when 24 repeats of the RNA aptamer are incorporated into a target RNA, allow for up to 48 MS2-GFP molecules to bind to the RNA, yielding single RNA sensitivity. Using

single fluorophore-based probes, such as molecular beacons, Vargas *et al*. (2005) only achieved single molecule sensitivity and the detection of single RNAs through the incorporation of 16 binding sites in a GFP coding mRNA. When 96 sites were included in the mRNA sequence, the mRNA were bright enough for dynamic imaging to be performed; single molecule sensitivity using native, nonengineered RNA was not possible.

The need for single RNA sensitivity is reflected in two branches of the RNA literature, the RNA *in situ* hybridization literature, as well as investigations interested in RNA turnover and copy number per cell. A recent review article discussing RNA imaging in fixed cells, as well as a close examination of the *in situ* hybridization literature (Itzkovitz and van Oudenaarden, 2011), clearly demonstrated the need for single RNA sensitivity for studying RNA in the cellular context. Two of the most well-known examples in the *in situ* literature are that of Femino *et al*. (1998) and Raj *et al*. (2006, 2008); both papers successfully utilize single molecule sensitive probes for RNA characterization in fixed cells and also applied the probes for gene expression profiling. In both cases, many (>10) short (<50 nt) deoxyoligonucleotides either labeled with multiple or single fluorophores are hybridized to a single RNA species. By using multiple short oligos, these methods have superior sequence specificity to full-gene length probes, and they are able to add a sufficient number of fluorophores to the target RNA in order to yield single RNA sensitivity. It is not surprising that after the Singer lab realized the need for single RNA sensitivity *in situ*, they would pursue other single RNA sensitive methods such as MS2-GFP for live cell imaging. Raj *et al*. developed their method in order to measure mRNA levels at the single molecule level and study the stochastic nature of mRNA synthesis (Raj *et al*., 2006). They theorized that mRNA levels were more sensitive to the overall process of gene expression because their half-lives are much shorter than that of proteins, and therefore their instantaneous numbers better represented the results of transcription. They primarily examined the expression of an integrated synthetic gene within the genome of CHO cells (Raj *et al*., 2006). From their results, they clearly showed that with only one integration of the plasmid into the genome the average number of mRNA generated was on the order of 100 molecules. They then found that with multiple integrations, mRNA expression increased, on average, to ~1000 mRNAs per cell (Raj *et al*., 2006).

A number of different biochemical approaches have been utilized to quantify mRNA copy number on a per cell basis such as serial analysis of gene expression (SAGE) (Velculescu *et al*., 1999), quantitative PCR (qPCR) (Bengtsson *et al*., 2008; Diercks *et al*., 2009; Kanno *et al*., 2006; Taniguchi *et al*., 2009). Velculescu *et al*., as early as 1999, published in *Nature Genetics* an analysis of the human transcriptome on a per cell basis using SAGE. In their analysis of colon cancer cell lines, they described the range of expression from 0.5 to 2672 copies per cell, where 61 transcripts, which were expressed at over 500 transcript copies per cell, made up one-fifth of the mRNA mass of the cell, and the most highly expressed 623 genes accounted for nearly one-half of the mRNA content. In contrast, most unique transcripts were expressed at low levels, with just under 23% of the mRNA mass of the cell comprising 90% of the unique transcripts expressed. A few examples include: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was found to be expressed at a level of 864 mRNAs per cell on average, with a range from 194 to 1985 copies per cell, cytoplasmic actin mRNA with an average of 560, and a range from 147 to 1203, and survivin mRNA (in cancer tissue) from 16 to 53 copies per cell.

Later in 2009, Taniguchi *et al*., while describing a single cell qPCR methodology, examined four targets, with gene expression ranging from tens to a few thousand copies per cell (Taniguchi *et al*., 2009). Bengtsson *et al*. examined three genes in pancreatic β cells; for two of the genes, the average copy numbers were well below 1000 copies per cell, while the gene for insulin 2 (Ins2) was much higher, in the 5000 copy range per cell (Bengtsson *et al*.,

2008). This is likely due to the fact that these cells are the major insulin producers in the pancreas and therefore large quantities are produced. In another study by Kanno *et al*., they focused on normalization methods for "per cell" data from qPCR and DNA microarray studies (Kanno *et al*., 2006). Data over a large number of genes were collected, over 14,000 genes in the mouse genome. It should be noted that the maximum copy number they "spiked" their samples with for reference, was 10,000, and they had very few genes approaching that, with the majority less than 1000, consistent with other results.

The most recent study, Schwanhausser *et al*., used pulse labeling of proteins and mRNAs to quantify gene expression control in 3T3 and MCF7 cells (Schwanhausser *et al*., 2011). Newly synthesized mRNAs were labeled with 4-thiouridines, isolated using standard techniques, and then sequenced the labeled mRNA using Solexa RNA sequencing. The results from this study were generally in agreement with previous studies, where most mRNAs were expressed at less than 1000 copies per cell, in both the cell types, and the median copy number in 3T3 cells was reported as 17.

Overall, it is clear that in order to study native, nonengineered mRNA, within the cellular milieu, whose target copy number will likely be under 1000, a single molecule sensitive technique is necessary.

4. Imaging Native, Nonengineered RNA in Live Cells with Single RNA Sensitivity Using Exogenous Probes

One way of increasing the sensitivity of nucleic acids based on exogenous probes is to multiply-label linear nucleic acids with organic fluorophores (Randolph and Waggoner, 1997). This approach has been used extensively for fixed cell *in situ* hybridization but not for live-cell imaging. In recent work by Santangelo *et al*. (2009), 2–4 fluorophores were added per ligand without experiencing self quenching, and by combining four of these multiply-labeled ligands using streptavidin, probe brightness was increased fourfold. In this way, for the first time, single molecule sensitivity was achieved using a small number (<20) of organic fluorophores and a physically small probe (~5 nm) (Santangelo *et al*., 2009); these probes were called multiply-labeled tetravalent RNA imaging probes (MTRIPs).

MTRIP ligands are typically composed of a 2′-*O*-methyl RNA/DNA chimera nucleic acid with four or five, amino-modified thymidines, a 5′ biotin modification, and a short (5–7 bases) polyT section to extend the ligands from the surface of streptavidin. The amino-modified thymidines are used to conjugate NHS-ester modified fluorophores to the ligand. Once tetramerized via streptavidin, the probes are approximately the size of a single RNA binding protein, and only two or three (200–300 kDa) probes are typically utilized to achieve single molecule sensitivity versus an average of 1.3 MD for MS2-GFP (Fusco *et al*., 2003). MTRIPs, when delivered via reversible cell membrane permeabilization with SLO, have been demonstrated to bind rapidly to RNA (<10min) and achieved single RNA sensitivity using conventional fluorescence microscopy techniques. Target RNA were identified by the enhanced signal-to-background ratio achieved through binding of multiple probes per RNA (Santangelo *et al*., 2009).

4.1. MTRIP ligand design

Ligands utilized for MTRIPs typically contain an antisense hybridization domain of 20–25 nucleotides, and an extension region of typically seven thymidine nucleotides on the 5′ end of the ligand. The extension region is included in order to extend the hybridization region from the surface of streptavidin or neutravidin allowing it to bind more readily to target RNA. The hybridization regions are chosen to contain a GC content of approximately 50%, and modified to contain approximately 15, 2′-*O*-methyl RNA nucleotides, with at least two

or three, C6-amino modified thymines, which are used as sites of conjugation for organic fluorophores. Ligand designs are folded using nucleic modeling software such as MFOLD or UNAFOLD to ensure that they do not contain secondary structures, and they are checked using BLAST to ensure they do not have significant homology with nontarget RNAs, as well as checked for overlap with miRNA and RNA binding protein binding sites. Typically, if there are less than four mismatches with another mRNA, then the sequence is discarded; more than four mismatches are not considered a problem. In addition, the 5′ ends are modified with biotin for avidin conjugation. Amine reactive organic fluorophores are conjugated using standard techniques, and typically 2–3 fluors are attached per ligand. In principle, any amine-reactive fluorophore can be used, but variability in hydrophobicity can affect ligand binding. Additionally, we tend to choose fluorophores with long fluorescent lifetimes and with good resistance to photobleaching. We have had excellent results using Cy3B (GE Healthsciences) and Dylight 650 (Thermo Scientific) as replacements for Cy3 and Cy5.

4.2. MTRIP assembly, purification, and deposition on coverglass

1. In order to bind ligands to streptavidin or neutravidin, combine streptavidin or neutravidin (Thermo Scientific) (typically 6 μ*M*) and ligands (>30 μ*M*) in nuclease free 1 × PBS at a 1:5 molar ratio with a total volume of 10–20 μl for 1 h. Do NOT dilute before they are bound together for 1 h.
2. Add 450 μl nuclease free 1 × PBS, and add the 500 μl mixture to a 30 K MWCO centrifuge filter.
3. Centrifuge at 14,000 rpm for ~5–10 min until 20–40 μl remain in the upper chamber of the filter, and repeat three times.
4. Resuspend probes in 30–60 μl of 1 × PBS yielding a final concentration of 1 μ*M*.

MTRIPs probes are initially characterized on glass coverslips (#1.5) at low concentrations, usually 1–2 n*M*. This is a typical methodology utilized to determine single probe sensitivity (Agrawal *et al*., 2008) and verify proper assembly and probe brightness. Probes are immobilized on a glass surface by adding them in growth media to a 10–12 mm coverslip within a 24-well plate, and incubating them for 10 min at 37 °C. The mixture is removed, and the glass surface imaged using either laser scanning confocal or widefield deconvolution microscopy. Individual batches of each probe, in addition to a mixture of probes labeled with different dyes, have been imaged on glass surfaces. Individual probes were identified, and the mean intensity within the diffraction limited spots was plotted as a histogram. From the images of the probe mixtures, the histograms of each probe, and three-dimensional plots of the intensity of individual probes, it was concluded that single probes and not aggregates were detected. If the probes were aggregating, the two-color mixture experiment would yield substantial colocalization of the probes, but in previous experiments with Cy3B and Atto647N this did not occur (Santangelo *et al*., 2009). This has also been the case with other dye combinations (Cy3B and Dylight 649, and 650). This procedure should be utilized to perform an initial characterization of all new probes. We typically image the probes on glass with a widefield microscope using a 63×, NA = 1.4 objective, and a Hamamatsu Orca-ER camera, with exposure times of ~300 ms. Z-stacks are often taken, and the images deconvolved using interative deconvolution software from Perkin Elmer (Volocity software). Deconvolution is not necessary to detect the probes though, as they can be observed in widefield.

4.3. Characteristics of probe sensitivity and delivery via SLO into live cells

In order to characterize probe sensitivity and delivery in live cells, MTRIPs were designed to target the genomic RNA of the wild-type strain A2 of human respiratory syncytial virus

(hRSV) and were delivered into noninfected A549 cells using SLO. The hRSV probes have no target in these cells and were utilized to characterize probe delivery and distribution within the cytosol. From a single optical plane within a live cell using widefield microscopy, individual probes were observed to be homogenously distributed within the cytoplasm. Localization or accumulation of probes was not observed. From this data (Santangelo *et al*., 2009), it was clear that MTRIPs were delivered to the cytoplasm efficiently, achieving a homogenous distribution within the cytoplasm, were sufficiently bright such that single probes were observed within a live cell, and did not change cell morphology. This can be performed with any "scrambled" probe (no target in the genome) and examined in the cell of choice. It is also suggested that this is done before performing experiments with probes targeted to a specific mRNA.

4.4. Standard protocol for probe delivery into live cells using SLO

This protocol is specifically for 24-well plates or 4-well chambered cover slips. It should be optimized for different size wells.

1. Dissolve SLO (Sigma #S5265-25KU) in molecular biology grade H_2O (Do NOT use DEPC treated water) to 25,000 U/ml, then aliquot in 2 U/ml and store at −20 °C. Do NOT freeze thaw, as this will decrease SLO activity.
2. Add 1–1.5 μl of 0.5 *M* TCEP solution (Pierce # 77720) to 100 μl of 2 U/ml SLO. Incubate the 100 μl solution at 37 °C for 60 min.
3. Dilute 2 U/ml of activated SLO to 0.2 U/ml in growth medium (without serum) or OptiMEM (Gibco) or PBS (w/o) Ca^{+2}; typically add 100 μl of SLO to 900 μl media. (Whether you use basal medium, OptiMEM, or PBS will depend on the cell type.)
4. Dilute probe to a final concentration of 5 nm–30 n*M* in 250 μl of this solution. (This concentration should be varied depending on the target.)
5. Remove growth medium from cells and wash 1 × with PBS (no Ca^{+2} or Mg^{+2}).
6. Add 250 μl of (basal medium/SLO/probe) to cells and incubate for 10–15 min at 37 °C.
7. Remove mixture by pipette and add 250–500 μl of normal growth medium.
8. Incubate at 37 °C for 10–15 min.
9. The cells, at this point, are ready for imaging or fixation.

This protocol has been used for probe delivery to Hela, MiaPaCa-2, U2OS, primary human dermal fibroblasts, Panc-1, HUVEC, primary bovine turbinates, Vero, Vero C1008, A549, primary chicken embryonic fibroblasts, LNCaP, DU145, MDBK, and MDCK cells. Further optimization of SLO concentration and dilution medium may be necessary for other cell types.

4.5. Targeting of native mRNAs, comparison with scrambled probe, and colocalization with known RNA binding proteins

In order to demonstrate the ability to image single RNAs using MTRIPs, two Cy3B-labeled MTRIPs designed to target two regions of the human β-actin mRNA coding sequence and an ATTO 647N-labeled "scrambled" probe were delivered via SLO simultaneously into living A549 cells each at 30 n*M* (Santangelo *et al*., 2009). Twenty minutes after live cell delivery, the cells were fixed in 4% paraformaldehyde, to facilitate quantification, and imaged. It was found that individual "unbound" probes, probes from the glass surface, as

well as localized granules with two times the intensity (β-actin mRNA), could be specifically identified.

β-Actin mRNA was prevalent in the perinuclear region of the cell but also localized to the leading edges, whereas the “scrambled” probe showed only perinuclear signals; it did not localize in abundance at the cell periphery but rather uniformly filled the volume of the cytoplasm, therefore, demonstrating β-actin probe specificity. Average single probe intensities, quantified from probes on the glass surface, were removed via thresholding, and the remaining granules detected and analyzed in software. Using this approach single β-actin mRNAs, containing approximately two times the single probe intensity could be observed within the cell and a total number of ~1455 granules were detected. This number was consistent with previous quantifications (~1500 in serum-stimulated cells), using a similar analysis for β-actin mRNA in epithelial cells (Femino *et al*., 1998). As an additional control, A549 cells were serum-starved for 48 h and the number of β-actin mRNA granules counted in cells fixed, postlive cell hybridization (Santangelo *et al*., 2009). A representative cell contained only 409 granules, as compared with 1455 granules detected in a cell grown with serum. In this cell, the standard deviation of the mean granule fluorescence intensity for all 409 granules was 21%. The relative difference in granule count was consistent with previous experiments (Femino *et al*., 1998). Recent experiments using siRNA to knock down β-actin mRNA and characterize probe specificity are discussed later in this chapter (Lifland *et al*., 2011). Imaging of clustered β-actin mRNAs within lamellae of both A549 cells and chicken embryonic fibroblasts was performed, and they were colocalized with the β-actin mRNA binding protein, ZBP1, in three dimensions (Santangelo *et al*., 2009) (see supplementary information for the images of the fibroblasts). In addition, simultaneous imaging of β-actin mRNA, Arp-2 mRNA, and ZBP1, in primary chicken embryonic fibroblasts was performed. This showed that multiplexing was possible using MTRIPs. Currently, for this type of experiment, we often utilize Cy3B (Amersham) and Dylight 650 (Thermo Scientific), but other dyes can be used.

5. Time-Lapse Imaging of Native, Nonengineered β-actin mRNA Granule Dynamics

In addition, MTRIPs can be implemented to analyze mRNA dynamics. Utilizing MTRIPs and real-time fluorescence microscopy, Lifland *et al*. accurately quantified the dynamics of native, nonengineered, β-actin mRNAs within the cytoplasm of epithelial cells and fibroblasts for the first time (Lifland *et al*., 2011). Applying up to six MTRIP probes, this enabled the imaging of mRNAs at 5 Hz for over 5 min, which is significantly faster and longer than ever before. This should easily be extended in future work through the use of electron-multiplying CCD (EMCCD) technology. Using single particle tracking and temporal analysis, it was determined that native β-actin mRNAs, under physiologic conditions, exhibit bursts of intermittent, processive motion on microtubules, interspersed between time periods of diffusive motion, characterized by nonthermal enhanced diffusivity. When transport processes were perturbed via ATP-depletion, temperature reduction, dynamitin overexpression, and chemical inhibitors, processive motion was diminished or eliminated and diffusivity was reduced. The data presented supported a model whereby processive, motor driven motion was responsible for long distance mRNA transport. This was accomplished using six Cy3B labeled MTRIPs targeted to both the coding and 3′-UTR region of β-actin mRNA, avoiding the ZBP1 binding site and all AU-rich regions. Imaging was performed using a Zeiss Axiovert 200 *M*, Hamamatsu ORCA-ER camera, Chroma Sedat filter set, and a 63×, NA = 1.4, objective. Cells were imaged either in a Bioptechs T4 system, or using 35 mm dishes and the Live Cell Instruments Chamlide system. Single particle tracking was accomplished using Volocity software, and standard deviation maps using ImageJ.

In this work, a number of new control experiments were performed. To demonstrate that MTRIPs did not induce cross-linking of mRNA, the number of mRNA granules were counted using six multiply-labeled linear probes, two MTRIPs, and six MTRIPs; the results indicated no statistical difference in the number of granules for each probe type (Lifland *et al*., 2011). Additional confirmation of probe specificity was achieved with a novel hybrid live and fixed cell assay. In this assay, Dylight 649 MTRIPs were delivered to live cells and hybridized. The cells were then fixed in paraformaldehyde and Cy3B probes against *different sites* on β-actin mRNA were hybridized to the mRNA. A high degree of colocalization was observed, confirming probe specificity. In addition, siRNA against β-actin mRNA were applied, and knockdown quantification was compared using MTRIP imaging and granule counting, and qPCR. Knockdown from imaging/granule counting and qPCR were approximately 80% and 90%, respectively, again confirming specificity. Last, the localization of mRNA labeled with MTRIPs was compared with endosomal and lysosomal localization, showing less than 5% overlap, confirming the dynamics observed were not of vesicles.

6. Imaging RNA-Protein Colocalization Using MTRIPs

It is well established that transacting factors, such as miRNA and RNA binding proteins, govern mRNA function. Due to the resolution limitations of most optical microscopy techniques, imaging RNA-transacting factor colocalization and correlation functions, does not guarantee but does lend support for their interactions. Performing this type of assay though can be very challenging. Often this is performed by first fixing cells in paraformaldehyde, and then following with both *in situ* hybridization for RNA, and immunofluorescence to detect the protein of interest (Grunwald *et al*., 2008). This seems straightforward, but in practice, it is fraught with difficulty. *In situ* hybridization buffers often contain formamide, which is used to adjust the stability of duplexes formed between the probe and its target. Formamide though, has been shown to alter antibody binding, sometimes causing a loss of signal or nonspecific binding. *In situ* experiments combined with immunofluorescence should always be performed separately first, to ascertain whether the formamide is causing any problems (Grunwald *et al*., 2008). One way of circumventing this problem is through the use of live cell hybridization assays using MTRIPs, followed by paraformaldehyde fixation, permeabilization, blocking, and a slightly modified immunostaining protocol (Santangelo *et al*., 2009; Zurla *et al*., 2011). Because probe hybridization occurs while the cells are living, harsh chemicals, such as formamide are not utilized. The only difference between these assays and conventional immunostaining is that nuclease free buffers are used. To date, we have had no problems with RNA degradation using this approach.

Recently, using this methodology, interactions of native, nonengineered β-actin mRNA with SGs, PBs, and the RNA exosome during translation initiation inhibition, via sodium arsenite and Pateamine A exposure, were characterized while respecting the physiological stoichiometry of protein/mRNA interactions because both native RNAs and proteins were observed (Zurla *et al*., 2011).

6.1. Protocol for RNA-protein colocalization imaging using MTRIPs

1. Deliver MTRIPs against RNA of interest as per above (these assays are typically performed on 10–12 mm, #1.5 coverslips inserted in a 24-well pate).
2. Remove media (no need to wash cells) and fix cells 4% paraformaldehyde (Electron Microscopy Science) in nuclease free 1 × PBS. (Typically volumes of 250 μl are used.)
3. Permeabilize cells with 0.2% Triton X-100 (Sigma) in nuclease free PBS for 5 min.

4. Block cells with 5% nuclease-free bovine serum albumin (Ambion) for 30 min at 37 °C.
5. Incubate cells with primary antibodies for 30 min at 37 °C and then with secondary antibodies for 30 min at 37 °C.
6. Stain cells with DAPI (Invitrogen) and mount using Prolong (Invitrogen), or another mounting medium of choice.

For microtubule staining, cells were first washed with BRB80 buffer (80 m*M* Pipes, pH 6.8, 1 m*M* $MgCl_2$, 1 m*M* EGTA) prior to fixation, and subsequently fixed in 4% paraformaldehyde in BRB 80 buffer. For γ-tubulin staining, cells were fixed in 100% methanol for 10 min at −20 °C and permeabilized using 100% acetone for 2 min at −20 °C.

7. Conclusions

Even though single RNA sensitivity has been achieved with exogenous probes, and cytoplasmic delivery is successful in many cell types, additional capability will be required to image RNA accurately throughout their life cycle. Future areas of research that are necessary to improve the use of exogenous probes for RNA imaging include: improving delivery methods to both the cytosol and nucleus, understanding the role of nucleic acid chemistry in probe interactions with RNA, and the addition of electron microscopy (EM) compatibility. EM compatibility would be useful because it would allow the probes to provide information over a wider range of resolution than current imaging technology can provide. Regarding the effects of probe chemistry on RNA function, it will be necessary to identify the correct mixture of nucleic acid types that will provide the right balance of affinity and noninvasiveness to monitor mRNA production during:

1. transcription
2. egress from the nucleus
3. translation
4. degradation in both p-bodies and the RNA exosome

To date, some information regarding these issues exists, but more detailed studies are needed. The hope is that once these probes can be easily and repeatedly delivered into the cytosol and nucleus of a multitude of cell types and are nimble enough to follow the lives of most RNAs, they will become the standard for future RNA biology studies.

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