RNA voyeurism: A coming of age story

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Abstract

Studies of gene expression are typically carried out by a molecular analysis that averages entire populations of cells in culture, or in a tissue. This approach cannot detect cell to cell variability, or collect subcellular information, such as spatial distribution. At the transcriptional level, it is evident that even a robust transcriptional response in ensemble measurements is not uniform among all cells in a population. At the post-transcriptional level, mRNAs and proteins can be trafficked to specific subcellular compartments allowing spatiotemporal regulation of gene expression, but these critical spatial relationships are lost with common molecular biology approaches. Through direct visualization of mRNA during the biogenesis process and analyzing the distribution of single mRNA molecules in cells we have gained a deeper understanding of gene expression at many levels. Recent technical advances have made these types of analysis more accessible than ever. The utility of this approach toward studying transcriptional events is underscored throughout many of the articles within this volume. Techniques such as fluorescent in situ hybridization (FISH) are being applied to single molecule studies in fixed cells with far-reaching results, but they are limited in their ability to provide information about the dynamic nature of mRNA *in vivo*, so methodology to visualize single mRNA molecules in living cells has become desirable. In this article, we will discuss the state-of-the-art tagging systems used for real-time imaging of mRNAs that have been developed. We will present an overview of how these approaches have been applied to impacting our view of gene expression.

Making mRNA visible in living cells

The first and most widely used technique to image mRNA in live cells uses the MS2 coat protein (MCP) -system from the MS2 bacteriophage of *E. coli*. This system exploits the high affinity specific interaction between the MCP and the MCP binding stem loop (MBS) within this bacteriophage's RNA genome (Bernardi and Spahr 1972). When a cell co-expresses the MCP fused to a fluorescent protein (MCP-FP) and an mRNA containing the MBS stem loops, this mRNA can be visualized through fluorescence microscopy in living cells (Bertrand et al. 1998, Singer 2015). A mutant MCP that dimerizes, but cannot further oligomerize to form a capsid is an important feature to the success of this approach (Peabody and Ely 1992). In practice, several copies of the MBS are added onto a specific reporter mRNA to ensure a strong enough signal (Figure 1) (Chao JA; Czaplinski, K; Singer, RH 2008, Querido and Chartrand 2008). A nuclear localization sequence (NLS) can be added to the MCP to lower background fluorescence during detection of single mRNAs in real time in the cytoplasm, however for transcription site visualization an NLS is not always a requirement if the MCP isn't excluded from the nucleoplasm. Combining an NLS and nuclear export sequence (NES) has been used to ensure access of the MCP to the nucleus while decreasing the nuclear background (Schmidt et al. 2011). Multiple copies of the GFP inserted in tandem have also been applied to increase fluorescence yield from the system (Haim et al. 2007). Since this landmark achievement demonstrated the feasibility of real time single molecule mRNA imaging, mRNA tagging systems that exploit other specific RNA-protein interactions have been developed. The ability to

label an mRNA with different tags has presented some very exciting opportunities to study the steps of gene expression at the single molecule level, including transcription, which is the focus of this issue of Methods.

Based on the success of the MS2 system, a similar tag was developed from a distant relative of the MS2 bacteriophage. Bacteriophage PP7 coat protein (PCP) has an overall similar structure to that of MCP, however this bacteriophage of *Pseudomonas aeruginosa* has diverged significantly from MS2, and the result is recognition of a distinct RNA binding sequence within the PP7 genome, the PCP binding stem loop (PBS)(Chao J. A. et al. 2008, Lim et al. 2001) In practice all the same considerations for live cell imaging apply to the PCP-PBS system as for the MCP-MBS system. A direct quantitative comparison of these two systems for *in vivo* mRNA labeling presented evidence that PCP was capable of occupying all of the 24 binding sites inserted into the 3' UTR of a co-transfected reporter in these tests, however the MCP did not occupy all 24 binding sites in an equivalent reporter (Wu et al. 2012). This study also revealed that the requisite dimerization of MCP for MBS binding lowered the *in vivo* labeling efficiency of mRNA quite significantly in the cytoplasm of cells. Fortunately, the amino-terminus of one MCP subunit is nearby the carboxy-terminus of the other MCP when the protein dimerizes (Chao J. A. et al. 2008, Ni et al. 1995). This allowed the construction of a tandem-dimerized MCP-FP (tdMCP-FP), or PCP-FP (tdPCP-FP) that overcomes this limitation and makes the labeling of individual mRNA molecules much more uniform (Figure 1). A recent improvement to the tandem MBS cassette should also be able to address the output from the tdMCP constructs (Wu et al. 2015b). While the

tdPCP-FP iteration of this system should provide future researchers with the best signal to noise ratio for mRNA detection, the improved tdMCP-FP should allow existing MBS based reporter systems be more robust.

Other RNA binding protein tags have been employed for *in vivo* studies as well. The specific interaction between an arginine-rich peptide within the bacteriophage λ N protein (BoxB binding peptide, BBP) and the BoxB stem loop from the N-utilization site of λ -phage genome has been used for tagging mRNAs *in vivo* (Lazinski et al. 1989). This tagging system was already being successfully used to target mRNAs specifically in reporter assays *in vivo* as well as for biochemistry *in vitro* (Baron-Benhamou et al. 2004, Czaplinski et al. 2005). For imaging of an mRNA, the BoxB sequence can be inserted in tandem into an RNA sequence analogous to the MBS and the BBP fused to one or more copies of GFP are co-expressed to visualize mRNA of interest (Daigle and Ellenberg 2007). In this setup, only 4 BoxB sequences were sufficient to observe Box-B reporter mRNA-dependent fluorescence in the cytoplasm of live cells, although the fluorescence observed did not show the same type of diffraction limited punctate appearance that MBS or PBS reporter mRNAs do (Daigle and Ellenberg 2007). Nonetheless, 4 BoxB sites add only 80 nt to a reporter, as opposed to the 1300 nt that 24 copies of an MBS or PBS requires. For imaging RNA substrates where adding a minimal size tag is a requirement, this could be an advantage. In studies using the corn fungus *Ustilago maydis* the authors applied 16 BoxB sites and the BBP fused to a triple-tandem-GFP to achieve particle labeling, and in studies of mRNA splicing in human cell lines, 25 Box-B sites were added to the intron of a β -globin (Baumann et al. 2014, Konig et al. 2009,

Martin et al. 2013). It is worth to note that one study indicated that for reliable single molecule detection in yeast, the BoxB system wasn't as effective as MS2 or PP7 labeling systems, however no description of the BoxB tagging arrangement they tried was described and no representative data was shown (Hocine et al. 2013). As with most single molecule detection systems, the strength of the particle signal to background ratio will be the most critical factor in being able to detect a single particle. It is likely that many factors, such as different mRNA stoichiometries within a particle or different organisms may play a role in how effective any particular labeling system is.

This general strategy for labeling mRNAs for live imaging has been successful with other combinations of RBP and cognate binding sites as well. An *E. coli* anti-terminator RNA binding protein called BglG binds to a stem loop within the bgl operon. 18 BglG binding stem loops were introduced into modified HIV1 genome RNA, and co-expressed with a different HIV1 genome modified to contain 24 MBS sites. These genomic RNAs were co-expressed in cells that expressed BglG-mCherryFP and MCP-YFP, and the viral particles that resulted were scored for genomic RNA dimerization state by the coincidence of mCherry and YFP labeling. Intracellular trafficking of single HIV1 genomic RNAs with a BglG tag wasn't reported, but may be feasible based on the success of the MS2 system. A non-bacteriophage stem-loop and cognate RNA binding protein has been used to tag mRNA in yeast. The U1A protein is a component of the U1 snRNP particle – a complex important for pre-mRNA splicing – and the amino terminus contains an RNA binding domain that specifically binds a stem loop within U1 snRNA (Hall and Stump 1992). U1A-fluorescent protein fusion (U1A-FP) can be used to

visualize an mRNA reporter containing multiple U1A stem loops (Takizawa and Vale 2000). This method would be riskier in mammalian cells as the system is derived from the human U1A protein, so this labeling system may interfere with U1 snRNA functions within the cell and endogenous U1A may interfere with labeling, although this has never been reported.

Having many possibilities to label mRNAs is more than just an opportunity to provide researchers freedom of choice in finding a favorite labeling system, but it has allowed the opportunity to label multiple mRNAs at any given time, or perhaps more interestingly to label an mRNA in different places (Lange et al. 2008). The combination of MS2 and PP7 has also been used to create a chimeric probe, where one half of a VenusFP is added to MCP and the other half is added to PCP (Wu et al. 2014). In this arrangement, there is no background fluorescence due to unbound probe, but when the target RNA contains multiple MBS-PBS pairs in tandem the juxtaposition of the two halves of VenusFP allows the protein to come together and fluoresce (Figure 1). The result is that only (Cheong and Hall 2006, Lu et al. 2009) when the target mRNA is occupied can you see fluorescence. This provides improved signal to background ratio, particularly for mRNA particles close to the nucleus where the unbound MCP-FP would accumulate and potentially interfere with imaging in this area of the cytoplasm. This approach, called bi-molecular fluorescence complementation (BiFC), strongly depends on rate that the two halves of the FP to come together and fold properly, which can be slow and have an unpredictable impact the results obtained (Miller et al. 2015).

A system for labeling unmodified mRNAs based on RBPs engineered to recognize specific sequences also employed BiFC. The RNA binding motif, called the Pumilio Homology Domain (Pum-HD) binds to a single-stranded sequence of specific bases, and this specificity is understood at the atomic level such that Pum-HDs can be engineered to recognize almost any sequence (Cheong and Hall 2006, Lu et al. 2009). This was exploited to recognize an endogenous RNA by creating one PumHD to recognize a sequence in the 3' UTR of the mt NADH dehydrogenase mRNA, and a second PumHD to recognize a sequence adjacent to the first. These were applied to visualize mRNA in the mitochondria by expressing them fused to complementary halves of GFP (Ozawa et al. 2007). A later study applied this methodology by fusing 2 separate PumHDs to the same GFP protein, one the N-terminus and other the C-terminus of GFP. This tri-partite GFP fusion protein contained two separate PumHD fusions targeting adjacent sequences within the 3' UTR of β -actin mRNA. This protein was designed to label endogenous β -actin mRNA through PumHD fusions targeting this mRNA's 3'UTR. There is a big difference in the number of fluorophores per mRNA for this system as compared to the MS2 system, which will likely limit the visibility of single mRNAs, but time-lapse imaging using TIRF microscopy reported observing individual GFP particles at 4 hours post-transfection (Yamada et al. 2011).

Some important considerations for the imaging of single mRNAs

There are several technical considerations to make before deciding which system to use when imaging mRNA in live cells, depending on what you want to study. Single mRNA is diffraction limited, therefore images must be acquired two times as fast as

objects can move to neighboring pixels to reliably track particles over time (Rodriguez et al. 2007). In other words if your exposure time is too long, you won't be able to image particles that move while the camera's shutter is open because they will be blurred out over many pixels within the image. This means long exposure times will bias images toward large and/or slow moving objects, and this is the reason that multiplying the fluorescent probes bound to the mRNA is a must to ensure that it is bright enough to be able to film it at frame rates that are fast enough to image all moving particles. Wide-field microscopes offer the maximum collection of light, and are often used with high speed electron multiplying (EM) CCD cameras with large pixel size to gain sufficient tracking resolution (Rodriguez et al. 2007). More advanced modes of microscopy, such as confocal or super-resolution, may be limited in their capacity to detect single mRNAs *in vivo* since they improve image quality at the cost of speed or brightness, and these tradeoffs are typically enough to lose the ability to see single mRNAs moving around the cell. But with continual improvements in microscopy at the level of speed and sensitivity, these approaches may be applicable to mRNA imaging in the future, and state of the art spinning disk confocal microscopes have been used to image mRNAs (Halstead et al. 2015).

In contrast to these constraints on imaging mRNA particles trafficking around the cell, mRNA at the site of transcription is locally restricted at the seconds to minutes time scale, and when transcription occurs, typically multiple nascent mRNAs are localized to the site of transcription. These two features have made transcription sites a robust place to visualize mRNA in living cells because the inherently bright single

mRNAs are present for an extended time in multiple copies in a very confined area (Figure 2). This makes them easier to detect in time lapse images because longer exposure times can be applied. Tracking of transcription sites even within motile cells such as *Dictyostelium* is possible when MS2 or PBS stem-loops are inserted, and putting them in the 5'UTR labels transcription sites most effectively (Figure 2) (Chubb et al. 2006). Real-time transcription dynamics of an endogenous yeast gene were recorded with 24xPBS stem loop repeats on the 5'-UTR of endogenous target genes (Larson et al. 2011). This allowed observation of the kinetics of RNA polymerase II (pol II) by imaging an increase in fluorescence when the nascent mRNA transcript begins to be transcribed and the rapid loss in fluorescence when the pol II finishes transcription and the transcript rapidly diffuses away from the transcription site. The addition of PBS sites to either the 5'-UTR or 3'-UTR of a large gene allowed the authors to track transcription initiation or termination kinetics in transcripts by quantifying the fluorescence profile at the site of transcription over time.

Imaging mRNA at the transcription site provides access to many steps of gene expression

There have been many advances in understanding the trafficking of mRNA just being able to observe it directly, but having more labeling tags has provided many more opportunities for quantifying steps of gene expression through two-color mRNA labeling. Distinct tagging of each allele of a gene in a diploid yeast cell, one allele with MBS the other with PBS, was used to directly observe the kinetics of pol II for a single gene *in vivo* (Hocine et al. 2013). With this it was demonstrated that the expression of

MDN1 alleles fluctuates independently over time (Hocine et al. 2013). In the same study, PBS stem loops were inserted into the 5'-UTR of *MDN1* and MBS stem loops were inserted into the 3'-UTR so that the time between the appearance of the 5' signal (PCP-FP) and the 3' signal (MCP-FP) provided a direct *in vivo* measurement of pol II elongation rates.

This direct observation of gene expression in model organisms is proving very powerful to demonstrate cellular properties that could previously only be inferred or hypothesized. In today's research climate, there is great interest to study transcription from individual genes in mammalian cells. This goal is complicated by the challenges of manipulating the genome in mammalian cells. For this purpose, retroviral vectors have proven effective for insertion of single copy transcription units of MBS reporter mRNA into a mammalian genome. A reporter system was developed that allowed visualization of the nascent pre-mRNA in a human cell line at a hormone activated locus (Larson et al. 2013). A parental cell line that stably expresses a chimeric ecdysone receptor along with the MCP-FP was first created. These cells could be induced to activate transcription from a promoter with an ecdysone response element (ERE) by application of the steroid ponasterone A (PA). A lentiviral reporter transcription unit with an ERE containing promoter driving an mRNA that contained MBS stem loops was transduced into these cells. When PA was added, the reporter's transcription site was visible following induction and could be quantified in their response to the hormone.

In addition to quantifying the appearance of RNA at the genetic locus as a readout of transcription, two-color labeling of an mRNA was used to determine the

temporal relationship to transcription and splicing (Coulon et al. 2014, Martin et al. 2013). In one study MBS stem loops were inserted into the upstream intron and BoxB stem loops were inserted into the downstream intron of a two-intron reporter construct. Fluorescence at the transcription site for both the MCP-FP and BBP-FP could be recorded over time, and the difference in the timing of appearance and disappearance were used to gather information on transcription rates. This study also showed that a change in the timing of fluorescence disappearance from the transcription site was sensitive to the strength of the RNA splicing signals, suggesting that splicing was occurring co-transcriptionally. In another study, PBS stem-loops were inserted into an intron of a reporter mRNA, and MBS stem loops were inserted into the 3'-UTR. When both PCP-mCherry and MCP-GFP were expressed in the cell background, labeling of different parts of the primary transcript was observed with different colors (Figure 2). Because the PBS was located within an intron, unspliced mRNAs were visible in both colors whereas spliced mRNAs were only visible with MCP-GFP labeling. This system allows real-time imaging of intron removal and showed that splicing is stochastic, occurring either before or after release from the transcription site, indicating no interdependence on either of these processes on this reporter. Moreover, the majority of transcripts were spliced after release from transcription sites, and splicing on freely diffusing transcripts is 10-fold faster than on chromatin-bound transcript, suggesting splicing post-release is more energetically favorable. These results differ from the Martin et al, and from another using a different

reporter mRNA with MBS labelled introns that indicated splicing was most frequently co-transcriptional (Schmidt et al. 2011).

Obtaining a robust system for mRNA detection in many different organisms

With these approaches you will clearly make an excess of the MCP-FP over the reporter mRNA. Therefore our use of the word robust means being extremely confident that the fluorescent particles you are quantifying are caused by binding to the tagged RNA (you must beware of the danger that fluorescence does not always equal mRNA in cells with MCP-FP). There are many ways to introduce a tagged reporter mRNA and the cognate protein-FP probe into cells. Transient co-transfection of these from a plasmid in cultured mammalian cells is just about the easiest, and it can work but is very tricky since appropriate levels of both MBS- probe RNA and MCP-FP are needed to get good quantifiable images (discussed in (Chao JA; Czaplinski, K; Singer, RH 2008)). Not surprisingly, genetically tractable systems like yeast have been most successful in allowing tagging of substrate mRNAs at physiological levels since the MBS probes can be added at single copy within the genome, even within the physiological target mRNA (Beach et al. 1999, Bertrand et al. 1998, Haim et al. 2007, Zipor et al. 2009). This live imaging system was used determine how ASH1 mRNA localizes to the bud tip to limit mating type switching to the daughter cell in yeast (Beach et al. 1999, Bertrand et al. 1998). Tagging of mRNAs that encode either peroxisome proteins or mitochondrial proteins with MBS sites has demonstrated that some of the mRNAs can be found directly associated to their corresponding organelle (Figure 2). This suggests that translation of organelle proteins directly at the organelle

may be an unrecognized aspect of organelle biogenesis and function (Gadir et al. 2011, Haim et al. 2007, Zipor et al. 2009).

The MBS system was also applied to image RNA in live *Drosophila* oocytes and embryos (Forrest and Gavis 2003). Many mRNAs that determine proper anterior-posterior body axis patterning mRNAs are synthesized by the ovarian nurse cells and are transported to the oocytes by mid-oogenesis, such as *nanos* (*nos*), *oskar* (*osk*), *gurken*, and *bicoid* (Becalska and Gavis 2009, Forrest and Gavis 2003, Weil et al. 2006, Zimyanin et al. 2008). These mRNAs are then localized within the oocyte asymmetrically. Live imaging of these mRNAs has been used to understand the function of the proteins required for localizing these mRNAs. This method provided direct evidence for two distinct steps to mRNA localization in these cells; trafficking, which can utilize microtubule motors, and anchoring, the latter requiring the actin cytoskeleton in *Drosophila*. By being able to image living cells over time it was possible to show that active trafficking of *nos* mRNA accounts for its posterior pole localization, rather than the local prevention of RNA degradation at the posterior pole as previously thought.

Although *Xenopus laevis* isn't a popular genetic model, the oocytes are a very powerful vehicle for studies of the *in vivo* processing and trafficking of microinjected *in vitro* synthesized RNAs. Injection of MBS labelled vegetal localizing mRNA directed a co-expressed MCP-mCherry protein to label the vegetal pole (Gagnon et al. 2013). By using a photo-activatable MCP-mCherryFP, the authors demonstrated that mRNA particles show different patterns of transport when the mRNPs are photo-activated within different regions of the cytoplasm. Given the constraints of microscopy in large

biological samples such as oocytes, the mRNA particles observed in these studies may not represent single RNA molecules. However, oligomerization of the trafficking RNAs in these systems likely provides enough signal to background to enable their detection. *Zebrafish* expressing MCP-FP have recently been created and embryos of these have been used to visualize mRNA produced from plasmid DNA injected into them (Campbell et al. 2015). This injected DNA was constructed to integrate into the embryonic genome and was used to study the zygotic genome activation through visualizing transcription sites. These embryos were also used to study the cytoplasmic localization of nanos reporter mRNAs in primordial germ cells of fish expressing this transgene.

MBS tagging has also been used to visualize mRNA in *E. coli* to study transcription response at an inducible promoter. This system employs even more copies of the MBS sites than in mammalian cells, either 48 or 96 (Golding and Cox 2004, Hakkinen and Ribeiro 2015). The increased MBS site content, combined with a regulatable GFP cassette that limits GFP levels from getting too high, ensures the strong fluorescence of the RNA over the background. Subcellular localization of bacterial mRNAs was also studied using only 6 copies of the MBS site, demonstrating that many *E. coli* RNAs contain sequence elements that localize these mRNAs to sites of their cellular function independently of translation (Nevo-Dinur et al. 2011).

Genomic integration of MBS site reporter mRNAs into mammalian cell lines through transfection and drug selection is possible (Janicki et al. 2004, Shav-Tal et al. 2004), however it is very labor intensive to screen for single clones that have the desired

properties and genomic insertion is not site-specific. There are cell lines that have been developed that streamline this process and lead to single copy insertion at a defined genomic locus much more rapidly (Yunger et al. 2013). Retroviral derived vectors are ideal for inserting a reporter in single copy into the mammalian genome that can be effectively applied to even difficult to transfect and non-dividing primary cells, but the repetitive MBS or PBS sites cause deletions during retroviral particle formation and integration such that this approach could not be used produce successful integration of reporters. A recent modification of the 24MBS cassette has created a cassette with 24 different stem loop sequences as well as 24 different linker regions that circumvents this deletion problem so this approach should become much more tractable in the near future (Wu et al. 2015b).

Site specific knock-in of MBS sites into the 3' UTR of the β -actin mRNA locus (Actb-MBS) within mice has been achieved through well established genome targeting approaches in mouse ES cells (Lionnet et al. 2011). The creation of Actb-MBS transgenic mice from these cells has facilitated visualization of many *in vivo* gene expression stages all from an endogenous genetic locus in a mammalian cell. The serum-responsive transcription of the β -actin gene was measured in real-time in living cells using this allele (Kalo et al. 2015, Lionnet et al. 2011). Creating the complementary mouse line with the MCP-GFP has made the analysis possible in primary cells and tissues directly, without introducing other exogenous probes, when these two lines were crossed (Park et al. 2014). β -actin mRNA leading edge localization in primary fibroblasts and

depolarization-dependent transcription of the β -actin allele was directly observable in brain slice cultures derived from these mice.

Capturing the spatio-temporal movements of mRNA within the cell in real time

Being able to observe the mRNA directly has allowed a direct description of how mRNA traffics around the cell (Figure 2). Initially single reporter mRNAs containing the coding sequence of LacZ, MBS stem loops, and the 3'-UTR of β -actin were tracked in the cytoplasm of mammalian cells after transient transfection and binned into movement profiles based on mean squared displacement (MSD) over short and long time frames (Fusco et al. 2003). The MSD measures the average distance displaced over time and four categories of mRNA movement were described: directed, static, corralled, and diffusive. This approach to quantify mRNA movements allowed study of determinants of the dynamics of mRNAs in the cytoplasm, and found that the 3' UTR of the chicken β -actin mRNA produced after transient transfection increased the number of LacZ reporter mRNA particles that showed directed movement in the cytoplasm. When the endogenous Actb-MBS mRNA was analyzed using these parameters, the majority of mRNAs in primary fibroblasts were diffusive (> 40%), while < 40% were corralled, < 20% were stationary, and < 1% exhibited directed movement (Park et al. 2014). In contrast the movement of Actb-MBS mRNAs was recorded in primary neurons and these demonstrated surprisingly different trafficking profiles from fibroblasts. In contrast to mRNA movements in fibroblasts, the majority of mRNA particles in primary neurons were stationary (> 40%) (Lionnet et al. 2011, Park et al. 2014). While the percentage of corralled particles was similar in both neurons and fibroblasts, there were

many fewer diffusive particles and many more directed particles in neurons, with these directed particles showing very long persistent directed runs that were not observed in fibroblasts. Directed particles were observed in both the anterograde and retrograde direction, with an average speed of 1.3 $\mu\text{m/s}$, falling in the range characterized when β -actin mRNA was first observed in cultured neurons *in vivo* (Lionnet et al. 2011, Park et al. 2014). Individual directed runs of mRNA in cultured neurons have been observed with transfected MBS reporter mRNAs, but the systematic comprehensive trafficking profile analysis of endogenous β -actin mRNAs was made possible through the genetic labeling process (Dynes and Steward 2007, Rook et al. 2000). mRNA trafficking through the nucleoplasm was also possible by following the release of inducible MBS reporter mRNAs from a genomically integrated reporter (Shav-Tal et al. 2004).

Co-localization with mRNAs; guilt by association

These advances in understanding the trafficking of mRNA come from just being able to observe its' behavior directly, but many more interesting opportunities are awaiting study by using live cell co-localization of heterologous mRNAs or of mRNAs with specific parts of the cell. In yeast, labeling different bud tip localized RNAs with separate tags and different colored-FPs has demonstrated multiple mRNA travelling toward the bud tip within the same particle (Lange et al. 2008). In mammalian cells, when nuclear pore complexes were fluorescently labeled with POM121-FP, single nuclear pores could be resolved and high-speed movies of NPCs and Actb-MBS mRNAs were taken simultaneously to track mRNA export events through an individual pore (Figure 2). Tracking the endogenous Actb-MBS mRNA in relationship

to this spatial marker allowed the authors to make several novel observations about nuclear mRNA export (Grunwald and Singer 2010). A role for the targeting of β -actin mRNA translation directly to focal adhesions has also been suggested by imaging Actb-MBS mRNA and focal adhesion proteins simultaneously in a living cell (Figure 2)(Katz et al. 2012). Labeling an mRNA encoding a fluorescent peripherin intermediate filament protein subunit with MBS sites has allowed Chang et al. to visualize movement of these mRNAs in relationship to particles of fluorescent peripherin. These experiments allowed them to propose a co-translational assembly model for incorporation of newly synthesized peripherin subunits into existing particles (Chang et al. 2006). Association of mRNA with P-bodies through live-imaging was first reported using the MS2 system in yeast (Sheth and Parker 2003), and recently Aizer et al studied the association of mRNA with cytoplasmic Processing bodies (P-bodies) during cellular stress in live mammalian cells (Figure 2) (Aizer et al. 2014).

Recently, two color mRNA labeling has facilitated direct observation of the onset of mRNA translation (Figure 2). When PBS stem-loops are located in the coding region and the MBS stem loops are located in the 3' UTR, PCP-GFP and MCP-RFP are co localized to an mRNA. Upon translation MCP-RFP persists in labeling the mRNA but the translocation of ribosomes through the coding region removes the PCP-GFP leaving the mRNA marked only in one color (Halstead et al. 2015). With further advances in live cell fluorescence analysis it is becoming possible to even study the binding of individual proteins to single mRNA molecules in living cells. Wu et al. used fluorescence fluctuation spectroscopy (FFS) in sub-femtoliter volumes of the cytoplasm

of living cells to quantify *in vivo* RNA-protein interactions and mRNA translation state (Figure 2) (Wu et al. 2015a). Upon FFS imaging of Actb-MBS simultaneously with an Actb mRNA binding translational repressor, ZBP1, the authors' data suggested that approximately half of all β -actin mRNAs are bound by ZBP1 in the perinuclear region, although there is less bound in the periphery of fibroblasts. When FFS imaging of Actb mRNA was performed along with a mutant ZBP1 that improves translational repression, increased ZBP1 binding to Actb mRNA in the periphery of the cell was observed. This data supports the proposed role for this protein in translational repression of the β -actin mRNA. In parallel the authors studied ribosome loading of the Actb-MBS mRNA using FFS against mCherryRPL10A (a protein in the large ribosomal subunit) and found that fewer ribosomes were bound to Actb-MBS mRNA in the perinuclear region, suggesting that β -actin mRNA is not as highly translated in this cytoplasmic region. This distribution was also dependent on ZBP1, as ZBP1^{-/-} cells exhibited increased ribosome association near the nucleus (comparable to that of the leading edge).

Image your mRNAs with caution

There are some caveats inherent in the application of the MS2 system. In yeast cells expressing target mRNA with 24xMBS stem loops, an accumulation of truncated tagged mRNAs were found, although the extent of this varied widely among different transcripts tested (Garcia and Parker 2015). This population of mRNAs can be detected on a northern blot by probes hybridizing 3' but not 5' of the MBS stem loops, and their formation is dependent on MCP-FP binding and on the 5'-3' exonuclease Xrn1.

Therefore MCP binding to MBS sites appear to impede progress of the Xrn1 5'-3' exonuclease in yeast. This leaves mRNA degradation intermediates that may be visible with the MCP-FP and localize to P-bodies, where the Xrn1 nuclease accumulates, although this wasn't been formally demonstrated. Addition of strong stem loops to trap decay intermediates was long known in yeast, although this approach hasn't ever been demonstrated to be effective in mammalian cells so tandem MBS sites are unlikely to have a similar effect there (Muhlrads et al. 1994). The studies that have observed interaction of mRNA with mRNA nuclease rich P-bodies allows us to raise a limitation for using live-cell mRNA imaging, the study of mRNA turnover. While mRNA is stable and detectable it provides a fluorescent signal that can be tracked. But the loss of fluorescence during microscopy can be the result of processes other than mRNA turnover. To make such studies convincing it would be a necessary, and difficult, task to show that loss of a fluorescent particle was the result of degradation and not some indirect effect that might dislodge the protein probes, or cause the mRNA to go out of focus. We look forward to someone taking up this challenge in the future.

mRNA tagging is the only means to watch an *in vivo* transcribed mRNA in real time, and these approaches have also been applied to visualization of retroviral RNA particles in living cells (Bann and Parent 2012). Nonetheless, the addition of the extra mRNA sequence to tag an mRNA combined with the added bulk of MCP-FP to an mRNA will always maintain the potential to affect mRNA behavior in unpredictable ways. Importantly, several experiments have addressed this possibility within the studies cited here that suggest this has negligible effects on the properties of an mRNP.

Moreover, many studies using MBS and the BoxB fusion proteins to specifically tether mRNA processing factors to reporter mRNAs have been published and these demonstrate that by and large these proteins do not impart side effects on synthesis or processing of reporter mRNAs (Clement and Lykke-Andersen 2008, Gehring et al. 2008, Keryer-Bibens et al. 2008). Most importantly, there is currently no robust means to fluorescently label an endogenous mRNA, so the MS2, PP7 and Box-B systems remain the methods with the best track record of success for single molecule *in vivo* mRNA imaging in real time. Molecular beacon technology remains a promising approach to label unmodified endogenous mRNAs, although they haven't been adopted on a wide scale for that purpose. It is also important to be aware that there are also other approaches for *in vivo* detection of endogenous unmodified mRNA that may eventually develop into robust single mRNA sensitive probes (Bratu et al. 2011, Catrina et al. 2012, Santangelo et al. 2009, Sato et al. 2015, Yamada et al. 2011, Yoshimura et al. 2012). Lacking a method for detecting endogenous mRNAs, further progress on reducing the footprint of the tagging complexes may come from making the RNA tag itself fluorescent, and advances on this goal have been recently reported (Dolgosheina et al. 2014, You and Jaffrey 2015).

Final thoughts for the future of real-time imaging of mRNA

The advances that have been made in single molecule mRNA imaging have been facilitated by the tagging of the endogenous β -actin mRNA as well as through extensive use of reporter approaches that exploit cis-acting regulatory elements. There remain many questions about gene expression that the existing tools will assist in answering,

but there are questions regarding the regulation of other endogenous genes these tools will not be able to directly address. Making a transgenic mouse similar to the Actb-MBS for each of these will likely be impractical for the majority of these genes. But the enormous impact that CRISPR/CAS gene targeting is having on the manipulation of the mammalian genome should make it easier and faster to develop human cells that have endogenous genes targeted with PBS or MBS sites so the next few years may bring even more advances.

Acknowledgements

K. C. is supported by a National Science Foundation CAREER award (IOS-1254146) and would like to thank Robert H. Singer and the too-many-to list colleagues from the Singer lab for the opportunity to be a part of the greater *in vivo* mRNA imaging community.

References

- Aizer A, Kalo A, Kafri P, Shraga A, Ben-Yishay R, Jacob A, Kinor N, Shav-Tal Y. 2014. Quantifying mRNA targeting to P-bodies in living human cells reveals their dual role in mRNA decay and storage. *J Cell Sci* 127:4443-4456.
- Bann DV, Parent LJ. 2012. Application of live-cell RNA imaging techniques to the study of retroviral RNA trafficking. *Viruses* 4:963-979.
- Baron-Benhamou J, Gehring NH, Kulozik AE, Hentze MW. 2004. Using the lambdaN peptide to tether proteins to RNAs. *Methods Mol Biol* 257:135-154.
- Baumann S, Konig J, Koepke J, Feldbrugge M. 2014. Endosomal transport of septin mRNA and protein indicates local translation on endosomes and is required for correct septin filamentation. *EMBO Rep* 15:94-102.
- Beach DL, Salmon ED, Bloom K. 1999. Localization and anchoring of mRNA in budding yeast. *Curr Biol* 9:569-578.
- Becalska AN, Gavis ER. 2009. Lighting up mRNA localization in *Drosophila* oogenesis. *Development* 136:2493-2503.
- Bernardi A, Spahr PF. 1972. Nucleotide sequence at the binding site for coat protein on RNA of bacteriophage R17. *Proc Natl Acad Sci U S A* 69:3033-3037.
- Bertrand E, Chartrand P, Schaefer M, Shenoy SM, Singer RH, Long RM. 1998. Localization of ASH1 mRNA particles in living yeast. *Mol Cell* 2:437-445.
- Bratu DP, Catrina IE, Marras SA. 2011. Tiny molecular beacons for in vivo mRNA detection. *Methods Mol Biol* 714:141-157.
- Campbell PD, Chao JA, Singer RH, Marlow FL. 2015. Dynamic visualization of transcription and RNA subcellular localization in zebrafish. *Development* 142:1368-1374.
- Catrina IE, Marras SA, Bratu DP. 2012. Tiny molecular beacons: LNA/2'-O-methyl RNA chimeric probes for imaging dynamic mRNA processes in living cells. *ACS Chem Biol* 7:1586-1595.
- Chang L, Shav-Tal Y, Trcek T, Singer RH, Goldman RD. 2006. Assembling an intermediate filament network by dynamic cotranslation. *J Cell Biol* 172:747-758.
- Chao JA, Patskovsky Y, Almo SC, Singer RH. 2008. Structural basis for the coevolution of a viral RNA-protein complex. *Nat Struct Mol Biol* 15:103-105.
- Chao JC, K; Singer, RH. 2008. Using the Bacteriophage MS2 Coat Protein-RNA Binding Interaction to Visualise RNA in Living Cells in LW M, ed. *Probes and Tags to Study Biomolecular Function*. Weinheim: Wiley-VCH.
- Cheong CG, Hall TM. 2006. Engineering RNA sequence specificity of Pumilio repeats. *Proc Natl Acad Sci U S A* 103:13635-13639.
- Chubb JR, Trcek T, Shenoy SM, Singer RH. 2006. Transcriptional pulsing of a developmental gene. *Curr Biol* 16:1018-1025.
- Clement SL, Lykke-Andersen J. 2008. A tethering approach to study proteins that activate mRNA turnover in human cells. *Methods Mol Biol* 419:121-133.
- Coulon A, Ferguson ML, de Turre V, Palangat M, Chow CC, Larson DR. 2014. Kinetic competition during the transcription cycle results in stochastic RNA processing. *Elife* 3.
- Czaplinski K, Kocher T, Schelder M, Segref A, Wilm M, Mattaj IW. 2005. Identification of 40LoVe, a *Xenopus* hnRNP D Family Protein Involved in Localizing a TGF-beta-Related mRNA during Oogenesis. *Dev Cell* 8:505-515.
- Daigle N, Ellenberg J. 2007. LambdaN-GFP: an RNA reporter system for live-cell imaging. *Nat Methods* 4:633-636.

Dolgosheina EV, Jeng SC, Panchapakesan SS, Cojocaru R, Chen PS, Wilson PD, Hawkins N, Wiggins PA, Unrau PJ. 2014. RNA mango aptamer-fluorophore: a bright, high-affinity complex for RNA labeling and tracking. *ACS Chem Biol* 9:2412-2420.

Dynes JL, Steward O. 2007. Dynamics of bidirectional transport of Arc mRNA in neuronal dendrites. *J Comp Neurol* 500:433-447.

Forrest KM, Gavis ER. 2003. Live imaging of endogenous RNA reveals a diffusion and entrapment mechanism for nanos mRNA localization in *Drosophila*. *Curr Biol* 13:1159-1168.

Fusco D, Accornero N, Lavoie B, Shenoy SM, Blanchard JM, Singer RH, Bertrand E. 2003. Single mRNA molecules demonstrate probabilistic movement in living mammalian cells. *Curr Biol* 13:161-167.

Gadir N, Haim-Vilmovsky L, Kraut-Cohen J, Gerst JE. 2011. Localization of mRNAs coding for mitochondrial proteins in the yeast *Saccharomyces cerevisiae*. *Rna* 17:1551-1565.

Gagnon JA, Kreiling JA, Powrie EA, Wood TR, Mowry KL. 2013. Directional transport is mediated by a Dynein-dependent step in an RNA localization pathway. *PLoS Biol* 11:e1001551.

Garcia JF, Parker R. 2015. MS2 coat proteins bound to yeast mRNAs block 5' to 3' degradation and trap mRNA decay products: implications for the localization of mRNAs by MS2-MCP system. *Rna* 21:1393-1395.

Gehring NH, Hentze MW, Kulozik AE. 2008. Tethering assays to investigate nonsense-mediated mRNA decay activating proteins. *Methods Enzymol* 448:467-482.

Golding I, Cox EC. 2004. RNA dynamics in live *Escherichia coli* cells. *Proc Natl Acad Sci U S A* 101:11310-11315.

Grunwald D, Singer RH. 2010. In vivo imaging of labelled endogenous beta-actin mRNA during nucleocytoplasmic transport. *Nature* 467:604-607.

Haim L, Zipor G, Aronov S, Gerst JE. 2007. A genomic integration method to visualize localization of endogenous mRNAs in living yeast. *Nat Methods* 4:409-412.

Hakkinen A, Ribeiro AS. 2015. Estimation of GFP-tagged RNA numbers from temporal fluorescence intensity data. *Bioinformatics* 31:69-75.

Hall KB, Stump WT. 1992. Interaction of N-terminal domain of U1A protein with an RNA stem/loop. *Nucleic Acids Res* 20:4283-4290.

Halstead JM, Lionnet T, Wilbertz JH, Wippich F, Ephrussi A, Singer RH, Chao JA. 2015. Translation. An RNA biosensor for imaging the first round of translation from single cells to living animals. *Science* 347:1367-1671.

Hocine S, Raymond P, Zenklusen D, Chao JA, Singer RH. 2013. Single-molecule analysis of gene expression using two-color RNA labeling in live yeast. *Nat Methods* 10:119-121.

Janicki SM, et al. 2004. From silencing to gene expression: real-time analysis in single cells. *Cell* 116:683-698.

Kalo A, Kanter I, Shraga A, Sheinberger J, Tzemach H, Kinor N, Singer RH, Lionnet T, Shav-Tal Y. 2015. Cellular Levels of Signaling Factors Are Sensed by beta-actin Alleles to Modulate Transcriptional Pulse Intensity. *Cell Rep* 11:419-432.

Katz ZB, Wells AL, Park HY, Wu B, Shenoy SM, Singer RH. 2012. beta-Actin mRNA compartmentalization enhances focal adhesion stability and directs cell migration. *Genes Dev* 26:1885-1890.

Keryer-Bibens C, Barreau C, Osborne HB. 2008. Tethering of proteins to RNAs by bacteriophage proteins. *Biol Cell* 100:125-138.

Konig J, Baumann S, Koepke J, Pohlmann T, Zarnack K, Feldbrugge M. 2009. The fungal RNA-binding protein Rrm4 mediates long-distance transport of *ubi1* and *rho3* mRNAs. *Embo J* 28:1855-1866.

Lange S, Katayama Y, Schmid M, Burkacký O, Brauchle C, Lamb DC, Jansen RP. 2008. Simultaneous transport of different localized mRNA species revealed by live-cell imaging. *Traffic* 9:1256-1267.

Larson DR, Fritzsche C, Sun L, Meng X, Lawrence DS, Singer RH. 2013. Direct observation of frequency modulated transcription in single cells using light activation. *Elife* 2:e00750.

Larson DR, Zenklusen D, Wu B, Chao JA, Singer RH. 2011. Real-time observation of transcription initiation and elongation on an endogenous yeast gene. *Science* 332:475-478.

Lazinski D, Grzadzińska E, Das A. 1989. Sequence-specific recognition of RNA hairpins by bacteriophage antiterminators requires a conserved arginine-rich motif. *Cell* 59:207-218.

Lim F, Downey TP, Peabody DS. 2001. Translational repression and specific RNA binding by the coat protein of the *Pseudomonas* phage PP7. *J Biol Chem* 276:22507-22513.

Lionnet T, Czaplinski K, Darzacq X, Shav-Tal Y, Wells AL, Chao JA, Park HY, de Turris V, Lopez-Jones M, Singer RH. 2011. A transgenic mouse for in vivo detection of endogenous labeled mRNA. *Nat Methods* 8:165-170.

Lu G, Dolgner SJ, Hall TM. 2009. Understanding and engineering RNA sequence specificity of PUF proteins. *Curr Opin Struct Biol* 19:110-115.

Martin RM, Rino J, Carvalho C, Kirchhausen T, Carmo-Fonseca M. 2013. Live-cell visualization of pre-mRNA splicing with single-molecule sensitivity. *Cell Rep* 4:1144-1155.

Miller KE, Kim Y, Huh WK, Park HO. 2015. Bimolecular Fluorescence Complementation (BiFC) Analysis: Advances and Recent Applications for Genome-Wide Interaction Studies. *J Mol Biol* 427:2039-2055.

Muhlrad D, Decker CJ, Parker R. 1994. Deadenylation of the unstable mRNA encoded by the yeast MFA2 gene leads to decapping followed by 5'→3' digestion of the transcript. *Genes Dev* 8:855-866.

Nevo-Dinur K, Nussbaum-Shochat A, Ben-Yehuda S, Amster-Choder O. 2011. Translation-independent localization of mRNA in *E. coli*. *Science* 331:1081-1084.

Ni CZ, Syed R, Kodandapani R, Wickersham J, Peabody DS, Ely KR. 1995. Crystal structure of the MS2 coat protein dimer: implications for RNA binding and virus assembly. *Structure* 3:255-263.

Ozawa T, Natori Y, Sato M, Umezawa Y. 2007. Imaging dynamics of endogenous mitochondrial RNA in single living cells. *Nat Methods* 4:413-419.

Park HY, Lim H, Yoon YJ, Follenzi A, Nwokafor C, Lopez-Jones M, Meng X, Singer RH. 2014. Visualization of dynamics of single endogenous mRNA labeled in live mouse. *Science* 343:422-424.

Peabody DS, Ely KR. 1992. Control of translational repression by protein-protein interactions. *Nucleic Acids Res* 20:1649-1655.

Querido E, Chartrand P. 2008. Using fluorescent proteins to study mRNA trafficking in living cells. *Methods Cell Biol* 85:273-292.

Rodriguez AJ, Condeelis J, Singer RH, Dichtenberg JB. 2007. Imaging mRNA movement from transcription sites to translation sites. *Semin Cell Dev Biol* 18:202-208.

Rook MS, Lu M, Kosik KS. 2000. CaMKIIα 3' untranslated region-directed mRNA translocation in living neurons: visualization by GFP linkage. *J Neurosci* 20:6385-6393.

Santangelo PJ, Lifland AW, Curt P, Sasaki Y, Bassell GJ, Lindquist ME, Crowe JE, Jr. 2009. Single molecule-sensitive probes for imaging RNA in live cells. *Nat Methods* 6:347-349.

Sato S, Watanabe M, Katsuda Y, Murata A, Wang DO, Uesugi M. 2015. Live-cell imaging of endogenous mRNAs with a small molecule. *Angew Chem Int Ed Engl* 54:1855-1858.

Schmidt U, Basyuk E, Robert MC, Yoshida M, Villemin JP, Auboeuf D, Aitken S, Bertrand E. 2011. Real-time imaging of cotranscriptional splicing reveals a kinetic model that reduces noise: implications for alternative splicing regulation. *J Cell Biol* 193:819-829.

Shav-Tal Y, Darzacq X, Shenoy SM, Fusco D, Janicki SM, Spector DL, Singer RH. 2004. Dynamics of single mRNPs in nuclei of living cells. *Science* 304:1797-1800.

Sheth U, Parker R. 2003. Decapping and decay of messenger RNA occur in cytoplasmic processing bodies. *Science* 300:805-808.

Singer RH. 2015. Reminiscences on my life with RNA: a self-indulgent perspective. *Rna* 21:508-509.

Takizawa PA, Vale RD. 2000. The myosin motor, Myo4p, binds Ash1 mRNA via the adapter protein, She3p. *Proc Natl Acad Sci U S A* 97:5273-5278.

Weil TT, Forrest KM, Gavis ER. 2006. Localization of bicoid mRNA in late oocytes is maintained by continual active transport. *Dev Cell* 11:251-262.

Wu B, Buxbaum AR, Katz ZB, Yoon YJ, Singer RH. 2015a. Quantifying Protein-mRNA Interactions in Single Live Cells. *Cell* 162:211-220.

Wu B, Chao JA, Singer RH. 2012. Fluorescence Fluctuation Spectroscopy Enables Quantitative Imaging of Single mRNAs in Living Cells. *Biophys J* 102:2936-2944.

Wu B, Chen J, Singer RH. 2014. Background free imaging of single mRNAs in live cells using split fluorescent proteins. *Sci Rep* 4:3615.

Wu B, Miskolci V, Sato H, Tutucci E, Kenworthy CA, Donnelly SK, Yoon YJ, Cox D, Singer RH, Hodgson L. 2015b. Synonymous modification results in high-fidelity gene expression of repetitive protein and nucleotide sequences. *Genes Dev* 29:876-886.

Yamada T, Yoshimura H, Inaguma A, Ozawa T. 2011. Visualization of nonengineered single mRNAs in living cells using genetically encoded fluorescent probes. *Anal Chem* 83:5708-5714.

Yoshimura H, Inaguma A, Yamada T, Ozawa T. 2012. Fluorescent probes for imaging endogenous beta-actin mRNA in living cells using fluorescent protein-tagged pumilio. *ACS Chem Biol* 7:999-1005.

You M, Jaffrey SR. 2015. Structure and Mechanism of RNA Mimics of Green Fluorescent Protein. *Annu Rev Biophys* 44:187-206.

Yunger S, Rosenfeld L, Garini Y, Shav-Tal Y. 2013. Quantifying the transcriptional output of single alleles in single living mammalian cells. *Nat Protoc* 8:393-408.

Zimyanin VL, Belaya K, Pecreaux J, Gilchrist MJ, Clark A, Davis I, St Johnston D. 2008. In vivo imaging of oskar mRNA transport reveals the mechanism of posterior localization. *Cell* 134:843-853.

Zipor G, Haim-Vilmovsky L, Gelin-Licht R, Gadir N, Brocard C, Gerst JE. 2009. Localization of mRNAs coding for peroxisomal proteins in the yeast, *Saccharomyces cerevisiae*. *Proc Natl Acad Sci U S A* 106:19848-19853.

Figure Legends

Figure 1 - The many configurations of the Bacteriophage Coat Protein- Fluorescent Protein (CP-FP) mRNA tagging system for fluorescence imaging of mRNA. (A) The coat protein of a bacteriophage (MS2 or PP7) is expressed as a chimera with a fluorescent protein. eGFP is depicted here, but many others, as well as multiple tandem GFPs, have been used successfully within the literature. An NLS can be added as described in the text, and many the original MCP constructs in use also contain an N-terminal HA epitope tag for biochemical detection purposes. The constructs shown contain the orientation of the tags that is most common throughout the literature, but there are no known constraints on the orientation of these elements. An MBS/PBS stem loop is found in tandem in an mRNA to be visualized, and an MCP dimer associates with a single stem loop, bringing two GFPs to each stem loop. **(B)** The CP can be expressed in tandem within the same polypeptide to overcome inefficient dimerization of the CP in the cytoplasm (tdCP). This reduces the number of GFPs bound to the stem loops, but in practice the more efficient stem loop binding makes up for the loss in signal intensity as compared to the single MCP-FP fusions. Wu et al. stated that this loss in signal intensity could be compensated by inserting the FP in tandem as well, although this approach would cause a concomitant increase in background that was due to unbound MCP (Wu et al. 2012). **(C)** TdMCP and tdPCP polypeptides can be co-expressed with either the N-terminal or C-terminal halves, respectively, of the Venus fluorescent protein, producing CP-FP fusion proteins that do not produce background

fluorescence. A target mRNA that has repeating adjacent MBS and PBS sites in tandem can be inserted into a reporter mRNA, and recruitment of the tdMCP and tdPCP next to each other will facilitate re-constitution of the active Venus-FP.

Figure 2 - mRNA tagging shines a light on the steps of gene expression like never before. Real-time single molecule imaging of mRNA has been applied to all of the steps of gene expression that are indicated in this figure: **1.** mRNA biogenesis as either RNA synthesis at the site of transcription (**1A**) or mRNA splicing (**1B**); **2.** export of mRNA from the nucleus; **3.** mRNA association with cellular structures (focal adhesions, P-bodies) or organelles (peroxisomes, mitochondria); **4.** onset of mRNA translation; **5.** mRNA-protein interaction or mRNA ribosome interaction; **6.** mRNA trafficking using real-time single mRNA tracking in the cytoplasm or nucleoplasm. See the text for more details and references.

A

B

C

Figure2

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Figure 2