Video Article

Real-time Imaging of Single Engineered RNA Transcripts in Living Cells Using Ratiometric Bimolecular Beacons

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Abstract

The growing realization that both the temporal and spatial regulation of gene expression can have important consequences on cell function has led to the development of diverse techniques to visualize individual RNA transcripts in single living cells. One promising technique that has recently been described utilizes an oligonucleotide-based optical probe, ratiometric bimolecular beacon (RBMB), to detect RNA transcripts that were engineered to contain at least four tandem repeats of the RBMB target sequence in the 3'-untranslated region. RBMBs are specifically designed to emit a bright fluorescent signal upon hybridization to complementary RNA, but otherwise remain quenched. The use of a synthetic probe in this approach allows photostable, red-shifted, and highly emissive organic dyes to be used for imaging. Binding of multiple RBMBs to the engineered RNA transcripts results in discrete fluorescence spots when viewed under a wide-field fluorescent microscope. Consequently, the movement of individual RNA transcripts can be readily visualized in real-time by taking a time series of fluorescent images. Here we describe the preparation and purification of RBMBs, delivery into cells by microporation and live-cell imaging of single RNA transcripts.

Video Link

The video component of this article can be found at https://www.jove.com/video/51544/

Introduction

The expression and regulation of RNA transcripts is a complex and dynamic process that is largely responsible for controlling cell behavior and fate. Although the importance of RNA in dictating cell function has been known for some time, it is often difficult to draw clear connections between the two since most RNA analysis tools lack the spatial and temporal resolution required to capture important regulatory events such as transcription bursting, RNA trafficking, and localized RNA processing. This has led to the advent of several techniques that allow individual RNA transcripts to be visualized in living cells in real-time[1]. Perhaps, the most prominent of these techniques utilizes a GFP-MS2 fusion protein to target RNA that has been engineered to contain tandem repeats of the MS2 binding site in the 3'-UTR[2,3]. By bringing multiple GFP molecules into close proximity, individual RNA transcripts appear as bright fluorescent spots via fluorescence microscopy. The GFP-MS2 system has provided unprecedented insight into RNA behavior, including direct visualization and measurements of transcriptional bursting[4,5], detection of unique sub-cellular localization and processing[6-9], and real-time imaging of RNA transport[10]. However, despite the tremendous potential of the GFP-MS2 system, unbound GFP-MS2 fusion proteins can create a high background fluorescent signal that limits the versatility and dynamic range of this technique. Several approaches have been developed to limit this background signal, including subcellular compartmentalization of unbound GFP-MS2[10], protein fragment complementation[11], and alternative RNA binding proteins and targets[12]. However, all of these approaches remain sensitive to the relative and total expression of the RNA target and GFP-MS2 fusion protein.

As an alternative to the GFP-MS2 systems, molecular beacons have also been used to detect engineered RNA transcripts with tandem repeats of the complementary binding site in the 3'-UTR[13,14]. Molecular beacons are hairpin-forming oligonucleotide probes that are labeled at one end with a quencher and at the other end with a fluorescent reporter. When not bound to target RNA the fluorescent reporter and quencher remain in close proximity, resulting in a low-fluorescent state. Upon hybridization, the fluorescent reporter and quencher are forced apart and fluorescence is restored. Similar to the GFP-MS2 system, the binding of multiple molecular beacons onto a single RNA transcript results in a bright fluorescent spot that can be identified by fluorescence microscopy; however, the background fluorescence is expected to be much lower, due to the quenched configuration of unbound molecular beacons. Unfortunately, despite the clever activation mechanism that is incorporated into the molecular beacon design, there is now growing evidence that unhybridized molecular beacons do not remain in the hairpin conformation when introduced into living cells. Consequently, they generate a false-positive signal that significantly reduces the signal-to-background. To overcome this shortcoming, we recently developed a new synthetic probe for imaging RNA in living cells, ratiometric bimolecular beacons (RBMBs; **Figure 1A**)[15,16]. RBMBs are composed of two 2'-O-methyl oligonucleotide strands that form a hybrid structure with features from both short hairpin RNA (shRNA) and molecular beacons. The loop and fluorescence activation mechanism is similar to the molecular beacon, while

the long double stranded domain with a 3'-UU overhang is more characteristic of shRNA. The shRNA features are designed to drive nuclear export, which we have found increases intracellular lifetime to >24 hr, with minimal observable degradation, and prevents non-specific opening of the loop. As a result RBMBs exhibit a significantly higher signal-to-background than molecular beacons.

It should be noted that RBMBs cannot be prepared using DNA oligonucleotides, since DNA-based probes do not possess the same nuclear export capabilities as RNA-based probes. Structurally, the RBMB loop is typically designed to be between 15 and 21 bases long, to create a balance between specificity and selectivity upon RNA hybridization. The short stem that forms from the two self-complementary domains is usually designed to be 4 bases. If a longer stem is selected, the rate of hybridization between the RBMB loop and target RNA is significantly slowed[17,18]. Conversely, when a shorter stem sequence is selected the melting temperature is often too low to sustain the stem-loop structure at 37 °C, leading to a high background signal. The specificity of the RBMB is also reduced as the stem length is shortened. Since the sequence of the RBMB stem-loop can also influence RBMB performance, it must be carefully selected[19]. In particular, ideal loop sequences should have minimal secondary structure, hybridize to RNA sequences with minimal secondary structure, avoid protein binding sites, and avoid off-target binding. Predictions of both RBMB and RNA secondary structure can be obtained using software such as mfold[20]. Complementary off-target sites can identified using a nucleotide Basic Local Assignment Search Tool (BLAST). However, because of inconsistencies in model predictions and the difficulty in identifying protein-biding sites, the specificity of all RBMBs must ultimately be validated experimentally.

If desirable, an unquenched reference dye that is insensitive to the hybridization state can be added to the RBMB[15]. The addition of a reference dye can provide a marker for probe delivery and be used for ratiometric imaging, if more accurate measurements of total cellular fluorescence are required. The reference dyes allows measurements to be adjusted for differences in background owing to cell-to-cell variations in delivery. However, when imaging individual RNA transcripts the reference dye is not necessary. Notably, some reference dyes can interfere with the export of RBMBs from the nucleus, leading to a slightly higher background signal in the nucleus.

When designed properly, RBMBs can be used to image individual RNA transcripts in single living cells, if the target RNA is engineered to contain at least four RBMB binding sites (**Figure 1B**)[16]. RBMBs can be efficiently delivered into a wide range of cells types via microporation, with little to no effect on cell viability[21], and quantitative measurements of gene expression can be acquired within 30 min. Moreover, the methodology is fairly insensitive to RBMB concentration and target RNA levels, since fluorescence from unbound RBMBs is efficiently quenched. Here we provide a detailed description of the methodology used to prepare and purify RBMBs, as well as a general procedure for the delivery of RBMBs into live-cells via microporation and the imaging of single RNA transcripts in real-time.

Protocol

In this protocol, one oligonucleotide (RBMB1) was labeled at its 5'-end with a CF640R reporter dye and has the sequence: 5'-**mCmUmUmC** mGmUmC mCmAmC mAmAmA mCmAmC mAmAmC mUmCmC mU **mGmAmAmG** mGmAmC mGmGmC mAmGmC mGmUmG mCmAmG mCmUmC mUmU-3'. Self-complementary domains, which drive the formation of the hairpin structure, are in bold. The second oligonucleotide (RBMB2) was labeled at the 3'-end with an Iowa Black RQ-Sp quencher and has the sequence: 5'-mGmAmG mCmUmG mCmAmC mGmCmU mGmCmC mGmUmC-3'.

1. Preparation of RBMBs

1. Perform a quick spin of the tubes containing the RBMB1 and RBMB2 oligonucleotides, to ensure the dried oligonucleotide is at the bottom of the tube, and resuspend in DNase and RNase-free water to a final concentration of 100 µM. For example, a 10 nmole sample of oligonucleotide should be resuspended in 100 µl of water.
2. Measure the exact concentration of RBMB1 and RBMB2 stock samples by UV-vis spectroscopy.
    1. Mix 3 µl of the RBMB sample with 117 µl DPBS (without calcium and magnesium) in a microcentrifuge tube.
    2. Blank the spectrophotometer with DPBS and measure the absorbance from 200-800 nm. Note: Peak absorbances at 260 nm and 650 nm should be visible.
    3. Calculate the stock concentration of the RBMB samples based on the absorbance at 260 nm (or 650 nm for RBMB1), using the equation:
    Stock Concentration (M) = A_{260} dilution factor/extinction coefficient where A_{260} is the absorbance at 260 nm and the Dilution factor is 40.*
    Note: The extinction coefficient for the RBMB can be found on the specification sheet provided by the oligonucleotide manufacturer. The extinction coefficient of CF640R at 650 nm is 103,000.
3. Mix 20 µl of 100 µM RBMB1, 30 µl of 100 µM RBMB2, 6 µl 10x phosphate buffer (10x phosphate buffer: 480 mM K_2HPO_4, 45 mM KH_2PO_4, 140 mM NaH_2PO_4, pH 7.2) and 4 µl DNase and RNase-free water. Incubate the mixture at room temperature for 30 min. Note: This is typically sufficient for approximately 50 studies.
4. Prepare a liquid chromatography column using 75 prep grade Superdex to remove any unhybridized RBMB2 oligonucleotides. Use an 8 mL (0.7 x 20 cm) liquid chromatography column with a ~4 ml bed volume to purify the small volume of mixed probes.
5. Wash and equilibrate the Superdex with ~50 ml of 1x phosphate buffer using a syringe pump running at a flow at a flow rate of 0.6 ml/min. Before all of the fluid enters the bed volume, stop the syringe pump, detach it, and let the remaining liquid go through the column by gravity.
6. Load the RBMB mixture (60 µl) onto the chromatography column.
    1. After the RBMB sample has completely entered the bed, slowly add another 250 µl of 1x phosphate buffer to the top of the bed to ensure that the entire sample has completely entered the column.
    2. Fill the column to the rim with 1x phosphate buffer. Close the top, and start the syringe pump - the syringe should be filled with ~50 ml 1x PBS and run at a flow rate of 0.6 ml/min.

3. Once the RBMB nears the bottom of the column – the RBMB sample can typically be readily visualized due to the color of the dye incorporated into the probe – collect the flow-through at 2 drops per microcentrifuge tube (1.5 ml). Stop collecting the sample once the color within the microcentrifuge tubes becomes clear.

7. Combine the tubes containing the colored RBMB sample – typically 5-6 tubes - and load into a centrifugal filter device (10,000 MW cutoff). Centrifuge the sample at 10,000 RCF for 20 min or until the desired volume. Note: These speeds and times will typically yield a final volume of ~30 µl.
8. Measure the exact concentration of the purified RBMB sample by UV-Vis spectroscopy.
    1. Take 3 µl of the concentrated RBMB sample and mix with 117 µl DPBS in a microcentrifuge tube.
    2. Blank the spectrophotometer with DPBS and measure the absorbance from 200-800 nm. Note: Peak absorbances at 260 nm and 650 nm should be visible.
    3. Calculate the stock concentration of the hybridized RBMB based on the absorbance at 650 nm, using the equation:
    Stock Concentration (M) = A_{650} dilution factor/extinct coefficient where A_{650} is the absorbance at 650 nm, the Dilution factor is 40.*
    Note: The extinction coefficient for CF640R at 650nm is 103,000 and the extinction coefficient for Iowa Black RQ is ~20,000. The extinction coefficients are additive and are not sensitive to hybridization. Therefore, the stock RBMB sample has a combined extinction coefficient of approximately 123,000 at 650 nm.

9. Label the tube appropriately with name and concentration and store at -20 °C for future use.

2. Preparation of Poly-d-lysine Coated 8-well Chambered Coverglass

1. Prepare a 0.2 mg/ml solution of Poly-D-lysine by dissolving 5 mg of Poly-D-lysine (lyophilized powder, g-irradiated) in 25 ml of sterilized water in a sterile environment.
2. Add 200 µl of the 0.2 mg/ml Poly-D-lysine solution to each well of an 8-well chambered coverglass, in a sterile environment.
3. Incubate at room temperature for 16-18 hr in the cell culture hood.
4. Aspirate the Poly-D-lysine and wash the well 3 times with sterile distilled water. Note: The coated chamber slides can be stored at 4 °C.

3. Probe Delivery

Note: The cell system used should contain an integrated gene construct that expresses RNA with at least 4-sequential binding sites for the RBMB in the 3'-untranslated region (UTR). The target sequences should be complementary to the loop of the RBMB. It is advised that as a negative control, the same cell line be engineered to express the same gene construct, but without the tandem repeats. In this protocol, a human fibrosarcoma cell line, HT1080, was engineered to express *gfp* RNA with 96-tandem repeats of the RBMB target sequence in the 3'-UTR. The control cell line was engineered to express wild-type *gfp* RNA.

1. Plate cells in a T25 flask at 40-50% confluency one day before probe delivery. Culture the cells with DMEM media supplemented with 1% pen/strep and 10% fetal bovine serum (FBS), and incubate at 37 °C with 5% CO_2.
2. The next day, turn on the microporator and set up the microporation parameters to 950 V, 2 pulses, 25 msec. Note: These parameters have been optimized for HT1080 cells, but they are cell-type dependent and can be adjusted for other cell types as described in the manufacturer's instructions.
3. Fill the microporation tube with 4 ml electrolytic buffer and place it on the microporation station.
4. Take out the stock sample of purified RBMB and defrost it. Dilute several microliters to a final concentration of 12 µM with 1x phosphate buffer. 1 µl is needed for each microporation.
5. Pipette 1 ml of culture medium with FBS but without antibiotics into a microcentrifuge tube. Note: This will be used to suspend the cells immediately after microporation and can be set aside, near the microporation device, for the time being. The inclusion of antibiotics in culture media will decrease the cell viability after microporation.
6. Remove the cell culture media from the engineered HT1080 cells (60-80% confluent), wash the cells with 1 ml Ca^{2+} and Mg^{2+}-free DPBS once, and incubate with 1 ml trypsin for 1-2 min.
7. Stop the trypsinization by adding 1 ml DMEM media supplemented with 10% fetal bovine serum, without antibiotics and phenol red, and transfer the cells into two 1.5 ml microcentrifuge tubes.
8. Spin down the cells in the microcentrifuge tube at 200 x g for 5 min. Remove the supernatant, resuspend and combine the cell pellets in a final volume of 1 ml DPBS.
9. Take 10 µl from the well-mixed cell suspension and count the cells.
10. Pipette the cells up and down several times to make sure they are well dispersed and transfer 300,000 cells with DPBS into a new 1.5 ml microcentrifuge tube and spin down at 200 x g for 5 min.
11. Remove the supernatant, being careful not to disturb the cell pellet. Resuspend the pellet in 11 µl resuspension buffer and pipette up and down several times to ensure that the cells are well dispersed. Be sure not to generate any air bubbles. Note: Air bubbles will cause a spark during microporation and result in poor RBMB delivery and cell death.
12. Add 1 µl of the diluted RBMB (12 µM) and mix well by pipetting up and down several times. Again be careful not to generate any air bubbles.
13. Aspirate 10 µl of the RBMB-cell mixture, using the microporation pipette, and insert the pipette into the microporation tube. Push the start button to start the microporation. Note: There should be no visible air bubbles in the tip.
14. When the screen of the microporator shows completion, remove the pipette from the station, expel the 10 µl mixture into the microcentrifuge tube that was prepared earlier, with 1 ml culture medium with FBS but without antibiotics. Mix gently by rocking the tube side-to-side several times.
15. Spin the cells at 200 x g for 5 min and wash the cells two more times, in 1 ml phenol red free culture medium with FBS but without antibiotics, to remove any RBMBs that were not delivered into the cells. Resuspend with 400 µl phenol red free culture medium with FBS but without antibiotics.
16. Plate the microporated cells into the Poly-D-lysine coated 8-well chambered coverglass at 200 µl per well or at the desired confluency.
17. Optional: If it is desirable to image the nucleus, add Hoescht 33342 to the cells at a final concentration of 0.01 mg/ml.

18. Place the chambered coverglass into a cell culture incubator. Incubate the cells for 1-2 hr prior to imaging, so that the cells have adequate time to settle down on the coverglass surface. Note: Imaging can be performed as soon as 30 min post-microporation.

4. Image Acquisition

1. Turn on the live cell stage top incubation system and equilibrate it until it reaches 37 °C, 5% CO_2, and 75% humidity.
2. Turn on the microscope and fluorescent light source and open the Metamorph software. Note: Other similar software packages for microscope control and image acquisition can also be used.
3. Apply Immersol oil to the objective.
4. Transfer the chambered coverglass with microporated cells to the live cell stage top incubation system. Incubate the stage top system until the temperature and CO_2 levels are stabilized.
5. Open the Acquire tab, in Metamorph software. Click the Show Live button to find the field, adjust the focus under white light and click the Stop Live button.
6. Under the Acquire tab, click and open the stream acquisition pop-up button, and set up the desired movie acquisition parameters. Acquire 150 frames using the Cy5/CF640R filter and save the images to the hard drive.

Representative Results

Shortly following the microporation of HT1080 cells in the presence of RBMBs, individual RNA transcripts that were engineered to contain multiple RBMB binding sites in their 3'-UTR appear as bright fluorescent spots when imaged by wide-field fluorescence microscopy (**Figure 2**). While individual RNA transcripts with as few as four RBMB binding sites can be imaged in live-cells, the more RBMB binding sites in the 3'-UTR, the stronger the fluorescent signal. Moreover, the more RBMBs that are bound to each transcript the longer the RNA can be visualized before the signal is lost due to photobleaching. In this protocol, the RNA was engineered to contain 96-tandem repeats of the RBMB target sequence in the 3'-UTR. The acquisition of streaming images allows individual RNA transcripts to be imaged in real-time (**Movie 1**). Individual RNA transcripts can readily be seen moving within the cytoplasm and nucleus of the cells. While most of the spots appear to undergo Brownian or sub-diffusive movements, in rare cases an RNA undergoing directed transport will be observed (**Movie 2**). Theses RNA transcripts typically move rapidly along a straight path (**Figure 3**); however, some RNA do follow curved paths or make abrupt changes in direction. These movements are in sharp contrast to Brownian movements, which are random in nature and exhibit small overall displacements within the short time-frames acquired here (*i.e.* <1 min). The directed RNA movements are consistent with the transport of RNA along microtubules and microfilaments. These experiments indicate that RBMBs provide a versatile and robust tool for imaging individual RNA transcripts in living cells.

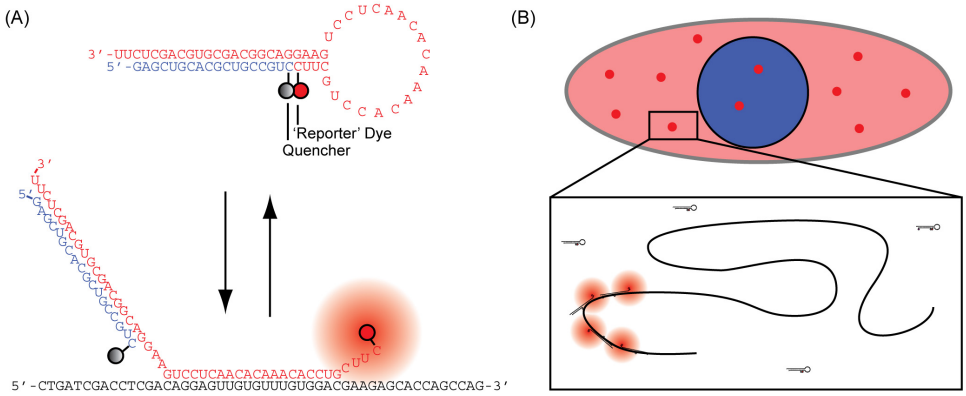

Figure 1. Schematic of RBMBs and the methodology used image individual RNA transcripts in living cells. **A)** RBMBs in the presence and absence of complementary target RNA. In the absence or target RNA, RBMB fluorescence is quenched. In the presence of target RNA, RBMB fluorescence is restored. **B)** Multiple RBMBs bind each RNA transcript creating a bright fluorescent spot that can be detected by wide-field fluorescence microscopy. Please click here to view a larger version of this figure.

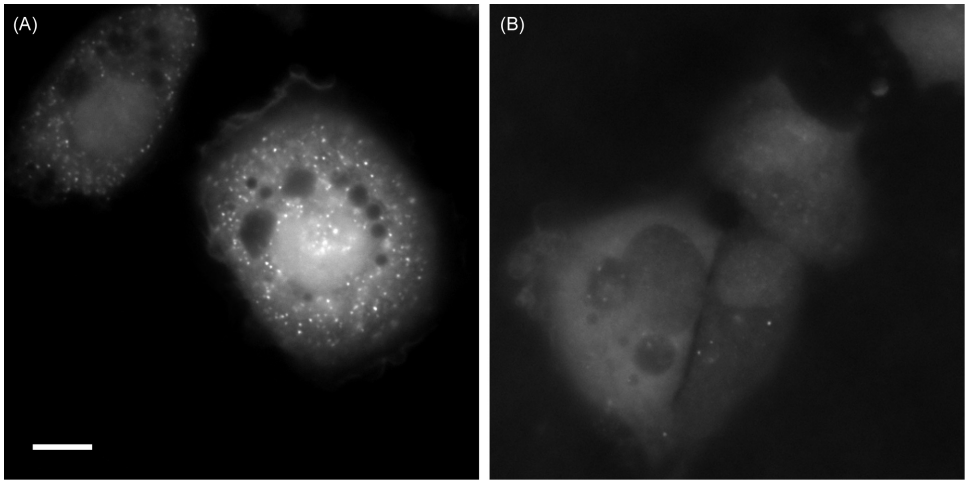

Figure 2. Representative image of HT1080 cells stably expressing RNA with **A)** 96-tandem repeats or **B)** 4-tandem repeats of the RBMB binding site in the 3'-UTR, following the intracellular delivery of RBMBs. The presence of 96-tandem repeats allows individual RNA transcripts to appear as bright fluorescent spots. RNA containing only 4-tandem repeats can also be visualized, but the intensity of the fluorescent spots is very dim and often only detectable in areas of exceptionally low background. The small number of particularly bright spots corresponds to RNA that is not moving. Scale bar: 10 μm. Please click here to view a larger version of this figure.

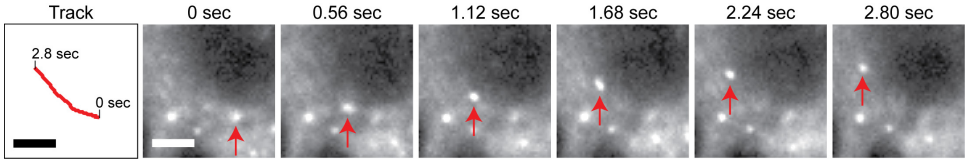

Figure 3. Montage of an RNA transcript undergoing directed transport. The trajectory of the transcript is shown in the left-most panel. The trajectory is overlaid over a single frame of the time series. Scale bar: 2.5 μm. Please click here to view a larger version of this figure.

Movie 1. Representative movie of HT1080 cells stably expressing RNA with 96-tandem repeats of the RBMB binding site in the 3'-UTR, following the intracellular delivery of RBMBs. Individual RNA transcripts appear as bright fluorescent spots. Scale bar: 10 μm. Click here to view video.

Movie 2. Representative movie of an RNA transcript undergoing directed transport. Scale bar: 10 μm. Click here to view video.

Discussion

The ability to image single engineered RNA transcripts in living cells using a conventional wide-field microscope requires a bright and photostable fluorescent signal to be associated with each RNA transcript and a low fluorescent background emanating from unbound fluorescent probes. In this method, a bright fluorescent signal is achieved by hybridizing multiple (up to 96) oligonucleotide-based fluorescent probes, *i.e.* RBMBs, onto each RNA transcript. However, as few as four bindings sites are sufficient[16]. The collective signals result in a bright fluorescent spot that can tracked in real-time. The presence of multiple probes also allows for longer imaging times, since a substantial fraction of the fluorescent dyes must be photobleached before the collective signal is lost. It is envisioned that this technique will provide unique insight into RNA biology and allow for the study of a wide range of RNA behaviors ranging from measuring the response of RNA trafficking to various external stimuli, observing unique subcellular localization patterns of target RNA, studying the fate and lifetime of RNA, and providing insight into RNA regulation. In addition it may be possible to track changes (*i.e.* up- and down-regulation) in RNA expression. Although this capability has not yet been validated, we have previously shown that RNA copy number can be fairly accurately quantified for up to 24 hr in live cells[22]. Moreover, RBMBs don't appear to affect the level of gene expression. Combined, these results provide initial evidence that changes in gene expression over this time period were accurately tracked, on a cell-by-cell basis, and that RBMBs did not lead to an increase in RNA stability or slowed RNA degradation. However, it is not clear to what extent gene expression actually changed during this time if at all.

In general, it is expected that any increase in gene expression would be easily identified; due to the abundance of unbound RBMB in the cell that is free to bind newly formed transcripts. However, what is less clear is how RBMBs dissociate from RNA during translation and degradation and if there is some lag between RNA expression/degradation and the imaging of these transcripts. Additional studies are still required to better understand RBMB performance in these scenarios.

Several alternative approaches to imaging single RNA transcripts have been reported previously. The earliest studies involved fluorescently labeling isolated RNA transcripts and re-introducing these transcripts into cells, typically by microinjection[23,24]. The signal-to-background of this approach is very high, due to the ability to remove any unbound fluorescent dyes, but there are concerns over whether the observed RNA behavior accurately represents true RNA processing. The most common solution to this shortcoming has involved engineering transgenes with tandem repeats in the 3'-UTR that can be bound by a fluorescent reporter, analogous to the RBMB approach presented here. Previous fluorescent reporters have included small molecules that only fluoresce upon binding RNA aptamers, commonly referred to as Spinach[25], and GFP. Spinach has not yet been used to imaging single RNA transcripts, but has been used to image global expression. In contrast,

GFP has been used to successfully image single RNA transcripts. In this approach, a GFP reporter is fused to the coat protein of bacterial phage MS2 (GFP-MS2) and binds to an engineered RNA construct with tandem repeats of the MS2 binding site in the 3'-UTR[2,10]. While this approach is very similar to the approach described here, RBMBs offer several advantages. For example, RBMBs allow organic fluorophores to be used for imaging, which provides a wider diversity of choices with superior photostability and superior brightness compared with fluorescent proteins. Further, there are many commercially available organic fluorophores that emit in the far to near-infrared. The benefit of choosing dyes with red-shifted emission spectrums stems from the lower cellular autofluorescence observed at these wavelengths.

Since high levels of autofluorescence can easily drown out any fluorescent signals associated with RNA hybridization, the ability to dramatically reduce autofluorescence provides an important boost in signal-to-background. Another important advantage of using RBMBs is that the signal from unbound probes is significantly quenched. Although, various approaches have been taken to reduce the background fluorescence of unbound GFP in the GFP-ms2 approach, *e.g.*, use of nuclear localization signals, controlling GFP expression levels, and split GFP complementation[1,10,11,26,27], the presence of an actual quenching moiety within the RBMB design provides users with much greater experimental flexibility. For example, the RBMB approach is relatively insensitive to both the relative and total level of RNA expression and probe concentration. In fact, individual transcripts can be imaged with RBMB concentrations that span an order of magnitude. The combined advantages of higher photostability, brighter signal, and lower background make real-time RNA imaging experiments with RBMBs far more accessible for microscopy users with limited expertise.

Perhaps, the most notable disadvantage of using exogenous probes such as RBMBs, in contrast to a molecular reporter such as GFP, is the need for intracellular delivery. Fortunately, a number of options exist, *e.g.*, microinjection[28], transfection agents[29], cell penetrating peptides[30], and streptolysin O (SLO)[31]. Personally, we have found that microporation is the most user friendly and versatile option, typically resulting in >95% viability and delivery efficiency[21]. This is likely because the microliter-volume electroporation process exhibits a reduction in the many harmful events often associated with electroporation, including heat generation, metal ion dissolution, pH variation and oxide formation. Importantly, microporation is also amenable to high-throughput studies, since RBMBs can be delivered into >100,000 cells in a single experiment.

Despite the many advantages of using RBMBs to image RNA localization and movement in living cells, a number of limitations and challenges still remain. Perhaps, the greatest challenge is the inability to track RNA for more than a few minutes under continuous exposure. This is a consequence of both photobleaching and the ability of RNA to move out of the focal imaging plane. Brighter and more photostable dyes can help alleviate both of these shortcomings, by increasing the number of times each dye can be excited and allowing the discrete fluorescent spots to be imaged at greater distances from the focal plane. One promising approach to increase photostability involves the use of self-healing dyes, which utilize triplet-state quenchers in proximity to the organic fluorophore to extend their lifetime[32]. Another option may be the use of fluorescent amplifying conjugated polymers, which are composed of a large number of connected fluorophores that do not self-quench. These polymers can be many times brighter than single organic fluorophores and yet still be efficiently quenched by a single quenching moiety[33,34]; however, additional work in this area is still needed. It should be noted that intermittent imaging, with a frame rate >1 frame per sec, cannot be performed to image a single RNA transcript over extended periods of time because it is difficult to ensure that it is the same RNA transcript being tracked from frame-to-frame. Of course, quantitative evaluation of gene expression at the single cell level is still possible over long time frames, through the counting of individual fluorescent spots on a per cell basis[16,35,36]. In this case, it is not necessary to keep track of individual transcripts. Overall, it is believed that RBMBs are capable of providing important insight into RNA function and enables the imaging of single engineered RNA transcripts with conventional microscopy equipment and limited expertise.

Disclosures

Dr. Mark Behlke and Dr. Ling Huang are employed by IDT which offers oligonucleotides for sale similar to some of the compounds described in the manuscript. IDT is, however, not a publicly traded company and they personally do not own any shares/equity in IDT.

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