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IMAGEtags: Quantifying mRNA transcription in real time with multiaptamer reporters

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Abstract

Cell communications are essential to the organization, development and maintenance of multicellular organisms. Much of this communication involves changes in RNA transcription and is dynamic. Most methods for studying transcription require interrupting the continuity of cellular function by sacrificing the communicating cells and capturing gene expression information by periodic sampling of individual cells or the population. The IMAGEtag technology to quantify RNA levels in individual living cells, demonstrated here in yeast, allows living cells to be tracked over time as they respond to different environmental cues. IMAGEtags are short RNAs consisting of strings of a variable number of tandem aptamers that bind small molecule ligands. The aptamer strings can vary in length and in configuration of aptamer constituents, such as to contain multiples of the same aptamer or two or more different aptamers that alternate in their occurrence. A minimum effective length is about 5 aptamers. The maximum length is undefined. The small molecule ligands are enabled for imaging as fluorophore conjugates. For each IMAGEtag, two fluorophore conjugates are provided, which are FRET pairs. When a cell expresses an RNA containing an IMAGEtag sequence, the aptamers bind their ligands and bring the fluorophores in to sufficiently close proximity to allow FRET. The background fluorescence of both fluorophores is minimal in the FRET channel. These features endow IMAGEtags with the sensitivity to report on mRNA expression levels in living cells.

Keywords

RNA imaging; IMAGEtags; FRET; fluorescence; yeast

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Introduction

Transcriptional regulation is central to cellular function and to the abilities of cells to respond to their environments. Cellular responses are rapid and cells in the same environment can respond differently. Thus, methods of tracking transcription in individual cells in real-time are required for developing a better understanding of the processes involved in cell regulation at the transcriptional level. Several RNA reporters have been developed for monitoring transcription in living cells. These RNA reporters fall in two classes. The first to be developed were the MS2-based reporters in which a series of identical viral RNA elements are present in the RNA sequence and the MS2 peptide to which they bind is expressed as a peptide tag for a fluorescent protein such as GFP. The RNA reporters are detected by their ability to concentrate the MS2-tagged GFP (Bertrand et al., 1998; Hocine, Raymond, Zenklusen, Chao, & Singer, 2013). This method requires computational deconvolution of the images and allows the analysis of RNA transcription and trafficking in single cells. The second class of aptamer applied as RNA reporters is a group light-up aptamers for which the ligands have greatly elevated fluorescence yield in the aptamer-ligand complex than when free in solution. The Spinach and Broccoli aptamers are examples of light-up aptamers that have recently been applied to visualizing RNA transcription in individual cells (Filonov, Moon, Svensen, & Jaffrey, 2014; Paige, Wu, & Jaffrey, 2011; Strack, Disney, & Jaffrey, 2013). These aptamers have been used to image rRNA transcription and to localize trinucleotide RNA sequences but have not been used to image mRNAs. The latter RNAs provide a big challenge for imaging because of their low levels of expression and the consequent requirement for very sensitive means of detection. To address this problem, we have used FRET (Förster resonance energy transfer) for mRNA detection. FRET only occurs when two molecules capable of interacting with energy transfer are co-located within 100 Å. These FRET pairs interact infrequently when moving randomly in a volume such as the cell cytoplasm. However, energy transfer is efficient when they are held within 100 Å or less of each other for extended periods. Thus, the major advantage of FRET is that the signal from appropriately tethered FRET pairs is much stronger than the signal from these molecules when they are moving randomly in solution.

IMAGETag (**I**ntracellular **M**ultiaptamer **G**enetic **tag**) reporters consist of strings of tandem aptamers which bind small molecule ligands that are conjugated with one or the other of a FRET pair. The FRET signal is produced by interaction of the FRET ligands with aptamers in the IMAGETag strings. When bound by the aptamers the fluorophore-conjugated ligands are in sufficiently close proximity to participate in FRET, thereby creating a fluorescent signal that reports on the presence of the IMAGETags (Fig. 1). The IMAGETag can consist of aptamers that recognize the same or different ligands. Two aptamers with different specificities would be coded in the IMAGETag as alternating tandem structures. However, the combinations of aptamers that we have so far tested for proper folding in alternating series have not given promising results. Instead, we created IMAGETags consisting of multiple copies of the same aptamer sequence in strings of at least five units. These IMAGETags function well for imaging promoter activity when the aptamer ligand is presented in two forms, each conjugated with one of a FRET pair (Muslum Ilgu et al., 2016; Shin et al., 2014). The effectiveness of these single-aptamer IMAGETags can be understood

when it is considered that, when an internal aptamer of the string binds one of the two ligands, there is a 50% chance that one or the other adjacent aptamer will bind the FRET pair and therefore a 75% chance that the central aptamer will have an adjacent FRET partner. Although homo-FRET can occur if two of the same ligand are closely located by the IMAGEtags, this output is much lower in intensity than from the hetero-FRET pairs. Thus, an IMAGEtag containing a single aptamer type will provide a signal that increases in efficiency with the number of tandem aptamers in the string. We have found that strings of 5 aptamers or more of a single type provide sufficient signal to detect transcribed mRNA (Muslum Ilgu, et al., 2016; Shin, et al., 2014).

IMAGEtags

We have so far made three IMAGEtag reporters, each consisting of a tandem series of the same aptamer (neomycin, tobramycin or PDC aptamers). Any aptamer that binds a target with the ability to enter a cell is a potential component of IMAGEtags. However, the following practical considerations limit the choice of aptamers. 1) The ligand needs to be nontoxic in the concentration range used for imaging. 2) The aptamer components of IMAGEtags must be capable of binding their targets in the cellular cytoplasmic environment. 3) The aptamers must fold appropriately for binding their targets when linked to additional sequence in the IMAGEtag that includes other aptamers and additional RNA sequence. The ability to fold appropriately depends on many aspects of the RNA sequence including the spacing between aptamers. We have found that a space of 4 nucleotides is sufficient for small aptamers such as those described here, which all fold as stem-loops and are in the range of 20–40 nt long. It is preferable that the IMAGEtags are as short as possible to minimize their effects on transcription rate. The ability to fold appropriately under intracellular conditions can be predicted for IMAGEtags by co-transcriptional folding programs such as Kinefold (Xayaphoummine, Bucher, & Isambert, 2005). 4) The aptamers should be tested for their abilities to bind ligand under similar conditions as present in the cytoplasm in which the free Mg^{2+} concentration is much lower than is used for selecting most aptamers and where the major monovalent cation is K^{+} rather than Na^{+} . We have found that aptamer affinities and ligand specificities can be quite different in buffers that approximate intracellular conditions compared with the buffers in which they were selected (Muslum Ilgu, et al., 2016; M. Ilgu, Wang, Lamm, & Nilsen-Hamilton, 2013).

When used as reporters of promoter activity as we have done, IMAGEtags can form the majority of the RNA sequence and should have a short half-life so that they report specifically on promoter activity. The IMAGEtags described here fit those characteristics, with measured half-lives in yeast of 6–8 min. If IMAGEtags are applied to measure longer term steady-state RNA levels in the cell or to track RNA trafficking, the optimal position of the IMAGEtags in the RNA may vary. Additional RNA sequence can change the transcribed reporter RNA abundance by altering characteristics such as RNA stability and transcriptional elongation rate. Therefore, the tagged RNA should be demonstrated to be regulated similarly and have the same rate of turnover as the endogenous RNA.

The IMAGEtag reporters have been demonstrated to function in yeast in which they have been tested for reporting transcription from the GAL1, ADH1, and ACT1 promoters (Shin,

et al., 2014). The ligands for each aptamer were conjugated with cyanine3 (Cy3) or cyanine5 (Cy5) as the FRET pair. With the GAL1 promoter driving RNA expression and in the presence of the inducer, galactose, or with the constitutive ADH1 and ACT1 promoters driving RNA expression, FRET signals are generated when the cells are incubated with the appropriate ligand pair (Fig. 2). An important aspect of this method is that the background created by each fluorophore is absent in the FRET channel (Fig. 2). The high specificity of the aptamers for their ligands can be used to validate that the IMAGEtag is the source of the FRET signal. Thus, when yeast cells expressing the 6xPDC IMAGEtags (6 copies of the PDC aptamer) were incubated with Cy3 or Cy5-conjugated PDC ligands, the FRET signals from cells that expressed the aptamer were significantly higher than obtained from cells that expressed a non-relevant RNA from the same promoter (Fig. 3A). Cells expressing the 5xTOB IMAGEtags (5 copies of the tobramycin aptamer) incubated with the PDC ligands gave FRET signals equal to the control level (Shin, et al., 2014). By contrast, when tobramycin ligands were provided, an increase in FRET was observed in cells that express the 5xTOB IMAGEtags from the GAL1 promoter compared with the control RNA (Fig. 4A).

The importance of imaging live cells is that real time changes in gene expression can be observed upon promoter induction. The increased transcriptional activity of the GAL1 promoter in response to galactose was monitored by the appearance of the IMAGEtag RNA with time in the cell population (Fig. 3B) and in individual cells (Fig. 3C). By comparison, only a decrease in FRET due to fluorophore quenching was observed in cells expressing the control RNA (Fig. 3B,C).

Visualizing gene expression with IMAGEtags

To ensure that IMAGEtag expression is responsible for the observed FRET signals, it is important to have a series of control experiments and conditions, which include: 1) Control cells that are imaged under the same conditions as the IMAGEtag-expressing cells, which express (from the same vector as used for the IMAGEtag RNA) a non-relevant RNA that does not bind the aptamer ligand. 2) Cells harboring the IMAGEtag-expressing plasmid that are not induced to express the IMAGEtag RNA, 3) Validation of FRET by acceptor photobleaching, and 4) validation of changes in mRNA levels in the cell population by RT-qPCR. In addition, it is very important in all studies that involve microscopy to ensure objective data collection and analysis. For example, the choice of field should be randomized and/or performed by a “blinded” operator. Similarly, the analysis should be performed by a “blinded” operator. Operator blinding can be readily achieved if another individual labels the tubes/plates of cells with a numerical or alphabetical system that can be disclosed after the images have been obtained and analyzed.

Materials and Instrumentation

1. PLATE solution [40% polyethylene glycol (PEG) 3350, 0.1 M lithium acetate, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5]
2. Dimethyl sulfoxide (DMSO)

3. SD-Uracil media; 6.7 g/L Difco™ Yeast nitrogen base without amino acids, 1.92 g/L Yeast synthetic drop-out medium supplement without uracil (add 20 g agar/L for preparing plates). Autoclave then add filter-sterilized glucose or raffinose to final concentrations of 2% (w/v).
4. 20% galactose in water
5. RNA extraction buffer (0.1 M NaCl, 5% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 7.5)
6. Phenol:chloroform:isoamyl alcohol (49.5:49.5:1). Note that the pH of this solution should be acidic (pH 4.5). Unlike dsDNA, which has a tendency to precipitate at lower pH and partitions into the organic phase (Sambrook & Russell, 2006), RNA remains water-soluble due to H-bonding between water and the protonated bases. Thus extraction at low pH minimizes contamination of the RNA preparation by chromosomal DNA.
7. Chloroform:isoamyl alcohol (24:1)
8. Fluorescent aptamer ligand conjugates. These will depend on the IMAGEtags that are used (see Fig. 5 for the fluorophore-conjugated ligands described here)
9. The plasmids used in this study can be obtained from the corresponding author, MNH.
10. In these studies, we have used yeast strain BY4735, Genotype: MAT α ade2⁻ hisGhis3⁻ 200leu2⁻ omet15⁻ 0trp1⁻ 63ura3⁻ 0.
11. Confocal microscope: In these studies we used a white light laser to excite and PMT for recording emissions from the fluorophores.

Transformation of yeast cells

1. Yeast expression plasmid pYES2 was used as the backbone to construct vectors from which RNA containing IMAGEtags could be expressed in yeast.
2. Grow *Saccharomyces cerevisiae* in YPD (1% yeast extract, 1% peptone, 2% glucose) medium at 30°C overnight with shaking at 250 rpm.
3. Take 0.5 mL of the culture and spin for 30 sec at 10,000 rpm. Remove the supernatant and resuspend the pellet in 0.5 mL PLATE solution. Add 10 μ L carrier DNA (100 μ g salmon testes DNA) and 10 μ L transforming plasmid DNA (1 μ g) and vortex. Add 57 μ L DMSO and vortex briefly. Leave for at least 30 min at room temperature (can be left on the bench overnight).
4. Heat shock at 42°C for 15 min. Centrifuge the cells at $13,200 \times g$ for 30 sec and carefully remove the supernatant. Resuspend the cells gently in 200 μ L of sterile distilled and deionized water by aspirating up-and-down with the pipette tip. Immediately spread the cells on SD-Uracil plates. Incubate the plates at 30°C until colonies appear.

Cell preparation for FRET measurements

Grow yeast cells containing the plasmids with expression capability for either the control RNA or the IMAGEtag RNA.

1. Pick a colony from the SD-Uracil plates containing the transformed cells. Grow the cells in SD-Uracil medium with 2% (w/v) glucose at 30°C with shaking at 250 rpm until they reach OD600 of 1.0.
2. Inoculate the culture to a fresh SD-Uracil medium with 2% (w/v) raffinose to an OD600 of 0.02. Grow the cells at 30°C with shaking at 250 rpm until the culture reaches an OD600 of 0.4–0.6.
3. Centrifuge the cells at $1000 \times g$ for 5 min. Remove supernatant and resuspend the pellet in 270 μ L of SD-Uracil medium with 2% raffinose. Divide the cell culture into 3 tubes and add one or the other or both fluorescent ligand conjugates (Shin, et al., 2014). The appropriate concentration of each ligand must be determined experimentally to account for differential uptake by the cells. This is done empirically by testing for optimal FRET signal from cells that express IMAGEtags and have been incubated with different ratios and concentrations of ligand pairs. For the PDC ligands, we have used final concentrations in the cell culture of 4 μ M Cy5-PDC and 10 μ M Cy3-PDC. For the tobramycin IMAGEtags we have used final concentrations in the cell culture of 25 μ M of each ligand conjugate and up to 40 μ M for the sulfonated ligands, which do not enter the cells as readily as ligands lacking sulphonates on the fluorophore due to their net negative charge. For this reason, we prefer to use nonsulfonated ligands although the cost of purchasing the precursors is higher for their synthesis than for the sulfonated derivatives.
4. Incubate the cells for 1 h at 30°C.
5. For a time course experiment, in which the promoter driving IMAGEtag RNA expression is GAL1, plate 45 μ L of cells on a poly-D-Lysine coated 35 mm glass bottom dish from MatTek Corporation (Ashland, MA). Place a lid over the dish and put it on a heated stage at 30°C under the confocal microscope. Allow the cells to settle down for about 10 min. Add 5 μ L of 20% (w/v) galactose very slowly (to avoid disturbing the cells) to a final concentration of 2%. Focus on the cells and start collecting data.
6. For a single time point experiment in which the promoter driving IMAGEtag expression is GAL1, add 10 μ L of 20% (w/v) galactose to a final concentration of 2% in each tube from step 4. Incubate for 60 to 90 min at 30°C. Place 50 μ L of cells on a 35 mm glass bottom MatTek dish and put it over a heated stage at 30°C under the confocal microscope. Put a lid over the dish to avoid evaporation. Allow the cells to settle for 10 min and then collect data.

Data acquisition and analysis

Sensitized emission data is collected from the control RNA-expressing cells or the IMAGEtag-expressing cells from samples containing individual ligands (either Cy3-PDC or

Cy5-PDC) and both the ligands together. For these experiments we have used a Leica SP5X laser scanning confocal microscope (Figs 2,3,4B) or a Nikon Eclipse 200 confocal microscope (Fig. 4A). The following instructions are for the Leica microscope, but similar protocols can be used with other confocal microscopes. Although a wide-field microscope could be used for measuring FRET, it is not a good choice as it suffers the problem of signals being averaged from many focal planes.

1. Place the dish with cells and medium containing either Cy3 ligand conjugate (donor) or Cy5 ligand conjugate (acceptor) under the confocal microscope. Image acquisition should be done in a sequential mode. In the first sequence illuminate the cells with a 550 nm white light laser and collect images from the donor emission channel (Cy3; 560 – 626 nm) and the FRET channel (Cy5; 660 – 754 nm). In the second sequence illuminate the cells with a 650 nm laser and collect images at the acceptor emission channel (Cy5; 660 – 754 nm). Collect the DIC image of the cells simultaneously. In our experiments, focus on the cells was achieved with a 63x oil immersion objective. The choice of field and initial focus is based on the bright field image as seen through the eyepiece. Additional fine Z-focusing is based on the fluorescence/confocal signal on the screen.
2. Four channels should be visible on the screen including the DIC image (Fig. 2C). Adjust the gains of each emission channel so that signals are below saturation. The donor-only and acceptor only control samples are essential to correct for cross-talk.
3. Using identical settings from steps 1 and 2, collect data from cells in medium containing the combination of donor and acceptor ligands.
4. Data collection can be done from a single focal plane or by Z stacking. For time course experiments, data can be collected manually or the microscope can be programmed to collect images at regular intervals (Fig. 3).
5. Images should be analyzed using the equation $FRET = (B - A \cdot b \cdot c) / C$ where B= FRET emission, A= donor emission, b= donor emission cross-talk ratio (B in donor-only sample/ A in donor-only sample), c=acceptor excitation cross-talk ratio (B in acceptor-only sample/ C in acceptor-only sample), C= acceptor emission (Wouters, Verveer, & Bastiaens, 2001). In our experiments we have found the FRET efficiency to be in a range of 0.1–0.8.
6. For FRET experiments, donor-only and acceptor-only controls are necessary (Fig. 2A,B) to calculate the cross-talk signals. FRET signals should be calculated by taking into consideration the emissions measured in the Cy3, FRET and Cy5 channels (Fig. 2C). As an example, the increase in FRET signals of cells expressing 6xPDC IMAGEtag RNAs and the FRET in control cells over a period of 40 to 99 min after induction of the GAL1 promoter with galactose are shown in figure 3 after quantification as described here.
7. Although the cells are attached to the dish by way of poly-D-lysine, some can still move in and out of the confocal field and in the horizontal direction. These movements are small and visual examination of consecutive images allows

tracking of single cells. To analyze many cells in an image is laborious, but can be achieved automatically with the application of an algorithm implemented in Matlab™ (Mitra D, Bouthcko R, Ray J, & Nilsen-Hamilton M, 2015).

Acceptor photobleaching to validate FRET.

FRET measurements should also be validated by the acceptor photobleaching method in which the acceptor is photobleached using a high intensity laser power. The donor emission is measured before and after photobleaching. This procedure can be performed using the Leica FRET-AB acceptor photobleaching wizard or equivalent software associated with other instruments.

1. Place the dish with cells containing both the donor and acceptor ligands. Focus and illuminate the cells with a 550 nm white light laser and record the emission from the donor channel (560–626 nm). Simultaneously excite the cells with a 650 nm laser and record emissions from the acceptor channel (660–754 nm). Adjust the gains of the channels to ensure that they are below saturation.
2. Select a specific region of interest (ROI) and illuminate the ROI with 100% power of the 650 nm laser to bleach the acceptor. Record the emissions from the donor and the acceptor channels. Repeat for multiple ROIs. The FRET efficiencies will be calculated using the formula:
3.
$$\text{FRET efficiency} = \left(\text{Donor}^{\text{post-bleach}} - \text{Donor}^{\text{pre-bleach}} \right) / \text{Donor}^{\text{post-bleach}}$$
4. Perform the same exercise for ROIs that do not include cells to measure background FRET efficiency.

Measurement of IMAGETag RNA level by RT-qPCR

As an additional control, the IMAGETag RNA levels can be quantified in the same cell populations as they are imaged. This control should be done when evaluating new IMAGETags or IMAGETag expression in different cell types. The observation of parallel increases in FRET of individual cells with an increase in IMAGETag RNA in the cell population supports the interpretation that the FRET signals emanate from the IMAGETags. This control can be done readily with yeast because they are liquid cultures from which some portion can be set aside for later analysis.

1. Harvest the cells in 0.6 mL RNA extraction buffer and 0.6 mL of phenol:chloroform:isoamyl alcohol (49.5:49.5:1) for 6 min at room temperature.
2. Add 0.2 g glass beads (0.45 mm diameter) and lyse the cells with vigorous agitation for 2 min.
3. Centrifuge at $2200 \times g$ for 5 min and transfer the upper aqueous phase to a new tube.
4. Add 1 volume of phenol:chloroform:isoamyl alcohol (49.5:49.5:1).
5. Vortex for 2 min and centrifuge at $2200 \times g$ for 5 min.
6. Transfer the aqueous phase to a new tube. Repeat steps 4–6.

7. Add 1 volume of chloroform:isoamyl alcohol (24:1).
8. Vortex for 2 min and centrifuge at $2200 \times g$ for 5 min.
9. Transfer the aqueous phase to a new tube.
10. Precipitate with 2.5 volumes of 100% ethanol (final = 73%) and 0.1 volume of 3 M sodium acetate, pH 5.2 (final = 73 mM).
11. Resuspend the RNA in DEPC-treated water.
12. Treat 1 μ g RNA with RNase-free DNase I (Life Technologies) at room temperature for 15 min and stop the reaction by bringing the solution to 2.5 mM EDTA.
13. Reverse transcribe with Superscript III reverse transcriptase (Life Technologies, Cat# 18068–015) with reverse primer 5'-CCTAGACTTCAGGTTGTCTAACTCC-3' in a final volume of 20 μ L.
14. Dilute the reaction to 50% and use 2 μ L of the diluted cDNA from the previous reverse transcription step for qPCR with SYBR green (Life Technologies, Carlsbad, CA, Cat# S7563), GoTaq Flexi polymerase (Promega, Madison, WI, Cat# M8291), forward primer 5'CTAGAGGGCCGCATCATGTAATTAG3' and reverse primer 5'CCTAGACTTCAGGTTGTCTAACTCC3'. These primers are located at the 3' end of the IMAGEtag sequence and part of the pYES2 vector. The endogenous ACT1 mRNA can be used as an internal control, which is amplified by the forward primer 5'ATTCTGAGGTTGCTGCTTT3' and reverse primer 5'GTCCCAGTTGGTGACAATAC3'. The qPCR was performed in an MJ Research Opticon 2 PCR machine with a protocol of 94°C for 5 min followed by 40 cycles (94°C for 15 sec, 60°C for 15 sec, 72°C for 15 sec) then a plate read. Standards consisting of each IMAGEtag sequence to be amplified or the ACT1 cDNA fragment defined by the stated primers should be on the same plate as the samples and subjected to the same PCR protocol. The amount of RNA of each type is calculated from the standard curves.

Synthesis of ligands

The aptamer ligand conjugates discussed in this article are shown in figure 5. The choice of ligand is important as the ligand must not be toxic to the cells under study in the range of ligand concentrations required for imaging. The nature of the ligand-fluorophore conjugate is also important because it should ideally readily enter the cells in order to be present in high enough concentrations in the majority of cells for achieving a FRET signal. A barrier to cell entry can come from the ligand or the conjugated fluorophore. For example, the Cy3 and Cy5 precursors for conjugate formation can be purchased as the sulfonated (Fig. 5A) or nonsulfonated (Fig. 5B–D) forms. The additional negative charge on the sulfonated ligands will reduce cell uptake (Fig. 4B) such that fewer cells in the population can be analyzed for FRET. Once in the cell, the ligand conjugate should interact as little as possible with cellular components other than the aptamers as this interaction may increase the background FRET and/or compete with the IMAGEtags for binding their ligands. Another very important

property of aptamer ligand conjugates is their toxicity. Some aptamers must be ruled out as IMAGETags due to the toxicity of their ligands (Muslum Ilgu, et al., 2016; Kraus et al., 2008). We have found that Cy3 and Cy5 conjugated ligands can be effectively used in a concentration range in which they are not toxic. The fluorophores are commercially available in forms that can be readily linked to a variety of aptamer ligands. Other FRET pairs could be incorporated into the IMAGETag protocol. However, it is important that the chosen fluorophores do not interact with the RNA independently such that their fluorescence is quenched. For example, guanine quenches the fluorescence of many fluorophores, but not Cy3 and Cy5 (Torimura et al., 2001).

The commercial sources of some reagents are listed for the syntheses described next. All other reagents were of reagent grade or higher and obtained from Sigma-Aldrich.

Synthesis of Cy5- and Cy3-tobramycin

1. Prepare a 20 mL vial equipped with a stirring bar and wrapped in aluminum foil to ensure complete darkness during the procedure
2. In the vial mix Cy3 NHS ester (1 equiv.), aminoglycoside (1.1 equiv.), and *N,N*-diisopropylethylamine (20 equiv.) and DMSO (1.0 mL). Tobramycin was from Sigma (cat #T4014). The sources of NHS esters are: GE Healthcare Life sciences (Cy3 NHS ester cat # PA13105 and Cy5 NHS ester, cat# PA15100) both sulfonated) and Lumiprobe (Cy3 NHS ester cat #41020 and Cy5 NHS ester #43020, both not sulfonated).
3. Stir at room temperature under an argon atmosphere in the dark for 12 h.
4. Purify by preparative TLC using a 5:4:1 mixture of DCM:MeOH:NH₄OH to resolve the product ($R_f = 0.4$).
5. Evaluate by mass spectrometry with the following results expected: 1) Cy5-Tobramycin: HRMS: calculated for C₅₀H₇₄ClN₇O₁₀ = 968.63, found: 967.5867 ($M^+ - H$); 2) Cy3-Tobramycin: HRMS: calculated for C₄₈H₇₂ClN₇O₁₀ = 942.59, found: 875.5918 ($M^+ - CH_2OH$) 3) Cy5-SO₃-Tobramycin: MS (ESI): m/z = 1104.46 (M^-); HRMS: calculated for C₅₁H₇₄N₇O₁₆S₂ = 1104.4633, found: 1104.4606; 4) Cy3-SO₃-Tobramycin: MS (ESI): m/z = 1078.44 (M^-); HRMS: calculated for C₄₉H₇₂N₇O₁₆S₂ = 1078.4477, found: 1078.4447.

Synthesis of Cy5-PDC

1. Prepare a 20 mL vial equipped with a stirring bar and wrapped in aluminum foil to ensure complete darkness during the procedure
2. In the vial combine Cy5-NHS ester (Jung & Kim, 2006), (50.0 mg, 71.0 μ mol), PDC-Gly (Kraus, Gupta, Mokhtarian, Mehanovic, & Nilsen-Hamilton, 2010) (34.0 mg, 71.0 μ mol) and triethylamine (0.5 mL) and DMF (5.0 mL).
3. Stir at room temperature under an argon atmosphere in the dark for 12 h
4. Purify by preparative TLC using 10% MeOH:DCM to give the pure product in 66% yield ($R_f = 0.3$).

5. Evaluate by mass spectrometry with the following results expected: MS for $C_{55}H_{59}Cl_2N_8O_3$: MS (m/z): 947.5 ($M^+ - 1$), 497.4, 383.3.

5.4.3 Synthesis of Cy3-PDC-Gly

1. Prepare a 20 mL vial equipped with a stirring bar and wrapped in aluminum foil to ensure complete darkness during the procedure
2. In the vial combine Cy5-NHS ester Cy3-NHS ester (Jung & Kim, 2006), (30.0 mg, 43.0 μ mol), PDC-Gly (Kraus, et al., 2010) (25.0 mg, 52.0 μ mol) and *N,N*-diisopropylethylamine (0.1 mL) in DMF (5.0 mL)
3. Stir at room temperature under an argon atmosphere in the dark for 12 h
4. Purify by preparative TLC using 10% MeOH:DCM to give the pure product in 72% yield ($R_f = 0.3$).
5. Evaluate by mass spectrometry with the following results expected: 1) Cy5-PDC-Gly: $C_{55}H_{57}Cl_2N_8O_3^+$, MS (m/z): 947.5 ($M^+ - 1$), 497.4, 383.3; Cy3-PDC-Gly: MS (m/z): 923.3910 ($M^+ + 1$), 471.2992; HRMS: calculated for $C_{53}H_{55}Cl_2N_8O_3^+$: 923.3931, found: 923.3915.

Cloning repetitive sequences

IMAGEtags can be created from many different aptamers for which the ligand is small and can enter the cell. However, cloning repetitive sequences can be challenging, in part because of the probability of circularization of the enlarging inserts. We have developed a successful method of cloning repeated sequences that is based on previously reported methods (Meyer & Chilkoti, 2002; Won & Barron, 2002). Although repeated sequences have been reported to be genetically unstable, we have found that most of the sequences cloned using the method described here were genetically stable in *E. coli* in which the plasmids are maintained (Shin, et al., 2014).

Prior to cloning a repetitive sequence of aptamers as an IMAGEtag, it is important to check the likelihood of the aptamers folding appropriately in the string to enable each aptamer to retain a structure capable of binding ligand. A co-transcriptional folding program should be used to simulate the folding of an IMAGEtag *in vivo* (Fig. 6A).

Cloning initial repetitive aptamer sequences from synthetic oligonucleotides

1. Digest pZerO-2 vector with *Afl*III (New England Biolabs-NEB, Ipswich, MA, Cat# R0541S) and *Apo*I (NEB, R0566S) restriction enzymes and isolate the 1854 base pair fragment using gel electrophoresis followed by extraction and purification by a method such as the QIAquick gel extraction kit (QIAGEN, Valencia, CA, Cat# 28704).
2. Use two pairs of complementary oligonucleotides to create inserts containing between two and seven repeats (150–250bp) of the sequences flanked by restriction sites for *Bsa*I (NEB, R0535S) and *Bsm*AI (NEB, R0529S) for subsequent multiplication (Fig. 6B).

3. Anneal the complementary oligonucleotides by slow cooling from 95°C to 25°C and phosphorylate using T4 polynucleotide kinase (Promega, Cat# M4101).
4. Ligate the double-stranded inserts into the digested vector with T4 DNA ligase (Promega, Cat# M1801) and transform into *E. coli* chemically competent strain NEB 5-alpha (NEB, Cat# C1987I) by heat shock for 1 min at 42°C.

Constructing longer repetitive sequences

1. Use *Bsm*AI to cut the initial repetitive sequences from the vector and purify the excised fragment by agarose gel electrophoresis.
2. *Bsa*I cuts at a single site at the 5' end of the multiaptamer, leaving the aptamers in the vector. Dephosphorylate the *Bsa*I-digested vector to eliminate self-ligation, and ligate the excised multiaptamer fragment with *Bsm*AI into the cut vector.
3. Analyze the clones for inserts by sequencing in each direction with primers 2819 (5'-GGCCTTTTACGGTTCCTGG-3') and 2820 (5'-GCCGCTCCCGATTGCGAG-3'). Clones can also be analyzed by digestion with *Bsm*AI followed by agarose gel electrophoresis to establish the size of the insert.
4. In general, several plasmids are obtained that appear, by electrophoretic mobility, to have the expected insert sizes. The number of repeats in each plasmid is confirmed by sequencing.

Multiplying the original repetitive sequences

The restriction enzymes *Bsa*I and *Bsm*AI are used to multiply the initial set of aptamer repeats. The strategy is based on the fact that the recognition sequences of these restriction enzymes are not palindromic and they cut DNA beyond their recognition sequences. The recognition sequence for *Bsm*AI is part of the longer *Bsa*I recognition sequence. Thus, a sequence cut by *Bsa*I is also cut by *Bsm*AI but not *vice versa*. Consequently, a region between the two restriction enzyme sites can be cut from the vector by *Bsm*AI, while digestion with *Bsa*I only cuts the vector at one end of the insert. This allows the *Bsm*AI fragment to be ligated back into the same vector but that has been digested at a single site with *Bsa*I. The result is multiplication of the number of repeats in the insert from the first round of cloning (Fig. 6C).

With each round of expansion we can expect to obtain cloned inserts with double the number of repeats of the previous insert. In addition to the expected inserts, some clones contained inserts with 3, 4 and up to eight multiples of the inserts.

Additional rounds of cloning as just described can be used to further multiply the number of repeated sequences. As for the first round, the number of repeat sequences in the additional round products can be even multipliers of the original number of repeats. However, more than the expected repeats are sometimes found because this strategy allows the insertion of multiple fragments in a single ligation reaction. In addition, odd-numbers of repeated sequences are occasionally obtained when an even number was expected.

Conclusions

The IMAGEtag technique provides a relatively simple way of monitoring promoter activity in living cells without requiring the expression of exogenous fluorescent proteins. The IMAGEtag reporters do not influence cell physiology as evidenced by the lack of change in cell growth rate or housekeeping mRNA levels with expression of RNAs containing IMAGEtags (Shin, et al., 2014). Currently optimized for monitoring yeast gene expression as described here and previously reported (Shin, et al., 2014), the IMAGEtag method is being developed for application in other eukaryotic cells. The considerations in adapting the method to other cell types for optimal signal/noise include: 1) ligand permeability, 2) ligand toxicity, 3) the promoter employed for aptamer expression, 4) 3' UTR modifications that promote aptamer stability, and 5) the effect of the IMAGEtag and its position in RNA on the steady state level of the cellular RNA in which it is incorporated (Hogg & Collins, 2007; Said et al., 2009).

The IMAGEtag approach to tracking gene expression in living cells in real-time provides an opportunity to observe rapid changes in gene expression in individual cells as they interact with their neighbors or in cell populations as they respond to environmental cues. The tags are small and readily integrated into any RNA sequence. The application of FRET provides the sensitivity required to detect mRNAs in individual cells using standard confocal microscope equipment. The analysis of FRET is straightforward and can be automated for analyzing FRET associated with many cells in a field, even when the cells are moving.

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Figure 1. The IMAGEtag technology.

RNAs containing IMAGEtag sequences are transcribed by the cell (top panel) and, when presented with their fluorescently conjugated ligands (o-o), will bind their ligand allowing the fluorophores to interact in FRET (lower panel) that enables a unique image of the IMAGEtags over the background fluorescence.

Figure 2. FRET signal acquisition from IMAGEtags.

A) Yeast cells expressing 6xPDC IMAGEtag RNA were incubated with only Cy3-PDC (Donor). Cells were excited at 550 nm with a white light laser and emissions were read with a PMT2 setting at 560–626 nm (Cy3) and with a PMT4 setting at 660–753 nm (Cy5/FRET channel). **B)** Yeast cells expressing 6xPDC IMAGEtag RNAs were incubated with only Cy5-PDC (acceptor). The cells were excited at 650 nm and emission read at 660–754 nm (Cy5/FRET channel). **C)** Yeast cells expressing 6xPDC IMAGEtag RNA were incubated with both Cy3-PDC (donor) and Cy5-PDC (acceptor). The cells were excited first at 550 nm with a white light laser and emissions are shown for the Cy3 (560–626 nm) and Cy5/FRET (660–754 nm) channels. The cells were then excited at 650 nm and emissions are shown for the Cy5/FRET channel (660–753 nm). The DIC image is shown with a FRET overlay.

A. Population response

B. Population time course

C. Individual cells

Figure 3. IMAGEtags track temporal changes in RNA expression levels.

Yeast cells were grown in 2% raffinose containing SD-Uracil and induced with 2% galactose. **A)** FRET images are shown of two populations of yeast expressing either control RNA or 6xPDC IMAGEtags under the control of the GAL1 promoter. **B)** Quantification of the FRET from 8 cells expressing control RNA and 13 cells expressing IMAGEtag RNA. The control cell FRET decreased with time due to cumulative exposure to laser light. To obtain the FRET efficiency values for IMAGEtag-expressing cells, the average control FRET emissions per cell at each time point were subtracted from the FRET emissions of individual IMAGEtag expressing cells. The data is represented as the range of values (areas of color) with the mean value as a line within the range of values. The circles show the time course of increase in FRET for the single cell expressing IMAGEtags shown in C. **C)** Images of a budding yeast cell that expresses IMAGEtags (upper panel) or that expresses control RNA (lower panel) are shown as a function of time after addition of galactose to the culture. Shown are merged DIC and FRET images.

Figure 4. Tobramycin IMAGEtags and the impact of ligand conjugate charge on cell uptake. **A)** Yeast cells expressing either the control RNA or 5xTOB IMAGEtags were grown in 1% raffinose containing SD-Uracil and incubated for 30 minutes with Cy3-tobramycin and Cy5-tobramycin and 2% galactose. The cells were washed and FRET images were acquired. Quantification of the images is shown by the intensity profiles below each image. **B):** Yeast cells expressing TOB IMAGEtags were incubated with either Cy5-tobramycin or Cy5-SO₃-tobramycin for 90 minutes, washed and then imaged in the Cy5 channel. The DIC

images show the locations of the cells in the field. The DIC for the Cy5-SO₃ has been overlaid by the image to its left from the Cy5 channel.

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Figure 5.

Cy3 and Cy5 conjugated ligands for the PDC and tobramycin IMAGEtags

Figure 6. Cloning multiple tandem sequences.

A) IMAGEtag sequence design involves checking for folding during transcription. The result of folding the 5xTOB IMAGEtag RNA by KineFold is shown. **B)** The method for cloning multimers to create IMAGEtags starts with oligonucleotide inserts that contain the sequence of a series of 4 or more aptamers for the initial insertion by ligation into the pZERO vector, which has been minimized in size to allow for maximum insert sizes. **C)** Aptamer repeats are expanded using *Bsa1* to cut the vector and *BsmA1* to create a new fragment for insertion.