Imaging intracellular RNA distribution and dynamics in living cells

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Powerful methods now allow the imaging of specific mRNAs in living cells. These methods enlist fluorescent proteins to illuminate mRNAs, use labeled oligonucleotide probes and exploit aptamers that render organic dyes fluorescent. The intracellular dynamics of mRNA synthesis, transport and localization can be analyzed at higher temporal resolution with these methods than has been possible with traditional fixed-cell or biochemical approaches. These methods have also been adopted to visualize and track single mRNA molecules in real time. This review explores the promises and limitations of these methods.

The use of genetically encoded fluorescent tags in live cells has revealed the fascinating dynamics of the expression and subcellular distribution of many proteins. *In vivo* imaging of mRNAs is less common, even though mRNAs undergo similarly intricate dynamics. The richness of mRNA dynamics is evident in the exquisitely timed and tightly controlled processes by which mRNAs from different genes are produced and degraded, in their export from the nucleus to the cytoplasm, and in their sorting and localization into different regions of the cytoplasm.

Much of the knowledge about intracellular RNA dynamics has come from either *in situ* hybridization in fixed cells or from the biochemical fractionation of subcellular components. However, these methods provide a static picture at the time of fixation or fractionation. In contrast, live-cell imaging methods promise a finer temporal and spatial resolution of RNA dynamics and powerful new analytical possibilities. Among the possibilities are selection, sorting and expansion of fractions of cells that have a particular gene expression pattern; perturbation of cells while observing the dynamics and transport of their mRNAs (for example, electrically stimulating neurons while studying mRNA traffic in their processes); and studies of how mRNA dynamics is correlated with protein or organelle dynamics (for example during a viral infection).

If intrinsically fluorescent RNA motifs existed, it would be simple to use them as genetically encoded tags for RNA imaging, but none have been discovered thus far. Instead, GFP-tagged proteins that bind to specific RNA motifs have been adopted for mRNA imaging. A second approach is to use sequence-specific oligonucleotide probes whose fluorescence changes upon binding to natural mRNA targets. A third approach, currently under development, is to tag the target mRNA with an RNA motif that binds to an externally provided small, nonfluorescent dye that becomes fluorescent upon binding to the target. While wrinkles in these methods are still being ironed out, as recently reviewed[1–3], they already yield new insights into the cell biology of mRNA. This review will describe the strengths and limitations of present methods and explore the promise of new methods that are under development.

IMAGING mRNAs WITH GFP

Tagging mRNAs with intact GFP

MS2 system. To tag an mRNA, an RNA-binding protein is fused to GFP and is expressed along with a target mRNA tagged with an RNA motif with which the RNA-binding protein can associate. An ideal RNA-binding protein for this purpose should bind to the cognate RNA motif with great specificity and affinity and neither the protein, nor the RNA motif, should be naturally found in the cells to be imaged. Early on, the coat protein of bacteriophage MS2, which binds to a unique hairpin in the genomic RNA of the phage with a strong affinity (dissociation constant, 5 nM)[4], was identified as an ideal tag. This protein and its cognate RNA motif have evolved

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to avoid cross-reactions with the vast universe of RNAs and proteins that are present in its *Escherichia coli* host. To image an mRNA, multiple copies of the phage hairpin motif are introduced into the untranslated region of the gene encoding the target mRNA, and the engineered mRNA is expressed in the cell along with the MS2 coat protein fused to GFP. The consequent binding of multiple copies of the MS2 coat protein–GFP to the target mRNA renders the mRNA considerably more fluorescent than the surrounding region of the cell, enabling its detection (**Fig. 1a**,**b**).

Since its introduction for imaging *ASH1* mRNA in the cytoplasm of yeast[5,6], this method has been used to image many different mRNAs in diverse biological contexts[2,3]. For example, it has been used to study the transport of *nanos, oskar, gurken* and *bicoid* mRNAs in oocytes of fruit flies[7–10], and *CamK* mRNA and *Arc* mRNA in the dendrites of hippocampal neurons[11,12]; to study transcriptional bursts at a gene locus in *Dictyostelium discoideum*[13]; to confirm the localization of many mRNAs identified in an intracellular localization screen[14] (**Fig. 1b**); to track the dispersal of single molecules of mRNA in protein complexes from the sites of their synthesis in *E. coli* and in mammalian cells[15,16]. Furthermore, a general method to tag and image any mRNA with the MS2 hairpin has become available in yeast[17].

λ_N system. Recently another RNA motif derived from bacteriophage λ, called boxB, and a 22-amino-acid peptide called λ_N that tightly and specifically binds to boxB RNA have been used[18]. As an alternative to the MS2 system, this development provides an opportunity to image two mRNA species simultaneously, as has been shown recently by studying the mobility of boxB-tagged *ASH1* and MS2 hairpin–tagged *IST2* mRNA in yeast[19].

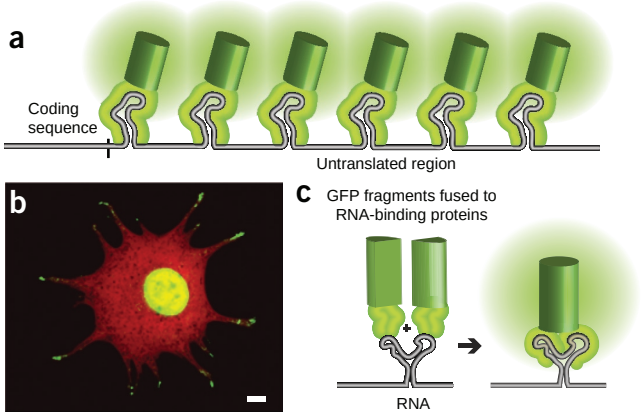

Figure 1 | Enlisting GFP to image mRNAs. (**a**) Schematic representation of the MS2 coat protein–GFP method. A tandem array of MS2 coat protein–binding hairpins is introduced into the 3′-untranslated region of the target mRNA. An MS2 coat protein–GFP molecule binds to each motif and renders that mRNA more fluorescent than the surrounding medium. (**b**) Fluorescence image of a fibroblast expressing MS2 coat protein–GFP and an mRNA containing a *RAB13* untranslated region (that directs the mRNA to cellular protrusions), and 24 copies of the MS2 coat protein–binding hairpin. The red marker outlines the cell. Scale bar, 10 μm. (Image is reprinted from ref. 14). (**c**) Eliminating the fluorescence background signal by using the split-GFP approach. Two motifs that bind to two different RNA binding proteins are placed next to each other on the target mRNA. The binding of two RNA binding proteins fused with two 'halves' of GFP in proximity on the target RNA mediates the assembly of functional GFP.

Poly(A)-binding protein. Some highly abundant mRNAs can be imaged without an MS2 hairpin tag if the cell produces a protein that specifically associates with them. This has been demonstrated by fusing GFP to poly(A)-binding protein to image the mobility of the general pool of mRNAs that are normally polyadenylated in mammalian cells[20].

Challenges in tagging with intact GFP. As GFP constructs are always fluorescent, GFP bound to target mRNA needs to be distinguished from unbound GFP. This is done by either expressing low amounts of the GFP fusion protein; by introducing a relatively large number of MS2 motifs into the target mRNA; or by attaching, for example, a nuclear localization signal to the GFP-fusion protein so that its unbound form resides in the nucleus rather than the cytoplasm where the mRNA is to be imaged[21]. However, the result then needs to be interpreted as the product of two opposing forces that would simultaneously operate on the mRNA GFP complex: the nucleocytoplasmic transport system operating on the nuclear localization signal in GFP would tend to draw the complex into the nucleus, whereas the mRNA export system would tend to draw it out of the nucleus. This tug of war may unduly influence the export of mRNA.

RNA-mediated reconstruction of GFP

It is desirable that the fraction of the GFP construct that is not bound to the target mRNA be nonfluorescent. This has recently been accomplished by adopting the 'split GFP' approach, based on the demonstration that GFP and related proteins can be split into two nonfluorescent fragments. The two fragments do not bind to each other, but if a pair of tags that have an affinity for each other are attached to these GFP fragments, they can assemble, producing a correctly folded two-part protein that is fluorescent[22].

To adopt this method for the detection of mRNAs, the two halves of the fluorescent protein are fused to two different RNA-binding proteins that bind strongly and specifically to two different RNA motifs, which are themselves placed at adjacent locations in the target mRNA. When the engineered RNA containing the two motifs is coexpressed with RNA-binding proteins fused to GFP fragments, the proteins bind to their target motifs, and owing to their proximity, reconstitute a fluorescent GFP (**Fig. 1c**). If the target mRNA is not present, no fluorescence is seen in the cell.

Three different groups have demonstrated the efficacy of the split GFP approach[23–25]. Their methods differ in the choice of RNA motifs and in the choice of the pair of proteins that are used to bind to these motifs.

MS2 coat protein and zip code–binding protein. In this approach, an MS2 coat protein–binding motif and a 'zip code'–binding motif derived from 3′ untranslated region of β-actin mRNA are introduced into an artificial mRNA construct. This RNA is then coexpressed along with two GFP fragments fused to the MS2 coat protein and the zip code–binding protein[23]. This approach has been used to detect a firefly luciferase reporter mRNA in Cos-7 cells[23].

eIF4A domains. A single RNA aptamer was isolated and used to bind to eukaryotic initiation factor 4A (eIF4A)[24]. eIF4A is a dumbbell-shaped protein with two globular domains, each domain possessing a strong affinity for one side of the aptamer. eIF4A was then split, and one part fused to the N-terminal, and the other part to the C-terminal of the GFP. When the two fragments of eIF4A bind to either side of

the aptamer, the GFP halves are brought in close proximity, creating a functional GFP. This method allowed the imaging of *LacZ* mRNA and 5S rRNA in *E. coli*[24].

PUMILIO1. In a third embodiment, which has the potential to detect endogenous mRNAs, a unique RNA-binding protein, PUMILIO1 is used[25]. This protein binds to stretches of RNA in a sequence-specific manner rather than recognizing secondary structures, which is the norm for RNA-binding proteins. The sequence of this protein can be engineered to alter its target-sequence specificity in a predictable manner. Two varieties of PUMILIO1 were created to bind to two adjacent eight-nucleotide stretches of an endogenous mRNA encoding NADH dehydrogenase subunit 6 and image it in the mitochondria of HeLa cells[25].

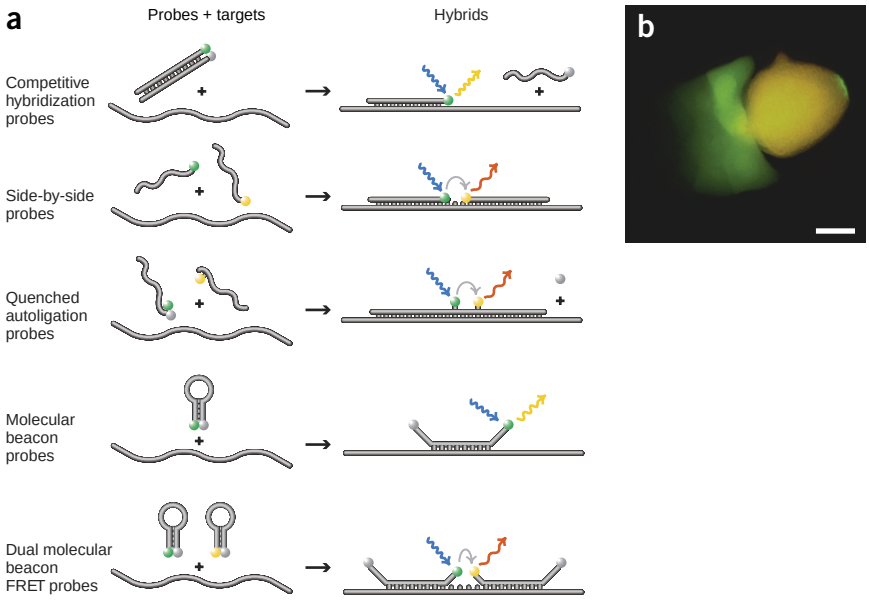

Figure 2 | Detecting mRNA with hybridization probes. (**a**) Schematic representation of different fluorogenic probes for the detection of mRNAs. Donor (green), quencher (gray) and acceptor (yellow) dyes are attached to the probes and interact as indicated. (**b**) Imaging native mRNA in a *Drosophila melanogaster* egg with molecular beacons (S.T.; unpublished data). *oskar* mRNA–specific molecular beacon (green) detects *oskar* mRNA in nurse cells where it is synthesized and at the posterior tip of oocytes where it accumulates. Control molecular beacon (orange) has no specific target. Scale bar, 50 μm.

Challenges in split GFP–based imaging. A potential limitation of the split GFP approach, pointed out by *in vitro* studies[26], is in the 'metastability' of the reconstituted GFP: once the two halves have assembled into a productive conformation, they do not dissociate, even if the molecule that initially brought them together disappears. As a result, it may be difficult to use this approach to image fast dynamic processes related to the synthesis and degradation of mRNAs. This concern is mitigated by observations in live cells in which dynamic events related to cytoskeletal dynamics could be recorded[27]. Furthermore, it may be possible to overcome this problem by using a GFP variant with a shorter half-life.

Advantages of GFP tags

As both the target mRNA and the reporter proteins are genetically encoded in the two approaches discussed above, stable transgenic cell lines and organisms can be engineered once and then be imaged over and over again. mRNA transport and dynamics can be studied in cells located in different organs and expressed during different stages of development.

Limitations of GFP tags

Engineered genes could be expressed in abnormal amounts; the binding of many MS2 coat protein–GFP molecules to target mRNAs may considerably impact their behavior; the genetic manipulations are often not practical for studies that require imaging unmodified endogenous mRNAs. Whereas MS2 coat protein–binding sites can be inserted relatively easily into endogenous loci in yeast, this is much more difficult in higher eukaryotes. The multiplexing potential of GFP tags is low owing to limited availability of well-characterized high-affinity RNA motifs; and the background fluorescence is high unless the split-GFP approach is used or GFP is targeted to a specific cellular compartment.

IMAGING ENDOGENOUS mRNAs WITH HYBRIDIZATION PROBES

To image endogenous mRNAs, several different 'fluorogenic probes' (whose fluorescent properties change upon sequence-specific hybridization) have been used (**Fig. 2a**). To obtain a signal that only occurs when the probes hybridize to their target RNA, these technologies use label moieties that interact either by fluorescence resonance energy transfer (FRET) or by contact-mediated quenching (**Box 1**).

Competitive hybridization. In one of the earliest of these methods, called 'competitive hybridization', a small double-stranded oligonucleotide is used, in which a fluorophore is attached to the 5′ end of one strand and a fluorescence quencher is attached to the 3′ end of the other strand[28]. The target-complementary strand bears the fluorophore, which remains quenched in the double-stranded state. However, as small double-stranded oligonucleotides exist in dynamic equilibrium with their component strands, the target mRNA, if it is present, gradually displaces the strand possessing the quencher, binds to the strand possessing the fluorophore and is labeled in the process. The kinetics of target labeling are rather slow, as it is a second-order process. However, these kinetics can be improved by making the target-complementary strand a little longer than the quencher strand, thereby creating an overhang that binds rapidly to the target, resulting in the ejection of the quencher oligonucleotide by strand displacement[29].

When competitive hybridization probes were initially used for live-cell mRNA detection in 1994, the detection of endogenous mRNAs proved elusive, but it was possible to detect synthetic oligonucleotides that had been injected into cells, showing that the intracellular environment is conducive for hybridization[30].

Side-by-side probes. The side-by-side probe–based method also uses a pair of labeled oligonucleotides, but both oligonucleotides are designed to bind to the target mRNA at adjacent positions. Two interactive fluorophores are used, one placed at the 5′ end of one probe and

BOX 1 LABELS IN PROBES INTERACT VIA FRET OR BY CONTACT

In various fluorogenic probes, the hybridization of the probes to the mRNA targets either split apart or bring together a pair of interactive dyes attached to the probes. This restores the fluorescence of a quenched fluorophore or allows resonance energy transfer (FRET) between a donor and an acceptor fluorophore residing on separate probes (**Fig. 2**).

FRET and quenching in oligonucleotide probes are related phenomena. In FRET, a fluorophore transfers the energy that it receives from the incident light to a nearby acceptor molecule, usually another fluorophore, which then emits the received energy as light of a longer wavelength. The efficiency of energy transfer is high when the emission spectrum of the donor substantially overlaps with the absorption spectrum of the acceptor and when the distance between them is 20–100 Angstroms. The efficiency drops steeply with increased distance[64]. At an optimal distance, emission from the donor is suppressed and emission from the acceptor is enhanced, leading to a shift in the color of the emitted light toward red. However, if the distance between the fluorophores is less than 20 Angstroms, emission from both dyes is suppressed, leading to a reduction in the intensity of emission but usually no change in the color[78]. The dye pair can absorb energy from light but then loses it as heat—a phenomenon often referred to as contact quenching[36,78,79]. The efficiency of contact quenching is independent of spectral overlap, which is an immensely useful feature for the construction of multicolor probes[36]. A reduction in light intensity is sometimes preferred over a change in color, as the analysis is simpler and more extensive multiplexing becomes possible. A reduction in light intensity instead of a color change can be obtained in FRET as well, through the use of nonfluorescent dyes as acceptors, which release the energy that they receive as heat rather than as light[36,80].

The blunt ends of competitive hybridization probes and molecular beacons bring the attached dyes so close to one another that quenching occurs predominantly by contact, whereas in side-by-side probes the target sequences for each of the probes is chosen so that the dye-bearing ends of the probes will be separated by a few nucleotides, so that they are close enough for FRET to occur, but not so close that quenching occurs by contact (**Fig. 2**). Dual molecular beacon FRET probes and quenched autoligation probes can use either contact quenching or FRET for mRNA detection.

the other placed at the 3′ end of the other probe, so that the interactive fluorophores are in close proximity to each other when the probes are hybridized to the target, enabling them to undergo FRET. Thus, only target-bound probes exhibit FRET leading to the detection of the target[31]. Side-by-side FRET probes were used successfully to image *c-fos* mRNA expressed from a plasmid in Cos7 cells[32].

In a variant of this scheme, called 'quenched autoligation', the 5′ end of one probe (containing a FRET donor and a quencher) is placed in the immediate vicinity of the 3′ end of the other probe (containing the FRET acceptor) when both are hybridized to the target. The two ends are functionalized such that they 'self-ligate' when in close proximity. Upon ligation, the quencher is severed from the probe, leading to the unquenching of the donor fluorophore, which transfers its energy to the acceptor fluorophore, whose emission is recorded[33,34]. Quenched autoligation probes have been used to detect ribosomal RNA in mammalian and bacterial cells[34].

Molecular beacons. These internally quenched oligonucleotide probes have small complementary sequences on either end, enabling the molecule to assume a hairpin configuration in which a fluorophore and quencher are held in close proximity. The formation of a probe-target hybrid disrupts the hairpin stem, removing the fluorophore from the vicinity of the quencher, restoring the probe's fluorescence and revealing the presence of the target[35–37]. To improve specificity, two molecular beacons that bind to adjacent locations on their target can be used to generate a FRET signal[37,38].

Molecular beacons have been used to image the distribution and movement of various RNAs in several different biological contexts. For example, *oskar* mRNA in fruit fly oocytes[37] (**Fig. 2b**); influenza virus mRNA in canine kidney epithelial cells[39]; β-actin mRNA in motile fibroblasts[40]; bovine respiratory syncytial virus RNA in bovine turbinate cells[41]; respiratory syncytial virus RNA in Vero cells[42]; *VegT* and *Xlsirts* mRNAs in *Xenopus laevis* oocytes[43]; polio virus RNA in Vero cells[44]; individual mRNA molecules of an artificial construct in the nucleus of Chinese hamster ovary cells[45]; and cell-to-cell dissemination of coxsackievirus RNA in cultured monkey kidney cells[46].

Singly labeled probes. Forgoing the benefits of higher signal-to-background ratios provided by the use of quenched fluorescent probes, oligonucleotides labeled with only a single fluorophore have also been used to image mRNAs in live cells. In these studies, just enough probes are introduced into the cells to saturate the available mRNA pool. For example, oligo(dT) or oligo(rU), labeled with a single fluorophore, have been used to bind to nuclear mRNAs and to study their mobility by fluorescence correlation spectroscopy or by fluorescence recovery after photobleaching[47,48]. Similar studies have enabled the labeling and tracking of a large mRNA, called Balbiani ring mRNA, that contains many repeat sequences, which serve as targets for the probes[49]. Another single-label strategy is to attach an intercalating dye to an oligonucleotide probe, with the idea that when the probe hybridizes to its nucleic acid target, it will provide an opportunity for the dye to intercalate in the target in a manner that markedly increases the fluorescence of the dye[50].

Challenges in probe-based imaging

Ideally, a probe for an intracellular target should be easy to deliver inside the cell; it should be stable once it is inside the cell; after binding, it should not destroy its target or otherwise perturb its function; it should be distributed homogeneously within the cell; and it should yield a signal only when and where the target is present. As different fluorogenic probes have been used for mRNA imaging, problems have been encountered on each of these accounts, resulting in the development of various solutions.

Introduction of probes into cells. Being medium-sized hydrophilic molecules, oligonucleotide probes cannot freely traverse plasma membranes. Among the techniques used to deliver probes across the plasma membrane barrier are microinjection[37,43,45], transfection

with cationic lipids such as Oligofectamine[51], membrane permeabilization using pore-forming agents such as streptolysin-O[38,52,53], the use of cell-penetrating peptides such as the HIV-derived peptide Tat[46,54,55] and electroporation[53]. Although microinjection is effective and yields high-quality data, it is tedious and can be used to deliver probes to only a few cells. Lipofaction, pore-forming agents and cell-penetrating peptides, in contrast, can be used to deliver probes into a large number of cells.

Subcellular distribution of probes. When probes of different backbone chemistries, labels and conformations are introduced into cells, they are not homogenously distributed. Free probes enter the nucleus through nuclear pores, perhaps passively, but once inside the nucleus, they are held there by nucleic acid binding proteins that are abundant in the nucleus[40,51,56]. In fact, the sequestration of probes in the nucleus is so rapid and thorough that if the probes are introduced into the cell in a single pulse, as occurs during microinjection, they do not have enough time to bind to cytoplasmic mRNAs before being sequestered in the nucleus[40,51].

This problem can be circumvented by delivering the probes slowly at a steady pace, for example, by including probes in the growth medium and using streptolysin-O or penetrating peptide to ferry the probes across the cell membrane. Nuclear sequestration of the probes can be turned into an advantage to label nascent mRNA in the nucleus and detect it in the cytoplasm (which is devoid of background signals) after export. When it is absolutely necessary to maintain a high concentration of probes in the cytoplasm, bulky moieties, such as streptavidin[32,40,53] or quantum dots[53] that cannot go through nuclear pores or tRNA transcripts[51] that localize in the cytoplasm, can be attached to the probes.

Stability of probes in the cell. Oligodeoxynucleotides with natural backbones are degraded rapidly inside the cell. The degradation products, including the fluorophores, are cleared from the cells with a half-life of 15–30 min[57]. Furthermore, hybrids formed by oligodeoxynucleotides and RNAs are targets for RNase H, a ubiquitous enzyme in the cell that degrades mRNAs in RNA-DNA hybrids[58].

Synthesizing the probes from modified nucleotides such as 2′-*O*-methylribonuclotides solves these problems: they are stable against nucleases, and the hybrids that they form are not digested by RNase H[37]. Probes with phosphorothioate backbones are also stable against nucleases, but their hybrids remain vulnerable to cellular RNase H digestion[59]. 'Locked nucleic acids' also show improved stability inside the cells[60]. However, it is important to recognize that backbone modifications alter the thermodynamics of probe hybridization. For example, the presence of phosphorothioate linkages decreases the affinity of a probe for its target, whereas the presence of 2′-*O*-methylribonucleotides and locked nucleic acid linkages increases a probe's affinity for its target.

Target selection. The target sequence in an mRNA may not be available for the binding of a probe owing to the presence of secondary structures in the RNA, or because of the presence of RNA binding proteins. Even though it is typical for an mRNA in a cell to have many proteins simultaneously bound to it, secondary structures are the greater impediment for the binding of the probe. A target region surrounded by strong hairpin stems is more difficult to access than a target region that is bound to a protein because intramolecular stems are more difficult to break apart than the weaker intermolecular associations between proteins and mRNAs. Underscoring this issue, a microarray-based study revealed that only about 5% of all possible oligonucleotide probes efficiently bound their complements in a full-length mRNA[61].

RNA folding algorithms improve the odds of designing a probe that will be able to access its target. In one procedure, the alternative folding patterns predicted by the Zuker RNA folding program (http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi/) had been analyzed to identify long stretches of sequence that remain single-stranded in most suboptimal folding[62].

Advantages of hybridization probes

Natural mRNAs can be imaged without needing to engineer genes for the tagged mRNA and GFP construct; a relatively smaller mass is added to the target mRNA; the system has higher multiplexing potential, limited only by number of dyes that can be spectrally distinguished; and sorting of cells based on mRNA expression is possible.

Limitation of hybridization probes

The sensitivity is relatively lower than that of GFP tags, which may be responsible for their slow adoption by cell biologists. Strictly on a per-molecule basis, organic fluorophores used in hybridization probes are expected to yield higher fluorescence than GFP reporters because their extinction coefficients (a measure of how much light a fluorophore collects) and quantum yields (ratio of light released to light absorbed) are higher. For example, the extinction coefficients and quantum yields of enhanced GFP and fluorescein, two commonly used reporters, are 56,000 and 79,000 cm^{-1} M^{-1}, and 0.6 and 0.92, respectively[63,64]. In practice, however, the MS2 coat protein–GFP system yields higher signal intensities. This is because a tandem array of 6–24 copies of MS2 hairpins is usually tagged to the RNA and each MS2 coat proten–GFP binds as a dimer to the hairpin. The hybridization probes have only one fluorophore in each molecule, which results in lower overall signals. These signals can be improved by either using multiple unique probes for the same target or by using one probe that binds to a repeated sequence in the target. Among other limitations are: need of delivering probes into cells, degradation of probes unless modified backbone is used, accumulation of probes in the nucleus and prevention of probe binding by mRNA secondary structures.

APTAMER TAGS THAT RENDER DARK DYES FLUORESCENT

To develop genetically encodable RNA reporters that do not rely on GFP constructs, several groups are pursuing a new strategy in which artificial RNA motifs (aptamers) are included in the RNA that can stably bind to small, cell-permeant, nonfluorescent dyes, whereupon the bound dyes become fluorescent (**Fig. 3**).

The principle of aptamer-induced fluorescence generation is based on the understanding that fluorescence is an unlikely property of a select group of dyes whose electronic configurations allow them to absorb energy from light, store it briefly and then re-emit the energy as light of a longer wavelength. Dyes possessing constituents that are free to rotate or vibrate are usually nonfluorescent because these motions dissipate stored energy as heat. However, restricting these motions by binding to larger molecules enables the dye to fluoresce[65]. For example, an aptamer originally selected for its ability to bind to the triphenylmethane dye, malachite green, increases the dye's fluorescence more than 2,000-fold[65] and binding of an antibody to the same dye renders it 18,000 times brighter[66].

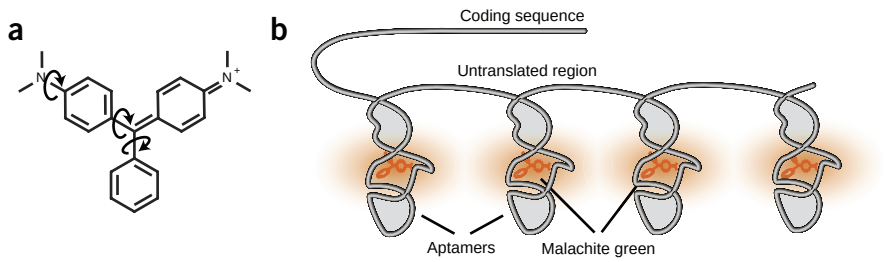

Figure 3 | A scheme for detection of mRNA using malachite green and an aptamer that binds to it and renders it fluorescent. (**a**) The chemical structure of malachite green. Malachite green is nonfluorescent because each of the phenyl rings in the triphenyl structure can rotate like a propeller and dissipate the energy that the dye absorbs from light. (**b**) Schematic representation of an mRNA with aptamer motifs bound to malachite green in its untranslated region.

An alternative strategy is to start with a brightly fluorescent molecule, render it nonfluorescent by the introduction of an energy dissipating group and then restore its fluorescence using an aptamer that binds to the group[67]. Following a simlar approach, Hoechst dye, a commonly used stain that becomes fluorescent after binding to DNA, has been modified to suppress its ability to bind to DNA, and an aptamer was selected to bind to it. This aptamer could serve as an effective RNA tag in combination with the Hoechst dye variant[68,69]. The availability of many apatmer dye combinations will allow imaging of multiple mRNAs simultaneously.

Once fully developed, the aptamer tagging will also permit stable expression and imaging of reporters in cell lines and small organisms. The advantages of this approach over GFP tagging are that fluorophore creation is faster than the maturation of GFP and there is no need to co-express GFP constructs. The advantage over hybridization probes is that the dyes permeate the cells more readily. A possible disadvantage of the malachite green approach (not relevant for other dyes), is that irradiation of malachite green creates free radicals that can destroy the RNA motif to which it binds[70].

IMAGING SINGLE mRNA MOLECULES

Imaging mRNAs with GFP and hybridization probes show their steady-state distributions. Although fluorescence recovery after photobleaching can be used to study the underlying dynamics of these distributions, it measures the average mobility of molecules in a defined subcellular zone and overlooks the variations and directionality in their motions. Such insights can be obtained by directly visualizing and tracking individual mRNA molecules. The single-molecule tracking reveals the range of behaviors of the individual molecules and defines characteristics of the cellular matrix in which they travel. Furthermore, as only a few copies of most mRNAs are expressed in each cell, methods for the detection of individual mRNA molecules provide the only means of seeing where these RNAs go and how they get there.

Common methods for single-molecule detection, such as total internal reflection microscopy, require the targets to be in extremely close proximity to the glass surface to which the cells are attached (100 nm). As most mRNA-related activities occur deep inside the cell (such as in the nucleus), strategies have been developed in which the fluorescence output of each mRNA molecule is enhanced. These methods incorporate 48 or 96 tandem repeats of either an MS2 coat protein–binding site or a probe-binding site into the untranslated region of the target mRNA. When many GFP or probe molecules bind to the RNA through these repeated motifs, each mRNA molecule becomes so intensely fluorescent that it can be visualized as a fine diffraction-limited spot. The signal from these repeats is sufficiently intense to enable the high-speed tracking of mRNA molecules and the subsequent analysis of their motion.

Analysis of the tracks of individual molecules of mRNA complexed with their natural entourage of proteins as they move within the nucleus has provided powerful insights into the mechanism of dispersal of mRNAs from the sites of transcription into the nucleoplasm[16,45,49] (**Fig. 4**). These analyses indicated that they move via simple Brownian motion through interchromatin spaces. Similar tracking studies have revealed the dynamics of synthesis and dispersal of an mRNA from its gene locus in *E. coli*[15].

NEW DEVELOPMENTS ON THE HORIZON

The current state of the art already allows the imaging of genetically tagged and natural mRNAs in many biological situations. Among additional desirable capabilities are the imaging of several different mRNAs at the same time in the same cell, improved sensitivity of detection, improved methods of probe delivery, new reporters to visualize the relatively fast dynamics of mRNA synthesis and degradation, and the capability to detect particular mRNAs in the organs, tissues and tumors of whole animals and people.

Imaging multiple mRNAs simultaneously with fluorescent protein–based methods requires several different fluorescent protein reporters, each with distinct excitation and emission spectra, and several RNA-binding proteins with different RNA motifs as their targets. The former set is already available in various hues of fluorescent proteins and the latter may be selected from a large set of known

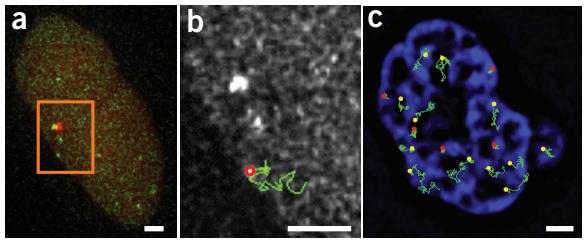

Figure 4 | Tracking single mRNA molecules in live cells using MS2-GFP and molecular beacons. (**a**) Individual mRNA molecules containing 48 repeats of the MS2 coat protein–binding hairpin bound to MS2 coat protein–YFP are visible as green fluorescent spots in the nucleus of a mammalian cell. (**b**) Each molecule was tracked by marking its location through successive frames of a time-lapse series. The track of one molecule (green line) and its last position (red circle) are overlaid on the enlargement of the portion of the image in **a** indicated by the orange rectangle. (**c**) Tracks of individual molecules of a reporter mRNA containing 96 molecular beacon binding sites, expressed in a mammalian cell and probed with molecular beacons. The tracks of some of the mRNA molecules (green lines) are laid over the chromatin density map (blue) of a nucleus. Analyses of these tracks in the two systems suggest that mRNA molecules move relatively freely (yellow spots) in the interchromatin spaces and get stuck (red spots) when they enter high-density chromatin. The data for these images were obtained from refs. 16 and 45. Scale bars, 2 μm.

RNA-binding proteins. For example, the coat proteins of RNA phages related to MS2, such as Qβ or PP7, would be eminently suitable. These coat proteins and the RNA hairpins to which they bind are sufficiently different from one another that cross-talk between them is unlikely. Similarly, the split GFP approach can be extended to include reporters for additional mRNA species.

As most mRNAs are expressed as only a few copies in each cell, substantial improvements in the sensitivity of detection are needed to image them. Some of this gain in sensitivity will come from optimization of the imaging hardware, such as the use of more sensitive cameras, but the most important advances will come from the use of brighter, more photostable reporters now under development. While the search for new proteins with greater fluorescence intensity than GFP continues in organisms living deep in the ocean, optimization of the GFP sequence has yielded more photostable versions[71]. Furthermore, brighter fluorogenic hybridization probes are being developed that incorporate quantum dots and silver dots[72], which yield stronger fluorescence and are more photostable than conventional organic dyes. Although, initial attempts to use quantum dots in molecular beacons yielded only a modest increase in signal-to-background ratio, improvements are likely to follow[73,74].

Nature has found powerful means of delivering hydrophilic cargo across plasma membranes. Two well-known examples are exocytosis of neurotransmitters at synapses that occurs within a millisecond of a stimulatory impulse and the highly efficient entry of viruses into cells. These and other natural processes are inspiring researchers in a wide variety of fields to develop methods that will be adopted for the more efficient delivery of probes into cells.

To study the dynamics of mRNA synthesis and degradation, it is desirable to use probes that respond rapidly to the appearance of target mRNA and are then degraded or turned off when the target mRNA is degraded. A prelude to what may be possible is the direct observation of random bursts of synthesis at a gene locus in *Dictyostelium*, using the MS2 coat protein–GFP system[13].

A transition from cellular imaging of mRNAs to organismal imaging is also being considered. Using correlation of mRNA expression profiles with particular disease states, such as the aggressiveness of tumor growth, it should be possible to develop a new generation of highly specific diagnostic imaging techniques. In addition to using various fluorogenic probes in the relatively transparent near-infrared window[75], such imaging may use smart bioluminescent reporters that cells would create when a particular mRNA appears in the cell[76,77]. Creative solutions for traversing blood vessel and plasma membrane barriers for delivering such imaging agents *in vivo* are under development.

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The author declares competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemethods/.

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