Advances in fluorescent tracking of nucleic acids in living cells

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Nucleic acids are typically detected in morphologically preserved fixed cells and tissues using in situ hybridization techniques. This review discusses a variety of established and more challenging fluorescence-based methods for the detection and tracking of DNA or RNA sequences in living cells. Over the past few years, various fluorescent in vivo labeling methods have been developed, and dedicated microscope and image analysis tools have been designed. These advances in technologies indicate that live-cell imaging of nucleic acids is likely to become a standard research tool for understanding genome organization and gene expression regulation in the near future. Recent live-cell imaging studies have already provided important insights into the dynamic behaviors of chromatin and RNAs in the cell.

INTRODUCTION

In situ hybridization techniques, and in particular its fluorescence-based variants, are routinely applied in a wide range of disciplines including genetics, developmental biology, pathology, and cell biology to study genome composition or gene expression in cells. This is primarily possible due to the many improvements in probes, labels, hybridization protocols, and microscope systems that have been realized since the introduction of the in situ hybridization technique in 1969. Since then, in situ hybridization studies have led to important advances in our understanding of the organization and composition of the genome and its aberrations as well as of the expression and localization of gene transcripts. One should realize, however, that in situ hybridization approaches have been developed mainly for the detection of nucleic acids in fixed, morphologically preserved specimens and consequently provide static rather than dynamic views on nucleic acid localization. Furthermore, specimen preparation and nucleic acid denaturation may cause redistributions or loss of target nucleic acid sequences, and these artifacts may hamper the interpretation of in situ hybridization data (1–3). The most important reason, however, for implementing live-cell imaging techniques in current research was to obtain a better understanding of complex cellular processes, including chromatin organization and transcription regulation. Some of these techniques have been developed to enable the overall in vivo labeling of chromatin or RNAs to obtain a more general view on nucleic acid behavior within a living cell. Other techniques, however, were designed to specifically label defined chromatin regions or particular RNA species.

To detect and track specific endogenous RNAs in a living cell, in vivo hybridization-based techniques have been developed that make use of a variety of different nucleic acid probe types and fluorescent detection methods. In essence, all these techniques are developed to pursue optimal detection sensitivity and specificity. Particularly over the past few years, we have witnessed the development of various probe types showing improved affinity and specificity for target sequences and resistance to cellular nucleases. At the same time, it became clear that an efficient delivery of probes into living cells is also an essential step in the visualization procedure. Most methods that pursue this have been previously described (4) and are not discussed here in detail.

Parallel to ongoing developments in nucleic acid-based probe technologies, approaches have been developed that take advantage of fluorescent proteins that specifically interact with DNA or RNA sequences. These approaches have the advantage that the fluorescent (fusion) proteins are made by the cell's own transcription and translation machinery, precluding the need for invasive techniques that may have an impact on the physiology of the cell. This review focuses on the various methods that are used to image the localization and mobility of nucleic acids in living cells, addresses technical limitations, and provides an outlook for future developments.

IN VIVO HYBRIDIZATION

DNA or RNA molecules can be visualized in living cells by incorporating fluorescent nucleotides using the cell's own replication or transcription machinery (5–7) or by binding DNA- or RNA-associating fluorescent dyes such as the membrane-permeable dyes Cyto 14 (8), dihydroethidium (9), and

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DRAQ5™ (10). These methods result in an overall labeling of cellular DNA or RNA in vivo, but do not allow for the detection of defined DNA sequences or specific RNA molecules in living cells. Fluorescence in vivo hybridization has long been the method of choice to detect specific endogenous RNA species in living cells (4,11). This is technically possible because RNAs are, at least partly, single-stranded molecules. Detection of DNA sequences by fluorescence in vivo hybridization requires denaturation of the double-stranded DNA molecule, which is not compatible with live-cell studies. To date, a plethora of different probe types and detection concepts have been used to visualize RNAs in living cells.

Linear Phosphodiester Oligodeoxynucleotide Probes

Phosphodiester oligodeoxynucleotides (ODNs) can be easily synthesized and fluorescently labeled, are inexpensive, and hybridize specifically to complementary RNA target sequences in vitro. For these reasons, ODNs were the first to be employed for the detection and tracking of RNA species in living cells. Indeed, it was shown that fluorescein-labeled ODNs are efficiently taken up by cells and delivered to the cell nucleus (12). These observations were followed by pioneering studies by Pedersons' group with regard to the visualization of endogenous poly(A) messenger RNA (mRNA) movement within living cells using ODNs (13). The question to be answered was how the poly(A) RNAs would travel through the cell nucleus before they were released into the cytoplasm of cells. By applying fluorescence correlation spectroscopy and fluorescence recovery after photobleaching (FRAP) measurements, it was suggested that a vast amount of poly(A) RNA molecules moves randomly throughout the nucleus without the need for energy consumption (13). In subsequent studies, these findings were confirmed using similar probes, labeled with a nonfluorescent caged fluorescein that fluoresced upon irradiation (14,15). Furthermore, these studies suggested that poly(A) RNA moves through interchromatin channels, avoiding chromatin dense regions. Together, these observations indicated that mRNAs do not travel along a directed pathway from their site of synthesis toward the cytoplasm. Instead, it was suggested that it is only by the process of diffusion, thus a matter of change, that an mRNA molecule reaches a nuclear pore, after which it can pass through the nuclear membrane and enter the cytoplasm of a cell.

These studies on poly(A) movement have been followed up with studies on the movement of rRNAs using ODN probes. For example, endogenous 28S rRNA has been visualized within nucleoli (16) and, more recently, the movement of 28S rRNA out of the nucleolus has also been studied in detail (17). Assuming that all 28S rRNA assembles in nascent ribosomal subunits, it was concluded that these subunits move in all directions within the nucleus by means of diffusion before they reach the cytoplasm. These results suggest that diffusion is a more common mechanism by which components exit the nucleus.

Despite the importance of these findings, the main problem associated with the use of ODNs in living cells is the poor signal-to-noise ratio that may lead to the misinterpretation of results and may hamper detection of less abundant or more widely distributed RNA species. A solution to these problems has been sought in the application of fluorescence resonance energy transfer (FRET) to eliminate fluorescence signals derived from nonhybridized probe sequences. In this approach, two fluorescently labeled ODNs, each labeled with a donor or an acceptor fluorophore, are hybridized to adjacent locations on the same target RNA. If both probes are hybridized and the distance between the donor and acceptor fluorophores becomes less than 10 nm, the donor fluorescence emission will decrease and the acceptor fluorescence emission will increase. In addition, the fluorescence lifetime of the FRET donor decreases as a result of FRET. The changes in fluorescence intensities or lifetimes can be measured and visualized using appropriate imaging facilities (18–20). Tsuji and colleagues (21) were among the first to test this FRET approach for imaging nucleic acids in living cells by visualizing the expression of c-*fos* mRNA in transfected COS-7 cells. A little later, they visualized the presence of c-*fos* mRNA in HeLa cells by FRET, measuring acceptor fluorescence decays with a time-resolved fluorescence microscope (22). Disappointingly, however, the application of FRET did not remove the limitations associated with the use of ODNs in in vivo hybridization studies. In fact, Sixou et al. (23) had predicted in 1994 that there would be problems using ODN probes because the probes are prone to degradation and lack sufficient affinity for complementary RNA sequences in living cells. Furthermore, it should be noted that measuring FRET can be quite problematic and requires specific expertise. Recently, various factors that can impair the accuracy of FRET measurements, including cell movement, have been reviewed (20). Thus, there is still a serious need for better detection approaches to image and quantify RNA localization and dynamics in live cells.

PNAs, RNAs, or LNAs?

Having distinguished the shortcomings of ODN probes, alternative probe types have been tested to monitor RNA target molecules in living cells with higher signal-to-noise ratios. Peptide nucleic acid (PNA) probes, which have peptide rather than sugar-phosphate backbones, were thought to be good candidates because they were shown to form extremely stable hybrids with complementary DNA as well as RNA target sequences in vitro (24). Nevertheless, PNAs have not yet been extensively explored to probe specific RNAs in living cells. This could be because of economical reasons but also because PNAs are known to be rather rigid molecules (25) and therefore are expected to have poor access to highly folded RNA structures, precluding hybridization to complementary sequences. Interestingly, however, PNA is the only nucleic acid-like probe type to date that allowed the detection and tracking of a specific DNA sequence in living cells. Using fluorescently

labeled PNAs, the spatial localization and dynamics of telomeres have been studied for the first time in living human osteosarcoma cells by Molenaar et al. (26). Shortly after the introduction of fluorescent PNAs into these cells, telomeres became visible as distinct dots that are distributed throughout the cell nucleus (Figure 1A). Employing time-lapse confocal microscopy in conjunction with quantitative image analysis software, most telomeres revealed a constrained motion within a small nuclear volume, and a few telomeres were shown to move over considerable longer distances. Noteworthily, it was also shown that some telomeres temporally associate with each other as well as with promyelocytic leukemia (PML) nuclear bodies that were visualized by a green fluorescent protein (GFP)-PML fusion protein (Figure 1B). However, the mechanism by which the PNAs bind to telomeric sequences remained elusive. The PNAs may bind by means of strand displacement, but it is also possible that they bind to the single-stranded overhang of a telomere sequence. The answer to this question may help to explain why attempts to label repetitive chromosomal regions other than telomeres in living cells using PNAs have failed.

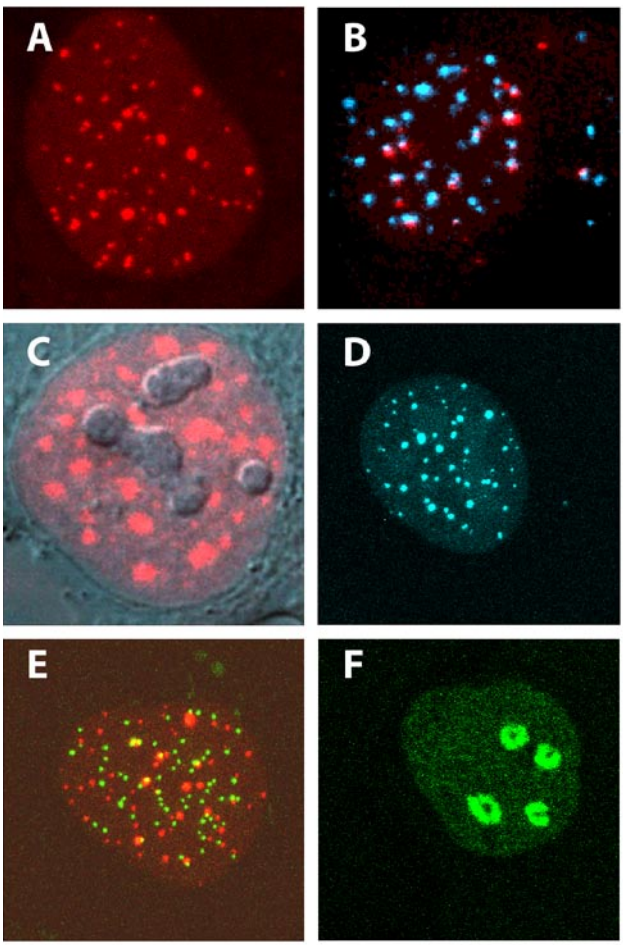

Figure 1. Visualization of nucleic acids in living osteosarcoma U2OS cells by fluorescence confocal microscopy. (A) A cell loaded with a Lissamine™-labeled (Invitrogen, La Jolla, CA, USA) PNA probe specific for telomeric DNA. Telomeric sequences light up as red dots, which are distributed throughout the cell nucleus excluding nucleoli. The dots are variable in size and intensity, reflecting telomere length differences and telomere associations. (B) A cell expressing cyan fluorescent protein (CFP) fused to promyelocytic leukemia protein (CFP-PML) loaded with Cy3™-labeled telomere PNA probe. Many telomeres (red dots) are associated with PML bodies (blue dots). (C) A cell microinjected with a 2′-*O*-methyl TAMRA-labeled (U)22 probe reveals discrete localization of poly(A) RNA in nuclear speckles and diffuse localization throughout the nucleoplasm excluding nucleoli. The figure shows an overlay of a fluorescence image with a differential interference contrast image of the same cell. (D) Distribution of telomeres (blue dots) in a cell nucleus expressing CFP-TRF2. The telomere binding protein TRF2 associates specifically with telomeres. (E) Distribution pattern of telomeres (red dots) and centromeres (green dots) in the nucleus of a living U2OS cell coexpressing the telomere binding protein CFP-TRF2 and the centromere binding protein GFP-CENPA. (F) Selective visualization of chromatin (green fluorescence) that is associated with nucleoli following photoactivation using a two-photon 820 nm laser beam in a cell expressing photoactivatable GFP histone H4. PNA, peptide nucleic acid; PML, promyelocytic leukemia: CENPA, centromere protein A.

Similar to PNAs, 2′-*O*-methyl RNA probes have been reported to form very stable hybrids with their target sequences while they are not degraded by cellular nucleases (27). Having initially been used in fluorescence in situ hybridization (FISH) studies to localize the distribution of some small nuclear RNAs, 2′-*O*-methyl RNA probes are now used to visualize RNAs within living cells as well. Recently, a 2′-*O*-methyl RNA probe has been used to study the kinetic properties of poly(A) RNA localization in the cell nucleus in great detail (28). While previous live-cell studies employing fluorescent ODN probes revealed that poly(A) RNA is evenly distributed throughout the nucleus and moves by free diffusion, it has been shown that poly(A) mRNA is in fact moving more slowly and is also located at speckle regions in the nucleus (Figure 1C). Interestingly, poly(A) RNA has been shown to move through the speckle regions that also contain RNA polymerase and various splicing factors. Furthermore, a direct comparison of the performance in living cells of an ODN with that of a 2′-*O*-methyl RNA probe, both specific for poly(A) mRNA tails, revealed that significantly better signal-to-noise ratios are obtained with a 2′-*O*-methyl RNA probe when similar amounts of probes have been microinjected in the cells (28). It has been reported that 2′-*O*-methyl RNA probes not only form more stable hybrids with complementary single-stranded RNA than ODNs (27,29) but also bind more efficiently to double-stranded regions of RNA molecules, probably by strand invasion (30). Therefore, 2′-*O*-methyl RNA probes may have the ability to efficiently hybridize to RNAs that are folded into higher order structures in living cells. That 2′-*O*-methyl RNA probes can also be used to visualize specific mRNAs in living cells has been illustrated by the visualization of mRNA transcripts synthesized from an inducible gene encoding a human cytomegalovirus immediate early antigen (31).

Apart from 2′-*O*-methyl modifications, other nucleic acid modifications may help to promote in vivo hybrid formation of probes. For example, locked nucleic acid (LNA) modifications may turn out to be useful in RNA detection. LNA nucleotides are nucleotide analogs that contain an

ethylene linkage between the 2′ oxygen and the 4′ carbon of the ribose ring and generate, upon RNA binding, the most stable hybrids ever measured. The melting temperature of the hybrids is increased up to 10°C per modification (32). Therefore, LNAs may also have the ability to hybridize to highly structured RNA targets, while they confer good protection against nuclease digestion (33).

In conclusion, the sensitivity and specificity of RNA detection in living cells has improved using fluorescently labeled high affinity probes instead of fluorescent ODNs. However, nonhybridized probe sequences will still contribute to a diffuse fluorescent background signal in living cells. This is not necessarily a problem when the target RNA sequences are localized at specific sites in a cell, which are morphologically distinguishable. However, when the target sequences are not concentrated at recognizable sites, the dispersed fluorescent signals of nonhybridized probe sequences may interfere with the visualization of particular target RNA sequences. Therefore, it was still a challenge to strongly reduce or eliminate these dispersed signals that lead to poor signal-to-noise ratios.

Shedding Light on Molecular Beacons

An elegant solution for eliminating the fluorescent signals derived from nonhybridized probes has been sought in the application of molecular beacons (34). Molecular beacons are ODNs that form a stem-loop hairpin structure and are dual-labeled with a reporter fluorophore at one end and a quencher at the other. In the absence of a complementary target, the molecular beacon is in a stem-loop configuration in which the fluorescence is quenched. Following hybridization to a complementary target, the hairpin structure is changed to an open configuration separating the fluorophore and quencher and restoring fluorescence. Importantly, it has been demonstrated that mRNAs hybridized to molecular beacons are still translated, indicating that probe binding does not necessarily interfere with the biological function of mRNAs (35). ODN molecular beacons have been applied in a number of studies to track the distribution of specific RNAs in living cells (36,37). The first reports that described the application of ODN molecular beacons appeared in 1998. In that year, Matsuo (38) described the localization of basic fibroblast growth factor mRNA in human trabecular cells, and Sokol et al. (39) described the real-time detection of *vav* proto-oncogene mRNA and *β-actin* mRNA in K562 human leukemia cells. Furthermore, Sokol et al. postulated that it would be feasible to visualize as few as 10 mRNA molecules in a single cell using molecular beacons. In practice, however, the ODN molecular beacon approach proved poorly reproducible and provided similar results to those obtained with linear ODNs (31). The in vivo hybridization efficiency of ODN molecular beacons is apparently strongly target-dependent. Furthermore, their stem-loop configurations may be forced to open prematurely, leading to nonspecific fluorescence signals that cannot be easily differentiated from specific hybridization signals (31,40). To overcome these limitations, molecular beacons have been synthesized possessing 2′-*O*-methyl ribonucleotide backbones (41). Indeed, when the thermodynamic and kinetic properties of 2′-*O*-methyl molecular beacon/RNA duplexes were analyzed, these duplexes revealed improved stability and hybridization kinetics compared with ODN molecular beacon/RNA duplexes (42). In living cells, however, 2′-*O*-methyl molecular beacons did not perform significantly better than linear 2′-*O*-methyl RNA probes. This is illustrated by the observation that the fluorescence signal intensity of molecular beacons hybridized to *β-actin* mRNA, one of the most abundant messengers in a cell, is only about 2.5-fold higher than the background fluorescence intensity (35).

To improve upon signal-to-noise ratios, a dual FRET molecular beacon approach has been developed (40,41). In this approach, two different molecular beacons, each possessing a different fluorophore that together forms a FRET pair, bind to the same target RNA at nearly adjacent positions. Only when both molecular beacons hybridize to their target will the donor fluorophore attached at the 5′ end of one molecular beacon be in close proximity (within 10 nm) to an acceptor fluorophore attached at the 3′ end of the other molecular beacon, allowing resonance energy transfer. FRET signals can then be measured by different imaging methods including fluorescence lifetime imaging microscopy and FRET filter microscopy. Using this dual FRET approach, the localization of *oskar* mRNA in *Drosophila* oocytes could be confirmed in vivo (41). A similar approach has been used to image *K-ras* and *survivin* mRNAs in cells employing unmodified dual-labeled molecular beacons (40). Both studies reported a significant reduction in background signals compared with the use of single molecular beacons and, according to Santangelo et al. (40), detection of only a few hundred copies of an endogenous mRNA in a single living cell would be feasible.

Similar to ODNs and 2′-*O*-methyl RNA oligonucleotide probes, molecular beacons are rapidly (typically within minutes) accumulating in the nucleus of cells regardless of the method by which they are delivered into the living cell, which could be an advantage for imaging nuclear transcripts. However, to image mRNAs in the cytoplasm of living cells, a strategy had to be developed to increase the residence time of the probe in the cytoplasm to build up a sufficiently high concentration of probe to allow target binding. To this end, linear ODNs were bound to the macromolecule streptavidin to prevent their passage to nuclear pores with the risk that they will bind to endogenous cytoplasmic biotin (35). Most recently, Mhlanga et al. (43) introduced an elegant approach by linking a molecular beacon to a transfer RNA (tRNA) transcript, preventing or at least delaying, nuclear sequestration of the probe. The applicability of this approach was demonstrated by the imaging of *β-actin* mRNA in the cytoplasm of chicken fibroblasts.

TRACKING RNA AND CHROMATIN BY FLUORESCENT PROTEINS

DNA and RNA Binding Proteins Are Fused to GFP

Current hybridization-based methods are generally incompatible with the detection and tracking of DNA sequences in living cells. Furthermore, these are not necessarily ideal tools for monitoring RNA expression in living cells. Alternative approaches took advantage of RNA or DNA binding proteins, which are ectopically expressed in a cell as a GFP fusion protein. The expressed fusion protein is meant to bind at specific endogenous DNA or RNA sequences in order to visualize them in living cells by fluorescence microscopy. For example, centromeres and telomeres, which are highly repetitive DNA sequences, have been visualized by exogenously expressed reporter proteins consisting of a centromere- and telomere-binding protein, respectively, fused to a GFP, or one of its color variants (Figure 1D). Simultaneous analysis of centromeres and telomeres also proved possible when both fusion proteins were coexpressed in the same cell (Figure 1E). By these means, it was observed that most centromeres and telomeres move by constrained diffusion within confined nuclear domains (26,44). In addition, the global movement of chromatin has been analyzed in living cells by the expression of GFP-histone fusion proteins that are stably incorporated into chromatin. For example, Kanda et al. (45) imaged the segregation of double minute chromosomes in cancer cells, and Manders et al. (46) studied the movement of chromatin domains during anaphase to G1 transition and observed remarkably little movement of chromatin domains relative to other chromatin. Interestingly, by tracking the in vivo behavior of chromatin, the long-standing question of whether the three-dimensional positioning of chromosomes would be inherited by the daughter cells after cell division could also finally be answered. By expressing GFP-tagged histone H2B and applying photobleaching, it turned out that the chromosome architecture is largely passed on to the daughter cells (47–49).

In the flow of developing various color variants of GFP, a photoactivatable GFP (paGFP) emerged (50). This paGFP happened to be very useful to track chromatin dynamics when fused to a histone protein. Similar to GFP, paGFP can be incorporated in chromatin when fused to a histone protein. This fusion protein is essentially nonfluorescent and starts to fluoresce only when irradiated with 410 nm light. So, by selective photoactivation of a nuclear region using a laser beam, it is possible to visualize and track specific chromatin sites in living cells. This approach has recently been applied to track fluorescently activated chromatin loci in *Drosophila*. Quantitative analysis revealed that these foci appeared to move by constrained diffusion (51). The same approach has also been applied to human cells to photoactivate specific chromatin regions (Figure 1F).

To date, fluorescent RNA binding proteins have been used less often than DNA binding proteins as a tool to monitor RNA localization in living cells. A reason could be that the binding of such proteins to target RNAs is often not very well characterized and thus may lack detection specificity and sensitivity. However, in *Drosophila* embryos, ribonucleoprotein (RNP) particles have been tracked during the anterior patterning by expressing GFP-Exu, which specifically binds *bicoid* mRNA (52). Later, fragile X mental retardation protein (FMRP)-GFP expression was used to track dendritic mRNA transport in PC12 cells (53) and GFP-tagged poly(A) binding protein II expression to analyze the mobility of poly(A) RNA containing RNP particles in living HeLa cells (54).

The lac Operator/Repressor System Marks Specific Chromatin Regions

An alternative approach to track discrete chromatin regions makes use of the lac operator and repressor system. The essence of this approach is to integrate bacterial lac operator repeats into the genome of cells and to express a lac repressor-GFP fusion protein that will associate with these repeats (55). The GFP-tagged loci can then be detected and tracked as distinct dots within the nucleus of living cells. Time-lapse movies of such dots taught us that chromatin moves generally within confined small nuclear regions (56–58). However, long-range chromatin mobility has been observed in particular stages of the cell cycle and in some organisms (59). To address more complex questions, various modifications of the lac operator/repressor system have been made. For example, by positioning the lac O repeat sequences in front of an inducible promoter regulating the expression of a cyan fluorescent protein (CFP), the dynamics of gene expression and related changes in chromatin structure have been monitored in living cells (60). One should be aware, however, that the introduction of tandem repetitive transgenes in the genome and the subsequent expression of the GFP-lac repressor protein might induce alterations in chromosome arrangements, including associations between homologous transgenic sequences (61).

In Vivo Visualization of RNA Containing Stem Loops

The elegant idea of inserting exogenous sequences in DNA, and thereby creating binding sites for GFP-tagged reporter proteins, has also evolved into an approach to visualize RNA synthesis and transport in vivo. In this approach developed by Bertrand and colleagues to investigate the localization of *ASH1* mRNA in yeast cells (62), several stem-loop structures that are binding sites for the bacteriophage coat protein MS2 are inserted into the RNA of interest without interference with the coding sequence. This RNA carrying the stem-loop structures is then expressed together with the GFP-MS2 fusion proteins that will bind these stem loops in the same cell. Furthermore, the GFP-MS2 protein is targeted to the nucleus so that it will associate with the nascent stem-loop-containing transcripts. Since its introduction, this approach has been applied to a diversity of cell types to image the kinetics of nascent transcript synthesis or to study mRNA movement within the nucleoplasm or cytoplasm (63,64).

Recently, the lac operator/repressor approach has been combined with the GFP-MS2 approach to visualize gene expression in all its aspects within a single living mammalian cell (65). Following induction of expression from an integrated inducible transgene array, chromatin was shown to decondense while nascent transcripts accumulated at the induced locus. At later time points, the transcripts were shown to move away from this site in all directions within the nucleus. This study illustrates the importance of taking an integrated approach to simultaneously visualize DNA, RNA, and protein and to further our knowledge of the regulation of gene expression at the level of the individual cell.

Conclusions

Many methods are currently available for the detection of DNA or RNA molecules in living cells. Still, an ideal detection system has not yet been developed. Each of the approaches described here have their advantages, but certainly have potential disadvantages as well. Hybridization-based methods that allow for the detection of specific endogenous RNAs are still further improved by facilitating probe access to cells and by gaining specificity and sensitivity. No doubt, GFP-based detection methods will gain in sensitivity and specificity, but interesting developments currently taking place are in the synthesis of chemical structures that have high affinity for specific DNA or RNA sequences and show fluorescence only upon binding. All together, these are most promising developments that, in conjunction with sensitive, quantitative, and multimodal imaging techniques, will make it possible to construct a realistic model that predicts how the behavior of chromatin and the localization of RNAs orchestrate the function and fate of cells.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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