

Nanostructured Probes for RNA Detection in Living Cells

PHILIP SANTANGELO, NITIN NITIN, and GANG BAO

Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, Atlanta, GA

(Received 5 September 2005; accepted 30 September 2005)

Abstract—The ability to visualize in real-time the expression level and localization of specific RNAs in living cells can offer tremendous opportunities for biological and disease studies. Here we review the recent development of nanostructured oligonucleotide probes for living cell RNA detection, and discuss the biological and engineering issues and challenges of quantifying gene expression *in vivo*. In particular, we describe methods that use dual FRET (fluorescence resonance energy transfer) or single molecular beacons in combination with peptide-based or membrane-permeabilization-based delivery, to image the relative level, localization, and dynamics of RNA in live cells. Examples of detecting endogenous mRNAs, as well as imaging their subcellular localization and colocalization are given to illustrate the biological applications, and issues in molecular beacon design, probe delivery, and target accessibility are discussed. The nanostructured probes promise to open new and exciting opportunities in sensitive gene detection for a wide range of biological and medical applications.

Keywords— Hairpin probe, Oligonucleotide, RNA detection, Live cell, Molecular beacon, Fluorescence resonance energy transfer, Peptide.

INTRODUCTION

The ability to image specific RNAs in living cells in real time can provide essential information on RNA synthesis, processing, transport, and localization, and on the dynamics of RNA expression and localization in response to external stimuli; it will offer unprecedented opportunities for advancement in molecular biology, disease pathophysiology, drug discovery, and medical diagnostics. As a central theme in biology, genetic information stored in DNA is translated into protein through messenger RNA.¹⁴ Over the last decade or so, there is increasing evidence to suggest that RNA molecules have a wide range of functions in living cells, from physically conveying and interpreting genetic information, to essential catalytic roles, to providing structural support for molecular machines, and to gene silencing. These functions are realized through control of the expression level and stability, both temporally and spa-

tially, of specific RNAs in a cell. Therefore, determining the dynamics and localization of RNA molecules in living cells will significantly impact on the molecular biology and medicine.

Many *in vitro* methods have been developed to provide a relative (mostly semiquantitative) measure of gene expression level within a cell population using purified DNA or RNA obtained from cell lysate. These methods include PCR,⁴¹ Northern hybridization (or Northern blotting),³ expressed sequence tag (EST),¹ serial analysis of gene expression (SAGE),⁶⁰ differential display,³⁰ and DNA microarrays.⁴⁴ These technologies, combined with the rapidly increasing availability of genomic data for numerous biological entities, present exciting possibilities for understanding human health and disease. For example, pathogenic and carcinogenic sequences are increasingly being used as clinical markers for diseased states. However, the detection and identification of foreign or mutated nucleic acids is often difficult in a clinical setting due to the low abundance of diseased cells in blood, sputum, and stool samples. Further, these methods cannot reveal the spatial and temporal variation of RNA within a single cell.

Various technologies and methodologies have been developed to study intracellular RNA biology by creating tagged full length RNAs or using RNA targeting probes. In most cases these tags are fluorescent or radioactive, although magnetic tags can be used as well. For example, tagged full-length RNA has been introduced into living cells using microinjection^{20,22,23} to monitor the localization of a specific mRNA or nuclear RNA. However, this approach cannot be used to measure endogenous RNAs in living cells.

Labeled linear oligonucleotide (ODN) probes have been used to study intracellular mRNA via *in situ* hybridization (ISH)⁵ in which cells are fixed and permeabilized to increase the probe delivery efficiency and unbound probes are removed by washing, therefore reducing background and achieving specificity.¹³ To enhance the signal level, multiple probes targeting the same mRNA can be used.⁵ However, fixation agents and other supporting chemicals can have considerable effect on signal⁷ and possibly on the integrity of certain organelles such as mitochondria.

Address correspondence to GANG Bao, Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, 313 Ferst Dr, Suite 2115, Atlanta, GA 30332. Electronic mail: gang.bao@bme.gatech.edu

Therefore, fixation of cells, by either cross-linking or denaturing agents, combined with the use of proteases in ISH may not provide an accurate description of intracellular mRNA localization. It is also difficult to obtain a dynamic picture of gene expression in cells using ISH methods.

In addition to oligonucleotide probes, tagged RNA-binding proteins such as those with GFP tags have been used to detect mRNA in live cells.¹¹ One limitation is that it requires the identification of a unique protein, which only binds to the specific mRNA of interest. To address this issue, recently a transgene with a binding site for the phage MS2 protein was synthesized.¹⁷ Generation of a GFP tagged phage MS2 protein in *Drosophila* eggs allowed the specific targeting of the nanos mRNA in a living egg system. The GFP-MS2 approach has also been used to track the localization and dynamics of RNA in living cells with single molecule sensitivity.⁴⁵ However, there is still a significant challenge in generating transgenes with the same functionality as endogenous mRNA.

One approach to tagging and tracking endogenous mRNA transcripts in living cells is to use fluorescently labeled oligonucleotide probes that recognize specific RNA targets via Watson–Crick base pairing. In order for these probes to truly reflect the mRNA expression *in vivo*, they must satisfy a number of criteria: they need to be able to distinguish signal from background, convert target recognition *directly* into a measurable signal, and differentiate between true and false-positive signals. Further, these probes are required to have high sensitivity for quantifying low gene expression levels and fast kinetics for tracking alterations in gene expression in real time. Although imaging assays can be performed by introducing fluorescently labeled linear oligonucleotide (ODN) probes into living cells for RNA tracking and localization studies,^{35, 36, 55} this approach lacks the ability to distinguish background from true signal. Two linear probes with a fluorescence resonance energy transfer (FRET) pair of (donor and acceptor) fluorophores have been used to enhance detection specificity and increase signal-to-background ratio.⁵⁵ However, the dual linear-probe approach may still have a high background signal due to direct excitation of the acceptor and emission detection of the donor fluorescence. Further, it is difficult for linear probes to distinguish targets that differ by a single base since the difference in free energy of the two hybrids (with and without mismatch) is typically rather small. This limits the application of linear ODN probes in disease studies (such as cancer detection).

Clearly, the detection of specific RNAs in living cells requires probes to have high specificity, sensitivity, and signal-to-background ratio, especially for low abundance genes and clinical samples containing a small number of diseased cells. Further, for detecting genetic alterations such as mutations and deletions, the ability to recognize single nucleotide polymorphisms (SNPs) is essential. It is also desirable to quantify the RNA expression level in individ-

ual cells with high accuracy. To satisfy all these requirements, it is necessary to have a good understanding of the structure–function relationship of the probes, the stability of the probes, RNA structural dynamics, and probe–target interaction in living cells. It is also necessary to achieve efficient cellular delivery of probes with minimal probe degradation, and to determine the possible effect of probe/target hybridization on gene translation and other processes in living cells.

In the remaining sections, we discuss the critical issues in living cell RNA detection using nanostructured hairpin oligonucleotide probes, including probe design, cellular delivery of probes, probe/target interaction dynamics, and mRNA detection sensitivity, specificity, and signal-to-background ratio. Emphasis is placed on the design and application of molecular beacons, although the issues are common for other hairpin probes.

MOLECULAR BEACONS

When designed properly, hairpin nucleic acid probes have the potential to be highly sensitive and specific in living cell gene detection. As shown in Fig. 1, one class of such probes is molecular beacons, which are dual-labeled oligonucleotide probes with a fluorophore at one end and a quencher at the other end.⁵⁸ They are designed to form a stem-loop structure in the absence of a complementary target so that fluorescence of the fluorophore is quenched. Hybridization with target nucleic acid opens the hairpin and physically separates the fluorophore from quencher, allowing a fluorescence signal to be emitted upon excitation. Under optimal conditions, the fluorescence intensity of molecular beacons can increase by >200-fold upon binding to their targets.⁵⁸ This enables a molecular beacon to function as a sensitive probe with a high signal-to-background ratio. The stem-loop hairpin structure provides an adjustable energy penalty for hairpin opening, which improves probe specificity.^{9, 53} The ability to transduce target recognition *directly* into a fluorescence signal with high signal-to-background ratio, coupled with an improved specificity, has allowed molecular beacons to enjoy a wide range of biological and biomedical applications, including multiple analyte detection, real-time enzymatic cleavage assaying, cancer cell detection, protein–DNA interactions, real-time monitoring of PCR, gene typing and mutation detection, and mRNA detection in living cells.^{8, 10, 15, 25, 29, 33, 36, 37, 38, 39, 47, 54, 56, 57, 61, 63}

A conventional molecular beacon has four essential components: loop, stem, fluorophore, and quencher, as illustrated in Fig. 1(a). The loop usually consists of 15–25 nucleotides and is selected based on target sequence and melting temperature. The stem, formed by two complementary short-arm sequences, is typically four to six bases long and is usually chosen to be independent of the target sequence [Fig. 1(b)]. Molecular beacons, however, can also

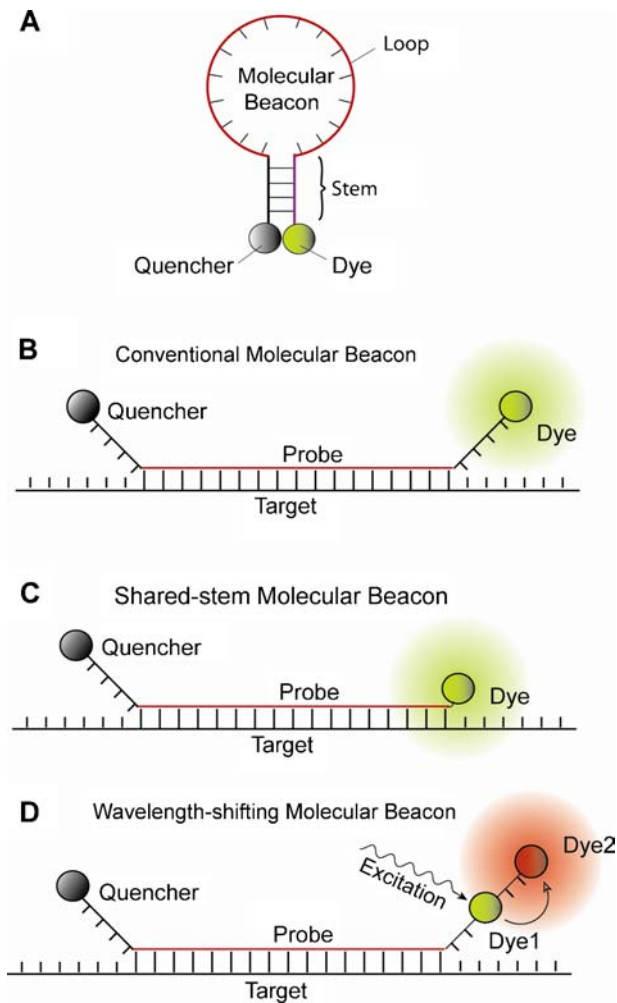


Figure 1. Illustrations of molecular beacons. (a) Molecular beacons are stem-loop hairpin oligonucleotide probes labeled with a reporter fluorophore at one end and a quencher molecule at the other end. (b) Conventional molecular beacons are designed such that the short complementary arms of the stem are independent of the target sequence. (c) Shared-stem molecular beacons are designed such that one arm of the stem participates in both stem formation and target hybridization. (d) Wavelength-shifting molecular beacons containing two fluorophores, one absorbs in the wavelength range of the monochromatic light source, and the other emits light at the desired emission wavelength due to FRET.

be designed such that one arm of the stem participates in both stem formation and target hybridization (shared-stem molecular beacons),⁵² as illustrated schematically in Fig. 1(c). Although a molecular beacon can be labeled with any desired reporter-quencher pair, proper selection of the reporter and quencher could improve the signal-to-background ratio and multiplexing capabilities.

A novel design of hairpin probes is the wavelength-shifting molecular beacons that can fluoresce in a variety of different colors.⁵⁹ As shown in Fig. 1(d), in this design, a molecular beacon contains two fluorophores: a first fluo-

rophore that absorbs strongly in the wavelength range of the monochromatic light source, and a second fluorophore that emits at the desired emission wavelength due to fluorescence resonance energy transfer from the first fluorophore to the second fluorophore. It has been demonstrated that wavelength-shifting molecular beacons are substantially brighter than conventional molecular beacons that contain a fluorophore that cannot efficiently absorb energy from the available monochromatic light source.

One major advantage of the stem-loop hairpin probes is that they can recognize their targets with higher specificity than linear ODN probes. Solution studies suggested that,^{9,53} using molecular beacons, it is possible to discriminate between targets that differ by a single nucleotide. In contrast to current techniques for detecting single nucleotide polymorphism (SNP), which are often labor-intensive and time-consuming, the use of molecular beacons may provide a simple and promising tool for the diagnosis of genetic diseases.

A major challenge in using conventional molecular beacons for living cell gene detection is that molecular beacons can be degraded by cytoplasmic nucleases or open due to nonspecific interaction with hairpin-binding proteins, causing a significant amount of false-positive signals. To overcome this difficulty, a pair of molecular beacons labeled with a donor and an acceptor fluorophore, respectively (dual FRET molecular beacons) are used^{43,54} (Fig. 2). The probe sequences are chosen such that this pair of molecular beacons hybridizes to adjacent regions on a single RNA target (Fig. 2). Since FRET is very sensitive to the distance between donor and acceptor molecules, it occurs stably only when the donor and acceptor beacons are both bound to the same RNA target. Thus, the sensitized emission of the acceptor fluorophore upon donor excitation serves as a positive signal in the FRET-based detection assay, which is readily differentiable from non-FRET false-positive signals due to probe degradation and nonspecific probe opening. This approach combines the low background signal and high specificity of molecular beacons with the ability of FRET assays in differentiating between true target recognition and false positive signals.

MOLECULAR BEACON DESIGN AND STRUCTURE-FUNCTION RELATIONS

There are three major design issues of molecular beacons: probe sequence, hairpin structure, and fluorophore/quencher selection. In general, the probe sequence is selected to ensure specificity, and to have good target accessibility. The hairpin structure as well as the probe and stem sequences are determined to have the proper melting temperature, and the fluorophore-quencher pair should give high signal-to-background ratio. To ensure specificity, for each gene to target, one can use the NCBI BLAST⁴⁹ or similar software to select multiple target sequences of

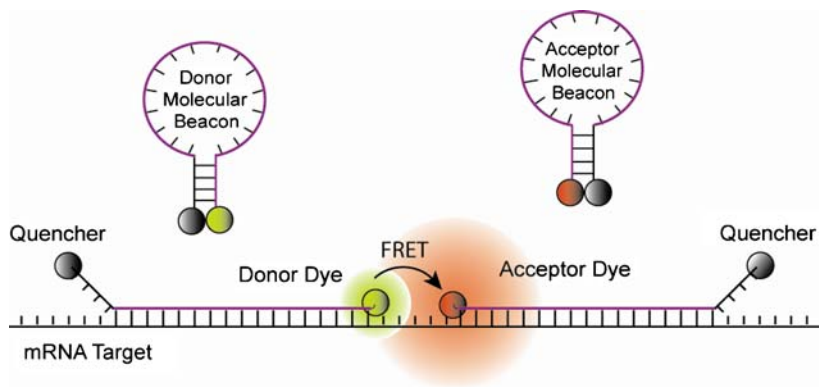


Figure 2. A schematic illustration showing the concept of dual FRET molecular beacons. Hybridization of donor and acceptor molecular beacons to adjacent regions on the same mRNA target results in FRET between donor and acceptor fluorophores upon donor excitation. By detecting FRET signal, fluorescence signals due to probe/target binding can be readily distinguished from that due to molecular beacon degradation and nonspecific interactions.

15–25 bases that are unique for the target RNA. Since the melting temperature of molecular beacons affects both the signal-to-background ratio and detection specificity, especially for mutation detection, it is often necessary to select the target sequence with a balanced G-C content, and to adjust the loop and stem lengths and the stem sequence of the molecular beacon to realize the optimal melting temperature. In particular, it is necessary to understand the effect of molecular beacon design on melting temperature so that, at 37°C, single-base mismatches in target mRNAs can be differentiated. This is also a general issue for detection specificity in that, for any specific probe sequence selected, there might be multiple genes in the mammalian genome that have sequences differ from the probe sequence by only a few bases. Therefore, it is important to design the molecular beacons so that only the specific target RNA would give a strong signal.

The loop, stem lengths, and sequences are critical design parameters for molecular beacons, since at any given temperature they largely control the fraction of molecular beacons that are bound to the target.^{9,53} In many applications, the choices of the probe sequence are limited by target-specific considerations, such as the sequence surrounding a single nucleotide polymorphism (SNP) of interest. However, the probe and stem lengths, and stem sequence, can be adjusted to optimize the performance (i.e., specificity, hybridization rate, and signal-to-background ratio) of a molecular beacon for a specific application.^{53, 54}

To demonstrate the effect of molecular beacon structure on its melting behavior, the melting temperature for molecular beacons with various stem-loop structures is displayed in Fig. 3(a). In general, it was found that the melting temperature increased with probe length but appeared to plateau at a length of ~20 nucleotides. It was also found that the stem length of the molecular beacon could strongly influ-

ence the melting temperature of molecular beacon–target duplexes.

While both the stability of the hairpin probe and its ability to discriminate targets over a wider range of temperatures increase with increasing stem length, it is accompanied by a decrease in hybridization on-rate constant, as shown in Fig. 3(b). For example, molecular beacons with a 4-base stem had an on-rate constant up to 100 times greater than molecular beacons with a 6-base stem. Changing the probe length of a molecular beacon may also influence the rate of hybridization, as demonstrated by Fig. 3(b).

From the thermodynamic and kinetic studies, it was found that, if the stem length is too large, it is difficult for the beacon to open upon hybridization. On the other hand, if the stem length is too small, a large fraction of beacons may open due to the thermal force. Similarly, relative to the stem length, a longer probe may lead to a lower dissociation constant; however, it may also reduce the specificity, since the relative free energy change due to one base mismatch would be smaller. A long probe length may also lead to coiled conformations of the beacons, resulting in reduced kinetic rates. Therefore, the stem and probe lengths need be carefully chosen in order to optimize both hybridization kinetics and beacon specificity.^{53, 54} In general, it has been found that molecular beacons with longer stem lengths have an improved ability to discriminate between wild-type and mutant targets in solution over a broader range of temperatures. This can be attributed to the enhanced stability of the molecular beacon stem-loop structure and the resulting smaller free energy difference between closed (unbound) molecular beacons and molecular beacon–target duplexes, which generates a condition where a single-base mismatch reduces the energetic preference of probe–target binding. Longer stem lengths, however, are accompanied by a decreased probe–target hybridization kinetic rate. Similarly, molecular beacons with short stems have faster hybridiza-

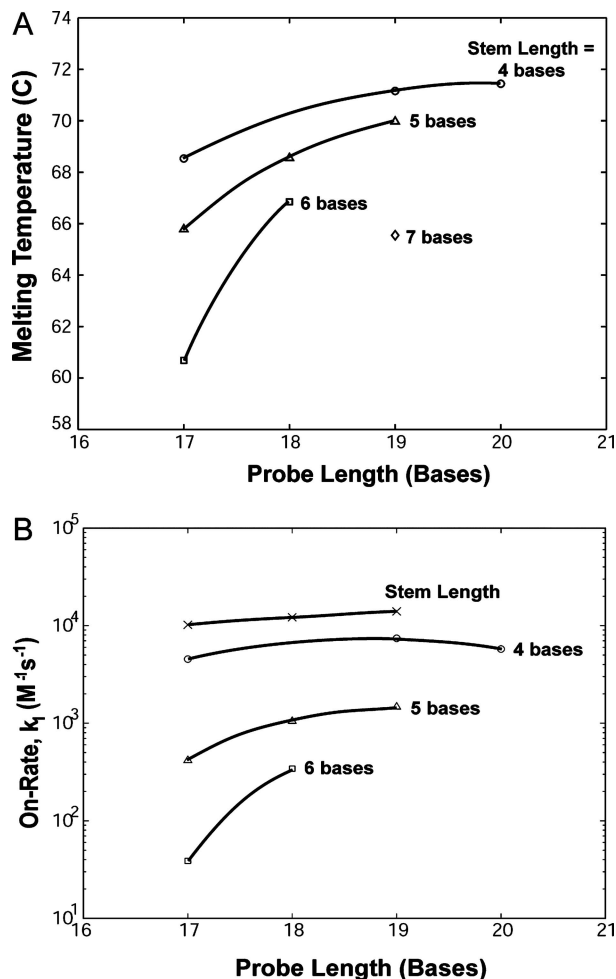


Figure 3. Structure–function relations of molecular beacons. (a) Melting temperatures for molecular beacons with different stem lengths in the presence of target. (b) The rate constant of hybridization k_1 (on-rate constant) for molecular beacons with various probe and stem lengths hybridized to their complementary targets.

tion kinetics but suffer from lower signal-to-background ratios compared with molecular beacons with longer stems.

Target accessibility is a critical issue of molecular beacon design and requires fundamental knowledge of RNA structure, RNA–protein interactions especially the formation of ribonucleoproteins (RNPs). Since mRNA molecules often have secondary (folded) structures, it is important to avoid targeting sequences where double stranded RNA is formed. Further, the target sequence may be occupied by RNA binding proteins. One difficulty in the molecular beacon design is that, although predictions of mRNA secondary structure can be made using software such as *Beacon Designer* (www.premierbiosoft.com) and *mfold* (<http://www.bioinfo.rpi.edu/applications/mfold/old/dna/>), they may be inaccurate due to limitations of the biophysical models used, and the limited understanding of protein–

RNA interaction. Therefore, for each gene to target, it may be necessary to select multiple unique sequences along the target RNA, and have corresponding molecular beacons designed, synthesized, and tested to select the best target sequence.

Selecting a fluorophore label for a molecular beacon as the reporter is usually not as critical as the hairpin probe design since many conventional dyes can yield satisfactory results. However, proper selection could yield additional benefits such as an improved signal-to-background ratio and multiplexing capabilities. Since each molecular beacon utilizes only one fluorophore it is possible to use multiple molecular beacons in the same assay, assuming that the fluorophores are chosen with minimal emission overlap.⁵⁷ Molecular beacons can even be labeled simultaneously with two fluorophores, i.e., “wavelength shifting” reporter dyes, allowing multiple reporter dye sets to be excited by the same monochromatic light source yet fluorescing in a variety of colors.⁵⁹ Clearly, multicolor fluorescence detection of different beacon/target duplexes can become a powerful tool for the simultaneous detection of multiple genes.

For dual FRET (fluorescence resonance energy transfer) molecular beacons, the donor fluorophores typically emit at shorter wavelengths compared with that of acceptor. Energy transfer occurs as a result of long-range dipole–dipole interactions between the donor and the acceptor. The rate of energy transfer depends upon the extent of the spectral overlap of the emission spectrum of the donor with the absorption spectrum of the acceptor, the quantum yield of the donor, the relative orientation of the donor and acceptor transition dipoles,²⁷ and the distance between the donor and acceptor molecules (usually four to five bases). In selecting donor and acceptor fluorophores, in order to have high signal-to-background ratio, it is important to optimize the above parameters, and to avoid direct excitation of the acceptor fluorophore at the donor excitation wavelength, as well as minimizing donor emission detection at the acceptor emission detection wavelength. Examples of FRET dye pairs include Cy3 (donor) and Cy5 (acceptor), TMR (donor) and Texas Red (acceptor), and fluorescein (FAM) (donor) and Cy3 (acceptor).

It is relatively straightforward to select the quencher molecules. Organic quencher molecules such as dabcy1, BHQ2 (blackhole quencher II) (Biosearch Tech), BHQ3 (Biosearch Tech), and Iowa Black (IDT) can all effectively quench a wide range of fluorophores by both fluorescence resonance energy transfer (FRET) and the formation of an exciton complex between the fluorophore and the quencher.³²

CELLULAR DELIVERY OF NANOPROBES

One of the most critical aspects of measuring the intracellular level of RNA molecules using synthetic probes is the ability to deliver these probes into cells through the

plasma membrane, which is quite lipophilic and restricts the transport of large or charged molecules. Therefore, it is a very robust barrier to polyanionic molecules such as hairpin oligonucleotides. Further, even if the probes enter the cells successfully, the efficiency of delivery in an imaging assay should be defined not only by how many probes enter the cell or how many cells have probes internalized, but also how many probes remain functioning inside cells. This is different from both antisense and gene delivery applications where the reduction in level of protein expression is the final metric used to define efficiency or success. For measuring RNA molecules (including mRNA and rRNA) in the cytoplasm, a large amount of probes should remain in the cytoplasm.

Existing cellular delivery techniques can be divided into two categories: endocytic and nonendocytic methods. Endocytic delivery typically employs cationic and polycationic molecules such as liposomes and dendrimers, while nonendocytic methods include microinjection, and the use of cell-penetrating peptides (CPP) or streptolysin O (SLO). Probe delivery via the endocytic pathway typically takes 2–4 h. It has been reported that ODN probes internalized via endocytosis are predominately trapped inside endosomes and often lysosomes, and being degraded there due to cytoplasmic nucleases.⁴⁰ Consequently, only 0.01–10% of the probes remain functioning after having escaped from endosomes and lysosomes.¹⁶

Oligonucleotide probes (including molecular beacons) have been delivered into cells via microinjection.²⁸ In most of the cases the ODNs exhibited a fast accumulation in the cell nucleus, preventing the probes from targeting mRNAs in the cell cytoplasm. Depletion of intracellular ATP or lowering the temperature from 37 to 4°C did not have a significant effect on ODN nuclear accumulation, ruling out active, motor-protein driven transport.²⁸ It is unclear if the rapid transport of ODN probes to nucleus is due to electrostatic interaction, or driven by microinjection-induced flow, or the triggering of some signaling pathway. There is no fundamental biological reason why ODN probes should accumulate in the cell nucleus. To prevent nuclear accumulation, streptavidin (60 kDa) molecules were conjugated to linear ODN probes via biotin.⁵⁵ After microinjection into cells, dual FRET linear probes could hybridize to the same mRNA target in the cytoplasm, resulting in a FRET signal. More recently, it was demonstrated that when tRNA transcripts are attached to molecular beacons with 2'-O-methyl backbone and injected into the nucleus of HeLa cells, the probes are exported into the cytoplasm. When these constructs are introduced into the cytoplasm, they remain cytoplasmic.³⁴ However, even without the problem of unwanted nuclear accumulation, microinjection is inefficient in delivering probes into a large number of cells.

Another nonendocytic delivery method is toxin-based cell membrane permeabilization. For example, streptolysin O (SLO) is a pore-forming bacterial toxin that has been used

as a simple and rapid means of introducing oligonucleotides into eukaryotic cells.^{4,18,19,48} SLO binds as a monomer to cholesterol and oligomerizes into a ring-shaped structure to form pores of approximately 25–30 nm in diameter, allowing the influx of both ions and macromolecules. It was found that SLO-based permeabilization could achieve an intracellular concentration of ODNs of approximately 10 times that of electroporation and liposomal-based delivery. Since cholesterol composition varies with cell types, the permeabilization protocol needs to be optimized for each cell type by varying temperature, incubation time, cell number, and SLO concentration. An essential feature of this technique is that the toxin-based permeabilization is reversible. This can be achieved by introducing oligonucleotides with SLO under serum-free conditions and then removing the mixture and adding normal media with serum.^{4,64}

Cell penetrating peptides (CPP) have been used to introduce proteins, nucleic acids, and other biomolecules into living cells.^{6,46,62} Among the family of peptides with membrane translocating activity are antennapedia, HSV-1 VP22, and the HIV-1 Tat peptide. To date the most widely used peptide are HIV-1 Tat peptide and its derivatives due to their small size and high delivery efficiency. The Tat peptide is rich in cationic amino acids especially arginines which is very common in many of the cell penetrating peptides. However, the exact mechanism for CPP-induced membrane translocation remains elusive.

A wide variety of cargos have been delivered into living cells both in cell culture and in tissue using cell penetrating peptides.^{12,63} For example, Allinquant *et al.*² linked Antennapedia peptide to the 5' end of DNA oligonucleotides (with biotin on the 3' end) and incubated both peptide-linked ODNs and ODNs alone with cells. By detecting biotin using streptavidin-alkaline phosphatase amplification, it was found that the peptide-linked ODNs were internalized very efficiently into all cell compartments compared with control ODNs. No indication of endocytosis was found. Similar results were obtained by Troy *et al.*⁵¹ with a 100-fold increase in antisense delivery efficiency when ODNs were linked to antennapedia peptides. Recently, Tat peptides were conjugated to molecular beacons using three different linkages (Fig. 4); the resulting peptide-linked molecular beacons (see Table 1) were delivered into living cells to target GAPDH and survivin mRNAs.³⁷ It was demonstrated that, at relatively low concentrations, peptide-linked molecular beacons were internalized into living cells within 30 min with nearly 100% efficiency. Further, peptide-based delivery did not interfere with either specific targeting by or hybridization-induced fluorescence of the probes, and the peptide-linked molecular beacons could have self-delivery, targeting, and reporting functions. In contrast, liposome- (Oligofectamine) or dendrimer-based (Superfect) delivery of molecular beacons required 3–4 h and resulted in a punctate fluorescence signal in the cytoplasmic vesicles and a high background in both cytoplasm and nucleus of cells.³⁷

TABLE 1. The design of peptide-linked molecular beacons.

Peptide	
TAT	(N terminus) TyrGlyArgLysLysArgArgGlnArgArgArg (C terminus)
Unmodified molecular beacon	
GAPDH	5'-Cy3-CGACG GAGTCCTTCCACGATACCACGTCG-BHQ2-3'
Modified molecular beacons	
GAPDH	5'-Cy3-CGACG GAGTCCTTCCACGATACCACG/thiol-dT/CG-BHQ2-3'
Survivin	5'-Cy3-CGACG GAGAAAGGGCTGCCACG/thiol-dT/CG-BHQ2-3'
Random	5'-Cy3-CGACG CGACAAGCGCACCGATACG/thiol-dT/CG-BHQ2-3'

It was clearly demonstrated that cellular delivery of molecular beacons using the peptide-based approach has far better performance compared with conventional transfection methods.

LIVING CELL RNA DETECTION USING MOLECULAR BEACONS

Sensitive gene detection in living cells presents a significant challenge. In addition to issues with detection specificity and probe delivery as discussed above, achieving high detection sensitivity and signal-to-background ratio requires not only careful design of the probes but also a better understanding of target accessibility and probe-target interactions. It is likely that different applications have different requirements on the properties of probes. For example, rapid determination of RNA expression level and localization requires fast probe/target hybridization kinetics, while long-time monitoring of gene expression dynamics requires probes having high intracellular stability.

To demonstrate the capability of molecular beacons in sensitive detection of specific endogenous mRNAs in living cells, dual FRET molecular beacons were designed to detect K-ras and survivin mRNAs in HDF and MIAPaCa-2 cells, respectively.⁴³ Each FRET probe pair consisted of two molecular beacons, one labeled with a donor fluorophore (donor beacon) and a second labeled with an

acceptor fluorophore (acceptor beacon). These molecular beacons were designed to hybridize to adjacent regions on an mRNA target so that the two fluorophores will lie within the FRET range (~ 6 nm) when probe/target binding occurs for both beacons. Excitation of the donor fluorophore then results in fluorescence emission at a wavelength characteristic of the acceptor fluorophore, which serves as a positive FRET signal readily differentiable from non-FRET false-positive signals due to probe degradation and nonspecific probe opening. As shown in Table 2, dual FRET molecular beacon pairs were designed in a shared-stem fashion,⁵² i.e., the sequence of the fluorophore-attached arm of the stem (Fig. 1) is complementary to the target so that it participates in both stem formation and target hybridization. This design was chosen to help fix the relative distance between the donor and acceptor fluorophores and improve energy transfer efficiency. For all FRET molecular beacon pairs, Cy3 (peak excitation at 545 nm) and Cy5 (peak emission at 665 nm) were used as the donor and acceptor fluorophores, respectively, and BHQ-2 and BHQ-3 were used as quenchers for the donor and acceptor molecular beacons, respectively. One pair of molecular beacons targets a segment of the wild-type K-ras gene (Table 2) whose codon 12 mutations are involved in the pathogenesis of many cancers. A negative control dual FRET molecular beacon pair was also designed ("random beacon pair") whose specific 16-base target sequence was selected using

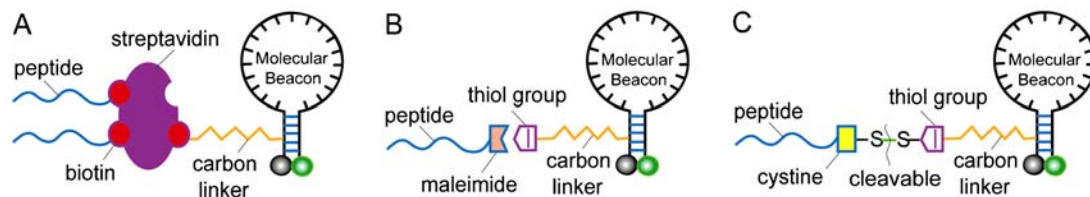


Figure 4. A schematic illustration of three different conjugation schemes for linking a delivery peptide to a molecular beacon. (A) The streptavidin-biotin linkage in which a molecular beacon is modified by introducing a biotin-dT to the quencher arm of the stem through a carbon-12 spacer. The biotin-modified peptides are linked to the modified molecular beacon through a streptavidin molecule, which has four biotin-binding sites. (B) The thiol-maleimide linkage in which the quencher-arm of the molecular beacon stem is modified by adding a thiol group which can react with a maleimide group placed to the C terminus of the peptide to form a direct, stable linkage. (C) The cleavable disulfide bridge in which the peptide is modified by adding a cysteine residue at the C terminus which forms a disulfide bridge with the thiol-modified molecular beacon. This disulfide bridge design allows the peptide to be cleaved from the molecular beacon by the reducing environment of the cytoplasm.

TABLE 2. Target sequences and the design of dual FRET molecular beacons.

Wild-type K-ras target (bases 1–78)
5'-ATGACTGAATATAAACTTGTGGTAGTT GGAGCTGGTGGCGTAGG
caag AGTGCCTTGACGATACAGC TAATTCAGAAT-3'
K-ras dual FRET molecular beacons
Donor MB: 5'-Cy3-CCTACGCCACCAGCTCCGTAGG-BHQ2-3'
Acceptor MB: 5'-BHQ3-AGTGCCTGTATCGTCAAGGCACT-Cy5-3'
Survivin target (bases 1–78)
5'-ATGGGTGCCCGACGTTGCCCCCTGCC TGGCAGCCCTTTCTC
aagg ACCACCGCATCTCTAC ATTCAAGAAGCTGGCCC-3'
Survivin dual FRET molecular beacons
Donor MB: 5'-Cy3-GAGAAAGGGCTGCCATTCTC/BHQ2-3'
Acceptor MB: 5'-BHQ3-ACCACGTAGAGATGCGGTGGT-Cy5-3'
“Random” sequence target
5'-ATCGGTGCGCTTGTGC-3'
“Random” sequence molecular beacon
Donor MB: 5'-Cy3-CACGTGCGACAAGCGCACCGATACGTG-BHQ2-3'
Acceptor MB: 5'-BHQ3-ACGTGCGACAAGCGCACCGATCACGT-Cy5-3'

random walk, thus having no exact match in the mammalian genome.

After delivering dual FRET molecular beacons into live HDF cells using SLO and incubating the cells for 1 h, the resulting fluorescence signal was imaged using FRET optics (excitation at 545 nm and emission detection using 665 nm filters). The signal from “random beacons” in cells served as the background. It was found that detection of the FRET signal significantly reduced false-positives, leading to sensitive imaging of K-ras and survivin mRNAs in live HDF and MIA PaCa-2 cells. For example, FRET detection gave a ratio of 2.25 of K-ras mRNA expression in stimulated versus unstimulated HDF, comparable to the ratio of 1.95 using RT-PCR, and in contrast to single-beacon result of 1.2. The detection of survivin mRNA also indicated that, compared with the single-beacon approach, dual FRET molecular beacons gave lower background signal, thus having a higher signal-to-background ratio.⁴³

An intriguing discovery in detecting K-ras and survivin mRNAs using dual FRET molecular beacons is the clear and detailed mRNA localization in living cells.⁴³ To demonstrate, in Fig. 5(a), a fluorescence image of K-ras mRNA in stimulated HDF cells is shown, indicating a filamentous localization pattern. The survivin mRNA, however, is localized in MIA PaCa-2 cell very differently. As shown in Fig. 5(b) in which the fluorescence image was superimposed with a white-light image of the cells, survivin mRNAs seemed to localize in a nonsymmetrical pattern within MIA PaCa-2 cells, often to one side of the nucleus of the cell. These mRNA localization patterns raise many interesting biological questions. For example, what is the biological implication of mRNA localization? How mRNAs are transported to their destination and how the destination is recognized? To what subcellular organelle might the mRNAs be colocalized?

The transport and localization of oskar mRNA in *Drosophila melanogaster* oocytes has also been visualized.¹⁰ In this work, molecular beacons with 2'-*O*-methyl backbone were delivered into cells using microinjection and the migration of oskar mRNAs were tracked in real time, from the nurse cells where it is produced to the posterior cortex of the oocyte where it is localized. Clearly, the direct visualization of specific mRNAs in living cells with molecular beacons will provide important insight into the intracellular trafficking and localization of RNA molecules.

Recently, Santangelo *et al.* revealed that K-ras and GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) mRNAs are colocalized with mitochondria inside live HDF cells.⁴² Specifically, the dual FRET molecular beacon pair targeting K-ras mRNA was delivered into HDF cells using SLO, and mitochondria in the same cell were labeled using MitoFluor Green. One hour after beacon and dye molecule delivery, the fluorescence signal from the acceptor molecular beacons and MitoFluor Green was detected using an epifluorescence microscope with different filter sets. As shown in Fig. 6(a–c), unexpectedly, the localization pattern of K-ras mRNA is strikingly similar to that of mitochondria, suggesting that there is a very close spatial correlation between K-ras mRNA and mitochondria (within the spatial resolution of optical imaging). Since K-ras proteins interact with proteins such as Bcl-2 in mitochondria to mediate both antiapoptotic and proapoptotic pathways, it seems that cells localize certain mRNAs where the corresponding proteins can easily bind to their partners.

A similar colocalization pattern was observed for GAPDH mRNAs in HDF cells. In this case single (unpaired) peptide-linked molecular beacons targeting GAPDH (see Table 1) were used for rapid cellular delivery of the probe and the fluorescence signal from molecular beacons and MitoFluor Green was detected using a confocal microscope. As shown in Fig. 6(d–f), most of the signals

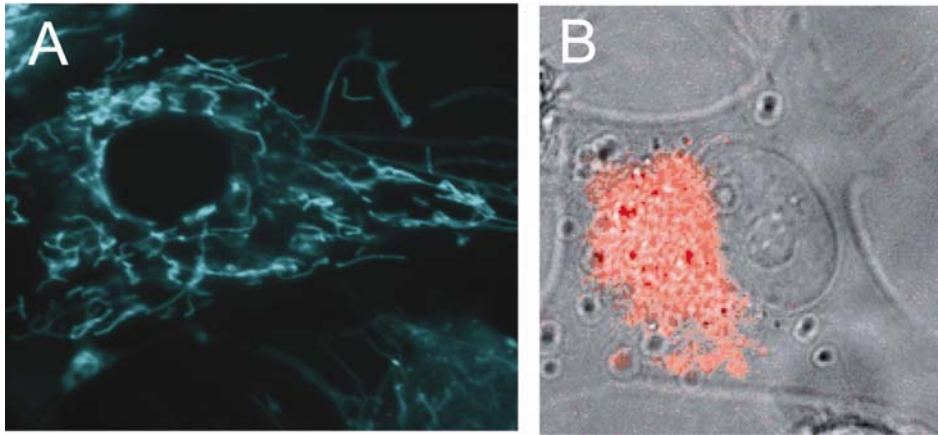


Figure 5. mRNA localization in HDF and MIAPaCa-2 cells. (a) Fluorescence images of K-ras mRNA in stimulated HDF cells. Note the filamentous K-ras mRNA localization pattern. (b) A fluorescence image of survivin mRNA localization in MIAPaCa-2 cells. Note that survivin mRNAs often localized to one side of the nucleus of the MIAPaCa-2 cells.

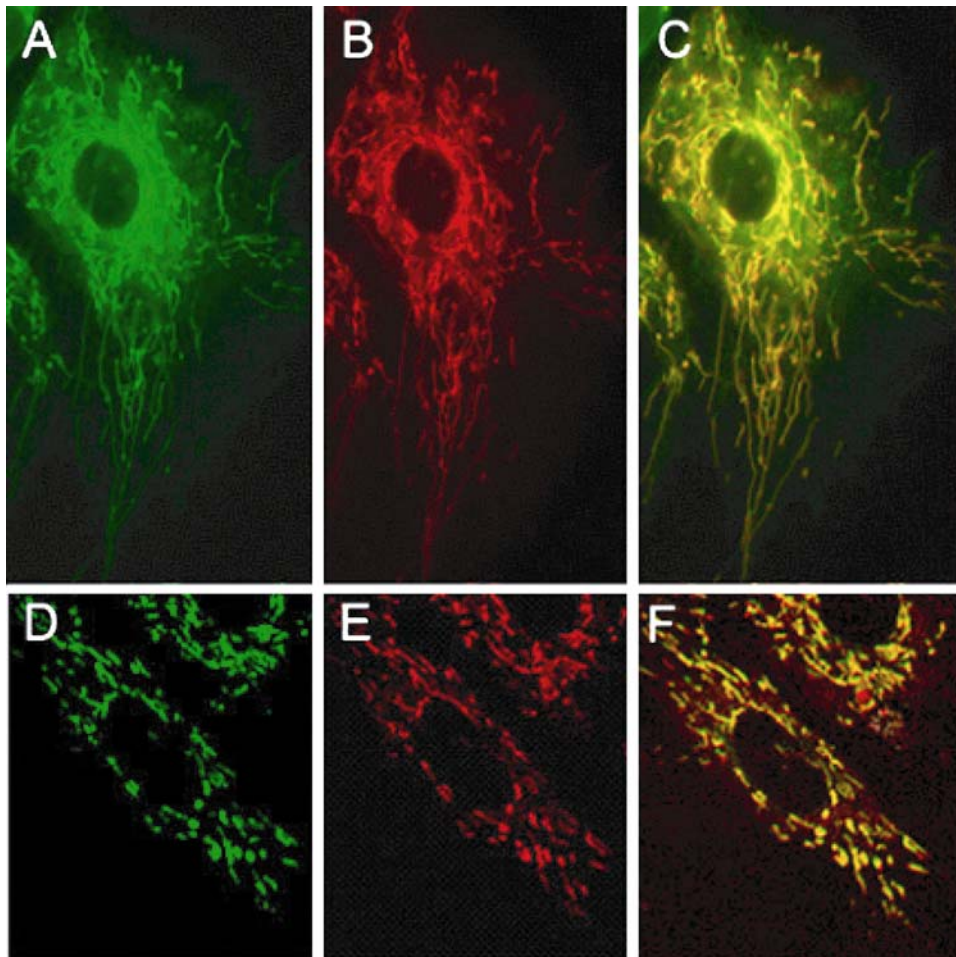


Figure 6. Colocalization of K-ras and GAPDH mRNAs with mitochondria in HDF cells. (a) and (b) display, respectively, the epifluorescence images of mitochondrial staining using MitoFluor Green and K-ras mRNA detected using dual FRET molecular beacons in the same HDF cell. Shown in (c) is the overlapping of the mitochondria and K-ras mRNA images, strongly suggesting that most of K-ras mRNA molecules are colocalized with mitochondria. The confocal images of mitochondrial staining and GAPDH mRNA detection in the same HDF cell are shown, respectively, in (d) and (e), and their overlap is shown in (f). It appears that GAPDH mRNA and mitochondria are colocalized as well.

from GAPDH-targeting molecular beacon and MitoFluor Green that labels mitochondria are clearly colocalized. This could be attributed to the association of GAPDH proteins with mitochondria in mediating glycolysis and the production of pyruvate. Here again, the localization of GAPDH mRNAs seems to be closely related to the function of GAPDH proteins. Therefore, the observation of mRNA colocalization with mitochondria may provide new insight into the dynamics and functions of mRNA and the associated protein.

BIOLOGICAL ISSUES AND ENGINEERING CHALLENGES

Nanostructured molecular probes such as molecular beacons have the potential to enjoy a wide range of applications that require sensitive detection of genomic sequences. However, to date molecular beacons are used mostly as a tool for the detection of single-stranded nucleic acids in homogeneous *in vitro* assays. For example, molecular beacons have been modified for solid phase studies.^{24,31} Surface-immobilized molecular beacons used in microarray assays allow for the high throughput parallel detection of nucleic acid targets while avoiding the difficulties associated with PCR-based labeling.^{31,50} Another novel application of molecular beacons is the detection of double-stranded DNA targets using PNA “openers” that form triplexes with the DNA strands.²⁶ Further, proteins can be detected by synthesizing “aptamer molecular beacon”^{21,65} which, upon binding to a protein, undergoes a conformational change that results in the restoration of fluorescence.

The most exciting application of nanostructured oligonucleotide probes, however, is living cell gene detection. As demonstrated, the dual FRET molecular beacons and peptide-linked molecular beacons can detect endogenous mRNA in living cells with high specificity, sensitivity, and signal-to-background ratio, and thus have the potential to provide a powerful tool for laboratory and clinical studies of gene expression *in vivo*. For example, molecular beacons can be used in high-throughput cell-based assays to quantify and monitor the dose-dependent changes of specific mRNA expression in response to different drug leads. The ability of molecular beacons to detect and quantify the expression of specific genes in living cells will also facilitate disease studies, such as viral infection detection and cancer diagnosis.

There are a number of challenges in detecting and quantifying RNA expression in living cells. In addition to issues of probe design and target accessibility, quantifying gene expression in living cells in terms mRNA copy-number per cell poses a significant challenge. For instance, it is necessary to distinguish true and background signals, determine the fraction of mRNA molecules hybridized with probes,

and quantify the possible self-quenching effect of the reporter, especially when mRNA is highly localized. Since the fluorescence intensity of the reporter may be altered by the intracellular environment, it is also necessary to create an internal control by, for example, injecting fluorescently labeled oligonucleotides with known quantity into the same cells and obtaining the corresponding fluorescence intensity. Further, unlike in RT-PCR studies where the mRNA expression is averaged over a large number of cells (usually over one million), in optical imaging of mRNA expression in living cells, only a relatively small number of cells (typically less than 1000) are observed. Therefore, the average copy number per cell may change with the total number of cells observed due to the (often large) cell-to-cell variation of mRNA expression.

Another issue in living cell gene detection using hairpin ODN probes is the possible effect of probes on normal cell function, including protein expression. As revealed in the antisense therapy research, complementary pairing of a short segment of an exogenous oligonucleotide to mRNA can have a profound impact on protein expression levels and even cell fate. For example, tight binding of the probe to the translation start site can block mRNA translation. Binding of a DNA probe to mRNA can also trigger RNase H-mediated mRNA degradation. However, the probability of eliciting antisense effects with hairpin probes may be very low when low concentrations of probes (<200 nM) are used for mRNA detection, in contrast to the high concentrations (typically 20 μM)¹⁹ employed in antisense experiments. Further, it generally takes 4 h before any noticeable antisense effect occurs, whereas visualization of mRNA with hairpin probes requires less than 2 h after delivery. However, it is important to carry out a systematic study of the possible antisense effects, especially for molecular beacons with 2'-*O*-methyl backbone, which may also trigger RNA interference.

As a new approach for *in vivo* gene detection, the nanostructured probes can be further developed to have enhanced sensitivity and a wider range of applications. For example, it is likely that hairpin ODN probes with quantum dot as the fluorophore will have a better ability to track the transport of individual mRNAs from the cell nucleus to the cytoplasm. Hairpin ODN probes with NIR dye as the reporter, combined with peptide-based delivery have the potential to detect specific RNAs in tissue samples, animals, or even humans. It is also possible to use lanthanide chelate as the donor in a dual FRET probe assay and perform time-resolved measurements to dramatically increase the signal-to-noise ratio, thus achieving high sensitivity in detecting low abundance genes. Although very challenging, the development of these and other nanostructured ODN probes will significantly enhance our ability to image, track, and quantify gene expression *in vivo*, and provide a powerful tool for basic and clinical studies of human health and disease.

ACKNOWLEDGMENT

This work was supported in part by National Institute of Health Grant UO1 HL80711-01 and by the Office of Science, Department of Energy Grant DE-FG02-04ER63785 to GB.

REFERENCES

- ¹Adams, M. D., M. Dubnick, A. R. Kerlavage, R. Moreno, J. M. Kelley, T. R. Utterback, J. W. Nagle, C. Fields, and J. C. Venter. Sequence identification of 2375 human brain genes. *Nature* 355:632–634, 1992.
- ²Allinquant, B., P. Hantraye, P. Mailleux, K. Moya, C. Bouillot, and A. Prochiantz. Downregulation of amyloid precursor protein inhibits neurite outgrowth *in vitro*. *J. Cell Biol.* 128:919–927, 1995.
- ³Alwine, J. C., D. J. Kemp, B. A. Parker, J. Reiser, J. Renart, G. R. Stark, and G. M. Wahl. Detection of specific RNAs or specific fragments of DNA by fractionation in gels and transfer to diazobenzyloxymethyl paper. *Methods Enzymol.* 68:220–242, 1979.
- ⁴Barry, M. A., and A. Eastman. Identification of deoxyribonuclease II as an endonuclease involved in apoptosis. *Arch. Biochem. Biophys.* 300:440–450, 1993.
- ⁵Bassell, G. J., C. M. Powers, K. L. Taneja, and R. H. Singer. Single mRNAs visualized by ultrastructural *in situ* hybridization are principally localized at actin filament intersections in fibroblasts. *J. Cell Biol.* 126:863–876, 1994.
- ⁶Becker-Hapak, M., S. S. McAllister, and S. F. Dowdy. TAT-mediated protein transduction into mammalian cells. *Methods* 24:247–256, 2001.
- ⁷Behrens, S., B. M. Fuchs, F. Mueller, and R. Amann. Is the *in situ* accessibility of the 16S rRNA of *Escherichia coli* for Cy3-labeled oligonucleotide probes predicted by a three-dimensional structure model of the 30S ribosomal subunit? *Appl. Environ. Microbiol.* 69:4935–4941, 2003.
- ⁸Bernacchi, S., and Y. Mely. Exciton interaction in molecular beacons: A sensitive sensor for short range modifications of the nucleic acid structure. *Nucleic Acids Res.* 29:E62–2, 2001.
- ⁹Bonnet, G., S. Tyagi, A. Libchaber, and F. R. Kramer. Thermodynamic basis of the enhanced specificity of structured DNA probes. *Proc. Natl. Acad. Sci. U.S.A.* 96:6171–6176, 1999.
- ¹⁰Bratu, D. P., B. J. Cha, M. M. Mhlanga, F. R. Kramer, and S. Tyagi. Visualizing the distribution and transport of mRNAs in living cells. *Proc. Natl. Acad. Sci. U.S.A.* 100:13308–13313, 2003.
- ¹¹Brodsky, A. S., and P. A. Silver. Identifying proteins that affect mRNA localization in living cells. *Methods* 26:151–155, 2002.
- ¹²Brooks, H., B. Lebleu, and E. Vives. Tat peptide-mediated cellular delivery: Back to basics. *Adv. Drug Deliv. Rev.* 57:559–577, 2005.
- ¹³Buongiorno-Nardelli, M., and F. Amaldi. Autoradiographic detection of molecular hybrids between RNA and DNA in tissue sections. *Nature* 225:946–948, 1970.
- ¹⁴Crick, F. H. On protein synthesis. *Symp. Soc. Exp. Biol.* 12:138–163, 1958.
- ¹⁵Dirks, R. W., C. Molenaar, and H. J. Tanke. Methods for visualizing RNA processing and transport pathways in living cells. *Histochem. Cell Biol.* 115:3–11, 2001.
- ¹⁶Dokka, S., and Y. Rojanasakul. Novel non-endocytic delivery of antisense oligonucleotides. *Adv. Drug Deliv. Rev.* 44:35–49, 2000.
- ¹⁷Forrest, K. M., and E. R. Gavis. Live imaging of endogenous RNA reveals a diffusion and entrapment mechanism for nanos mRNA localization in *Drosophila*. *Curr. Biol.* 13:1159–1168, 2003.
- ¹⁸Giles, R. V., C. J. Ruddell, D. G. Spiller, J. A. Green, and D. M. Tidd. Single base discrimination for ribonuclease H-dependent antisense effects within intact human leukaemia cells. *Nucleic Acids Res.* 23:954–961, 1995.
- ¹⁹Giles, R. V., D. G. Spiller, J. Grzybowski, R. E. Clark, P. Nicklin, and D. M. Tidd. Selecting optimal oligonucleotide composition for maximal antisense effect following streptolysin O-mediated delivery into human leukaemia cells. *Nucleic Acids Res.* 26:1567–1575, 1998.
- ²⁰Glotzer, J. B., R. Saffrich, M. Glotzer, and A. Ephrussi. Cytoplasmic flows localize injected oskar RNA in *Drosophila* oocytes. *Curr. Biol.* 7:326–337, 1997.
- ²¹Hamaguchi, N., A. Ellington, and M. Stanton. Aptamer beacons for the direct detection of proteins. *Anal. Biochem.* 294:126–131, 2001.
- ²²Huang, Q., and T. Pederson. A human U2 RNA mutant stalled in 3' end processing is impaired in nuclear import. *Nucleic Acids Res.* 27:1025–1031, 1999.
- ²³Jacobson, M. R., and T. Pederson. Localization of signal recognition particle RNA in the nucleolus of mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.* 95:7981–7986, 1998.
- ²⁴Kambhampati, D., P. E. Nielsen, and W. Knoll. Investigating the kinetics of DNA–DNA and PNA–DNA interactions using surface plasmon resonance-enhanced fluorescence spectroscopy. *Biosens. Bioelectron.* 16:1109–1118, 2001.
- ²⁵Kostrikis, L. G., S. Tyagi, M. M. Mhlanga, D. D. Ho, and F. R. Kramer. Spectral genotyping of human alleles. *Science* 279:1228–1229, 1998.
- ²⁶Kuhn, H., V. V. Demidov, J. M. Coull, M. J. Fiandaca, B. D. Gildea, and M. D. Frank-Kamenetskii. Hybridization of DNA and PNA molecular beacons to single-stranded and double-stranded DNA targets. *J. Am. Chem. Soc.* 124:1097–1103, 2002.
- ²⁷Lakowicz, J. R., Principles of Fluorescence Spectroscopy, 2nd ed. New York: Plenum Publishing, 1999.
- ²⁸Leonetti, J. P., N. Mehti, G. Degols, C. Gagnor, and B. Lebleu. Intracellular distribution of microinjected antisense oligonucleotides. *Proc. Natl. Acad. Sci. U.S.A.* 88:2702–2706, 1991.
- ²⁹Li, J. J., R. Geyer, and W. Tan. Using molecular beacons as a sensitive fluorescence assay for enzymatic cleavage of single-stranded DNA. *Nucleic Acids Res.* 28:E52, 2000.
- ³⁰Liang, P., and A. B. Pardee. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* 257:967–971, 1992.
- ³¹Liu, X., and W. Tan. A fiber-optic evanescent wave DNA biosensor based on novel molecular beacons. *Anal. Chem.* 71:5054–5059, 1999.
- ³²Marras, S. A., F. R. Kramer, and S. Tyagi. Efficiencies of fluorescence resonance energy transfer and contact-mediated quenching in oligonucleotide probes. *Nucleic Acids Res.* 30:e122, 2002.
- ³³Medley, C. D., T. J. Drake, J. M. Tomasini, R. J. Rogers, and W. Tan. Simultaneous monitoring of the expression of multiple genes inside of single breast carcinoma cells. *Anal. Chem.* 77:4713–4718, 2005.
- ³⁴Mhlanga, M. M., V. D. Fung, F. R. Kramer, and S. Tyagi. tRNA-linked molecular beacons for imaging mRNAs in the cytoplasm of living cells. *Nucleic Acids Res.* 33:1902–1912, 2005.
- ³⁵Molenaar, C., A. Abdulle, A. Gena, H. J. Tanke, and R. W. Dirks. Poly(A)+ RNAs roam the cell nucleus and pass through speckle domains in transcriptionally active and inactive cells. *J. Cell Biol.* 165:191–202, 2004.

- ³⁶Molenaar, C., S. A. Marras, J. C. Slats, J. C. Truffert, M. Lemaitre, A. K. Raap, R. W. Dirks, and H. J. Tanke. Linear 2' *O*-Methyl RNA probes for the visualization of RNA in living cells. *Nucleic Acids Res.* 29:E89–9, 2001.
- ³⁷Nitin, N., P. J. Santangelo, G. Kim, S. Nie, and G. Bao. Peptide-linked molecular beacons for efficient delivery and rapid mRNA detection in living cells. *Nucleic Acids Res.* 32:e58, 2004.
- ³⁸Peng, X. H., Z. H. Cao, J. T. Xia, G. W. Carlson, M. M. Lewis, W. C. Wood, and L. Yang. Real-time detection of gene expression in cancer cells using molecular beacon imaging: New strategies for cancer research. *Cancer Res.* 65:1909–1917, 2005.
- ³⁹Piatek, A. S., T. S. Pol, A. C. Telenti, L. P. Miller, F. R. Kramer, and D. Alland. Molecular beacon sequence analysis for detecting drug resistance in *Mycobacterium tuberculosis*. *Nat. Biotechnol.* 16:359–363, 1998.
- ⁴⁰Price, N. C., and L. Stevens. *Fundamentals of Enzymology: The Cell and Molecular Biology of Catalytic Proteins*, 3rd ed. New York: Oxford University Press, 1999.
- ⁴¹Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. Arnheim. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230:1350–1354, 1985.
- ⁴²Santangelo, P. J., N. Nitin, and G. Bao. Direct visualization of mRNA colocalization with mitochondria in living cells using molecular beacons. *J. Biomed. Opt.* 10: 44025, 2005.
- ⁴³Santangelo, P. J., B. Nix, A. Tsourkas, and G. Bao. Dual FRET molecular beacons for mRNA detection in living cells. *Nucleic Acids Res.* 32:e57, 2004.
- ⁴⁴Schena, M., D. Shalon, R. W. Davis, and P. O. Brown. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 270:467–470, 1995.
- ⁴⁵Shav-Tal, Y., X. Darzacq, S. M. Shenoy, D. Fusco, S. M. Janicki, D. L. Spector, and R. H. Singer. Dynamics of single mRNPs in nuclei of living cells. *Science* 304:1797–1800, 2004.
- ⁴⁶Snyder, E. L., and S. F. Dowdy. Protein/peptide transduction domains: Potential to deliver large DNA molecules into cells. *Curr. Opin. Mol. Ther.* 3:147–152, 2001.
- ⁴⁷Sokol, D. L., X. Zhang, P. Lu, and A. M. Gewirtz. Real time detection of DNA:RNA hybridization in living cells. *Proc. Natl. Acad. Sci. U.S.A.* 95:11538–11543, 1998.
- ⁴⁸Spiller, D. G., R. V. Giles, J. Grzybowski, D. M. Tidd, and R. E. Clark. Improving the intracellular delivery and molecular efficacy of antisense oligonucleotides in chronic myeloid leukemia cells: A comparison of streptolysin-O permeabilization, electroporation, and lipophilic conjugation. *Blood* 91:4738–4746, 1998.
- ⁴⁹States, D. J., W. Gish, and S. F. Altschul. Improved sensitivity of nucleic acid database searches using application-specific scoring matrices. *Methods* 3:66–70, 1991.
- ⁵⁰Steeners, F. J., J. A. Ferguson, and D. R. Walt. Screening unlabeled DNA targets with randomly ordered fiber-optic gene arrays. *Nat. Biotechnol.* 18:91–94, 2000.
- ⁵¹Troy, C. M., D. Derossi, A. Prochiantz, L. A. Greene, and M. L. Shelanski. Downregulation of Cu/Zn superoxide dismutase leads to cell death via the nitric oxide-peroxynitrite pathway. *J. Neurosci.* 16:253–261, 1996.
- ⁵²Tsourkas, A., M. A. Behlke, and G. Bao. Structure–function relationships of shared-stem and conventional molecular beacons. *Nucleic Acids Res.* 30:4208–4215, 2002.
- ⁵³Tsourkas, A., M. A. Behlke, S. D. Rose, and G. Bao. Hybridization kinetics and thermodynamics of molecular beacons. *Nucleic Acids Res.* 31:1319–1330, 2003.
- ⁵⁴Tsourkas, A., M. A. Behlke, Y. Xu, and G. Bao. Spectroscopic features of dual fluorescence/luminescence resonance energy-transfer molecular beacons. *Anal. Chem.* 75:3697–3703, 2003.
- ⁵⁵Tsuji, A., H. Koshimoto, Y. Sato, M. Hirano, Y. Sei-Iida, S. Kondo, and K. Ishibashi. Direct observation of specific messenger RNA in a single living cell under a fluorescence microscope. *Biophys. J.* 78:3260–3274, 2000.
- ⁵⁶Tyagi, S., and O. Alsmadi. Imaging native beta-actin mRNA in motile fibroblasts. *Biophys. J.* 87:4153–4162, 2004.
- ⁵⁷Tyagi, S., D. P. Bratu, and F. R. Kramer. Multicolor molecular beacons for allele discrimination. *Nat. Biotechnol.* 16:49–53, 1998.
- ⁵⁸Tyagi, S., and F. R. Kramer. Molecular beacons: Probes that fluoresce upon hybridization. *Nat. Biotechnol.* 14:303–308, 1996.
- ⁵⁹Tyagi, S., S. A. Marras, and F. R. Kramer. Wavelength-shifting molecular beacons. *Nat. Biotechnol.* 18:1191–1196, 2000.
- ⁶⁰Velculescu, V. E., L. Zhang, B. Vogelstein, and K. W. Kinzler. Serial analysis of gene expression. *Science* 270:484–487, 1995.
- ⁶¹Vet, J. A., A. R. Majithia, S. A. Marras, S. Tyagi, S. Dube, B. J. Poiesz, and F. R. Kramer. Multiplex detection of four pathogenic retroviruses using molecular beacons. *Proc. Natl. Acad. Sci. U.S.A.* 96:6394–6399, 1999.
- ⁶²Wadia, J. S., and S. F. Dowdy. Protein transduction technology. *Curr. Opin. Biotechnol.* 13:52–56, 2002.
- ⁶³Wadia, J. S., and S. F. Dowdy. Transmembrane delivery of protein and peptide drugs by TAT-mediated transduction in the treatment of cancer. *Adv. Drug Deliv. Rev.* 57:579–596, 2005.
- ⁶⁴Walev, I., S. C. Bhakdi, F. Hofmann, N. Djonder, A. Val-eva, K. Aktories, and S. Bhakdi. Delivery of proteins into living cells by reversible membrane permeabilization with streptolysin-O. *Proc. Natl. Acad. Sci. U.S.A.* 98:3185–3190, 2001.
- ⁶⁵Yamamoto, R., T. Baba, and P. K. Kumar. Molecular beacon aptamer fluoresces in the presence of Tat protein of HIV-1. *Genes Cells* 5:389–396, 2000.