Published in final edited form as:

Nanomedicine (Chichester). 2010 ; : 143–165. doi:10.1002/9783527628155.nanotech054.

NANOSTRUCTURED PROBES FOR IN VIVO GENE DETECTION

Gang Bao, Phillip Santangelo, Nitin Nitin, and Won Jong Rhee

Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, Atlanta, GA 30332

Abstract

The ability to visualize in real-time the expression dynamics and localization of specific RNAs *in vivo* offers tremendous opportunities for biological and disease studies including cancer detection. However, quantitative methods such as real-time PCR and DNA microarrays rely on the use of cell lysates thus not able to obtain important spatial and temporal information. Fluorescence proteins and other reporter systems cannot image endogenous RNA in living cells. Fluorescence *in situ* hybridization (FISH) assays require washing to achieve specificity, therefore can only be used with fixed cells. Here we review the recent development of nanostructured 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 oligonucleotide probes, combined with novel delivery strategies, 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 are given to illustrate the biological applications, and issues in probe design, 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 probe; RNA detection; live cell; molecular beacon; fluorescence resonance energy transfer

1. INTRODUCTION

The ability to image specific RNAs in living cells in real time can provide essential information on RNA synthesis, processing, transport, and localization, as well as 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. 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 spatially, of specific RNAs in a cell. Therefore, determining the dynamics and localization of RNA molecules in living cells will significantly impact on molecular biology and medicine.

Many *in vitro* methods have been developed to provide a relative (mostly semi-quantitative) measure of gene expression level within a cell population using purified DNA or RNA obtained from cell lysate. These methods include PCR [1], Northern hybridization (or Northern blotting) [2], expressed sequence tag (EST) [3], serial analysis of gene expression (SAGE) [4], differential display [5], and DNA microarrays [6]. 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, using *in vitro* methods to detect and identify 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.

Labeled linear oligonucleotide (ODN) probes have been used to study intracellular mRNA via *in situ* hybridization (ISH) [7] in which cells are fixed and permeabilized to increase the probe delivery efficiency. Unbound probes are removed by washing to reduce background and achieve specificity [8]. To enhance the signal level, multiple probes targeting the same mRNA can be used [7]. However, fixation agents and other supporting chemicals can have considerable effect on signal level [9] and possibly on the integrity of certain organelles such as mitochondria. Thus, fixation of cells, by either cross-linking or denaturing agents, and the use of proteases in ISH assays may prevent from obtaining an accurate description of intracellular mRNA localization. It is also difficult to obtain a dynamic picture of gene expression in cells using ISH methods.

Of particular interest is the fluorescence imaging of specific messenger RNAs (mRNAs), both their expression level and subcellular localization, in living cells. As shown schematically in Figure 1, for eukaryotic cells a pre-mRNA molecule is synthesized in cell nucleus. After processing (including splicing and polyadenylation) the mature mRNAs are transported from cell nucleus to cytoplasm, and often localized at specific sites. The mRNAs are then being translated by ribosome to make specific proteins, and degraded by RNases after a certain amount of time. The limited lifetime of mRNA enables a cell to alter protein synthesis rapidly in response to its changing needs. During the entire life cycle of an mRNA, it is always complexed with RNA-binding proteins to form a ribonucleoprotein (RNP). This has significant implications to the live-cell imaging of mRNAs (as discussed later).

In order to detect RNA molecules in living cells with high specificity, sensitivity and signal-to-background ratio, especially for low abundance genes and clinical samples containing a small number of diseased cells, the probes need to recognize RNA targets with high specificity, convert target recognition *directly* into a measurable signal, and differentiate between true and false-positive signals. It is important for the probes to quantify low gene expression levels with high accuracy, and have fast kinetics in tracking alterations in gene expression in real time. For detecting genetic alterations such as mutations, insertions and deletions, the ability to recognize single nucleotide polymorphisms (SNPs) is essential. To achieve this optimal performance, it is necessary to have a good understanding of the structure-function relationship of the probes, probe stability, and RNA target accessibility in living cells. It is also necessary to achieve efficient cellular delivery of probes with minimal probe degradation.

In the remaining sections, we review commonly used fluorescent probes for RNA detection, discuss the critical issues in living cell RNA detection, including probe design, target accessibility, cellular delivery of probes, and detection sensitivity, specificity and signal-to-background ratio. Emphasis is placed on the design and application of molecular beacons, although some of the issues are common to other oligonucleotide probes.

2. FLUORESCENT PROBES FOR LIVE CELL RNA DETECTION

Several classes of molecular probes have been developed for RNA detection in living cells, including: (1) tagged linear oligonucleotide (ODN) probes; (2) oligonucleotide hairpin probes; (3) probes using fluorescent proteins as reporter. Although probes composed of full length RNAs (mRNA or nuclear RNA) tagged with a fluorescent or radioactive reporter have been used to study the intracellular localization of RNA [10-12], these probes are not discussed here since they cannot be used to measure the expression level of specific RNAs in living cells.

Tagged linear ODN probes

Single fluorescently labeled linear oligonucleotide probes have been developed for RNA tracking and localization studies in living cells [13-15]. Although these probes may recognize specific endogenous RNA transcripts in living cells via Watson-Crick base pairing and reveal subcellular RNA localization, this approach lacks the ability to distinguish background from true signal, since both bound probes (i.e., probes hybridized to RNA target) and unbound probes give fluorescence signal. It may also lack detection specificity since partial match between the probe and target sequences could induce probe hybridization to RNA molecules of multiple genes. A novel way to increase signal-to-noise ratio and improve detection specificity is to use two linear probes with a fluorescence resonance energy transfer (FRET) pair of (donor and acceptor) fluorophores [13]. 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 few bases 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 biological and disease studies.

ODN hairpin probes

Hairpin nucleic acid probes have the potential to be highly sensitive and specific in live-cell RNA detection. As shown in Figure 2a-2b, 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 [16]. They are designed to form a stem-loop hairpin structure in the absence of a complementary target so that fluorescence of the fluorophore is quenched. Hybridization with the target nucleic acid opens the hairpin and physically separates the fluorophore from quencher, allowing a fluorescence signal to be emitted upon excitation (Fig. 2b). Under optimal conditions, the fluorescence intensity of molecular beacons can increase by > 200-fold upon binding to their targets [16]. 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 [17, 18]. 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, real-time monitoring of PCR, genotyping and mutation detection, viral infection studies, and mRNA detection in living cells [14, 19-32].

As illustrated in Fig. 2a, a conventional molecular beacon has four essential components: loop, stem, fluorophore, and quencher. The loop usually consists of 15-25 nucleotides and is selected to have a unique target sequence and proper melting temperature. The stem, formed by two complementary short-arm sequences, is typically 4-6 bases long and chosen to be independent of the target sequence (Fig. 2a).

A novel design of hairpin probes is the wavelength-shifting molecular beacons that can fluoresce in a variety of different colors [33]. As shown in Fig. 2c, in this design, a molecular beacon contains two fluorophores: a first fluorophore 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 [17,18], 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, molecular beacons may provide a simple and promising tool for detecting SNPs in disease diagnosis.

Figure 3 compares the basic features of molecular beacon versus fluorescence in-situ hybridization (FISH). Specifically, molecular beacons are dual-labeled hairpin probes of 15-25 nt, while FISH probes are dye-labeled linear oligonucleotides of 40-50 nt. The molecular beacon based approach has the advantage of detecting RNA in live cells without the need of cell fixation and washing. However, it requires cellular delivery of the probes and has low target accessibility (discussed in later sections). The advantage of FISH assays is the ease of probe design due to better target accessibility. Although FISH assays can be used to image the localization of mRNA in fixed cells, they rely on stringent washing to achieve signal specificity and do not have the ability to image the dynamics of gene expression in living cells.

In the conventional molecular beacon design, the stem sequence is typically independent of the target sequence (Fig. 2b), although sometimes two end bases of the probe sequence, each adjacent to one arm sequence of the stem, could be complementary with each other, thus forming part of the stem (light blue base of the stem shown in Fig. 2a). Molecular beacons can also be designed such that all the bases of one arm of the stem (to which a fluorophore is conjugated) are complementary to the target sequence, thus participating in both stem formation and target hybridization (shared-stem molecular beacons) [34] (Fig. 2d). The advantage of this shared-stem design is to help fix the position of the fluorophore that attached to the stem arm, limiting its degree-of-freedom of motion, and increasing the fluorescence resonance energy transfer (FRET) in the dual FRET molecular beacon design, as discussed below.

A dual FRET molecular beacons approach was developed [26-28] to overcome the difficulty that, in live-cell RNA detection, molecular beacons are often degraded by nucleases or open due to non-specific interaction with hairpin-binding proteins, causing a significant amount of false-positive signal. In this dual probe design, a pair of molecular beacons labeled with a donor and an acceptor fluorophore, respectively are employed (Fig. 4). The probe sequences are chosen such that this pair of molecular beacons hybridizes to adjacent regions on a single RNA target (Figure 4). Since FRET is very sensitive to the distance between donor and acceptor fluorophores and typically occurs when the donor and acceptor fluorophores are within ~10 nm, FRET signal is generated by the donor and acceptor beacons only if both probes are 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 differentiable from non-FRET false-positive signals due to probe degradation and non-specific 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, leading to an enhanced ability to quantify RNA expression in living cells[28].

Fluorescent protein based probes

In addition to oligonucleotide probes, tagged RNA-binding proteins such as those with GFP tags have been used to detect mRNA in live cells [35]. 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, a coat protein of the RNA bacteriophage MS2 was tagged with GFP and a RNA sequence corresponding to several MS2 binding sites was introduced to the mRNA of interest, which allowed for the specific targeting of the nanos mRNA in live *Drosophila* eggs[36]. The GFP-MS2 approach has been used to track the localization and dynamics of RNA in living cells with single molecule sensitivity [37,38]. However, since unbound GFP-tagged MS2 proteins also give fluorescence signal, the background signal in the GFP-MS2 approach could be high, leading to a low signal-to-background ratio in live-cell imaging of RNA.

An interesting fluorescent-protein based approach that overcomes this problem is to utilize the fluorescent protein complementation[39,40]. In this approach (split-GFP), a RNA-binding protein is dissected into two fragments, which are respectively fused to the split fragments of a fluorescent protein. Binding of the two tagged fragments of the RNA-binding protein to adjacent sites on the same mRNA molecule (or two parts of an aptamer sequence inserted to the mRNA sequence) brings the two halves of the fluorescent protein together, reconstituting the fluorescent protein and restoring fluorescence[40]. Alternatively, two RNA binding proteins that bind specifically to adjacent sites on the same mRNA molecule can be tagged with the split fragments of a fluorescent protein, and their binding to the target mRNA results in the restoration of fluorescence[39]. The advantage of this novel approach is that background signal is low – no fluoresce signal unless the RNA binding proteins (or protein fragments) are bound to the target mRNA. The split-GFP method, however, may have difficulties in tracking the dynamics of RNA expression in real-time, since the reconstitution of the fluorescent protein from the split fragments typically takes 2-4 hours, during which the RNA expression level may change. Transfection efficiency could also be a major concern in the GFP-based approaches in that usually only a few percent of the cells express the fluorescent proteins following transfection. This limits the application of the split-GFP methods in detecting diseased cells using mRNA as a biomarker for the disease.

3. PROBE DESIGN AND STRUCTURE-FUNCTION RELATIONS

Target specificity

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 BLAST [41] or similar software to select multiple target sequences of 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.

Several approaches can be taken to validate the signal specificity. For example, one could up- or down-regulate the expression level of a specific RNA, quantify the level using RT-PCR, and comparing the PCR result with that of molecular beacon based imaging of the same RNA in living cells. However, complications may arise when the approach used to change the RNA expression level in living cells has an effect on multiple genes, leading to some ambiguity even when the PCR and beacon results match. Perhaps the best way to down-regulate the level of a specific mRNA in live-cells is to use siRNA treatment, which typically leads to >80% reduction of the specific mRNA level. Since the effect of siRNA treatment varies depending on the specific siRNA probes used, the siRNA delivery method, and the cell type, optimization of the protocol (probe design and delivery method/ conditions) is often needed.

Molecular beacon structure-function relations

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 [17,18]. 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 [17,34].

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 Figure 5a. 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 influence 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 Figure 5b. 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. 5b.

From the thermodynamic and kinetic studies, it was found that, if the stem length is too large, it is be 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 MB specificity[17,34]. 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 hybridization kinetics but suffer from lower signal-to-background ratios compared with molecular beacons with longer stems.

Target accessibility

A critical issue in molecular beacon design is target accessibility, as is the case for most oligonucleotide probes for live-cell RNA detection. It is well known that a functional mRNA molecule in a living cell always has RNA-binding proteins on it, forming a ribonucleoprotein (RNP). Further, an mRNA molecule often has double stranded portions and forms secondary (folded) structures (Fig. 6). Therefore, in designing a molecular beacon, it is necessary to avoid targeting mRNA sequences that are double-stranded, or occupied by RNA-binding proteins, for otherwise the probe has to compete off the RNA strand or the RNA-binding protein in order to hybridize to the target. Indeed, molecular beacons designed for targeting a specific mRNA often show no signal when delivered to living cells. 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 in living cells to select the best target sequence.

To uncover the possible molecular beacon design rules, the accessibility of BMP-4 mRNA was studied using different beacon designs[42]. Specifically, molecular beacons were designed to target the start codon and termination codon regions, the siRNA and anti-sense oligonucleotide probe sites identified previously, and sites that were randomly chosen. All the target sequences are unique to BMP-4 mRNA. Of the eight molecular beacons designed to target BMP-4 mRNA, it was found that only two beacons gave strong signal, one targets the start codon region, and the other targets the termination codon region. It was also found that, even for a molecular beacon that works well, shifting its targeting sequence by just a few bases towards the 3' or 5' ends would significantly reduce the fluorescence signal from beacons in a live-cell assay, indicating that the target accessibility is quite sensitive to the location of the targeting sequence. These results, together with molecular beacons validated previously, suggest that the start codon and termination codon regions and the exon-exon junctions are more accessible than other locations in an mRNA.

Fluorophores and quenchers

With proper backbone synthesis and fluorophore/quencher conjugation, in theory, a molecular beacon can be labeled with any desired reporter-quencher pair. However, proper selection of the reporter and quencher could improve the signal-to-background ratio and multiplexing capabilities. 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 [19]. Molecular beacons can even be labeled simultaneously with two fluorophores, i.e., “wavelength shifting” reporter dyes (Fig. 2c), allowing multiple reporter dye sets to be excited by the same monochromatic light source yet fluorescing in a variety of

colors [33]. 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 (Fig. 4), 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 efficiency 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 [43], and the distance between the donor and acceptor molecules (usually 4-5 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), fluorescein (FAM) (donor) and Cy3 (acceptor).

It is relatively straightforward to select the quencher molecules. Organic quencher molecules such as dabcyl, 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 [44].

4. 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 and 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 non-endocytic methods. Endocytic delivery typically employs cationic and polycationic molecules such as liposomes and dendrimers, while non-endocytic 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 hours. 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 [45]. Consequently, only 0.01-10 percent of the probes remain functioning after escaped from endosomes and lysosomes [46].

Oligonucleotide probes (including molecular beacons) have been delivered into cells via microinjection [47]. 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°C to 4°C did not have a significant effect on ODN nuclear accumulation, ruling out active, motor-protein driven transport [47]. 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 [13]. After microinjected 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[48]. However, even without the problem of unwanted nuclear accumulation, microinjection is inefficient in delivering probes into a large number of cells.

Another non-endocytic 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 [49-51]. 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 [50,52].

Cell penetrating peptides (CPP) have been used to introduce proteins, nucleic acids and other biomolecules into living cells [53-55]. 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 to living cells both in cell culture and in tissue using cell penetrating peptides [56,57]. For example, Allinquant et al.[58] 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.[59] 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 different linkages (Fig. 7); the resulting peptide-linked molecular beacons were delivered into living cells to target GAPDH and survivin mRNAs[29]. 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 hours and resulted in a punctate fluorescence signal in the cytoplasmic vesicles and a high background in both cytoplasm and nucleus of cells[29]. It was clearly demonstrated that cellular delivery of molecular beacons using the peptide-based approach has far better performance compared with conventional transfection methods.

5. LIVING CELL RNA DETECTION USING NANOSTRUCTURED PROBES

Sensitive gene detection in living cells presents a significant challenge. In addition to issues with target accessibility, 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 and advanced fluorescence microscopy imaging, but also a better understanding of RNA biology 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 [28]. K-ras is one of the most frequently mutated genes in human cancers[60]. A member of the G-protein family, K-ras is involved in transducing growth-promoting signals from the cell surface. Survivin, one of the inhibitor of apoptosis proteins (IAPs), is normally expressed during fetal development but not in most normal adult tissues [61], thus can be used as a tumor biomarker for several types of cancers. Each FRET probe pair consisted of two molecular beacons, one labeled with a donor fluorophore (Cy3, donor beacon) and a second labeled with an acceptor fluorophore (Cy5, acceptor beacon). These molecular beacons were designed to hybridize to adjacent regions on an mRNA target so that the two fluorophores lie within the FRET range (~ 6 nm) when probe/target hybridization occurs for both beacons. 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 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 random walk, thus having no exact match in the mammalian genome. 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 MIAPaCa-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 indicted that, compared with the single-beacon approach, dual FRET molecular beacons gave lower background signal, thus having a higher signal-to-background ratio [28].

Biological Significance

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 [28]. To demonstrate, in Fig. 8a, a fluorescence image of K-ras mRNA in stimulated HDF cells is shown, indicating an intriguing filamentous localization pattern. The localization pattern of K-ras mRNA was further studied and found to be co-localized with mitochondria inside live HDF cells [62]. Since K-ras proteins interact with proteins such as Bcl-2 in mitochondria to mediate both anti-apoptotic and pro-apoptotic pathways, it seems that cells localize certain mRNAs where the corresponding proteins can easily bind to their partners.

The survivin mRNA, however, is localized in MIAPaCa-2 cell very differently. As shown in Fig. 8b in which the fluorescence image was superimposed with a white-light image of the cells, survivin mRNAs seemed to localize in a non-symmetrical pattern within MIAPaCa-2 cells, often to one side of the nucleus of the cell. These mRNA localization patterns raise many interesting biological questions. For example, how mRNAs are transported to their

destination and how the destination is recognized? To what subcellular organelle might the mRNAs be co-localized? What is the biological implication of mRNA localization? Although mRNA localization in living cells is believed to be closely related to post-transcriptional regulation of gene expression, much remains to be seen if such localization indeed targets a protein to its site of function by producing the protein ‘right on the spot’.

The transport and localization of oskar mRNA in *Drosophila melanogaster* oocytes has also been visualized [26]. 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.

As another example of targeting specific genes in living cells, molecular beacons were used to detect the viral genome and characterize the spreading of bovine respiratory syncytial virus (BRSV) in living cells [63]. It was found that molecular beacon signal could be detected in single living cells infected by RSV with high detection sensitivity, and the signal revealed a connected, highly three-dimensional, amorphous inclusion-body structure not seen in fixed cells. Shown in Fig. 9 is the molecular beacon signal indicating the spreading of viral infection at days 1, 3, 5 and 7 post-infection (PI), which demonstrates the ability of molecular beacons to monitor and quantify in real-time the viral infection process. Molecular beacons were further used to image the viral genomic RNA (vRNA) of human RSV (hRSV) in live Vero cells, revealing the dynamics of filamentous virion egress, and providing insight into how viral filaments bud from the plasma membrane of the host cell [64].

6. ENGINEERING CHALLENGES IN NEW PROBE DEVELOPMENT

Nanostructured molecular probes such as molecular beacons have the potential to enjoy a wide range of applications that require sensitive detection of genomic sequences. For example, molecular beacons are used as a tool for the detection of single stranded nucleic acids in homogeneous *in vitro* assays [65,66]. 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 [65,67]. Another novel application of molecular beacons is the detection of double-stranded DNA targets using PNA “openers” that form triplexes with the DNA strands [68]. Further, proteins can be detected by synthesizing “aptamer molecular beacon” [69,70] 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, molecular beacons can detect endogenous mRNA in living cells with high specificity, sensitivity, and signal-to-background ratio, thus having 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 one thousand) 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 may 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 μ M; [51]) employed in antisense experiments. Further, it generally takes 4 hours before any noticeable antisense effect occurs, whereas visualization of mRNA with hairpin probes requires less than 2 hours 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 unwanted 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.

There are many possibilities for nanostructured probes to become clinical tools for disease detection and diagnosis. For example, molecular beacons could be used to perform cell-based early cancer detection using clinical samples including blood, saliva and other bodily fluids. In this case cells in the clinical sample are separated and molecular beacons designed to target specific cancer genes are delivered to the cell cytoplasm for detecting mRNAs of the cancer biomarker genes. Cancer cells having a high level of the target mRNAs (such as survivin) or mRNAs with specific mutations that cause cancer (such as K-ras codon 12 mutations) would show high level of fluorescence signal, while normal cells would show just low background signal, allowing cancer cells to be distinguished from normal cells. In this approach, the target mRNAs would not be diluted compared with approaches using cell lysate, such as PCR. Thus, molecular beacon based assays have the potential to positively identify cancer cells in a clinical sample with high specificity and sensitivity. It may also be possible to detect cancer cells *in vivo* by using NIR-dye labeled molecular beacons in

combination with endoscopy. Nanostructured probes could be used for cell-based detection of other diseases as well. As illustrated above, well designed molecular beacons can rapidly detect viral infection in living cells with high specificity and sensitivity. Another possibility is to analyze the vulnerability of atherosclerotic plaques by designing nanostructured probes to image biomarkers (mRNAs or proteins) of vulnerable plaques in blood samples. Although there remain significant challenges, imaging methods using nanostructured probes have a great potential in becoming a powerful clinical tool for disease detection and diagnosis.

Acknowledgments

This work was supported by the National Heart Lung and Blood Institute of the NIH as a Program of Excellence in Nanotechnology (HL80711), by the National Cancer Institute of the NIH as a Center of Cancer Nanotechnology Excellence (CA119338), and by the NIH Roadmap Initiative in Nanomedicine through a Nanomedicine Development Center award (PN2EY018244).

REFERENCES

1. Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N. Science. 1985; 230:1350. [PubMed: 2999980]
2. Alwine JC, Kemp DJ, Parker BA, Reiser J, Renart J, Stark GR, Wahl GM. Methods Enzymol. 1979; 68:220. [PubMed: 94421]
3. Adams MD, Dubnick M, Kerlavage AR, Moreno R, Kelley JM, Utterback TR, Nagle JW, Fields C, Venter JC. Nature. 1992; 355:632. [PubMed: 1538749]
4. Velculescu VE, Zhang L, Vogelstein B, Kinzler KW. Science. 1995; 270:484. [PubMed: 7570003]
5. Liang P, Pardee AB. Science. 1992; 257:967. [PubMed: 1354393]
6. Schena M, Shalon D, Davis RW, Brown PO. Science. 1995; 270:467. [PubMed: 7569999]
7. Bassell GJ, Powers CM, Taneja KL, Singer RH. J Cell Biol. 1994; 126:863. [PubMed: 7914201]
8. Buongiorno-Nardelli M, Amaldi F. Nature. 1970; 225:946. [PubMed: 5416464]
9. Behrens S, Fuchs BM, Mueller F, Amann R. Appl Environ Microbiol. 2003; 69:4935. [PubMed: 12902289]
10. Huang Q, Pederson T. Nucleic Acids Res. 1999; 27:1025. [PubMed: 9927735]
11. Glotzer JB, Saffrich R, Glotzer M, Ephrussi A. Curr Biol. 1997; 7:326. [PubMed: 9115398]
12. Jacobson MR, Pederson T. Proc Natl Acad Sci U S A. 1998; 95:7981. [PubMed: 9653126]
13. Tsuji A, Koshimoto H, Sato Y, Hirano M, Sei-Iida Y, Kondo S, Ishibashi K. Biophys J. 2000; 78:3260. [PubMed: 10828002]
14. Dirks RW, Molenaar C, Tanke HJ. Histochem Cell Biol. 2001; 115:3. [PubMed: 11219605]
15. Molenaar C, Abdulle A, Gena A, Tanke HJ, Dirks RW. J Cell Biol. 2004; 165:191. [PubMed: 15117966]
16. Tyagi S, Kramer FR. Nat Biotechnol. 1996; 14:303. [PubMed: 9630890]
17. Tsourkas A, Behlke MA, Rose SD, Bao G. Nucleic Acids Res. 2003; 31:1319. [PubMed: 12582252]
18. Bonnet G, Tyagi S, Libchaber A, Kramer FR. Proc Natl Acad Sci U S A. 1999; 96:6171. [PubMed: 10339560]
19. Tyagi S, Bratu DP, Kramer FR. Nat Biotechnol. 1998; 16:49. [PubMed: 9447593]
20. Li JJ, Geyer R, Tan W. Nucleic Acids Res. 2000; 28:E52. [PubMed: 10871351]
21. Molenaar C, Marras SA, Slats JC, Truffert JC, Lemaitre M, Raap AK, Dirks RW, Tanke HJ. Nucleic Acids Res. 2001; 29:E89. [PubMed: 11522845]
22. Sokol DL, Zhang X, Lu P, Gewirtz AM. Proc Natl Acad Sci U S A. 1998; 95:11538. [PubMed: 9751701]
23. Vet JA, Majithia AR, Marras SA, Tyagi S, Dube S, Poesz BJ, Kramer FR. Proc Natl Acad Sci U S A. 1999; 96:6394. [PubMed: 10339598]
24. Kostrikis LG, Tyagi S, Mhlana MM, Ho DD, Kramer FR. Science. 1998; 279:1228. [PubMed: 9508692]

25. Piatek AS, Tyagi S, Pol AC, Telenti A, Miller LP, Kramer FR, Alland D. *Nat. Biotech.* 1998; 16:359.
26. Bratu DP, Cha BJ, Mhlanga MM, Kramer FR, Tyagi S. *Proc Natl Acad Sci U S A.* 2003; 100:13308. [PubMed: 14583593]
27. Tsourkas A, Behlke MA, Xu Y, Bao G. *Anal Chem.* 2003; 75:3697. [PubMed: 14572032]
28. Santangelo PJ, Nix B, Tsourkas A, Bao G. *Nucleic Acids Res.* 2004; 32:e57. [PubMed: 15084672]
29. Nitin N, Santangelo PJ, Kim G, Nie S, Bao G. *Nucleic Acids Res.* 2004; 32:e58. [PubMed: 15084673]
30. Tyagi S, Alsmadi O. *Biophys J.* 2004; 87:4153. [PubMed: 15377515]
31. Peng XH, Cao ZH, Xia JT, Carlson GW, Lewis MM, Wood WC, Yang L. *Cancer Res.* 2005; 65:1909. [PubMed: 15753390]
32. Medley CD, Drake TJ, Tomasini JM, Rogers RJ, Tan W. *Anal Chem.* 2005; 77:4713. [PubMed: 16053280]
33. Tyagi S, Marras SA, Kramer FR. *Nat Biotechnol.* 2000; 18:1191. [PubMed: 11062440]
34. Tsourkas A, Behlke MA, Bao G. *Nucleic Acids Res.* 2002; 30:4208. [PubMed: 12364599]
35. Brodsky AS, Silver PA. *Methods.* 2002; 26:151. [PubMed: 12054891]
36. Forrest KM, Gavis ER. *Curr Biol.* 2003; 13:1159. [PubMed: 12867026]
37. Shav-Tal Y, Darzacq X, Shenoy SM, Fusco D, Janicki SM, Spector DL, Singer RH. *Science.* 2004; 304:1797. [PubMed: 15205532]
38. Haim L, Zipor G, Aronov S, Gerst JE. *Nature Methods.* 2007; 4:409. [PubMed: 17417645]
39. Ozawa T, Natori Y, Sato M, Umezawa Y. *Nature Methods.* 2007; 4:413. [PubMed: 17401370]
40. Valencia-Burton M, McCullough RM, Cantor CR, Broude NE. *Nature Methods.* 2007; 4:421. [PubMed: 17401371]
41. States DJ, Gish W, Altschul SF. *Methods.* 1991; 3:66.
42. Rhee WJ, Santangelo PJ, Jo H, Bao G. *Nucleic Acids Res.* 2007; 36:e30. [PubMed: 18276638]
43. Lakowicz, JR. *Principles of Fluorescence Spectroscopy.* 2 ed.. Plenum Press; New York, NY: 1999.
44. Marras SA, Kramer FR, Tyagi S. *Nucleic Acids Res.* 2002; 30:e122. [PubMed: 12409481]
45. Price, NC.; Stevens, L. *Fundamentals of Enzymology: The Cell and Molecular Biology of Catalytic Proteins.* 3rd ed.. Oxford University Press; New York: 1999.
46. Dokka S, Rojanasakul Y. *Adv Drug Deliv Rev.* 2000; 44:35. [PubMed: 11035196]
47. Leonetti JP, Mechti N, Degols G, Gagnor C, Lebleu B. *Proc Natl Acad Sci U S A.* 1991; 88:2702. [PubMed: 1849273]
48. Mhlanga MM, Vargas DY, Fung CW, Kramer FR, Tyagi S. *Nucleic Acids Res.* 2005; 33:1902. [PubMed: 15809226]
49. Giles RV, Ruddell CJ, Spiller DG, Green JA, Tidd DM. *Nucleic Acids Res.* 1995; 23:954. [PubMed: 7731809]
50. Barry MA, Eastman A. *Arch Biochem Biophys.* 1993; 300:440. [PubMed: 8424678]
51. Giles RV, Spiller DG, Grzybowski J, Clark RE, Nicklin P, Tidd DM. *Nucleic Acids Res.* 1998; 26:1567. [PubMed: 9512525]
52. Walev I, Bhakdi SC, Hofmann F, Djonder N, Valeva A, Aktories K, Bhakdi S. *Proc Natl Acad Sci U S A.* 2001; 98:3185. [PubMed: 11248053]
53. Snyder EL, Dowdy SF. *Curr. Opin. Mol. Ther.* 2001; 3:147. [PubMed: 11338927]
54. Wadia JS, Dowdy SF. *Curr. Opin. Biotechnol.* 2002; 13:52. [PubMed: 11849958]
55. Becker-Hapak M, McAllister SS, Dowdy SF. *Methods.* 2001; 24:247. [PubMed: 11403574]
56. Wadia JS, Dowdy SF. *Adv Drug Deliv Rev.* 2005; 57:579. [PubMed: 15722165]
57. Brooks H, Lebleu B, Vives E. *Adv Drug Deliv Rev.* 2005; 57:559. [PubMed: 15722164]
58. Allinquant B, Hantraye P, Mailleux P, Moya K, Bouillot C, Prochiantz A. *J Cell Biol.* 1995; 128:919. [PubMed: 7876315]
59. Troy CM, Derossi D, Prochiantz A, Greene LA, Shelanski ML. *J Neurosci.* 1996; 16:253. [PubMed: 8613791]

60. Minamoto T, Mai M, Ronai Z. *Cancer Detect Prev.* 2000; 24:1. [PubMed: 10757118]
61. Altieri DC, Marchisio PC. *Lab Invest.* 1999; 79:1327. [PubMed: 10576203]
62. Santangelo PJ, Nitin N, Bao G. *J. Biomed. Opt.* 2005; 10:44025. [PubMed: 16178658]
63. Santangelo P, Nitin N, LaConte L, Woolums A, Bao G. *J Virol.* 2006; 80:682. [PubMed: 16378971]
64. Santangelo PJ, Bao G. *Nucleic Acids Res.* 2007; 35:3602. [PubMed: 17485480]
65. Liu X, Tan W. *Anal Chem.* 1999; 71:5054. [PubMed: 10575961]
66. Kambhampati D, Nielsen PE, Knoll W. *Biosens Bioelectron.* 2001; 16:1109. [PubMed: 11679296]
67. Steemers FJ, Ferguson JA, Walt DR. *Nat Biotechnol.* 2000; 18:91. [PubMed: 10625399]
68. Kuhn H, Demidov VV, Coull JM, Fiandaca MJ, Gildea BD, Frank-Kamenetskii MD. *J Am Chem Soc.* 2002; 124:1097. [PubMed: 11829619]
69. Hamaguchi N, Ellington A, Stanton M. *Anal Biochem.* 2001; 294:126. [PubMed: 11444807]
70. Yamamoto R, Baba T, Kumar PK. *Genes Cells.* 2000; 5:389. [PubMed: 10886366]

Figure 1. The mRNA life cycle

Messenger RNA (mRNA) that encoding the chemical “blueprint” for a protein is synthesized (transcribed) from a DNA template, and the pre-mRNA is processed (spliced) to have mature mRNA, which is transported to specific locations in the cell cytoplasm. The coding information carried by mRNA is being used by ribosomes to produce proteins (translation). After a certain amount of time, the message is degraded. mRNAs are almost always complexed with RNA-binding proteins to form ribonucleoprotein (RNP) molecules.

Figure 2.

Illustrations of molecular beacon Designs. (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) 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. (d) Shared-stem molecular beacons are designed such that one arm of the stem participates in both stem formation and target hybridization.

Figure 3.
Comparison of molecular beacon and FISH approaches.

Figure 4.

A schematic 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 non-specific interactions.

Figure 5. Structure-function relations of molecular beacons. (a) Melting temperatures for molecular beacons with different structures 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.

Figure 6.

A schematic illustration of a segment of the target mRNA with a double stranded portion and RNA binding proteins. A molecular beacon has to compete off an mRNA strand or RNA-binding protein(s) in order to hybridize to the target.

Figure 7.

A schematic of peptide-linked molecular beacons. (A) A peptide-linked molecular beacon using 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. (B) A peptide-linked molecular beacon with a 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.

Figure 8. 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 9.

Live-cell fluorescence imaging of the genome of bovine respiratory syncytial virus (bRSV) using molecular beacons shows the spreading of infection in host cells at days 1, 3, 5 and 7 post-infection (PI). Primary bovine turbinate cells were infected by a clinical isolate of bRSV, CA-1, with a viral titer of $2 \times 10^{3.6}$ TCID₅₀/ml. Molecular beacons were designed to target several repeated sequences of the gene-end-intergenic-gene-start signal within the bRSV genome, with a signal-to-noise ratio of 50-200.