 MCB, vol.1, no.1, pp.23-36, 2004

Engineering Nanostructured Probes for Sensitive Intracellular Gene Detection

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Abstract: The ability to detect, localize, quantify and monitor the expression of specific genes in living cells in real-time will offer unprecedented opportunities for advancement in molecular biology, disease pathophysiology, drug discovery, and medical diagnostics. However, current methods for quantifying gene expression employ either selective amplification (as in PCR) or saturation binding followed by removal of the excess probes (as in microarrays and in situ hybridization) to achieve specificity. Neither approach is applicable when detecting gene transcripts within living cells. Here we review the recent development in engineering nanostructured molecular probes for gene detection *in vivo*, describe probe design approaches and its structure-function relations, and discuss the critical issues and challenges in performing living cell gene detection with high specificity, sensitivity and signal-to-background ratio. The underlying biological and biochemical aspects are illustrated.

1 Introduction

Quantitative methods to measure gene expression such as real-time PCR and microarrays have revolutionized molecular biology and drug development. Such approaches, however, are generally used with purified DNA or RNA obtained from cell lysate. The ability to detect and quantify the expression of specific genes in living cells in real-time will offer tremendous opportunities for biological and disease studies, provide another leap forward in our understanding of cell and developmental biology, disease pathophysiology, and significantly impact medical diagnostics (Tsien, 2003). However, currently available technologies for gene detection in intact cells such as in-situ hybridization (ISH) are not capable of detecting and quantifying gene expression in living cells. Unlike ISH studies and solid-phase *in vitro* assays in which probes not hybridized to their target (i.e., background) can be removed by washing (Femino et al, 1998; Raap et al, 1997), gene detection in living cells requires more sophisticated methods to distinguish signal from background. Probes must be able to recognize the target with high specificity, convert target recognition *directly* into a measurable signal with high signal-to-background ratio, and allow for differentiation between true and false-positive events. To meet these challenges and definitive needs, recently, extensive studies have been conducted to develop novel nanostructured molecular probes with high specificity, sensitivity, and signal-to-background ratio.

Gene expression in a living cell can be quantified through the level of either mRNA expression or protein expression; the former is controlled by transcription, the latter by translation. Although both are dictated by the regulation of the specific gene, their expression levels can be very different, since an mRNA molecule can produce multiple proteins. In this article, we focus on the detection of mRNA molecules based on Watson-Crick base pairing, which is one of the most specific molecular interactions in a living cell. 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 both the expression level of specific RNAs, and their spatial distribution. Therefore, there is an urgent need to image specific mRNAs in living cells in real-time in order to provide essential information on mRNA synthesis, processing, transport, localization, and on the dynamics of mRNA expression and localization in response to external stimuli. However, existing methods cannot reveal the spatial and temporal variation of RNA within a single cell.

One approach to tagging and tracking endogenous

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mRNA transcripts in living cells is to use fluorescently labeled oligonucleotide probes that recognize specific RNA targets (Tsourkas and Bao, 2003). To facilitate such development, in this article, we review the recent approaches in engineering nanostructured molecular probes for gene detection *in vivo*, describe probe design issues and the structure-function relations, and discuss the biological and biochemical challenges in performing living cell gene detection with high specificity, sensitivity and signal-to-background ratio.

2 Nanoprobe Design Issues

The detection and quantification of specific mRNAs require probes to have high sensitivity and specificity, especially for low abundance genes and with a small number of diseased cells in clinical samples. Further, for detecting genetic alterations such as mutations and deletions, the ability to recognize single nucleotide polymorphisms (SNPs) is essential. When designed properly, hairpin nucleic acid probes have the potential to be highly sensitive and specific, with fast probe-target hybridization kinetics.

Molecular Beacons. As shown in Figure 1a, molecular beacons are single-stranded oligonucleotide probes (ODN) with a fluorophore (dye) at one end and a fluorescence quencher at the other end. They are designed to form a stem-loop hairpin structure in the absence of target mRNA such that the fluorescence of the fluorophore is quenched due to FRET and direct contact between the fluorophore and the quencher end (Tyagi & Kramer, 1996). The loop portion has a probe sequence complementary to a target mRNA molecule. The arm sequences near each end of the loop (Fig. 1a) are complementary to each other; they anneal to form the stem of a molecular beacon. When a molecular beacon encounters a target mRNA molecule, the loop (and possibly a part of the stem) hybridizes to the target mRNA, causing a conformational change that forces the stem apart (Fig. 1b). The quencher moves away from the fluorophore, leading to the restoration of fluorescence (Tyagi & Kramer, 1996). Thus, molecular beacons enable a homogenous assay format where background is low, without the need to wash away unbound probes. The design of the hairpin structure provides an independently adjustable energy penalty for hairpin opening that improves probe specificity (Bonnet et al, 1999; Tsourkas et al, 2003a). 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.

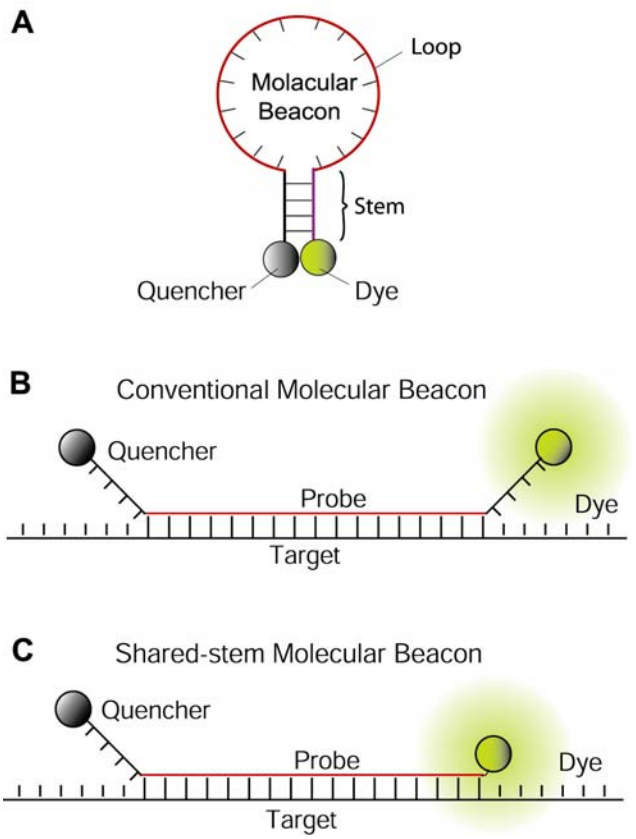

Figure 1 : Illustrations of molecular beacons. (a) Molecular beacons are stem-loop oligonucleotide probes labeled with a reporter dye at one end and a quencher molecule at the other end. In the closed hairpin conformation the fluorescence is quenched. Upon binding to target, the fluorophore is separated from the quencher and fluorescence is restored. (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.

The underlying mechanics for the conformational transition of a molecular beacon is the large difference in bending stiffness between single-stranded and double-stranded nucleic acids. Assume that a segment of a nu-

cleic acid can be taken as a thin elastic rod, its bending stiffness is given by

$$\kappa = EI = \xi k_B T \quad (1)$$

where E is Young's modulus, I is moment of inertia of the rod, ξ is the persistence length, k_B is the Boltzmann constant and T the absolute temperature.

Note that the persistence length of a single-stranded DNA is $\xi \approx 1$ nm, and that for double-stranded DNA is $\xi \approx 50$ nm. Linear elasticity theory implies that the energy ΔG required to bend a short segment of nucleic acid through an angle $\Delta\theta$ is (Bustamante et al, 2003)

$$\Delta G = k_B T (\Delta\theta)^2 (\xi/2L) \quad (2)$$

where L is the loop length of the molecular beacon. This suggests that, after binding to a complementary single-stranded DNA target, in keeping a 20-base loop in a circular confirmation at 37^{o}C, the stem must have a binding energy of at least $\sim$90 kcal/mol. Since the stem of a molecular beacon is typically very short ($\sim$ 4-6 bases), it has a much lower binding energy even if the interaction energy of fluorophore/quencher is included. As a result, a molecular beacon opens up on hybridization with a target.

One major advantage of the stem-loop hairpin probes is that they can recognize their targets with higher specificity than linear ODN probes. Properly designed molecular beacons can discriminate between targets that differ by as little as a single nucleotide (Bonnet et al, 1999). In contrast to current techniques for detecting single nucleotide polymorphism (SNP), which are often labor-intensive and time-consuming, molecular beacons provide a simple and promising tool for the diagnosis of genetic diseases. Since its initial development in 1996, the molecular beacon technology has been utilized in a variety of applications in DNA, RNA, and protein studies, including multiple analyte detection, real-time enzymatic cleavage assaying, protein–DNA interactions, real-time monitoring of PCR, gene typing and mutation detection, and mRNA detection in living cells (Sokol et al, 1998; Vet et al, 1999; Kostrikis et al, 1998; Fang et al, 2000; Bratu et al, 2003; Santagelo et al, 2004; Nitin et al, 2004).

Major Design Issues. There are three major design issues of nanostructured molecular probes: probe sequence selection, hairpin structure, and fluorophore/quencher pair (Santangelo et al, 2004a). In general, the probe sequence is selected to ensure specificity, and to have good accessibility. The hairpin structure as well as the sequence are determined to have the proper melting temperature, and the fluorophore/quencher pair should give high signal level and the optimal signal-to-background ratio. To endure specificity, for each gene to target, one can use BLAST to select 16-20 base target sequences that are unique for the specific gene to target. Since mRNA molecules often have secondary (folded) structures, it is important to avoid targeting sequences where double stranded RNA is formed. One difficulty in molecular beacon design is that, although predictions of mRNA secondary structure can be made using existing software, they may not be accurate due to limitations of the biophysical models used, and the limited understanding of protein-RNA interaction. The target sequence may also be occupied by RNA binding proteins. Therefore, for each gene to target, it is often necessary to select multiple unique sequences along the target RNA, and have corresponding probes designed, synthesized and tested to select the best target sequence. It is also important to adjust the G-C content of the probe and stem sequences to realize the desired melting temperature. However, if SNP detection is involved, there is no other choice but targeting the sequence containing the mutation. The target accessibility is an important issue for nucleic acid hairpin probe design and requires more fundamental biological studies of mRNA structure, RNA-protein interactions especially the formation of ribonucleoprotein complexes (RNPs).

A conventional molecular beacon has four essential components: loop, stem, fluorophore and quencher, as illustrated in Figure 1a. The loop usually consists of 15-25 nucleotides and is selected based on target sequence, melting temperature, and the secondary structure of the target mRNA. The stem, formed by two complementary short-arm sequences, is typically 4-6 bases long and is usually chosen to be independent of the target sequence (Figure 1b). Molecular beacons, however, can also be designed such that one arm of the stem participates in both stem formation and target hybridization (shared-stem molecular beacons)(Tsourkas et al, 2002b), as illustrated schematically in Figures 1c. 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.

The loop, stem lengths and sequences are the critical de-

sign parameters for molecular beacons, since at any given temperature they largely control the fraction of molecular beacons in each of three different conformational states: bound-to-target, stem-loop, and random-coil (Bonnet et al, 1999). 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 (Tsourkas et al, 2003). Detailed discussions on the effects of probe and stem lengths on molecular beacon performance, i.e., structure-function relations, will be given in the next section.

Reporter Selection. 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 (Tyagi, et ak, 1998). 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 (Tyagi, et al. 2000). Specifically, a "harvester" fluorophore absorbs the excitation light and transfers the energy to an "emitter" fluorophore which emits fluorescence. The same "harvester" fluorophore is used with various emitter fluorophores to generate multiple colors. Another possibility is to use quantum dots (QDs) with different emission wavelengths as the reporter dye. Similar to wavelength-shifting dyes, many QDs can be excited with a single UV lamp light source (Chan and Nie, 1998**)**. However, it remains to be seen if QDs can be effectively quenched and if their functional size can be reduced to be useful when conjugated to a molecular beacon.

Clearly, multicolor fluorescence detection of different beacon/target duplexes can become a powerful tool for the simultaneous detection of multiple genes. For example, almost all cancers are caused by multiple genetic alterations in cells and the detection of cancer cells in a clinical sample would requires the use of multiple tumor markers. Thus, the use of multiple molecular beacons is potentially a powerful tool in the early detection and diagnosis of cancer.

Quencher Selection. Similar to fluorophore selection, choosing the optimal quencher can also improve the signal-to-background ratio of molecular beacons. Organic quencher molecules such as dabcyl, BHQ-II (blackhole quencher), BHQ-III and Iowa Black can all effectively quench a wide range of fluorophores by both fluorescence resonance energy transfer (FRET) and the formation of an exiton complex between the fluorophore and the quencher (Marras et al, 2002). In addition to organic quenchers, gold nanoparticles can also be used as a quencher (Dubertret et al, 2001). It should be noted, however, that the interaction between the gold particle and the fluorophore could significantly affect the performance of molecular beacons.

3 Structure-Function Relations

In order to design molecular beacons with optimal specificity and kinetic rates, it is necessary to establish their structure-function relationships. Specifically, a systematic study was performed of thermodynamic parameters ΔH_{12} and ΔS_{12} determining the phases of a molecular beacon and the effects of probe length and stem length on the binding specificity and kinetic rates of molecular beacons (Tsourkas el al, 2003). In this study a series of GAPDH-targeting molecular beacons with different probe and stem lengths were designed and synthesized. Herein, probe length is defined as the portion of the molecular beacon that is complementary to the target nucleic acid and stem length is defined as the end portion of the molecular beacon that is self-complementary. Conventional molecular beacons are designed such that the target-specific probe domain is located between short self-complementary stems that are independent of the target-specific domain. In this study, however, shared-stem molecular beacons with one stem complementary to the target sequence were considered (Fig. 1c) (Tsourkas et al, 2002b).

Molecular beacons in solution can have at least three distinct conformations: molecular beacon/target duplex (phase 1), stem-loop (phase 2) and random coil (phase 3). Following Bonnet et al (1999), the dissociation constant K_{23} characterizing the transition between stem-loop conformation and random coils is given by $K_{23}(\theta)$ =

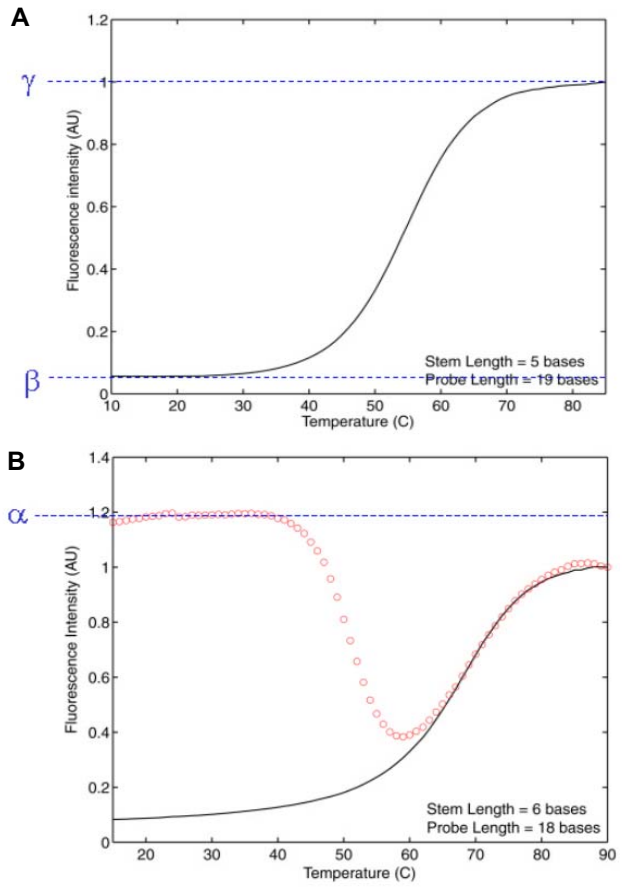

Figure 2 : Typical molecular beacon thermal denaturation profiles. In the absence of target, the fluorescence of molecular beacons is low at low temperatures; as temperature increases the molecular beacons melt into random coils with high fluorescence emission, as shown in (a). In the presence of perfectly complementary targets, molecular beacons emit a maximal signal at low temperatures indicating that the molecular beacons are bound to target; as temperature increases the molecular beacons melt away from the target, as illustrated in (b).

$(\Phi-\beta)/(\gamma-\Phi)$ where Φ is the fluorescence intensity as a function of temperature θ (i.e., the thermal profile) shown in Fig. 2a, β is the fluorescence emitted by the molecular beacons when they are all in the stem-loop conformation, obtained from the fluorescence intensity measured at 10 $^\circ$C, and γ is the fluorescence emitted by the molecular beacons when they are all randomly coiled, obtained from the fluorescence intensity measured at 80 $^\circ$C (Fig. 2a). Further,

$$Rln[(\Phi-\beta)/(\gamma-\Phi)] = -\Delta H_{23}/\theta + \Delta S_{23} \quad (3)$$

where R is the gas constant (1.9872 cal mol^{-1} K^{-1}), θ is the temperature in Kelvin, ΔH_{23} and ΔS_{23} are respectively the changes in enthalpy and entropy of the system. The dissociation constant K_{12} corresponding to the transition between molecular beacons bound to target and those in the stem-loop conformation can be determined from the fluorescence data describing the thermal profile of molecular beacons in the presence of target, $K_{12}(\theta) = [(\alpha-\Psi)T_o]/[(\Psi-\beta)+(\Psi-\gamma)K_{23}]$ where Ψ is the thermal profile for molecular beacon-target duplexes as shown in Fig. 2b, T_0 is the initial concentration of targets, β is the same as before, α is the fluorescence of bound molecular beacons at 10 $^\circ$C (Fig. 2b). Typical thermal denaturation profiles of molecular beacons only and molecular beacon-target duplex are shown in Figures 2a and 2b. The changes in enthalpy and entropy due to this transition are given by

$$Rln(T_0 - 0.5B_0) = -\Delta H_{12}/\theta_m + \Delta S_{12} \quad (4)$$

where the melting temperature θ_m of each molecular beacon-target mixture is defined as the temperature at which half of the molecular beacons are bound to target and B_0 is the initial concentration of molecular beacons. For molecular beacons alone and molecular beacon-target duplexes, the dissociation constants K_{23} and K_{12}, the corresponding changes in enthalpy and entropy of the system, and the melting temperature θ_m can be quantified using the measured thermal profiles and the above equations.

The effects of molecular beacon structure (probe and stem lengths) on probe/target binding kinetic rates can be quantified by performing hybridization assays using a stopped-flow accessory (Tsourkas et al, 2003a). The reaction kinetics of binding/unbinding between molecular beacons (B) and targets (T) is governed by (Lauffenburger and Linderman, 1993)

$$B + T \underset{k_2}{\overset{k_1}{\rightleftarrows}} C, \quad \frac{dC}{dt} = k_1 BT - k_2 C \quad (5)$$

where B(t), T(t) and C(t) are respectively the instantaneous concentrations of molecular beacon, target and the

beacon-target duplex, k_1 is the rate constant of hybridization between molecular beacon and target (on-rate constant), and k_2 is the rate of dissociation (off-rate constant) (Tsourkas et al, 2003a). The exact solution to Equation 5 is given by

$$1 - C(t)/C_{eq} = e^{-\Delta k_1 t}\,[1 - \lambda C(t)/C_{eq}] \qquad (6)$$

where $C_{eq} = B_0 + T_0 + K_{12} - \Delta$, $\lambda = C_{eq}^2/B_0T_0$, $\Delta = \sqrt{(B_0+T_0+K_{12})^2 - 4B_0T_0}$, B_0 and T_0 are respectively the initial concentration of molecular beacon and targets, and $K_{12} = k_2/k_1$ is the dissociation constant defined above. For each beacon-target duplex the fluorescence intensity $F(t)$ as a function of time t due to probe-target hybridization can be measured. Assuming that $C(t)/C_{eq}$ = *(F(t)-F*$_0$*)*$/(F_{eq} - F_0)$ where F_{eq} is the fluorescence intensity as t $\rightarrow \infty$ and F_0 is the initial fluorescence intensity, the on-rate k_1 (and $k_2 = k_1 \quad K_{12}$) can be obtained from the F vs. t curves using Equation 6.

To illustrate the effect of molecular beacon structure on its melting behavior, the melting temperature for molecular beacons with various stem-loop structures are displayed in Figure 3a. It can be seen that the melting temperature increases with probe length but appear to plateau at a length of $\sim$20 nucleotides. It is also clear 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 3b. 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 Figure 3b. Although molecular beacons with a 4-base stem have a larger on-rate constant than molecular beacons with longer stems, they also give rise to a higher background fluorescence (Tsourkas et al, 2003a).

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

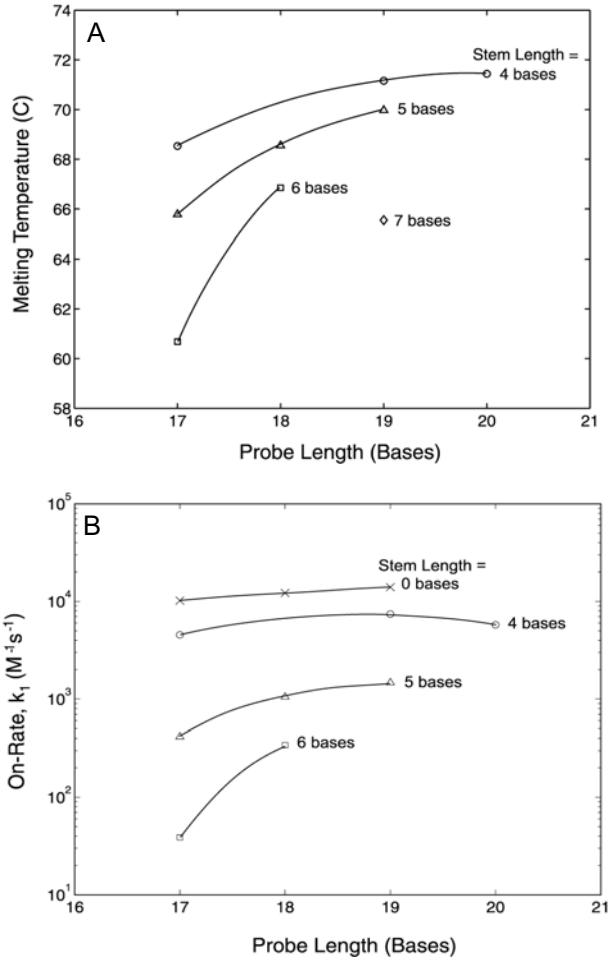

Figure 3 : Structure-function relations of molecular beacons. (a) Melting temperatures for molecular beacons with different structures in the presence of target. (b) The rate constant of hybridization k_1 (on-rate constant) for molecular beacons with various probe and stem lengths.

condition where a single-base mismatch reduces the energetic preference of probe-target binding. The competition between the two stable conformations of a molecular beacon (i.e., closed and bound to target) also explains why it has an enhanced specificity compared with linear probes. 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. It is interesting to note, however, that stem-less molecular beacons, which lack the short com-

plementary arms and rely solely on the random-coiled nature and interactions between the dye and quencher to maintain a dark state, are still able to differentiate between bound and unbound states (Kuhn et al., 2002).

Increasing the probe length of molecular beacons results in improved target affinity and increased kinetic rates, but leads to a reduced specificity. The effect of probe length on the behavior of molecular beacons is typically less dramatic compared with that of stem length and can be used to fine-tune functionality (Tsourkas et al, 2003a). A detailed description of the thermodynamic parameters of probe-target hybridization as determined by the structure of molecular beacons can be found in Tsourkas et al (2003).

4 Intracellular Stability of Probes

Although oligonucleotide hairpin probes such as molecular beacons have the potential to detect mRNA in living cells, these probes can be degraded by endonucleases or opened by hairpin binding proteins, resulting in a large amount of false-positive signals and thus significantly limiting the detection sensitivity. It has been reported that unmodified phosphodiester oligonucleotides may possess a half-life as short as 15-20 minutes in living cells (Dirks et al, 2001; Molenaar et al, 2001). To overcome this difficulty, molecular beacons can be made with more nuclease-resistant backbone chemistries, such as phosphorothioate, peptide nucleic acid (PNA) (Kuhn et al, 2001), and 2'-*O*-methyl modifications (Dirks et al, 2001). In particular, 2'-*O*-methyl oligoribonucleotides have been found to exhibit higher affinities for RNA, faster hybridization kinetics (Majlessi et al, 1998), better nuclease resistance (Cummins et al, 1995), and the ability to avoid degradation of target RNA by RNase-H upon hybridization (Zamaratski et al, 2001). Compared with unmodified oligodeoxyribonucleotides, 2'-*O*-methyl oligoribonucleotides have a methoxy group at the 2' position of the sugar moiety instead of a hydrogen atom. This allows the 2'-*O*-methyl oligoribonucleotides to confer a RNA-like 3'-endo conformation upon hybridization with RNA or DNA due to the Gauche effect between O_4'-O_2' (Teplova et al, 1999; Manoharan, 1999). One possible drawback of using 2'-*O*-methyl oligoribonucleotides for *in vivo* hybridization studies is that such probes tend to localize in the nucleus and therefore become less available for hybridization to cytoplasmic RNAs (Dirks et al, 2001; Molenaar et al, 2001). Another well-studied oligodeoxynucleotides system is the phosphorothioate oligos (PS-oligos) in which one of the non-bridging oxygen atoms in the phosphate backbone is replaced by a sulfur atom (Agrawal, 1999). Compared with unmodified DNA backbone, PS-oligos have a greater resistance to nuclease degradation: it was found that 70% of PS-oligos in living cells are still intact after 24 hr (Fisher et al, 1993). Further, the affinity of PS-oligos for mRNA targets was found to be higher than the unmodified DNA probes (Stein, 1996). However, there were conflicting reports as to whether PS-oligos are more likely to induce RNase-H cleavage when hybridized to mRNA targets (Dirks et al, 2001; Agrawal, 1999). It may also be beneficial to use mixed-backbone oligonucleotides (Agrawal et al, 1997). Other possible backbone modifications in oligonucleotides include phosphoroamidates, phosphotriesters, and phosphorodithioates (Mesmaeker, et al. 1995; Verma and Eckstein; 1998). The challenge is to find a backbone modification so that the resulting probes are not only nuclease resistant and capable to avoid RNase-H degradation, but also have a low binding affinity to nucleic acid binding proteins and high binding affinity to RNA targets.

Molecular beacons can be synthesized using peptide nucleic acids (PNAs), which are structural analogues of DNA with N-(2-aminoethyl)glycine linkages in place of the deoxyribose phosphate ester backbone (Egholm et al. 1993). The uncharged backbone of the PNA allows it to bind complementary targets without the electrostatic repulsion present in unmodified nucleic acids duplexes. As a result, PNA-DNA and PNA-RNA duplexes have been found to have an improved stability compared with the corresponding DNA-DNA duplexes (Jensen et al. 1997; Ratilainen et al. 1998; Kambhampati, 2001). Further, PNAs are resistant to nuclease degradation. There are, however, several shortcomings in using PNAs for *in vivo* gene detection. PNA probes show little sequence specificity when detecting RNA in living cells (Dirks et al, 2001). PNAs may also inhibit the expression of the gene they target by inducing RNase-H activity (Doyle et al, 2001), which is problematic for monitoring the cellular activity of RNA. Although molecular beams can be made with PNA (Kuhn et al, 2001), the uncharged backbone may cause difficulties in cellular delivery using transfection.

We have performed thermodynamic and kinetic studies of the 2'-*O*-methyl modified molecular beacons in

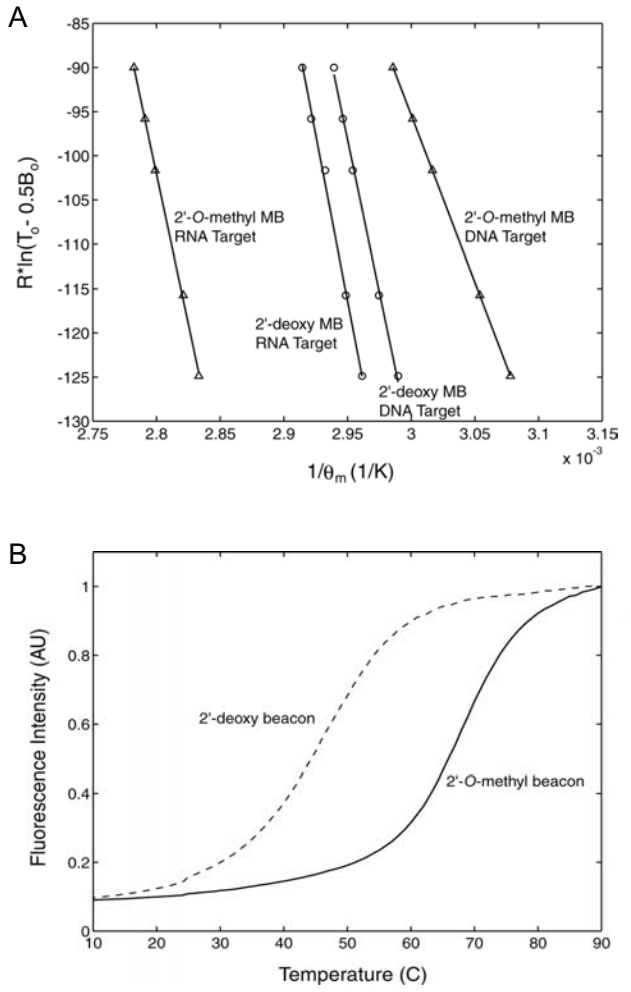

Figure 4 : The effects of 2'-*O*-methyl modification of molecular beacon backbone. (a) Reciprocal of melting temperature for the transition between bound to RNA or DNA targets and free in the hairpin conformation and (b) thermal denaturation profiles for 2'-*O*-methyl and 2'-deoxy molecular beacons (MBs) with stem length of 5 bases and probe length of 18 bases.

the presence of RNA and DNA targets (Tsourkas et al, 2002a). We found that in terms of molecular beacon/target duplex stability 2'-*O*-methyl/RNA $>$ 2'-deoxy/RNA $>$ 2'-deoxy/DNA $>$ 2'-*O*-methyl/DNA, as shown in Fig. 4a. The improved stability of the 2'-*O*-methyl/RNA duplex was accompanied by a slightly reduced specificity compared with the duplex of 2'-deoxy molecular beacons and RNA targets. We also found that the 2'-*O*-methyl molecular beacons hybridized to RNA faster than 2'-deoxy molecular beacons. For the pairs tested, the 2'-deoxy-beacon/DNA-target duplex showed the fastest hybridization kinetics. As an added advantage, the 2'-*O*-methyl molecular beacons exhibited lower background fluorescence at 37^{o}C compared with the 2'-deoxy molecular beacons, as demonstrated in Fig. 4b.

Recently, evidence suggests that not all of the 2'-*O*-methyl molecular beacons retain a stem-loop structure in living cells, possibly due to nucleic acid binding proteins that disrupt the hairpin (Dirks et al, 2001; Molenaar et al, 2001). Therefore, other chemical modifications may be beneficial in this setting, such as phosphorothioate internucleoside linkages or a combination of phosphorothioate chemistry with 2'-*O*-methyl RNA. These molecular beacon backbone modifications may significantly reduce the false-positive signal and thus increase the signal-to-background ratio and detection sensitivity.

5 Energy Transfer Based Dual Probes

To drastically reduce the false-positive signals in living cell gene detection, two different approaches have been taken. The first is to modify the backbone of molecular beacons to make them more resistant to endogenous nucleases, as discussed above. The second is a dual molecular beacons approach which measures the fluorescent signal due to fluorescence resonance energy transfer (FRET) or luminescence resonance energy transfer (LRET) as a result of the direct interaction between two molecular beacons (Tsourkas et al, 2003b). FRET (fluorescence resonance energy transfer) refers to the non-radiative transfer of energy from one fluorophore, called the donor, to another fluorophore, called the acceptor (reviewed in Clegg, 1995). The efficiency (E) of FRET is extremely sensitive to the distance r between the donor and acceptor molecules, and is given by $E = [R^6/(R^6 + r^6)]$ where R is the Förster energy transfer distance. For typical fluorophores R = 1-5 nm. When conducting FRET experiments, the efficiency of energy transfer between the donor and acceptor probes can vary significantly depending on the fluorophores chosen. Ideally, the fluorophores used should have a high extinction coefficient (i.e. the ability to absorb energy) and a high quantum yield (i.e. the ability to emit energy that was absorbed). Further, FRET efficiency relates to the degree of overlap between the emission spectrum of the donor and the excitation spectrum of the acceptor. However, acceptor and donor pairs should be carefully matched not just

for the degree of spectral overlap but also for the separation of excitation spectra. Two fluorophores have a large overlap between donor emission and acceptor excitation wavelengths can nevertheless perform poorly in a FRET assay if the acceptor is directly excited by the light source used for donor excitation, resulting in a low signal-to-background (S:B) ratio.

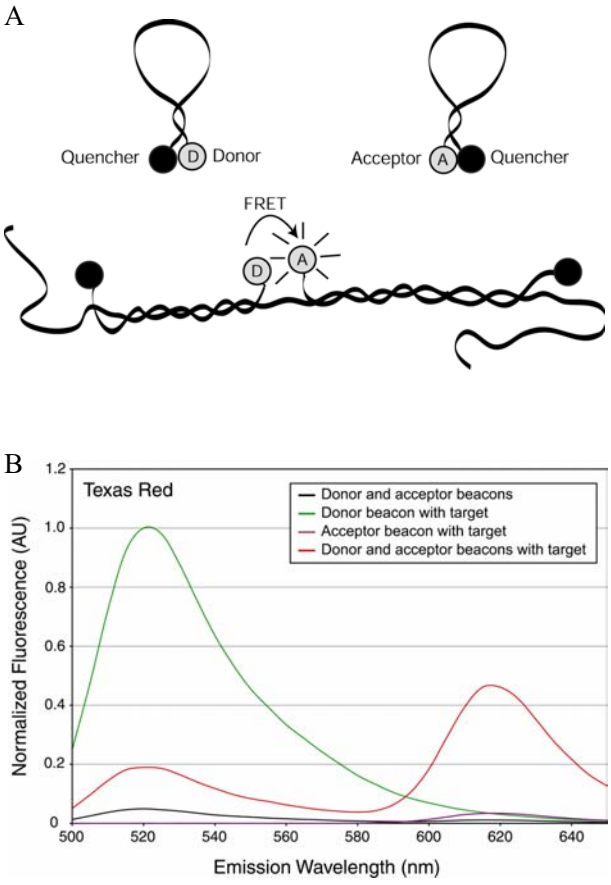

Figure 5 : Dual FRET molecular beacons. (a) Hybridization of the donor and acceptor molecular beacons to adjacent regions on the same mRNA target results in FRET. By detecting FRET, fluorescence signals due to probe/target binding can be distinguished from that due to beacon degradation and non-specific interactions. (b) Emission spectra for dual FRET molecular beacons with a Fam-Texas Red FRET pair. The samples were excited at a wavelength of 475 nm.

As shown in Figure 5a, the dual FRET/LRET molecular beacons approach utilizes a pair of molecular beacons, one with a donor fluorophore and a second with an acceptor fluorophore. Probe sequences are chosen such that the molecular beacons hybridize adjacent to each other on a single nucleic acid target, bringing the respective fluorophores into close proximity and promoting FRET (Tsourkas et al, 2003b). The sensitized emission from the acceptor fluorophore then serves as a positive signal in the FRET based detection assay. When the donor and acceptor fluorophores are properly chosen (e.g., with minimal spectral overlap), a strong fluorescence signal will emit from the acceptor only when both molecular beacons are hybridized to the same target and FRET occurs. The fluorescence emitted from molecular beacons that are degraded or opened by protein interactions will be substantially lower than the signal elicited by the donor/acceptor FRET interaction. Thus, a true positive signal owing to probe/target binding events can be readily distinguished from false-positive signals.

For dual FRET molecular beacons, both steady-state and time-resolved measurements can be performed to monitor fluorescence emission. For steady-state measurements the sample is continuously illuminated with a light of constant intensity; they are often limited by background fluorescence due to scattering and autofluorescence. For time-resolved measurements, the sample is excited by a pulse of light. This allows for the discrimination of fluorescence sources by both emission and lifetime. On short time scales ($<$10 ns), time-resolved measurements may not give a higher S:B ratio compared with steady-state measurements. However, for dye molecules that have a longer lifetime ($>$100 ns), it is possible to use time-gating techniques to begin the recording of emission after the undesired background fluorescence has decayed. Unfortunately, since most common fluorophores have fluorescence lifetimes of $\sim$10 ns, special probes must be synthesized for this type of experiment.

The most common long-lifetime probes synthesized are lanthanide (i.e. terbium and europium) chelates, which exhibit lifetimes of more than 1 ms (Lemmetyinen *et al.* 2000; Diamandis, *et al.* 1989). Lanthanide ions alone do not emit photons very efficiently due to their low absorption cross-section ($<$1 M^{-1} cm^{-1}) and generally require an aromatic ligand in close proximity that can capture the excitation light and transfer the absorbed energy to the lanthanide ion (Chen and Selvin, 2000; Xiao and Selvin. 2001; Rodriguez-Ubis et al,2000). Lanthanide probes also require a chelate that can tightly bind the lanthanide

ion while providing binding sites for the aromatic ligand and the probe molecule. Ideally, the chelate should also prevent water molecules from interfering with the energy transfer from the triplet state of the aromatic ligand to the lanthanide ion (Ando et al, 1992). Since lanthanide emission does not involve singlet-to-singlet transition, the use of lanthanide chelate as a donor results in luminescence resonance energy transfer (LRET). In addition to the long lifetime, the emission of lanthanide chelates exhibits a sharply spiked spectrum, reducing donor and acceptor emission overlap during LRET. Further, the Förster transfer distance of an LRET pair can be greater than 6 nm, and LRET does not depend on the orientation of the donor and acceptor [69]. Therefore, lanthanide chelates are becoming a powerful tool for energy transfer experiments in biological studies (Selvin, 2002).

To demonstrate the potential of dual FRET molecular beacons approach, we have carried out an in-solution spectroscopy study to demonstrate the potential of dual FRET molecular beacons (Tsourkas et al, 2003). Specifically, a series of molecular beacons were designed and synthesized which are in antisense orientation with respect to exon 6 and exon 7 of the human GAPDH gene, and a dabcyl quencher was attached to the 5'-end and a 6-Fam fluorophore to the 3'-end of donor molecular beacons; a dabcyl quencher was attached to the 3'-end and either a Cyanine 3 (Cy3), 6-carboxyrhodamine (ROX), or Texas Red fluorophore was attached to the 5'-end of acceptor molecular beacons. The stem sequence was designed to participate in both hairpin formation and target hybridization, as shown in Figure 1c. This was adopted to help fix the relative distance between the donor and acceptor fluorophores and improve energy transfer efficiency (Tsourkas et al, 2002b). Both the donor and acceptor moleculars were designed with a probe length of 18 bases and a stem length of 5 bases. The synthetic wild-type GAPDH target has a 4-base gap between the donor dye and the acceptor dye. Gap spacing was adjusted to 3, 5, and 6 bases by either removing a guanine residue or adding 1 or 2 thymine residues (Tsourkas et al, 2003b).

FRET measurements were carried out using a Safire microplate fluorometer (Tecan) to excite the donor beacons and detect resulting acceptor beacon fluorescence emission (500 nm to 650 nm) of a sample with equal amount (200 nM) of target, donor and acceptor molecular beacons. As shown in Fig. 5b, when 6-FAM (peak excitation at 494 nm) was used as the donor dye and Texas Red (perk emission at 620 nm) as the acceptor dye, a signal-to-background ratio of about 50 was achieved. Clearly, the FRET signal should allow differentiation between the signal emitted upon target detection and false-positive signals.

When the distance between the donor and acceptor molecular beacons was increased from 3 to 6 bases, there was a slight increase in the FRET signal intensity (data not shown). This trend was found to be the same for all the acceptor fluorophores studied. It seems that a gap size of 4 or 5 bases is desirable whereas a 3-base gap is unfavorable due to possible interference between the donor and acceptor dyes.

To demonstrate the ability of molecular beacons in sensitive detection of mRNA in living cells, we designed and synthesized dual-FRET molecular beacons targeting wild-type K-ras mRNA, and delivered them into normally-growing and stimulated human dermal fibroblasts (HDF cells) using a reversible permeabilization method (Faria M et al. 2001). We also designed a control molecular beacon (random beacon) whose sequence has no perfect match in the mammalian genome. Both the K-ras-targeting dual FRET molecular beacon pair, and control molecular beacons have a 16-base target sequence and 5-base stem ((Santangelo et al, 2004b). For both molecular beacon pairs, Cy3 and Cy5 fluorophores were used as the donor and acceptor, respectively. One hour after the delivery of molecular beacons, the HDF cells were excited at 545 nm (Cy3 maximum excitation), and the resulting fluorescence signal was observed at 665 nm (Cy5 maximum emission). We found that the dual-FRET molecular beacons approach can provide fascinating images of mRNA localization (Santangelo et al, 2004b). As an example, Fig. 6 shows a fluorescence image of K-ras mRNA localization in a few stimulated HDF cells. Evidently, K-ras mRNA molecules are not randomly distributed in the cytoplasm, but localized, possibly to a cytoskeletal component. To our knowledge, this is the first direct visualization of mRNA localization in single living cells with such fine details revealing the intriguing filamentous pattern.

6 Concluding Remarks

Nanostructured molecular probes have the potential to become a powerful tool in living cell gene detection with high specificity, sensitivity and signal-to-background ra-

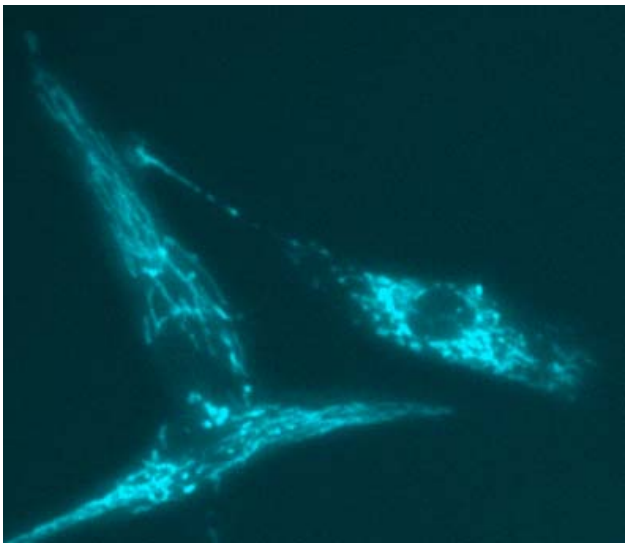

Figure 6 : Localization of K-ras mRNA in stimulated human dermal fibroblast (HDF) cells imaged using dual FRET molecular beacons. Note the intriguing filamentous pattern of mRNA localization.

tio (Santangelo et at, 2004b). It is easy to envision these probes such as molecular beacons being used on a regular basis in a research laboratory to study basic cell and molecular biology, including monitoring in real-time the changes in mRNA expression level due to disease states, toxic assaults, stimuli, and drug molecules; studying mRNA processing, localization, and transport; and quantifying the knock-down effect of RNA interference. These probes can also be used to perform disease detection and diagnosis.

To date nanostructured hairpin probes are used mostly as a tool for the detection of single stranded nucleic acids in homogeneous assays. However, new and novel applications are constantly being developed that maximize the potential of these probes, especially in living cell gene detection (Tsourkas and Bao, 2003; Santangelo et all, 2004b). For example, molecular beacons have recently been modified for solid phase studies (Kambhampati et al, 2001; Ratilainen et al, 1998). In particular, 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 (Doyle et al, 2001; Kuwahara et al, 2001; Ortiz et al, 1998). New applications of this powerful technology continue to emerge and the possibilities seem limitless.

Other novel applications of nanostructured probes include the detection of double-stranded DNA targets and proteins. Double-stranded DNA targets were identified by designing a pair of PNA "openers" that form triplexes with the DNA strands flanking the site of interest, resulting in strand displacement (Fang et al, 1999). DNA or PNA molecular beacons were then used to bind to the exposed sequence. Further, proteins can be detected by synthesizing "aptamer molecular beacons" (Steemers et al, 2000; Liu and Tan, 1999). Aptamers are oligonucleotides, usually with secondary structure, that have a high affinity for proteins. Similar to typical molecular beacons, upon association with the target molecule, in this case a protein, the molecular beacon aptamer undergoes a conformational change that results in the restoration of fluorescence. The aptamer beacons may provide a powerful tool for proteomic studies both *in vitro* and *in vivo*.

The detection and quantification of gene expression in living cells poses a significant challenge to researchers. For example, it is very challenging to obtain an accurate measure of the mRNA copy number in single living cells using molecular beacons or any other imaging methods. To achieve this one needs to: (a) distinguish true and background signals; (b) create an internal control (i.e., inside the cell) of the fluorescence intensity of a single reporter molecule, since the intracellular environment may have a strong effect on fluorescence intensity of the reporter; (c) quantify the possible self-quenching effect of the reporter, especially when mRNA is highly localized; (d) determine the fraction of mRNA molecules hybridized with probe. Further, unlike in RT-PCR studies where the mRNA expression is averaged over a large number of cells (usually over one million), in living cell mRNA visualization assays, in optical imaging of mRNA expression in living cells, only a relatively small number of cells (less than one hundred) are observed. Therefore, the average copy number changes with the total number of cells studied due to the cell-to-cell variation of mRNA expression. It is necessary to address these issues in order to accurately quantify the number of mRNA copies per cell using molecular beacons.

Acknowledgement: This work was supported in part by NSF (BES-0222211), and by DARPA/AFOSR (F49620-03-1-0320). GB and PJS thank helpful discus-

sions with Nitin Nitin and Leslie LaConte.

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