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IMAGING RNA IN LIVING CELLS WITH MOLECULAR BEACONS: CURRENT PERSPECTIVES AND CHALLENGES

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There is a growing realization that cell-to-cell variations in gene expression have important biological consequences underlying phenotype diversity and cell fate. Although analytical tools for measuring gene expression, such as DNA microarrays, reverse-transcriptase PCR and *in situ* hybridization have been widely utilized to discover the role of genetic variations in governing cellular behavior, these methods are performed in cell lysates and/or on fixed cells, and therefore lack the ability to provide comprehensive spatial-dynamic information on gene expression. This has invoked the recent development of molecular imaging strategies capable of illuminating the distribution and dynamics of RNA molecules in living cells. In this review, we describe a class of molecular imaging probes known as molecular beacons (MBs), which have increasingly become the probe of choice for imaging RNA in living cells. In addition, we present the major challenges that can limit the ability of MBs to provide accurate measurements of RNA, and discuss efforts that have been made to overcome these challenges. It is envisioned that with continued refinement of the MB design, MBs will eventually become an indispensable tool for analyzing gene expression in biology and medicine.

Keywords: Gene expression; fluorescence; probes; molecular imaging; biosensor.

1. Introduction

Since the discovery of nucleic acids over more than a century ago, the role of RNA has been unraveled in many biological events, ranging from synthesis of proteins, silencing of gene expression, to the catalysis of various biochemical reactions. In addition, there is also a growing realization that slight variations in RNA synthesis, processing and/or transport may drastically alter the behavior of the cells and errors in their coordination in time and space can lead to irreversible biological complications. Consequently, much effort has been devoted to developing techniques capable of providing a complete spatial-temporal profile of RNA. A robust method should help us decipher the role of RNA function in cellular fate and disease evolution, allow us to predict the onset and stage of disease progression, and thereby provide early diagnosis and effective treatment plans that are tailored to individual patients.

Currently, there are numerous methods capable of measuring gene expression *in vitro*, such as northern blotting, reverse-transcriptase (RT) PCR, and DNA microarrays; however, these methods are generally used to provide the relative change in gene expression for a population of cells. In some cases, global analysis of gene expression via averaging the RNA expression across a population of cells may

overlook the aberrant behavior exhibited by just a small fraction of cells. Further, numerous studies have shown that the behavior of only a few cells can dictate the dynamics of the entire population and that the stochastic nature of RNA expression can drive phenotypic diversity and cell fate.[1–4] While strategies have been developed to perform RT-PCR at the single cell level (i.e., single-cell RT-PCR), these techniques are laborious and are only practical for examining a limited number of cells. In addition, recent reports have suggested that up to 90% of transcripts can be lost during RNA purification, cDNA synthesis, and other steps required for PCR.[5] These complications are likely to be even further exacerbated when working at the single-cell level.

As a complementary approach to PCR, single-cell analysis of RNA expression (and localization) has also been carried out by *in situ* hybridization (ISH). Recent advances in ISH have allowed for the visualization of single RNA transcripts in intact cells with spatial resolution at the single-cell level;[6–9] however, these techniques remain extremely laborious and time consuming. Heterogeneity between samples and sample integrity is also a constant concern with ISH since these protocols rely on fixation and permeabilization steps. Another significant shortcoming of ISH is its limited temporal resolution.

To overcome the limitations intrinsic to current *in vitro* RNA detection assays, much effort has been devoted to developing methods for imaging endogenous RNA in living cells (see Ref. 10 for review). Live cell imaging not only eliminates the need for cell lysis, RNA handling, cell fixation, and cell permeabilization, but also provides an opportunity to *rapidly* analyze gene expression at the single cell level. Currently, the majority of live cell imaging approaches have utilized "molecular beacons" (MBs) to detect intracellular RNA (Fig. 1).

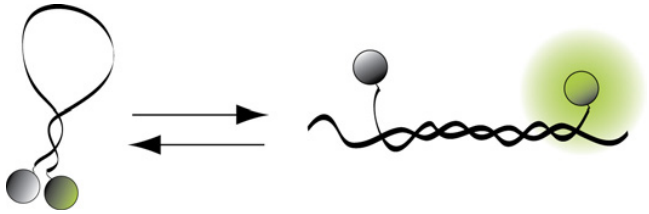

Fig. 1. Schematic drawing of a molecular beacon (MB) in the absence and presence of a complementary nucleic acid target. In the absence of target, MB fluorescence is quenched; however, upon hybridization fluorescence is restored.

MBs are dual-labeled oligonucleotide probes labeled with a "reporter" fluorophore at one end and with a quencher at the other end.[11] In the absence of complementary nucleic acid targets the MBs assume a hairpin conformation, which brings the fluorophore and quencher into close proximity and creates a low-fluorescence or "dark" state. Upon hybridization with complementary targets, the fluorophore becomes separated from the quencher and fluorescence is restored. The unique ability of MBs to convert target recognition into a measurable fluorescent signal has led to their use in a variety of live cell applications, from imaging the up-regulation of specific oncogenes in live cancer cells to following the distribution and transport of β-actin mRNAs in motile fibroblasts.[12–31]

Despite being widely employed as the probe of choice for imaging RNA in living cells, MBs are yet to be truly adopted as a common laboratory tool for measuring endogenous gene expression. This is largely because MBs face several challenges that have yet to be completely resolved. In the sections below, we will describe these challenges and steps that have been taken or need to be taken to overcome these obstacles.

2. Sensitivity of Molecular Beacons

The ability of MBs to sensitively detect specific RNA in living cells is highly contingent upon both the signal-to-background (S:B) ratio and the signal-to-noise (S:N) ratio. The signal-to-background ratio of the MB is defined as the maximum fluorescence of the reporter dye in the hybridized (unquenched) state divided by the maximum fluorescence of the reporter dye in the unhybridized (quenched) state. It is generally observed that the S:B drops off significantly when the MBs are moved from test tube assays to the intracellular environment, where autofluorescence, nonspecific interactions and nuclease degradation can all contribute to background enhancement. The S:N ratio, on the other hand, suffers most from the limited fluorescence intensity of organic fluorophores and a very low enhancement in MB fluorescence due to the presence of only a small number of RNA targets in each cell and a low percentage of hybridized MBs. To date, the low S:N ratio of MBs has limited their use to only a handful of highly over-expressed RNAs. It has been estimated that the lower detection limit may be as high as ~2 000 transcripts of RNA per cell.[27] Of course, this number is likely

to be much lower if the target RNA is concentrated within subcellular domains. Since it is commonly agreed that most endogenous mRNAs exist in less quantity than can be detected by MBs, they would clearly benefit from further improvement in sensitivity for live-cell applications. In theory, this may be achieved through several different mechanisms.

Perhaps the most straightforward way to improve the sensitivity of intracellular RNA detection is to target each RNA with multiple MBs. Presumably, the total fluorescent signal increases linearly with each additional MB; however, the drawbacks of this approach are the increased cost and the increased likelihood of interfering with translation and normal RNA function. Nonetheless, the power of this approach was recently demonstrated by targeting RNA that was engineered to possess 96 tandem repeats of the same MB binding site in its 3′-untranslated region.[32] It was found that with the combined fluorescence of 96 MBs per RNA, even single molecule sensitivity could be achieved.

Another straightforward approach to improving MB sensitivity simply involves selecting a far to near-infrared (NIR) fluorophore as a reporter dye. In the NIR regime (emission wavelength >600 nm), cells and media both exhibit lower levels of autofluorescence. Further, at these wavelengths we have generally observed a reduction in noise, presumably also due to the lower level of autofluorescence. Some of the far-red dyes, including Texas Red[12–14,17,20,27] and Alexa594[32] have already been incorporated into the MB design for live-cell applications. We have observed that MBs designed with reporter dyes >600 nm generally exhibit significantly higher S:B and S:N ratios compared with MBs designed with blue-shifted dyes, such as tetramethylrhodamine (TMR). In one related study, the lower detection limit for Cy5 dyes (Emission: 668 nm) was determined to be as low ∼500 molecules on a conventional inverted fluorescent microscope, compared with ∼15,000 molecules for TMR (Emission: 574 nm).[33]

As an alternative to using conventional organic fluorophores as the reporter dye for MBs, several recent studies have shown that it may also be possible to use quantum dots (QD).[34] In contrast to organic dyes, QDs have bright luminescence, excellent photostability, flexible wavelength for excitation, and narrow and symmetric emission peaks.[35] However, incorporation of QDs into the MB design has thus far been limited by inefficient quenching. One study (using dabcyl as the quencher) reported that quantum dot–conjugated MBs could only elicit a S:B ratio of 5:1,[34] which is significantly lower than the >20:1 S:B ratios observed with many of the organic fluorophore-quencher pairs.[36] Nonetheless, perhaps the loss in S:B may be compensated by implementing time-resolved imaging strategies to image QDs that can exhibit long fluorescence lifetime.

Although approaches that lead to brighter MBs are generally preferred, S:B ratio can also be improved by using more efficient quenchers. Dubertret *et al.* [37] showed that when 1.4 nm-diameter gold nanoparticles are incorporated into the MB design, they can quench fluorescein as much as 100 times better than the organic quencher DABCYL. Furthermore, gold nanoparticles were also found to exhibit a higher quenching efficiency for NIR dyes. MBs designed with multiple quenchers, called superquenchers (SQs), have also exhibited remarkable signal-to-background enhancement.[38] When the number of quenchers was increased from one to three, MBs (fluorescein as the reporter and DABCYL as the quencher) exhibited 14-, 81-, and 320-fold signal enhancement upon hybridization to complementary targets.

Based on the discussion above, clearly there is room for improvement when it comes to enhancing MB sensitivity; however, several technologies on the horizon may hold promise. One exciting technology is known as Conducting Polymers (CPs), which exhibit a unique property called fluorescence superquenching.[39–41] The fluorescence superquenching effect is attributed to a combination of delocalization of the electronic excited state and fast migration of the excited state along the CP chain. As a result, if the fluorescence of any single repeat unit is quenched, the entire polymer chain responds in the same fashion. It has been shown that an entire polymer chain with about 1,000 repeating units can be quenched by a single methyl violet molecule.[42] Ideally, when the MB is in its closed state, the CP will be brought close to the quencher, resulting in quenching of the fluorescence of the CP in a cascade manner. After the MB binds to its RNA target, the fluorescence of the CP will be restored and the entire polymer will light up. It is envisioned that incorporation of the CP into the MB design may significantly improve S:B and S:N ratios and thus the lower detection limit.

3. Molecular Beacon Structure–Function Relationships

An important feature of MBs that sets them apart from other imaging probes is their improved ability to differentiate between perfectly complementary targets and targets with a single base mismatch. This level of specificity is offered by the stem-loop (hairpin) design of the MB, which increases the energy penalty of a mismatch.[43–45] We should stress the point, however, that when designed incorrectly, MBs can still elicit a similar signal in the presence of perfectly complementary targets and single nucleotide polymorphisms (SNPs) at 37°C (Fig. 2).[44,45] Therefore, proper MB design is critical for highly sensitive studies in living cells. To design a MB, three main structural elements may be optimized: the length of the stem, the length of the targeting domain (i.e., loop length), and the sequence of the loop and stem.

In general, MBs with longer stem lengths have an improved ability to discriminate between wild-type and mutant targets over a broader range of temperatures.[44,45] However, the increase in specificity offered by lengthening the stem is mitigated by a decrease in the rate of MB-target hybridization. For example, MBs with a 4-base stem have an on-rate constant up to 100 times greater than MBs with a 6-base stem. Conversely, if the stem length is too short, then a larger fraction of beacons may open due to thermal fluctuations, increasing the overall background fluorescence and decreasing the sensitivity of target detection. These factors must all be taken into consideration when designing a MB for a particular application.

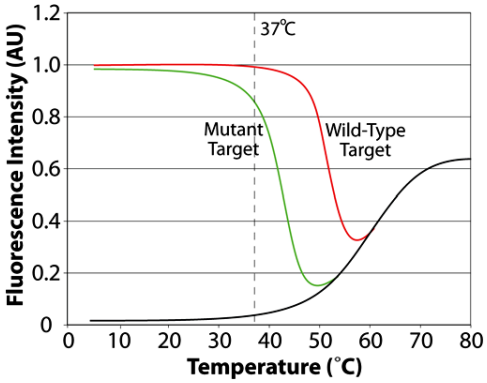

Fig. 2. Representative melting curves of a MB alone (black line) and in the presence of wild-type (red line) and mutant targets (green line). MBs are known to have a higher specificity than linear oligonucleotides as illustrated by the large difference in melting temperature between MB-wild-type and MB-mutant targets; however, if designed incorrectly, MBs can still bind to mutant targets at 37°C and elicit a bright fluorescent signal.

In addition to the stem length, the length of the targeting domain also has a significant influence over MB specificity. Specifically, as the length of the targeting domain is reduced, the ability to discriminate wild-type targets from targets with SNPs is improved. Of course, a shorter targeting domain will also result in reduced affinity (i.e., melting temperature) and will make it more challenging to select a unique target sequence. However, considering that statistically the targeting domain only has to be $\geq$13 bases in order to identify a unique sequence in the human genome[46] and that DNA oligonucleotides of this length can have melting temperatures above 37°C, a targeting domain somewhere between 13 and 18 bases is likely preferred and probably the shorter the better. If the affinity of the MB is a concern, this can be improved through the incorporation of various chemical modifications into the MB design (e.g., 2′-O-methyl, locked nucleic acids). These modifications will also lead to an improved structural stability against nucleases (discussed below).

It should be noted that a longer targeting domain does have the benefit of increasing the rate of hybridization (on-rate constant), particularly for MBs with longer stems (e.g., 6-bases); however, for most MB designs the improvement is marginal.[44] Further, increasing the length of the targeting domain increases the probability of unwanted secondary structure within the loop, potentially interfering with MB hybridization.

The choice of MB sequence can also influence the performance of a MB. Because G/C base pairs bond more strongly than A/U base pairs, high GC content can result in high stability of the stem–stem and/or the probe–target hybrid. Consequently, the sensitivity and specificity of a MB for its target can be fine-tuned by adjusting the sequence accordingly.

Although the improved specificity of hairpin-forming MBs has clearly been delineated *in vitro*, it is still unclear whether this outweighs the associated sacrifice in hybridization kinetics for live-cell applications. Recently, several in cellulo studies have used stemless MBs and claim that they have more favorable hybridization kinetics and consequently a better signal-to-noise ratio than stem-loop probes.[47] It is likely that in order to truly determine the optimal MB design for accurate measurements of

RNA in living cells, a comparative study of MBs with a range of stem and loop lengths must be conducted directly in living cells. Unfortunately, this is not trivial, as this would require that the intracellular concentration of MBs and targets be the same for each study, and that MBs report only true hybridization signals and not false-positive signals, which can result from nuclease degradation and/or nonspecific interactions.

4. Molecular Beacons Elicit False-Positive Signals in Living Cells

While *in vitro* studies have long indicated that MBs can elicit false-positive signals as a result of degradation by nucleases and/or nonspecific interactions with cellular proteins,[48,49] it has only recently been confirmed that false-positives are also generated in living cells.[13] Specifically, it was found that when nonsense MBs (i.e., not complementary to any known endogenous RNA) were microinjected into the cytoplasm of cells, they were not only rapidly sequestered into the nucleus, but they also elicited a bright false-positive signal once they passed through the nuclear pores (Fig. 3). We have found this to be true for a wide range of cell lines including Hela, MCF-7, NIH3T3, and MDA-MB-231 cells. We have also found this to be true regardless of whether the MBs were delivered via microinjection, transfection (Lipofectamine 2000, Invitrogen), or microporation.[14,50] The presence of false-positive signals in the nucleus has significant implications when trying to use MBs to measure gene expression. For example, nonspecific opening of MBs can easily be confused with MB hybridization and lead to ambiguous results. Further, nonspecific opening results in a significant loss in the sensitivity and dynamic range of MBs in living cells.

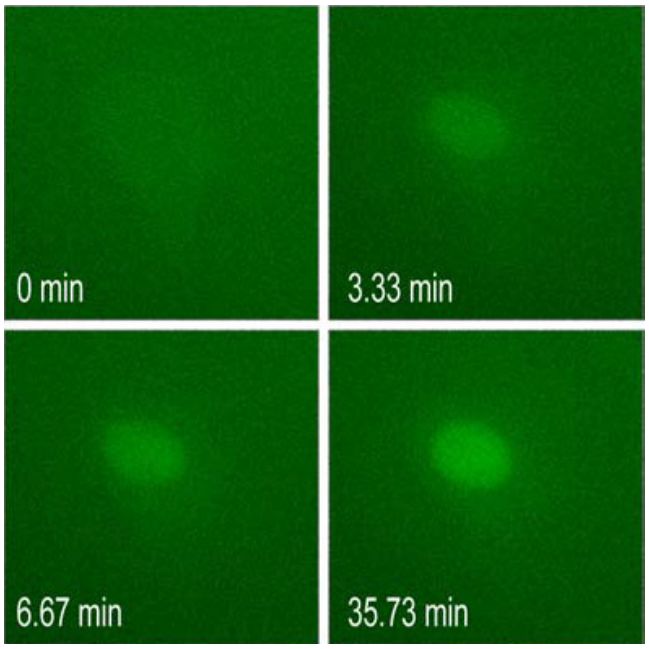

Fig. 3. Fluorescence microscopy images of nonsense MBs at various times after being microinjected into the cytoplasm of living cells. Immediately after injection the MBs localize to the nucleus and emit a bright false-positive signal.

In general, there are two approaches that offer hope of eliminating the nonspecific opening and/or degradation of MBs in living cells: (1) they can be confined to the cytoplasm, or (2) their backbone can be chemically modified (discussed in the next section). Recently, we showed that when MBs are confined to the cytoplasm they do not elicit any detectable false-positive signal, even when nuclease-sensitive DNA backbones are utilized.[13] Two strategies for keeping MBs out of the nucleus have been reported in the literature. The first approach involves attaching MBs to a macromolecule (or nanoparticle), which prevents passage of the MBs through the nuclear pores due to size exclusion.[13] When this approach was implemented, no false-positive signals could be detected in living cells for at least one hour.[13] The second approach that can be used to retain MBs in the cytoplasm involves attaching MBs to tRNAs, which can drive nuclear export.[17] Although it has not been confirmed that this latter approach can prevent the generation of false-positives, presumably it will at least reduce the extent of false-positives. However, one concern with using tRNAs is that MBs must at least transiently enter the nucleus prior to export.

5. Molecular Beacons Can Be Chemically Modified to Reduce/Prevent Nonspecific Interactions and/or Nuclease Degradation

Although it has been widely reported in the literature that MBs with phosphodiester backbones (DNA-MBs) can be used to image RNA in live cells, it is now increasingly accepted that DNA-MBs are highly susceptible to nuclease degradation and nonspecific interactions, both of which can lead to the generation of false-positive signals. In an attempt to reduce the impact of probe degradation,

MBs have been developed with a number of chemical modifications. The most popular modification involves the use of 2′-O-methyl RNA (2′-O-methyl MB), which exhibits improved nuclease resistance, higher specificity, faster hybridization kinetics, and a superior affinity for targets.[51] Surprisingly, despite these reported attributes, recent evidence has suggested that even 2′-O-methyl MBs are highly susceptible to nonspecific opening and generate false-positive signals.[14,50,52] This has recently spurred the evaluation of numerous alternative backbone modifications. In one design, 2′-O-methyl RNA bases were combined with phosphorothioate internucleotide linkages. Despite the reported improvement in structural stability, this was accompanied by an apparent increase in nonspecific binding to cellular proteins, which also resulted in false-positives.[50]

The incorporation of Locked Nucleic Acids (LNA) into the MB backbone presumably eliminated false-positive signals resulting from nuclease degradation and nonspecific interactions; however, hybridization kinetics were significantly slowed due to the high energy barrier of opening the LNA–LNA stem.[53] To alleviate this problem, MBs have recently been designed with a mixture of both LNA and DNA nucleotides in the stem and loop domains (i.e., LNA/DNA MB).[29,54] Initial reports have suggested that these MBs can avoid false-positives while still maintaining reasonable hybridization kinetics.

Potentially, another suitable backbone modification involves preparing MBs with an L-DNA stem.[55] L-DNAs are unnatural bases that do not hybridize with natural nucleic acids. It has been reported that L-DNA MBs exhibit reduced intra- and inter-molecular stem invasions, enhanced selectivity for the target, and improved biostability. Results thus far look promising, although additional testing is needed.

As an alternative to MBs with phosphate-based backbones, MBs have also been prepared with Peptide Nucleic Acid backbones (PNA-MB).[56] In addition to being highly resistant to nuclease degradation, PNAs are neutrally charged and, therefore, are thought to have a lower tendency to interact with intracellular biomolecules.[57] However, neutrally charged PNA backbones are known to experience other complications, most notably a tendency to self-aggregate.[58,59] This has thus far significantly limited the use of PNA-MBs in live cell applications.

6. Cell-to-Cell Variations in MB Delivery Can Interfere with Measurements of Gene Expression

It is likely that before MBs become widely adopted by the scientific community, they must be able to provide accurate information on cell-to-cell variations in gene expression. Unfortunately, this is not currently possible with conventional MBs. This deficiency stems from the inability to accurately account for the large variations in cellular fluorescence that results from heterogeneous delivery (Fig. 4). For example, cells that have no or low amounts of internalized MBs could easily be mistaken for cells with low gene expression, thus resulting in a false-negative. Conversely, cells that have high levels of internalized MBs generally exhibit a measurable background that can easily be mistaken for probe hybridization, i.e., false-positive. Heterogeneous delivery across a population of cells is a common feature of most (likely all) nucleic acid delivery methods. The inability to measure the efficiency of MB delivery with current MB designs limits their use to studying highly expressed RNA, i.e., studies where fluorescence enhancement upon MB hybridization is significantly greater than the cell-to-cell variability in fluorescence that results from heterogeneous delivery.

Several groups have tried to account for variations in MB delivery by simultaneously introducing both MBs and optically distinct fluorescently labeled oligonucleotides (i.e., a reference probe) into living cells[12,15,16]; however, unless microinjection

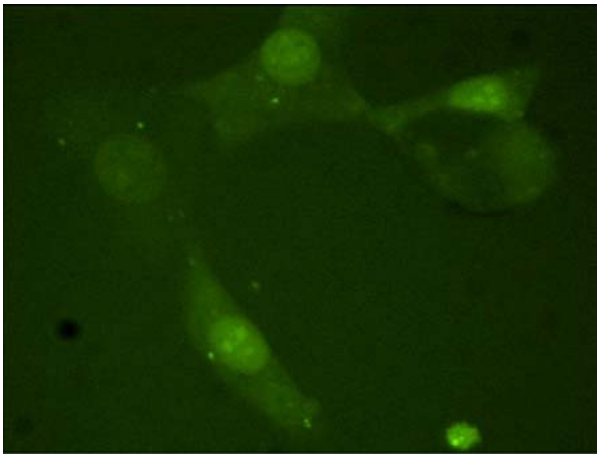

Fig. 4. Fluorescence microscopy images of cells transfected with nonsense MBs. A large cell-to-cell variability in MB background fluorescence is evident.

is utilized, it is unlikely that an equal amount of both probes will be delivered into every cell. Further, even if equal quantities are delivered into every cell, there is a very low probability that both probes would exhibit the same intracellular localization pattern and lifetime. This is problematic since any variation in the ratio of MBs to the reference probe, that is not a consequence of MB hybridization, negates the benefits of performing ratiometric measurements. In theory, these problems can be resolved by attaching a reference dye directly to MBs, but unfortunately this results in unwanted interactions between the reference fluorophore, reporter fluorophore, and/or quencher (i.e., FRET and/or quenching). We generally observed $\geq$90% quenching of the reference fluorophore regardless of the attachment site and fluorophore selected (unpublished data). Recently, it has been shown that this problem may be overcome by using quantum dots (QD) as the reference emitter, since QDs are not easily quenched and are photostable (Fig. 5).[13] Alternatively, the reference fluorophore can be first conjugated to NeutrAvidin and then subsequently linked to the biotinylated MBs. In this approach the presence of the NeutrAvidin–biotin binding complex prevents quenching of the reference dye by increasing the physical separation between the reference fluorophore and the quencher.[14] Although these strategies do show promise, it should be noted that the attachment of a macromolecule/nanoparticle to the MB does complicate and limit the number of viable options for efficiently delivering MBs into the cytoplasm of living cells. Therefore, live cell imaging applications could still benefit from a novel MB design, with an attached reference fluorophore that does not significantly increase the size of the probe.

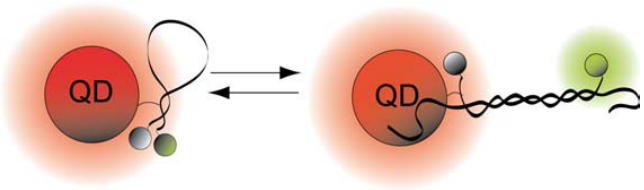

Fig. 5. Schematic drawing of a MB–quantum dot (QD) conjugate in the absence and presence of a complementary nucleic acid target. The QD elicits a bright fluorescent "reference" signal regardless of the hybridization state of the MB. The presence of an unquenched reference signal can be used to normalize against cell-to-cell variability in MB delivery, allowing for more accurate measurements of MB hybridization.

7. Sensitivity of the Molecular Beacon Fluorescence to the Environment

In order to use MBs to accurately measure gene expression in living cells, it is important that the fluorophores that are incorporated into the MB design not be sensitive to the intracellular environment. Unfortunately, it is generally acknowledged that many commercially available fluorophores are highly sensitive to their environment. Specifically, factors including pH and nonspecific protein interactions have already been found to influence fluorescence properties of some fluorophores,[60] either by enhancing or quenching their fluorescence. Ideally, when MBs are designed to measure RNA in living cells, they should only report signal resulting from target recognition, with minimal influence from the environment. If this design constraint can be met, then it is potentially even possible to acquire absolute measurements of RNA hybridization within a compartment/cell based on the total MB signal emitted. Recently, we developed a strategy capable of assessing the extent to which the emission of commercially available fluorophores is altered within the cytoplasm of single living cells.[33] Specifically, changes in fluorescent emission were detected by comparing the fluorescent signal intensity of each fluorophore to that of a fluorescent reference probe (i.e., TMR-labeled dextran encapsulated liposome) that is insensitive to pH and shielded from the cytoplasmic environment. Comparing the fluorescence ratio ($F_{\mathrm{DYE}}/F_{\mathrm{REF}}$) in living cells and in aqueous buffer, we were able to identify a number of environment-insensitive fluorophores that may be incorporated into the design of MBs for RNA quantification in living cells.

8. Concluding Remarks

The ability to accurately monitor the spatial-temporal profile of RNA expression could have a tremendous impact on our understanding of gene regulation and control in health and disease. The unique ability of MBs to transform nucleic acid target recognition into a signal that can be detected by standard epifluorescence microscopes has positioned MBs as the probe of choice for visualizing RNA in living cells. However, although MBs have already been used in a variety of live cell applications, many challenges must still be overcome before they can truly provide accurate measurements of

gene expression. Currently, numerous groups are working to refine the MB structure, chemistry, and optical characteristics to improve their versatility and it is only a matter of time before a consensus is reached on a robust design that can meet the needs of the broader scientific community.

Acknowledgments

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