Peptide-linked molecular beacons for efficient delivery and rapid mRNA detection in living cells

Nitin Nitin, Philip J. Santangelo, Gloria Kim, Shuming Nie and Gang Bao*

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

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ABSTRACT

Real-time visualization of specific endogenous mRNA expression in vivo has the potential to revolutionize medical diagnosis, drug discovery, developmental and molecular biology. However, conventional liposome- or dendrimer-based cellular delivery of molecular probes is inefficient, slow, and often detrimental to the probes. Here we demonstrate the rapid and sensitive detection of RNA in living cells using peptide-linked molecular beacons that possess self-delivery, targeting and reporting functions. We conjugated the TAT peptide to molecular beacons using three different linkages and demonstrated that, at relatively low concentrations, these molecular beacon constructs 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. We could therefore detect human GAPDH and survivin mRNAs in living cells fluorescently, revealing intriguing intracellular localization patterns of mRNA. We clearly demonstrated that cellular delivery of molecular beacons using the peptide-based approach has far better performance compared with conventional transfection methods. The peptide-linked molecular beacons approach promises to open new and exciting opportunities in sensitive gene detection and quantification in vivo.

INTRODUCTION

Quantitative methods to measure gene expression in vitro, such as real-time PCR and microarray analysis, have revolutionized molecular biology and drug development (1–4). These approaches, however, are generally used with purified DNA or RNA from cell lysates. The ability to detect and quantify the expression of specific endogenous mRNA in living cells and tissues in real time will offer tremendous opportunities for biological and disease studies, and will significantly impact drug discovery and medical diagnostics. However, currently available technologies for gene detection in intact cells such as in situ hybridization (ISH) cannot be used in vivo, since ISH studies rely on washing away unbound probes to reduce background signal (5,6). To perform gene detection in living cells, the probes must be able to recognize the target with high specificity, convert target recognition directly into a measurable signal with high signal-to-background (S/B) ratio, and allow for differentiation between true and false positive events (7). Probes must also be delivered into living cells with high efficiencies.

Among the technologies currently under development for living cell gene detection and quantification, the most promising one is perhaps molecular beacons. Molecular beacons are dual-labeled antisense oligonucleotide (ODN) probes with a fluorophore at one end and a quencher at the other end (8,9). In contrast, to fluorescently labeled linear ODN probes, molecular beacons are designed to form a stem–loop (hairpin) structure in the absence of complementary target so that fluorescence of the fluorophore is quenched. Hybridization with target mRNA opens the hairpin and physically separates the reporter from quencher, allowing a fluorescence signal to be emitted upon excitation. Thus, molecular beacons enable a homogenous assay format where background is low without the need to wash away free probes. However, to detect mRNA in vivo, one needs to deliver molecular beacons into living cells with high efficiencies and fast kinetics. Conventional delivery approaches are based on DNA transfection techniques, such as those employing liposomes or dendrimers. These approaches are inefficient (<80%) (10), slow (delivery times ~4 h), and can potentially trap molecular beacons in the endosomes and degrade them in the lysosomes, significantly increasing the background signal (11). The long delivery time also gives rise to significant degradation of cytoplasmic molecular beacons by nuclease, further increasing false positive detection (12). Although the reversible membrane permeabilization method using streptolysin O (SLO) is faster (~2 h) and not based on endocytosis (13), it can only be used in ex vivo cellular assays. Other delivery methods, such as microinjection (14) and electroporation (15) are invasive and may cause severe damage to cells. Further, microinjection is labor-intensive and only practical for studying a small number of cells.

To overcome these difficulties, we have developed novel peptide-linked molecular beacons that can quickly and efficiently enter living cells without the need of any other delivery reagent. Recent research has identified several small regions (9–16 amino acids) of proteins called protein transduction domains (PTDs) or cell penetrating peptides

*To whom correspondence should be addressed. Tel: +1 404 385 0373; Fax: +1 404 894 4243; Email: gang.bao@bme.gatech.edu

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The peptide-linked molecular beacon consisted of the quencher arm of the stem through a carbon-12 spacer. Introducing a modified oligonucleotide, biotin-dT, to the delivery peptide to molecular beacons, as illustrated in Figure 1.

In the first approach (Fig. 1A), peptides were linked to a delivery peptide to molecular beacons, as illustrated in Figure 1. Three conjugation strategies were developed in attaching the peptide (TAT-1) to molecular beacons yields a multifunctional probe that can enter into living cells with nearly 100% efficiency, fast (~30 min) delivery kinetics, and the ability to localize in cell cytoplasm. Further, these peptide-linked molecular beacons function well for mRNA detection in living cells. This novel molecular beacon design can thus provide a powerful means for rapid detection of gene expression in living cells and tissues in real time, with high specificity and sensitivity.

**MATERIALS AND METHODS**

**Design of peptide-linked molecular beacons**

We designed and synthesized peptide-linked molecular beacons targeting the human GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and survivin mRNAs, as well as molecular beacons with a ‘random’ probe sequence. The specific design of these molecular beacons, and the sequence of the 11 amino-acid TAT-1 peptide used in the study are shown in Table 1. GAPDH is a glycolytic protein that has a diverse range of activities in mammalian cells including membrane fusion, microtubule bundling, nuclear RNA export, DNA replication and repair (19). These activities suggest that GAPDH is involved in apoptosis, age-related neurodegenerative disease, prostate cancer and viral pathogenesis. Survivin is a member of the inhibitor of apoptosis protein (IAP) family and also regulates cell division (20). The GAPDH beacon is comprised of a 19-base probe domain targeting the Exon 6 region of GAPDH gene flanked by complementary 5-base sequences that hybridize to form the stem. The survivin beacon has a 16-base target sequence with a similar design of the stem. The ‘random’ beacon was designed as a negative control, with a 17-base probe sequence that does not have any match in the entire human genome. To ensure good target accessibility, we typically design and test molecular beacons complementary to different segments of the same mRNA molecule to avoid targeting sequences that are occupied by RNA-binding proteins or where double-stranded RNA is formed.

Three conjugation strategies were developed in attaching the delivery peptide to molecular beacons, as illustrated in Figure 1. In the first approach (Fig. 1A), peptides were linked to a molecular beacon through a streptavidin–biotin bridge by introducing a modified oligonucleotide, biotin-dT, to the quencher arm of the stem through a carbon-12 spacer. The peptide-linked molecular beacon consisted of the biotin-modified molecular beacon, a streptavidin molecule and biotin-modified TAT-1 peptides. Since each streptavidin molecule has four biotin-binding sites, we were able to link biotin-modified molecular beacons and delivery peptides on the same streptavidin molecule. The stoichiometry was controlled so that the probability of having more than one molecular beacon linked to the same streptavidin is small. In the second design (Fig. 1B), we placed a thiol group on the quencher arm of the molecular beacon stem through a carbon linker; the thiol group then reacted with a maleimide group placed to the C terminus of the peptide to form a direct, stable linkage. (C) The cleavable disulfide bridge in which the peptide is modified by adding a cysteine residue at the C terminus which forms a disulfide bridge with the thiol-modified molecular beacon. This disulfide bridge design allows the peptide to be cleaved from the molecular beacon by the reducing environment of the cytoplasm.
We and others have demonstrated that, for certain cell types, CPPs including TAT-1 peptide tend to carry cargos into cell nucleus (18,21). While this may be useful in detecting nuclear RNA targets such as polyadenylated nuclear RNA (PAN), in most applications however, the target mRNAs are in the cytoplasm, so should be the molecular beacons. Thus, as the third approach, we functionalized the TAT-1 peptide by adding a cysteine residue at the C terminus which forms a disulfide bridge with the thiol-modified molecular beacon as shown in Figure 1C. This cleavable design was based on the rationale that the reducing environment of the cytoplasm will cleave the disulfide bond once the construct enters the cell, thereby separating peptide from probe. This has been demonstrated by Hallbrink et al. using a TAT-peptide linked cargo–quencher construct (22). Specifically, a peptide-conjugated, fluorescently-labeled cargo was linked to a fluorescence quencher through a disulfide bond, resulting in a reduction-sensitive construct. Upon internalization into Bowes human melanoma cells, the disulfide bond was quickly reduced, as indicated by the increased fluorescence intensity. Since fast cleavage of disulfide bridge inside living cells is well documented (23), we believe that, once the cleavable, peptide-linked molecular beacons (Fig. 1C) are inside the cell, the disulfide bridge will be reduced, leaving the molecular beacons in the cytoplasm.

Peptide conjugation

In the design shown in Figure 1A, streptavidin was chosen as a linker molecule because of its high affinity for biotin and availability of multiple biotin-binding sites. The modified nucleotide (dT) at the third base from the 3’ end on the quencher arm of the stem was linked to biotin through a 12-carbon spacer, and the biotin-modified peptides were conjugated to the biotin-modified molecular beacon through binding between biotin and streptavidin. The peptide-linked molecular beacon complex was dialyzed with PBS (1×) overnight using a Slide-A-Lyzer Dialysis Unit, 10K MWCO (Pierce Biotech Inc., Rockford, IL) to exchange the buffer and to remove the unconjugated peptide. The modified molecular beacons were synthesized at Integrated DNA Technologies, Inc. (Coralville, IA) and MWG Biotech, Inc. (High Point, NC), and the modified peptides were synthesized by Invitrogen Inc. (Carlsbad, CA) and Synpep Corporation (Dublin, CA).

In the direct linkage approaches shown in Figure 1B and C, the same position on the quencher arm of the stem bore a modified nucleotide dT-amine group with a 6-carbon spacer. To generate a direct, stable linkage between the peptide and molecular beacon, the peptide was modified with a maleimide group at its C terminus. Amine-modified molecular beacons were reacted in PBS buffer with a 2-fold molar excess of the heterobifunctional crosslinker SPDP, N-Succinimidyl 3-(2-pyridyldithio)propionamide (Sigma–Aldrich) for 2 h, followed by reduction with a 20-fold molar excess of TCEP, 3,3’,3’’-Phosphinidyne-tripropionic acid hydrochloride (Sigma–Aldrich), to create a free thiol (–SH) functional group. This thiolated beacon was then reacted with maleimide-modified peptide to form a stable chemical bridge between the beacon and the peptide. The peptide-linked molecular beacon complex was dialyzed overnight using Slide-A-Lyzer Dialysis Unit, 10K MWCO (Pierce Biotech Inc.) to remove the unconjugated peptide. A similar approach was used in forming a cleavable disulfide bridge between the cysteine-modified peptide and the amine-modified molecular beacon. To reduce the probability of forming beacon–beacon conjugates, a higher (1.5×) concentration of peptides was used compared with that of beacons. Further, the positive charge of peptides helped prevent homodimerization of peptides.

Solution assays of hybridization kinetics and S/B ratio

Measurement of hybridization kinetics and S/B ratio of peptide-linked molecular beacons was carried out using a SAFIRE microplate monochromator reader (TECAN, Austria). For kinetic studies, 200 nM peptide-linked molecular beacons was mixed with 1 μM complementary oligonucleotide target at 37°C and the fluorescence intensity was recorded as a function of time for conventional molecular beacons, direct linked (stable and cleavable) peptide–molecular beacon complexes, and streptavidin-linked peptide–molecular beacon complex. To determine the S/B ratios, 200 nM conventional and peptide-linked molecular beacons were mixed with 200 nM of complementary target, respectively in the microplate reader, and the fluorescence intensity at equilibrium was recorded. The fluorescence signal of each molecular beacon type (conventional and peptide-linked) in the absence of target was recorded as the background signal. All solution assays were performed in 1× PBS buffer.

Cellular delivery of peptide-linked molecular beacons

Primary human dermal fibroblast (HDF) cells (Cambrex, NJ) and a pancreatic cancer cell line MiaPaca-2 (ATCC, VA) were used for this study. These cells were cultured in an eight-well Nalge Nunc culture plate with a glass coverslip bottom in their respective cell culture media for 24 h prior to experiments. Delivery assays were performed by incubating cells at 37°C with the media containing peptide-linked molecular beacons. For molecular beacons targeting GAPDH, peptide-linked molecular beacons with three different concentrations (0.25, 0.5 and 1.0 μM) were incubated with HDF cells for 30, 60 and 90 min. For molecular beacons targeting survivin, 0.5 μM of peptide-linked molecular beacons were incubated with HDF and MiaPaca-2 cells for 30 min. For all assays, the cells were washed twice with PBS to remove the incubation medium and placed in fresh medium for fluorescence imaging, which was carried out using a confocal microscope (Axiovert LSM-100, Zeiss).

Fluorescence in situ hybridization

Normal human dermal fibroblast cells were cultured in eight-well chambered cover slides for 24 h in normal growth medium (FGM-2 Cambrex Co.) and then washing with 1× PBS (without Ca or Mg). The slide was fixed in 100% methanol at −20°C for 10 min. After removing the methanol, the slides were allowed to air dry and stored overnight at −80°C. In situ hybridization assays were then performed by first washing the slides for 5 min in 1× PBS and hybridizing them overnight at 37°C in 1× PBS (no Ca or Mg) containing 200 nM of unmodified GAPDH-targeting molecular beacons. After removing the hybridization solution with washing and adding 1× PBS, the cells were imaged using an Axiovert 100 epi-fluorescent microscope.
Delivery of molecular beacons using commercial transfection reagents

To compare the efficiency and functionality of different delivery methods, we used three commercially available transfection reagents: Effectene (Qiagen), Superfect (Qiagen) and Oligofectamine (Invitrogen). Transfection assays were carried out according to the procedure recommended by respective suppliers; both primary HDF cells and MiaPaca-2 pancreatic cancer cells were incubated with conventional (unmodified) molecular beacons for 0.5, 2 and 3.5 h.

RESULTS AND DISCUSSION

Hybridization kinetics of peptide-linked molecular beacons

To determine the effect of peptide conjugation on molecular beacon function, in-solution hybridization assays were carried out for the binding kinetics of peptide-linked molecular beacons with different conjugation methods. One concern was that, when the positively charged TAT-1 peptide is conjugated to a molecular beacon, it might interact with the negatively charged hairpin oligonucleotide, thus interfering with proper probe–target binding. Shown in Figure 2A are normalized fluorescence intensity versus time curves as a result of probe–target hybridization for unmodified GAPDH-targeting molecular beacons and peptide-linked molecular beacons with the streptavidin–biotin linkage, the stable thiol–maleimide linkage, and the cleavable disulfide bridge. Peptide-linked molecular beacons with the thiol–maleimide linkage had almost exactly the same probe–target hybridization kinetics as unmodified molecular beacons (black and green curves, respectively in Fig. 2A), indicating that the conjugation of peptide using the thiol–maleimide linkage has essentially no effect on the functionality of molecular beacons. Molecular beacons with the cleavable disulfide bridge also behaved similarly to the unmodified ones. With streptavidin–biotin linkage, the hybridization kinetics of peptide-linked molecular beacons was slightly slower, but it did not affect the signal level, as can be seen from Figure 2A. It is possible that the streptavidin molecule, whose size is comparable to that of the molecule beacon, may have sterically hindered binding between the target and the hairpin probe, leading to a slightly reduced hybridization kinetic rate. The effect of different conjugation methods on S/B ratio is relatively small (Fig. 2B), although the S/B for peptide-linked molecular beacons was slightly lower than that of the unmodified ones. Taken together, the results shown in Figure 2 clearly indicate that conjugation of peptide to molecular beacons did not impair their normal function.

Detection of GAPDH mRNA using peptide-linked molecular beacons

To demonstrate the self-delivery and mRNA targeting functions of peptide-linked molecular beacons, we first detected mRNA of a housekeeping gene human GAPDH in normal human dermal fibroblast (HDF) cells. After just 30 min of incubation with TAT-peptide conjugated GAPDH-targeting molecular beacons, we observed clear and localized fluorescence signal in HDF cells as a result of molecular beacon-target mRNA hybridization for all three conjugation schemes, i.e. thiol–maleimide (Fig. 3A), disulfide bridge (Fig. 3B) and streptavidin–biotin (Fig. 3C). In contrast, peptide-linked random-sequence molecular beacons with streptavidin–biotin conjugation gave essentially no signal 30 min after delivery (Fig. 3D). Similar results were obtained using random-sequence molecular beacons with thiol–maleimide and disulfide linkages for peptide (data not shown). This demonstrates that peptide-linked molecular beacons remained highly specific in living cells after internalization. Further, we found that GAPDH mRNAs displayed a very intriguing filament-like localization pattern in HDF cells, with a clear tendency of surrounding the cell nucleus and following the cell morphology (Fig. 3A–C). Interestingly, molecular beacons with the cleavable (thiol–cysteine disulfide bridge) design seemed...
to give better localization patterns than those with the thiol–maleimide linkage, and the latter seemed to perform better than molecular beacons with the streptavidin–biotin linkage. Cleavage of the delivery peptide from the construct may have provided molecular beacons a better access to target mRNA molecules, although more studies of this phenomenon are required to validate this assumption. It is likely that a molecular beacon with a relatively bulky streptavidin molecule is less able to penetrate into the secondary structure of the GAPDH mRNA, thus reducing its ability to seek out its targets. Almost all the HDF cells exposed to GAPDH peptide-linked molecular beacons showed a strong fluorescence signal, implying a near 100% delivery efficiency (data not shown).

Similar results were obtained after 60 min of incubation (Fig. 3E–H). About the same level of fluorescence was observed for GAPDH-targeting molecular beacons with different linkages for peptide, whereas the random sequence molecular beacons did not give much signal. Even after 90 min, there was essentially no increase in the signal level (data not shown), indicating that most of the peptide-linked molecular beacons entered the HDF cells within the first 30 min. Further, fluorescence signal levels and mRNA localization patterns in HDF cells were similar for experiments at three different nominal molecular beacon concentrations (0.25, 0.5 and 1.0 \( \mu M \)). As a result of the peptide conjugation process, it was estimated that, with 0.25 \( \mu M \) nominal molecular beacon concentration, the actual concentration of peptide-linked molecular beacons used in the assay was about 150–200 nM.

### Comparison with in situ hybridization

To correlate the results of the present method with a traditional method, we performed fluorescence in situ hybridization (FISH) assays targeting GAPDH mRNA in fixed HDF cells. The probes used in the FISH assays were fluorescently labeled linear probes (5’-Cy5-GAGTCCTTCCACGATACCA-3’) that have the same probe sequence as the GAPDH-targeting molecular beacon. As demonstrated in Figure 4A, the fluorescence image obtained in FISH assays of GAPDH mRNA in HDF cells gave a filamentous localization pattern similar to that revealed in living cell assays, although the signal was not as sharp. Note the diffused fluorescence signal in cell nuclei. A negative control study of the FISH assay using fluorescently labeled linear Poly-A probes resulted in very low background.

![Figure 3](image-url). Detection of GAPDH mRNA in HDF cells using 0.5 \( \mu M \) of peptide-linked molecular beacons. (A–C) Fluorescence images of HDF cells after 30 min of incubation with TAT-peptide conjugated, GAPDH-targeting molecular beacons, with (A) thiol–maleimide, (B) disulfide bridge and (C) streptavidin–biotin linkages. (D) With peptide-linked random-sequence molecular beacons, there was essentially no detectable signal 30 min after delivery. (E–G) Fluorescence images of HDF cells after 60 min of incubation of peptide-linked, GAPDH-targeting molecular beacons with (E) thiol–maleimide, (F) disulfide bridge and (G) streptavidin–biotin linkages. The signal level of peptide-linked random-sequence molecular beacons after 60 min is shown in (H). Clearly, the fluorescence signal level did not increase when incubation time was doubled, indicating that most of the molecular beacons internalized within the first 30 min. Note the intriguing GAPDH mRNA localization patterns shown in (A–C) and (E–G).

![Figure 4](image-url). Control studies using fluorescence in situ hybridization (FISH). (A) Detection of GAPDH mRNA in fixed HDF cells. The filamentous localization pattern of GAPDH mRNA was similar to that revealed in living cell assays, although the signal was not as sharp. Note the diffused fluorescence signal in cell nuclei. (B) A negative control study of the FISH assay using fluorescently labeled linear Poly-A probes resulted in very low background.
pH-dependent cellular delivery of molecular beacons with three commercially available transfection reagents: Superfect (dendrimer-based), Oligofectamine (liposome-based), and Effectene (liposome based), and performed delivery studies of unmodified GAPDH-targeting molecular beacons with three commercially available transfection reagents: Superfect (dendrimer-based), Oligofectamine (liposome-based), and Effectene (liposome based), and followed all the manufacturers’ instructions. When fluorescence in HDF cells was observed after 30 min of GAPDH beacon delivery using Superfect, Oligofectamine or Effectene, there was essentially no signal (data not shown). We found that, after 3.5 h of transfection with Superfect, the molecular beacons gave strong signal in HDF cells. However, the fluorescence image had different characteristics from that of peptide-linked molecular beacons. Specifically, the fluorescence signals were concentrated in random ‘bright spots’ in both cytoplasm and nucleus (Fig. 6A), suggesting that molecular beacons were trapped and degraded by endosomes, lysosomes and nucleus. Even more disturbing is that the random-sequence molecular beacons showed similar fluorescence signal levels and ‘bright spots’ in HDF cells (Fig. 6D), indicating that the signals in both cases were largely due to molecular beacon degradation. With Oligofectamine transfection, the results after 3.5-h incubation were even worse: only highly concentrated bright spots were present in the HDF cells, with a similar signal level generated by GAPDH-targeting and random-sequence molecular beacons (Fig. 6B and E). Using Effectene for molecular beacon delivery gave fluorescence images (Fig. 6C and F with GAPDH-targeting and random-sequence molecular beacons, respectively) that resembled the corresponding images shown in Figure 6A and E. Clearly, caution must be taken in using these transfection methods in delivering molecular beacons for living cell gene detection assays.

We have clearly demonstrated that cellular delivery of molecular beacons using the peptide-based approach has far better performance compared with conventional transfection methods. This opens new opportunities that become possible only with peptide-linked molecular beacons. For example, the fast delivery kinetics (~30 min) and high efficiency in probe internalization, combined with the high specificity and S/B ratio of molecular beacon makes the peptide-linked molecular beacon probes a unique and novel system for studying gene expression in living cells in real time. We and other have shown that when delivered through the endocytic pathway (e.g. liposome-based transfection), antisense oligonucleotide probes tend to be trapped inside endocytic vesicles and degraded in the endosomes and lysosomes by nucleases (25). Peptide-based internalization has the potential to avoid the endocytic pathway, therefore reducing false-positive signals due to nuclease degradation. Consequently, ODN backbone modifications of the probe such as the use of 2’-O-methyl modified molecular beacons may not be necessary. As demonstrated in our study, combining fast probe delivery with prompt optical imaging, a high S/B can be realized in live cell mRNA detection. Although the dual FRET molecular beacons approach (26,27) can also significantly reduce the false-positive signal and give rise to enhanced sensitivity in mRNA detection in living cells, it requires simultaneous hybridization of two probes on the same target and typically takes much longer than 30 min. The peptide-linked molecular beacons approach, on the other hand, is faster (~30 min) and simpler, which may be more suitable for certain applications, including basic biological studies of mRNA expression level in living cells in response to drug molecules, toxin and external stimuli, the knock-down effect of RNAi, and disease detection and diagnosis. Using NIR fluorophores as the reporter, peptide-linked molecular beacons have the potential to become a powerful tool for shallow tissue molecular imaging with high sensitivity and spatial resolution.

Although our results demonstrated the high efficiency of peptide-based probe delivery, the underlying mechanism of cell membrane penetration remains largely unknown. Some studies suggest that cell entry is independent of the endosomal pathway (28) with the notion that the internalization is much...
Figure 6. Cellular delivery using conventional transfection methods. (A–C) Fluorescence signal in HDF cells after 3.5 h transfection of unmodified GAPDH-targeting molecular beacons with (A) Superfect, (B) Oligofectamine and (C) Effectene. Note the concentrated ‘bright spots’ in both cytoplasm and nucleus. (D–F) Similar fluorescence signal levels were observed after 3.5 h delivery of random-sequence molecular beacons with (D) Superfect, (E) Oligofectamine and (F) Effectene. The resulting ‘bright spots’ in HDF cells indicate that the fluorescence signals in (A–F) were largely due to molecular beacon degradation.

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faster than known endocytic processes (29), whereas others suggest that the CPP-induced cargo internalization is due to classical endocytosis (30,31). One possibility is that the initial interaction between the peptide and cell surface is due to electrostatic forces, but subsequent cell membrane penetration is mediated by glycosaminoglycans (32). However, many questions still remain open. For example, for certain cell lines the TAT-1 and other peptides have the ability to deliver cargos into cell nuclei, suggesting a directed motion in the cytoplasm. It is still unclear whether different CPPs follow a common mechanism or have different pathways. More biochemical and biophysical studies of the CPP-based delivery mechanism are clearly warranted.

There are many challenges in quantifying mRNA expression in living cells, including the need to create an internal control for fluorescence intensity of the reporter fluorophore in the intracellular environment, and determine the fraction of mRNA molecules hybridized with probes. The detection sensitivity is also affected by the properties of fluorophore, fluorescence detection method, the optical imaging instrumentation used and background signal. It has been suggested that the molecular beacon-based approach could detect as low as 10 mRNA molecules in a single cell (14). Alternatively, the average mRNA expression over a large number of cells can be obtained using FACS (Fluorescence Activated Cell Sorter) to yield more reproducible and statistically more significant results.


