

Real-Time Monitoring of Intracellular mRNA Hybridization Inside Single Living Cells

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A molecular beacon, an oligonucleotide probe with inherent signal transduction mechanisms, is an optimal tool for visualizing real-time mRNA hybridization in single living cells. Each molecular beacon (MB) consists of a single-stranded DNA molecule in a stem-loop conformation with a fluorophore linked to the 5' end and a quencher at the 3' end. In this study, we demonstrate real-time monitoring of mRNA-DNA hybridization inside living cells using molecular beacons. A MB specific for β -actin mRNA has been designed and synthesized. After micro-injection into the cytoplasm of single living kangaroo rat kidney cells (PtK2 cells), the MB hybridizes with β -actin mRNA as shown by fluorescence measurements over time. Hybridization dynamics have been followed. Strict control experiments have been carried out to confirm that the fluorescence signal increase is indeed due to the hybridization of mRNA inside single living cells. Variation in the MB/mRNA hybridization fluorescent signal has been observed for different PtK2 cells, which indicates the amount of mRNA in different cells is different. We have also monitored the β -1 adrenergic receptor mRNA inside the PtK2 cells. These studies demonstrate the feasibility of using MBs and the ultrasensitivity achieved in our fluorescence imaging system for real-time detection of mRNA hybridization and for the visualization of oligonucleotide/mRNA interactions inside single living cells.

Monitoring gene function and activity in living tissue has long been a problem of great importance and difficulty in biomedical and basic life science research. Determining if and when genes are active, what control mechanisms are available to regulate gene function, and mechanisms of modulating genetic activity represent but a few of the many potential interests surrounding this field of research. Here we report a means of monitoring, in real time, genetic activity by targeting steady-state levels of specific mRNA molecules in the cytoplasm of single living cells using molecular beacons, a novel class of nonradiolabeled, fluorescent oligonucleotide probes.

Molecular beacons have become a leading oligonucleotide probe used in a variety of applications involving DNA-DNA and DNA-RNA hybridization studies and protein/DNA interactions^{1–7} in solution or at an interface. Each molecular beacon (MB)

consists of a single-stranded DNA molecule in a stem-loop conformation with a fluorophore linked to the 5' end and a quencher at the 3' end. The stem of the MB consists of a double-stranded DNA of five to seven bases in length with each strand of the stem complementary to the other. This stem structure holds the fluorophore and the quencher in close proximity to one another, preventing the fluorophore from emitting a signal as a result of fluorescence resonance energy transfer (FRET).⁸ The loop typically consists of 15–30 bases and forms the basis for specifically binding the target of interest.¹ Once the MB binds to its target, the stem is torn apart and the fluorophore and the quencher are spatially separated. This separation restores the ability of the fluorophore to emit a fluorescent signal when excited with the proper wavelength of light.

There are three primary advantages to using MBs for detection of hybridization to specific targets of interest over traditional fluorescent probes, namely, an inherent signal transduction mechanism, the ability to detect target hybridization without separation of hybridized and nonhybridized probes, and enhanced specificity over traditional DNA probes. With the ability to increase its fluorescence intensity up to 200 times^{1,2} after hybridizing to its target, MBs serve as a highly sensitive, extremely selective, nonradioactive, easily detectable probe for monitoring real-time hybridization, making them an optimal probe for intracellular studies. Moreover, MBs require no supplementary manipulation after initial delivery and subsequent hybridization for detection, unlike other hybridization techniques⁹ that require intercalating reagents, blotting or staining, autoradiography, or other laborious and costly procedures. MB can be used for homogeneous detection of DNA/RNA targets. In solution, MBs have been shown to discriminate between oligonucleotide targets that differ by as little as a single base pair mismatch.¹ Additionally, MBs have a wider temperature range to anneal to targets than linear DNA probes as a result of the stem-loop conformation of the mol-

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Table 1. MB Sequences

MB	sequence
β -actin MB1	GCGAGCGGGGATATCATCATCCATAACCTCGC
β -actin MB2	GCTTGACGGCGATATCATCATCCATAACTCAAGC
control MB1	CGTCCGAATGCTCGGCCAGCTGCACGGCGGACG
control MB3	CCGTGCAATGGTCCGCCAGCTGCACGG
control MB4	CCGTGCAGAATGTCTCGGCCAGCTGCACGG
control MB5	CCTAGTCTAAATCGCTATGGTCCGGCTAGG
β -1 AR MB	AAGCTTGCCGACGACAGGTTTTTAAGCTT

ecule.¹⁰ This imparts greater specificity for the MB than conventional linear DNA probes.^{2,10}

In a previous study, Sokol et al.⁴ demonstrated the feasibility of performing microinjection of molecular beacons into living cells to target specific molecules such as β -actin mRNA. Confocal microscopy was used to monitor a group of cells injected with MB after 15 min of hybridization. Two images were obtained for data analysis. The study suggested that visualization of oligonucleotide/mRNA interactions was possible. Here we demonstrate real-time hybridization kinetics and detailed analysis of MB/mRNA hybridization at the single living cell level. We will also use a series of strict control experiments to confirm that intracellular mRNA hybridization is specific in the living cellular environment.

MATERIALS AND METHODS

Cell Line. Kangaroo rat kidney cells (PtK2 cells) were grown in 10 \times minimum essential medium supplemented with 10% fetal bovine serum and 2% v/v penicillin/streptomycin at 37 °C in a 5% CO₂ atmosphere to near confluence. Culture medium was replaced every 3–4 days, and cells were subcultured as necessary. Prior to microinjection, cells were transferred to cell dishes for use on the microscope and grown in the microincubator under conditions described previously for a minimum of 2 days after transfer to ensure adhesion to the bottom of the culture dish.

Molecular Beacons. All MBs in this study employ the fluorophore tetramethylrhodamine (TAMRA) at the 5' end of the structure, followed by a C6–NH linker. This was bound to a variable stem–loop sequence (sequences listed in Table 1), which was then coupled to the universal quencher 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL) at the 3' terminus. Stem–loop sequences of the MBs synthesized by TriLink BioTechnologies, Inc. are provided in Table 1. Each of these beacons was RP-HPLC and PAGE purified; 10⁻⁴ M stock solutions of each MB were made from 1 mM Tris-HCl, pH 7.7, containing 3 mM MgCl₂. Dilutions of MBs were made in this same buffer solution for the cellular microinjection experiments.

Fluorescence Microscopy Imaging System. The system mainly consists of a microscope, an intensified charge-coupled device (ICCD), an argon ion laser, and a temperature control cell on the microscope stage. The system has been used for single molecular imaging and single living cell calcium flux dynamics studies.^{11,12} Ultrasensitivity in bioanalysis has been achieved with the setup. An inverted microscope (Olympus, model 75F) was

used for optical measurements. The microscope was equipped with a 100 \times (Olympus, LM Plan F1, 0.8 NA) and a 60 \times (Olympus, UplanFI, 0.75 NA) objective. An ICCD (Princeton, EEV 512 \times 1024 FT) was mounted on the top entrance port of the microscope. To excite the TAMRA fluorophore, a 514-nm argon ion laser operating at 50 mW was passed through a beam expander into the rear port of the microscope and up to the stage. Fluorescent signals were collected by the objective and then directed to the ICCD. An Olympus WG dichroic mirror was used to collect the appropriate signal produced by the excited fluorophores while excluding the laser light from the image detection device. To specifically select the fluorescent signal, a 580-nm interference filter was put in front of the ICCD camera. The ICCD was controlled by a PC computer with WinView software (Princeton Instruments Inc., Princeton, NJ). Images were taken using either full frame (512 \times 512) or different sizes of subframes such as 150 \times 150 or 250 \times 200 pixels. The exposure time used in all the experiments for the collection of an image of living PtK2 cells was 750 ms.

Single-Cell Microinjection. Cells were injected with an Eppendorf microinjection system (Micromanipulator 5171, Transducer 5246). Although this system provides the capability of performing numerous cell injections in a short time period, the number of injections at any given time was generally limited to five cells. Eppendorf Femtotips were used to deliver picoliter amounts of MB solution directly into the cytoplasm of the cells, which were manually injected while being viewed in the microscope at 60 \times magnification.

Imaging and Data Collection. Cell culture dishes containing PtK2 cells were secured to the stage of the Olympus inverted microscope. Cells were visualized using a 60 \times power objective. Images of the illuminated fluorophores within the cytoplasm were captured using the cooled ICCD mounted directly on the c-clamp of the microscope. Prior to injection, optical and fluorescence images of the cells were taken for location determination and background measurement, respectively. Depending on the density of the cells in the viewing area, typically up to five cells were injected with picoliter volume of MB solution. The versatility of our imaging system allows for analysis of individual cells as shown in Figures 1 and 4. Subsequent fluorescence images were taken at 3-min intervals to 15 min. A shorter time interval could be used depending on the hybridization rate of the MB inside the living cells. The microscope shutter was opened only long enough to allow the laser to illuminate the injected cells while a fluorescence image was collected at each of the required time points to avoid unnecessary photobleaching of the TAMRA dye molecules. After the final fluorescence image at 15 min, an optical image of the cells was captured to identify which cells had been injected. Injected cells were morphologically distinguishable from cells that had not been injected, as they tended to be more round as opposed to flat. A rectangular region surrounding each of the injected cells was then fixed and used to determine the average fluorescence intensity in that region at each of the illumination time points. These average intensities for each cell at each time point were recorded and plotted against the appropriate time value at which each measurement was made.

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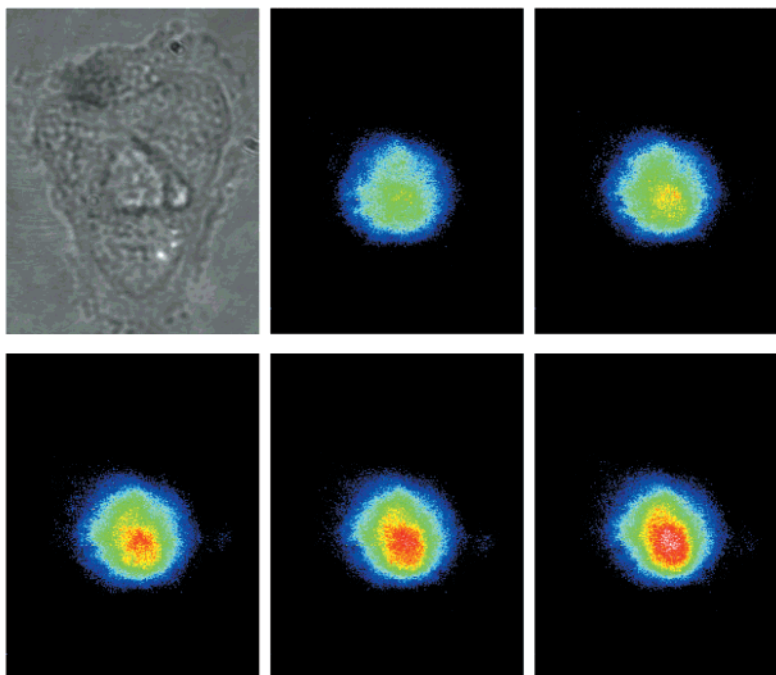


Figure 1. Typical images of PtK2 cells after microinjection of MB specific for β -actin mRNA: optical image; fluorescence images at 3-min intervals to 15 min following injection of 10^{-6} M β -actin MB2.

RESULTS

MB for Intracellular Monitoring of Target mRNA inside Single Living Cells. Imaging experiments were performed using β -actin MB1 to determine whether a reliable and reproducible increase in signal intensity after microinjection of this probe into the cytoplasm of the PtK2 cell line was detectable in single living cells as shown in Figure 1. The hallmark of a hybridization event for a fluorescently labeled oligonucleotide probe is a consistent, reproducible increase in signal intensity over a given time interval, as illustrated in Figure 1. Solution studies indicate MBs hybridize to their complementary, antiparallel targets over several minutes with maximum intensities generally achieved within 8–12 min, depending on experimental conditions. Therefore, we chose to monitor signal increase at 3-min intervals to 15 min following microinjection of MB into the cytoplasm. Before we carried out cellular hybridization experiments, blank experiments (only the cells and no injection; no cells but an injection into the cell dish; only the cDNA solution is injected into the cells, etc.) were done to make sure that the subsequent images were indeed due to the hybridization of mRNA inside living cells.

Previous studies⁴ indicate that hybridization of MB for β -actin mRNA is most likely to occur in the cytoplasm of mammalian cells. The concentration of MB in these studies was reported to be 150×10^{-6} M. Using our instrumental setup, which is capable of single-molecule imaging,¹¹ we first determined that PtK2 cells injected with β -actin MB1 solution (10^{-5} M) in the injection capillary gave a consistent increase in fluorescent signal intensity over time in the cytoplasm when individually analyzed as described in the Materials and Methods section. An example of this phenomenon is presented in the fluorescent images of Figure 1. It is clear that there is a continuous fluorescence increase in the first 15 min. To further determine if this increase in fluorescence intensity over time represented hybridization to β -actin mRNA as opposed to inherent DNase activity⁶ or other mechanisms of MB destruction

within the cell that might result in undesired fluorescence restoration, we repeated this experiment using control MB5, which has no sequence similarity to β -actin MB1, at 10^{-5} M in the injection capillary. None of the cells injected with this particular MB gave a consistent increase in signal intensity during the course of this experiment.

To quantify the data, a linear regression on the plot of intensity versus time for individual cells was performed, analyzing the average intensity at 3, 6, 9, 12, and 15 min. For control MB5, the average of the regression slopes for the individual cells was negative, which indicates that this particular MB did not produce a consistent increase in signal intensity over the time course of the experiment. However, the average of the regression slopes for the individual cells was positive for β -actin MB1, which clearly suggested the potential for the MB to locate and hybridize to its target within a living cell.

To determine the lowest concentration of MB specific for β -actin mRNA that could be detected on a consistent basis, the cell growth medium was removed from the cell dish just prior to the microinjection experiment and replaced with ~ 2 mL of MB buffer (1 mM Tris-HCl, pH 7.7, containing 3 mM MgCl_2). By changing to a translucent solution, we were able to more easily detect the fluorophore within the cell at the given laser beam intensity. The injection pressure that drives the MB solution from the capillary was lowered by a factor of 3, which significantly reduces the volume of MB injected into the cell. Experiments between β -actin MB1 and β -actin MB2 at 10^{-6} M in the injection capillary under these new conditions indicated β -actin MB2 gave more consistent and reliable results (data not shown). However, it is a difficult task to detect a consistent signal from β -actin MB2 at 10^{-7} M in the injection capillary.

Figure 1 shows a set of typical images collected for β -actin MB2 at 10^{-6} M in the injection capillary. In the optical image,

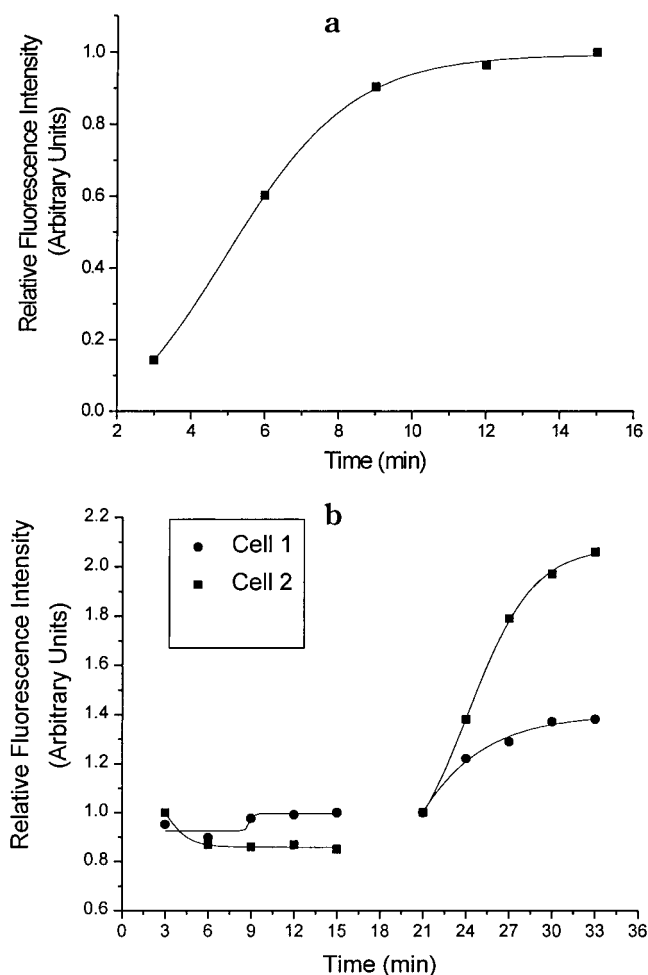


Figure 2. (a) Typical time course measurement of fluorescence intensity for β -actin MB2 inside a single living PtK2 cell. The injected MB2 concentration is at 10^{-6} M. (b) The first segment represents two typical time course measurements of the fluorescence intensity of two PtK2 cells after control MB5 solution at 10^{-6} M is injected into the cells. The second segment illustrates the increase in fluorescence produced for these two cells after introducing cDNA into the cytoplasm at 5×10^{-5} M concentration.

PtK2 cells are shown clustered together. One individual cell in the cluster was successfully targeted for microinjection, as indicated in the subsequent fluorescence images. Lack of signal in or on the adjacent cells from this cluster in the fluorescence images demonstrates the ability to microinject individual cells in such an arrangement as well as the ability to accurately deliver the probe to the cytoplasm of only the targeted cell. Injected cells are viable immediately after MB injection and during the process of hybridization. This has been confirmed by the optical images taken before and after the monitoring experiments.

Hybridization Kinetics inside Living Cells. The fluorescence images in Figure 1 clearly demonstrate the ability of β -actin MB2 to locate and hybridize to β -actin mRNA in the cytoplasm of the PtK2 cells. The transition in the color images in this figure represents the increase in fluorescence intensity as MBs locate and hybridize to their specific target. Figure 2a shows the kinetics of a typical β -actin MB2 microinjection experiment at 10^{-6} M in the injection capillary and also clearly demonstrates the increase in signal intensity with respect to time as β -actin MB2 hybridizes

to β -actin mRNA. It seems that, after 10 min, most of the β -actin mRNA has reacted in the hybridization process.

To determine if our assay to 15 min provided sufficient time to monitor the increase in signal intensity, we injected β -actin MB2 at 10^{-6} M and captured images at 2-min intervals to 30 min and then waited 10 min to capture a final image at 40 min. Analyses at each of these time points showed that the maximum intensity was generally attained within 15 min and remained relatively constant thereafter to 40 min.

Selective Hybridization of β -Actin mRNA in Living Cells.

Having obtained consistent and reproducible results for β -actin MB2 at 10^{-6} M in the injection capillary, control experiments were conducted under the same conditions used in the comparison between β -actin MB1 and β -actin MB2 outlined above. In separate experiments, microinjection of control MB4, control MB3, control MB5, and control MB1 at 10^{-6} M in the injection capillary into the PtK2 cells consistently resulted in no increase of signal intensity over time, as more than 97% of all cells injected and analyzed with a control MB showed no increase in signal intensity. Quantitative analysis of the images obtained from these control experiments showed no signal increase over time. This indicates that these beacons did not hybridize to any target in appreciable quantities in the cells.

Further experiments were performed to demonstrate that the control molecular beacons remained viable in the cytoplasm for more than 15 min after injection into the PtK2 cells. As previously done in the control experiments, control MB5 at 10^{-6} M in the injection capillary was injected into the cytoplasm of the cells and the fluorescence signal was monitored at 3-min intervals to 15 min. Figure 3a is a typical set of fluorescence images demonstrating the lack of signal intensity increase for cells injected with control MB5 in the injection capillary. After the 15-min monitoring, a cDNA solution for MB5 at 5×10^{-5} M in the injection capillary was subsequently injected into the cytoplasm of these same cells that were previously injected with 10^{-6} M control MB5. Changes in signal intensity at 3-min intervals to 15 min following this second injection were monitored. In this study, the cDNA is a complementary, antiparallel, exact base pair matched target for the control MB of interest. The cDNA for control MB5 was able to hybridize to the previously injected MB5, which remained in closed form, indicated by the consistent increase in signal intensity after the second injection event shown in the fluorescence images of Figure 3b and further illustrated in Figure 2b quantitatively. These results clearly demonstrate that the control MBs remained closed for the duration of the original control experiments due to lack of a specific target in the cytoplasm of the PtK2 cells. However, the control MB5 was able to hybridize to its cDNA target and thus to restore its fluorescence after the introduction to the living cell.

In Figure 3b, it is worth noting that the disparity in fluorescence signal enhancement after introduction of the cDNA target arises from the manner in which these cells were analyzed for signal intensity. Rather than selecting a region that surrounds the entire cell, we selected a region within the cell that lies inside the apparent "hot spot" of each cell as indicated in Figure 3a. As such, we are able to report the maximum signal enhancement inside each individual cell as a result of hybridization of the MB to its synthetic oligonucleotide target.

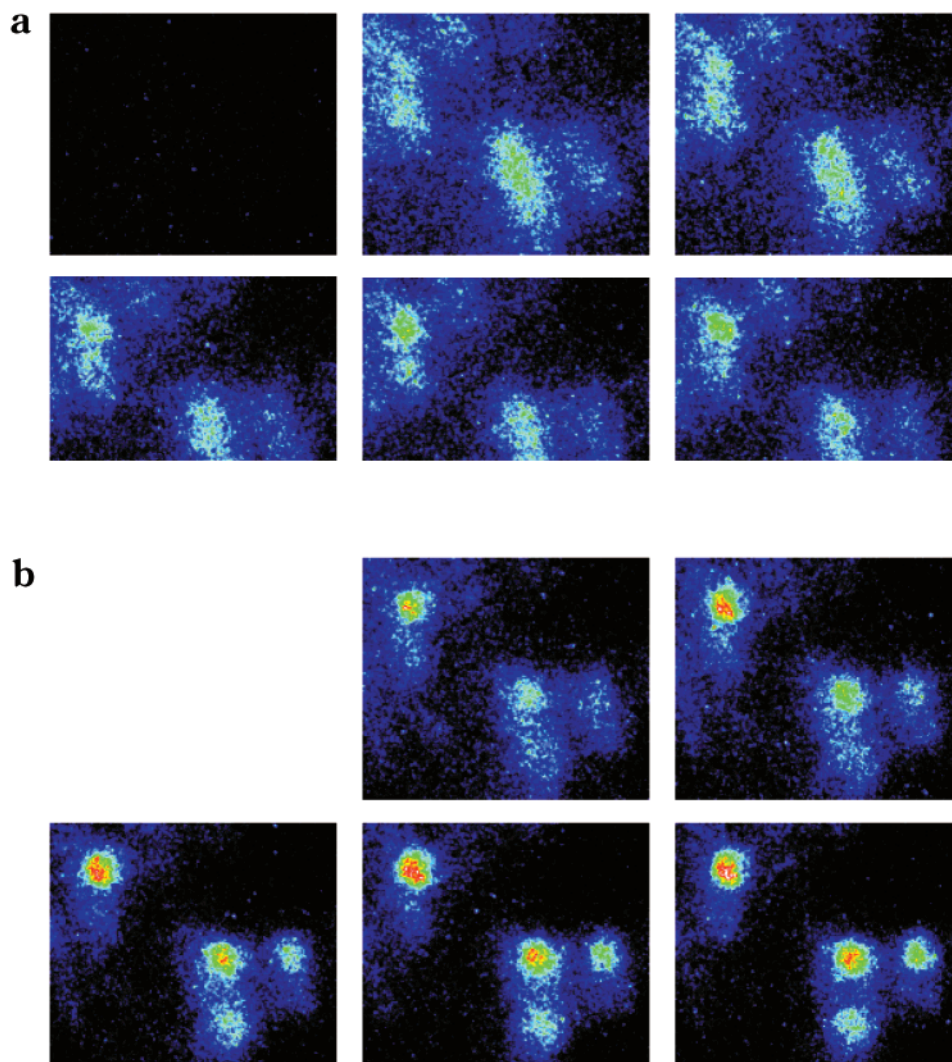


Figure 3. (a) Typical images after microinjection of control MB5 at 10^{-6} M in the injection capillary: background fluorescence; fluorescence images at 3-min intervals to 15 min. (b) Images of control MB5 fluorescence restoration after introduction of cDNA at 5×10^{-5} M into the cytoplasm of cells previously injected with MB5. These are the same cells shown in Figure 3a.

Imaging β -1 Androgenic Receptor mRNA inside Living Cells. To further demonstrate the generality of this approach in living cell hybridization and the ability to locate a different mRNA target in the PtK2 cell line, we designed a MB specific for β -1 androgenic receptor mRNA and repeated the same experiment as outlined previously for β -actin MB2 at 10^{-6} M in the injection capillary. The experimental protocol remained unchanged. Figure 4 illustrates a typical series of images captured after injection of β -1 AR MB at 10^{-6} M in the injection capillary into the cytoplasm of a PtK2 cell. These images clearly demonstrate the ability of the β -1 AR MB to restore fluorescence after hybridization to the specific target of interest.

DISCUSSION

In their 1998 report, Sokol et al.⁴ demonstrated that mRNA hybridization had occurred to an antisense MB based on one single fluorescence measurement taken approximately 15 min after introduction of the molecular beacon into the cell. Comparisons between intensities from single measurements were made between MBs designed to locate specific target mRNA molecules and control MBs. As explained above, the major advancement of

this work over the 1998 paper is in the following four areas. First, we have been able to use much lower MB concentration for mRNA monitoring inside living cells (1×10^{-6} M). The concentration of our MBs injected into living cells is 150-fold lower than that used by Sokol et al. The capability to use low concentrations of MB to monitor low levels of mRNA inside living cells is critically important for a number of studies. If the injected MB concentration is too high, the fluorescence increase brought by the hybridization of a small amount of mRNA molecules inside a single living cell will be buried under the relatively strong background signal. It is therefore necessary to use low concentrations of MB for the elucidation of minute amounts of mRNA inside living cells. We explicitly use optimally determined laser beam excitation to detect only the signal of interest, as opposed to UV excitation which may generate autofluorescence or other unwanted signals from within the cell.

Second, we are able to follow the hybridization kinetics. Based on our experience in carrying out the experiments, it is necessary to analyze multiple image intensities over time of individual cells injected with MB to determine whether hybridization has occurred

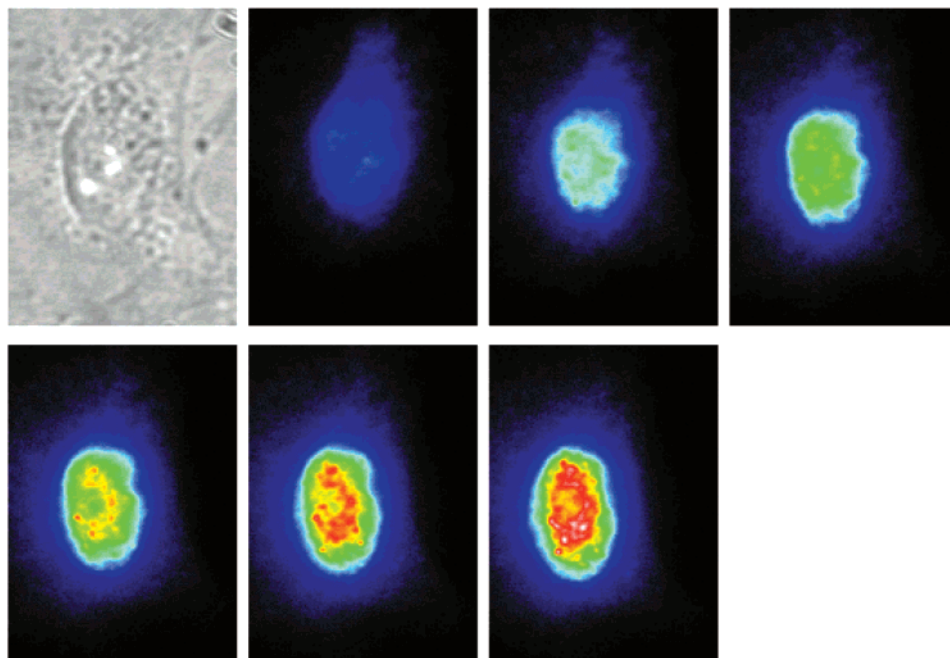


Figure 4. Typical images during microinjection of MB specific for β -1 andrenergic receptor mRNA in PtK2 cell: optical image; fluorescence images at 3-min intervals to 18 min following injection of β -1 AR MB at 10^{-6} M.

between a given MB and its mRNA target. Sokol et al. suggested that a difference in intensity levels between an antisense and control MB at one time point is sufficient to demonstrate that the control did not hybridize to its target while the antisense MB did. We have tracked the intensity signal in individual living cells over 15 min for MB designed to hybridize to β -actin mRNA as well as control MBs. We then analyze the data on an individual cellular basis and determine whether hybridization had occurred in individual cells for each MB tested. We thus have solid data to demonstrate not only the feasibility of MB hybridization in living cells but also more detailed kinetic investigations of MBs in the intracellular environment. The dynamics can be monitored at much shorter time interval if needed.

Third, we carried out strict control experiments in our study to validate that the aforementioned signal enhancement represented hybridization of MBs to their target in the cellular environment. Consideration in the scope of this work not addressed by Sokol et al. is the integrity of the control MB after microinjection into the cell. It is worth noting that control MBs used in this study exhibit very low levels of background fluorescence within single living cells. This background signal is likely due to incomplete quenching of the fluorophore. However, unlike the MBs designed to specifically hybridize to β -actin mRNA and to the β -1 andrenergic receptor mRNA, the resulting signal from all control MBs did not increase with respect to time, indicating a lack of hybridization with any target in the cells. The integrity of the control MB after microinjection into the cell was examined. This is an important issue that demands additional attention. In addition to lack of a suitable target in appreciable quantity, potential reasons for lack of signal increase with respect to time for a given MB include photobleaching of the beacon solution, inadequate conditions within the cell for hybridization, or a beacon whose design prohibits hybridization to a target. To test the assumption that our control MBs were indeed viable beacons that remained inside the cytoplasm during the course of the experi-

ment, we injected cDNA into the cytoplasm of cells that had previously been injected with a control MB. Our results clearly demonstrate that control MBs remained within the cytoplasm, predominantly in the closed stem-loop form, and maintained their ability to specifically hybridize to their complementary targets in the cellular environment.

Fourth, we are able to image single living cells in this work, while in the 1998 paper a group of cells was used for data analysis to show the hybridization of mRNA by injected MB. We have analyzed multiple images over time of individual cells injected with MBs to accurately determine whether hybridization between a given MB and its mRNA target can be successfully monitored in real time. Single-cell imaging gives us the capability to study variations among a group of cells. The results we obtained in this work clearly show that at the single-cell level we are able to (i) successfully monitor, in real time, the fluorescence restoration from molecular beacons designed to hybridize to specific mRNA molecules in the cellular environment and (ii) differentiate between background fluorescence due to incomplete quenching of control MBs and true signal resulting from MBs designed to hybridize to specific targets. There are, however, difficulties in performing and analyzing these types of cellular experiments. For example, in the early stages of our experiments, microinjection of β -actin MB2 at 10^{-6} M in the injection capillary gave an increase in intensity over time for $\sim 15\%$ of all cells injected with this MB. It is expected that advanced equipment such as computer-controlled micromanipulators could greatly enhance this success rate. Factors that complicate quantitative analysis of the data include small but noticeable variations in fluorescence signal intensities from cells injected with a particular MB.

An interesting phenomenon observed while measuring relative fluorescent intensities of cells injected with β -actin MB2 was the variability in signal intensity change from 3 to 15 min in various cells. Figure 5 shows the change in intensity from 3 to 15 min for individual cells injected with 10^{-6} M β -actin MB. The most likely

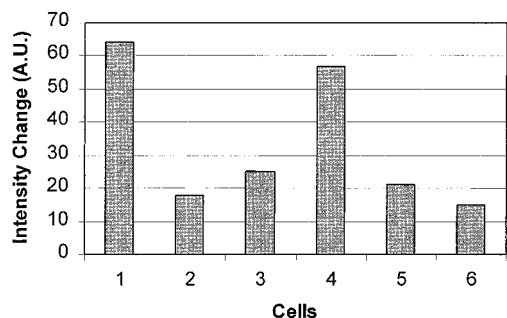


Figure 5. Variations in fluorescence restoration of different cells in the same dish during hybridization studies. The amount of β -actin MB2 has been kept constant. The intensity change shown here is the absolute intensity difference of the fluorescence measurements at 3 and 15 min after β -actin MB2 was injected into the cell.

explanation for this variation in signal intensities between cells can be attributed to different concentrations of β -actin mRNA present in the cytoplasm of individual cells. It would be of great interest to be able to quantitate the number of MBs that actually hybridize to a specific oligonucleotide target within single living cells. At the moment we are addressing this challenge by combining innovative approaches to MB design and taking advantage of the superior imaging ability afforded by the present instrumentation imaging setup to create a quantitative system. Using two fluorophores to label the MB, we will be able to use the ratio of fluorescence intensities at different wavelengths of the spectrum to quantify genetic material inside living cells.

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Preliminary results in this area have shown great feasibility and promise.¹³ Such an achievement could allow this system to be used as a diagnostic tool in several areas of current research, specifically gene therapy. With the completion of the human genome project, a vast array of genetic data lies waiting to be screened for potential therapeutic use. Practical applications of such a quantitative system include monitoring of induced gene expression or repression by observing changes between steady-state and induced mRNA concentrations in single living cells for a specific gene of interest. Also, one could readily achieve rapid, high-throughput screening of various cell lines for potential antisense hybridization activity for a given antisense sequence in the form of a MB. Development of the ability to quantify, in real time, the hybridization between MB and specific targets of interest should prove to be extremely beneficial to many areas of cell biology and genetic research.

ACKNOWLEDGMENT

PtK2 cells were generously donated from the laboratory of Dr. Frederick Southwick and Dr. Daniel Purich in the College of Medicine at the University of Florida. Additional thanks goes to Monde Qhobosheane for assistance with optical and fluorescence imaging. This work is partially supported by the NIH R01 NS39891 and by NSF Career Award 9733650.

Received for review June 7, 2001. Accepted August 31, 2001.

AC010633B