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# Nucleic Acid Beacons for Long-term Real-time Intracellular Monitoring

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## Abstract

Real time intracellular monitoring of biological molecules inside living cells is important in many biomedical studies and reveals valuable information unobtainable by conventional molecular biology techniques. A variety of methods and molecular probes have been developed, but long term (from a few hours to days) intracellular monitoring with high sensitivity and selectivity is impossible and has not been accomplished. We have used locked nucleic acids (LNA) to engineer novel molecular beacons (MBs) for long term intracellular monitoring. The LNA-MBs were made of a mixed LNA and DNA bases to have extremely high biostability. The new beacons were tested with MDA-MB-231 cancer cells and used effectively to monitor mRNA expression levels in real-time for 5–24 hours. After 24 hours inside living cells, the LNA-MBs were still functional, demonstrating a greatly enhanced stability enabling the measurement of intracellular gene expression over an extended period of time.

## Introduction

Advanced neurological studies (e.g. associated to learning and memory mechanisms), differentiation, injury and regeneration we well as drug discovery protocols presuppose the need for super stable molecular probes for use in long-term real-time gene monitoring inside living cells, as well as *in vivo* monitoring inside animals. Such probes should be able to resist nuclease digestion and avoid cellular protein binding. One probe, the molecular beacon (MB),[1] promises to meet these requirements. The MB is a short hairpin oligonucleotide probe which produces a fluorescence signal upon hybridizing to specific nucleic acids. Although MBs have been used for real-time intracellular detection, DNA MBs are known to yield false positive signals. This results from multiple intracellular interactions that degrade DNA MBs, or change their conformation, through processes such as endogenous nuclease degradation and/or stem-loop structure disruption by nucleic acid binding proteins.[2] Consequently, a biostability problem arises which has been addressed by incorporating nuclease-resistant building blocks, such as phosphorothioate,[3] 2′-O-methyl RNA bases,[4] peptide nucleic acids,[5] and locked nucleic acids (LNA),[6] into MB designs. Among these candidates, MBs with LNA bases have demonstrated adequate biostability *in vitro*.[6, 7] In this paper, we report the design of LNA MBs for long-term real-time gene monitoring inside living cells.

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Supporting Information Available. Listing including the experimental procedures about hybridization kinetics, DNase I sensitivity and RNase H sensitivity tests, three *in-vitro* hybridization kinetics graphs of β-actin LNA-MB, MnSOD LNA-MB, control LNA-MB upon the addition of 5-fold target cDNA, and *in vivo* hybridization kinetics of β-actin LNA-MB and control LNA-MB inside the living cells. This material is available free of charge via the Internet at http://pubs.acs.org.

# EXPERIMENTAL SECTION

## Chemicals and Reagents

MBs prepared are listed in Table 1. DNA and LNA synthesis reagents were purchased from Glen Research (Sterling, VA). Deoxynuclease I, Ribonuclease H, and Single-Stranded Binding Protein were purchased from Fisher.

## Equipment

An ABI3400 DNA/RNA synthesizer (Applied Biosystmes, Foster City, CA) was used for all MB probes and DNA target preparation. A ProStar HPLC (Varian, Walnut Creek, CA) with a C18 column (Econosil, 5μ, 250×4.6 mm) from Alltech (Deerfield, IL) was used for probe purification. A Cary Bio-300 UV spectrometer (Varian, Walnut Creek, CA) was used to measure absorbance for probe quantitation. Fluorescence measurements were performed on a Fluorolog-Tau-3 spectrofluorometer (Jobin Yvon, Inc., Edison, NJ). The protein concentrations in the cell lysate were determined with Bio-Rad protein assay dye reagent concentrate (Bio-Rad Laboratories Inc.) by comparing with Bovine Serum Albumin (BSA) calibration curve. Cell images were taken with a confocal microscope setup consisting of an Olympus IX-81 inverted microscope with an Olympus FluoView 500 confocal scanning system and tunable Argon Ion laser (458 nm, 488 nm, 514 nm) and a green HeNe laser (543 nm) with two separate photomultiplier tubes (PMT) for detection. A 40× 0.6 NA air objective was used. A Leiden microincubator with a TC-202A temperature controller (Harvard Apparatus, Holliston, MA) was used to keep the cells at 37°C during injection and monitoring. An EXFO Burleigh PCS-6000-150 micromanipulator was used for positioning the injector tip. An Eppendorf Femtojet microinjector with 0.5 μm Femtotips was used to inject the MBs into the cells. Image acquisition and analysis were conducted with the FluoView software.

## Cell Culture

MDA-MB-231 breast carcinoma cells (American Type Culture Collection, Manassas, VA) were maintained in Leibovitz's L-15 Medium (American Type Culture Collection, Manassas, VA) with 10% fetal bovine serum (Invitrogen, Carlesbad, CA) and 0.5 mg/ml Penicillin-Streptomycin (American Type Culture Collection, Manassas, VA) at 37°C. Cells were plated in 35-mm glass bottom culture dishes (MatTek Corp., Ashland, MA) and grown to 80% confluency for 48 h before microinjection. To stimulate MnSOD mRNA expression and real-time monitor signal change, cell medium was immediately replaced by fresh cell medium with 1 μg/mL lipopolysaccharide (LPS) from *E. coli* serotype 055:B5 (Sigma) during the imaging. For long-time monitoring, fresh cell medium was added at times to keep cells alive.

## Cell Lysate Preparation

A 75 $cm^2$ flask of ~95% confluent MDA-MB-231 and was washed with serum-free medium (Leibovitz's L-15 medium with L-Glutamine, ATCC) for 1 hour in the incubator. After removing the SFM, the cell cultures were suspended in 3mL ice-cold detergent-free buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 2 mM EGTA, 0.33 M sucrose, 1 mM dithiothreitol) containing a broad-range protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA). The cells were then scraped off the flask using a plastic cell scraper and passed five times through a 27½-gauge needle. The samples were fractionated by centrifuge at 1000 *g* for 10 min at 4°C to isolate the nuclei, and the supernatant was cytosolic fraction. After one more washing with the detergent-free buffer, the nuclei pellet was resuspended in a lysis buffer (20 mM HEPES, 1 mM EDTA, 2 mM EGTA, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% Igepal and 0.5%

deoxycholic acid, pH 7.5) containing a broad-range protease inhibitor cocktail (Roche Molecular Biochemicals) and mixed for 90 min at 4°C to ensure full membrane lysis. After the centrifuge at 1000 g, the supernatant was nuclear fraction. Each fraction's protein concentration was determined, using the Bradford protein assay, and was stored at −80°C until use.

### Biostability Study with Cell Lysate

With the fluorometer, the fluorescence intensity change of MBs was monitored upon the addition of 2 μL 4 ug/mL cell lysate fractions (nuclear or cytosolic fraction) into the 100nM MB solutions. The buffer used was 20 mM Tris-HCl (pH 7.5) containing 5 mM $MgCl_2$ and 50 mM NaCl.

### Imaging and Data Collection

All the cellular fluorescent images were collected by the confocal microscope using laser excitation. The control MB with AF488 was excited at 488nm and collected at 520 nm. The MnSOD MB and β-actin MB with Cy3 was excited at 543 nm and collected at 570 nm. The probe solution used in the experiments contains 4 μM concentration of each MB in 20 nM Tris, 50 mM NaCl and 5 mM $MgCl_2$ buffer. Images were taken every minute or every other minute. 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 dye photobleaching and any damage to the cells. A region surrounding each of the injected cells was used to determine the average fluorescence intensity for each channel at each time point. These average intensities for each cell at each time point were recorded and plotted against the time frame. For long-time imaging, some cells might change their shapes or move slightly. The signal region for analysis was accordingly adjusted to achieve more accurate data. The data collection was stopped when the injected cell changed its shape, a circumstance which raised some concerns about cell viability. The relative fluorescence intensities were calculated by directly comparing the current intensity with its intensity at the beginning state for each case. For data given in Figure 2c, the relative fluorescence intensity ratios were calculated by the average ratios from the final five points divided by the monitoring starting point (for the cases without LPS treatment), or by the data points right before and LPS treatment (for the cases with LPS treatment). The error bars represent the standard derivatives from the calculated five data points for each case.

## RESULTS AND DISCUSSION

### MB Design and *in vitro* Characterization

We demonstrated that MBs made entirely of LNA improves biostability and sequence selectivity greatly *in vitro* [6]. However, we discovered that the overall effectiveness of fully modified LNA MBs can be compromised by extremely slow hybridization rates. To overcome the limitations of MBs in intracellular measurements, we made design modifications by mixing LNA and DNA bases in both stem and loop of a MB, resulting in MBs that can effectively measure intracellular events with extremely high biostability (Table 1). As shown in the supplementary materials *in vitro* testing (Supplementary Figure S1), these MBs combine long term stability with excellent sensitivity, selectivity and fast hybridization kinetics.

Our design strategy consisted of two steps. First, in order to maintain the fast hybridization rate, the MB stem was consistently composed of 50% LNA in an alternating fashion. Such optimized LNA MBs would then have a fast response to excess complementary target DNA (Supplementary Fig. 2, online). When compared to DNA MBs, it is precisely this

hybridization behavior which makes it possible to use these LNA MBs to track gene expression levels in real-time. Second, we gradually increased the LNA percentage in the loop starting from 50% until the LNA MB satisfied the biostability screening criteria (Supplementary Fig. 1, online).

The biostability screening criteria are to test LNA-MBs with various amounts of LNA by three types of experiments: single-stranded DNA binding protein (SSB) interaction, DNase I and Ribonuclease H (RNase H) digestion. The *in vitro* biostable LNA-MBs, which passed all three *in vitro* biostability tests, were called the optimized LNA-MBs listed in Table 1, which were then used for further study inside living cells

## MB *in vitro* testing with cellular samples

Before we attempted to use the LNA-MBs for intracellular measurements, we tested them with cytosolic and nuclear fractions of MDA-MB-231 cells. DNA-MB was used for a comparison, and the results are shown in Figure 1. When the cell lysate was added to the DNA-MB solution, we saw a significant fluorescence increase over time. In contrast, there was no such fluorescence increase for any of the optimized LNA-MBs, clearly demonstrating that the LNA-MB can sustain biostability for a long period of time, even with cell lysate samples. This finding enabled us to test the feasibility of the LNA-MB as a long-term molecular probe for mRNA monitoring inside living cells. To accomplish this, the LNA-MB was microinjected into living cells and their hybridization with nucleic acid targets was monitored through fluorescence by confocal microscopy. The LNA sequences showed little nucleus accumulation problem inside MDA-MB-231 cancer cells, therefore the average fluorescence intensities around the injected cells were determined for data analysis.

## Long-term monitoring inside living cells

The LNA MB was observed to monitor mRNA expression inside a living cell for more than five hours. To further evaluate the biostability and mRNA detection ability of LNA-MBs in living cells, we used LNA bases to synthesize both control MB and MnSOD MB, which were co-injected into MDA-MB-231 cancer cells. The control LNA-MB and MnSOD LNA-MB were made with donor fluorophores (Alexa Fluor (AF488) and Cyanine (Cy3), respectively). Since their excitation and emission wavelengths do not overlap, both beacons could be imaged simultaneously in our confocal system.[8] In this experiment, lipopolysaccharide (LPS) was used to stimulate MnSOD mRNA expression inside living cells.[9] The control LNA-MB had no complement inside the cells. Therefore, we determined that the signal from the control LNA-MB would be stable, as long as the MB was not degraded by nucleases or nonspecifically opened by protein binding. As shown in Figure 2a and 2b, the fluorescence signal of the control MB did not change noticeably, even after 5.5 hours (1.5 hours before LPS treatment plus another 4 hours after treatment). At the same time, the MnSOD LNA-MB was effective in monitoring MnSOD mRNA expression over a 5.5 hour period. Also as shown in Figure 2a, there is a slow fluorescence increase in the MnSOD LNA-MB before LPS induction, while the fluorescence stays flat for the control MB. As the basal level of MnSOD is very low inside the cell, the number of newly formed hybrids due to hybridization is slowly increased over time. We tested multiple cells, which showed the same trend as that discussed above, and the relative fluorescence signal changes from the injected cells are plotted in Figure 2c. Overall, these results showed that the basal MnSOD expression level in MDA-MB-231 cells was low, but that MnSOD mRNA could be highly expressed with LPS treatment. In addition, the hybridization kinetics of LNA MBs inside the cells (with either synthetic complement DNA or native mRNA; Supplementary Figure S2–3, online) was fast (within minutes) The prolonged signal increase seen in Figure 2a suggests a continuously induced mRNA expression. Since the signal for the control MB inside the living cells during the same time period was not changed, the long term stability

of the LNA MBs was further confirmed. The small fluctuation of signal enhancement ratios among these cells only reflects a cell-to-cell gene expression variation. These cell-to-cell differences might have been a consequence of some membrane ruffling and cell morphology changes during the monitoring period, as indicated in the time-lapse images shown in Figure 2b.

### MB's long-term stability

The LNA MBs are also shown to be effective for nucleic acid monitoring, even after 24 hours inside living cells. To demonstrate this, the control LNA-MB was injected into a cell and was incubated for 24 hours before the cell was used for nucleic acid hybridization studies. A target complementary DNA solution was injected into the cell 24 hours after the original control LNA-MB injection. Time-lapse fluorescence images (Figure 3) show that the control LNA-MB remains functional, even after incubating inside the cells for one full day. We also tested the enhancement of the LNA-MB by adding cDNA for the LNA-MB, both immediately after MB injection and 24 hours later. The signal enhancements in both cases are about the same, 2.26 for immediate second-injection (standard deviation is 32.9%) and 2.02 for delayed second-injection (standard deviation is 4.24%). The fact that the LNA-MB showed the same response to complementary DNA after 24 hours of incubation inside a living cell proves that the LNA MB can be used for long term monitoring of gene expression inside living systems.

## CONCLUSIONS

We have created LNA-MBs that have superior resistance to enzymatic cleavage and protein binding and that retain their functions inside cells, even after 24 hours of incubation. To put this into perspective, DNA-MBs degrade after about 30 minutes in the cellular environment.[8] In contrast, the newly designed LNA-MBs provide outstanding biostability which extends their application to long-term real-time intracellular gene monitoring and possible *in vivo* monitoring inside living animals. In particular, we will be able to study gene expression levels within a single cell, making it possible to carry out experiments in which specific cells within a tissue or tumor can be monitored over long time periods. Examples include 1) following gene expression in a single cell as it differentiates, 2) observing specific cells during development, 3) measuring cellular responses to drugs and 4) studying specific cellular reorganization processes in cancer, i.e., tumor cell migration and angiogenesis. The super stability of the newly designed LNA MBs makes them an effective tool for many *in vivo* studies and monitoring where stability of the molecular probe is needed for a protracted period of time.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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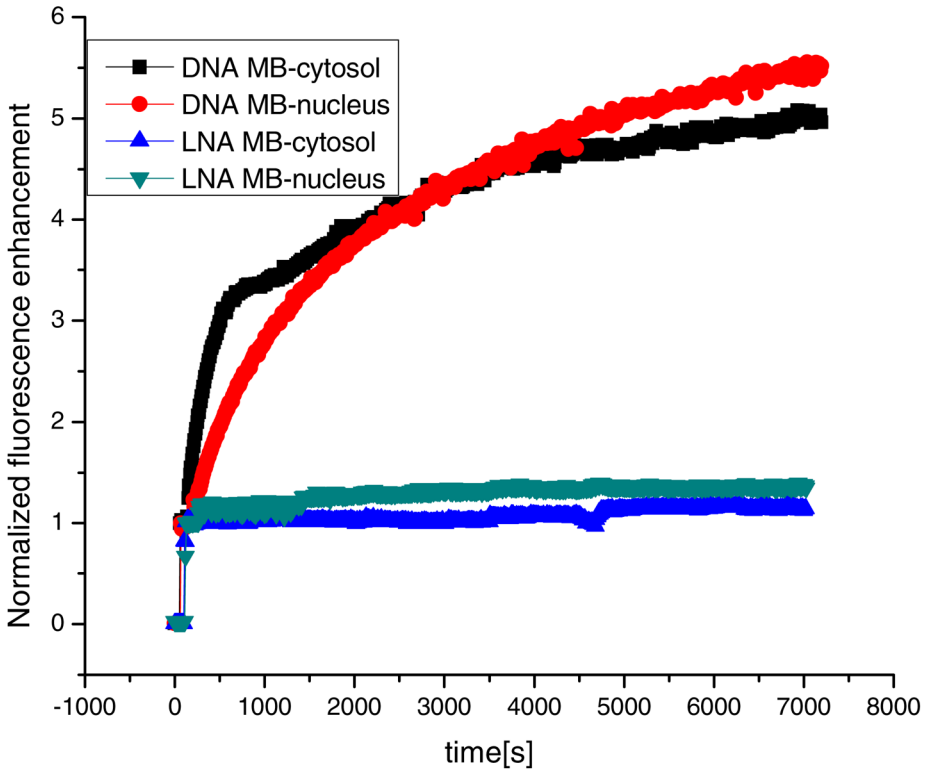


**Figure 1.**

Normalized representative fluorescence intensity changes of DNA MB and LNA MB with cytosolic and nuclear fractions from MDA-MB-231 cell lysate. The DNA MBs had significant fluorescence increase over time, while the LNA MBs did not have any significant fluorescence changes.

**Figure 2 a.**

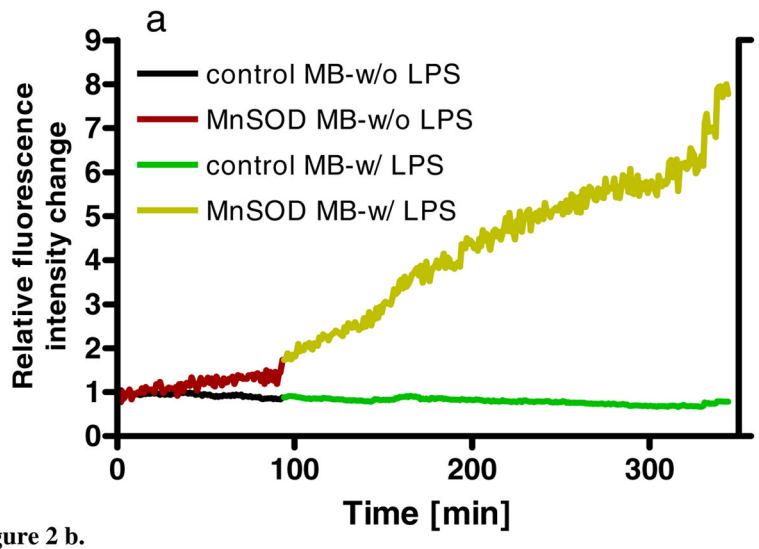


**Figure 2 b.**

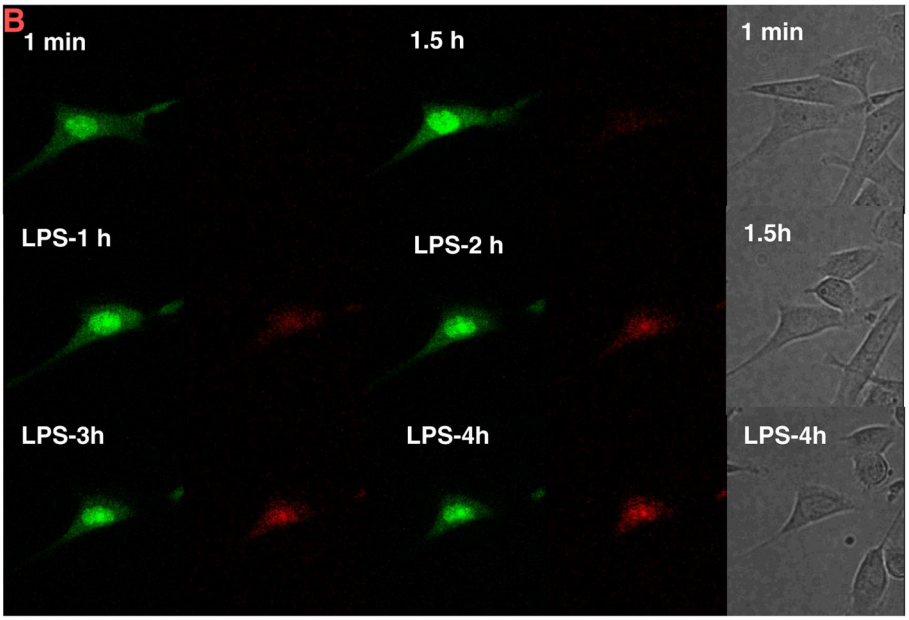

**Figure 2 c.**

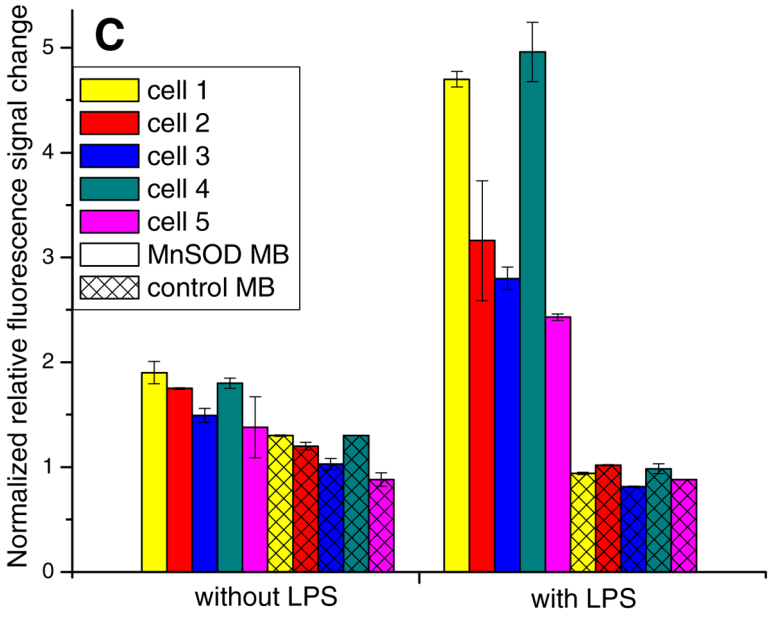


**Figure 2. Representative microinjection experiments**

**(a)**. Relative fluorescence signal changes of control MB and MnSOD MB during 1.5 hours without LPS treatment and during 4 hours with LPS treatment. The y-axis represents the signal changes relative to the initial fluorescence signal directly after microinjection (Supplementary Materials and Methods, online). **(b.)** Time-lapse of control MB (green) and MnSOD MB (red) inside of a MDA-MB-231 cell. The control MB fluorescence did not change over time, but that for MnSOD changed significantly as the gene was induced by LPS. **(c.)** Histograms of relative fluorescence signal change of MnSOD MBs (no pattern) and control MB (cross-hatched pattern) without LPS and with LPS treatments within different cells.

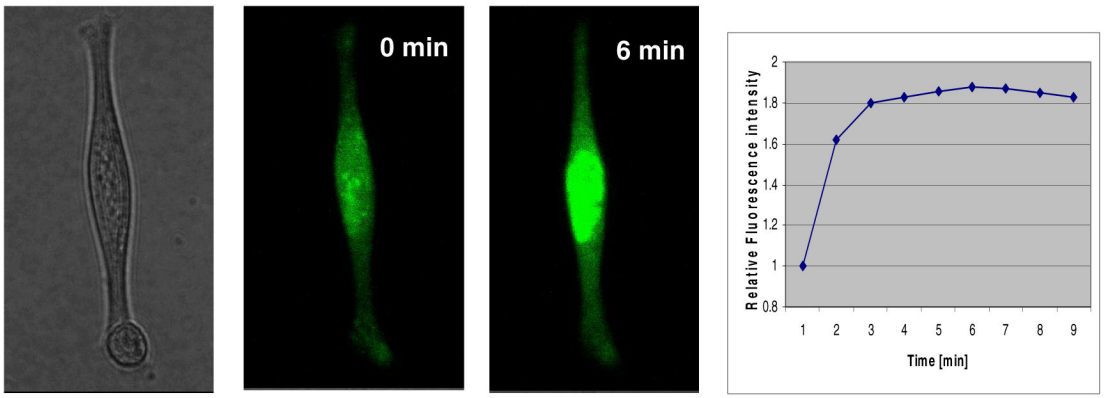


**Figure 3.**
Time-lapse fluorescence images after microinjection of excess cDNA into the same cell which has been injected by the optimized control MB after 24 hours.

**Table 1**

The optimized LNA-MBs for intracellular experiments.

| | | |
|---|---|---|
| Optimized LNA MBs | β-actin MB | 5'-Cy3-CAGTCGAGGAAGGAAGGCTGGAAGAGCGACTG-BHQ2-3' |
| | MnSOD MB | 5'-Cy3-CCTAGCCAGTTACATTCTCCCAGTTGATTGCTAGG-BHQ2-3' |
| | Control MB | 5'- AF488 -CTAGCTCTAAATCACTATGGTCGCGCTAG-BHQ1 -3' |

Red letters represents LNA bases, and underlined letters are bases for MB stem.