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# Molecular beacons

Weihong Tan<sup>1,2\*</sup>, Kemim Wang<sup>1,2</sup> and Timothy J Drake<sup>1</sup>

This opinion covers the field of molecular beacons (MBs), in which nucleic acids are molecularly engineered to have unique functions for the investigation of biomolecules. Molecular beacons have been used in a variety of formats, and this review discusses four: first, *in vitro* RNA and DNA monitoring; second, biosensors and biochips based on MBs; third, real-time monitoring of genes and gene expression in living systems; and finally, the next generation of molecular beacons that will be highly useful for studies with proteins, molecular beacon aptamers. These unique applications have shown that MBs holds great potential in genomics and proteomics where real-time molecular recognition with high sensitivity and excellent specificity is critical.

## Addresses

<sup>1</sup> Center for Research at the Bio/nano Interface, Department of Chemistry and McKnight Brain Institute, University of Florida, Gainesville, Florida 32611-7200 USA

<sup>2</sup> Biomedical Engineering Center, College of Chemistry and Chemical Engineering, Hunan University, Changsha, Hunan, 410082, China  
\*e-mail: tan@chem.ufl.edu

**Current Opinion in Chemical Biology** 2004, **8**:547–553

This review comes from a themed section on Analytical techniques  
Edited by Renato Zenobi and Fred Regnier

Available online 21st August 2004

1367-5931/\$ – see front matter  
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DOI 10.1016/j.cbpa.2004.08.010

## Abbreviations

**FRET** fluorescence resonance energy transfer  
**MB** molecular beacon  
**MBA** molecular beacon aptamer  
**RET** resonance energy transfer

## Introduction

In the post-genomic and proteomic era, a continued demand exists for biomolecular recognition probes with high sensitivity and selectivity for use in quantitative studies of genomic and proteomic information. These are particularly important for fundamental biomedical studies, disease diagnosis and drug discovery. Since the first report in 1996 [1], molecular beacons (MBs) (shown in Figure 1) have become a class of DNA probes that is widely used in chemistry, biology, biotechnology and medical sciences for biomolecular recognition [2–8], due in part to their ease of synthesis, unique functionality, molecular specificity and structural tolerance to various

modifications. In the traditional format, MBs act like switches that are normally closed — or ‘off’. Binding induces conformational changes that open the hairpin and, as a result, the fluorescence is turned ‘on’. The 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 resonance energy transfer (RET) [1,9]. Once the single stranded loop portion of the MB hybridizes to the target, the stem melts and the resulting spatial separation of the fluorophore from the quencher leads to an enhancement in fluorescence signal.

MBs can be used in a variety of applications where studies were initially not possible. These have included solution-based RNA–DNA interaction investigations, living systems measurements, biosensor designs, as well as protein–DNA interaction studies. While the basic function of MBs is similar for most of the bioanalytical methods that employ them, each MB is individually tailored to meet the needs of the application. Because of the unique structural and thermodynamic properties of MBs, these probes offer several advantages for biotechnology method development, one of which is their ability to differentiate between two target DNA sequences that differ by as little as a single nucleotide [10,11]. This degree of molecular specificity is very advantageous in a variety of bioanalytical applications, such as real-time PCR monitoring [11,12], DNA array development [13,14] and DNA analysis. Another advantage of MB probes is their inherent signal transduction mechanism. With appropriate fluorophore and quencher pairs and MB designs [15], very high signal-to-background ratios of more than 200 are possible when binding occurs under optimal conditions [9]. The signal transduction of MBs also enables the detection of target DNA in situations where it is not possible or desirable to isolate the probe–target hybrids from an excess of the unhybridized probes. These characteristics make them particularly attractive to biologists as well as chemists for bioanalytical applications. While there have been many interesting applications and fundamental studies on MBs, this review focuses on the recent bioanalytical applications of MBs, mainly the aforementioned four areas.

## *In vitro* RNA and DNA monitoring

One common use for MBs has been for real-time monitoring of DNA or RNA amplification during PCR. Because the non-hybridized MB has minimal fluorescence, the increasing fluorescence signal after each cycle is representative of the increasing concentration of the amplified sequence. MBs have also provided an



enables real-time monitoring of the ligation reaction and its kinetics in homogeneous solutions. Given the generality, simplicity, speed and sensitivity of such approaches to monitor DNA cleavage and ligation mechanisms, these methodologies could be easily adapted to a high-throughput format and become a new method of choice in modern drug discovery to screen for novel protein-based or small-molecule-derived DNA cleavage agents.

### Biosensing applications

While solution applications of MBs have been widely applied, their use in biosensor development has become popular as well. Various applications of surface-immobilized MB sensors have been described including miniaturized hybridization assays developed with positional separation in fiber optic bundles [19] and microarrays [12–14]. Using commercially available aldehyde-coated glass slides, the detection of *Francisella tularensis* has been accomplished with amine functionalized MB in a microarray format [20]. The array demonstrated characteristic MB specificity toward complementary oligonucleotide sequences, single-nucleotide mismatch sequences and multiple nucleotide mismatches. In human disease diagnostics, gene expression studies, and gene profiling, MB-immobilized magnetic nanoparticles, or genomagnetic nanocaptors, have been developed to collect, separate and detect trace amounts of DNA or RNA molecules with one single-base difference [21]. As a result, this newly developed technique could be useful for a variety of sample sources in forensic, medical and biotechnological fields.

Although the majority of surface immobilized probes incorporate a single dye-based quencher molecule into the signal transduction mechanism and the material to which the probe is attached serves only a passive role, some attention has been paid to developing alternative approaches. Gold offers particularly attractive molecular characteristics and has been extensively used in biotechnology and nanotechnology for signal transduction purposes [22]. At the core of these approaches is a gold surface that has the ability to quench fluorescent molecules. By immobilizing a fluorophore-functionalized hairpin onto a gold surface, in the absence of target, the fluorescence is quenched due to the close proximity of the fluorophore to the gold surface [23]. With improvements, these methods hold promise in the area of microarray technology because of the surface enhancement capabilities of roughened metal substrates.

In most biosensor applications, the sensitivity of the MBs immobilized on a silica surface is usually low. New MB probes have been designed to enable a larger separation between the surface and the surface-bound MBs by adding a poly-T linker at one stem end of the MB probe [24]. Using these MB probes, a DNA array has seen limited improvement in analytical sensitivity. On the

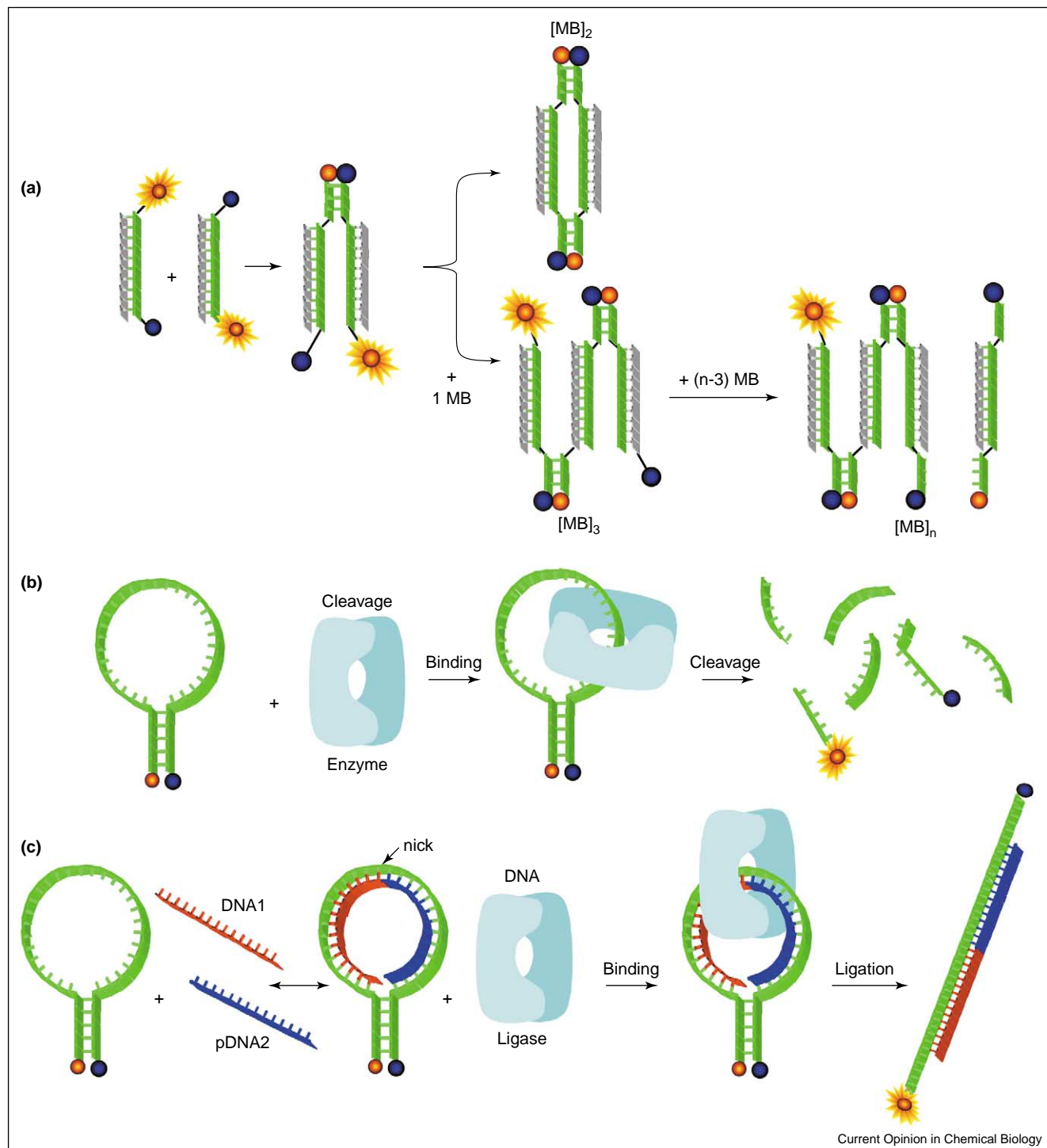
other side, ways to improve MB biosensing capability are under current investigation. For example, the reaction kinetics of single MB molecule on a liquid–solid interface has been studied to better understand how an MB is hybridized on a surface [25]. As shown in Figure 3, single MB molecules were imaged during the hybridization to individual cDNA probes. Among 400 MB DNA probes that were tracked, 349 MBs (87.5%) were hybridized quickly and showed an abrupt fluorescence increase, whereas 51 probes (12.5%) reacted slowly, resulting in a gradual fluorescence increase. This ratio stayed about the same when varying the concentrations of cDNA in MB hybridization on the liquid–surface interface. Statistical data of the 51 single-molecule hybridization images showed that there was a multi-step hybridization process. The results enabled a better understanding of DNA hybridization processes using single molecule techniques, which will improve biosensor and biochip development.

### Gene monitoring in living systems

The ability to detect and quantitate changes in gene expression, especially in real-time and with a degree of sensitivity suitable to monitor minor changes at a single cell level, will have considerable value in functional genomics. RNA analysis within a living cell has proven to be a tremendous challenge to biologists. With the rise of functional genomics, our fundamental understanding of RNA synthesis, transport pathways, and subcellular localization is increasingly important. Real-time measurements of mRNA *in vivo* and *in vitro* have allowed new directions to be investigated.

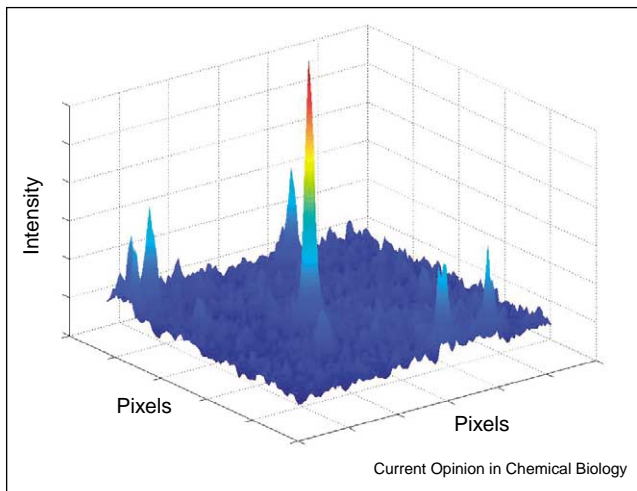
MBs have been reported for the detection of basic fibroblast growth factor mRNA in human trabecular cells [26], human c-fos mRNA in transfected Cos7 cells [27], and  $\beta$ -actin mRNA in K562 human leukemia cells [28] and in PTK2 cells [29]. These early studies have shown the promise of MB's applicability in monitoring genes in living systems, but the results were mostly preliminary. Successful implementation of MBs for mRNA detection depends on a combination of rational sequence design, efficient probe insertion into the cell, and optimized fluorescence imaging conditions. It is also important to choose an appropriate portion of the mRNA target sequence that will be accessible to the probe and a loop sequence and will be non-complementary to other possible interfering sequences. This is often accomplished using software to predict secondary structures of the mRNA sequences, the NCBI Gene Bank, and intuitive engineering. Synthesizing several MBs for a single mRNA is frequently done. It is expected that not all of the MBs will yield fluorescence enhancements when testing *in vitro* and *in vivo*. Computer programs that predict secondary structures of DNA are also useful for optimizing the MB design. An excel algorithm has recently been developed to aid in MB design by

Figure 2



A wide variety of sensitive assays can be developed based on the molecular beacon structural conformation changes in the presence of both proteins and nucleic acids. **(a)** MBs can exhibit a substantial amount of intermolecular interactions as a result of sticky-end pairing of the MB stems in the presence of target nucleic acids. Two complementary sticky ends from two MB hybrids can pair to form a short double helix, leading to association of the two hybrids at one end. These two MB hybrids can form a closed structure,  $[MB]_2$ , by pairing the other two sticky ends or polymerizing into a multimolecular structure,  $[MB]_n$  ( $n > 3$ ), by pairing with more hybrids. With sticky-end pairing, fluorophore (orange circle) and quencher (blue circle) are drawn together again, causing fluorescence quenching. **(b)** DNA cleaving enzymes, or nucleases, can also be monitored by using the ability of the enzymes to cut the MB into short sequences. Eventually, the enzyme cleaves the stem sequence and destabilizes the hybrid in such a manner to restore the fluorescence of the fluorophore. **(c)** Ligase activity can also be monitored, where the MB is

Figure 3



Single molecular beacon DNA hybridization at a liquid–solid interface. Individual MB DNA probes and their hybridization have been followed at an interface using fluorescence imaging microscopy. Each one of the major peaks represents one individual molecular hybridization event.

generating a series of possible MB sequences based on a 20 base loop [30]. Once an MB has been found to yield an appropriate response, it can be inserted into the cell by means of liposome delivery or by direct microinjection. Both of these methods have been demonstrated to maintain the physiological and structural integrity of the cells.

Recently, strategic MB and experimental design resulted in the visualization, distribution and trafficking of mRNA in *Drosophila melanogaster* oocytes [30]. DNA and peptide nucleic acid MBs were also successfully used to detect rRNA in solution and cells [31]. Solution-based DNA analysis assays have been demonstrated and are promising for intracellular measurements. The first example is a reversible MB design that can be used to determine the concentration of single-stranded DNA in solution by a ratiometric fluorescence measurement caused by fluorescence resonance energy transfer (FRET) [32]. Also, two MB beacons used simultaneously to hybridize adjacent regions of the same target, resulting in FRET, has also been investigated [30]. These two approaches reduce false-positive signals in detecting target nucleic acids because of an increased signal-to-background ratio compared with that seen in single MB assays. The dual FRET MBs provide an interesting technique for sensitive RNA detection and quantitation in living cells.

Intracellular measurements using fluorescent intensity often do not yield reproducible results. Fluorescent ratiometric measurements can be better. To solve this problem, the microinjection of two probes, one MB probe for the target sequence and one reference probe for optical signal calibration, proves to be more reproducible, reliable and quantifiable for the target nucleic acids in the cytoplasm of single living cells. With these new approaches for MB intracellular measurements, it is possible to more effectively and reliably monitor and quantify in real-time mRNA inside living cells, as shown in Figure 4.

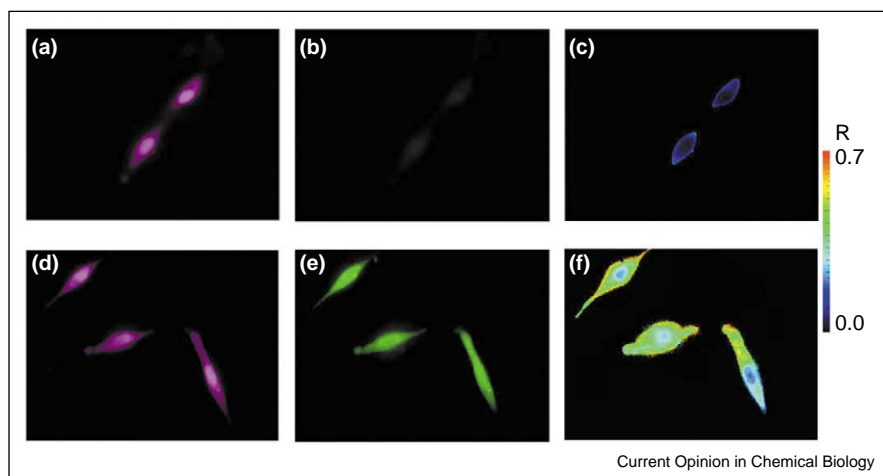
### Protein study

Although MBs were originally designed to bind and recognize specific nucleic acids, these probes can also lead to increased fluorescence upon binding to certain proteins. The protein recognition ability of MBs was first recognized with an *E. coli* single-stranded DNA-binding protein [33]. These results demonstrated that while MBs are sensitive and somewhat selective to DNA-binding proteins, they are not specific enough to be capable of distinguishing a particular protein, and a more selective protein recognition mechanism is required.

The combined sensitive signal transduction mechanism of MBs with the specific protein-binding capability of aptamers results in a novel class of analytical probes termed molecular beacon aptamers (MBAs) [34]. Aptamers are single-stranded DNA or RNA molecules, generally 25–60 nucleotides in length, that have been selected in a process termed SELEX from a combinatorial library by their ability to bind a specific target [35–37]. The MBAs developed to date can be described by three general classifications: FRET fluorophore/quencher pairs, FRET donor/acceptor pairs, and single labeled anisotropy probes. An example of a FRET-based quenching MAB is the one developed using the known and well-studied thrombin aptamer. When bound to thrombin, the aptamer exists primarily in its quadruplex form containing two G-quartet structures, but in free solution, it can adopt either conformation, depending in part on the ionic strength and temperature. This conformation shift provides the basis for the MBA. By labeling the two ends of the aptamer with a fluorophore and quencher pair, aptamer binding of thrombin forces the quencher adjacent to the fluorophore, resulting in a substantial decrease in fluorescence. Similarly, a donor and acceptor fluorophore can be positioned on either end to create a two fluorophore FRET probe [34,38]. Binding of target protein can be detected by monitoring the fluorescence of F2 directly or preferably by the ratio of fluorescence of F2/F1. The

(Figure 2 Legend continued) designed to be complementary to the ligated product. Initially, DNA1 and pDNA2 (5' phosphorylated DNA) can individually bind to the probe, creating two adjacent bases that are not connected by a phosphodiester bond, or a nick. As a result of the two sequences binding to the MB, the fluorescence slightly increases. Once the nick is formed, the DNA ligase can bind and join the two target sequences. The full complementary strand then has the ability to fully restore the fluorescence of the MB.

Figure 4



Intracellular imaging of single cells using MB DNA probes. A ratiometric approach has been used to minimize experimental variations and enable more reliable data collection. On the top row are the cellular response for 'closed' MBs. On the bottom row are the cellular responses for 'open' MBs. (a) and (d) are the fluorescence emission images of a reference probe. (b) and (e) correspond to fluorescence emission images of the MB probe response. (c) and (f) are representative ratiometric images of the MB responses by dividing the image from the MB by the image of the reference probe.

two fluorophore approach also enables the use of ratiometric imaging measurements. As a result, a much improved signal to background ratio and therefore much higher sensitivity in protein imaging are obtained. The third class of molecular aptamer probes is based on monitoring the change in fluorescence anisotropy upon protein binding [38–40]. Anisotropy probes are constructed by labeling an aptamer with a single fluorophore. When the labeled aptamer is bound with its target protein, the rotational motion of the fluorophore becomes much slower as a result of the larger molecular weight of the aptamer–protein complex. This results in a significant increase in fluorescence anisotropy that can be monitored using plane-polarized light. One particular advantage of anisotropy probes is that they are more universal than the FRET-based probes. With further development, MBAs are expected to be useful as intracellular protein recognition agents to probe proteins in different environments and to monitor protein–DNA/RNA interactions.

### Conclusions

With the completion of the human genome project, there has been a rapid shift in focus from simply collecting and archiving genomic data to utilizing genetic analysis for prediction and discovery. The development and utilization of new quantitative tools for research across disciplinary interfaces will prove vital in achieving these objectives. MBs are ideally suited for and hold great promise in genomics and proteomics. More research is expected for three areas. First, MB application in mutation detection for a variety of disease diagnostics and disease mechanism studies. Gene expression under different conditions will

also be studied with precise quantitation. The extraordinary target-specific capability along with the availability of different fluorophore–quencher pairs makes MB probes extremely useful for multiplex analysis applications as well. These excellent properties also make MBs exquisitely suited for use in the identification of genetic alleles or particular strains of infectious agents. Second, MBAs are in the early stages of development, but their potential will be further explored, especially in their application to proteomics. As the first target in cell-based aptamer selection, cancer proteomics will prove to be an interesting area to investigate. A third area is the improvement of MB design. There are three important areas where improved MB design must improve before many more practical applications of MBs and MBAs become easily accomplishable with unique features and important advantages: first, MBs should be made with lower background signal and higher signal enhancement, especially for surface immobilizable MBs in biosensor and biochip applications; second, MBs should be made more robust against components of the cellular environment such as nuclease enzymatic cleavage; thirdly, MBs should be made with multiple functions to realize recognition and then functionalization. All of these developments will open the possibility of using easily obtainable and designer DNA molecules for genomics and proteomics studies, for molecular diagnosis of diseases, for molecular therapy and for new drug development.

### Acknowledgements

This work was partially supported by NIH grants, by an NSF NIRT Award and by National Key Basic Research Program (2002CB513100) of China Natural Science Foundation.

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