Selective probing of mRNA expression levels within a living cell

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We report on a selective and nondestructive measurement of mRNA (messenger ribonucleic acid) expression levels within a living cell. We first modify an atomic force microscope tip to create a tapered nanoscale coaxial cable. Application of an ac (alternating potential) between the inner and outer electrodes of this cable creates a dielectrophoretic force attracting mRNA molecules toward the tip-end which is pretreated with gene specific primers. We selectively extracted and analyzed both high (~ 2500) and extremely low (10^1) copy number mRNA from a living cell mRNA in less than 10 s. © 2009 American Institute of Physics. [DOI: 10.1063/1.3213343]

Atomic force microscopes (AFMs) have been used for both injecting¹ and manipulating^{2–5} molecules. We present initial results on the use of an AFM for determining very low and high copy number messenger ribonucleic acid (mRNA) expression levels within a living cell. The technique is based on the use of a modified AFM probe—a dielectrophoretic nanotweezer (DENT)—for attracting mRNA molecules toward its end. It gives us the flexibility to extract mRNA from within compartments of a single cell—in our case the nucleus. The DENT can be viewed as a nanoscale, tapered, coaxial cable integrated into an AFM probe. Application of an ac electric field between the inner and outer electrodes of the DENT creates a large electric field gradient at the probe tip resulting in a dielectrophoretic attractive force on mRNA molecules. Selective mRNA extraction was achieved by combining the dielectrophoretic attractive force with chemical derivatization of the probe surface—using gene specific oligonucleotide primers tailored to hybridize specific target mRNAs of interest.

DENTs were built using commercially available conical, highly doped (resistivity 4–6 Ω cm) silicon AFM probes ($k \sim 1.5$ N/m). We start by growing a 20 nm thick layer of thermal SiO₂ on the AFM probes. This serves to electrically insulate the entire silicon probe including the AFM cantilever and handling chip. In the second step, we e-beam evaporate a 10 nm titanium adhesion layer followed by a 20 nm film of platinum onto the side of the cantilever that contains the probe tip. In the final step, we polish the end of the Pt coated tip until the doped silicon tip is just exposed. The last step was performed by scanning the tip in contact with a flat sapphire surface for about 5 min using a loading force of 7.5×10^{-7} N in the AFM setup. Figure 2(a) shows the scanning electron micrograph of the tip in its final form.

Chemical modification of the probe surface was achieved by immobilizing gene specific primers complementary to the mRNA of interest using standard biotin-streptavidin chemistry.⁶ First the cantilevers were cleaned using solvents for 5 min and air dried. Next, the samples were transferred to an ultraviolet (UV) chamber and irradiated for 60–75 min. A mixture of 5% [aminopropyltriethoxysilane

(APTES)] in ethanol was prepared, the samples were immersed in the APTES solution for 3 h, then washed in 100% ethanol solution and air dried. Samples were then incubated with 10 mM of EZ-Link Sulfo-NHS-LC-Biotin (sulfo-succinimidyl-6-[biotin-amido]hexanoate) from Pierce, Rockford, IL, USA) for 45–60 min at room temperature. The biotininated substrates were then incubated with 100 mM Streptavidin (Sigma, St. Louis, MO, USA) solution for 60–75 min at room temperature. After incubation, the samples were washed with 1X phosphate buffered solution (pH 7.4, Fisher, Pittsburgh, PA, USA). Finally, the samples were incubated with the appropriated single strand deoxyribonucleic acid (ssDNA) primers for specific mRNA capture. The incubation was done at 4 °C overnight and then washed with 1X tris-acetate-ethylenediaminetetraacetic acid (EDTA) buffer (Sigma, St. Louis, MO, USA).

mRNA expression experiments were performed by inserting the modified AFM probes into the nucleus of the cell, applying a 120 KHz, 5 V peak-to-peak (pp) ac voltage signal across the inner and outer electrodes of the DENT for a duration of 60–75 s, extracting the probe tip with the captured mRNA, melting the captured mRNA, and finally analyzing it using gel electrophoresis or quantitative polymerase chain reaction (qPCR). Figure 1 illustrates the complete sequence.

Single cell mRNA expression experiments were performed using rat fibroblast cells (*Rattus norvegicus*) and cultured following the procedures described in Ref. 4. Three mRNA populations were analyzed; a 125 bp portion of β -actin [forward primer 5'-AGA GAA GCT GTG CTA TGT TGC CCT-3' and β -actin reverse primer 5'-ACC GCT CAT TGC CGA TAG TGA TGA-3']. Biotinilated 5'-/5Biosg/ACC GCT CAT TGC CGA TAG TGA TGA-3' DNA was attached to the Si substrate as the mRNA binding primer.], a 141 bp portion of GAPDH (glyceraldehyde 3-phosphate dehydrogenase) [forward primer 5'-TGA CTC TAC CCA CGG CAA GTT CAA-3' and reverse primer 5'-ACG ACA TAC TCA GCA CCA GCA TCA-3']. Biotinilated 5'-/5 Biosg/TGA CTC TAC CCA CGG CAA GTT CAA-3' DNA was attached to the Si substrate as the mRNA binding primer.] and a 166 bp portion of HPRT (hypoxanthine phosphoribosyltransferase) [forward primer 5'-AAG AGT CCT GTT GAT GTG

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FIG. 1. (Color online) (a) AFM modification to accommodate gene specific primers. (b) Insertion of the tip into the nucleus of a cell. (c) Target mRNAs are hybridized on to the probe. (d) Tip is withdrawn from the cell. (e) Melting of the hybridized mRNA. (f) Quantification of extracted mRNA.

GCC AGT-3' and reverse primer 5'-CTG CCT ACA GGC TCA TAG TGC AAA-3'. Biotinilated 5'-/5Biosg/CTG CCT ACA GGC TCA TAG TGC AAA-3' DNA was attached to the Si substrate as the mRNA binding primer.]

AFM tips were prepared and the appropriate ssDNA-mRNA binding primers corresponding to the mRNA to be extracted were immobilized. Rat fibroblast cells were cultured on regular glass microscope slides ($2 \times 2 \text{ cm}^2$). The AFM tip was moved to a randomly selected cell on the slide; it was operated in the contact mode so that contact with the cell membrane could be easily detected through a change in cantilever deflection amplitude. Once contact was established, a short voltage pulse was applied to the z -piezo in order to penetrate the cell membrane. A similar procedure was followed for penetrating the nuclear membrane. Once the probe was inserted into the nucleus, 1 ms duration, 120 kHz, and 5 V pp voltage bursts at 50% duty cycle were applied across the inner and outer electrodes of the DENT. This facilitates many encounters of the target molecules with the probe surface. After 60–75 s, the ac voltage was turned off and the tip was removed from the cell. The probe was then immersed in de-ionized water in ice for 45 min in order to release the captured mRNA into solution. The iScript cDNA (complementary deoxyribonucleic acid) Synthesis Kit (Bio RAD, Hercules, CA, USA) was used to make cDNA from the isolated mRNA molecules. Finally, the cDNA was used to perform qPCR analysis of the samples. cDNA was prepared according to the instructions of iScript cDNA synthesis kit. For real-time qPCR cycling, SYBR green was used as the probe in the Chromo4 qPCR system from Bio-RAD. The following cycling protocol was used: 40 cycles of 94 °C for 15 s, 57 °C for 30 s, and 72 °C for 45 s for each mRNA. A melt curve was generated by increasing the temperature from 45 to 95 °C and holding for 30 s after each 1° temperature increment.

To gain a better understanding of the parameters for molecular extraction using the dielectrophoretic force probe, we performed a series of two-dimensional (2D) field and force calculations using finite element analysis (COMSOL AB). We computed the potential distribution by solving the 2D electrostatic problem. The outer Pt layer of the tip was kept at ground potential while the inside Si probe was kept at 10 V. The electric fields and their gradients were then derived from

FIG. 2. (Color online) (a) Scanning electron microscope image of structurally modified tip (top view). (b) 2D finite element simulation showing the capture range for molecules. (c) Calculated $(\nabla|E|^2)$. [(d)–(f)] Time lapse images showing the attraction of Cy5 labeled BSA molecules in 1X PBS solution. Scale bars indicate 200 nm.

the calculated potential distributions. In order to estimate the capture range during mRNA extraction, we applied the condition that molecules satisfying $[(\alpha E^2)/2] \geq 100kT$ will be attracted toward the probe. Here, α is the dielectric polarizability of the mRNA molecules, E is the applied electric field, k is Boltzmann constant, and T is the absolute temperature of the surrounding medium. Figure 2(b) illustrates the capture range at $T=300 \text{ K}$ for 2000 base length mRNA molecules within the cell (approximate value for $\alpha=8.94 \times 10^{-30}$).⁸ Figure 2(c) shows $(\nabla|E|^2)$ with the DENT inserted within the cell. A large gradient is present at the end of the probe as expected. Since the dielectrophoretic force given by $\vec{F}=[(\nu\alpha)/2]\vec{\nabla}|E|^2$ (Ref. 7) where ν is the volume of the particle, α is the polarizability, and E is the external applied electric field, we note that mRNA molecules are preferentially attracted toward regions of high $(\nabla|E|^2)$ —i.e., toward the interface between the Si and SiO₂ in our case as illustrated in Fig. 2(c).

Initial experiments were focused on attracting fluorescently (Cy5) labeled bovine serum albumin (BSA) molecules. The probe was immersed in a solution of BSA molecules, an ac voltage (5 V pp at 120 KHz) was applied across the tweezers electrodes and the molecules were observed in real time using an Olympus fluorescence confocal microscope. Figures 2(d)–2(f) show a time sequence of fluorescence images following the application of the ac voltage. We clearly observe BSA molecules being attracted toward the highest gradient regions of the probe tip.

In our first series of experiments we extracted β -actin mRNA from a single living cell. The DENT tips were chemically modified. The cells were grown on microscope slides, mRNA extracted and cDNA generated followed by the qPCR quantification reaction. Figure 3(a) shows the fluorescence intensity of the sample as a function of cycle number. We performed two separate sets of experiments. In the first set we applied the ac electric voltage pulse sequence as described earlier. Detectable fluorescence intensity was consistently observed after the 20th cycle and leveled off after the 35th cycle. The second set of experiments was performed without the application of the ac electric voltage pulse sequence. Detectable fluorescence intensity was observed only after the 26th cycle [see Fig. 3(a)]. From these results we conclude that the application of the ac electric voltage pulse sequence increases the molecular collection efficiency by approximately 100 fold. To verify that we are indeed detecting β -actin mRNA, we performed both a melt curve analysis and

FIG. 3. Capturing high (β -actin, ~ 2500), medium (GAPDH, 1500) to extremely low copy number (HPRT, ~ 10) mRNA. (a) Figure indicates fluorescence vs cycle number for two separate experiments: field on and field off for β -actin mRNA. (b) Melt curve and gel electrophoresis (inset), of samples used in (a) observed using 100 bp ladder. (c) Detection GAPDH and HPRT; figure shows the fluorescence vs cycle number for 10-fold dilutions of GAPDH and HPRT. (d) Melt curve analysis and gel electrophoresis (inset) for HPRT.

gel electrophoresis. Figure 3(b) shows the melt curve. Note that a single peak was observed as expected. The inset shows the gel electrophoresis analysis; note that one β -actin band at about 125 bp (between the 100 and 200 bp) was observed for both samples.

We also performed similar experiments where a cw voltage of 5 V pp at 120 KHz was applied across the tweezers electrodes instead of a pulse sequence to collect the mRNA molecules of interest. Although one might expect that many repeated encounters with the chemically modified probe surface would increase the collection efficiency of a specific target mRNA, surprisingly, we obtained similar collection efficiencies in both cases. This is probably due to the fact that our current probes have large enough surface area to attract and sample a range of mRNA molecules from within the nucleus without masking the surface and preventing the hybridization of the target molecules. All subsequent experiments were therefore performed using a cw voltage across the DENT.

In the second and third series of experiments, we explored the validity and limits of our technique by extracting GAPDH and HPRT mRNA, respectively. In these experiments, we made 10-fold dilutions of the cDNA and used it for qPCR analysis. Figure 3(c) shows the results. We ob-

served equally spaced fluorescence versus cycle plots for GAPDH as expected. For HPRT however, detectable fluorescence was only observed at the highest concentration level of cDNA. Figure 3(d) shows the melt curve and gel electrophoresis analysis of the samples that contained HPRT. A single peak was observed in the melt curve as expected. Gel electrophoresis showed a 166 bp HPRT band. After each experiment, cell viability was confirmed using the standard Trypan blue test.

In summary, we have demonstrated a technique for the extraction and analysis of a range of mRNA species from a single living cell. As compared with other technologies,^{9–11} the approach presented here is highly specific to the type of mRNA being analyzed and, therefore, much more sensitive. It is fast, simple and nondestructive and does not require cell lysing, purification of mRNA from the contaminants. Our technique would enable one to monitor the mRNA expression within a single cell as a function of time in response to various stimuli. We believe that the technique will have a myriad of application areas ranging from systems biology to cancer research.

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¹D. Nawarathna, K. Unal, and H. K. Wickramasinghe, *Appl. Phys. Lett.* **93**, 153111 (2008).

²K. Unal, J. Frommer, and H. K. Wickramasinghe, *Appl. Phys. Lett.* **88**, 183105, (2006).

³M. B. Viani, L. L. Pietransanta, J. B. Thompson, A. Chand, I. C. Gebeshuber, J. H. Kindt, M. Richter, H. G. Hansma, and P. K. Hansma, *Nat. Struct. Biol.* **7**, 644 (2000).

⁴T. Osada, U. Hironori, K. Hyonchol, and I. Atsushi, *J. Nanobiotechnology* **1**, 2 (2003).

⁵H. Uehara, Y. Kunitomi, A. Ikai, and T. Osada, *J. Nanobiotechnology* **5**, 7 (2007).

⁶G. Barbany, A. Hagberg, E. Waldenström, and U. Landegren, *Biomol. Eng.* **16**, 105 (1999).

⁷H. A. Pohl, *J. Electrochem. Soc.* **155**, 155C (1968).

⁸A. Ajdari and J. Prost, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 4468 (1991).

⁹T. Nolan, R. E. Hands, and S. A. Bustin, *Nat. Protoc.* **1**, 1559 (2006).

¹⁰J. Eberwine, H. Yeh, K. Miyashiro, Y. Cao, S. Nair, R. Finnell, M. Zettl, and P. Coleman, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 3010 (1992).

¹¹J. S. Marcus, W. F. Anderson, and S. R. Quake, *Anal. Chem.* **78**, 3084 (2006).