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Targeted mRNA Profiling of Transfected Breast Cancer Gene in a Living Cell

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Abstract

Selective mRNA profiling of transfected breast cancer gene expression in a living cell is demonstrated. Atomic Force Microscope (AFM) probe tips are structurally modified to create a dielectrophoretic force that attracts mRNA molecules within the cell nucleus. The tip end is chemically modified to hybridize only to the target mRNA from a pool of molecules within the nucleus. We successfully combined this scheme with standard assay techniques to develop an assay technology that can be used for early disease detection and basic studies in cell biology.

Keywords

mRNA; Atomic Force Microscope; Dielectrophoretic force

Reverse Transcription-Quantitative Real Time Polymerase Chain Reaction (RT-qPCR) and micro-array analysis are the gold standards for gene profiling [1,2,3]. These techniques synthesize cDNA from collected mRNA sample using a reverse transcription reaction. Their success relies on the ability to obtain fresh and pure RNA samples. Degraded RNAs or RNAs with impurities produce degraded cDNA. Even with freshly prepared, intact RNA, contaminants in the samples (e.g. phenol or CsCl₂) can interfere with the enzymatic reactions. Some contaminants that are co-purified with RNA may introduce unwanted products during downstream applications (e.g. contamination with genomic DNA could introduce an unwanted background signal in a RT-qPCR assay)[1]. In addition, rRNA that constitutes more than 80% of total RNA may compete for reagent in the cDNA preparation reaction [1,4]. There is a need for clean, highly sensitive techniques for non-destructive quantitative analysis of mRNA levels within living cells.

Atomic Force Microscope (AFM) techniques have recently been explored for several single cell manipulations. For example, the introduction of Bovine Serum Albumin molecules into cells by local electroporation [5] and the detection of β -actin molecules (copy number 2000-3000) adsorbed on AFM probes inserted into living cells; although very interesting, these experiments were limited to high copy number detection since the AFM probe picks up all molecules that are non-specifically and randomly adsorbed to its surface [6].

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We have developed a selective and ultra fast method to extract mRNA molecules from a single living cell down to very low copy numbers (10 molecules per cell). The technology builds on the Atomic Force Microscope (AFM) platform. We modified the structure of a commercially available AFM probe tip to accommodate the electric field gradients necessary for extraction of the mRNA molecules through dielectrophoresis. Target-specific short mRNA capture primers (20-30 base pairs long) were immobilized on the modified AFM probes using Biotin-Streptavidin chemistry [7]. Cells were either plated on regular glass cover slides, micropallet arrays (MPA) or deposited on cover slides after trypsinization. Upon inserting the modified probe tip into a selected single living cell, the target mRNA was captured by generating a near-field dielectrophoretic force at the end of the tip, to attract and hybridize target mRNA onto the tip. Following hybridization, the tip is withdrawn from the cell, and the extracted mRNA is collected and analyzed using conventional RT-PCR.

We performed computer simulations to determine the strength and distribution of the dielectrophoretic force on ssDNA molecules as shown in Figure 1(c) [7] ; note that molecules are attracted towards the structurally and chemically modified area of the tip where capture primers are immobilized.

We modified commercially available AFM tips ($k \sim 1.5\text{N/m}$, tip length $\sim 10\text{ }\mu\text{m}$, end curvature $\sim 20\text{nm}$ and $\sigma \approx 0.001\text{ }\Omega\text{-cm}$ from Veeco probes, model: 1930-00) in the following manner. A 20nm thick layer of SiO₂ was grown in a conventional oxidation furnace (60 min at 900° C) to electrically insulate the entire probe tip surface including the AFM cantilever. 10nm of Ti and 10 nm of Pt were e-beam evaporated on the oxidized tips. The Pt coated tip end was then sectioned until it barely exposed the SiO₂. Figure 1(b) shows the final version of a tip after processing. In a second step, we chemically modified the AFM tip and cantilever surface with a 3-Aminopropyltriethoxysilane (5% APTES for 3 h) film followed by biotinylation for 45 min at room temperature. The biotinylated tips were incubated with 100mM streptavidin (Sigma, St. Louis, MO, USA) solution for 60-75 min at room temperature. Finally, the samples were incubated with the appropriate biotinylated ssDNA primers for mRNA capture (4°C overnight). We verified that the surface chemistry was optimized to immobilize target mRNA capture primers onto the SiO₂ coated Silicon tips, by performing a separate test to immobilize fluorescently labeled DNA primers (24 bp) onto a planar SiO₂ coated silicon substrate.

Figure 1 (a) shows the schematic of our set-up for mRNA extraction. We employed an integrated Confocal Optical and Atomic Force Microscopy system (MFP-3D-CF, Asylum Research, Santa Barbara, CA). Cells were cultured on regular glass microscope slides (2'x2') or MPA and mounted on the microscope stage (Olympus America, Center Valley, PA) for experiments. Cell selection was achieved by observing the cell sample population through a 20X objective lens. The modified AFM tip was approached toward the surface of a randomly selected cell by operating the AFM in contact mode (see Figure 1(a)). The AFM probe was then inserted 2-3 μm into the cell while an AC electric field was applied to the tip. We used the force distance feature of the AFM software to determine when cell penetration occurred. The applied voltage was 5Vpp at 120 kHz. In all experiments, the tip was kept within the cell for 1-2 min with the field turned on. The field was then turned off and the tip retracted from the cell. Finally, the entire tip was immersed in DI water (at 4°C) to dissociate the captured mRNA into solution. After each mRNA experiment cell viability was measured using the trypan blue test [7].

The iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) was used to synthesize cDNA from the isolated mRNA molecules. A reaction volume of 20 μl was prepared using 4 μl of 5x iScript Reaction Mix, 1 μl of iScript Reverse Transcriptase and 15 μl of mRNA template.

The reaction sample was incubated in a thermocycler for 5 min at 25°C, 30 min at 42°C and 5 min at 85°C. Freshly made cDNA was used in qPCR experiments. qPCR analysis was performed from the cDNA in the following manner: Three 10-fold dilutions (X, X/10, X/100, X/1000) with 3 replicates of each cDNA sample were used in all experiments. For real-time qPCR cycling, SYBR green was used as the double-stranded DNA intercalating dye in the Chromo4 qPCR system from Bio-Rad. The following thermal cycling protocol was used: 40 cycles of 94°C for 15 sec, 57°C for 30 sec and 72°C for 45 sec for each cDNA dilution. A melt curve was generated by increasing the temperature from 45°C to 95°C and holding for 30 sec after each 1°C temperature change.

In our first experiment, we experimentally demonstrated our ability to capture mRNA molecules from Rat fibroblast cells (see supplementary information for details) with and without the application of a dielectrophoretic force. Two identical AFM probes with capture probes were used. We captured beta-actin mRNA both with and without the application of a dielectrophoretic force. cDNA was synthesized and qPCR was performed. Figure 2 (a) shows the qPCR results. Note that approximately a 100-fold improvement (based on C_t) was achieved by applying the dielectrophoretic force. Figure 2 (b) shows the melt curve analysis. One peak was observed as expected (from both samples). Finally we used some of the qPCR products to perform gel electrophoresis. qPCR products were electrophoresed in 2% agarose gel and visualized with ethidium bromide staining. We used a 100bp DNA ladder (New England Biolabs, Ipswich, MA) to determine the size of qPCR products. The operating conditions were 150V for 30 min and imaging was done in a Bio-Rad gel imaging system (Chemi Doc, Hercules, CA). Inset (Figure 2(b)) shows the gel electrophoresis data. The runs show a band at 125bp as expected from both samples. Negative control experiments were performed in parallel. Structural and chemical modifications of the tip were performed by following the same procedure as used for the positive control experiments. For negative controls, the AFM probes were immersed into fresh cell culture medium that did not contain any cells. qPCR data showed no fluorescence (data not shown).

For our second series of experiments we studied the capture of two mRNA molecules (GAPDH and Uts2r - Urotensin 2 receptor) using a single tip. The AFM probes were structurally modified and *two* different biotinylated capture primers were immobilized on a single tip (following the same protocol used to immobilize one capture primer). The target mRNAs were captured as before and cDNA was synthesized following the protocol mentioned in our first experiment. qPCR was performed separately for the two different cDNA reactions following the protocols used before. Figure 2(c) shows the qPCR data (fluorescent vs. cycle number) for GAPDH and Uts2r. Note that detectable fluorescence was observed at different cycle numbers for GAPDH ($C_t = 25$) and Uts2r ($C_t = 30$). Finally, we performed melt curve analysis for each sample and observed a single peak in each case (data not shown).

In the last series of experiments, we used our mRNA profiling technique to develop a new assay technology that could be useful in many areas of biology including stem cell research and diagnostics. In this experiment, we used a NIH 3T3 fibroblast cell line that was transfected with rat *neu*. We then mixed the 3T3 transfected cells with 3T3 wild type cells (1:1 ratio) to generate a bimodal distribution of cells. The experiment was divided into two parts. In the first part, the mixture of cells was applied to a specially prepared MPA that immobilizes the cells. Following immobilization, target cells were identified using a monoclonal antibody that recognizes an antigen on the cell surface of rat *neu* transfected target cells followed by a fluorescently tagged secondary antibody [see supplementary materials for details [8,9,10].

In the second part, rat *neu* transfected target cells were recognized by detecting the Alexa Fluor 488 fluorescence of MPA immobilized cells in our inverted microscope. Rat *neu* mRNA was extracted from the target cells (see Figure 1(d)). After extraction, the mRNA was dissociated into solution and cDNA was synthesized. The cDNA was then used in a qPCR reaction as described earlier. Negative control experiments were performed using wild type 3T3 cells. Figure 2(d) shows the fluorescence vs. cycle number for our target cells compared with the negative control experiments. A detectable fluorescence signal was only observed from the rat *neu* transfected target cells confirming that combining MPA with our mRNA extraction technique can be a powerful tool to selectively target and interrogate single cells from a complex mixture of cells.

In summary, we introduced a new technique for single cell mRNA assays of living cells. Initial experiments were performed using a single AFM probe tip. In principle one can extend the technique to an array of probes in order to profile mRNA expression levels in an array of 10,000 cells. Our profiling technique can be easily integrated into other well-established techniques such as fluorescent activated cell sorting (FACS) thereby providing a powerful tool for mRNA analysis of target cells (stem cells or diseased cells) at very low concentrations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Schematic representation of the experimental set-up used in mRNA extraction (not to a scale). (B) Scanning Electron Microscope (SEM) image of the modified tip. Note that inner Si and outer Pt are seen in the picture. An electric field is applied between inner Si and out Pt to establish a dielectrophoretic force. Scale bar is 100 nm (C) FEM of mRNA capturing force. Direction of the force is shown in arrows. Scale bar is 100nm. (D) Indicates the cells immobilized on the MPA for mRNA extraction. This is combination of two images (bright field image and corresponding fluorescence image). Note that target cells (rat *neu* injected cells) are fluorescently labeled. Scale bar indicates 10um.

Figure 2.

(A) qPCR results from β -actin mRNA samples collected with and without electric field. (B) Melt curve analysis and gel electrophoresis results (inset) from the two samples from part (A). (C) qPCR results from GAPDH and Utsr2 mRNA samples collected from a single cell. (D) Fluorescence vs. cycle number from samples that has rat *neu* mRNA positive and negative.