QUANTITATIVE MEASUREMENT OF CELL SURFACE EXPRESSION VIA MAGNETOPHORETIC CYTOMETRY

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ABSTRACT

Identification of membrane antigens and measurement of their expression within a cell population is of fundamental importance to medical and biological studies. In this work, we present a cytometry approach that is based on magnetophoresis and distributed Coulter sensing in a microfluidic system. Our magnetophoretic cytometer offers quantitative analysis of cell membrane antigens on a portable and disposable platform compared to conventional flow cytometers, which are complex, expensive and large systems. Our tests with human breast cancer cells show the utility of our microfluidic device and its potential as a point-of-care instrument for biomedical testing.

KEYWORDS

Microfluidics, antigen expression, magnetophoresis, flow cytometry, Microfluidic CODES

INTRODUCTION

Cell membrane antigens are routinely utilized as diagnostic and prognostic markers in biomedicine, hence their qualitative and quantitative analysis is widely sought in applications ranging from immunophenotyping to drug screening. Fluorescence-based flow cytometry is an established method for the characterization of these membrane antigens at the single cell level. Despite their well acknowledged utility, high cost, bulkiness and operational complexity of flow cytometers impede their adoption in environments with limited resources [1]. We previously introduced a projectile-based cytometry approach by coupling magnetophoresis with distributed Coulter sensing in a microfluidic device to electrically profile the surface expression in a cell population in a highly-portable and low-cost microfluidic platform [2]. While this device could quantify fractions of magnetically sorted cell subpopulations, it could not estimate the surface expression of individual cells.

In this work, we demonstrate an integrated microfluidic flow cytometer that quantitatively measures the surface expression of individual cells by electrically tracking the trajectory of magnetically sorted cells via integrated sensors [3, 4] and computational modeling of on-chip magnetophoresis. Our cytometry approach relies on the fact that magnetophoretic trajectory of an immunomagnetically labeled cell is a function of (1) its magnetic load, which is proportional to cell surface expression [5, 6], (2) its size due to Stokes' drag forces acting in the fluid, and (3) the properties of the magnetic components such as magnetic beads used in labeling and the external magnet.

DEVICE DESIGN

In our device (Figure 1), immunomagnetically labeled cells are driven into a free-flow magnetophoresis chamber, where they are differentially sorted under a magnetic field gradient generated by an externally positioned neodymium magnet. The cell population is introduced from a designated inlet, and 1X phosphate buffered saline (PBS) is supplied from a bifurcating inlet to create a uniform sheath-flow hydrodynamically focusing cells at the inlet. The magnetophoresis chamber leads to 8 uniformly spaced fluidic channels for electrical detection and quantification.

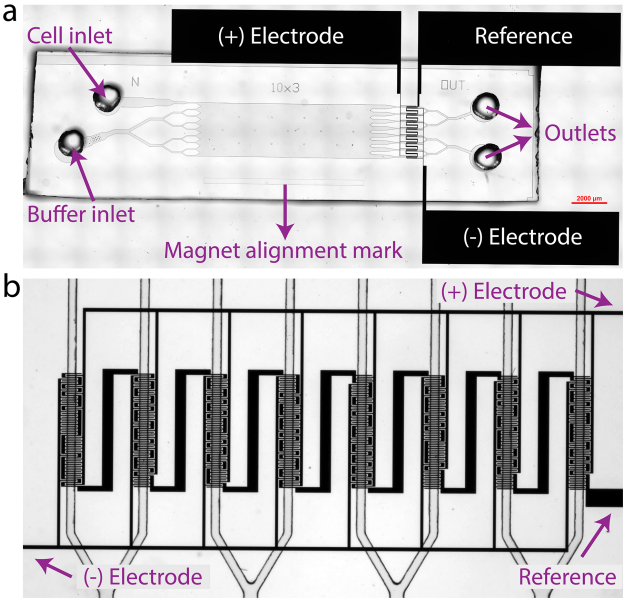

Figure 1: Images of our device. (a) Microscope image of the whole magnetophoretic cytometry chip. The neodymium magnet is later placed along the microfluidic channel using the photolithographically defined alignment marks. (b) Close up image of the sensor network. Each sensor in the network is encoded with a distinct 31-bit Gold sequence for multiplexed electrical readout.

To count cells sorted into each outlet, we embedded a network of code-multiplexed Coulter sensors [3, 4] into our device, allowing us to electrically determine the sorted cell distribution from a single electrical waveform. The sensors were formed using 3 electrodes: a reference electrode for excitation, a positive sensing electrode representing a "1", and a negative sensing electrode representing a "0". Each sensor was designed to produce a distinct 31-bit Gold code [7, 8] that is orthogonal to the codes produced by the other sensors in the device. Besides the identity of the fluidic channel that cells were sorted into, the electrical waveform provides the size of each cell from the amplitude of the sensor code signal. Once calibrated, this multi-dimensional data provides sufficient information to calculate the surface expression in the form of magnetic load for each cell.

FABRICATION

The magnetophoretic cytometry chip consists of 2 layers and an external magnet. The microfluidic features reside on the polydimethylsiloxane (PDMS) layer. The PDMS layer contains 2 inlets, one for the sample, and one for the buffer. The magnetophoresis chamber is 3mm-wide and 1cm-long, leading to 8 channels with 30 µm width. This layer was fabricated via soft-lithography, where a 4-inch silicon wafer was spin-coated with 35 µm-thick SU-8 2035 photoresist (MicroChem). The wafer was exposed through a chrome mask by a mask aligner (Karl Suss MA6) to pattern the photoresist. Once the mold was created, it was treated with trichloro(octyl)silane (MilliporeSigma) for effortless detachment of PDMS layer from the mold. A 10:1 mixture of PDMS with its crosslinker was prepared, poured on the mold and degassed in a desiccator. PDMS layer was cured at 65ºC and peeled-off from the mold.

The sensor electrodes were fabricated on a glass substrate using a lift-off process. 1.5 µm-thick NR9-1500PY photoresist (Futurrex) was spun on a 1-inch by 3-inch glass slide. Then, the glass slide was exposed with a maskless aligner (Heidelberg MLA-150) to transfer the features. A 500 nm Au/Cr film layer was deposited on the glass slide using an electron beam evaporator (Denton Explorer), and a subsequent lift-off process was performed. The glass substrate and the previously fabricated PDMS layer were treated under oxygen plasma for surface activation, and they were permanently bonded on a hot plate at 65°C after alignment.

As the last stage, the neodymium magnet (B848, K&J Magnetics) was placed underneath the glass layer, aligned with a microscope and fixed into its position with epoxy.

RESULTS AND DISCUSSION

Device Calibration

To calculate the magnetic load (i.e. number of magnetic beads) on a cell from the corresponding sensor data, we developed a model of the magnetophoresis process in our microfluidic system with COMSOL. Based on the manufacturer's specifications together with results from Tarn et al. [9] for the magnetic beads (Dynabeads MyOne Streptavidin C1, Invitrogen) and the data sheet of the neodymium magnet (B848, K&J Magnetics), we simulated cell trajectory under 30 mbar drive pressure. In those simulations, we first swept the magnetic load (Figure 2a) with one bead increments for a fixed cell size to exclusively investigate the effects of cell surface expression on the particle trajectory. As expected, the magnetophoretic deflection was observed to increase with the cell surface expression. The simulated final deflections of the cells were then stored to create a look-up table.

Cells vary in size and the size of a cell affects both the maximum number of magnetic beads that can be accommodated on the membrane and the Stokes' drag force experienced during magnetophoresis. Given the cell size is an important parameter for the calibration, we simulated cell trajectories for different-sized particles in 1 µm increments (Figure 2b) and recorded resulting deflections for each magnetic load. Larger cells were confirmed to deflect less than smaller cells due to higher Stokes' drag in the transverse direction, demonstrating the importance of the size information in the interpretation of the electrical sensor data for quantitative results.

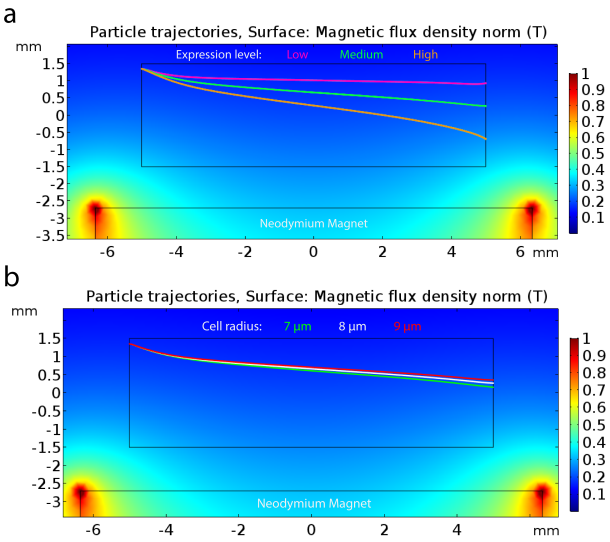

Figure 2: Simulation of particle trajectories in magnetophoresis. (a) Trajectories of a low, a medium and a high expresser cell with 8µm radius. Transverse deflection increases with higher surface expression. (b) Trajectories of 3 cells with the same magnetic load but with different radii (r=7µm, r=8µm, r=9µm). Transverse deflection decreases with increasing cell size due to larger drag forces.

In order to convert the electrical sensor signal into a distribution of surface expression for cells, we used the data obtained from previously described computer simulations on magnetophoresis. Specifically, we created a calibration look-up table (Figure 3) mapping the cell size, magnetic load and sensor identity (i.e., the receiving outlet of the sorted cell) for a given input pressure used to drive the sample through the microfluidic device. The flat top region of the calibration curve is due to the sensor saturation, since beyond certain magnetic load – size combinations, all cells would be collected by the furthest (i.e. 8th) outlet irrespective of their individual differences.

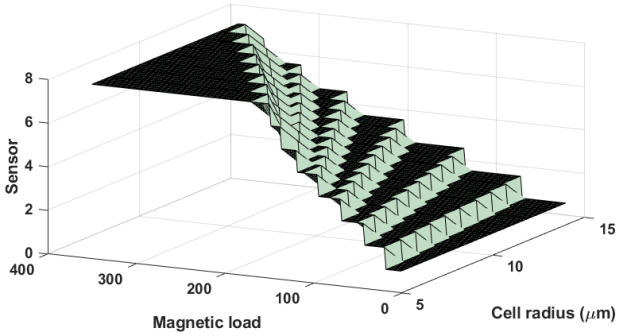

Figure 3: Calibration look-up table at 30 mbar drive pressure. The look-up table was obtained by combining results from a series of finite element analyses covering a range of cell sizes and magnetic loads.

Sample Preparation

We tested our device with a suspension of SK-BR-3 (ATCC HTB-30) breast cancer cells. The cells were

cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (Seradigm) and 1% penicillin/streptomycin (AMRESCO) in an incubator at 65ºC and 5% CO_2. Once the culture reached 80% confluence, the cells were detached from the culture flask via 3-minute treatment with 0.25% trypsin-EDTA (Gibco).

For magnetic labeling, 1µm-beads (Dynabeads MyOne Streptavidin C1, Invitrogen) were pelleted and washed. Then, 12-unit volume of magnetic beads (at 7-10x10^9 beads/mL concentration) were conjugated with 10-unit volume of biotin-anti-EpCAM antibody (Product ID: 324216, BioLegend) at 4°C for 15 minutes. These functionalized beads were mixed with the cell suspension at a ratio of 300 beads per cell to ensure surface saturation. The mixture was incubated at room temperature on a rocker for 45 minutes.

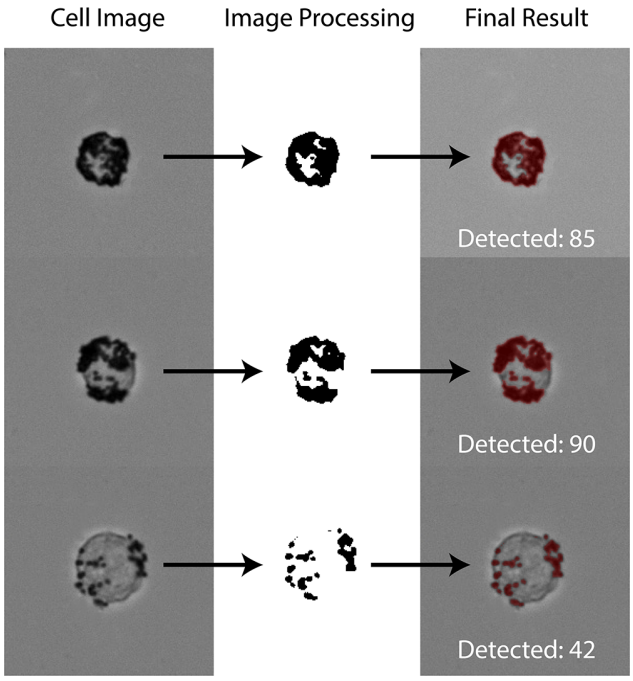

Figure 4: Optical characterization of magnetic load on individual cells. Custom image processing software calculates the number of magnetic beads on the cell surface.

Optical characterization of sample

Prior to processing on the chip, magnetically labeled SK-BR-3 cells were examined under a microscope (n=500) for image-based quantification of magnetic load through a custom image processing software. Specifically, the recorded photos of the individual cells were imported into this image processing program where the beads were segmented out from the cells and the number of dark pixels generated after morphological operations were counted. Using previously determined pixel-per-bead information, the magnetic load of each cell was calculated for comparison (Figure 4). As the microscopy images of the cells represented a 2-dimensional projection of a transparent spherical object, the bead count was underestimated using this method. It was also noted that the error rate of image-based measurements increased with the amount of magnetic load.

Signal Acquisition and Data Analysis

Immunomagnetically labeled cells were pneumatically driven through the cytometry chip at 30 mbar supplied by a software-controlled pressure regulator (Fluigent). For the electrical measurement, the sensor network was excited with a 500 kHz sine wave, and electrical current from the positive and negative electrodes (i.e. sensing electrodes) were first converted to voltage signal through transimpedance amplifiers. Signals from the sensing electrodes were subtracted from each other to generate a bipolar signal, whose amplitude was subsequently measured with a lock-in amplifier (HF2LI, Zurich Instruments). The demodulated differential signal was sampled at 50 kHz into a computer via a data acquisition system.

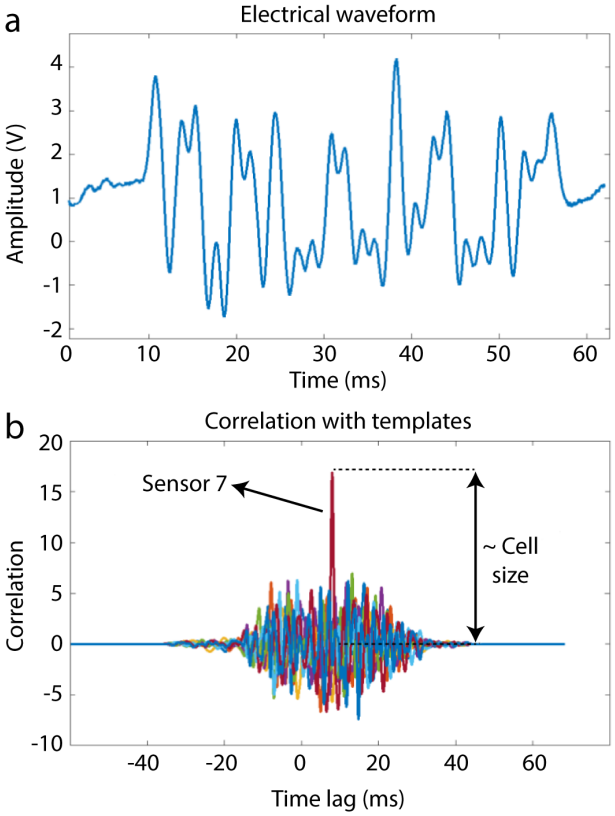

Figure 5: Electrical sensor signal processing. (a) A representative 31-bit code waveform generated by a cell in the 7th fluidic channel, encoded by the 31-bit Gold code of 1011001101000110001001100010111. (b) The correlation of the signal with the templates corresponding to all 8 sensors on the microfluidic chip. Signal cross-correlation exclusively yields a match for the template corresponding to the sensor 7. Amplitude of the correlation peak was used to estimate the cell volume.

The recorded waveform was decoded using a template library that contained all the codes implemented in the device. As the code-set was specifically designed to be orthogonal [7], cell signals (Figure 5a) yielded a distinctive correlation peak for the matching template revealing the sensor identity (Figure 5b). In cases when multiple cells were coincidently detected by the same or different sensors, we used a successive interference cancellation algorithm to recover individual events from the signal by

iteratively subtracting the identified signals from the raw signal until there were no residual signal [3]. Besides providing the sensor identity, the decoding algorithm also calculated cell size, following calibration, from the amplitude of correlation with the template library. The sensor identity and cell size data obtained from the microfluidic device were finally mapped to simulated magnetic load for each cell using the calibration look-up table constructed earlier.

In our test, we processed 632 SK-BR-3 cells, immunomagnetically labeled against epithelial cell adhesion molecule (EpCAM), with our magnetophoretic cytometry chip. Our analysis led to a magnetic load distribution with a mean of (128 beads/cell) for the processed population (Figure 6). These results matched with results from microscopic characterization of the same sample with a correlation coefficient of 0.844 and a p-value of 2.9E-6. The mismatch between the two distribution is expected to be due to the underestimation of the magnetic load in optical characterization as described earlier. This was also supported by the fact that the mismatch was more pronounced for the cells carrying a large number of magnetic beads (Figure 6).

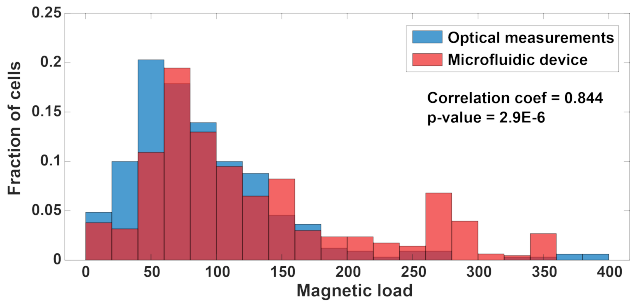

Figure 6: Experimental results obtained from SK-BR-3 breast cancer cells (n=632). The results from our microfluidic device agrees well with the microscopic characterization of the cells.

CONCLUSION

We demonstrated a magnetophoretic cytometry technique to quantitatively compute the surface expression of cells. Our integrated microfluidic device electrically tracks the trajectories of magnetically manipulated cells and utilizes computer simulations to estimate the surface expression of a cell from its deflection. Our results from the on-chip characterization of human breast cancer cells closely match with independent optical characterization of the sample, validating our technique. As our device is built on a highly-portable, low-cost and easy-to-operate platform, it can provide a compelling electronic alternative to fluorescence-based flow cytometry for point-of-care testing of biological samples.

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