On-chip Manipulation and Sorting of Cancer Cells for Next-Generation Diagnostic Technologies

by

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Department of Electrical and Computer Engineering
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Abstract

Cancer is a leading cause of death and disability. Early detection can significantly improve long-term survival in cancer patients. The advent of point-of-care technologies, which are typically based on lab-on-chip tools, enables convenient and real-time healthcare at or near the patient bedside. Development of point-of-care cancer testing can keep the advantages of, but overcome the high cost of, existing expensive genetic analysis methods. It can do so while providing the results promptly to physicians as they seek to customize and improve disease treatment.

The central aim of this thesis is to develop new strategies to detect cancer prior to the spread of cancer cells to the distant organs. Metastasis relies on the release of migratory cancer cells, and is responsible for as much as 90% of cancer associated mortality. The factors that determine the invasiveness of these circulating cells remain poorly defined, and it is difficult to distinguish cancer cells having high versus low metastatic potential. New technologies are required that sort heterogeneous cancer cells into relevant subpopulations, and profile thereby small numbers of cells according to their phenotypes.

Herein we describe a powerful new capability for the monitoring of cancer progression. We developed a novel fluidic chip that selectively isolates rare cancer cells that exhibit different levels
of phenotypic surface markers. We show that the device successfully profiles the surface expression of very small numbers of cells; and it accomplishes this directly from whole blood. We couple the surface marker profiling approach with a migration platform with single cell resolution: this allows us to characterize more deeply, still on-chip, the biological behavior of invasive cancer cells. We deploy these new techniques to reveal the dynamic phenotypes of these rare cells. We prototype the system and prove it out using samples of unprocessed blood from mice. We characterize the samples as a function of tumor growth and aggressiveness and prove that the new profiling technology provides powerful and relevant information that correlates with tumor stage and aggressiveness. The strategies presented offer to guide the development of sensitive and specific approaches for cancer diagnosis that provide new information not available using prior methods.
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List of Abbreviations

3DSTEs – Three dimensional sharp-tipped electrodes

BSA – Bovine serum albumin

CK – Cytokeratin

CTC – Circulating tumor cells

DNA – Deoxyribonucleic acid

EGF – Epidermal growth factor

EMT – Epithelial to mesenchymal transition

EpCAM – Epithelial cell adhesion molecule

FACS – Fluorescence-activated cell sorting

MagRC – Magnetic ranking cytometry

PBS – Phosphate buffered saline

PDMS – Polydimethylsiloxane

PI – Propidium iodide

POC – Point of care

qPCR – Quantitative polymerase chain reaction

RIN – RNA integrity number

RNA – Ribonucleic acid

TMP – Transmembrane potential

WBC – White blood cell
1 Introduction

1.1 Point-of-care diagnostics

The term point-of-care (POC) diagnostics refers to *in vitro* diagnostic (IVD) tests that provide results without reliance on complex laboratory facilities and expert technical staff. The tests require only elementary instruction and seek to enable the detection of multiple markers\(^1\). Reading the results may be as simple as viewing a stripe or spot of color. POC measurements can indirectly lower medical costs by reducing interference of personnel and reducing sample mislabeling and mishandling.

Existing diagnostic tests that are performed in central laboratories cannot provide results quickly to physicians. Samples are taken at the doctor’s office, shipped to the centralized laboratory and the results are reported to the patient during a second follow-up visit several days later. This delays treatment and increases healthcare costs. However, POC tests provide results promptly, enabling more effective treatment of rapidly progressing afflictions, even making a life-or-death difference with highly mortality rate diseases such as cancer\(^2\). New technologies to rapidly detect cancerous cells could improve patient survival and bring cancer diagnostics to the point-of-care.

1.2 Sample processing

One of the essential steps that must be integrated with any POC device is sample processing. While significant progress has been made in developing cell analysis technologies, sample preparation techniques have been limited in light of the complexities\(^4\). Sample preparation is defined as a series of cellular and molecular separation steps such as cell capture, cell pre-concentration and purification, and cell lysis and nucleic acid extraction. In most laboratories, sample preparation is performed off-chip by technicians. Off-chip methods usually require large volume of samples, preventing single cell analysis, and increasing the cost of tests. Recently, on-chip methods have attracted great attention since they can be integrated with downstream analysis. However, due to the lack of development in the area of sample preparation, only a few complete lab-on-chip systems capable of handling raw samples have been developed. Practical applications in clinical analysis require processing of samples as complex as whole blood, serum, and urine\(^4,5\).
On-chip sample preparation methods have taken advantage of microfluidic devices. The use of microfluidics provides numerous benefits. These advantages include reduced consumption of reagents; analysis with high precision and high throughput; and on-chip diagnosis\cite{6-8}. The unique advantages of microfluidic systems which make them a superior candidate for portable point-of-care diagnostic devices are the capability of measuring from small volumes of fluidics and integrating with downstream analysis\cite{5}.

Figure 1.1 Two sample preparation steps usually performed are target cell capture and nucleic acid extraction. One approach to capture cells of interest is to use magnetic tag conjugated with specific marker on the target cells, and apply magnets. Afterwards, the captured cells could be analyzed with microscope or used for nucleic acid extraction. As top pictures demonstrate off-chip sample preparation requires performing the steps manually while all the operations could be integrated within a microfluidic chip. On-chip methods decrease the chance of cell loss while preserve sample integrity.

Off-chip sample handling steps include centrifugation, pipetting, and cell immunostaining. These operations are manually performed in clinical labs by skilled technicians. They usually must aliquot, centrifuge and pipette to make samples. Errors can result from experimental mishandling or subjective interpretation of results. Automation of these steps to minimize sample handling leads to overall less error while preserving sample integrity. Figure 1.1 depicts off-chip sample preparation steps. All of these operations could be integrated to a miniature microfluidic chip.
1.3 Rare cell capture

The preliminary step in sample preparation is isolation of target cells from non-target ones. Cell capture is important, particularly when target cells are rare. The batch process for rare cell analysis involves enrichment steps such as pipetting, changing of the sample container, and chemical treatments that may contribute to the loss of cells. To decrease the rate of rare cell loss, scientists are working towards making rare cell isolation platforms using microfluidic devices\textsuperscript{[9]}. Circulating tumor cells or CTCs shed into the bloodstream during cancer progression offer the prospect of a liquid biopsy from the blood. These cancer biomarkers may replace metastatic tissue biopsies in the prediction and monitoring of therapeutic responses and tumor recurrence\textsuperscript{[10]}. While CTCs may have highly potential clinical application for cancer diagnosis, their rarity in the blood stream makes CTC capture challenging.

Recent advances in rare cell capture technology\textsuperscript{[11,12]} have made it possible to isolate CTCs with high sensitivity and specificity. Affinity capture\textsuperscript{[13]}, negative selection\textsuperscript{[14]} and physical parameter-based separation\textsuperscript{[15]} have been reported as powerful approaches to count rare cells. These capture devices developed to date isolate a bulk population of CTCs and do not differentiate subpopulations that may have varying phenotypes with different levels of clinical relevance. Advanced rare cell profiling tools\textsuperscript{[16]} enable fingerprinting of genomic and proteomic properties; however, even the most advanced techniques reported to date must perform these analyses offline and do not phenotypically analyze the low numbers of cancer cells found in clinical specimens in situ. Specialized techniques are highly demanded that integrate CTC analysis platforms with enrichment techniques, enabling to develop POC techniques for early cancer diagnosis. Some of the techniques developed for CTC enrichment are summarized in the following.

1.3.1 Physical parameter-based separation

Separation based on physical parameters isolates CTCs from normal blood cells by relying on intrinsic properties of the CTCs (such as size, density, deformability, and electrical charges)\textsuperscript{[17]}. Size-based isolation of CTCs from whole blood was introduced in 1960s, and has been revisited more recently\textsuperscript{[18]}. Utilizing the well-known characteristic that cancerous cells are larger than surrounding normal blood cells, CTCs are isolated by using filters fabricated with a defined pore
size that allows passing of smaller blood cells while capturing larger CTCs. Density gradient centrifugation for the enrichment of cells is based on the differences in cellular density\textsuperscript{19}. Although filter based approaches are not limited by cell types, these techniques cannot differentiate between cancer cells and normal blood cells that are physically similar in size or density\textsuperscript{17}. Moreover, when passing through filtration systems, CTCs may potentially become damaged or lost due to the increased shear stress.

1.3.2 Affinity capture

In affinity mediated immunoassays, the capture force is provided by affinity ligands. Due to the nature of antibody-antigen interaction, these assays usually display better selectivity than physical separation. The first demonstration of affinity immunoassays uses three-dimensional antibody-coated micropost structures for the capture of CTCs from whole blood\textsuperscript{12}. Afterwards, a number of devices with different engineered structures were developed to enhance the separation efficiency\textsuperscript{20}. However, the affinity mediated immunoassays are limited by the relatively slow transport of target cells to the capture surface. An example of affinity-based separation of CTCs is the commercially available CellSearch\textsuperscript{TM} System (Veridex, Raritan, NJ, USA). The system is based on the enumeration of epithelial cells from whole blood. Separation is achieved by antibody-antigen conjugation that targets epithelial cell adhesion molecule, EpCAM, and subsequently identifies CTCs with fluorescently labeled antibodies against cytokeratin (CK 8, 18, 19) and a nuclear stain. The CellSearch definition of a CTC is a nucleated cell lacking CD45 (white blood cell marker) and expressing cytokeratin and EpCAM.

1.3.3 Negative selection

In negative depletion, normal hematopoietic cells are targeted and subsequently removed, thereby enriching the blood cell suspension for the rare tumor cells. Some reported assays first remove red blood cells (RBCs) via the use of RBC lysis buffer or gradient separation; it is followed by CD45 expressing cell removal for CTC enrichment\textsuperscript{14}.

1.4 Rare cell lysis

After cell capture, it is required to release biomarkers such as DNA and RNA from the interior of cells for downstream analysis. The release of biomarkers from cells is called cell lysis that represents a key step in biomarker analysis. This is typically performed by permeabilizing the
cell membrane\(^5\). Nucleic acid sample preparation requires several steps including collecting and purification of DNA or RNA from raw samples such as whole blood, urine, saliva, and serum, which is highly time consuming and requires extensive training\(^{21–23}\). To solve these challenges and simplify the steps, microfluidics provides a simple platform for nucleic acid processing. On-chip micro-techniques for nucleic acid preparation usually reduce the total analysis time and cost by reducing the samples and consumed reagents.

1.5 Thesis objectives and overview

The objective of this thesis is to develop new approaches for rapid sample processing and profiling of rare cells using microfluidic systems to manage the inherent trade-offs that arise in point-of-care applications. We will explore on-chip methods for overcoming limitations on the sensitivity and specificity of phenotypic profiling of rare cells. We showcase these new approaches with clinical applications including monitoring cancer progression.

The remainder of this thesis will be organized as follows:

1.5.1 Chapter 2: Amplified micro-magnetic field gradients for profiling of rare cells

In this chapter, we focus on new strategies for rapid isolation and enumeration of circulating tumor cells from whole blood sample. We seek to not only capture these rare cells, but also profile them according to their levels of invasiveness. We take the view that, to meet the challenge of high-resolution characterization of rare cells, while at the same time providing the highest achievable sensitivity, we would require a distinct phenotypic sorting strategy. We present a fluidic chip, termed magnetic ranking cytometry or MagRC, that achieves immunomagnetic capture of CTCs. It features one hundred zones that selectively isolate cells with different levels of surface marker expression. We explore the capability of the device to profile the surface expression of very small numbers of cells directly from whole blood. We show the use of MagRC approach to track a variety of different surface expression markers. In order to evaluate the utility of the device for the analysis of CTCs and their dynamic properties, we analyze blood from mice bearing xenografted tumors as a function of tumor growth. This approach provides a new means to monitor CTC dynamic phenotypes and their involvement in metastasis in cancer models.
1.5.2 Chapter 3: Magnetic and drag force engineering for sorting of rare cells

In this chapter, we seek to develop a simplified version of MagRC approach for rare cell profiling, with the goal of improving the rate of fabrication. We present a microfluidic device that incorporates both magnetic and drag forces engineered to enumerate and profile cancer cells. Magnetic force engineering is achieved via the integration of nickel micro-magnets into the microfluidic device, while channel width increment leads to drag force manipulation. We show that this new design increases the yield of fabrication threefold, allowing the production of devices that can be used in different clinical studies.

1.5.3 Chapter 4: 2D-phenotypic profiling of rare cells

In this chapter, we further analyze the invasiveness of heterogeneous CTC subpopulations using a 2D-phenotypic profiling approach. We investigate the migration behavior of cells having different levels of epithelial marker expression. First cancer cells are sorted according to their levels of EpCAM expression into one of four zones, generating a profile of relative surface marker expression. In the second dimensional profiling, cell subpopulations extracted from each zone are subjected to chemotactic phenotype binning. Finally, the 2D system is challenged with blood from mice bearing xenografted tumors to monitor 2D migration phenotypes of CTCs in cancer models. Using this 2D phenotypic assay, we show that we can extract 20 cell subpopulations that have different migratory behaviors.

1.5.4 Chapter 5: Rapid electrical release of biomarkers from cells

In this chapter, we explore new means for electrical release of intact biomarkers from interior of a cell. We seek to release RNA with high integrity from bacterial cells. Bacterial cell lysis is challenging due to the high voltage requirement. We optimize the lysis efficiency by incorporating three dimensional, sharp-tip electrodes that generate a high electrical field at lower applied potentials. We showcase that this approach preserves RNA quality for further downstream analysis.
1.6 References


2 Amplified micro-magnetic field gradients for profiling of rare cells

In this chapter, we investigate front-end sample processing techniques for efficient capture and profiling of rare cells. Circulating tumor cells or CTCs are rare cancer cells that have shed from the primary tumor into the bloodstream. CTCs are rare and outnumbered by peripheral blood cells by a factor of $10^6$. Profiling the phenotypes of CTCs in whole blood is critical to unraveling the complex and dynamic properties of these potential clinical markers. As CTCs enter the bloodstream, they may change their phenotypes and possess heterogeneous subpopulations. Some CTCs may possess benign properties while others exhibit much higher metastatic potential. Here we explore strategies for profiling heterogeneous tumor cell subpopulations using microfluidic approach.

This chapter has been submitted as a journal publication:


All experiments in this chapter were performed by M. Poudineh and S. Ahmed unless otherwise indicated.

2.1 Introduction

The metastasis of cancerous tumors relies on the release of circulating cells that migrate to distant sites and form secondary tumors. The factors that determine the invasiveness of these circulating tumor cells remain poorly defined, and it is not currently possible to distinguish CTCs having high versus low metastatic potential. Studying CTCs directly collected from unprocessed blood samples is a challenge given their rarity (parts per billion) in the bloodstream. Moreover, multiple cell phenotypes can exist within a given tumor, and their properties evolve dynamically once they leave a tumor and enter the bloodstream.
The current method for characterizing and sorting heterogeneous cell subpopulations relies on Fluorescence-Activated Cell Sorting (FACS). Although FACS has many applications for cancer cell sorting in buffers, it is not compatible with analysis of undiluted whole blood and can simply miss rare cells.

Microfluidics opens a new venue to study CTCs. A variety of microfluidic approaches have been reported for rare cell isolation including devices for size-based selection\textsuperscript{[1,2]}, immunoaffinity-based capture\textsuperscript{[3,4]}, and immunomagnetic capture of nano or micro particle labeled cells. Immunomagnetic approach possesses several advantages compared to the other CTC separation methods: (a) selectivity, due to antibody-antigen binding, the immunomagnetic separation is remarkably sensitive\textsuperscript{[5]}; (b) tunability, the applied magnetic field can be modulated accurately to adjust the required field intensity for rare cell isolation\textsuperscript{[6]}; (c) Integration, Immunomagnetic assay can be integrated with other separation methods such as size-based isolation to enhance capture efficiency\textsuperscript{[7]}.

In immunomagnetic isolation techniques, magnetic force is applied to separate CTCs labeled with magnetic tags\textsuperscript{[8,9]}. Magnetic tags are magnetic nano-beads conjugated specifically to cancer cells through antibody–antigen binding without affecting normal blood cells. The movement of CTCs flowing in a microfluidic channel, under an external magnetic field, is affected by two main forces: magnetic force and hydrodynamic drag force\textsuperscript{[10]}. Cells will be captured within a fluidic device when the retaining magnetic force overcomes the drag force that opposes capture.

Different antigens in the cell surface can be conjugated to magnetic beads. One of the universal surface markers used as a target for CTC capture is epithelial cell adhesion molecule (EpCAM). It is known that CTCs will lose EpCAM through epithelial to mesenchymal transition (EMT) during cancer progression\textsuperscript{[11,12]}. Cells with low levels of EpCAM expression are known to be more aggressive and speculated as metastasis initiators.
Figure 2.1 A) Velocity Valley device that features four different regions with different linear velocities. Cells having high magnetic loadings are trapped in zone I and cells with medium to low levels of magnetic tags are captured in later zones. B) Spatial distribution of linear velocities in the first zone of the device. Blue regions correspond to velocity valleys.

In our group, we developed the Velocity Valley (VV) chip\cite{13}, which incorporates ‘X’-shaped structures in a microfluidic channel to generate pockets of locally low flow velocity, enabling efficient capture of CTCs labeled with the magnetic nanoparticles (Figure 2.1). By changing the cross-sectional area of the microfluidic channel, the linear velocity (and thus the drag force acting on the CTCs) could be manipulated to capture CTCs with different levels of surface marker expression in one of four zones, generating a CTC profile. Here we developed a second generation of VV chip that generates a profile with much higher resolution, sorting CTCs into one hundred different compartments. Rather than sorting based on varying the drag force as in the VV chip, this method (termed magnetic ranking cytometry or MagRC) introduces micromagnets with increasing sizes to generate local amplifications to an external magnetic field. These amplifications allow us to sort heterogeneous CTC subpopulations into different bins according to surface marker expression levels. The micro-magnets in the MagRC chip are round nickel structures centered around the same style of ‘X’-structure found in the VV chip (Figure 2.2A). Nickel is a ferromagnetic material that can be used to amplify magnetic fields. Other studies made use of this property of nickel to capture CTCs, successfully employing nickel micro pillars\cite{14}, nickel lines\cite{15,16}, and nickel nanoparticles\cite{17}. By gradually increasing the size of the nickel micro-magnets along the length of a microfluidic channel having a fixed width (to maintain a constant linear velocity), the MagRC chip creates localized regions with very high
magnetic field gradients (Figure 2.2C), yielding the ability to magnetically rank cells with different levels of surface marker expression. Inside the channel, micro-magnets range from 136 µm in radius to 235 µm (100 different sizes). Each of the 100 zones has two rows of ‘X’-structures with the same size of nickel sites. This nickel-based CTC capture device has the ability to not only enumerate CTCs, but also bin the cells into subpopulations. This makes it possible to examine the progression of the disease as the CTC profile changes over time.

**Figure 2.2** A) A microfluidic channel with an array of X-shaped structures generates regions of locally low velocity; circular nickel micro-magnets are patterned within the channel to enhance an external magnetic field. B) Arrays of cylindrical permanent NdFeB magnets applied to the top and bottom of the microfluidic chip generate the external magnetic field. C) Inside the microfluidic channel, magnetically labeled cells are subjected to both the external magnetic field (left) and high-field gradient amplifications generated by the micro-magnets (right).

### 2.2 Results and discussion

In our MagRC device, a flow channel is sandwiched between arrays of magnets that generate a high field gradient inside the chip (Figure 2.2B). Cells are labeled with magnetic nanoparticles coated with EpCAM, a marker which is commonly overexpressed in epithelial tumors. Key to this idea is the use of nickel micro-magnets to generate regions of high field gradient. As the size of micro-magnets increases along the channel, a broader area of the chip is impacted by the heightened magnetic forces acting near the nickel\[^{18}\]. As cells pass through the channel, they are deflected towards regions exhibiting high magnetic field strengths and sharp field gradients. The amount of deflection depends on the number of bound magnetic nanoparticles, which in turn relates directly to the EpCAM expression level of a cell. Cells with lots of surface EpCAM are deflected easily by the magnetic forces acting on the nanoparticles, whereas cells which express less EpCAM deflect only if they are close to high magnetic field gradient regions.
2.2.1 Computational modeling of the MagRC fluidic device

A quantitative physical model of the device (see supplementary material, Figure S7.1 – S7.4) was developed to explore how cells exhibiting varied expression levels would generate different MagRC profiles that reflected their individual phenotypes. A capture volume was defined as a region in which the magnitudes of the magnetic and drag forces are comparable. As a result, those cells that pass through a capture zone will be deflected to a channel wall and be captured. For a cell with an abundance of bound magnetic nanoparticles, the capture zone generated by even the smallest micro-magnets is sufficient to ensure substantially complete capture in the earliest zones of the MagRC chip (Figure 2.3A). Cells with low surface marker expression are deflected only if they are close to edges of the micro-magnets, where the magnetic force acting on the nanoparticles is highest (Figure 2.3B). Since each micro-magnet is positioned concentrically with an ‘X’-structure, the regions in the MagRC chip exhibiting the highest magnetic forces and field gradients also correspond to the regions exhibiting the slowest flows. This has the benefit of creating localized regions with favorable capture dynamics (low drag and high magnetic forces), while also contributing to the high-resolution sorting capability of the chip.

![Figure 2.3](image.png)

**Figure 2.3** Capture regions of high and low magnetic loading cells generated by three different sizes of micro-magnets are shown. Cells having high levels of surface marker expression are more likely to encounter a capture region as they flow through the chip, since they require less field amplification to generate comparable magnetic forces. Cells with high levels of surface marker (e.g. EpCAM) expression (and thus high magnetic loading) are captured in the earliest zones where the micro-magnets are small (A), while for low expression cells, larger micro-magnets are required to generate a sufficiently large capture region (B).

For each cell in each zone, the probability of that cell encountering a capture region was calculated and reported as the capture parameter. Since the nickel micro-magnets generate amplified magnetic fields near the bottom of the microfluidic channel, the capture parameter of the cells within the chip is strongly dependent on their vertical position. The vertical dependency of the parametric model for cells having different levels of magnetic loading is illustrated in
Figure 2.4A. The overall modeling results presented in Figure 2.4B show the predicted capture locations for three types of cells having high, medium and low levels of magnetic loading. (See supplementary material for a detailed explanation of the parametric model).

Figure 2.4 Numerical modeling of cell capture in the MagRC device. A) Normalized capture parameter as a function of height and zone in the chip, for three different inlet heights. B) A parametric model predicts where high, medium and low magnetically loaded cells will be captured in the MagRC chip. See supplementary information and Figures S7.1 – S7.4 for an explanation of the model. (We wish to acknowledge Peter M. Aldridge for developing computational modeling and performing COMSOL simulations.)
2.2.2 Validation of the performance of the MagRC approach

As a first suite of experiments, we challenged the capture and sorting capabilities of the MagRC chip with three cell lines with known levels of EpCAM expression. The level of EpCAM expression of the cell lines was confirmed via flow cytometry. Three different target cell lines—VCaP (a human prostate cancer cell line), SKBR3 (a breast adenocarcinoma cell line), and MDA-MB-231 (a breast cancer cell line with mesenchymal characteristics)—were incubated with 50 nm magnetic particles coated with anti-EpCAM in buffered solution for half an hour. We then ran samples through the chip using a syringe pump at a flow rate of 500 µL/h. A nuclear stain was performed on the captured cells and capture efficiency was assessed by counting the capture cells using fluorescence microscopy. Experiments for each cell line were repeated three times.

Figure 2.5A shows the bright field and fluorescence microscope images of an SKBR3 cell captured at the edge of a nickel micro-magnet (where the magnetic field and field gradients are at a maximum). The three different cell lines exhibited a distinctly different and highly reproducible profile of distribution within the chip (Figure 2.5B and Figure S7.6). VCaP cells with the highest level of EpCAM expression were found primarily in first 10 zones. SKBR3 cells which exhibit an approximately 10-fold lower level of EpCAM expression than VCaP, and hence retain a lower number of bound magnetic tags, were captured mainly after zone 10. MDA-MB-231 cells, which have the lowest level of EpCAM expression, were found generally after zone 70, near the outlet of the chip where the micro-magnets are the largest. The relative levels of EpCAM expression of the cell lines were confirmed via flow cytometry (Figure 2.5B inset).

T-test analysis was used to assess statistical significance of MagRC profiles obtained from different cell lines (Table S7.1, S7.2 and S7.3). The calculated P-values (<0.0001) confirm the statistical significance of the uniqueness of the MagRC profiles and that the resolution of this technique is high. In sum, magnetic ranking cytometry successfully sorts cells according to their expression level of surface markers. Importantly, high recoveries of the cells injected into the device are achieved (VCaP 96±4%, SKBR3 93±4%, MDA-MB-231 94±5%) (Figure 2.5C). The efficient capture of MDA-MB-231 cells, which have a low level of EpCAM, indicates that cells with lowered epithelial character would still be visualized with this approach. Control experiments using only an external magnetic field and no nickel micromagnets yielded less useful profiling information, highlighting the crucial role of the internal magnetic structures (Figure S7.7).
We also evaluated the MagRC chip by screening the SKBR3 cell line for three different surface markers that are often over expressed in epithelial cancer cells: human epidermal growth factor receptor 2 (HER2)/neu, EpCAM, and N-Cadherin (Figure 2.5D). The inset in figure 2.5D shows the level of these three surface markers in SKBR3 cells measured by flow cytometry. HER2 is known to be highly overexpressed in this cell line, and indeed, experiments with magnetic nanoparticles coated with anti-HER2 led to cell capture within the very earliest zones of the chip. In contrast, capture with anti-N-Cadherin coated nanoparticles showed most cells being captured in the later zones of the chip. EpCAM levels are intermediate for these cells, a fact also reflected in the MagRC profile.

We also evaluated whether magnetic ranking cytometry could monitor dynamic phenotypes in cancer cells, and in particular changes induced by EMT. Using an in vitro model for EMT – CoCl$_2$ induced hypoxia$^{[19,20]}$ – we studied SKBR3 cells that were untreated versus those in which EMT had been induced. Following 72 hours of CoCl$_2$ treatment, we used MagRC to assess control and treated samples using EpCAM as a profiling marker. The inset in Figure 2.5E shows flow cytometry data that confirm the down-regulation of EpCAM in treated samples. The shift observed for treated cells sorted in the magnetic ranking device also confirms EpCAM down regulation (Figure 2.5E).

The data presented indicate that magnetic ranking cytometry produces profiles comparable to those reported by flow cytometry (FCM). FCM is a powerful and robust approach useful in analyzing protein expression and heterogeneity in living cells. It is limited in its sensitivity; however, and requires cell numbers of $10^4$ or higher for accurate results. As shown here, MagRC reports on protein expression with similar resolution, but using much smaller collections of cells. It is also noteworthy that the MagRC approach is a gentle analysis method that allows high recoveries of viable cells. As shown in Figure S7.8, 92% of captured cells can be recovered, and 98% of the recovered cells are viable.
Figure 2.5 Binning cells with different surface marker expression levels in the MagRC chip. A) Bright field and fluorescent microscope images (left & right, respectively) of a captured, immunostained SKBR3 cell. Captured cells are stained for nuclei (blue) and CK (red). Due to the high field gradients generated along the edges of the micro-magnets, cells typically come to rest along the perimeter of the micro-magnets where the magnetic force is the highest. B) Distribution of VCaP, SKBR3, and MDA-MB-231 cells in the MagRC chip. Different levels of EpCAM expression yielded different capture zones for each cell line. The inset figure shows EpCAM expression for the three cell lines; cells were fluorescently labeled with anti-EpCAM, and expression levels were measured using flow cytometry. C) Capture efficiency for cells that have different levels of EpCAM expression. High recovery of low EpCAM cells (MDA-MB-231) proves the suitability of the MagRC approach to monitor cells with lowered epithelial markers. D) The SKBR3 cell line was profiled for different cancer biomarkers using three capture antibodies: EpCAM, HER2, and N-Cadherin. The inset figure shows expression of three markers on SKBR3 cells measured by flow cytometry. E) The capture efficiency of the MagRC chip was tested by spiking different numbers of SKBR3 cells in buffer solution before flowing them through the chip. A low number of cells (n=10) can be captured using the MagRC chip. Error bars show standard errors, n=3.

2.2.3 Performance of MagRC in unprocessed blood samples

We then proceeded to challenge the system using unprocessed whole blood samples, and, as reported below, we found that MagRC retains its sensitivity and profiling capability. When whole blood samples (1 mL) containing between 10 and 40 cells were profiled using EpCAM as
a target marker, reproducible profiles were obtained (Figures 2.6A and 2.6C). We compared the performance of the MagRC approach with the CTC gold standard, FDA-cleared CellSearch assay (Figure 2.6B). Spiked blood samples containing 100 SKBR3, PC3 (metastatic human prostate cancer cell line) and MDA-MB-231 cells per milliliter were prepared and analyzed using both MagRC chip and CellSearch. High recoveries of the spiked samples injected into the MagRC chip are achieved (SKBR3=97±3%, PC3=90±2%, MDA-MB-231=90±3%). The efficient capture of MDA-MB-231 and PC3 cells, which have a low level of EpCAM, indicates that low EpCAM cells presented in whole blood sample would still be visualized with this approach. However, CellSearch system is inefficient to recover low EpCAM cells. The purity of cancer cells recovered during MagRC profiling was assessed by counting the numbers of WBCs non-specifically captured within our devices. The MagRC chip depletes up to 99.98% of the WBCs, with approximately 2000 WBCs found in the chip after processing 1 mL of blood. While much of this contamination is derived from non-specific binding of WBCs to the device, we wondered if non-specific binding of magnetic beads could also contribute to the capture of these cells. We used flow cytometry to compare the specific binding of particles to MDA-MB-231 cells and the non-specific binding to WBCs (Figure S7.5). The data from this experiment indicated that the level of non-specific binding of the magnetic nanoparticles to WBCs is ~ 10x lower than that occurring on low EpCAM cells, indicating that WBCs would not be captured within our device via this mechanism. The level of WBC contamination found in MagRC chip is comparable to other microfluidic capture approaches, including the micropost CTC chip with approx. 640 WBCs/mL\textsuperscript{[21]}, the microvortex-generating herringbone-chip with 4500 WBCs/mL\textsuperscript{[3]}, and the tunable nanostructured coating approach with 1200 WBCs/mL\textsuperscript{[22]}. We performed head-to-head studies of blood samples containing 100 cancer cells where both MagRC and FCM were used for profiling. MagRC profiled cells in the presence of normal blood cells (Figure 2.6C, 2.6D, and 2.6E), while FCM was unable to report a specific signal (Figure 2.6E). Even in the presence of 10,000 cells spiked into blood, a specific signal was not obtained with FCM. Spiked cancer cells could be visualized only after the blood was treated to lyse red blood cells. Unfortunately, this processing step eliminates over 50% of the cancer cells (Figure S7.9), and therefore creates the significant potential for false negatives. In contrast with FCM, the MagRC approach provides accurate profiling even with very low levels of cancer cells in unprocessed blood. This is a requirement for the evaluation of CTCs. It is noteworthy that the exact shape of the profile returned with MagRC is affected by the presence of blood cells (Figure 2.6E);
Figure 2.6 Magnetic Ranking Cytometry applied to rare cells in whole blood. A) Specific immunostaining of cancer cells. After capture, cancer cells are stained for DAPI, CK, and CD45. SKBR3 cells were identified as DAPI+/CK−/CD45− and white blood cells were identified as DAPI+/CK+/CD45+. B) Head-to-head comparison of the MagRC chip with CellSearch. 100 cells of SKBR3, PC3 and MDA-MB-231 cells were spiked into whole blood. MagRC and CellSearch were used to count cells. CellSearch was inefficient to recover low EpCAM cells while
MagRC retains cells with efficiency more than 90%. C) Different numbers of SKBR3 cells were spiked in 1 mL of whole blood and the MagRC chip was used to profile the spiked samples for surface expression of EpCAM. Experiments were repeated three times. D) MagRC was used to count rare cells in unprocessed whole blood samples and RBC-lysed samples. A significant proportion of cells were lost when this sample processing step was used. In unprocessed blood, MagRC shows high levels of sensitivity and linearity. See Figure S7.9 for raw data. Cells were spiked into 1 mL of human blood for all trials shown. Error bars show standard errors, n=3. E) Both flow cytometry and the MagRC chip were used to monitor cells in PBS, whole blood, and RBC-lysed blood. The MagRC chip was able to accurately profile cells accurately in all three solutions. However, the background signal for whole blood samples overwhelmed the signals collected via FCM; only cells in PBS and RBC-lysed blood samples were accurately measured using the technique. Due to the inability of FCM to accurately count low (~100) numbers of spiked cells (inset), samples with a higher level of SKBR3 cells ($10^4$) were measured and counted using FCM. Profiling with both FCM and MagRC was repeated three times.

however, since it is affected in a consistent and predictable fashion by the increased drag acting on the tumor cells that arises from interactions with the blood cells, it gives reproducible data for a given type of sample (e.g. whole blood).

2.2.4 Monitoring dynamic CTC phenotypes using MagRC in an animal model of cancer

The utility of MagRC approach for the monitoring dynamic CTC phenotypes was investigated using analysis of blood from mice in a xenografted model. To generate the model, MCF-7/Luc human breast cancer cells were implanted into the mammary fat pad of immunodeficient mice. We analyzed blood of two groups of mice. One group of mice received an estrogen pellet prior to tumor implantation ($E^+$), as estrogen stimulates MCF-7 tumor growth. The other group of mice was not treated with estrogen prior to tumor implantation and tumor growth was inhibited. After tumor cell injection, blood was collected from each mouse every 10 days and analyzed using MagRC. We used EpCAM as the capture agent with the aim of studying changes in the level of epithelial marker expression during the tumor growth. After capture and fixation, immunostaining was carried out to distinguish between CTCs and mouse blood cells (Figure 2.7A) to ensure an accurate profile. Cancer cells were identified by a triple stain for cytokeratin ($\text{CK}^+$), a nuclei stain ($\text{DAPI}^+$), and by confirmation that they were missing any staining for mouse H-2Kd. Tumor growth was visualized by imaging the bioluminescence generated by the luciferin-tagged MCF-7 cells (Figure 2.7B).
Figure 2.7 A) Representative images of a captured CTC and a normal mouse cell. Nuclei are stained with DAPI (blue), CTCs are stained for CK (red), and mouse cells for mouse H-2k (green). B) Bioluminescence images of mice implanted with MCF-7 tumors in estrogen positive (E⁺) and estrogen negative (E⁻) groups during the course of tumor progression.

As tumor growth progressed in the xenografted animals, a marked change was visualized in the CTCs detected. In both the estrogen-positive and -negative animal groups, CTC levels rose as the study progressed. In the estrogen-positive group, as expected, the CTC levels increased to a much higher level than in the estrogen negative group. CTC profiles of two representative mice in E⁺ group were shown in Figure 2.8. A clear shift in CTC phenotypic profile could be noticed in the more aggressive cancer model. The CTC profiles in these mice shifted to later zones within the MagRC microfluidic chip relative to early CTCs and cultured MCF-7 cells (Figure S7.10). This observation indicates that their phenotypes were changing and EpCAM levels were decreasing. The profiles of the CTCs from two estrogen negative mice were shown in Figure 2.9. Any shift was not observed in the CTC profiles of this group. At the end of the study, mouse lungs were extracted and sent for histopathology to compare invasiveness of the tumors in the two groups. Mouse lung sections of both groups were analyzed by ex-vivo imaging for micrometastasis detection. Micrometastases were found in lungs of the E⁺ group (Figure 2.10); however, there were no micrometastases in the E⁻ group. The presence of the metastases along with the altered CTC profile observed by MagRC is consistent with the hypothesis that the CTCs produced by the estrogen-positive tumor possess a more invasive profile.
Figure 2.8 CTC distribution profiles of two mice (A&B) in E+ group. Bar graphs show the total number of CTCs found in each day. Each black circle denotes one CTC. The red zone represents the distribution area for cultured MCF-7 cells. The curved diagrams show the scaled normal distribution profiles of CTCs extracted at each time point, centered at the median CTC zonal position. CTC profiles in the E+ model show a shift toward less epithelial phenotypes at the later stages of the disease.
**Figure 2.9** CTC distribution profiles of two mice (A&B) in E’ group. Bar graphs show the total number of CTCs found in each day. Each black circle denotes one CTC. The red zone represents the distribution area for cultured MCF-7 cells. The curved diagrams show the scaled normal distribution profiles of CTCs extracted at each time point, centered at the median CTC zonal position. CTC profiles in the E’ model do not show any shift during the course of study.

**Figure 2.10** A) Bioluminescence image of whole lung of a mouse in the E’ group. Visible luminescence indicates the presence of metastases in lung. B) Histopathology image of lung section of a mouse from the E’ group confirming the presence of micrometases.

### 2.3 Conclusions

We showed a new technique, magnetic ranking cytometry that is able to profile the properties of small collections of CTCs in unprocessed blood. Using a microfluidic chip with 100 discrete
capture zones, CTCs were isolated from blood and trapped in fluidic zones according to the levels of protein markers displayed on the surface of the cancer cells. The use of antibody-functionalized nanoparticles enables this approach to achieve high levels of resolution, as 1000s of binding events can be leveraged to separate cells with varying levels of protein expression. Any surface marker that can be recognized by an antibody can be used with this approach, allowing samples to be profiled for levels of multiple markers.

Magnetic ranking cytometry is able to provide accurate profiles of low levels of CTCs in unprocessed blood samples. MagRC provides information similar to the gold standard method, flow cytometry, but it is compatible with much lower cell numbers and is not affected by normal blood cells.

The high level of sensitivity obtained and compatibility with whole blood makes this technique a powerful tool for the analysis of rare circulating tumor cells. CTCs collected from mice with xenografted tumors were monitored as a function of tumor growth, and an emerging phenotypic profile was acquired for these cells. These results suggest that magnetic ranking cytometry is an effective and accessible approach for CTC profiling that allows the heterogeneity and evolving phenotypes of CTCs to be monitored. CTC profiles can now be more concretely connected with the progression of cancer and the formation of metastatic lesions.

Future work on this device involves validating the approach with patient samples that is critical before it can be translated to the clinic. However, low fabrication rate of the MagRC chip hinders mass production of the device for further use in the clinical studies. In the next chapter, we provide some solutions than can overcome the low fabrication rate problem.

2.4 Material and methods

MagRC microfluidic chip fabrication:
Glass substrates obtained from EMF-Corp (Ithaca, NY) were used to fabricate the MagRC chip. A 1.5 µm Ni layer was sputtered onto the glass slides. The micro-magnet structures were patterned using standard contact lithography processes. First, a positive photoresist layer (S1811) was spin-coated onto the Ni coated glass. The photoresist was exposed to UV light for 10 seconds before being developed in photoresist developer. This was followed by Ni wet etching to reveal micro-magnets, after which the remaining photoresist was stripped away. To pattern the
‘X’-structures on top of Ni micro-magnets, a thick negative photoresist, SU-8 3050 (Microchem, Newton, MA) was spin-coated on top of the nickel coated glass substrates followed by 30 minutes soft-baking. The final thickness of SU-8, and thus the height of channel, was 50 µm. After exposing for 20 seconds, the SU-8 layer was developed using SU-8 developer. Once the micro-magnets and channel structures were completed, the channel was topped with a flat layer of cured polydimethylsiloxane (PDMS). Holes were punched in the PDMS layer, and Teflon tubing was inserted to act as inlet and outlet ports.

Simulation and modeling:
All magnetic field and flow simulations were carried out using COMSOL Multiphysics®. Simulation results were incorporated into a parametric model predicting the capture locations of cells having different levels of magnetic loading. Details of the simulations and modeling are presented in the supporting information.

Cancer cell lines:
MDA-MB-231, SKBR3 and VCaP cell lines were obtained from American Type Culture Collection (ATCC). MDA-MB-231 cells were cultured in Leibovitz’s L-15 medium (ATCC), SKBR3 cells were cultured in McCoy’s 5a Medium Modified (ATCC) and VCaP cells were cultured in DMEM (ATCC). All of the media were supplemented with 10% fetal bovine serum (FBS). MCF-7/Luc human breast cancer cells were purchased from Cell Biolabs Inc. and grown in DMEM (High Glucose) supplemented with 10% FBS, 0.1mM MEM Non-Essential Amino Acids (NEAA) and 2mM L-glutamine. Human prostate cancer cells PC3 (a kind gift from Dr. Alison Allan, London Health Sciences Centre, London,ON,) were cultured in F12K media (ATCC) supplemented with 10% FBS. All cell lines were authenticated and checked for microbial contaminations.

Assessing the level of magnetic beads adsorption to WBC
Fresh blood was collected from healthy donors and RBCs were lysed using 0.5M EDTA, pH 8. MDA-MB-231 and SKBR3 cells also were prepared with the concentration of $10^5$ cells/mL in PBS plus 1%BSA. Then 10µL of anti-EpCAM Nano-Beads (MACS) and 20µL of FcR Blocking Reagent (MACS) were added to the samples (MDA-MB-231 cells, SKBR3 cells and RBC-lysed blood) and incubated for 30 minutes. After washing step, the samples were incubated for another 30 minutes with 1.5 µL of Anti-mouse H-2Kd- Alexa Fluor 488 that served as the secondary
antibody. It was followed by injection of samples into the flow cytometer. Counts versus fluorescence intensity measurements were made in the green channel of the flow cytometer.

**Spiking of tumor cells in whole blood:**
Fresh blood was collected from healthy volunteers, and immediately used for experiments. Different numbers of SKBR3 cells were spiked into whole blood. After this step, some samples underwent an additional RBC lysis step; 1 mL of RBC lysis buffer was used, and this was followed by two washing steps with PBS. Lastly, both whole and RBC-lysed blood samples were run through the MagRC chip and analyzed via flow cytometry.

**EMT induction model:**
SKBR3 cells were seeded in 6-well plates (4×10^5 cells/well). After 24 hours, cells were treated with CoCl$_2$ solution at the final concentration of 150 µM. Cells were incubated for 72 hours in a conventional incubator (37°C; 5% CO$_2$). After this period, cells were harvested using trypsin.

**Orthotropic tumor xenograft model and CT imaging:**
All animal experiments were carried out in accordance with the protocol approved by the University of Toronto Animal Care Committee. 6- to 8-week-old female SCID-beige mice were purchased from Charles River and maintained at the University of Toronto animal facility. 2 days prior to tumor implantation, the subset of mice received a subcutaneous pellet of 60-d sustained release 17-β-estradiol (0.72 mg/pellet; Innovative Research of America). Tumor xenografts were generated by injecting 5 ×10^6 cells suspended in 50 µL of Matrigel (BD Biosciences) orthotopically into the 4th left inguinal mammary fat pad. Mice were anaesthetized by isoflurane before injection. Tumor growth was measured both by caliper and by imaging using a Xenogen IVIS Spectrum imaging system (Caliper Life Sciences). If tumor growth was not observed in a mouse, it was excluded from the rest of study. Prior to imaging, mice were injected intraperitoneally with 100 µL of phosphate-buffered saline containing D-Luciferin substrate (PerkinElmer). At the end of the experiment, animals were euthanized and selected tissues were analyzed by ex-vivo imaging for micro-metastasis detection.

For intermediate CTC capture from tumor bearing mice, 50 – 100 µl of blood was collected from the saphenous vein and for the terminal studies 0.5 ml -1 ml blood was collected from each mouse by cardiac puncture. All blood samples were collected in K2EDTA tubes (Microvette, Sarstedt). During the animal study, randomization was not applied.
Profiling of mouse CTCs and immunostaining:
Collected mouse blood was diluted with PBS-EDTA (100 µL of PBS-EDTA was added to 50 µL of blood). This was followed by adding 10 µL of anti-EpCAM Nano-Beads (MACS) to 150 µL of diluted blood. After 30 minutes incubation with the magnetic beads, blood was pumped through the MagRC Chip at a flow rate of 500 µL/h. Next, 200 µL PBS-EDTA was introduced to flush away any non-magnetically captured non-target cells. Captured cells were then fixed with 4% paraformaldehyde, and then permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) in PBS. Anti-CK-APC (GeneTex) antibody was used to stain CTCs, and mouse cells were marked by anti-mouse-H-2K-FITC antibody to distinguish with CTCs. All antibodies were prepared in 100 µL of PBS and pumped through the chip at a flow rate of 50 µL/hr for 2 hrs. After immunostaining, chips were washed using 0.1% Tween 20 in PBS. Cell nuclei were stained with DAPI ProLong Gold reagent (Invitrogen, CA) at 500 µL/h. After completion of staining, all chips were washed with PBS and stored at 4 °C before scanning.

Image scanning and analysis:
After immunostaining, chips were scanned using a Nikon microscope under 10X objective, and images were acquired with NIS-Elements AR software. Bright field, red (APC channel), green (FITC channel) and blue fluorescence images were recorded. The captured images were then analyzed manually and target and non-target cells were counted.

Histopathology of mouse tumors:
After terminal blood collection, animals were euthanized and lungs, liver, lymph nodes were extracted and fixed in 10% buffered formalin. Fixed tissues were then embedded in paraffin for histological examination with hematoxylin and eosin (H&E) staining. The pathologist was blinded to the different groups of animals during the study.

Antibodies:
The following antibodies were used in this study: CD326 (EpCAM) MicroBeads (130-061-101, MACS-dextran ferrite colloids beads with a diameter of 50 nm, purchased from Miltenyi Biotec), pan-Cytokeratin antibody [C-11] -APC (GTX80205); CD45 Mouse Anti-Human mAb (clone HI30)-Alexa Fluor 488 (MHCD4520); anti-human CD326 (EpCAM)-Alexa Fluor 647 (324212); Anti-mouse H-2Kd-Alexa Fluor 488 (116609); DAPI nuclear stain (R37605), FcR Blocking Reagent (130-059-901).
2.5 References


3  Magnetic and drag force engineering for sorting of cancer cells

In Chapter 2, we demonstrated that the MagRC device is able to profile the low number of cells present in whole blood. Highly accurate phenotypic profiling of circulating tumor cells or CTCs was achieved using the MagRC device. In addition, the efficiency of the device in the analysis of whole blood makes this technique highly amenable to the analysis of rare CTCs. However, fabrication of the MagRC device is time-consuming, and this could hinder mass production of the device for further use in diverse clinical studies.

In this chapter, we present a new device that incorporates both magnetic force and drag force manipulation for high efficiency cell profiling. Compared to the MagRC device, fabrication yield is improved threefold, allowing the production of devices that can be used in different clinical studies. In order to increase the yield of fabrication, we optimized the minimum number of zones required for full resolution. The new design contains 10 discrete zones rather than 100 zones in the MagRC device.

3.1  Results and discussion

3.1.1  Overview of 10-zone device

The 10-zone device leverages immunomagnetic separation\cite{1,2} for profiling rare cells as a function of their surface marker expression. In a manner to the MagRC approach, we used circular nickel micro-magnets patterned within the channel to enhance the externally applied magnetic field\cite{3}. In the MagRC device, the long length of a chip leads to long residence times and the potential for cells to settle towards the bottom of the chip. However, the long length of the device significantly reduced the rate of chip fabrication. In order to increase the rate of chip fabrication while keeping the long residence times of cells in the channel, we opted to increase the width of zones steadily which in turn could reduce the length of device by half.

The device design was engineered to incorporate nickel micro-magnets in a fluidic channel whose width increases systematically. Different designs were simulated and fabricated to select the optimum design for high efficiency cell profiling. The optimal design (Figure 3.1A)
Figure 3.1 A) Schematic of 10-zone device. The microfluidic chip contains 10 distinct zones with varied magnetic and drag forces. B) The new design leads to fabrication of six 10-zone chips per one 4”×4” nickel slide.

contains 10 zones, each of which features nickel micro-magnets and X-structures\textsuperscript{[2]}. The nickel radii increase by 10 \( \mu \text{m} \) sequentially from \( r=145 \mu \text{m} \) to \( r=235 \mu \text{m} \). On the other hand, increasing the width of the channel in the later zones of the device reduces the drag force acting on cells,
allowing the efficient capture of cells with low levels of surface marker expression. This new design made it possible to fabricate of six 10-zone chips per one 4”×4” nickel slide while spending equal time in cleanroom resulted in fabrication of two MagRC devices (Figure 3.1B). Increasing the size of the micro-magnets coupled with the channel width increment increases the area of regions subjected to high magnetic force and low drag force, which subsequently leads to efficient rare cell capture. Similar to MagRC device, a capture zone is defined as the region where the magnitudes of the magnetic and drag forces are comparable, meaning that any cells that pass through a capture zone are expected to be captured. For a cell coated with many magnetic nanoparticles, the capture zone generated by small micro-magnet in regions with high linear velocity is sufficiently large to ensure capture in earlier zones within the device. Therefore, cells having high magnetic loadings are captured in earlier zones, near the inlet, where the small micro-magnets are positioned in zones having small width. However, cells coated with the low number of magnetic nanoparticles are deflected only if they are close enough to the bottom of the chip and the edges of the micro-magnets, where the magnetic force acting on the nanoparticles is highest. At the final zones of the device, large micro-magnets and slow flow create large enough capture zones for capturing of cells with low levels of surface marker expression. In order to determine the size of a capture zone for the cells having high, medium, low levels of magnetic loading, the radius (measured from the center of the ‘X’-structure) of the capture zone was measured at the height of 10µm along the length of the chip (Figure 3.2).

![Graph showing calculation of capture zone radius versus zone number.](image-url)

**Figure 3.2** Calculation of the capture zone radius versus zone number.
3.1.2 Validation of the performance of 10-zone device

Seeking a first suite of experiments to challenge the performance of this device, we explored the profiling capabilities of the 10-zone chip with three cancer cell lines. We selected EpCAM as an initial profiling marker, since it is a well-characterized marker present on the surface of many different types of cancer cells\(^{[4]}\). Three different cell lines, VCaP (a human prostate cancer cell line), SKBR3 (a breast adenocarcinoma cell line), and MDA-MB-231 (a breast cancer cell line with mesenchymal characteristics) were incubated with anti-EpCAM antibodies functionalized with magnetic nanoparticles and analyzed using the 10-zone device. One hundred cells in buffered solution were introduced into the device at a flow rate of 400 µL/hr, captured, and stained using a nuclear marker. The cells trapped in different capture zones were then enumerated using fluorescence microscopy. The three different cell lines exhibited markedly different distributions within the device (Figure 3.3A). High recoveries of the cells injected into the device were achieved (VCaP=93±2%, SKBR3=91±5%, MDA-MB-231=89±2%). VCaP cells, which have the highest level of EpCAM expression, were found primarily in the earlier zones. However, MDA-MB-231 cells which have the lowest level of EpCAM expression were only captured after they were slow enough and encountered the large micromagnets near the outlet of the chip. The relative levels of EpCAM expression of the cell lines were confirmed via flow cytometry (Figure 3.3B). Based on these results, we can conclude that the 10-zone chip is able to sort cells according to the expression level of a targeted surface marker. Moreover, it efficiently captures cells exhibiting even low levels of a targeted surface marker.

![Graph A](image1.png)

![Graph B](image2.png)
Figure 3.3 Profiling cells with different levels of surface marker expression in the 10-zone chip. A) Distribution of VCaP, SKBR3, and MDA-MB-231 cells in the 10-zone chip; EpCAM was used as the profiling marker. 100 cells suspended in 100 µL of buffer were used in these trials. Profiling experiments for each cell line were repeated 3 times. B) EpCAM expression measured by flow cytometry for the three cell lines.

Control experiments were performed to investigate the effect of both channel width increment and nickel micro-magnets for capturing cells with varied levels of EpCAM expression. As a first control experiment, we explored the capture capabilities of a 10-zone chip lacking nickel micro-magnets. One hundred of VCaP, SKBR3, and MDA-MB-231 cells were suspended in 100 µL of buffered solution and introduced into the chip (Figure 3.4A). Capture experiments without nickel illustrate that VCaP cells that have the highest level of magnetic loading were captured at initial zones regardless of using micro-magnets. However, SKBR3 (medium magnetic loading) cells were distributed randomly along the device. The capture efficiency of MDA-MB-231 cells that have the lowest level of EpCAM expression was very low (9%) without incorporating micro-magnets. Control experiment using a 10-zone chip with a channel of fixed width (same width as MagRC device, W=13.6 mm) yielded less useful profiling information, highlighting the crucial role of the channel width increment for capturing low EpCAM cells (Figure 3.4B). Channel width of 13.6 mm is large enough for VCaP and SKBR3 cell capture, and similar to MagRC device, using nickel micro-magnets resulted in an efficient capture of cells having high and medium magnetic loadings. However, longer residence time is required for settling of MDA-MB-231 cells, confirming the requirement of channel width increment for efficient recovery of cells with the low levels of magnetic loading.

Figure 3.4 Control experiments were carried out using a microfluidic chip lacking nickel micro-magnets (A) and using a chip with a channel of fixed width (B).
3.1.3 Model of capture efficiency

A quantitative model was developed to explore the capture efficiency of cells exhibiting varied expression levels. The capture probability at a zone can be calculated as:

\[ P_{\text{capture}} = j \times \frac{A_{Fm>Fd}}{Q} \times \alpha \]  

Where \( j \) is the number of rows of capture structures in each zone, \( Q \) is the flow rate (\( \mu \)L/hr) at each zone, \( A_{Fm>Fd} \) is the average percentage of area surrounding a X-structure in which magnetic force and the drag force are comparable, and \( \alpha \) is an experimentally determined proportionality constant with unit set to ensure \( P_{\text{capture}} \) is unitless (unit is hr/\( \mu \)L).

The capture efficiency in the \( i \)th zone can be calculated as:

\[ E_i = P_i[N - (E_1 + E_2 + \cdots + E_{i-1})] \quad i = 1, 2, \ldots, 10 \]  

In this equation, \( E_i \) and \( P_i \) are defined as capture efficiency and capture probability in the \( i \)th zone, and \( N \) is the total number of loaded cells. Capture efficiency of each zone can be calculated by substituting the capture efficiency terms of the prior zones. In the following, capture efficiencies of zone 1, 2, and 3 have been written as an example:

\[ E_1 = NP_1, \quad E_2 = P_2[N - NP_1] = NP_2[1 - P_1], \quad E_3 = NP_3[1 - P_1 - P_2 + P_1P_2] \]  

The total capture efficiency is the sum of capture efficiencies in each individual zone:

\[ E_T = E_1 + E_2 + E_3 + \cdots + E_{10} \]  

By substituting capture efficiency terms of zones, we get:

\[ E_T = N[P_1 + P_2 + \cdots + P_{10} - P_1P_2 - P_1P_3 - \cdots + P_1P_2P_3 + \cdots] \]  

**Calculation of** \( A_{Fm>Fd} \). Using the capture zone radius calculation (Figure 3.2), the average percentage of area surrounding a capture structure in which the magnetic force and the drag force are comparable, was calculated for cells having high, medium and low levels of magnetic loading. We simulated the spatial distributions of net force acting on a cell and used COMSOL
to calculate the capture zone radii and $A_{F_m>F_d}$. Table 1 summarizes this percentage for VCaP, SKBR3, and MDA-MB-231 cells at different zones.

Table 3.1 Calculation of the average percentage of area surrounding a capture structure in which the magnetic force exceeds the drag as a function of cell line

<table>
<thead>
<tr>
<th>Zone number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A_{F_m&gt;F_d}$ (VCaP)</td>
<td>7.81</td>
<td>7.84</td>
<td>8.81</td>
<td>9.03</td>
<td>11.38</td>
<td>13.44</td>
<td>19.1</td>
<td>35.5</td>
<td>73.9</td>
<td>93.1</td>
</tr>
<tr>
<td>$A_{F_m&gt;F_d}$ (SKBR3)</td>
<td>4.52</td>
<td>4.47</td>
<td>4.89</td>
<td>6.14</td>
<td>6.39</td>
<td>8.57</td>
<td>10</td>
<td>17.6</td>
<td>39</td>
<td>62.9</td>
</tr>
<tr>
<td>$A_{F_m&gt;F_d}$ (MDA-MB-231)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.89</td>
<td>1.47</td>
<td>1.12</td>
<td>1.57</td>
<td>2.05</td>
<td>3.74</td>
<td>7.81</td>
</tr>
</tbody>
</table>

Calculation of $\dot{Q}$. The initial flow rate in the device is set to 400 µL/hr. The flow rates in the successive zones have been calculated according to the width of each zone (Table 4.2).

Table 3.2 Calculation of the flow rates in different zones of the device

<table>
<thead>
<tr>
<th>Zone number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate (µL/hr)</td>
<td>400</td>
<td>311</td>
<td>255</td>
<td>219</td>
<td>151</td>
<td>136</td>
<td>125</td>
<td>88</td>
<td>57</td>
<td>51</td>
</tr>
</tbody>
</table>

Fitting the model to the experimental data ($\alpha$). The data was fit to the VCaP capture efficiency data, and we found the model best fit the data using a proportionality constant of 0.48. For SKBR3 and MDA-MB-231 we found the model best fit the data using a proportionality constant of 0.56 and 0.95, respectively.

Table 3.3 Model parameters used to validate capture efficiency as a function of cell line

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Model parameter ($\alpha$)</th>
<th>Predicted capture Efficiency ($E_{\text{model}}$)</th>
<th>Experimentally measured capture efficiency ($E_{\text{experimental}}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VCaP</td>
<td>0.48</td>
<td>75%</td>
<td>93%</td>
</tr>
<tr>
<td>SKBR3</td>
<td>0.56</td>
<td>74%</td>
<td>91%</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>0.95</td>
<td>86%</td>
<td>89%</td>
</tr>
</tbody>
</table>
3.2 Conclusions

In this chapter, we demonstrated a simplified version of MagRC chip that profiles the properties of small collections of cells. A fluidic chip for immunomagnetic capture of rare cells was developed that selectively isolates cells with different levels of surface markers. The device was engineered to incorporate nickel micro-magnets in a fluidic channel with increasing circumference. The device efficiently recovers cells that exhibit even low levels of surface marker expression. Using control experiments, we confirmed the requirements of incorporating nickel micro-magnets and channel width increment for efficient cell capture and profiling. This new chip design increases the fabrication rate threefold, making it more suitable for different research projects and clinical studies. By analyzing the magnetic and drag forces acting on a cell, we also calculated the capture zone radius for cells having different levels of EpCAM expression and developed a model that investigates cell capture efficiency.

In Chapter 2 and 3, we showed two microfluidic devices that allow surface marker profiling of CTCs. However, further study would assist in the deconstruction of the heterogeneity in CTC subpopulations. Toward this aim, we present in the following chapter a microfluidic approach that profiles cell subpopulations in 2D platform.
3.3 References


4 2D-phenotypic profiling of rare cells

Although CTCs originate from the same primary tumor, they may have heterogeneous phenotypes. Some CTCs possess benign properties while others might have much higher metastatic potential. Deconstructing the heterogeneity of CTCs is challenging and new methods are needed that can sort small numbers of cancer cells according to their phenotypic properties. In Chapter 2 and 3, we showed that MagRC approach and 10-zone device profile rare cells according to their surface marker expression. We argued that low EpCAM cells have more mesenchymal properties and contribute to the formation of metastatic lesions.

Here we further explore the mesenchymal characteristics of cells having different levels of EpCAM expression via a 2D microfluidic approach. An integrated chip was designed that can deconvolute properties of heterogeneous rare cell subpopulations. Cancer cells are first profiled according to their surface marker expression. Cells having different levels of EpCAM expression are separated into four subsets. In the second dimension, the subsets are further separated into 20 subpopulations, according to their migratory behaviors toward a chemotactic agent. We chose chemotaxis-based sorting as the second dimension profiling since it has been proven that chemotaxis is involved in all steps of tumor cell dissemination. This system provides an important new means for characterizing circulating tumor cell subtypes.

This chapter has been submitted as a journal publication:


All experiments in this chapter were performed by M. Poudineh, M. Labib and S. Ahmed unless otherwise indicated.

4.1 Introduction

Overt metastasis that is responsible for as much as 90% of cancer associated mortality is the end results of a multistep process[1-4]. To successfully metastasize, a carcinoma cell must invade, intravasate, extravasate and grow at a distant site[5,6]. It has been thought that chemotaxis plays
an important role in each step of tumor cell dissemination\textsuperscript{[7]}. Cancer cell migration is mediated by chemokine and growth factors\textsuperscript{[8,9]}. Behavior of heterogeneous cancer cell populations is usually dramatically different and only those of cells that can respond fast enough to chemokines are thought to be involved in metastatic pathway\textsuperscript{[10]}. As cancer cells enter into the bloodstream, they start to lose their epithelial markers and gain mesenchymal ones\textsuperscript{[11,12]}. A means to monitor a tumor’s metastatic potential in real time and in a non-invasive way is to profile circulating tumor cells (CTCs) based on their surface marker expression\textsuperscript{[13–15]}. The loss of epithelial characteristics may engender CTCs with greater motility and other characteristics that would support invasion of secondary sites. The high motility of the metastatic CTCs along with their prompt respond to the chemokines helps them to migrate to distance sites and form secondary tumors\textsuperscript{[5,7]}.

Microfluidics opens a new venue to study CTCs\textsuperscript{[15–19]}, however, most of the existing techniques simply capture and enumerate CTCs and do not phenotypically analyze the heterogeneous cancer cell subpopulations encountered in clinical specimens \textit{in situ}. In some recent studies, microfluidic devices have been employed to study the migration of cancer cells toward a chemoattractant. Some endeavors were devoted to study the effect of geometry on cell migration using different channel cross-sectional sizes\textsuperscript{[20,21]}. In order to simulate the cancer invasion process, Chung et al\textsuperscript{[22]} filled the migration channels with hydrogel and extra-cellular matrix components. Zhang et al\textsuperscript{[23]} reported a high-throughput microfluidic device with 3120 microchambers to monitor mesenchymal migration. Chen et al\textsuperscript{[24]} studied individual cells’ migration behavior via a single-cell migration platform. These previous microfluidic approaches study cancer cell migration behaviors, but did not investigate the migration of CTC subpopulations presented in whole blood samples.

Here we developed a new 2D microfluidic approach that allows sorting of heterogeneous cell subpopulations based on both surface marker expression and their migratory behavior toward a chemotactic agent. Cancer cell populations are first profiled according to their surface marker expression. We leverage a fluidic device for immunomagnetic capture of CTCs\textsuperscript{[14]}. ‘X’-shaped structures are incorporated in a microfluidic channel to generate localized areas of low flow velocity, enabling efficient capture of CTCs labeled with magnetic nanoparticles. Linear velocity modulation helps to capture CTCs with different levels of surface marker expression in one of four zones, generating a profile of relative surface marker expression\textsuperscript{[13,14]}. In the second dimensional profiling, cell subpopulations extracted from each zone are subjected to chemotactic
phenotype binning. This approach allows sorting of CTCs in one of five regions of a migration channel. First, we characterized the chemotactic profiling approach. Utilizing different types of cancer cell lines, we showed that the chemotaxis chip is amenable to differentiate between highly chemotactic and non-chemotactic cells. Then we proceeded to extract 20 cell subpopulations using the integrated system. We showed herein using blood samples from mice bearing human prostate carcinoma xenografts, that 2D phenotypic profiling approach provides new information not available using prior methods.

4.2 Results and discussion

4.2.1 Overview of 2D-phenotypic profiling approach

Our sorting approach relies on fluidic capture of heterogeneous subpopulations of cancer cells. In the first sorting dimension, cells having different levels of surface marker expression are profiled into one of four zones (Figure 4.1A and Figure S7.11). Epithelia cell adhesion molecule (EpCAM) was chosen as an initial profiling marker since it has levels that are known to vary according to the invasiveness of cancer cells and their progression through the epithelial to mesenchymal transition (EMT)[1,25]. The sorting is achieved according to levels of bound aptamer-functionalized magnetic nanoparticles that in turn report on EpCAM expression[26]. The zones that are patterned within a microfluidic device feature with microfabricated X-structures to adjust the required drag force for cell capture. Changing the cross sectional area of microfluidic channel could manipulate the linear velocity (and thus the drag force acting on the CTCs) to capture distinct subpopulations of cancer cells exhibiting different levels of surface marker expression. The cross sectional area increases from zone 1 to zone 4. The first zone that has the highest linear velocity retains cells having high levels of surface marker. The succeeding three zones exhibit reduced linear velocities, each decreasing the velocity by a factor of 2. Consequently, the lowest linear velocity at the forth zone helps the accumulation of low surface marker cells. After binning the subpopulations into four successive zones, the cells are released using the antisense DNA strand complementary to the capture aptamer[26]. During the course of first dimensional sorting, the chemotaxis chips serving as the second sorting dimension are disconnected using integrated valves. The chemotactic sorting relies on the migration of extracted cell subpopulations toward a specific chemoattractant. Four chemotaxis chips are connected to the four zones of surface marker expression sorting device with the aim of studying
the migration behavior of extracted cell subpopulations (Figure 4.1B). Each chemotaxis chip contains one cell loading channel that is connected to two chemoattractant reservoirs through the migration channels. Microfabricated traps inside the cell loading channel are used as the capture sites (Figure 4.1C, right). Multiple parameters such as the shape of the trap sites and the gap between the traps and the migration channels were considered in design of the cell loading channel. The parameters were adjusted to have high capture efficiency. These capture sites allow us to monitor single cell migration behavior. When a cell is captured at one trap site, the captured cell blocks the flow through the one side of channel. Thus, the remaining cells migrate through the other side of cell loading channel and are subsequently captured at the downstream capture sites (Figure 4.1D). Migration channels (width=8 µm, height=30 µm, length=1 mm) connect cell loading channel and chemo-reservoirs. Prior to the loading of cells, desired chemoattractant was loaded into chemo-reservoirs that are disconnected from cell loading and migration channels via incorporated valves (Figure S7.11). These valves are used to prevent chemoattractant diffusion into the migration and cell loading channels during cell capture. After chemoattractant loading, released cells flew toward the cell loading channel and were captured at the ensuing trap sites. Subsequently, captured cells started to migrate toward chemical gradient generated in migration channel. To study single cell migration behavior more efficiently, each migration channel is divided into three regions (Figure 4.1C, left). This channel division makes it possible to distinguish between highly mobile cells and cells that do not tend to move fast. In other words, cells that are closer to the chemoattractant reservoir (M3-migrating region) can be more mobile than cells proximal to the cell loading channel (M1-migrating region).

We examined the formation of chemical gradient in the migration channels positioned in the chemotaxis chip. The chemoattractant was loaded into chemo-reservoirs prior to the cell loading. Microfabricated valves were integrated to prevent chemoattractant diffusion into the migration channels. During chemoattractant loading and cell capture, these valves were at closed position (Figure 4.2A). The valves were open once the cells were loaded, allowing the chemical gradient formation. When the valves are open, the concentration of chemoattractant increases linearly along the migration channel as confirmed by simulation (Figure 4.2C and Figure S7.12). Figure 4.2A and 4.2B show the formation of linear gradient profile after 3 hours that was measured experimentally using fluorescein dye.
Figure 4.1 Schematic of the 2D profiling approach. A) Cells are first sorted according to the level of EpCAM expression. High and low EpCAM cells are captured in Z1 and Z4, respectively. Marker expression binning is based on an aptamer-mediated isolation approach. B) After EpCAM sorting, cell subpopulations extracted from each zone are subjected to chemotactic phenotype sorting. Cells are captured in cell loading channel and chemoattractant is loaded into the chemo-reservoirs. C) Magnified image of the chemotaxis chip. i) The cell loading channel is connected to the chemo-reservoir through migration channels. Cells migrate from the cell loading channel toward the chemoattractant reservoir. The migration channel is divided into three zones to study the migration of different cell subpopulation more effectively. Here cells that are closer to the chemoattractant reservoir (M3) can be more invasive than cells proximal to the cell loading channel (M1). ii) Microfabricated traps located in cell loading channel are used as the capture sites. When a cell is captured at one trap site, the captured cell blocks the flow through one side of the channel. Thus, the remaining cells travel through the other side of cell loading channel and are subsequently captured at the downstream capture sites. D) Image of captured cells in the cell loading channel. Prior to the cell loading into the device, cells are incubated with a green fluorescent cell tracker.
Figure 4.2 A) Chemical gradient generation in the migration channel. i) Integrated valves are used to disconnect chemoattractant reservoirs and migration channels, preventing diffusion of chemoattractant to migration channel during cell trapping step. ii) Subsequent to the chemoattractant loading and cell capture, the valves are set to the opened position. After 3 hours, a linear chemical gradient is generated in the migration channel. Fluorescence image of generated chemical gradient in the migration channels is shown. B) Chemical gradient is estimated through the measurement of the fluorescent dye intensity in the migration channel. C) Chemical gradient generation simulation obtained by COMSOL.

The flow distribution was simulated around the capture sites to find the optimal gap size between the capture sites and the migration channels. Our goal is to have high capture efficiency and single cell capture. Two critical parameters were considered: the amount of flow going through the gap 1) before cell capture and 2) after cell capture. The gap size should be large enough to have certain amount of flux going through it while it must be small enough to capture only one cell (average size~10 µm) and block the flow after cell capture. As shown in Figure 4.3A, the size of the gap was varied from 3 to 10 µm and the ratio of percentage of flow through the gap over the total flow before and after cell capture was calculated. This ratio shows the possibility of single cell capture at the trap site. We chose the gap size of 5 µm as the optimal that is small enough for not allowing the cells to pass through while certain amount of flux can flow through it. Figure 4.3B shows the flow distribution in the channel before and after cell capture where the size of the gaps is 5 µm. Utilizing the gap size of 5 µm between the capture sites and the migration channels allows retaining more than 80% of introduced cells (Figure S7.13).
4.2.2 Validation of chemotactic profiling approach

Seeking a first suite of experiments, we explored the performance of the chemotaxis chip. Two model breast cancer cell lines and two model prostate cancer cell lines were chosen to study their migration behaviors. The cells were serum-starved overnight before experiments. Desired chemoattractant with a specific concentration was prepared in the serum-free media and loaded into chemo-reservoirs before cell injection. Cells were injected into the chemotaxis chip subsequent to the chemoattractant loading. In order to verify the effect of chemoattractant on cell migration, control experiments were performed by using a serum free medium instead of the chemoattractant. Then, both control and chemotaxis chips were placed into a cell culture incubator. Cell migration was monitored after 20 hours of incubation. Prior to the incubation, chips were imaged for the initial cell position using a Nikon microscope.

Migration results of PC3 (highly metastatic prostate cancer cell line) and LNCaP (a prostate adenocarcinoma cell line) cells are shown in Figure 4.4. We chose CXCL16 as a chemoattractant to study prostate cancer cells migration behavior. It has been shown that CXCL16 induces prostate cancer cell migration and may play an important role in prostate cancer progression\(^{27–29}\). We explored the migration behavior of both cell lines when 1 µg/mL and 100 ng/mL of CXCL16 were used as the chemoattractant. PC3 cells migration results (Figure 4.4A) showed...
that 100 ng/mL of CXCL16 induces more migration of cells than 1 µg/mL concentration. When 100 ng/mL of CXCL16 was applied as the migratory stimulator, more than 40% of PC3 cells either migrated completely toward chemo-reservoirs or were found in the last one-third of migration channel (M3). However, more than 60% of cells did not migrate or stopped in the first one-third of migration channel (M1) upon stimulation with 1 µg/mL of CXCL16. LNCaP migration in response to both concentrations was found negligible (Figure 4.4B). We also monitored both PC3 and LNCaP cells migration at different time points (0 hr, 5 hr, 10 hr, 15 hr, and 20 hr after cell loading) using 100 ng/mL of CXCL16 as the chemoattractant. The position of 13 cells was measured at each time point. Each red circle denotes the cell position at one time point. Results show that more than 35% of PC3 cells migrated in the first 5 hours of incubation while less than 25% of LNCaP cells migrated toward chemoattractant. Figure 4.4C shows time lapse images of PC3 cells migration. Single-cell distribution right after cell loading is illustrated in the left image. The middle and right images show the cell distribution after 4 and 20 hours incubation, respectively.

Epidermal growth factor (EGF) is an important growth factor associated with the development and spread of breast cancer. We explored the migration profile of MCF-7 (a breast adenocarcinoma cell line), and MDA-MB-231 (a breast cancer cell line with mesenchymal characteristics) in response to 100 ng/mL of EGF\[^{30}\]. Migration results of MDA-MB-231 and MCF-7 cells are presented in Figure 4.5A and 4.5B, respectively. Compared to the control, MDA-MB-231 cells show highly amplified migration upon stimulation with EGF. More than 60% of cells were migrated completely toward the chemo-reservoirs when EGF was used as the chemoattractant. However, MCF-7 migratory was insignificant in response to EGF.

Both prostate and breast cancer cells migration results reveal that metastatic characteristics increases the motility of cells. The two mesenchymal cell lines (PC3 and MDA-MB-231) showed higher mobility than the epithelial ones (LNCaP and MCF-7). Moreover, PC3 cell migration behaviour in response to 100 ng/mL of CXCL16 exhibited heterogeneity in this cell line. While 31% of cells migrated completely toward the chemo-reservoirs, more than 40% of the cells did not migrate or were found in the M1 region of migration channels. These results demonstrate that not only is the proposed chemotaxis chip amenable to profile cells according to their levels of invasiveness, but also it can be used to study single cell migration behavior.
Figure 4.4 Chemotactic phenotype sorting of model prostate cancer cell lines. Serum-starved cells were suspended in serum-free media and captured in the cell loading channel. Prior to the capture, chemoattractant was loaded into the chemo-reservoirs. Control experiments were carried out without the chemoattractant and migration was observed after 20 hours incubation. **A)** Chemotactic migration of aggressive (PC3) prostate cancer cell lines in response to CXCL16 as a chemoattractant. *i)* PC3 migration results show that 100 ng/mL of CXCL16 induces more migration than 1 µg/mL. Here migration was observed after 20 hours incubation. *ii)* PC3 migration was monitored at different time points (0 hr, 5 hr, 10 hr, 15 hr, and 20 hr after cell loading). Position of 13 cells was measured at each time point. Each red circle denotes the cell position at one time point. Results show that more than 35% of PC3 cells migrated in the first 5 hours of incubation. **B)** Chemotactic migration of non-aggressive (LNCaP) prostate cancer cell lines in response to CXCL16 as chemoattractant. *i)* Results demonstrate that LNCaP migration in response to both concentrations is negligible. *ii)* LNCaP migration was observed at different time points. Less than 25% of cells migrated towards chemoattractant. **C)** Images of the single-cell migration assay. PC3 cells were loaded in cell loading channel and 100 ng/mL of CXCL16 was used as chemoattractant. The left image illustrates the single-cell
distribution after cell loading. The middle and right images show the cell distribution after 4 and 20 hours incubation, respectively.

![Graph A: MDA-MB-231 migration](image1)

![Graph B: MCF-7 migration](image2)

**Figure 4.5 Chemotactic phenotype sorting of model breast cancer cell lines.** Serum-starved cells were suspended in serum-free medium and captured in the cell loading channel. Prior to the capture, the chemoattractant was loaded into chemo-reservoirs. Control experiments were carried out without the chemoattractant and migration was observed after 20 hours incubation. **A&B** Results of chemotaxis migration of two model breast cancer cell lines-MDA-MB-231 (A) and MCF-7 (B) in response to 100 ng/mL of EGF as a chemoattractant. Compared to MCF-7, MDA-MB-231 cells exhibited increased migration tendency upon stimulation with EGF.

In order to prove the ability of the chemotaxis chip to differentiate between epithelial and mesenchymal cells, we used an *in vitro* model for EMT. Using CoCl₂ treatment that induces hypoxia[^31], EMT was induced into SKBR3 (a breast adenocarcinoma cell line) cells. Following 24 and 72 hours of CoCl₂ treatment, we used the chemotaxis chip to assess the migration of control and treated samples using 100 ng/mL of EGF as the chemoattractant. The shift observed for treated cells (Figure 4.6) confirms the increased motility of cells that underwent through the EMT treatment compared to the untreated ones.

**Figure 4.6 Analysis of cells representing an *in vitro* EMT model.** Migration of untreated SKBR3 cells toward 100 ng/mL of EGF is minor while CoCl₂-treated cells tend to move after 24 and 72 hours of treatment.
4.2.3 Validation of 2D-phenotypic profiling

Subsequent to the validation of chemotaxis chip, we proceeded to challenge the entire integrated chip. PC3 cells were first sorted according to their levels of EpCAM expression. First dimensional sorting was carried out after tagging the cancer cells with magnetic nanoparticles-labeled EpCAM specific aptamer\[^{26}\]. Prior to the cell tagging, magnetic nanoparticles functionalized with streptavidin were conjugated to the biotinylated aptamer. Further details are provided in material and methods section. Serum-starved PC3 cells were suspended in a serum free medium to a final concentration of $10^3$ cells/mL. Then 100 μL of the magnetically labeled cells was introduced into the four zone device to sort cells according to their levels of EpCAM expression. The first zone captured highly EpCAM cells while low EpCAM cells were captured in the fourth zone. The EpCAM profile of PC3 cells is shown in Figure S7.15. EpCAM profiling was followed by cell release using 200 μM AS-EpCAM1, the corresponding antisense DNA. Later the released cells were subjected to chemotactic phenotype sorting using 100 ng/mL of CXCL16 as the chemoattractant. The 2D sorting profile of PC3 cells presented in Figure 4.7 (See Figures S7.16 for data with error analysis) demonstrate that the cells are distributed in the microfluidic chip according to their levels of EpCAM expression and migratory behavior.
Figure 4.7 Validation of 2D sorting approach. Prior to the experiment, PC3 cells were serum-starved for 18 hours. Serum-starved cells were suspended in serum free media. First dimensional sorting was performed based on EpCAM expression using an EpCAM specific aptamer tagged with magnetic nanoparticles. After releasing the cells using the corresponding antisense DNA, AS-EpCAM1, cell subpopulations extracted from each zone were subjected to chemotactic phenotype sorting. CXCL16 (100 ng/mL) was used as the chemoattractant. Migration of cells was monitored after 20 hours incubation.

4.2.4 2D-phenotypic profiling of spiked samples

We evaluated whether the integrated system could monitor 2D phenotypic properties of cells present in unprocessed whole blood sample. 1 mL of whole blood was spiked with 1000 PC3 cells. Following EpCAM sorting and releasing four different cancer cell subpopulations, 100 ng/mL of CXCL16 was used to stimulate chemotactic migration of extracted cell subpopulations. After 20 hours incubation, chemotactic migration was monitored. In order to distinguish between target cells and WBCs, immunostaining was carried out (Figure 4.8B). According to the position of cells in the migration channels, cancer cells were allocated to distinct regions of migration channel (non-migrated, M1-migrating, M2-migrating, M3-migrating and migrated) (Figure 4.8A). Results shown in Figure 4.8 confirm that cancer cell subpopulations with different levels of EpCAM expression exhibit disparate migration demeanor. Compared to the cell subpopulations extracted from high EpCAM zone, low EpCAM cells demonstrate increased migration tendency upon stimulation with the chemoattractant.

Figure 4.8 2D-phenotypic sorting chip was applied to cells in whole blood sample. A) PC3 cells were spiked into 1 mL of whole blood. Prior to the experiment, blood cells depletion was performed. After EpCAM sorting, cell
subpopulations were extracted from each zone and subjected to chemotactic sorting. Results show the cell migration after 20 hours incubation. B) Immunofluorescence images of a migrating cell (inside the channel) and a migrated cell (all the way to the chemo-reservoir). Staining was performed with DAPI and anticytokeratin (CK) to identify cancer cells.

4.2.5 Monitoring 2D migration phenotypes of CTCs in cancer xenograft model

The integrated system was challenged with blood from mice bearing xenografted tumors to evaluate the utility of system for the analysis of CTCs. Three prostate cancer cells (LNCaP, PC3 and PC3M) were implanted orthotopically into immunodeficient mice. Tumor growth was visualized by MRI imaging (Figure S7.17). Mice were sacrificed 4 weeks after tumor cell injection and 700 µL of their blood was collected for analysis using 2D-phenotypic profiling approach. Surface marker sorting of CTCs was performed according to their levels of EpCAM expression. After binning the subpopulations into four sequential zones, we released CTCs using the antisense DNA Strand. The released CTCs were subjected to the chemotactic migration using 100 ng/mL CXCL16 as the chemoattractant. Then, the chips were placed into a cell culture incubator. Prior to the incubation, chips were imaged for the initial cell position. Subsequent to 20 hours of incubation, immunostaining specific for the implanted human cancer cells was performed (Figure 4.9A).

2D-phenotypic profiles of CTCs extracted from three xenograft models are shown in Figure 4.9. PC3 and PC3M cells are known to have metastatic characteristics while LNCaP cells possess benign properties. In both invasive and non-invasive animal groups, CTC levels increased. Total numbers of captured CTCs from mice with xenografted tumors are as follows: LNCaP xenograft (Figure 4.9B): i) 27 and ii) 45, PC3 xenograft (Figure 4.9C): i) 42 and ii) 60, and PC3M xenograft (Figure 4.9D): i) 66 and ii) 73. As results show CTCs were mostly captured at the later zones of EpCAM sorting chip (zone 3 and 4) as tumor growth progresses in both invasive and non-invasive groups (EpCAM profiles of CTCs are shown in Figure S7.18). This indicates down regulation of EpCAM expression on the surface of CTCs. Metastatic xenograft results (Figure 4.9B and 4.9C-Table S7.4) suggest that more than 40% of the extracted CTCs were highly chemotactic and migrated toward chemo-reservoirs upon stimulation with CXCL16. These results confirm the high mobility of metastatic CTCs. However, compared to the invasive groups, migration of CTCs that originate from LNCaP tumor (Figure 4.9D) is negligible that in
turn suggests less mobility of non-metastatic CTCs (percentages of migratory CTCs in each group are provided in Table S7.4).

Figure 4.9 2D-phenotypic profiling of CTCs in cancer xenograft models. First, CTCs were sorted based on their EpCAM expression. Subsequent to releasing the CTCs using the corresponding antisense, CTC subpopulations extracted from each zone were subjected to chemotactic phenotype sorting. CXCL16 (100 ng/mL) was used as the chemoattractant. Migration of CTCs was monitored after 20 hours incubation. In order to distinguish between CTCs and mouse cells, immunofluorescent staining was performed A) Representative images of a captured CTC and a normal mouse cell. Nuclei are stained with DAPI (blue), CTCs are stained for CK (red) and Vimentin (orange) and mouse cells for mouse CD45 (green). B) 2D-phenotypic profiles of CTCs extracted from two mice in LNCaP xenograft. Total numbers of captured CTCs are: i) 27 and ii) 45. C) 2D phenotypic profiles of CTCs extracted from two mice in PC3 xenograft. Total numbers of captured CTCs are: i) 42 and ii) 60. D) 2D-phenotypic profiles of CTCs extracted from two mice in PC3M xenograft. Total numbers of captured CTCs are: i) 66 and ii) 73.
4.3 Conclusions

Here we demonstrated a 2D technique that is successfully used to isolate 20 different cell subpopulations defined by the expression levels of a surface marker and chemotactic migration. This approach provides accurate phenotypic profiles of low levels of CTCs in unprocessed blood samples. The high levels of sensitivity obtained and compatibility with whole blood samples makes this technique a powerful tool for the analysis of rare circulating tumor cells. CTCs collected from mice with xenografted tumors were monitored for both losing epithelial characteristics and migratory behaviors, and an emerging phenotypic profile could be collected for these cells. 2D phenotypic profiling approach therefore is an effective approach for CTC profiling that allows the heterogeneity phenotypes of CTCs to be monitored.

Future work on this device involves performing downstream analysis on extracted cell subpopulations such as gene expression; with the aim of better understanding of CTC behaviors. However, analyzing the molecular signature of cancerous cells requires high quality of biological markers. In the following chapter, we describe a technique that allows extracting RNA from interior of a cell, providing biomarkers with high integrity for further molecular analysis.

4.4 Material and methods

2D chip fabrication:
Polydimethoxysilane (PDMS, Dow Chemical, US) soft lithography was applied to fabricate 2D chips. First, Silicon masters were fabricated using 5 different masks. Four layers of SU8 (Microchem, US) with different heights were patterned to make EpCAM sorting part of the device. Each layer of SU8 was patterned separately to make different zones of the chip. Then chemotaxis chips layer was fabricated. SU8 with the height of 30 μm was used to serve as the second dimensional sorting part of the device. Integrated valves master was made separately. PDMS and curing agent with the ratio of 5:1 was poured on the chip master and the ratio of 20:1 was spun on the valve master. Both masters were baked at 80°C for 40 mins. PDMS of chip master was peeled and re-attached to the PDMS of valve master, allowing formation of integrated PDMS layer. It was followed by baking of PDMS at 67°C for 2.5 hours. After we peeled the replicas, we pierced holes to connect the tubing. PDMS replicas were attached to glass cover slips using a 30s plasma treatment and left to bond overnight. Afterward, the silicon tubing was attached to the inlet and outlet of the device. Prior to use, devices were conditioned with 1%
Pluronic F68 (Sigma-Aldrich, US) in phosphate-buffered saline (PBS) for 1 hour in order to reduce nonspecific adsorption.

Cancer cell lines:
MDA-MB-231, SKBR3 and MCF-7 human breast cell lines were obtained from American Type Culture Collection (ATCC). MDA-MB-231 cells were cultured in Leibovitz’s L-15 medium (ATCC), SKBR3 cells were cultured in McCoy’s 5a Medium Modified (ATCC). All of the media were supplemented with 10% fetal bovine serum (FBS). MCF-7 cell line was cultured in ATCC formulated Eagle's Minimum Essential media supplemented with 0.01 mg/ml human recombinant insulin (Invitrogen) and 10% FBS. Human prostate cancer cells, PC3, LNCaP, and PC3M were obtained from Dr. Alison Allan, London Health Sciences Centre, London, ON. PC3 cells were cultured in F12K media (ATCC) supplemented with 10% FBS while PC3M and LNCaP cells were cultured in RPMI-1640 (ATCC) supplemented with 10% FBS. All cell lines were authenticated and checked for microbial contaminations.

Single cell migration assay:
Cells were serum-starved for overnight prior to the experiments. In order to improve the imaging quality, cells were incubated with green fluorescent (Invitrogen, Cell tracker Green C2925) dye for 45 mins. Then, the cells were harvested and re-suspended in serum free culture media to a concentration of 104 cells/mL. Chemotaxis chips were conditioned with 1% Pluronic F68 in phosphate-buffered saline (PBS) for 1 hour to reduce nonspecific adsorption, prior to use. It was followed by applying the desired chemoattractant prepared in serum free media while the valves are in the closed position. Subsequently, 10 µL of the cell solution was injected into the cell loading, and cells were captured at the trapping sites. After cell capture, the valves are set to the opened position, allowing formation of chemical gradient. Then, the entire chip was placed into a cell culture incubator. Migration distance was measured based on the final cell position after 20 hours of incubation without media replenishment.

Cell tagging with magnetic nanoparticle-labeled aptamer:
Briefly, 100 µL of 20 µM of the aptamer solution in Dulbecco's phosphate-buffered saline (DPBS, Sigma-Aldrich, US) was first denatured for 5 min at 95°C then renatured on ice for 10 min. Afterward, the aptamer solution was added to the wells of the microtitre plate and incubated with 1 µL of 10 mg/mL of streptavidin coated magnetic nanoparticles (100µm, Chemicell, US)
for 1 hour at room temperature. Subsequently, the nanoparticles were deposited using a magnetic stand (Thermofisher, US) and washed twice with DPBS. Prior to loading into the fluidic device, the aptamer-labeled magnetic nanoparticles were incubated with the cells either in 1% BSA in DPBS or in blood for 1 hour at room temperature.

Table 4.1 Sequence of the nucleic acids (Integrated DNA Technologies, US), utilized in the experimental setup

<table>
<thead>
<tr>
<th>Nucleic acid</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>EpCAM1</td>
<td>Biotin–(TEG)–5' TGA AGG TTC GTT GTG GTG GTA GAC TCT TTA GAA GAG ATA CAG ATT TTG GGA ATG 3'</td>
</tr>
<tr>
<td>AS–EpCAM1</td>
<td>5' CAT TCC CAA AAT CTG TAT CTC TTT TAA AGA GTC TAC ACC CAC CGA AAC AAC CAA CCT TCA 3'</td>
</tr>
</tbody>
</table>

Spiking of tumor cells in whole blood:
Fresh blood was collected from healthy volunteers, and immediately used for experiments. PC3 cells were spiked into whole blood. The red blood cells were lysed for 5 min using a red blood cell lysis solution. Afterward, the blood was depleted from leukocytes using magnetic nanoparticles-labeled anti-CD15 antibodies. The nanoparticles and the bound leukocytes were subsequently separated using a magnetic stand. The supernatant was mixed with 75 µM mercaptoethanol (nuclease inhibitor) and 20 µM EpCAM1 aptamer solution in DPBS for 1 hour at room temperature. Then spiked samples were run through the 2D phenotypic profiling chip and analyzed to extract 20 different subpopulations.

EMT induction model:
SKBR3 cells were seeded in 6-well plates (4×10⁵ cells/well). After 24 hours, cells were treated with CoCl₂ solution at the final concentration of 150 µM. Cells were incubated for 24 and 72 hours in a conventional incubator (37°C; 5% CO₂). Then, cells were harvested using trypsin.

Orthotropic tumor xenograft model and MRI imaging:
All animal experiments were carried out in accordance with the protocol approved by the University of Toronto Animal Care Committee. 6- to 8-week-old male athymic nude mice were purchased from Envigo and maintained at the University of Toronto animal facility. Tumor xenografts were generated by injecting 10⁶ cells suspended in 25 µL Hank's buffered salt solution (HBSS; Life Technologies), orthotopically into the right dorsolateral lobe of the
prostate. Tumor growth was monitored by MRI imaging. Four weeks after tumor injection, whole blood was collected from each mouse by cardiac puncture under anaesthesia, for chip run. All blood samples were collected in EDTA tubes (BD Microtainer, Franklin Lakes, NJ).

**Profiling of mouse CTCs and immunostaining:**

700 µL of collected mouse blood was run through 2D phenotypic profiling chip. First red blood cells lysis was performed and it was followed by mouse cell depletion using anti-CD15. Then the sample was mixed with 75 µM mercaptoethanol (nuclease inhibitor) and 20 µM EpCAM1 aptamer solution in DPBS for 1 hour at room temperature. The prepared sample was pumped through the first dimensional sorting chip at a flow rate of 25 mL/h to separate CTC based on the level of EpCAM expression. Then CTCs were released using the antisense DNA Strand. The released CTCs were subjected to the chemotactic migration using 100 ng/mL CXCL16 as the chemoattractant. Then, chips were placed into a cell culture incubator. Prior to the incubation, chips were imaged for the initial cell position. Subsequent to 20 hours of incubation, migrated cells were fixed with 4% paraformaldehyde, and then permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) in PBS. Anti-CK-APC (GeneTex), CK19-Alexa 647 (Biolegend), and Vimentin-Alexa F-547 (Abcam), antibodies were used to stain CTCs, and mouse cells were marked by mouse anti-CD45-Alexa488 (Bioscience) to distinguish with CTCs. All antibodies were prepared in 50 µL of PBS and pumped through the chip at a flow rate of 50 µL/hr for 1 hr. After immunostaining, chips were washed using 0.1% Tween 20 in PBS. Cell nuclei were stained with DAPI ProLong Gold reagent (Invitrogen, CA) in PBS at flow rate of 300 µL/h. After completion of staining, all chips were washed with PBS and stored at 4 °C before scanning.

**Image scanning and analysis:**

After immunostaining, chips were scanned using a Nikon microscope under 20X objective, and images were acquired with NIS-Elements AR software. Bright field, red (APC and TRITC channels), green (FITC channel) and blue fluorescence images were recorded. The captured images were then analyzed manually to define the positions of migrated, migrating and non-migrated cells.
4.5 References


5 Rapid electrical release of biomarkers from cells

In previous chapters, we demonstrated some microfluidic techniques that allow capture and profiling of rare cells. However, further understanding of cell behavior necessitates genetic analysis in molecular levels. Biomarkers including nucleic acids, proteins, and small molecules provide a wealth of information pertinent to diagnosis and treatment ranging from cancer to infectious disease. The release of these molecules from within cells, called cell lysis, is a crucial step in biomarker analysis. This is typically performed by lysing or permeabilizing the cell membrane. Much effort has been put toward the realization of devices for automated cell lysis and molecular diagnostics that are miniaturized, cost effective, and operable at the point-of-care. In particular, the use of microfluidics provides numerous benefits to scientists. Reduced consumption of reagents, offering to perform analysis with high precision and high throughput, and allowing making integrated device for diagnosis of diseases are some of the advantages of using of microfluidics. In this chapter, we seek to develop an approach to develop pure, high-throughput electrical lysis with the goal of retaining high quality RNA. One main drawback of electrical lysis is its need for high voltage, especially, when lysing of bacteria is the goal. We show that if three-dimensional structures with very sharp, extended features are generated, then it is possible to produce a high field at lower applied potentials than used previously.

This chapter appeared as a journal publication:


All experiments in this chapter were performed by M. Poudineh unless otherwise indicated.

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5.1 Introduction

The release of biomarkers from the interior of a cell represents a key step in biomarker analysis. This is typically performed by lysing or permeabilizing the cell membrane. On-chip cell lysis strategies include chemical, thermal, mechanical, electrochemical, and electrical methods that
compromise membranes. One highly effective means of cell rupture is electrochemical lysis, where application of an electric field leads to hydrolysis of H$_2$O and concomitant production of hydroxyl radicals as well as hypochlorite ions\cite{1}. While this approach facilitates cell membrane rupture, it may also degrade the biological molecules that are detection targets\cite{2}. Specifically, the alkaline environment and the by-products of electrolysis at the anode degrade RNA and can negatively affect downstream analysis\cite{3}. Since RNA is an important biomarker for disease diagnosis, development of techniques to efficiently release RNA that is of sufficient quantity, purity, and integrity from cell lysates is an important challenge. With the goal of overcoming these limitations of electrochemical lysis, we focus herein on the development of a device that seeks to facilitate purely electrical lysis. Here the goal is rapid rupture of prokaryotic cell walls accompanied by the preservation of the intracellular components with minimal negative effects on subsequent analytical analysis.

Electrical lysis proceeds via electroporation, where the application of the electric field transiently produces small pores in the cell membrane. A drawback of electrical lysis has historically been the need for a high electric field (e.g. 10 kV/cm), which typically implies the use of high bias voltage\cite{4}. To address electric field requirement, Wang et al\cite{5} took advantage of a geometry modification that locally amplified the electrical field; however, the required voltage was still high (500 V), and the applied DC voltage in solution hydrolyzed water and thereby created a high concentration of hydroxyl radicals. The application of an alternating current (AC) voltage offers an avenue to purely electrical lysis, since water electrolysis can be decreased under rapidly-modulated electrical field\cite{6}. Previously-reported microfluidic systems for electrical lysis have employed frequencies in the range of 10 kHz; however, these systems suffered low throughput\cite{7}. Further, at these low frequencies, the pulse duration remains sufficient to electrolyze the water and create unwanted hydroxyl radicals and other by products that degrade RNA\cite{8}.

Here we describe an approach to purely electrical, high-throughput lysis with the goal of retaining high quality RNA. We hypothesized that if we could generate three-dimensional structures with very sharp, extended features, then we could generate a high field at lower applied potentials than used previously. Metal electrodeposition is an ideal approach to generate such structures. A significant body of prior work\cite{9-14} has shown that metal electrodeposition is a highly tunable process that can be used to generate structures on many different length scales.
We used electrodeposition to generate arrays of large structures with sharp, extended features within a channel, and used these structures in conjunction with high AC modulation frequencies to prevent electrolysis. This approach was shown to produce efficient bacterial lysis. When planar electrodes are employed, they fail to produce biomarker release, highlighting the need for three-dimensional structures to generate a high field. We found that only by employing three-dimensional, sharp-tipped electrodes (3DSTEs), readily fabricated by bottom-up growth via electrodeposition, were we able to achieve the combination of acceptable driving voltages, efficient biomarker release, and preservation of RNA quality. We feature the 3DSTEs in a microfluidic platform and fabricate a high-throughput device for the front-end processing of cells and bacteria for bioanalysis. Using 3DSTE in multiple parallel microfluidic channels, we process 1 mL of sample in less than 20 minutes. The feasibility of this method in microfluidic channels will allow for automation and integration of the lysis with downstream on-chip sensing.

5.2 Results and discussion

5.2.1 Overview of electrical lysis approach

We showed our device concept in Figure 5.1. The sample to be processed flows over and between the 3DSTEs used to apply an electric field with the goal of biomarker release (Figure 5.1A). A thick insulating photoresist (SU-8) serves as a spacer to define the vertical height of the channel. A thin chromium adhesion layer ensures reliable adhesion of Au to the surface of the glass chip. This Au layer serves as a site for gold electrodeposition and the growth of 3DSTEs. Similar structures were previously developed and used as electrochemical sensors for nucleic acids\cite{15,16}, proteins\cite{17} and small molecules\cite{18}, where the generated high surface areas facilitated ultrasensitive detection. Here it is the sharp features of these structure that are attractive, coupled with a bottom-up fabrication strategy that permits large structures to be arrayed across the flow path of a fluidic channel. The dimensions of the channel accommodate flow rates as high as 100 μL/min, excellent throughput even for large-volume samples such as blood, where typical patient samples are in the range 5–10 mL. The 3DSTEs are visualized using SEM as shown in Figure 5.1C. The structures are electrodeposited from a solution of gold ions. Electrodeposition growth conditions (see material and methods) were selected: i) to ensure that the 3DSTEs were as tall as they were wide (20–40 μm) to maximize reach into the solution being processed, and ii) to maximize the sharpness (minimize the radius of curvature) of the 3DSTE tips.
5.2.2 Electrical lysis of bacteria

In order to evaluate whether the 3DSTEs could dramatically lower the voltage required for biomarker release, we turned to a combination of modelling and experiments. The transmembrane potential (TMP) is the key figure that determines whether membrane electroporation will occur, with 1 V being the typical estimated threshold for electroporation of bacterial cell walls. The dependence of the TMP on modulation frequency and the cell’s properties can be calculated with the expression\(^{[4,5,14]}\):

\[
\Delta \varphi = 1.5 RE_0 \cos \theta / (1 + (2\pi f \tau)^2)^{1/2}
\]

(1)

where \(\tau\) is

\[
\tau = RC_m \left( \rho_{int} + \frac{\rho_{ext}}{2} \right)
\]

(2)
In these equations, $f$ is the frequency of AC signal, $C_m$ is the cell membrane capacitance, and $R$ is the radius of the cell. Based on these equations and literature parameters of bacteria $^{[19]}$ ($C_m = 1.5 \ \mu\text{f/cm}^2$, $\rho_{\text{int}} = 4.5 \ \Omega\cdot\text{m}$, $\rho_{\text{ext}} = 9 \ \Omega\cdot\text{m}$, and $R = 1 \ \mu\text{m}$), we could conclude that electrical field of 10 kV/cm is necessary to produce 1 V TMP required for bacteria cell membrane electroporation. We plotted the transmembrane potential as a function of frequency in figure 5.2A for the case of an applied electric field of 10 kV/cm. There is a modulation frequency window out to about 1 MHz over which the transmembrane potential can be maintained at a high level. We also used simulation to explore whether structuring of electrodes could dramatically increase the local electric field for a given AC applied bias. Figure 5.2B shows the simplified structured electrodes schematic used for the electrical field simulation. These electrodes mimic the morphologies of the 3DSTEs, but given the complexity of the fractal structures, only represent an approximation. It is apparent from the model that the x-y plane demonstrates higher electrical field concentration near the sharp tips of the electrodes. The peak field of structured electrodes near the sharp tips showed hot spots of 20 kV/cm under the bias conditions we considered, but the planar electrodes field was limited to 8 kV/cm (Figure 5.2C)
Figure 5.2 Modeling electrical fields produced at sharp-tipped electrodes. A) Modeling of transmembrane potential (TMP) of bacteria caused by electrical field of 10 kV/cm. For frequencies higher than 1 MHz, TMP drops dramatically. B) Electrical field distribution at voltage V = 40 V (corresponding to the maximum for an AC voltage of 80 Vpp). Regions shown in red represent high field density and regions in blue show low field density. C) Electrical field generated by 3DSTEs versus planar electrodes. The peak field of structured electrodes showed hot spots of 20 kV/cm while the planar electrodes field was limited to 8 kV/cm.

5.2.3 Validation of bacterial lysis via 3DSTEs

We explored, via experiment, the role of modulation frequency and voltage in the efficiency of biomarker release, and also in achieving a low electrochemical current indicative of minimal hydroxyl generation. As seen in Figure 5.3A, by operating at 500 kHz, we were able to suppress by over one order of magnitude (compared to lower frequencies) the current associated with RNA-degrading radical production. To identify a boundary condition for electrical and electrochemical lysis, we measured the current generated at a voltage of 80 Vpp as a function of frequency. As shown in the inset to Figure 5.3A, for any frequencies higher than 500 kHz, the current decreases by more than one order of magnitude while for lower frequencies, significant current is generated. We determined that 500 kHz represents an approximate boundary frequency between electrical and electrochemical lysis. While all frequencies greater than 500 kHz result in very low current, we opted to stay at 500 kHz to study electrical lysis as transmembrane potential drops dramatically when nearing 1MHz (Figure 5.2B).

The original hypothesis behind the design of the 3DSTE device was that the sharp tips of the electrodes would create a strong electrical field that could not be achieved with planar electrodes. To investigate the role of the morphology of the 3DSTEs directly, we compared a set of planar electrodes versus the 3DSTEs for an AC voltage of 80 Vpp. In Figure 5.3C, results from flow cytometry trials are shown that evaluate the levels of propidium iodide\textsuperscript{[20]} taken up in the differently treated samples. Under these conditions, the uptake of the dye by bacteria processed using planar electrodes was essentially the same as for the case of the unprocessed (no field applied) control. In contrast, when 3DSTEs were employed, uptake consistent with complete lysis (i.e. comparable to a heat-lysed reference sample) was observed. These results confirm that the morphology of the 3DSTEs is a requisite feature of a device that can perform high-throughput electrical lysis.
We sought also to demonstrate that the flow rates of our system could provide clinically-relevant sample processing speeds. The multi-channel devices we created achieved an aggregate sample processing rate of 50 µL/min combined with an electrical lysis efficiency of 95% (Figure 5.3D). This corresponds to 1600 cells/s, notably higher than in prior reports[6].

![Figure 5.3](image)

**Figure 5.3 Identification of conditions causing electrical versus electrochemical lysis.** A) Current versus voltage at different frequencies (50 kHz, 250 kHz, and 500 kHz. B) Lysis efficiency versus voltage at different frequencies. C) Flow cytometry measurements of cells that were unlysed (green), plain (orange) or structured (blue) electrodes, or thermally lysed (red). The conditions for lysis are f=500 kHz, V_{pp}=80 V, and lysis/flow rate =1600 cell/s or 50 µL/min. D) Electrical lysis efficiencies of high throughput device that were measured at different flow rates.

5.2.4 Characterization of the integrity of RNA isolated from cells

The main advantage of pure electrical lysis is the release of intact biomarker. We performed qPCR (quantitative polymerase chain reaction) and RIN (RNA integrity number) measurements to characterize the integrity of extracted RNA with the identified conditions that triggered
electrochemical versus electrical lysis. As shown in Figure 5.4, cells were subjected to conditions producing the different types of lysis. The rupture of the cells was confirmed using flow cytometry and growth of bacteria on agar plates. The RNA isolated from each sample was then analyzed to extract a RNA integrity number. RIN evaluates the 23S to 16S rRNA ratio\textsuperscript{[17]}, and translates it to a number between 1 and 10, where 10 correspond to the highest RNA quality. As seen in Figure 5.4C, the RIN of RNA from a sample subjected to electrical lysis samples is excellent (RIN=8.5), while the RNA is strongly degraded when electrochemical lysis and the RIN decreases (RIN=4).

![Figure 5.4 Evaluation of RNA integrity via RIN measurement. A) Flow cytometry uptake and growth of unlysed sample. Flow cytometry measurement, growth of bacteria and RIN measurement results of B) electrochemically and C) electrically lysed samples.](image)

In order to further characterize the integrity of the nucleic acids released using electrical versus electrochemical lysis, we investigated qPCR efficiencies for five reference genes specific to E. coli (Figure 5.5). The RNA was isolated from the cells using both methods, reverse transcribed
into DNA, and amplified. As shown in Figure 5.5, there was a significant increase in the copy number of RNA transcripts (non-degraded RNA) available in electrically lysed E. coli cells in comparison to electrochemically lysed samples for all five genes as shown by the differences in the average Ct values (30.4 ± 0.2 and 24.5 ± 0.1 for electrochemical and electrical lysis methods, respectively). Thus, using electrical lysis instead of electrochemical lysis is preferred, especially when rare transcripts are analyzed.

Figure 5.5 Assessment of mRNA integrity by qPCR. Analysis of qPCR efficiency for five E. coli RNA transcripts in cells lysed via electrical (grey) or electrochemical (black) treatment. (We wish to acknowledge Laili Mahmoudian for performing qPCR experiments.)

5.3 Conclusions

Herein we demonstrated a microfluidic device for the preparation of samples for subsequent molecular bioanalysis. The approach leverages three-dimensional, sharp-tipped gold electrodes created using electrodeposition to generate a high local electric field at sharp tips. This allowed operating at practical voltages, and also at AC modulation frequencies at which a vastly lower rate of current, and thus radical production, was achieved. Multiple-channel device was used to increase the flow rate up to 50 µL/min that makes the device suitable for high throughput biomarker extraction.
5.4 Material and methods

Bacterial preparation:
E. coli (Invitrogen, Carslbad, CA) was cultured in an LB Miller medium in an incubating shaker at 37°C for 14 hours. Prior to experimentation, the growth medium was replaced with 1× PBS. To assess the bacterial concentrations, optical absorption was measured at 600 nm.

Chip fabrication:
Glass substrates obtained from Telic Company (Valencia, CA) were used to fabricate lysis chips. A 5 nm Cr (adhesion layer) followed by a 50 nm Au were sputtered on to the glass substrate. A top-coat of positive photoresist (AZ1600) was used. Contact lithography was used to pattern the interdigitated electrodes; the width and distance between electrodes were both equal to 50 µm. These electrodes were used as substrate for electrodeposition of gold structures. After exposing for 10 seconds, photoresist was developed. This was followed by Au and Cr wet etching and removal of the top resist. A negative photoresist, SU-8 2002 (Microchem, Newton, MA), was spin-cast at 4000 r.p.m for 40 seconds to create the apertures for electrodeposition. Line apertures (W=5µm) were imaged into the interdigitated electrodes covered by SU-8. To fabricate the lysis chamber, a thick SU-8 layer (3025) was spin-cast at 2000 r.p.m for 40 seconds. The final thickness of the SU-8 layer was 40 µm. Lastly, the chamber mask was imaged on the surface and the two SU-8 layers were developed using SU-8 developer for 5 minutes.

3DSTE fabrication:
After dicing, chips were washed with acetone, IPA and dH₂O, and etched in O₂ plasma using Samco-RIE-1C reactive ion etcher at 75 W for 60 seconds to clean the surface completely. The structured electrodes were fabricated by electroplating using a standard 3 electrodes system with Ag/AgCl as the reference electrode, a platinum auxiliary electrode and the 5 µm line gold aperture as the working electrode. The working solution was 50 mM HAuCl₄ and 0.5 M HCl and electrodes were electrodeposited for 100 seconds at 0 mV.

Chip assembly:
After electrodeposition, chips were washed again using dH₂O and etched in O₂ plasma to make a hydrophilic surface for subsequent covering with polydimethylsiloxane (PDMS). PDMS was used as a lid to cover the lysis chamber. In PDMS layer, holes were punched as inlet and outlet.
Flow cytometry:
Following lysis, samples were incubated with propidium iodide at concentration of 25 µg.mL⁻¹ for 30 minutes in the dark. After incubation, samples were injected into a BD FACS Canto flow cytometer and measurements were plotted as histograms of fluorescence intensity.

RNA integrity number (RIN) measurement:
The integrity of RNA was assessed at TCAG Microarray Facility of The Hospital for Sick Children (HSC) by analysing a 1 µL aliquot using a RNA6000 Nano Lab Chip on an Agilent 2100 bioanalyzer. The RIN calculation software was applied to the fluorescence profiles after separation of RNA by capillary electrophoresis, and RIN values were calculated on a scale of 1–10 (low to high RNA integrity) for each sample.

Quantitative reverse transcription-polymerase chain reaction (qPCR):
Five E. coli-specific reference genes polA (gene for E. coli DNA polymerase I), polB (gene for E. coli DNA polymerase II), GAPDH (D-glyceraldehyde-3-phosphate dehydrogenase), CycG (uroporphyrin III C-methyltransferase) and rpoB (RNA polymerase β) were selected to test the efficiency of RNA obtained from electrochemical and electrical lysis devices. Table 5.1 shows the names and sequences of primers selected for the experiment. RNA was extracted from electrically and electrochemically-lysed samples using an RNeasy mini kit (Qiagen). For synthesis of cDNA from electrically and electrochemically lysed samples, an equal amount of total RNA was used (10.8 ng total RNA in 20 µL of RT reaction). Reverse transcription (RT) was performed using high capacity cDNA reverse transcription kit from Applied Biosystems. Q-PCR reactions were performed on an Applied Biosystems 7500 machine using Ssofast Eva Green Supermix. Briefly, 1µL of cDNA and 500 nM of gene primers were used in a total volume of 20 µL. Conditions used for qPCR included an initial step of 2 minutes at 50°C followed by 30 seconds at 95°C and 40 cycles of 5 seconds at 95°C and 30 seconds at 60°C. A no-template negative control was included for each sample tested.

Table 5.1 Sequences of primers used for quantitative real time PCR

<table>
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<tr>
<th>Target</th>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
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<td>polA</td>
<td>polA-F</td>
<td>ATGGTTTCAGATCCCCCAA</td>
<td>22</td>
</tr>
<tr>
<td>Gene</td>
<td>Primer</td>
<td>Sequence</td>
<td>Length</td>
</tr>
<tr>
<td>-------</td>
<td>---------</td>
<td>---------------------------------</td>
<td>--------</td>
</tr>
<tr>
<td>polA</td>
<td>R</td>
<td>TTCTACGCCAGAAACCGCCA</td>
<td>22</td>
</tr>
<tr>
<td>polB</td>
<td>F</td>
<td>TGGCATCGTCGATCACCAT</td>
<td>22</td>
</tr>
<tr>
<td>polB</td>
<td>R</td>
<td>TGGTTGGCATCAGAAAACCGGC</td>
<td>22</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F</td>
<td>TTCCGTGCTGCTCAGAAA</td>
<td>22</td>
</tr>
<tr>
<td>GAPDH</td>
<td>R</td>
<td>TGTGTTACGAGCAGTTT</td>
<td>22</td>
</tr>
<tr>
<td>rpoB</td>
<td>F</td>
<td>TGAGCCAGTTCTGGTCAAGAC</td>
<td>23</td>
</tr>
<tr>
<td>rpoB</td>
<td>R</td>
<td>CTCGAACAGGCTTCCGCTG</td>
<td>23</td>
</tr>
<tr>
<td>CysG</td>
<td>F</td>
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<tr>
<td>CysG</td>
<td>R</td>
<td>ATGCGGTGAACTGTGGAATAAC</td>
<td>24</td>
</tr>
</tbody>
</table>
5.5 References


6 Conclusions and future outlook

6.1 Thesis findings

In this thesis, we reported the development of new techniques for rapid sample processing and profiling of rare cells.

Broadly: We found that the phenotypic profiling of circulating tumor cells can indeed enable early diagnostically-relevant information that could improve monitoring of cancer progression.

In Chapter 2, we developed a new technique, magnetic ranking cytometry that profiles the properties of small collections of cells. Using a microfluidic chip with a series of discrete capture zones, we developed a strategy that isolates cells within zones as a direct function of the levels of protein markers present on their surface. The use of antibody-functionalized nanoparticles enables this approach to achieve high levels of resolution, as thousands of binding events can be exploited to enable populations of cells to be separated on the basis of varying levels of protein expression. We showed that any surface marker recognized by an antibody can be used with this approach, allowing samples to be profiled for levels of multiple markers. In this study, we presented results using three markers of interest, EpCAM, N-Cadherin and Her-2 to illustrate proof-of-concept. We performed head to head comparison of MagRC approach with gold standard techniques for cell sorting and CTC detections. We showed that the MagRC approach produces profiles comparable to those reported by flow cytometry (FCM), a powerful technique useful in analyzing heterogeneity in living cells. FCM was inefficient to profile low number of cells present in whole blood sample and requires cell numbers of $10^4$ or higher for accurate results. However, we showed that MagRC reports on protein expression with similar resolution, but using much smaller collections of cells. Comparison of the performance of the MagRC approach with the CTC gold standard FDA-cleared CellSearch assay showed that low EpCAM cells present in whole blood sample would still be visualized with MagRC approach. However, in contrast, the CellSearch system exhibits significantly suppressed capture efficiencies with low EpCAM cells. We used MagRC technique for monitoring CTC phenotypes during cancer progression in xenograft models. We showed that animals with aggressive tumors have dynamic EpCAM profiles that change as tumors progress, while animals with less aggressive tumors exhibit static profiles.
In Chapter 3, we developed a simplified version of MagRC approach for rare cell profiling. We showed that increasing channel width along with incorporating micro-magnets could be used for cell profiling while improving the rate of fabrication. Using control experiments, we confirmed the crucial roles of both channel width increment and nickel micro-magnets for tracking cell phenotypes.

In Chapter 4, we developed a 2D-phenotypic profiling approach that allows sorting of rare cell subpopulations according to both surface marker expression and chemotactic migration. We showed that this approach enables us to distinguish between highly mobile cells and cells that do not tend to move. We explored migration behaviors of mesenchymal and epithelial cancer cell lines and confirmed high mobility of mesenchymal cells, highlighting their roles in cancer dissemination. Challenging 2D-phenotypic profiling assay with model cell line revealed the increased motility of cells that were found in low EpCAM regions. We showed that the 2D approach could analyze the rare cells present in whole blood sample with high specificity. In order to evaluate the utility of system for the analysis of CTCs, the integrated system was challenged with blood from mice bearing xenografted tumors. Metastatic and non-metastatic prostate cancer cells were implanted orthotopically into immunodeficient mice, generating different xenograft tumor models. The 2D profiling results suggested that CTCs extracted from metastatic xenografts were highly chemotactic and migrated completely toward chemo-reservoirs upon stimulation with the chemoattractant. However, migration of CTCs that originate from a non-metastatic tumor is negligible, suggesting less mobility of non-metastatic CTCs.

In Chapter 5, we reported a new technique for electrical release of intact biomarkers from the interior of bacterial cells. We showed that three-dimensional sharp tip electrodes produce a high field that enables us to extract intact RNA at much lower applied potentials than used previously. Using both qPCR and RIN measurement, we confirmed high integrity of RNA extracted using pure electrical lysis approach.
6.2 Future outlook

This work lays the foundation for the development of tools to demonstrate new sample processing. The emphasis of this thesis is on the design of proof-of-concept devices to analyze the heterogeneity in cancer cell subpopulations. We showed that MagRC device is amenable to provide accurate in-line profiles of low levels of CTCs in unprocessed blood samples. The approach uses fluorescence staining to distinguish between target and non-target cells. In order to eliminate expensive fluorescence microscope requirement, stain-free detection methods such as electrochemical sensing can be incorporated to identify target cells. Future work on this device involves further analyzing the isolated cell subpopulations using downstream analysis such as gene expression profiling. Since high purities of recovered cells are required for downstream analysis, improvements should be considered that will lower the number of WBCs trapped in the device. In Chapter 5, we reported the electrical release of intact biomarkers from the interior of cells. In order to develop a fully automated sample processing platform, electrical lysis approach can be integrated with cell profiling system. This integration allows retaining intact RNAs of isolated cell subpopulations for downstream analysis. Furthermore, it would be invaluable to incorporate biosensing approaches for target nucleic acid detection. Validating this device with patient samples will also be critical before it can be translated to the clinic.

In Chapter 3, we showed the simplified version of MagRC device. The ease of 10-zone device fabrication allows using the platform in different projects and clinical studies. However, device performance in whole blood sample should be validated prior handling patient samples. Similar to MagRC approach, the capability of the device to profile CTC subpopulations can be investigated via metastatic and non-metastatic xenografted models. In the conducted animal study, we only investigated the loss of epithelial markers; however, in order to acquire more information, mesenchymal markers such as N-Cadherin should be explored as well.

In Chapter 4, we remarked that cell subpopulations with varied levels of EpCAM expression show different migratory behaviors. Modification in the design of migration channels can allow the release of cells from different regions of the channels for further downstream analysis. The flow rate used for cell trapping in the chemotaxis chip is low (75 µL/hr). Further design improvement should be considered to increase the flow rate; with the goal of decreasing the overall experiment time. We used off-chip WBC depletion in order to increase the purities of
recovered cells. However, microfabricated filters can be employed in the design to deplete non-target WBCs that reduces the off-chip sample processing steps. Similar to other platforms, device performance should be validated with patient samples.

6.3 Final remarks

Prior to this work, the following question could have been posed: would it be possible to sort cancer cells according to their levels of invasiveness for early cancer diagnosis using a simple-to-operate instrument?

Previously, the answer to this question was: it is unknown if and when this would be possible. The only standard method for cell sorting was flow cytometry – a method inefficient when called upon to profile low numbers of cells in whole blood samples.

With the advances reported herein, the MagRC provides accurate in-line profiles of low levels of CTCs in unprocessed blood samples. While techniques developed previously have leveraged surface-bound magnetic particles for CTC enumeration, none have achieved the level of sensitivity and resolution that MagRC exhibits. Further, none have provided the ability to report a protein expression profile for CTCs. MagRC provides information consistent with that provided by the existing gold standard method, flow cytometry, and, further, allows vastly lower cell numbers to be queried. Translating MagRC device to the clinic will enable point-of-care cancer testing. Efficient phenotypic profiling of CTC subpopulations may replace metastatic tissue biopsies in the prediction and monitoring of therapeutic responses and tumor recurrence. CTC profiles extracted from MagRC device will assist physicians to customize disease treatment which in turn may improve early diagnostic technologies.
7 Appendix A- Supporting information

7.1 Supporting information for Chapter 2

Modeling cell capture in the MagRC device. In order for cells to be captured in the MagRC device, the magnetic forces acting on the cells must be large enough to induce a significant transverse velocity, drawing the cells across the flow streamlines and towards the walls of the chip. Once cells are brought into contact with the walls, capture will occur if the combination of magnetic, friction, adhesion and normal forces acting on the cells is large enough to balance the drag force generated by the flow.

To determine where capture will occur in the chip for cell lines having high, medium and low levels of magnetic loading, magnetic and flow field simulations were carried out in COMSOL Multiphysics®, with the goal of comparing the magnitude of the flow velocity at each point in the chip with the magnitude of the velocity expected to be generated by the magnetic force acting on the cells at that point (with the magnetically induced velocity determined from Stokes law).

\[ V_m = \left( \frac{F_m}{6\pi \mu r} \right) \]

Since the height of the chip is very small compared to its length and width, even a moderate deflection induced by magnetic forces in the path of a cell will result in that cell being brought into contact with the walls of the chip. As a result, any region where the magnitude of the magnetically induced velocity was comparable to (or greater than) the flow velocity was deemed a “capture region”. Capture regions for different cell lines in zones 1 & 100 at a height of 10 \( \mu \text{m} \) are highlighted in Figure 7.1.1. Since the nickel micro-magnets are round, they generate annular regions with high magnetic forces, and thus the capture regions in Figure 7.1.1 appear, in some cases, as capture ‘rings’ rather than capture ‘surfaces’; locations in the chip with enclosed by a capture ‘ring’ were included as capture regions for all modeling, as a cell would necessarily pass through a capture region to find itself in a capture ‘ring’.

Two important characteristics are evident in Figure 7.1.1; first, the capture regions increase in size with increasing nickel radius, and second, the radius of the capture region extends further from the front and back of the ‘X’-shaped structures than from the sides (where the front, back and sides of the ‘X’-structures are defined by their orientation in the flow). The asymmetry in the
extent of the capture region is caused by asymmetry in the flow profile around the ‘X’-structures, with stagnation points generated at the front and back of the Xs (Figure 7.1.3. A).

**Figure 7.1 Analysis of capture regions for cell lines with varied expression levels and nanoparticle loading (High, Medium and Low).** In the first and last zones of the chip at a height of 10 µm from the bottom of the chip. In each case, the radius of the capture region increases with increasing nickel radius. Capture radius was evaluated at heights ranging from 5 µm to 45 µm. Regions in the interior of X-shaped structures with unfavorable capture dynamics were treated as capture regions if they were enclosed by a region with favorable capture dynamics.

In order to determine the size of a capture region for the three model cell lines in each zone of the chip at every vertical position, the radius (measured from the center of the ‘X’-structure) of the capture region for both the front/back and sides of the Xs was measured at 5 µm height increments (from 5 µm to 45 µm) at multiple zones along the length of the chip. Linear functions were then fitted to the capture radius data at each vertical position in the chip; a sample of these linear functions, plotted against nickel radius, are shown in Figure 7.1.2. Linear interpolation was used to determine the radius of a capture region for any height between the measured 5 µm height increments.

To quantify the likelihood that a cell flowing through the MagRC device will encounter a capture region, the flow field around an ‘X’-structure was modelled using COMSOL Multiphysics® (Figure 7.1.3A). A series of concentric control surfaces were defined every 5 µm from the innermost to the outermost radial positions from the center of the Xs (Figure 7.1.3B). The fluid
Figure 7.2 Calculation of the capture radius versus nickel radius for the front/back and the sides of the X-shaped structures (left and right, respectively) at a height of 10 µm. The dotted lines represent linear functions fitted to the data for each model cell line.

Volume crossing each control surface was determined by integrating the dot product of the velocity vector at the surface with the surface unit normal vector over the control surface area (Figure 7.1.3C). Since the middle area of each X is a dead end, the net volume flux across each control surface was necessarily zero; however, by evaluating only the positive contributions to the volume flux, we determined the unidirectional volume flux, essentially the amount of fluid changeover at a given radial position from the center of a ‘X’. The unidirectional volume flux at different radial positions is plotted in Figure 7.1.3D (as a percentage of the total flow rate).

A parametric model incorporating the above capture region and flow analysis was developed and implemented in MATLAB® to identify the likely capture location of a cell in the MagRC device. Thousands of model cells were simulated, each having a randomly assigned initial height ranging from 5 µm to 45 µm at the inlet of the microfluidic chip. For each cell in each zone, the percentage chance of that cell encountering a capture region was calculated and reported as the capture parameter (with the size of the capture region calculated using the cell’s vertical position and zone number). Cells with a capture parameter of 25% or greater in a given zone were eligible for capture. Once a cell’s capture parameter was above the 25% threshold, a random number generator was used to determine whether that cell would be captured in the given zone (with the
Figure 7.3 Calculating unidirectional volume flux as a function of radial position from the center of X-shaped structures. A) Velocity field around X-shaped structure at a height of 25 µm (mid-plane) in the chip. B) Control surfaces used during volume flux calculations (only two sides were necessary due to symmetry). C) Surface integral calculation of unidirectional volume flux. D) Unidirectional volume flux as a function of radial position from the center of X-shaped structures.

chance of capture directly proportional to the capture parameter). Introducing an element of chance into the parametric model was desirable and helped to mimic variabilities in cell magnetization within cell lines, inconsistencies in the flow field, and cell-cell collisions.
The capture parameter within the MagRC device for the three model cell lines (normalized so that the 25% threshold for capture is equal to unity) is presented in Figure 7.1.4. The capture parameter of the cells within the chip is strongly dependent on their vertical position since the nickel micro-magnets generate amplified magnetic fields near the bottom of the microfluidic channel. Additionally, the long length of the chip relative to its height (8.75 cm vs. 50 µm, respectively) leads to long residence times and the potential for cells to settle towards the bottom of the chip. To account for gravitational settling, a linear settling function was incorporated which imposed a 0.5 µm/zone drop in height for uncaptured cells\textsuperscript{[1]}.

![Figure 7.4](image)

**Figure 7.4** Normalized capture parameter for high, medium and low magnetically loaded model cell lines. Cells having a normalized capture parameter ≥1 were eligible for capture in the parametric model.
Figure 7.5 Comparison of the levels of non-specific adsorption of magnetic beads to WBCs relative to MDA-MB-231 and SKBR3 cells. EpCAM magnetic beads adsorption was assessed via flow cytometry. The median of MDA-MB-231 cells profile is 225 while the one of WBCs profile is 17. Since MDA-MB-231 cells have the lowest levels of EpCAM expression and are captured at the final zones of MagRC chip, the WBCs are not captured in the chip via magnetic forces.
Figure 7.6 Reproducibility of MagRC verified using model cell lines. Three runs using the same cell line produced a similar pattern of capture in the MagRC. In each trial, buffer solution were spiked with MCF-7 cells (A), SKBR3 cells (B), and MDA-MB-231 cells (C). The first three runs in each row are replicates, and the final panel shows the overlay of the fits of the capture profiles.
Control experiments were carried out using a microfluidic chip lacking nickel micromagnets. Capture experiments without nickel illustrate that VCaP cells that have highest level of magnetic loading were captured at initial zones regardless of using micro-magnets. However, SKBR3 (medium magnetic loading) cells were distributed randomly along the device and capture efficiency of MDA-MB-231 cells that have lowest level of EpCAM expression was zero without incorporating micro-magnets.

Assessment of the release efficiency of MagRC chip and the viability of released cells. SKBR3 cells captured in MagRC chip were released and cultured. Live cell imaging was conducted after 24 hours. The efficiency of cancer cell release was 92%. Viability of cells was determined via live-dead cell staining (Invitrogen near-IR fluorescent reactive dye (L10119)) and manually counting the live cells. The cell viability was 98%. Viable cells (left) and a dead cell (right) are shown. Scale bars are 50 um.
Figure 7.9 Profiling of SKBR3 cells in RBC-lysed blood using MagRC. After spiking blood with different numbers of SKBR3 cells, RBC lysis buffer was used to lyse RBCs. After lysis, a solution that contained SKBR3 cells and WBCs was analyzed using MagRC. As some cells were lost during washing steps, numbers of captured cells were less than numbers of loaded cells.

Figure 7.10 Profiling of MCF-7 cells in PBS (A) whole blood (B).
Table 7.1 T-test analysis results for the capture profiles of MCF-7 and SKBR3 cells

<table>
<thead>
<tr>
<th></th>
<th>MCF-7</th>
<th>SKBR3</th>
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<tr>
<td>Mean</td>
<td>6.1</td>
<td>18.1</td>
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<tr>
<td>Variance</td>
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<td>1832</td>
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<td>Paired t test</td>
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<td>Correlation coefficient (r)</td>
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Table 7.2 T-test analysis results for the capture profiles of SKBR3 and MDA-MB-231 cells

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<th>SKBR3</th>
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<tr>
<td>Variance</td>
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<td>Correlation coefficient (r)</td>
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Table 7.3 T-test analysis results for the capture profiles of MCF-7 and MDA-MB-231 cells

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<th>MDA-MB-231</th>
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<tr>
<td>Mean</td>
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<tr>
<td>Variance</td>
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7.1.1 References

Figure 7.11 The integrated device for surface marker and chemotactic sorting of cancer cell subpopulations. The microfluidic device contains two sets of valves, which are used to control the direction of flow. The sample is introduced along the first axis of separation to initiate the surface marker separation. During first dimension sorting, the Valves#1 are set to the closed position, allowing for cell sorting based on the surface marker. Valves#2 have two functions: 1) Separating chemoattractant reservoirs and migration channels to prevent chemoattractant diffusion to migration channels prior to the cell loading. 2) Disconnecting zones of 4-zone device. After the first dimensional sorting, set of Valve#2 will be closed and chemoattractant will be loaded into chemo-reservoirs. Then set of Valve#1 will be opened and cells will be directed to chemotaxis chips. At the end, Valves#2 will be opened to allow formation of chemical gradient.
Figure 7.12 Simulations of chemical gradient generated in the chemotaxis chip carried out by COMSOL multiphysics. Red color corresponds to the regions that contain 1 M chemoattractant while blue color shows region with no chemoattractant.

Figure 7.13 Capture efficiency of chemotaxis chip. Recovery efficiency of prostate (LNCaP and PC3) and breast (MCF-7 and MDA-MB-231) cancer cell lines were investigated.
Figure 7.14 Viability of cells in chemotaxis chip. PC3 cells were subjected to chemotactic migration. After 20 hours incubation, SYTOX Orange Dead Cell (Invitrogen, CA) Stain was used to identify viable cells in the device. Viable migrating (A), migrated (B and C) cells are shown. D) A dead cell that is stained with SYTOX.

Figure 7.15 EpCAM profiling of PC3 and LNCaP cells. A) Distribution of LNCaP and PC3 cells in 4-zone device using EpCAM as the profiling marker. Cell capture was performed at the flow rate of 25 mL/hr. B) EpCAM expression measured by the flow cytometry for the two cell lines. These results indicates that at the optimal flow rate of 25 mL/hr, high EpCAM cells (LNCaP) were captured mostly at the initial zone while PC3 cells with a
relatively low EpCAM expression were captured at all zones. Capture profile of PC3 reveals the heterogeneity in this cell line.

![Bar chart showing cell sorting](chart.png)

**Figure 7.16 2D sorting of PC3 cell subpopulations.** First, 100 cells were tagged with magnetic nanoparticles labeled EpCAM1 aptamer and captured in the zones 1, 2, 3, and 4 of the fluidic device according to their EpCAM expression level. After releasing the cells using AS-EPCAM1, cell subpopulations extracted from each zone were subjected to chemotactic phenotype sorting. CXCL16 (100 ng/mL) was used as chemoattractant. Migration of cells was monitored after 20 hours incubation.

**Figure 7.17 MRI images of mice implanted with PC3 tumor (A) and PC3M tumor (B) after 4 weeks tumor injection.** Red circles denote tumor tissue area.
Figure 7.18 CTC Distribution profiles of mice in different xenograft models in 4-zone device using EpCAM as the profiling marker.
Table 7.4 Percentages of migratory CTCs in different xenograft models

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<th>Xenograft tumor</th>
<th>% of invasive CTC (found in migrated or M3-region)</th>
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<td>PC3-mouse#2</td>
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<td>LNCaP-mouse#1</td>
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<td>LNCaP-mouse#2</td>
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8 Appendix B-Publications

8.1 Refereed journal publications


8.2 Patents

A Method for Analysis of Rare Cells Using Magnetic Ranking Cytometry (10002986).

8.3 Conference presentations


