MICROFLUIDIC DEVICES FOR ISOLATION OF CIRCULATING TUMOR CELLS

By

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To my beloved parents, sisters, and wife
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Cancer induces high death rate because of the high probability of metastasis. During the progression of metastasis, cancer cells detach from primary tumors or metastatic sites and enter the bloodstream, becoming circulating tumor cells (CTCs). CTCs are thus responsible for the spreads of cancer to distant organs, which lead to cancer-induced death. The level of CTCs can provide valuable information for monitoring cancer status and predicting survival rate of cancer patients. However, CTCs are extraordinarily rare (only a few CTCs in 1 mL blood with billions of blood cells), making their isolation and characterization a formidable technological challenge. Therefore, the objective of this research is to develop microfluidic system-based approaches for efficient isolation of CTCs from blood.

Firstly, we developed an aptamer-mediated micropillar-based microfluidic device, for efficiently capturing and enriching rare cancer cells. High-affinity DNA aptamers were used as an alternative capturing agent to antibodies for targeting cancer cells. The device consisted of >59,000 micropillars, which greatly enhanced the interactions between cells and the aptamer-coated surface. With optimized device geometry and
flow rate, rare tumor cells were captured from whole blood with high efficiency, purity, throughput, and cell viability.

Secondly, we incorporated nanoparticles in microfluidic devices for the enhanced capture of cancer cells. Simultaneous attachment of ~95 DNA aptamers onto each gold nanoparticle surface (forming DNA nanospheres) created an assembly of multivalent binding ligands, with significant enhancement of cell capture efficiency and throughput. The enhanced cell capture also accrues from the increased surface roughness, surface area and ligand density. A high-throughput flat channel device and micromixing device were developed for cancer cell isolation from lysed blood and whole blood, respectively.

Finally, we developed a microfluidic geometrically enhanced mixing device for isolation of CTCs from pancreatic cancer patients. We demonstrated the potential utility of the device in monitoring the response to anti-cancer drug treatment in cancer patients.

In summary, the microfluidic devices developed in this dissertation provide new means for efficient CTC isolation and accurate CTC enumeration. Since the methods are minimally invasive, the microfluidic devices show great potential for cancer diagnosis, monitoring disease progression and treatment response.
1.1 Cancer and Circulating Tumor Cells

Cancer is a major cause of death worldwide and is a leading public health problem.\(^1\) In the United States, one in four deaths is caused by cancer. It is estimated that >1.6 million new cases of cancer patients will be diagnosed and ~580,000 cancer-related deaths (both men and women) will occur in the United States in 2013.\(^2\) The direct medical care costs for all cancers are >$100 billion, according to the National Cancer Institute.

A primary reason of cancer-induced death is metastasis, the spread of cancer cells from the primary tumor to other organs (e.g., lungs, bones and liver are the most common sites of metastasis).\(^3\) During the progression of metastasis, cancer cells detach from the primary tumor, penetrate the blood vessels and enter into the bloodstream, becoming circulating tumor cells (CTCs).\(^4\)\(^6\) CTCs circulate through the lymphatic system and the bloodstream and migrate to other parts of the body. Eventually, the CTCs extravasate from blood vessels at a distant location and start to proliferate and stimulate angiogenesis, thereby spreading the cancer to other locations and tissues in the body.\(^7\)\(^8\) Hence, CTCs hold the key to understanding cancer metastasis and can serve as a potential cancer biomarker.

The clinical significance of CTCs towards non-hematologic cancer has been widely demonstrated.\(^9\)\(^-\)\(^12\) Specifically, CTCs can be used for: 1) early detection of metastasis; 2) monitoring of treatment response; 3) therapeutic design; 4) discovery of biomarkers and drugs; 5) cancer prognosis. Isolation and enrichment of CTCs will
enable the subsequent cellular and genetic study of CTCs, thus providing a means for discovery of biomarkers and understanding the biology of metastasis.

The high death rate of cancer is associated with the difficulty in cancer diagnosis. Currently, a variety of cancer diagnostic methods have been used in clinics, including medical imaging (e.g., X-ray computed tomography, or CT scans), endoscopy, and tumor biopsy. However, most of these diagnostic methods are invasive and expensive, and some of them are inaccurate. For example, biopsy, the current gold standard of cancer diagnosis, involves removal of tissue or cells from the body and examination by experienced surgeons and pathologists. The invasive nature of biopsy prevents patients from being tested in an ongoing or repetitive basis. CTC enumeration and examination, on the other hand, is much less invasive, with only 5-10 mL of patient blood needed; it is like a blood test for cancer. CTC monitoring is thus regarded as “liquid biopsy” or “live biopsy” for cancer, which enables noninvasive cancer diagnosis and real-time monitoring of therapeutic response.

However, CTCs are extraordinarily rare in nature. Typically, there are only around 5 CTCs in 7.5 mL of a patient’s blood, among tens of billions of blood cells. Thus, the isolation and characterization of CTCs is a major technological challenge. Recently, microfluidic devices provide unique opportunities for rare cell isolation and detection, and they have been used for size-based separation, affinity-based cell sorting and flow cytometry.

1.2 BioMEMS, Lab-on-a-chip and Microfluidics

1.2.1 Terminology

Microelectromechanical systems (MEMS), also called Microsystem Technology (MST) in Europe or Micromachines in Japan, are miniaturized systems or devices that
combine electrical and mechanical components with sizes from the sub-micrometer to millimeter level. MEMS extend the fabrication technology for semiconductor devices and integrated circuits, with the addition of mechanical components such as beams, springs and diaphragms. Examples of MEMS are inkjet-printer cartridges, accelerometers, gyroscopes, microphones, etc.\textsuperscript{18,19} MEMS technology also includes BioMEMS (biological or biomedical MEMS), which are used in biological and chemical analysis.

While engineers like to use the term BioMEMS to stress its microfabrication technology, chemists prefer to use Lab-on-a-chip and micro total analytical system (\(\mu\)TAS)\textsuperscript{20} to emphasize that multiple laboratory functions are integrated on a single chip. Microfluidics, however, is an even broader term that describes all fluid handling on the sub-millimeter scale, including microchannels, micropumps, microvalves, microdroplets, DNA chips, etc. Microfluidics was defined by Whitesides as “the science and technology of systems that process or manipulate small \(10^{-9}\) to \(10^{-18}\) litres) amounts of fluids, using channels with dimensions of tens to hundreds of micrometres”.\textsuperscript{21} Hence, microfluidics is now much more than a subset of MEMS, and is becoming a multidisciplinary field itself, uniquely identified by its materials and fabrication technologies, encompassing the fields of engineering, physics, chemistry and biotechnology.

\textbf{1.2.2 Materials and Fabrication of Microfluidic Devices}

Initially, traditional MEMS materials, such as silicon, were used for microfluidic device fabrication. Microfabrication and micromachining techniques for MEMS were applied for fabrication of such silicon microfluidic devices.\textsuperscript{22,23} Photolithography was used for the patterning required in microfabrication; and microstructures were made by subsequent etching.\textsuperscript{24} Later, glass was extensively used because of its easy availability and optical transparency. However, because the cost for fabrication of silicon and glass
device was too high, polymer or plastic materials, such as polydimethylsiloxane (PDMS)\textsuperscript{25} and thermoplastics,\textsuperscript{26} have been playing an increasingly important role for microfluidic devices, because of their low cost, mass production, optical clarity and biocompatibility.

Soft lithography has been rapidly developed for PDMS device fabrication.\textsuperscript{27-29} Soft lithography is “a collection of techniques based on printing, molding and embossing with an elastomeric stamp”,\textsuperscript{30} which can rapidly prototype devices on various substrates, including planar, curved, flexible and soft substrates, with different kinds of elastomeric materials. The name “soft” comes from its use of elastomeric materials such as PDMS. Four major steps are generally involved in a soft lithography procedure. 1) Pattern design. The pattern can be designed precisely using a number of computer aided design (CAD) programs (e.g., AutoCAD from Autodesk). 2) Fabrication of the mask. Generally, there are two kinds of masks: transparency mask, a photomask printed on transparency film, and chrome mask, a glass or quartz plate patterned with opaque chrome on its surface. A transparency mask with resolution up to 25,400 d.p.i can be obtained from high-resolution printing companies (e.g., CAD/ART Services, Inc.). The smallest feature a transparency mask can produce is ~10 µm. A chrome mask can produce even smaller features (~1 µm), but with substantially higher cost. Chrome masks are also available to be ordered from commercial suppliers such as Photo Sciences Inc. 3) Fabrication of the master. Conventional photolithography is the primary technique for fabricating a master that contains patterned relief structures on the surface, typically with feature sizes larger than 1 µm. The fabrication of silicon master starts with spin-coating of a thick photoresist (e.g., SU-8) on a wafer, followed by
UV light exposure under the mask, and the subsequent development. 4) Fabrication of the PDMS device. An elastomeric slab with patterns as relief structures on its surface is the key element of soft lithography. The slab is typically fabricated by casting a liquid PDMS precursor against the master with the complementary structures patterned on its surface. Sylgard 184 from Dow Corning has been most commonly used commercially available PDMS. Since one silicon master can be used hundreds of times for rapidly fabricating many PDMS devices, soft lithography results in much lower cost than traditional photolithography in mass production.

1.2.3 Advantages of Microfluidic Devices

With their small size, microfluidic devices provide many advantages for biological and chemical analysis,\(^\text{31}\) including: 1) small quantities of fluids required, resulting in lower cost of reagents, lower sample volumes for diagnostics, and less waste; 2) small dimensions leading to low power consumption and versatility in design; 3) high resolution and detection sensitivity, because of the high surface area-to-volume ratio, rapid mass transfer and interaction, and short diffusion distances; 4) potential for parallel operation with high throughput and capability to integrate with other miniaturized devices or components (e.g., mixers and detectors); 5) low cost, mass production allowing disposability, and resulting in elimination of cross contamination; and 6) portability, promising for point-of-care diagnostics. These advantages have propelled microfluidic devices from research laboratories to clinical research and industry in the past 20 years.

1.3 Isolation of CTCs Using Microfluidic Devices

Currently, the most commonly used cell isolation and sorting methods are fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS).
In FACS or flow cytometry, which is widely used in clinical diagnosis, cells are fluorescently labeled with specific dyes or antibodies and then sorted based on light scattering and fluorescence intensity.\textsuperscript{32} However, flow cytometry produces many false negatives, which increase the limit of detection, a severe problem when detecting rare cells. Also flow cytometry equipment is large and quite expensive (>\$250,000), requiring trained personnel for instrument operation and sample preparation. MACS is also widely used to isolate cells using antibody-coated magnetic beads. Target cells conjugated with magnetic beads are separated using magnetic force.\textsuperscript{9, 33-35} The FDA-approved commercial CellSearch system from Veridex\textsuperscript{36, 37} is based on this immunomagnetic beads-based cell isolation. Other cell isolation methods include sorting based on physical properties such as size and density, and micromachine-enabled isolation.\textsuperscript{38, 39} However, these methods have the disadvantage of low sensitivity, low purity or high cost.

Microfluidic devices, on the other hand, provide an inexpensive means to isolate CTCs with high sensitivity. Considerable research has been performed with microfluidic devices using size-based,\textsuperscript{40, 41} dielectrophoresis-based,\textsuperscript{42} fluorescence-activated\textsuperscript{43, 44} and magnetic-based\textsuperscript{45, 46} cell sorting. Recently, microfluidic devices with high-affinity ligands, primarily antibodies, have emerged as a distinctive method for isolation of rare cells.\textsuperscript{47, 48} In general, a microchannel was first functionalized with specific antibodies; cell suspension or whole blood was then driven through the device, resulting in target cell capture. Topographic features such as microposts,\textsuperscript{49} sinusoid shapes,\textsuperscript{48} silicon nanopillars\textsuperscript{50} and chaotic mixers\textsuperscript{51} have been applied to enhance the interaction between cells and the antibody-coated device surfaces.
**1.4 CTC Targeting Ligands**

### 1.4.1 Antibodies

Circulating tumor cells express certain receptors which can be used as CTC biomarkers, the molecules that can be used for detecting and isolating CTCs. With the biomarker information, corresponding antibodies can be developed for CTC isolation. The most notable CTC biomarker is epithelial cell adhesion molecule (EpCAM), since CTCs are essentially epithelial cells detached from primary tumor with epithelial tissue. EpCAM is a trans-membrane protein expressed on most of normal epithelial cells and is highly expressed in the majority of epithelial cancers, including breast, colorectal, prostate, and pancreatic cancers.\(^5\) Thus, monoclonal anti-EpCAM antibody has been widely used for CTC isolation. However, it is believed that a significant number of CTCs go through the epithelial-mesenchymal transition (EMT) during the progression of metastasis and lose epithelial characteristics and become mesenchymal cells, thus losing EpCAM receptors on their cell membrane.\(^5\) Other CTC biomarkers include human epidermal growth factor receptor 2 (HER2), epidermal growth factor receptor (EGFR), and the calcium-dependent cadherins.\(^5\) For prostate cancer, prostate-specific antigen (PSA) and prostate-specific membrane antigen (PSMA) are highly specific prostate cancer biomarkers and have been extensively used.\(^5\)

### 1.4.2 Aptamers

While only a few antibodies have been identified for tumor cells and cancer cell lines, a number of DNA aptamers with high affinity and excellent selectivity have already been selected for numerous cancers. Aptamers are short (usually 20-100 bases) single-stranded DNA or RNA oligonucleotides (or sometimes peptide molecules) that bind to a specific target molecule. Aptamers are generated using an in vitro selection process
termed SELEX (Systematic Evolution of Ligands by EXponential enrichment). The SELEX procedure usually begins with a random library of $10^{13}$-$10^{15}$ DNA or RNA sequences, followed by an iterative process with positive selections and counter selections for specific amplification of sequences having high binding affinity to the targets. By folding into unique secondary or tertiary structures, aptamers can bind with their targets with high affinity (with dissociation constants from µM to pM) and recognize their targets with a specificity that is comparable to antibodies. Aptamers have been selected for a broad range of targets including metal ions, small organic molecules, proteins, biological cells, viruses and bacteria.

Compared with antibodies, aptamers provide significant advantages, including: 1) rapid and reproducible production by chemical synthesis; 2) capability of selecting for virtually any target at reasonable cost; 3) long term stability; 4) easy modification with different functional groups or dyes. Because of these advantages, aptamers are becoming the next generation antibody-like molecular probes for diagnostic and clinical application.

Based on the SELEX process, cell-SELEX has been developed in the laboratory of Dr. Weihong Tan, our collaborator, to generate a panel of aptamers targeting different types of cancer cells, including leukemia, liver cancer, small cell lung cancer and colorectal cancer. These aptamers have the additional advantage of selection without preknowledge of the biomarkers on the cancer cell surface, while making it possible to identify the biomarkers after selection. For example, an aptamer called sgc8 has been selected for CEM cells (human acute lymphoblastic leukemia) with a dissociation constant $K_d=0.79 \pm 0.15$ nM. Human protein tyrosine kinase-7 (PTK7)
was later identified as the membrane receptor which binds with sgc8 aptamer. It was found that CEM cells have a high expression level of PTK7 with 1300 ± 190 receptors per 1 µm² of cell surface.64

1.5 Objective and Organization of This Dissertation

Isolation and enumeration of circulating tumor cells (CTCs) are of great importance for cancer diagnosis and disease monitoring. Analysis of this “liquid biopsy” holds the promise to usher in a new era of personalized therapeutic treatments and real-time monitoring for cancer patients. But the extreme paucity of CTCs in blood makes their isolation a formidable technological challenging. The major objective of this research is to study and develop novel microfluidic systems to address the challenges of CTC isolation: 1) maximizing the capture of target cells (i.e., CTCs); 2) minimizing the capture of non-target cells (i.e., leukocytes and erythrocytes). The research aims to develop inexpensive microfluidic chips for high-performance CTC capture, with accurate CTC counting capability, for noninvasive cancer diagnosis and monitoring. This dissertation also aims to use novel DNA aptamer-based cancer biomarkers and integrate them with microfluidic system for sensitive cancer cell isolation.

The significance of this work mainly lies in three aspects. First, we developed two generations of high-performance microfluidic devices for efficient tumor cell capture: a unique isotropically-etched elliptical micropillar array device with optimized channel geometry, and a geometrically enhanced mixing microfluidic device. Second, DNA aptamers with high affinity and excellent selectivity were used for cancer cell capture, while most researchers are using antibodies; also multivalent DNA nanospheres were used for the first time for enhanced cancer cell capture. Third, the geometrically enhanced mixing chip enabled efficient CTC isolation and accurate CTC enumeration.
from patients with metastatic pancreatic cancer, a disease where biopsies are difficult and the commercial CellSearch system is inefficient. The microfluidic systems developed in this dissertation are highly novel and enabled efficient isolation of CTCs with high efficiency, high purity, high throughput and high cell viability.

The rest of this dissertation is outlined as follows.

In chapter 2, an aptamer-mediated, micropillar-based microfluidic device is developed, which is able to efficiently isolate tumor cells from unprocessed whole blood. High-affinity aptamers were used as an alternative to antibodies for cancer cell isolation. The microscope-slide-sized device consists of >59,000 micropillars, which enhanced the probability of the interactions between aptamers and target cancer cells. The device geometry and the flow rate were investigated and optimized by studying their effects on the isolation of target leukemia cells from a cell mixture. The device yielded a capture efficiency of >95% and a purity of ~81% at the flow rate of 600 nL/s. Then, the device was exploited for isolating colorectal tumor cells from non-processed whole blood; as few as 10 tumor cells were captured from 1 mL of whole blood. The problem of low throughput of a typical microfluidic device was also addressed by processing 1 mL of blood within 28 minutes. In addition, ~93% of the captured cells were found viable, making them suitable for subsequent molecular and cellular studies. Furthermore, captured cells were efficiently released using toehold-mediated complementary DNA sequences which competitively hybridize with cell-bound DNA aptamers.

Chapter 3 describes the development of a platform combining multivalent DNA aptamer nanospheres with microfluidic devices for efficient isolation of cancer cells from blood. Gold nanoparticles (AuNPs) were used as an efficient multivalent platform for
assembling a number of aptamers for high-efficiency cell capture. Up to 95 aptamers were attached onto each AuNP, resulting in enhanced molecular recognition capability. An 39-fold increase in binding affinity was confirmed by flow cytometry for AuNP-aptamer conjugates (AuNP-aptamer) when compared with aptamer alone. With a laminar flow flat channel microfluidic device, the capture efficiency of human acute leukemia cells from a cell mixture in buffer increased from 49% using aptamer alone to 92% using AuNP-aptamer. AuNP-aptamer was also utilized in a microfluidic device with herringbone mixing microstructures for isolation of leukemia cells in whole blood. The cell capture efficiency was also significantly increased with the AuNP-aptamer over aptamer alone, especially at high flow rates. The results show that the platform combining DNA nanostructures with microfluidics has a great potential for sensitive isolation of CTCs, and is promising for cancer diagnosis and prognosis.

Chapter 4 describes the development of a geometrically enhanced mixing (GEM) chip for high-efficiency and high-purity tumor cell capture. The release and culture of the captured tumor cells were also successfully demonstrated. The high-performance microchip is based on geometrically optimized micromixer structures, which enhance the transverse flow and flow folding, maximizing the interaction between CTCs and antibody-coated surfaces. With the optimized channel geometry and flow rate, the capture efficiency reached >90% with a purity of >84% when capturing spiked tumor cells in buffer. The system was further validated by isolating a wide range of spiked tumor cells (50-50,000) in 1 mL of lysed blood and whole blood. With the combination of trypsinization and high flow rate washing, captured tumor cells were efficiently released. The released cells were viable and able to proliferate, and showed negligible difference
compared with intact cells that were not subjected to the capture and release process. Furthermore, the device was applied for detecting CTCs from metastatic pancreatic cancer patients’ blood; and CTCs were found in 17 out of 18 samples (>94%). The potential utility of the device in monitoring the response to anti-cancer drug treatment in pancreatic cancer patients was also tested, and the CTC numbers correlated with the clinical computed tomograms (CT scans) of tumors. The presented technology shows great promise for accurate CTC enumeration, biological studies of CTCs and cancer metastasis, as well as for cancer diagnosis and treatment monitoring.

Finally, the work is summarized in chapter 5 and future work and directions are discussed.
CHAPTER 2
APTAMER-ENABLED EFFICIENT ISOLATION OF CANCER CELLS FROM WHOLE BLOOD USING A MICROFLUIDIC DEVICE

2.1 Background

Metastases from primary tumors are the leading causes of death for non-hematological cancers.\(^6^5\) During the progression of metastasis, cancer cells shed from solid tumors and enter the bloodstream, becoming circulating tumor cells (CTCs), which has a potential to serve as important biomarkers for early diagnosis of cancer metastases.\(^6^6, 6^7\) While most current methods for cancer diagnosis require invasive biopsy followed by molecular analysis, CTC enumeration is less invasive and provides a means for cancer diagnosis and prognosis, as well as for monitoring the progress of treatment. However, CTCs are extremely rare, comprising only a few out of \(>10^9\) hematological cells in 1 mL of blood, making their isolation and characterization a significant technological challenge.

Recently, a variety of techniques have been developed for CTC isolation and detection,\(^6^8\) ranging from the use of immunomagnetic beads (e.g., CellSearch from Veridex), and size-based filtration systems, to microfluidic devices.\(^4^0, 4^5, 6^9-7^1\) Among these methods, microfluidic devices with high-affinity ligands, primarily antibodies, have provided unique opportunities for detecting CTCs from patient blood.\(^4^9, 7^2\) However, only a few antibodies have been identified for tumor cells and cancer cell lines. In contrast, a number of DNA aptamers with high affinity and excellent selectivity have been selected for numerous cancers.\(^7^3\) Aptamers are single-stranded oligonucleotides that can recognize and bind to their target cells by folding into unique secondary or tertiary

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structures. They can be easily generated using an in vitro selection process termed cell-SELEX (Systematic Evolution of Ligands by EXponential enrichment). Previously, our groups reported a flat channel polydimethylsiloxane (PDMS) device and utilized it to capture target cancer cells from a mixture of target and control cancer cells (1:1 ratio), with ~80% capture efficiency.\textsuperscript{74,75} To realize clinical utility, however, a device must be capable of isolating a few tumor cells from milliliters of whole blood (>10\textsuperscript{9} cells). As a result, the device must possess high capture efficiency (the percentage of tumor cells isolated relative to total tumor cells present), satisfactory cell purity (the percentage of target tumor cells in the cells isolated), and sufficient throughput (the amount of blood processed in a certain period of time).\textsuperscript{76} In addition, the captured cells are preferred to remain viable so that they can be further analyzed at the cellular and molecular level\textsuperscript{77} (e.g., to study apoptosis of tumor cells).

Herein, we report our development of an aptamer-functionalized, micropillar-based microfluidic device that isolates cancer cells from unprocessed whole blood with the required metrics mentioned above. Aptamers with specific binding to cancer cells of interest are used as an alternative to antibodies that have been often used for CTC isolation. The micropillars in the microchannel enhanced the probability of the interactions between the cells and the aptamers coated on the channel/pillar surfaces, resulting in high capture efficiency. After optimizing the geometry of the micropillars, efficient isolation of a few tumor cells from whole blood was achieved with sufficient throughput and high cell viability.\textsuperscript{78}
2.2 Device Design, Fabrication and Surface Functionalization

2.2.1 Device Design

The glass micropillar device was designed to be the size of a microscope slide, consisting of eight parallel channels with an array of >59,000 isotropically-etched, elliptical micropillars as shown in Figure 2-1. The geometric design of the micropillar array was inspired by the deterministic-lateral-displacement-based particle separation,\textsuperscript{79-81} in which the flow streamlines are distorted to enhance cell-micropillar interactions. The dimension of the elliptical pillars is 30 µm (major axis) × 15 µm (minor axis) × 32 µm (height), with an interpillar distance of 80 µm (center to center) and an 80-µm shift after every 3 rows in the direction of the minor axis.

To characterize the design of the micropillar device, computational fluid dynamics simulation was performed using COMSOL Multiphysics (COMSOL, Inc, Burlington, MA) to study the effects of the micropillar geometry and the arrangement of micropillars. The streamlines of the flow across the micropillar array are shown in Figure 2-2, with assumptions of an incompressible fully developed laminar flow, initial velocity at 1 mm/s, and Reynolds number (Re) at 0.1. Figure 2-3 shows the comparison of streamlines between the micropillar array with shifts and without shifts.

2.2.2 Device Fabrication

The glass devices were fabricated according to the procedures reported previously.\textsuperscript{82, 83} In brief, the layout of the device was designed in AutoCAD and then sent to Photo Sciences (Torrance, CA) to produce a chrome photomask. Glass substrates coated with chromium and photoresist layers were purchased from TELIC (Valencia, CA). The pattern on the photomask was transferred to the glass substrate via photolithography. The glass substrate was then chemically etched to a channel depth of...
24 to 44 µm using a mixture of HF, HNO₃ and H₂O. The channel depth was measured using a Dektak 150 profilometer, and the depth was controlled by the etching time. The glass substrate was then sealed with a 5 mm thick PDMS sheet, fabricated from Sylgard 184 reagents (Dow Corning, Midland, MI) according to the instructions of the manufacturer. Inlet and outlet wells were created at the channel ends by punching holes in the PDMS sheet.

2.2.3 Surface Functionalization

The device was functionalized with aptamers through a two-step surface modification (Figure 2-4): physical adsorption of avidin onto the glass surface (15 minute incubation) and immobilization of biotinylated aptamers via biotin-avidin interaction (15 minute incubation). Target cancer cells are captured due to the specific binding between cell surface receptors and aptamers.

To demonstrate the immobilization of DNA aptamers onto the surface of a microchannel, biotinylated aptamers labeled with fluorescein isothiocyanate (FITC) was introduced into the channel and confocal microscope images were taken to measure the fluorescence intensity on the surface. As shown in Figure 2-5, the fluorescence signal increased with increasing FITC-aptamer concentration (after thorough washing), proving that aptamers were successfully immobilized on the avidin-modified surfaces and the amount of aptamers immobilized is dependent on the aptamer concentration.

2.2.4 Comparison of Surface Modification Methods

The above mentioned physical adsorption-based surface treatment method was compared with a silane based-method, one commonly used surface modification for silicon/glass/PDMS. For the silane-based method, shown in Figure 2-6, the microchannels were first treated with 4% (v/v) 3-mercaptopropyl trimethoxysilane
(MPTMS) (Sigma-Aldrich, St. Louis, MO) in ethanol at room temperature for 1 hour, followed by incubation with 0.2 mM N-y-maleimidobutyryloxy succinimide ester (GMBS) (Pierce Biotechnology, Rockford, IL) in ethanol for 30 min at room temperature. GMBS serves as the crosslinker for the sulphydryl group on MPTMS to the amine group on streptavidin or NeutrAvidin. Then, the device was incubated with 10 µg/mL streptavidin in PBS for 30 min to attach streptavidin to the GMBS. Afterwards, biotinylated aptamers labeled with FITC were introduced into the channel and fluorescence microscopy image was taken to measure the fluorescence intensity.

Then we compared the amount of aptamers immobilized between this silane-based method and the above physical adsorption-based method. Results show that silane based method is similar as physical adsorption for glass surface, while physical adsorption gives higher signal than silane-based method on PDMS surface, as shown in Figure 2-7. The silane-based surface modification is widely used and proved to be effective. However, it involves 2-4 hours of surface treatment with multiple incubation and rinsing steps. In addition, the plasma treatment of the device surface made the device permanently bonded, and the device was not able to be cleaned and reused. On the other hand, surface modification using avidin physical adsorption was facile and robust, with reusable devices, thus it was chosen for future experiments.

2.3 Cell Isolation and Assay

2.3.1 Cell Culture

CCRF-CEM cells (CCL-119, T cell line, human acute lymphoblastic leukemia), Ramos cells (CRL-1596, B-cell, human Burkitt’s lymphoma), DLD-1 cells (Dukes’ type C colorectal adenocarcinoma) and HCT 116 cells (colorectal carcinoma) were purchased from American Type Culture Collection (ATCC). CEM, Ramos and DLD-1 cells were
cultured in RPMI medium 1640 (ATCC) supplemented with 10% Fetal bovine serum (FBS, heat-inactivated; GIBCO) and 100 units/mL penicillin-streptomycin (PS, Cellgro, Manassas, VA); HCT 116 cells were cultured in McCoy's 5A medium containing the same concentration of FBS and PS. All cultures were incubated at 37°C under 5% CO₂ atmosphere. The colorectal cancer cell lines were grown as adherent monolayers in 100 mm × 20 mm culture dishes to 95% confluence. Cells were washed in the dish with Dulbecco's Phosphate buffered saline (DPBS) (Sigma-Aldrich, St. Louis, MO), dissociated by 0.25% trypsin treatment (2 min) and seeded into culture dishes at a low concentration.

2.3.2 Aptamers

DNA aptamers were synthesized in-house. Aptamer sequences were as follows, sgc8, 5’-ATC TAA CTG CTG CGC CGC CGG GAA AAT ACT GTA CGG TTA GAT TTT TTT TTT T- 3’-biotin; TD05, 5’- AAC ACC GTG GAG GAT AGT TCG GTG GCT GTT CAG GGT CTC CTC CCG GTG TTT TTT TTT T- 3’-biotin; KDED2a-3, 5’-TGC CGA AAA CTG CTA TTA CGT GTG AGA GGA AAG ATC ACG CGG GTT CGT GGA CAC GGT TTT TTT TTT T-3’-biotin; KCHA10, 5’-ATC CAG AGT GAC GCA GCA GGG GAG GCG AGA GCG CAC AAT AAC GAT GGT TGG GAC CCA ACT GTT TGG ACA CGG TGG CTT AGT TTT TTT TTT T-3’-biotin. For flow cytometry and fluorescence microscopy, the 5’ end of an aptamer was labeled with fluorescein isothiocyanate (FITC) or carboxytetrame-thylrhodamine (TAMRA). All aptamers were synthesized using an ABI3400 DNA/RNA synthesizer (Applied Biosystems, Carlsbad, CA) with reagents purchased from Glen Research (Sterling, VA). DNA purification was performed with a ProStar HPLC (Varian, Walnut Creek, CA) using a C18 column (Econosil, 5U,
250 × 4.6 mm) from Alltech Associates (Deerfield, IL). DNA concentration was
determined by UV-Vis measurements using a Cary Bio-300 UV spectrometer (Varian).

The specific binding of aptamers and target cells was verified using confocal
fluorescence microscopy and flow cytometry. For confocal fluorescence microscopy,
cells were first incubated with 250 nM TAMRA-labeled aptamers in the binding buffer.
After washing three times with the washing buffer (DPBS with 4.5 g/L glucose and 5 mM
MgCl₂), fluorescence microscope images were taken using a confocal microscope. Flow
cytometry was performed with a FACScan cytometer (BD Immunocytometry Systems,
San Jose, CA). Briefly, 200,000 cells were incubated with FITC-labeled DNA aptamers
at 250 nM for 15 min in 200 µL of the binding buffer (or the capturing buffer as specified
later), and 10,000 counts were measured in the flow cytometer.

2.3.3 Cell Capture in a Buffer

Immediately before experiments, cells were rinsed with the washing buffer and
resuspended at 10⁶ cells/mL. By following the manufacturer’s instructions, the cells
were treated with Vybrant DiI or Vybrant DiD cell-labeling solutions (Invitrogen,
Carlsbad, CA) for 5 min at 37 °C, then rinsed with the washing buffer, and resuspended
at 10⁶ cells/mL in the capturing buffer. Labeled cells were stored on ice and further
diluted to the desired concentrations before experiments. Target CEM cells were spiked
into the control Ramos cells to form a final concentration of 10,000 cells/mL for CEM
cells and 10⁶ cells/mL for Ramos cells.

To initiate cell capture experiments, one channel volume of 1 mg/mL avidin
(Invitrogen, Carlsbad, CA) in phosphate buffered saline (PBS) was first introduced into
the device, followed by incubation for 15 min and then three rinses with the binding
buffer [washing buffer supplemented with yeast tRNA (0.1 mg/mL; Sigma-Aldrich),
bovine serum albumin (BSA) (1 mg/mL). Then, one channel volume of 30 μM biotinylated sgc8 aptamer with a poly-thymine (10-T) linker was introduced into the device and incubated for 15 min, followed by three times of rinsing with the binding buffer. Finally, 1 mL of a mixture of CEM cells (target) and Ramos cells (control) in the capturing buffer was pumped into the channel at a flow rate of 600 nL/s (or other flow rates specified in the text). To prevent cells from settling in the cell suspension while continuously pumping, a capturing buffer was prepared by mixing 1:1 volume ratio of the binding buffer and Histopaque-1119 (Sigma-Aldrich). The density of the capturing buffer was approximately 1.06 g/ml, which was close to the density of blood and cells. Histopaque-1119 increased the viscosity of the capturing buffer to that of whole blood to mimic the situation of isolating cells from whole blood. The BSA in the capturing buffer can passivate the surfaces to reduce the nonspecific adsorption of cells in the channel. At the end of the experiment, the microchannel was washed three times with the binding buffer, followed by taking fluorescent images for the determination of the cell concentrations. For the study of capturing Ramos cells using TD05 aptamer, CEM cells were used as control cells. For the study of capturing DLD-1 cells and HCT 116 cells using aptamers KDED2a-3 and KCHA10, Ramos cells were again used as the control cells. The concentration of all target cells was at 10,000 cells/mL, and the concentration of all control cells was at 10^6 cells/mL for all cell capturing experiments in the buffer.

2.3.4 Tumor Cell Isolation from Whole Blood

The single donor human whole blood was obtained from Innovative Research (Novi, MI), with anticoagulant of Ethylenediaminetetraacetic acid (EDTA). Colorectal cancer cells DLD-1 and HCT 116 were then spiked into the blood. The protocol of avidin adsorption and aptamer immobilization was performed as described in the previous
section. For capture study, to avoid damage by trypsin to the proteins on the cell surfaces, non-enzymatic cell dissociation reagent (1×) (MP Biomedicals, Solon, OH) was used (instead of trypsin) to detach cells from the culture dishes. Immediately before introducing into the device, cells were filtered using a 40 µm BD Falcon cell strainer (Becton, Dickinson and Company, Franklin Lakes, NJ) and then spiked into whole blood at a predetermined concentration of 10, 100, 1,000, 10,000 cells/mL.

2.3.5 Instrument Setup

The cell suspension (or whole blood) was introduced into the device by pumping. As shown in Figure 2-8, a Micro4 syringe pump (World Precision Instruments, Sarasota, FL) with a 1 mL syringe was connected to the inlet of the device via polymer tubing and a female luer-to-barb adapter (IDEX Health & Science, Oak Harbor, WA). The outlet of the device was connected to a waste collector. For tumor cell isolation from whole blood, no buffer additive (Histopaque-1119) was used to avoid the property change of whole blood. Instead, we used a tiny magnetic stirring bar inside the 1 mL syringe, with a stir plate beneath the syringe, to avoid cell settling. The magnetic stirring bar kept cells in suspension while blood was being pumped through the device. The device was placed on the stage of an Olympus FV500-IX81 confocal microscope (Olympus America, Melville, NY) for detecting cells captured.

To determine cell concentrations, a set of three images corresponding to the red fluorescent cells, blue fluorescent cells, and transmission images was acquired at eight positions in each channel. As shown in Figure 2-9, images were then imported into ImageJ (NIH), and cell counts were obtained using the Analyze Particles function after setting an appropriate threshold. Cell numbers were further verified by comparing fluorescent images with transmission images; only those with appropriate cell
mortality in the transmission images will be counted. Capture efficiency was
determined by dividing the number of the target cells captured by the number of total
target cells introduced into the channel. The purity of cells captured was determined by
dividing the target cells captured by the total cells captured.

2.3.6 Cell Viability

To determine the viability of cancer cells captured in the device, we performed
two assays on these cells: 1) propidium iodide (PI) and acridine orange (AO) staining
(Invitrogen) and 2) MTS assay (CellTiter 96® AQueous Non-Radioactive Cell
Proliferation Assay, Promega, Madison, WI). PI is a membrane-impermeant stain, thus
it labels only the dead cells with red fluorescence by penetrating the membranes of
dead cells and binding to their DNA. AO is a membrane-permeable dye that binds to
nucleic acids of all cells, resulting in green fluorescence. By PI/AO staining, nonviable
cells and viable cells can be differentiated by their difference in fluorescent images
under a microscope. We followed the instructions of the manufacturer to carry out the
assay. In brief, 200 µL of PI/AO working solution was prepared to contain 2 µM PI and 2
µM AO in PBS. After cell capture in the microfluidic device, one channel volume of the
PI/AO solution was introduced into the device and incubated for 10 min. The cells were
then examined under the confocal microscope and fluorescent images were taken to
evaluate the viability of captured cells.

MTS assay is a colorimetric method for determining the number of viable cells in
proliferation assays. To implement the assay, cells were first released from the device
by high flow rate washing and introducing air inside channel, followed by rinsing with the
washing buffer. These cells were then collected and quantified. Typically, 20,000
captured cells in 100 µL of fresh cell culture medium were seeded in each well of a 96-
well plate, and 3 repeats were simultaneously carried out. For comparison, cells without going through the capture experiment were seeded at the same concentration into other wells (3 repeats) of the same microplate. After the incubation of these cells at 37˚C under 5% CO₂ for 48 h, 20 µL of the MTS assay reagent was added to each well and incubated for another 3 h. A plate reader was used to measure the absorbance at 490 nm to evaluate the cell viability.

2.4 Results and Discussion

2.4.1 Cell-Aptamer Binding Using Flow Cytometry

To ascertain if a poly(T)-modified aptamer preserves its binding affinity and specificity to its target cells, flow cytometry analysis was carried out. Figure 2-10A shows the histogram of CEM cells from the flow cytometer. Compared to cells only, a large shift in the fluorescence signal was observed for those cells conjugated with sgc8 aptamers. The result suggests that poly(T)-appended sgc8 aptamers still have specific binding with CEM cells. A random single strand DNA library or TD05 aptamer did not have specific binding with CEM cells, showing a tiny shift in the fluorescence signal compared to cells only.

Similarly, Figure 2-10B shows that poly(T)-modified TD05 aptamers bound selectively with Ramos cells while sgc8 aptamer or a DNA library did not have specific binding. Figure 2-10C shows that the comparison of the flow cytometry between the cell-aptamer binding in the binding buffer and that in the capturing buffer. As detailed in the previous section, the capturing buffer was prepared by adding Histopque-1119 to the binding buffer for matching the density of blood. The flow cytometry results indicate that the addition of Histopque-1119 to the binding buffer did not have any adverse effect on the binding of sgc8 aptamer with CEM cells.
Confocal microscopy images also shows the strong binding of CEM cells with fluorescent-sgc8 aptamer (Figure 2-11A), while there is no binding between Ramos cells and sgc8 aptamer (Figure 2-11B), demonstrating the strong and specific binding of aptamers with their target cells.

### 2.4.2 Isolation of Lymphocytes in Device

The performance of the microfluidic device was demonstrated first by sorting leukemia cells: CCRF-CEM cells (human acute lymphoblastic leukemia) that function as target cancer cells and Ramos cells (human Burkitt’s lymphoma) that function as control cells. Biotinylated sgc8 aptamers have specific binding with CCRF-CEM cells, and they were immobilized onto the micropillars/microchannels. A cell mixture containing $10^4$ CEM cells and $10^6$ Ramos cells in 1 mL of the capturing buffer was used as a sample. To differentiate these two types of cells during imaging, CEM and Ramos cells were pre-stained with Vybrant Dil (red) and DiD (blue), respectively.

Figure 2-12A shows an image of the cancer cell mixture prior to sorting in the device and it is essentially all Ramos cells in blue. Figure 2-12B shows an image of cells captured after processing 1 mL of the cell mixture, and the majority of cells are now target CEM cells in red. These images show qualitatively that significant enrichment of the cancer cells was obtained through the microfluidic device. Figure 2-12C shows a single cancer cell captured on a micropillar in the device. To quantify the enrichment factor, we counted cells before and after sorting. Cell labeling and confocal fluorescence detection enabled the counting of the number of the target cells introduced into the device ($Ti$), the number of the target cells captured ($Tc$), and the number of the control cells captured ($Cc$). The cell capture efficiency ($E$) can be calculated by $E = Tc / Ti$ and the cell purity ($P$) in the captured cells can be calculated by $P = Tc / (Tc + Cc)$.
As high as 98% capture efficiency was obtained from the cell mixture when the flow rate was at 300 nL/s as discussed in detail below. The high capture efficiency partially arose from the specific binding of sgc8 aptamers with CEM cells, with a dissociation constant of $K_d = 0.8 \pm 0.09$ nM. In addition, a poly-T linker at the end of the biotinylated aptamer sequence should minimize the steric effects of the device surface on the aptamers, preserving the aptamers’ binding affinity to the target cells.

The specific and strong binding of sgc8 aptamers to CEM cells was verified by confocal fluorescence microscopy. Figure 2-12D shows the fluorescent micrograph of TAMRA-labeled sgc8 aptamer specifically bound to unstained CEM cells, but these aptamers did not bind to Ramos cells (Figure 2-11B). Fluorescence on cell surfaces not only demonstrated the binding of the aptamers to the target cells, but also showed a possible way to identify unstained CTCs captured from a sample via a fluorescently-labeled aptamer.

2.4.3 Effects of Channel Depth and Flow Rate

To optimize the performance of the microfluidic device, we investigated the effects of the channel depth and flow rate on capture efficiency and cell purity. By changing channel depth (pillar height), the size of micropillar sizes and the interpillar gap were altered simultaneously due to isotropic etching. As a result, the geometry of the micropillar device can be studied by varying the channel depth. Figure 2-13A shows that the capture efficiency reduced slightly with the increasing channel depth from 24 µm to 44 µm, whereas the cell purity in Figure 2-13B improved significantly with the same change in the channel depth. The decreased capture efficiency with the increasing channel depth is likely due to the reduction in the probability of cell encounters with the top and bottom surfaces in a deeper channel as well as cells
interaction with aptamers on pillars with wider inter-pillar gap. Nevertheless, the results show that our design of the pillar row shift and the curved surfaces of micropillars enabled sufficient interaction opportunities of cells with the surfaces, essentially maintained the capture efficiency when the channel depth was increased. However, the increased channel depth drastically reduced the non-specific binding, particularly geometric trapping of control cells, therefore significantly improving the cell purity. Based on the data in Figures 2-13 A and B, we chose a depth of 40 µm as the best trade-off between the capture efficiency and cell purity.

Figures 2-13C and D show the effects of the flow rate on the capture efficiency and the cell purity. The capture efficiency reduced with the increasing flow rate because of a larger shear force at a higher flow rate and the reduced interaction time between cells and surfaces. The cell purity improved with the increasing flow rate due to the fact that non-specifically-bound cells were washed away with a stronger shear force at a higher flow rate. Based on these results, we chose 600 nL/s as the best compromise between the capture efficiency and cell purity; and this flow rate results in sufficient throughput.

Using a device with the optimal channel depth of 40 µm and at a flow rate of 600 nL/s, we obtained cell purity of (81 ± 3)% with capture efficiency of (95 ± 2)%. These experimental conditions were used for all subsequent experiments. To compare this device with the previous efforts using a flat channel device, we plotted both results in Figure 2-14A. It shows that the capture efficiency of the device in this work is significantly better the flat channel in the previous work. The results verify the design in which 1) the increased surface area via micropillars enhanced the loading capacity of
aptamers, and 2) the row shift of micropillars and channel geometry significantly increased the probability of cell encounters with aptamers on the surfaces.

Using the optimal channel geometry and flow rate, we also studied 3 other types of cancer cells in the device as shown in Figure 2-14B. They were captured in a cell mixture using their respective aptamers. To study the isolation of Ramos cells, an aptamer called TD05 was used as it has specific binding with Ramos cells (with a dissociation constant of $K_d = 74.7 \pm 8.7 \text{nM}$). A cell mixture containing 10,000 Ramos cells and $10^6$ CEM cells in 1 mL buffer was used and the capture efficiency was $(93 \pm 2\%)$. Two carcinoma cells, HCT 116 cells (Dukes’ type C colorectal adenocarcinoma) and DLD-1 cells (colorectal carcinoma) were also studied. HCT 116 cells have strong affinity with KCHA10 aptamer ($K_d = 21.3 \pm 1.7 \text{nM}$) while DLD-1 cells show specific binding with KDED2a-3 aptamer ($K_d = 29.2 \pm 6.4 \text{nM}$).\textsuperscript{86,87} Using a cell mixture containing 10,000 of each type of carcinoma cell and $10^6$ Ramos cells (as the control), we obtained capture efficiency of $(97 \pm 3\%)$ for HCT 116 cells and $(91 \pm 1\%)$ for DLD-1 cells, respectively.

2.4.4 Tumor Cell Isolation from Whole Blood

To mimic the isolation of CTCs from patient blood, we spiked colorectal carcinoma cells, HCT 116 cells, into whole blood that was used as received. One mL of unprocessed whole blood, spiked with 100 HCT 116 cells, was introduced into the microfluidic device at a flow rate of 600 nL/s. Using the experimental procedures described in the previous section, we obtained capture efficiency of $(96 \pm 8\%)$ as shown in Figure 2-14C. A similar experiment using another carcinoma cells, DLD-1 cells, resulted in capture efficiency of $(92 \pm 6\%)$ (note that a different aptamer was used).
To illustrate the potential of the device for clinical applications, we evaluated the isolation of HCT 116 cells from whole blood at concentrations of 10,000, 1,000, 100 and 10 cells/mL. Capture efficiencies of >95% were achieved in all cases, and a calibration curve between the number of the cells spiked and the number of the cells captured is shown in Figure 2-14D. Comparable results were obtained for capturing HCT 116 cells from the capturing buffer. The results show that the device has a potential to detect CTCs in clinical samples since the number of CTCs in 1 mL of peripheral blood of cancer patients is often in the range of 1-100.\(^8^8\)

In addition, we addressed the problem of low throughput of a typical microfluidic device by connecting 8 microchannels through bifurcation (Figure 2-1). The width of each channel is 2 mm. With the optimal flow rate of 600 nL/s, the time required to process 1 mL of whole blood in the device is 28 minutes, which is favorable compared with hours of operation required in the benchmark instrument.

2.4.5 Cell Viability

Isolation and enumeration of tumor cells in peripheral blood of cancer patients is important for medical diagnostics and prognosis. However keeping the cells viable during the isolation process is important for subsequent molecular and cellular studies, so that potential therapeutic treatment can be derived after understanding the metastasis mechanisms.\(^8^9\)

The viability of the cancer cells captured was examined with PI/AO assay and MTS assay as described in the Experimental Section. Figure 2-15 shows fluorescent microscope image of all cells captured from the mixture of CEM and Ramos cells using PI/AO cell viability assay. The majority of cell colors are in green, indicating that the most of the captured cells are still viable. We obtained cell viability of (94 ± 2)% for CEM
cells when they were processed through the device under the optimal flow rate and channel depth. In addition, we used cell proliferation (MTS assay) as an alternative, and we found cell viability of (93 ± 3)%, which is statistically the same as in the PI/AO assay. These results indicate that the cells captured using our device are suitable for subsequent cell culture and molecular analysis.

2.5 Release of Captured Cells

2.5.1 Motivation

After tumor cells captured in the microfluidic device, a primary challenge is to detach the captured cells. For ligand-based surface capture methods, cells are firmly captured with antibodies or aptamers. Release of the cells of interest is very important for subsequent cell culture and further cellular and molecular analysis. In addition, because that the detection of captured cells relies on the time-consuming task of scanning the entirety of a microfluidic device using a microscope to generate statistically valid data, efficient release of captured cells will enable rapid detection methods such as flow cytometry. Several groups have reported the use of trypsin-based, DNase-facilitated and temperature-mediated methods for cell release. However, the efficiency of these approaches is not satisfactory. In addition, some of them are even detrimental to cells. Therefore, efficient cell release with negligible cell damage is of great significance. Herein, we have developed methods for efficient release of captured cells while kept cells viable.

2.5.2 Cell Release Using Complementary DNA

For ligand-based cell capture, DNA aptamers provide many advantages over antibodies for cell release. Here, we developed a release method using complementary DNA sequences, which can competitively hybridize with the cell-bound DNA aptamers.
After hybridization, aptamers were released from the cells, leading to cell detachment, shown in Figure 2-16. Results indicate that the complementary DNA can release ~60% of captured cells on the channel surface. Flow cytometry results (Figure 2-17B) also show that part of the aptamers were released from cell surface after incubating with complementary sgc8 (c-sgc8) for 30 min with subsequent washing (with decreased fluorescence intensity).

Aptamer usually has a hairpin structure (with a loop and a stem) after binding with its target proteins on the cell surface, making the hybridization difficult to initiate. To enhance DNA hybridization, a toehold sequence was incorporated to the end of the aptamer and the complementary DNA. The DNA aptamer sequences used for cell capture and release are listed in Table 2-1. As shown in Figure 2-17A, the toehold sequences displaced the base pair in the stem part, promoted the opening of a typical hairpin structure of aptamer, thus enhancing the DNA hybridization reaction. Flow cytometry analysis also showed enhanced DNA hybridization (Figure 2-17B) with the toehold-mediated DNA strand displacement. This DNA engineering method increased the release efficiency of the target cells from 60% to 82% (Figure 2-17C). Figure 2-18 shows representative cell images before and after release, demonstrating the effective release of captured CEM cells from the device using toehold-mediated DNA hybridization.

2.5.3 Alternative Cell Release Methods

Besides the complementary DNA based method, the following are potential methods for aptamer-mediated cell release. One method for cell release will involve the synthesis of a disulfide bond on the aptamer. After cell capture, cleavage of the disulfide bond can be induced by a biocompatible reducing agent (sodium 2-
sulfanylethanesulfonate, or Glutathione), which cuts the aptamer and releases the cells. Since the reducing agent is a small molecule, it has very low steric effect, allowing much more interaction between the reducing agent and the aptamers compared with DNase. Another method for cell release will involve using a photocleavable (PC) biotin or PC linker conjugated aptamer. The cell release is also feasible for PC-biotin conjugated antibodies. PC-biotin or PC-linker modified aptamer will be used for cell capture, and the aptamer can be cleaved upon UV light irradiation. After capture, cells can be released by UV irradiation for ~10 min. With a photomask, this photocontrollable method can also be used for cell micropatterning.

**2.6 Bonding and Surface Functionalization of Cyclic Olefin Copolymer Microchip for Aptamer-Based Cancer Cell Capture**

**2.6.1 Motivation**

While silicon and glass have been widely used for lab-on-a-chip applications, polymers have been increasingly used in diagnosis because of their inexpensive fabrication. Among them, polydimethylsiloxane (PDMS) has enjoyed popularity because of its fast prototyping using soft-lithography, and easy surface modification similar to silicon and glass. However, PDMS is poorly suited to mass production, which limits its disposability. In addition, the elastomeric PDMS suffers from mechanical softness and gas permeability, which limits its application for many diagnostic purposes. Recently, thermoplastics, most notably, polymethyl methacrylate (PMMA) and cyclic olefin copolymer (COC) have played an increasingly important role for microfluidic and lab-on-a-chip device fabrication because of their low cost, mass production capability and disposability. Thus thermoplastic microfluidics is invaluable for in vitro diagnosis, such as circulating tumor cell (CTC) detection.
Cyclic olefin copolymers (COCs) or Cyclic olefin polymers (COPs) are increasingly popular because of their excellent mechanical, thermal, chemical, and optical properties. Compared with other thermoplastics, COC devices have excellent optical transparency, high chemical resistance and wide range of glass-transition temperature ($T_g$), which makes COC ideal for lab-on-a-chip applications. However, COC has not been widely used because of its inert surface for chemical modification and biomolecule immobilization.

Herein, we developed a facile surface modification method for COC to immobilize biomolecules, such as aptamers. Antibodies can also be immobilized on COC surface with the similar protocol. We also developed a pressure-free bonding method for fabricating robust COC device. Then we employed the fabricated COC microchip for efficient capture of cancer cells using aptamers.

### 2.6.2 COC Device Design and Fabrication

The micropillar device design is the same as the device reported in section 2.2. A glass device was first fabricated using the same protocol reported in section 2.2. Afterwards, the glass device was sent to a vendor (NiCoForm, Inc., Rochester, NY) for fabrication of a metal mold using electroplating. With the metal mold (also called E-form), a COC device can be easily fabricated using compression molding. Figure 2-19A shows a picture of the fabricated COC device, with micropillar array shown in the SEM images (Figure 2-19 C &D).

### 2.6.3 Pressure-Free Bonding

Thermoplastic devices were often fabricated by bonding a cover film with a substrate containing microchannels or other microfeatures. Typical bonding techniques include thermal fusion, solvent bonding, surface treatment and adhesives.
Most bonding methods involve pressure (e.g., lamination), which leads to collapse of wide channels (e.g., the inlet and outlet area of our device shown in Figure 2-19A). Here, a COC substrate with microfeatures was bonded with another COC film by spincoating a layer of PDMS membrane (1500 rpm, 30 seconds) on the COC film, without applying any pressure. Both the COC substrate (Zeonor 1020R, Zeon Chemicals L.P., Louisville, KY) and PDMS-coated COC film (TOPAS 8007, TOPAS Advanced Polymers, Inc., Florence, KY) were treated with UV/Ozone before being attached together for the bonding. A scheme of the bonding process is shown in Figure 2-19B.

### 2.6.4 One-Step Surface Immobilization of Aptamers on COC

The surface functionalization of COC is difficult because of the saturated hydrocarbon structure of COC which has only C-C and C-H bonds. Methods of COC surface functionalization include direct oxygen plasma treatment, photografting, silanization, etc. Among these methods, silanization provides a robust immobilization of biomolecules by forming covalent bonds, but it usually involves using cross-linkers such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS), 1,4-Phenylenediisothiocyanate (PDITC), toluene-2,4-diisocyanate (TDI), and succinimidyl-4-((N-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC) for immobilization of biomolecules. The cross-linking step makes the surface modification complicated and less efficient. Herein, we developed a method of COC functionalization using 3-Isocyanatopropyl triethoxysilane (IPTES), which avoided the time-consuming cross-linking. The –N=C=O group on IPTES end can directly react with –NH₂ group on amino-aptamers without any catalyst, making our surface modification efficient and facile. Figure 2-20 shows the process of surface modification of COC device for immobilizing aptamers and capturing cancer cells. FITC-modified amino-aptamer was
used to determine the immobilization efficiency, with confocal laser scanning microscope for the detection. Results showed that a strong fluorescent signal was detected on IPTES-treated COC surface after washing (Figure 2-21A), which indicated that fluorescent DNA aptamers were immobilized on the surface of COC. Control experiments without using IPTES showed very weak fluorescence as shown in Figure 2-21B & C.

2.6.5 Cancer Cell Capture in COC device

For the IPTES-treated COC channel, amine-modified aptamer was used for immobilization on the device surface. Then, target cancer cells were introduced into the channel through pumping at a flow rate of 300 nL/s. The micropillar-based COC device achieved ~92% capture of CEM cells using amino-sgc8 aptamer, and ~90% capture of Ramos cells using amino-TD05 aptamer, as shown in Figure 2-21D.

2.7 Conclusion

In this chapter, a DNA aptamer-enabled, micropillar-based microfluidic device was demonstrated for the isolation of cancer cells in unprocessed peripheral blood. The unique geometry of the micropillar array in the device resulted in the high-performance cell isolation. High-affinity aptamers were used as an alternative to antibodies for cancer cell isolation. This microfluidic device enabled the isolation of as few as 10 tumor cells from 1 mL of untreated whole blood with >95% capture efficiency within 28 minutes. A cell release method was developed and a thermoplastic device was fabricated for cell capture. The advantages of such a device over the benchmark methods include rapid analysis, no pre-treatment of blood samples, and low detection limit. As a result, the device has a potential to be used for clinical applications such as cancer diagnosis, prognosis, and monitoring the progress.
Figure 2-1. Layout of the microfluidic device. A) Picture of the device consisting of 1 inlet, 1 outlet, and 8 channels connected through bifurcation. The size of the device is 3 in. × 1 in., the same size of a microscope slide. B) Optical micrograph (10X) of a portion of micropillar array in a channel. C) Scanning electron microscope (SEM) image of isotropically etched elliptical micropillars in the glass substrate.
Figure 2-2. COMSOL simulation for the micropillars. Streamline and velocity magnitude of flow across through the micropillar array: A) at the top of the micropillars; B) at the center of the micropillars. Since the micropillars were isotropically etched, the pillar size varies from the top to the bottom. Distorted streamlines indicated enhanced interactions between cells and device surfaces.
Figure 2-3. Comparison of streamlines through different micropillar arrays using COMSOL simulation. A) The micropillar array with a shift after every three rows, with distorted streamlines for enhancing cell-micropillar interactions; B) The micropillar array without any shifts, limited streamline distortion is observed.
Figure 2-4. Scheme of surface functionalization and cancer cell capture in the device. Avidin is immobilized on the surface of the microchannels/micropillars via physical adsorption, followed by immobilization of biotinylated aptamers through biotin–avidin chemistry. Target cancer cells are then captured via the specific interaction between the aptamers and the receptors on cell surfaces.

Figure 2-5. Confocal fluorescence images of FITC-modified aptamers with different concentrations immobilized on the surfaces of a microfluidic channel. 1 mg/mL of avidin was introduced into the channel and incubated for 15 min, followed by three times of washing using the binding buffer. FITC-labeled sgc8-poly(T)-biotin aptamers were then introduced into the channel, incubated for 15 min, and washed three times with the binding buffer. The concentrations of aptamers are: A) 2.5 µM, B) 25 µM, C) 50 µM, and D) 100 µM.
PDMS/glass substrate → Corona discharge/Air plasma → Hydrophilic surface

Oxidation

Device bonding; 4% MPTMS in ethanol for 1h

0.2 mM GMBS in ethanol for 0.5h

10 µg/mL streptavidin in PBS for 0.5h

Streptavidin modified surface

Biotinylated aptamer or antibody

Cell immobilized on surface

SA

Figure 2-6. Scheme of surface modification of glass or PDMS substrate with streptavidin using silane-based method. With streptavidin modified surface, biotinylated aptamer was then immobilized on the surface, followed by cell captured.

Figure 2-7. Comparison of avidin physical adsorption based method (Adsorption) and silane based method (Silane) toward the immobilization of aptamers, on PDMS and glass surface, respectively.
1 mL whole blood
Spiked with CTCs

Pumping

Microfluidic device

Blood in

Blood out

CTCs captured inside channel

SEI     5.0KV     X300     10µm

Figure 2-8. Scheme of cancer cell capture experiment setup. 1 mL whole blood spiked with CTCs was introduced into the 3” × 1” sized microfluidic device through pumping. CTCs were captured among the aptamer-functionalized micropillars. The bottom picture shows the scanning electron microscope (SEM) image of the micropillar array.
Figure 2-9. Counting of cell numbers using ImageJ. A) The intensity threshold, cell size and circularity can be appropriately selected for accurate cell counting. B) With display of the overlay outline, each cell count can be confirmed; adjustment can be made for cluster of cells.
Figure 2-10. Flow cytometry histograms showing the selective binding of target cells with corresponding aptamers. A) CEM cells selectively bind with sgc8 aptamers. B) Ramos cells selectively bind with TD05 aptamers. C) Comparison of cell-aptamer binding in the binding buffer (BB) and in the capturing buffer (CB).
Figure 2-11. Confocal laser scanning microscopy images showing the selective binding of target CEM cells with sgc8 aptamers. A) Fluorescence image of CEM cells selectively bind with TAMRA-labeled sgc8 aptamers (Red fluorescence). B) Ramos cells do not bind with sgc8 aptamers. Left panels are TAMRA fluorescence pseudo-colored red, and right panels are the overlay of TAMRA fluorescence and the bright-field image (scale bar = 20 µm).
Figure 2-12. Fluorescence images of cancer cells captured in device. A) Representative image of low-abundant target CEM cells (stained with a red fluorescent dye) among high-abundant control Ramos cells (blue) before sorting. B) Representative image of CEM cells (red) among Ramos cells (blue) after sorting (1 mL of the cell mixture was enriched through the microfluidic device). Scale bar = 50 µm. C) Image of a CEM cell captured on the wall of an elliptical micropillar in the device (scale bar = 20 µm). D) Microscopy image of unstained CEM cells bound with fluorescently-labeled aptamers, with color only on the surface of target cells (scale bar = 20 µm).
Figure 2-13. Capture efficiency and purity as a function of channel depth and flow rate. A) The capture efficiency as a function of the channel depth. B) The purity of cells captured as a function of the channel depth. The flow rate is 600 nL/s for both A) and B). C) The capture efficiency as a function of the flow rate. D) The purity of cells captured as a function of the flow rate. The channel depth is 40 µm for C) and D). In all experiments, CEM cells were used as the target cells and Ramos Cells as the control cells. The error bars represent one standard deviation of 3 repeated experiments.
Figure 2-14. Tumor cell capture from whole blood using the microfluidic device. A) Comparison of the capture efficiency between a flat channel device reported previously and the micropillar device in this work. B) Capture efficiencies of 4 types of cancer cells in the microfluidic device with micropillars. A different aptamer with specific binding with cells of interest was used for each type of cancer cells. C) Capture efficiencies of DLD-1 cells and HCT 116 cells in whole blood. D) Regression analysis of the number of the cells captured by the microfluidic device versus the number of the cells spiked into 1 mL of samples. HCT 116 cells at different concentrations were spiked either into the capturing buffer with Ramos cells as the control or into whole blood. Two calibration curves overlap with each other, reflecting no significant difference between buffer and blood samples. The error bars represent one standard deviation of 6 repeats for 10-cell samples and 3 repeats for other cell numbers.
Figure 2-15. Fluorescent microscope image shows the viability of captured cells. Image of CEM cells captured in the microfluidic device after PI/AO staining. The red color indicates nonviable cells (PI staining) while the green only color indicates viable cells (AO staining), scale bar = 50 µm.

Figure 2-16. Scheme of cell release using complementary DNA sequence. A) After cells captured inside microchannel, complementary DNA was added to the channel. B) Complementary DNA competitively hybridized with the aptamers, thus releasing the captured cells.
Figure 2-17. Release of captured cells using toehold-mediated DNA hybridization. A) Schematic of toehold-mediated DNA hybridization by DNA strand displacement. The complementary DNA competitively hybridizes with sgc8 aptamer, the toehold facilitates the opening up of the hairpin structure, thus enhancing the DNA hybridization. B) Flow cytometry assay shows the binding of fluorescent-sgc8 with CEM cells, with a high fluorescence signal observed (green and cyan histogram). After adding complementary DNA, the aptamer is released by competitive hybridization, and the signal shifts back (blue histogram). With the toehold sequence, the signal shifts back further (purple histogram). C) Comparison of releasing cells using hybridization alone and toehold-mediated hybridization.
Figure 2-18. Efficient release of captured cells. A) Representative microscopy image of the captured cells before release. Target CEM cells (red) were efficiently captured with a few control Ramos cells (blue) nonspecifically captured. B) Representative microscopy image of the captured cells after treatment with Toehold-based complementary DNA for 30 min and washing; most of the cells were released. Scale bar = 50 µm.
Figure 2-19. Picture of COC microchip and its bonding process. A) Picture shows the 3"x1" sized COC device after pressure-free bonding; B) The bonding process of COC device; C) 100× SEM image of the COC micropillar array; D) 600× SEM image of the COC micropillars.
Figure 2-20. One-step surface immobilization of aptamers on COC surface for cell capture. UV/Ozone treated COC was first incubated with IPTES, followed by washing, then amino-modified aptamer was incubated for 2h for immobilization, finally, target cancer cells were specifically captured on the COC device.
Figure 2-21. Surface coating of amino-aptamers on COC substrate for cell capture. A) With IPTES coating, fluorescent amino-aptamers were efficiently immobilized on COC surface; B) Without IPTES coating, few aptamers were immobilized; C) Comparison of the fluorescence intensity for A and B. D) Cell capture efficiency of CEM cells and Ramos cells using NH$_2$-sgc8 aptamers and NH$_2$-TD05 aptamers, respectively.
<table>
<thead>
<tr>
<th>Name</th>
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<td>sgc8</td>
<td>5’-ATC TAA CTG CTG CGC CGC CGG GAA AAT ACT GTA -poly T-biotin-3’</td>
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<tr>
<td>toehold-sgc8</td>
<td>5’-GAG TGA GGT TTT TAT CTA ACT GCT GCG CCG CCG (-poly T-biotin-3’</td>
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<tr>
<td>complementary</td>
<td>5’-TCT AAC CGT ACA GTA TTT TCC CGG CGG CGC AGC</td>
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<td>toehold-sgc8</td>
<td>AGT TAG ATA AAA ACC TCA CTC-3’</td>
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CHAPTER 3
MULTIVALENT DNA NANOSPHERES FOR ENHANCED CAPTURE OF CANCER CELLS IN MICROFLUIDIC DEVICES

3.1 Background

The isolation of rare cells in peripheral blood such as circulating tumor cells (CTCs) is highly important but challenging.\textsuperscript{13, 35, 100} CTCs are cancer cells shed from either primary tumors or metastatic sites and are highly related to the initiation of metastasis and the spread of cancer to distant organs. Thus CTCs hold the key for understanding metastasis, diagnosing cancer and monitoring treatment response.\textsuperscript{10, 12, 67} However, the extraordinary rarity of CTCs makes their isolation and characterization technically challenging. Traditionally, methods based on flow cytometry have been used in clinics, but with a considerable number of false negatives and low detection sensitivity.\textsuperscript{32, 101} The only FDA-approved CTC enumeration method is CellSearch Assay, which uses antibody-coated magnetic beads for CTC isolation. However, it suffers from low CTC-capture efficiency.\textsuperscript{37, 102} Recently, microfluidic devices with monovalent capture ligands, including antibodies\textsuperscript{17, 47, 51, 103} and nucleic acid aptamers,\textsuperscript{74, 75, 78} have been extensively used for immunocapture of rare tumor cells. However, most efforts for increasing the sensitivity of cell capture are based on engineering complicated structures inside the microfluidic devices, such as microposts, sinusoidal channels, and silicon nanopillars, etc., for enhancing ligand-cell interactions.\textsuperscript{48-50, 72, 104} These structures make the device fabrication time-consuming and induce significant nonspecific cell capture, causing low specificity.

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Part of this chapter has been published in “Sheng, W. et al., Multivalent DNA Nanospheres for Enhanced Capture of Cancer Cells in Microfluidic Devices, ACS Nano, 2013, 7, 7067-7076”
Herein, we have investigated use of nanotechnology-based multivalent binding to enhance cell capture in microfluidic devices. Multivalent binding, the simultaneous interaction of multiple ligands on one entity with the complementary receptors on another, has been widely used for achieving high-affinity molecular recognition in biological processes. The multivalency-enhanced binding between the ligands and targets in those biological systems has been extensively investigated. To achieve multivalent binding, scaffolds from numerous nanoscale structures, such as dendrimers, nanorods, nanoparticles, polymers and proteins, have been used by researchers for assembling multiple ligands. And dendrimer-mediated multivalent binding have been used for enhanced surface capture of cells. Recently, nucleic acid aptamers have been selected for targeting numerous cancers, and nanomaterial-aptamer conjugates have been extensively used for enhanced molecular recognition, but none of them have been used for enhancing capture of cancer cells. Here, we hypothesize the nanoparticle-aptamer conjugates could greatly improve the efficiency of capturing cancer cells. We chose gold nanoparticles (AuNPs) as the multivalent ligand scaffolds to assemble multiple DNA aptamers (DNA nanospheres) owing to their easy synthesis and conjugation with DNA. Our flow cytometric analysis demonstrated the multivalent binding between aptamers and cells through AuNP-conjugation. Then we developed a flat channel microfluidic device which is able to capture cancer cells from buffer or lysed blood with high efficiency and high throughput using the AuNP-aptamer conjugates (AuNP-aptamer). The enhanced binding affinity afforded by the AuNP-aptamer modified surface significantly increased the capture efficiency of target cancer cells. And the AuNP-aptamer maintained high
capture efficiency with increased flow rate, which considerably improves the sample throughput of the microfluidic device.\textsuperscript{127}

The scheme of the AuNP-aptamer mediated cell capture is shown in Figure 3-1. The microfluidic device surface is first coated with avidin by physical adsorption.\textsuperscript{75, 78} Then, biotinylated aptamer-conjugated AuNPs are immobilized onto the channel through biotin-avidin interaction. When a sample containing target cancer cells passes through the channel, cells are captured via the specific interaction between the aptamers and the target cell receptors. Since each AuNP is conjugated with ~95 aptamers, we hypothesize that the AuNP-aptamer binds to cell surface markers in a cooperative manner, leading to multivalent effect and resulting in enhanced cell capture efficiency. Besides the multivalent binding, the AuNP-aptamer modified surface increases the surface roughness\textsuperscript{104} and allows enhanced local topographic interactions between the AuNP-aptamers and nanoscale receptors on the cell surface,\textsuperscript{50, 128, 129} contributing to the increased cell capture.

### 3.2 Methods

#### 3.2.1 Synthesis and Characterization of Gold Nanoparticle-Aptamer Conjugates

Hydrogen tetrachloroaurate (III) (HAuCl\textsubscript{4}), trisodium citrate dihydrate, tris-(2-carboxyethyl) phosphine hydrochloride (TCEP), tris-(hydroxymethyl) aminomethane (Tris), and sodium acetate were obtained from Sigma-Aldrich (St. Louis, MO). Acetate buffer (500 mM, pH 5.2) was prepared using a mixture of sodium acetate and acetic acid. Tris acetate buffer (500 mM, pH 8.2) was prepared using Tris and acetic acid.

AuNPs were prepared using the protocols reported previously.\textsuperscript{130} Briefly, 100 mL of 1 mM HAuCl\textsubscript{4} solution was heated till reflux. Then, 10 mL of 38.8 mM sodium citrate was added and reflux was continued for another 20 min. The diameter of such prepared
AuNPs was ~13 nm, measured by transmission electron microscopy (TEM). The concentration of the AuNPs was ~13 nM, determined by UV-Vis measurement at 520 nm using a Cary Bio-300 UV spectrometer (Varian) (Figure 3-2A).

DNA aptamers were synthesized in-house. Thiol modified-sgc8 aptamer sequence was: 5’-thiol- PEG-ATC TAA CTG CTG CGC CGC CGG GAA AAT ACT GTA CGG TTA GA-biotin-3’. The sequences of all aptamers used are listed in Table 3-1. For flow cytometric analysis, a fluorescein isothiocyanate (FITC) modifier was used to replace the biotin linker. All DNA aptamers were purified using a ProStar HPLC (Varian, Walnut Creek, CA) with a C18 column (Econosil, 5U, 250 × 4.6 mm) from Alltech Associates (Deerfield, IL), with triethylammonium acetate-acetonitrile as eluent. DNA concentration was determined by UV-Vis measurement at 260 nm.

Thiol-modified aptamers were conjugated on AuNPs using the reported protocols.131,130,132 Aptamers (9 µL, 1 mM) were added with acetate buffer (1 µL, 500 mM) and TCEP (1.5 µL, 10 mM) and incubated for 1 h at room temperature to activate the thiol group. Then the TCEP-treated aptamer was added to 3 mL of as-prepared AuNPs and incubated for 16 h. Finally, Tris acetate buffer (30 µL, 500 mM) and NaCl (300 µL, 1M) were added, and the mixture was incubated for 24 h. Unconjugated aptamers was then removed by centrifugation at 14,000 rpm for 15 min.

The aptamer concentration in the supernatant was measured, and the final conjugated aptamer concentration in the AuNPs was determined by subtracting the supernatant concentration from the previous aptamer concentration. The final AuNP concentration was 12.7 nM with an aptamer concentration was 1.2 µM, giving an average of approximately 95 aptamers on each AuNP. Dynamic light scattering (DLS)
measurement was performed to evaluate the hydrodynamic diameter of the AuNPs before and after conjugation with aptamers using Zetasizer Nano ZS, (Malvern, Worcestershire, United Kingdom) (Figure 3-3). Zeta-potential measurements were performed using the same instrument. Fluorescence spectroscopy (Figure 3-2B) also demonstrated the successful conjugation of aptamer on the AuNP. The fluorescence signal of each AuNP-aptamer conjugate is much higher than that of individual aptamer.

**3.2.2 Device Design and Fabrication**

A single flat channel device was initially used for proof-of-concept studies, and then eight flat channels were parallelized to form a high throughput device. As shown in Figure 3-4A, the single flat channel device was designed with a length of 50 mm, width of 2 mm, depth of 100 µm, and with single inlet and outlet. Three independent devices can be incorporated within one microscope slide size (3 in. × 1 in.). To increase the throughput, eight channels were connected through parallelization, and uniform flow was maintained in the eight channels. The size of the high throughput device is also 3 in. × 1 in., as shown in Figure 3-4B. Both of the two devices were made of polydimethylsiloxane (PDMS), and bonded to a 3 in. × 1 in. glass slide.

PDMS devices were fabricated according to the procedures reported by Whitesides’ group. The layout of the device was designed in AutoCAD and then sent to CAD/Art Services, Inc. (Bandon, OR) to produce a high resolution transparency photomask. Silicon wafers (Silicon Inc., Boise, ID) were first spin-coated with SU-8 2035 photoresist (MicroChem, Newton, MA) using a spin coater (Laurell Tech., North Wales, PA). Then the pattern on the photomask was transferred to the silicon substrate via UV exposure. After development, a silicon master patterned with the complementary structures was obtained. PDMS devices were fabricated by casting a liquid PDMS
precursor against the master using Sylgard 184 reagents (Dow Corning, Midland, MI) according to the instructions of the manufacturer. To prevent the cured PDMS from sticking to the silicon master, TFOCS (Tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane) (Sigma-Aldrich) was vacuum vaporized to the surface of the master. The channel depth, which was controlled by the spin speed of the SU-8, was measured using a Dektak 150 profilometer. The PDMS substrate was then sealed with a glass microscope slide, and inlet and outlet wells were created at the channel ends by punching holes in the PDMS sheet.

The design of herringbone mixer-device was inspired by several works in the literature, and the dimensions were chosen for optimal cell capture, as shown in Figure 3-9A. The mixer device was fabricated as described above, but using a two-layer SU-8 fabrication technique, with two coating and exposure steps and a single developing step. The silicon mold consists of a first layer as the main channel and the second layer as the herringbone ridges, which become grooves after transfer to the PDMS substrate. As shown in Figure 3-5, enhanced mixing occurs with the herringbone groove structure inside the PDMS microchannel, which generates transverse flow and microvortex.

3.2.3 Cell Lines and Buffers

T-cell human acute lymphoblastic leukemia cells (CCRF-CEM cells, CCL-119) and B-cell human Burkitt’s lymphoma cells (Ramos cells, CRL-1596) were purchased from American Type Culture Collection (ATCC). CEM and Ramos cells were cultured in RPMI medium 1640 (ATCC) supplemented with 10% fetal bovine serum (FBS; heat-inactivated; Gibco) and 100 units/mL penicillin-streptomycin (Cellgro, Manassas, VA). Both cultures were incubated at 37°C under 5% CO₂ atmosphere. Dulbecco’s
phosphate buffered saline with calcium and magnesium (PBS) (Fisher Scientific, Hampton, NH) was used to wash cells. A solution of 50 mg/mL (5%) bovine serum albumin (BSA) (Fisher) and 0.1% Tween-20 (Fisher) in PBS was used for rinsing the unbound molecules on the surface, and resuspending cells for the cell capture. BSA and Tween-20 in PBS can fully passivate the surfaces to reduce nonspecific adsorption of cells in the channel.

3.2.4 Flow Cytometric Analysis

Flow cytometry was used to evaluate the targeting capabilities of AuNP-aptamer conjugates toward specific cells. Fluorescence measurements were made with a FACScan cytometer (BD Immunocytometry Systems, San Jose, CA). Briefly, 200,000 cells were incubated with FITC-labeled free aptamer or AuNP-aptamer conjugates in 200 μL of PBS (containing 0.1% BSA) for 30 min on ice. After incubation, the cells were washed three times by centrifugation with 200 μL PBS, and 10,000 counts were measured in the flow cytometer to determine the fluorescence. Varying concentrations of free sgc8 and AuNP-sgc8 aptamers were used to determine their binding affinities. The FITC-labeled random DNA library was used as a negative control to determine nonspecific binding. All of the experiments for the binding assay were repeated three times. The mean fluorescence intensity of target cells labeled by aptamers was used to calculate the specific binding by subtracting the mean fluorescence intensity of nonspecific binding from random DNA library. The equilibrium dissociation constants ($K_d$) of the aptamer-cell interaction were obtained by fitting the dependence of fluorescence intensity of specific binding on the concentration of the aptamers to the equation $Y = B_{max}X/(K_d + X)$ using SigmaPlot (Jandel, San Rafael, CA), where $Y$ is the fluorescence intensity and $X$ is the concentration of aptamers.
### 3.2.5 Cell Capture Assay in Microfluidic Devices

Immediately before cell capture experiments, cells were washed with PBS and resuspended at \(10^6\) cells/mL. By following the manufacturer's instructions, CEM and Ramos cells were stained with Vybrant Dil (red) and DiD (blue) cell-labeling solutions (Invitrogen, Carlsbad, CA), respectively, then washed with PBS, and resuspended at \(10^7\) cells/mL in the PBS containing BSA and Tween-20. Labeled cells were stored on ice and further diluted to the desired concentrations before cell capture.

The single donor human whole blood was obtained from Innovative Research (Novi, MI), with anticoagulant of ethylenediaminetetraacetic acid (EDTA). Lysed blood was obtained by treating whole blood with red blood cell lysing buffer (Sigma-Aldrich) (containing \(\text{NH}_4\text{Cl}\)) according to manufacturer's instructions. Different concentrations of CEM cells were then spiked in whole blood or lysed blood.

To start cell capture experiments, one device volume (~100 µL) of 1 mg/mL avidin (Invitrogen) in PBS was first introduced into the device, followed by incubation for 15 min and then three rinses with PBS. Then, 100 µL of sgc8 aptamer or AuNP-sgc8 aptamer was introduced into the device and incubated for 15 min, followed by three rinses with the PBS containing BSA and Tween-20. Finally, 1 mL of cell mixture or blood sample spiked with cancer cells was continuously pumped into the device at a flow rate of 1.2 µL/s (or other flow rates specified in the text). For cell capture using antibody, anti-PTK7 biotin (Miltenyi Biotec, Auburn, CA) was used instead of sgc8 or AuNP-sgc8 aptamer. Afterwards, the device was washed three times with PBS to remove nonspecifically captured cells, followed by acquiring fluorescent images to determine the cell numbers. To test the purity of captured cells from lysed blood or whole blood, DAPI (Invitrogen) was introduced into the device to label the
nonspecifically captured white blood cells. By following the manufacturer’s instructions, 300 nM DAPI was incubated with cells for 10 min, followed by rinsing with PBS.

The cell suspensions or blood samples were introduced into the device by pumping. A Micro4 syringe pump (World Precision Instruments, Sarasota, FL) with a 1 mL syringe was connected to the inlet of the device via FEP (Fluorinated ethylene propylene) tubing and a female luer-to-barb adapter (IDEX Health & Science, Oak Harbor, WA). To avoid cell settling, a tiny magnetic stirring bar was placed inside the 1 mL syringe, with a stir plate beneath the syringe. The magnetic stirring bar kept cells in suspension while cell mixture or blood was being pumped through the device. The device was placed on the stage of an Olympus IX71 fluorescence microscope (Olympus America, Melville, NY) for detecting captured cells. To determine cell numbers, sets of images corresponding to the red fluorescent cells, blue fluorescent cells, and transmission images were acquired at different positions in each channel. Images were then imported into ImageJ (NIH), and cell counts were obtained using the Analyze Particles function after setting an appropriate threshold. Cell counts were further confirmed by comparing fluorescent images with transmission images; only those with appropriate cell morphology in the transmission images were counted.

3.3 Results and Discussion

3.3.1 Synthesis and Characterization of AuNP-Aptamer Conjugates

AuNPs were prepared following the methods detailed in the experimental section. Figure 3-1C shows the transmission electron microscopy (TEM) image of the AuNPs, with an average diameter of 13.6 nm. The as-prepared AuNPs were then functionalized with thiol-modified DNA aptamers, and the TEM image is shown in Figure 3-1D, with average size of 13.7 nm. A 24-unit polyethylene glycol (PEG) spacer
between AuNP surface and aptamers was added to minimize the steric effects of the particle surface on aptamers and to increase the loading of DNA on AuNPs.\textsuperscript{131} Figure 3-1C & D show that the properties of AuNPs remained unchanged after conjugation with aptamers, without any aggregation. Dynamic light scattering (DLS) measurements showed that the hydrodynamic diameter of AuNPs was 17.4 nm. After conjugation with aptamers, the hydrodynamic diameter increased to 61.8 nm, demonstrating the successful conjugation of aptamers onto AuNPs (Figure 3-3). Zeta-potential measurements indicated that the AuNPs had a zeta-potential of -12.5 mV. After modification with aptamers, the zeta-potential became -23.2 mV, which is attributed to the negative charges carried by DNA aptamers. The comparison of properties between AuNPs and AuNP-aptamers is made in Figure 3-1E.

### 3.3.2 Flow Cytometric Analysis Demonstrating High Affinity Binding

To investigate the AuNP-aptamer mediated multivalent binding, we directly measured the binding behaviors of AuNP-sgc8 aptamer conjugates (AuNP-sgc8) and free sgc8 aptamer (sgc8) using flow cytometry. Sgc8 is an aptamer that has specific binding with CEM cells (human acute lymphoblastic leukemia), with a nanomolar (nM) dissociation constant (K\textsubscript{d}).\textsuperscript{63} Ramos cells (human Burkitt’s lymphoma) that do not bind with sgc8 aptamer were used as control cells here. Figure 3-6A shows a noticeable increase in fluorescence signal for both AuNP-sgc8 and free sgc8 aptamer compared to the random DNA library (Lib) and AuNP-Lib, proving that both have strong binding with their target cells. Besides, AuNP-sgc8 produces a higher fluorescence signal than free sgc8, even with 10 times lower concentration. As shown in Figure 3-6B, neither free sgc8 nor AuNP-sgc8 shows a signal increase when incubated with control Ramos cells, demonstrating the specificity of both free aptamers and AuNP-aptamers. Furthermore,
the binding affinity of sgc8 and AuNP-sgc8 to CEM cells was measured quantitatively by studying their binding with varying concentrations of sgc8 and AuNP-sgc8 aptamers. As demonstrated in Figure 3-6C & D, AuNP-sgc8 shows a 39-times higher binding affinity ($K_d = 0.10 \pm 0.02\ nM$) than that of free sgc8 ($K_d = 3.9 \pm 0.5\ nM$). The lower dissociation constant of AuNP-sgc8 suggests a multivalent-mediated enhancement in binding affinity when multiple aptamers on the AuNP surface bind to multiple receptors on the cell membrane. Note that the concentration of AuNP is used instead of the concentration of aptamer when measuring the dissociation constant of AuNP-aptamer.

**3.3.3 Enhanced Cancer Cell Capture in a Flat Channel Microdevice**

To study the cancer cell capture using AuNP-aptamer, we first developed a microfluidic laminar flow device with flat channels (Figure 3-4B), which allowed us to directly compare the capture performance between AuNP-aptamer and aptamer alone. After coating surfaces with AuNP-sgc8 aptamer, a cell mixture containing $10^5$ target CEM cells and $10^6$ control Ramos cells (1:10 ratio) in 1 mL of phosphate buffered saline (PBS) was introduced into the channel. Note that the cell solution was continuously pumped into the device without any interruption. CEM and Ramos cells were pre-stained with Vybrant DiI (red) and DiD (blue), respectively. Figure 3-7A shows a representative image of cells captured using AuNP-aptamer, a high percentage of target CEM cells (red) was captured, while most control Ramos cells (blue) were washed away. In another set of experiments with the same conditions, sgc8 alone was used instead of AuNP-sgc8. Figure 3-7B shows a typical image of cells captured after washing using aptamer alone (without the nanoparticle conjugation). The results in Figure 3-7A & B clearly indicate that much more target CEM cells were captured using AuNP-aptamer than with aptamer alone, demonstrating that enhanced cell capture was
achieved by the AuNP conjugation. The capture efficiency using AuNP-aptamer and aptamer alone was also studied at different flow rate conditions (with different shear stresses). We found that AuNP-aptamer exhibited more enhancement in the capture efficiency at higher flow rates, as shown in Figure 3-7C. At a flow rate of 1.2 µL/s, AuNP-aptamer maintained a capture efficiency of (92 ± 4)%, while aptamer alone yielded a capture efficiency of only (49 ± 6)%. The capture efficiency was defined as the ratio of the number of the target cells captured to the number of the target cells initially seeded. The AuNP-aptamer enables significant increase in capture efficiency for the target cells. We also studied the purity of the captured cells and found that the capture purity is not affected by the AuNP conjugation. The purity was defined as the ratio of the number of the target cells captured to the number of total cells captured. As shown in Figure 3-7D, similar purity was obtained for AuNP-aptamer and aptamer alone when the same flow rate was used; this suggests that AuNP-aptamer does not introduce more nonspecific binding relative to aptamer alone, which is consistent with flow cytometry results on Figure 3-6B. However, the AuNP-aptamer allows us to use higher flow rates to maintain the capture efficiency, higher purity can thus be obtained because nonspecifically adsorbed cells are more easily washed away with a stronger shear force at a higher flow rate.\(^{78}\)

In addition to the DNA nanosphere-mediated multivalent binding, the enhanced cell capture also accrues from the nanosphere-modified surface. The increases in the surface roughness and total surface area compared with plain surface, allowed enhanced local topographic interactions between the aptamer-coated nanoparticle and nanoscale components on the cell surface.\(^{128}\) Moreover, the nanoparticle surfaces
packed the aptamers in a highly dense manner, accommodating more aptamers to be immobilized than a plain surface, which is an additional advantage of using AuNP-aptamer. The increased ligand density also contributes to the enhanced interaction between cells and aptamers. Furthermore, the enhanced binding strength afforded by the multivalency effect lowers the detachment ratio of immobilized cells, thus increasing the capture efficiency compared to aptamer alone. To evaluate the versatility of our system, we also applied the system for capturing Ramos cells using AuNP-TD05 aptamer conjugates. TD05 is an aptamer with specific binding to Ramos cells. A capture efficiency of 90% was obtained with AuNP-TD05, while TD05 aptamer alone yielded only 41% capture, showing significant enhancement in capture efficiency as a result of using DNA nanosphere.

The reduced capture efficiency at higher flow rates (shown in Figure 3-7C) is due to increased flow-induced shear stress and the decreased interaction time between cells and aptamers on surfaces. We further characterized the distribution of captured cells at different locations of the 50 mm long microchannel with different flow rates. As shown in Figure 3-8A, at flow rate of 1.2 µL/s (with a shear stress of 0.4 dyn/cm²), 65% of the cells were captured in the first 25% of the channel coated with AuNP-aptamer. With an increased flow rate of 2.4 µL/s (Figure 3-8B), the cells captured were distributed along the channel because cells needed longer flow distance (travel length) to have an opportunity to interact with aptamers coated on the surfaces, and the attached cells experienced proportionally increased shear stresses. The cell-surface interaction is due to the ligand-receptor binding as well as gravitational force. With the AuNP-conjugation, the PEG spacer extends the aptamer strands into the 3D space of flow, increasing the
accessibility and frequency of interactions between aptamers and cells to permit more efficient cell capture under higher flow rates.

To explore the clinical utility of the system, we assessed the isolation of CEM cells from lysed blood (blood with red blood cells lysed) at concentrations ranging from $10^5$ to 100 cells/mL. As shown in Figure 3-8C, as few as 100 cells were efficiently isolated from 1 mL of lysed blood within 14 min. However, when we tried to capture cancer cells from unprocessed whole blood directly, the capture efficiency was significantly lower (even at a low flow rate), as shown in Figure 3-8D. The relatively low capture was primarily due to the reduced interaction chances between target cells and AuNP-aptamer, which is caused by abundant red blood cell blockage.

3.3.4 Efficient Isolation of Cancer Cells from Whole Blood Using DNA Nanospheres in Micromixer Devices

Although the laminar flow flat channel device achieved high efficiency when capturing cells in PBS or lysed blood, it showed a low capture efficiency (<60%) when capturing cells from whole blood. To enable the efficient capture of CTCs from whole blood, we integrated the AuNP-aptamer system into a herringbone groove-based micromixer device (Figure 3-9A). The staggered herringbone mixer generates microvortex and chaotic mixing inside the microchannel, which significantly enhances the cell-surface interactions, leading to higher capture efficiency.$^{51,72,133}$ We first evaluated the isolation of $10^4$ CEM cells (pre-stained by Dil, red) spiked in 1 mL of whole blood at a flow rate of 1 µL/s. After cell capture and rinsing, 4,6-diamidino-2-phenylindole (DAPI) was introduced into the device to test the purity of the target cells. DAPI stained all the cancer cells and leukocytes with blue color and verified that captured cells retain intact nuclei. As shown in Figure 3-9B, cells positive to both DAPI
and Dil were target CEM cells (blue merged with red), while cells positive to DAPI only were white blood cells (blue only). A purity of (70 ± 6)% was obtained when capturing CEM cells from whole blood, with a capture efficiency of (95 ± 3)%. This capture purity from whole blood is much higher than those reported in literature (~50% & 14%). Further, we tested the capture efficiency over a wide range of flow rates from 0.5 μL/s to 3 μL/s. Control experiments using identical device and conditions with aptamer alone (no AuNP-conjugation) were then conducted. Much higher capture efficiencies were obtained using AuNP-aptamer than aptamer alone, especially at high flow rates (Figure 3-9C). The combined effect of high affinity binding from AuNP-aptamer with the passive mixing provided by the herringbone structures enabled high capture efficiency from whole blood (93%) at high flow rate (1.5 μL/s). To compare the AuNP-aptamer based cell capture with traditional antibody based cell capture, anti-protein tyrosine kinase 7 (PTK7) antibody was used for capturing CEM cells with identical device and conditions. For the binding between CEM cells and sgc8 aptamer, our previous study identified PTK7 as the marker for CEM cells. As shown in Figure 3-9C, the capture efficiency of CEM cells using anti-PTK7 is comparable with aptamer alone, but significantly less than AuNP-aptamer. To test the limit of detection for the AuNP-aptamer based cell capture system, cell spike numbers from 10^5 to 100 were explored, and >90% capture efficiency were obtained for all cases at the flow rate of 1.5 μL/s. Regardless of whether the red blood cells are intact or lysed, high capture efficiency is always obtained by the integration of AuNP-aptamer with a herringbone mixer (Figure 3-9D). In addition, with the flow rate of 1.5 μL/s (5.4 mL/h), 1 mL of blood sample can be processed in 11 minutes, which gives sufficient throughput for clinical applications. The system gives
more benefit at higher flow rates, maintains a target cell capture efficiency of >75% for all flow rates up to 3 µL/s. With this flow rate, only 42 min is needed for processing 7.5 mL blood, the amount of blood needed to detect clinical relevant CTC number. Compared with reported work, this AuNP-aptamer modified mixer device enables >90% capture at a flow rate 5.4 mL/h, 2 to 4 fold higher than reported aptamer-alone-based micropillar device (2.2 mL/h)\textsuperscript{78} and antibody-coated herringbone device (1.2 mL/h).\textsuperscript{51} The results show that the AuNP-aptamer-modified herringbone device has a great potential for clinical CTC isolation and enumeration.

### 3.4 Conclusion

In this chapter, we demonstrated the use of gold nanoparticles as an efficient high affinity vehicle for molecular assembly of aptamers for target cancer cell capture in microfluidic devices. Up to 95 aptamers were attached onto each AuNP, resulting in enhanced aptamer molecular recognition capability. Flow cytometry results demonstrated the high affinity binding effect using AuNP-aptamer conjugates. The capture efficiency for target cancer cells was significantly increased using the AuNP-aptamer conjugates because of the cooperative, multiple ligand-receptor interactions, as well as the increased surface roughness and ligand density. With the AuNP-aptamer surface immobilization, a flat channel microfluidic device was able to capture 100 cancer cells from 1 mL of lysed blood with ~90% capture efficiency within 14 min (4.3 mL blood/h). Using the integration of the AuNP-aptamer with a herringbone mixer design, efficient capture of rare cancer cells from whole blood was achieved, with a throughput of processing 1 mL of blood in 11 min. The high efficiency, throughput and purity make the system suitable for clinical isolation of CTCs from patient blood.
The use of leukemia cell-targeting aptamers allows the platform to be suitable for minimal residual disease (MRD) detection. MRD is the small amount of leukemia cells remaining in patient blood during or after treatment when the patient is at remission, which is the major cause for cancer relapse.\textsuperscript{136, 137} Our system capable of efficient isolation of rare cells is suitable for sensitive detection of MRD, which will be promising for monitoring treatment response and predicting cancer relapse. However, aptamers are currently not as widely used as antibodies, and limited numbers of aptamers have been developed for targeting CTCs in patient bloods. Our future efforts will include incorporating AuNPs with CTC-marker-binding aptamers [e.g., anti-EpCAM aptamer (epithelial cell adhesion molecule),\textsuperscript{138} anti-PSMA aptamer (prostate specific membrane antigen)]\textsuperscript{139} for capturing CTCs from cancer patients, as well as exploring release and culture of captured CTCs.

Spherical DNA nanostructures have been well developed and widely used for cancer cell detection; however, to our knowledge, this is the first use of aptamer nanospheres for enhancing cancer cell capture. Our results show that the combination of nanotechnology with a microfluidic device\textsuperscript{140} has a great potential for sensitive isolation of cancer cells from patient blood, and is promising for cancer diagnosis and monitoring treatment response.
Figure 3-1. Illustration of enhanced cell capture using AuNP-aptamer modified surface. A) With AuNP conjugation, multiple aptamers on the AuNP surfaces bind with multiple receptors on the cell membrane, leading to cooperative, multivalent interactions. B) Without AuNP, aptamer alone binds with receptors via monovalent interaction, with much less interactions. C) Transmission electron microscopy (TEM) image of AuNPs. D) TEM image of AuNPs conjugated with aptamers, scale bar = 100 nm. E) Comparison between AuNP and AuNP-aptamer in terms of particle diameters from TEM images, hydrodynamic diameters from dynamic light scattering (DLS) measurements, and zeta-potential measurements.

<table>
<thead>
<tr>
<th>Name</th>
<th>AuNP</th>
<th>AuNP-aptamer</th>
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<tbody>
<tr>
<td>TEM Diameter (nm)</td>
<td>13.6 ± 1.3</td>
<td>13.7 ± 1.5</td>
</tr>
<tr>
<td>DLS Hydrodynamic diameter (nm)</td>
<td>17.4 ± 1.9</td>
<td>61.8 ± 4.2</td>
</tr>
<tr>
<td>Zeta-potential (mV)</td>
<td>-12.5 ± 0.6</td>
<td>-23.2 ± 1.4</td>
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Figure 3-2. Adsorption and fluorescence spectrum of AuNPs and AuNP-aptamer conjugates. A) Adsorption spectrum of AuNPs, ($\lambda_{\text{max}} = 520$ nm), using a molar absorptivity of $2.7 \times 10^8$ L mol$^{-1}$ cm$^{-1}$, the concentration of the AuNP is $\sim 13$ nM. B) Fluorescence spectrum of fluorescein-labeled aptamers at (a) 10 nM and (b) 1 µM; (c) the fluorescence of AuNP-aptamer conjugates at 10 nM. Around 95 fluorescein-labeled aptamers were conjugated to each AuNP. Thus, the fluorescence signal of each AuNP-aptamer is much higher than individual aptamer, as shown when comparing (a) and (c).
Figure 3-3. Dynamic light scattering (DLS) analysis of DNA nanospheres. A) DLS of AuNPs. B) DLS of AuNP-sgc8 aptamer conjugates. The hydrodynamic diameter of AuNP increased from 17.4 nm to 61.8 nm after conjugation with aptamers.
Figure 3-4. Pictures of the flat channel microdevices. A) The single flat channel device; B) The parallelized flat channel device with 8 channels connected. The size for both devices is 3 in. × 1 in. The single flat channel device was used for proof-of-concept studies; data from this device is not shown. All the data presented in this chapter are from eight channel devices.
Figure 3-5. Comparison between a flat channel device and a herringbone groove device for flow mixing. 

A) With a flat channel device, the flow is laminar, with minimal mixing between the green dye and red dye, and mixing is only caused by diffusion; 

B) With a herringbone groove structures inside the microchannel, transverse flow and chaotic mixing occurred, with enhanced mixing between the red dye and green dye.
Figure 3-6. Flow cytometry shows the strong and specific binding of AuNP-sgc8 aptamer conjugates with target CEM cells. A) CEM cells selectively bind with free sgc8 and AuNP-sgc8 aptamers; negligible signal change was observed for cells incubated with random DNA library (Lib) or AuNP-Lib conjugates (NP-Lib) compared with cells only. B) Control Ramos cells did not bind with either AuNP-sgc8 or sgc8 alone (with no signal shift for either case), demonstrating the specificity of free sgc8 and AuNP-sgc8 aptamers to CEM cells. C-D) Flow cytometry analysis determines the binding affinity of AuNP-sgc8 (C) and sgc8 alone (D) to CEM cells.
Figure 3-7. Comparison of AuNP-aptamer and aptamer alone based CEM cell capture in a flat channel device. A-B) Representative image of the target CEM cells (red) and control Ramos cells (blue) captured in the flat channel device using (A) AuNP-sgc8 aptamer conjugates; (B) sgc8 aptamer alone. Cell suspensions were continuously pumped into device without interruption. Scale bar = 50 µm. C) Comparison of CEM cell capture efficiency in PBS between AuNP-aptamer and aptamer alone when they were coated in a flat channel device, at flow rates from 0.4 µL/s to 2.4 µL/s. D) Comparison of the capture purity of target CEM cells between AuNP-aptamer and aptamer alone at the same flow rate; no statistical difference was observed. Error bars represent standard deviations (n=3).
Figure 3-8. Capture of CEM cells from blood using DNA nanospheres in the flat channel device. A-B) Spatial distribution of surface-captured CEM cells along the 50 mm-long microchannel in the flat channel device at different flow rates of (A) 1.2 µL/s and (B) 2.4 µL/s. C) Capture efficiency for 100,000, 10,000, 1000 and 100 CEM cells spiked in 1 mL of lysed blood, with flow rate of 1.2 µL/s. D) CEM cell capture efficiency from lysed blood or whole blood at the same flow rate (1.2 µL/s); 1000 CEM cells were spiked in 1 mL lysed blood or whole blood. Error bars represent the standard deviations of triplicate experiments.
Figure 3-9. Isolation of cancer cells from whole blood using DNA nanospheres in micromixer device. A) Device layout and dimensions of a microfluidic device containing herringbone mixers. B) Representative image of captured CEM cells (DiI+, DAPI+) from whole blood; the DAPI+ cells (blue only) are nonspecifically captured white blood cells; scale bar = 50 µm. C) CEM cell capture efficiency in whole blood at various flow rates using AuNP-aptamer, aptamer alone and anti-PTK7 antibody, respectively. D) Calibration plot of cancer cell capture from whole blood and lysed blood with different cell concentrations at 1.5 µL/s, solid lines represent linear fitting. Error bars represent standard deviations (n=3).
Table 3-1. Detailed aptamer sequence information. Underscore indicates the full sequence of sgc8 aptamer or TD05 aptamer; for flow cytometric test, fluorescein isothiocyanate (FITC) is used instead of biotin linker.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>sgc8</td>
<td>5'–ATC TAA CTG CTG CGC CGC CGG GAA AAT ACT GTA CGG TTA GAT TTT TTT-biotin-3'</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiol-sgc8</td>
<td>5'-thiol-(PEG)_{24}-ATC TAA CTG CTG CGC CGC CGG GAA AAT ACT GTA CGG TTA GA -biotin-3'</td>
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<tr>
<td>TD05</td>
<td>5'-AAC ACC GTG GAG GAT AGT TCG GTG GCT GTT CAG GGT CTC CTC CCG GTG TTT TTT T-biotin-3'</td>
</tr>
<tr>
<td>Thiol-TD05</td>
<td>5'-thiol-(PEG)_{24}-AAC ACC GTG GAG GAT AGT TCG GTG GCT GTT CAG GGT CTC CTC CCG GTG -biotin-3'</td>
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CHAPTER 4
A MICROFLUIDIC GEOMETRICALLY ENHANCED MIXING CHIP FOR CAPTURE, RELEASE AND CULTURE OF CIRCULATING TUMOR CELLS FROM PANCREATIC CANCER PATIENTS

4.1 Background

Pancreatic cancer is the fourth leading cause of cancer deaths in the United States, with the poorest 5-year survival rate (6%) for all cancer stages. Over 90% of pancreatic cancers progress to become metastatic.\textsuperscript{2,141} The poor prognosis of pancreatic cancer patients is related to the early dissemination of the disease and the lack of early detection.\textsuperscript{142} Circulating tumor cells (CTCs) are tumor cells disseminated from primary tumors which subsequently travel through the blood circulation to distant organs. CTCs are thus responsible for the initiation of metastasis and the in transit spread of cancer to distant sites.\textsuperscript{67,143} Therefore, CTCs hold the key to track metastasis, and they can be used for cancer diagnosis and monitoring of cancer status. Clinical studies have demonstrated that CTCs are correlated with disease progression for a wide range of cancers, such as breast, colorectal and prostate cancer.\textsuperscript{12,144} While biopsy is the current gold standard of cancer diagnosis, it involves removal of tissues or cells from the body and examination by experienced surgeons and pathologists.\textsuperscript{145} The invasive nature of biopsy prevents patients from being tested in an ongoing or repetitive basis. CTC examination, on the other hand, is much less invasive, with only 5-10 mL of patient blood needed; it is like a blood test for cancer. CTC monitoring is regarded as “liquid biopsy” or “live biopsy” of a tumor,\textsuperscript{146} which enables noninvasive cancer diagnosis and real-time monitoring of therapeutic response.

Part of this chapter has been published in “Sheng, W. et al., Capture, Release and Culture of Circulating Tumor Cells from Pancreatic Cancer Patients Using an Enhanced Mixing Chip, Lab on a Chip, 2014, 14, 89-98”
However, CTCs are extraordinarily rare, with only a few CTCs circulating amidst billions of blood cells, making their isolation and characterization a tremendous technical challenge. Thus, high-efficiency and high-purity isolation of CTCs from patient blood is urgently needed to obtain accurate information of CTCs. Currently, the only FDA-approved technology is the CellSearch system (Janssen Diagnostics, LLC, Raritan, NJ). Unfortunately, this system is limited by low efficiency, low purity and high cost, and does not fully address the issue of isolating the extremely low-abundance of CTCs. Recently, microfluidic devices with high affinity ligands, including antibodies and aptamers have provided distinctive opportunities for efficient and specific isolation of CTCs from patient blood. Microfluidic devices, due to their large surface area-to-volume ratio and short diffusion distance, can substantially increase the interaction between cells and the ligand-coated surface.

Staggered herringbone micromixers have been developed for fluid mixing in microchannels and have been exploited for enhancing the cell capture. Yet, limited research has been reported on the optimization of herringbone mixers for high-performance cell capture. Different from mixing solutions through transverse flow, inducing cell-surface interactions requires cells with nearly zero diffusivity for advection to microchannel surface. Herein, we have developed a geometrically enhanced mixing (GEM) chip for high-performance CTC capture (high efficiency, purity, throughput and cell viability). With experimental optimization of the herringbone micromixers, we achieved capture of spiked tumor cells with >90% capture efficiency and >84% purity. In addition, the time required to process 1 mL blood sample is <17 min, much faster than those reported in literature. Since very limited work has been...
done on cellular studies after CTC capture,\textsuperscript{46} we have investigated the release, the viability and the culture of the captured cells. Captured cells can be efficiently released with the combined methods of trypsinization and high flow rate washing. Experiments also showed that released cells grew as well as intact cells that had not been subjected to the capture and release process. Further, we applied the device for isolation of CTCs from pancreatic cancer patients, with CTCs observed in 17 of 18 patient samples. We also demonstrated the potential of using CTC enumeration as a surrogate for radiographic monitoring of chemotherapy response in pancreatic cancer patients. Our device sensitivity enables isolation and enumeration of CTCs from pancreatic cancer patients, a disease where invasive biopsies are difficult and the commercial CellSearch system has proven to be inefficient.\textsuperscript{151} Compared with reported efforts,\textsuperscript{47-49, 51, 72, 103} this work demonstrated a systematic study of the following aspects: geometric optimization of micromixer for enhanced target CTC capture, release and re-culture of captured tumor cells, cell viability before and after release, cell binding behaviors after release and re-culture, isolation and counting of understudied pancreatic CTCs, comparison of CTC enumeration with CT scans for monitoring chemotherapy response in pancreatic cancer patients. A comprehensive study of these aspects would further improve CTC isolation performance, help understand post-capture processing of CTCs and push forward CTC isolation for cancer diagnosis.

In this study, we first developed a geometrically enhanced mixing chip (GEM chip) based on patterned herringbone or chevron structures. The mixer design was inspired by several groups,\textsuperscript{51, 133, 149} and the dimensions were optimized for high-efficiency and high-purity cell capture. As shown in Figure 4-1, the GEM chip is the same size as a
microscope slide (3 in. × 1 in.), having 8 parallel channels with uniform flow to form a high throughput device. Each channel is 2.1-mm wide, 50-µm deep, with 50-µm deep herringbone grooves repeating over a total length of 50 mm. The staggered herringbone grooves disrupt streamlines and induce chaotic mixing, which maximize collisions and interactions between target cells and device surface, leading to increased cell capture efficiency. The groove width and the groove pitch were carefully selected for high-performance cell capture, as discussed in detail in Results and Discussion.

4.2 Experimental Section

4.2.1 Microfluidic Device Fabrication

The microfluidic GEM chip consists of a polydimethylsiloxane (PDMS) structure bonded to a 3" × 1" glass microscope slide. The PDMS structure was fabricated using two-layer soft lithography, according to literature.\textsuperscript{30,134} The two-layer SU-8 structure (a main channel layer and a herringbone mixer layer) was fabricated via two spin-coating and exposure steps and a single developing step. The device layout was designed in AutoCAD and then sent to CAD/Art Services, Inc. (Brandon, OR) to produce a high resolution transparency photomask (Figure 4-2). As shown in Figure 4-3, silicon wafers were first spin-coated with 50-µm thick SU-8 2035 photoresist (MicroChem, Newton, MA) as the main channel layer. After soft baking, UV light exposure, and post exposure baking, another layer of SU-8 was added to form the herringbone mixer layer. With precise alignment between the main channel and the mixer, a second exposure was performed to create the herringbone mixer pattern (Figure 4-4). After development, a silicon master patterned with the complementary structures was obtained. PDMS structures were fabricated by casting a liquid PDMS precursor against the master using Sylgard 184 silicone elastomer kit (Dow Corning, Midland, MI), according to the
manufacturer’s instructions (10:1 ratio of base to curing agent). Inlet and outlet wells were created at the channel ends by punching holes in the PDMS sheet. The channel depth, which was controlled by the spin speed of the SU-8, was measured using a Dektak 150 profilometer.

A high-aspect ratio micropillar based PDMS device was also fabricated for cell capture, as shown in Figure 4-5. Further experiments show that mixing device is better than the micropillar device in terms of purity when processing blood samples, thus mixing device was selected for future experiments. Study of flow and shear stress in microchannel is shown in Figure 4-6, as detailed in Appendix A.

4.2.2 Cell Culture

L3.6pl cells\textsuperscript{152} (human pancreatic cancer) were obtained from Dr. Jose Trevino’s lab (Department of Surgery, University of Florida). BxPC-3 cells (CRL-1687, human pancreatic adenocarcinoma) and MIAPaCa-2 cells (CRL-1420, human pancreatic carcinoma) were purchased from American Type Culture Collection (ATCC). Cells were cultured in DMEM medium (ATCC) supplemented with 10% fetal bovine serum (FBS; heat-inactivated; GIBCO) and 100 units/mL penicillin-streptomycin (PS, Cellgro, Manassas, VA) and incubated at 37°C under 5% CO\textsubscript{2} atmosphere. Cells were grown as adherent monolayers in 60 mm × 15 mm culture dishes to 90% confluence, subsequently detached with 0.05% Trypsin-0.53 mM EDTA (0.05%, Cellgro) and re-seeded at a lower concentration.

4.2.3 Reagents and Buffers

Biotinylated anti-EpCAM (Anti-Human CD326, eBioscience, San Diego, CA) immobilized on device surface was used as the CTC capture agent. Anti-cytokeratin FITC (CAM 5.2, conjugated with fluorescein isothiocyanate, BD Biosciences, San Jose,
CA) and anti-CD45 PE (conjugated with phycoerythrin, BD Biosciences) were used to label CTCs and white blood cells, respectively. DAPI (4’,6-diamidino-2-phenylindole, Invitrogen, Carlsbad, CA), which stains DNA in cell nuclei, was used to label all nucleated cells bound to the device (i.e., white blood cells and CTCs). Dulbecco’s phosphate buffered saline with calcium and magnesium (PBS, Fisher Scientific, Hampton, NH) was used to wash cells. A buffer containing 10 mg/mL (1%) bovine serum albumin (BSA, Fisher Scientific) and 0.05% Tween-20 (Fisher Scientific) in PBS was used for rinsing the unbound molecules from the channel surface, and resuspending cells for cell capture. BSA and Tween-20 in PBS was used to fully passivate the surfaces to reduce nonspecific adsorption of cells in the channels.

Flow cytometry analysis was used to test the binding capabilities of anti-EpCAM to pancreatic cancer cell lines. Fluorescence measurements were performed with a FACScan cytometer (BD Immunocytometry Systems, San Jose, CA). Briefly, 200,000 cells were incubated with 10 µg/mL biotinylated anti-EpCAM in 200 µL of PBS (containing 0.1% BSA) for 20 min on ice. After incubation, the cells were washed three times with PBS. Then streptavidin phycoerythrin (SA PE)-Cy5 (Invitrogen) was added and incubated for another 20 min. After washing, 10,000 counts were measured in the flow cytometer to determine the fluorescence. The cells incubated with SA PE-Cy5 alone were used as a negative control to determine nonspecific binding. Figure 4-7A & B show the strong binding of the anti-EpCAM antibody with L3.6pl cells and BxPC-3 cells, respectively. Figure 4-7C, which shows no binding between anti-EpCAM and MIAPaCa-2 cells, indicates that MIAPaCa-2 cells can be used as a negative control.
4.2.4 Capture of Spiked Tumor Cells in Microfluidic Device

Immediately before experiments, cells were detached from the culture dish and then rinsed with PBS and resuspended at $10^6$ cells/mL. By following the manufacturer’s instructions, the target cells and control cells were stained with Vybrant DiI (red) and Vybrant DiD (blue) cell-labeling solutions (Invitrogen), then rinsed with PBS, and resuspended at $10^6$ cells/mL in the PBS containing BSA and Tween-20. Labeled cells were stored on ice and further diluted or spiked into blood to the desired concentrations before experiments.

Anti-coagulant-containing human whole blood from healthy participants was commercially obtained from Innovative Research (Novi, MI), and used for all “spike-in” experiments. For some experiments, CTC capture from whole blood samples was preceded by red blood cell lysis performed as previously described. Briefly, lysed blood was obtained by treating whole blood with red blood cell (RBC) lysing buffer, prepared by adding 155 mM (8.3 g/L) ammonium chloride in 0.01 M Tris-HCl buffer, with pH=7.5. Different concentrations of cancer cell lines were then spiked in whole blood or lysed blood. The detailed RBC lysis procedure is shown in Appendix B.

To initiate cell capture experiments, one channel volume (~100 µL) of 1 mg/mL avidin (Invitrogen) in PBS was first introduced into the device, followed by incubation for 15 min and then three rinses with PBS. Then, one channel volume of biotinylated anti-EpCAM (20 µg/mL) was introduced into the device and incubated for 15 min, followed by three rinses with the PBS containing BSA and Tween-20. Finally, 1 mL of cell mixture or blood sample was pumped into the device at a flow rate of 1 µL/s (or other flow rates specified in the text). At the end of the experiment, the microchannel was
washed three times with PBS, followed by acquiring fluorescent images for the
determination of the number of cells captured.

4.2.5 Instrument Setup

The cell suspension or blood sample was introduced into the device by pumping
using a syringe pump (KD Legato 111, KD Scientific, Holliston, MA) with a BD syringe
connected to the inlet of the device via polymer tubing and a female luer-to-barb
adapter (IDEX Health & Science, Oak Harbor, WA). To avoid cell settling, a tiny
magnetic stirring bar was placed inside the BD syringe, with a stir plate beneath the
syringe. The magnetic stirring bar kept cells in suspension while the cell mixture or
blood was being pumped through the device. An Olympus IX71 fluorescence
microscope (Olympus America, Melville, NY) with an automated ProScan stage (Prior
Scientific, Rockland, MA) was used to image and count the captured cells on the device.

4.2.6 Cell Release and Re-culture

Cell release was achieved by trypsin and high flow rate washing. After cells
captured inside the channel, proteolytic enzyme trypsin (0.25%) was introduced into the
device and incubated for 5 min at 37 °C. Then, cell culture medium was pumped into
the device at a flow rate of 5 µL/s to dislodge the bound cells. The release flow rate was
much higher than the cell capturing flow rate of 1 µL/s. Released cells were collected in
a new cell culture dish (60 mm × 15 mm size), with a total volume of 4 mL culture
medium. Then the cells were put into the incubator for propagation in culture.

To test the viability of cells captured by the device, propidium iodide (PI) and
acridine orange (AO) staining (Invitrogen) assays were performed. PI is a membrane-
impermeant stain that labels only dead cells with red fluorescence. AO is a membrane-
permeable dye that binds to nucleic acids of all cells and induces green fluorescence.
By following the manufacturer’s instructions, PI/AO working solution was prepared to contain 2 µM PI and 2 µM AO in PBS. After incubating the working solution with cells for 10 min, fluorescent images were taken to evaluate the viability of the captured cells (Figure 4-12C).

4.2.7 Patient Blood Specimen Collection and Processing

Blood samples of patients with metastatic pancreatic cancer were obtained from the University of Florida Health Cancer Center after informed consent through a University of Florida Institutional Review Board (IRB)-approved protocol. Blood samples from normal healthy participants were obtained through the Gainesville LifeSouth Community Blood Center following a University of Florida IRB-approved protocol. Specimens were collected into BD Vacutainer tubes containing anticoagulant sodium heparin and were processed within 6 hours after being drawn. The blood processing safety protocol is shown in Appendix C.

CTC capture was performed by the same protocols as described above. Unlike the pre-stained tumor cells spiked in blood, CTCs from patients’ blood were not labeled. Three-color immunocytochemistry (DAPI, FITC anti-cytokeratin, PE anti-CD45) was conducted to identify CTCs from nonspecifically captured blood cells. Cell staining began with cell fixation and permeabilization by incubation for 20 min with 4% paraformaldehyde and 0.2% Triton X-100, respectively. Then, a mixture of 10 µg/mL PE anti-CD45, 10 µg/mL FITC anti-cytokeratin and 500 nM DAPI were introduced into the device and incubated for 20 min. After washing, the microfluidic device was examined under the fluoresce microscope. Only cells that were DAPI positive, CD45 negative, cytokeratin positive, with the appropriate size and morphology were counted as CTCs (DAPI+, CD45−, cytokeratin+). Cell debris, red blood cells (DAPI−), white blood cells
(DAPI+, CD45+) and “double positive” cells (both CD45+ and cytokeratin+, with DAPI+) were excluded from counting. CTC capture purity was defined as the ratio of the number of CTCs captured to the total number of nucleated cells (DAPI+) bound to the device. For another sets of experiments, we released the specifically captured CTCs along with nonspecifically captured leukocytes into culture dish (instead of staining and counting). And fresh medium was added once a week (with leukocytes washed away). We observed a few cells (probably CTCs) adhered to the culture dish after 1 week of culture. However, these adhered cells did not proliferate, even after 4 months of culturing (unlike the spiked tumor cells which grew into clusters within 2 weeks).

4.3 Results and Discussion

4.3.1 Target Cell Capture from a Homogenous Cell Mixture

The performance of the device was first evaluated by sorting a mixture of pancreatic cancer cell lines: target L3.6pl cells (EpCAM+) and control MIAPaCa-2 cells (EpCAM-). Flow cytometry results show that L3.6pl cells bind strongly with anti-EpCAM, while MIAPaca-2 cells do not bind with anti-EpCAM (Figure 4-7). This means that L3.6pl cells express a significant number of EpCAM receptors, while MIAPaCa-2 cells express negligible surface EpCAM, which is consistent with data already reported in literature. To start the cell capture, biotinylated anti-EpCAM was first immobilized on the surface of microchannel. Then a cell mixture containing $10^6$ L3.6pl cells (stained with Vybrant Dil, red) and $10^6$ MIAPaCa-2 cells (stained with Vybrant DiD, blue) per mL sample were introduced into the microchannel. Figure 4-8A shows a representative image of the cell mixture prior to sorting, with same number of target cells and control cells. Figure 4-8B shows a typical image after the cell mixture was processed through the device, with L3.6pl cells in the majority, while most control MIAPaCa-2 cells were removed by
washing. Figure 4-8A & B clearly indicate that significant enrichment of target cells can be achieved using the antibody-coated microfluidic device.

After the initial experiments, different flow rates were used to study the effects of flow rate on cell capture efficiency, defined as the ratio of the number of target cells captured to the number of target cells initially introduced. As shown in Figure 4-9A, the capture efficiency of L3.6pl cells was >90% at low flow rates, but decreased dramatically at flow rates above 1 µL/s, primarily due to the reduced interaction time between the cells and antibody-coated surfaces as well as the increased shear stress at higher flow rates. To obtain both efficient capture and sufficient throughput, an optimal flow rate of 1 µL/s was chosen, with a flow velocity of 0.75 mm/s and maximum shear stress of 0.38 dyn/cm² at the wall. As shown in Figure 4-9B, the capture efficiency was (90 ± 2)% for L3.6pl cells and (92 ± 4)% for BxPC-3 cells at 1 µL/s.

4.3.2 Micromixer Device Optimization for High-Performance Cell Capture

When we used the traditional micromixer design dimensions (HB chip, Figure 4-1C) for pancreatic tumor cell capture, we found that non-target cells were easily trapped in the device (causing low CTC capture purity) and cells were not captured on the same focus plane (making imaging and counting difficult). We suspected that cell trapping took place in narrow grooves (with high aspect ratio) as illustrated in Figure 4-1C, and hypothesized that an increased groove width would give better purity. Thus we made two new designs by increasing the groove width from 50 µm (narrow groove, Figure 4-1C) to 80 µm and 120 µm (wide groove, Figure 4-1D). Experimental results proved that a wider groove with increased groove pitch achieved high purity cell capture, while maintaining cell capture efficiency. As shown in Figure 4-10, with a groove width of 120 µm, we obtained a capture purity of 84%, while the traditional 50-µm groove
width yield only 61% purity. In addition, the capture efficiency for the wide groove design was not reduced, even slightly higher, which agrees with simulation study by Forbes et al.\textsuperscript{149}

\textbf{4.3.3 Tumor Cell Capture from Lysed Blood and Whole Blood}

To test cell capture under more physiological conditions and to mimic CTC capture from patient blood, we conducted a series of experiments in which labeled L3.6pl cells were spiked in lysed or whole blood. Samples were prepared by spiking 50-50,000 L3.6pl cells in 1 mL lysed blood or whole blood. After being pumped through the micromixer device, as many as \~92\% of L3.6pl cells were captured from lysed blood (Figure 4-11A), and \~89\% of L3.6pl cells were captured from whole blood (Figure 4-11B), proving that the device and the conditions are suitable for capturing CTCs from patient blood specimens with or without prior red blood cell lysis.

\textbf{4.3.4 Cell Release and Cell Viability}

The detachment and release of captured cells in antibody-coated microchannels was achieved by using a combination of trypsinization (enzymatic release)\textsuperscript{48} and high flow rate washing (high shear stress).\textsuperscript{71} Detached cells were collected in a cell culture dish with fresh medium for propagation in cell culture. As shown in Figure 4-12A, the release efficiency of L3.6pl cells increased to \textgreater 60\% by using the combined releasing method, while high flow washing alone gave only \~30\% release. The release efficiency is defined as the ratio of the number of cells released to the number of cells captured. The trypsin release and shear stress-based release procedures cause minimum cell damage as proved by cell viability assay and flow cytometry. PI/AO assay was used to test the viability of released cells, with \textgreater 85\% cells remaining viable after the capturing and release process (Figure 4-12B & C), making the isolated tumor cells suitable for
subsequent cellular analysis. Flow cytometry tests also showed that released L3.6pl cells retain their binding with anti-EpCAM, as shown in Figure 4-12D.

4.3.5 Re-culture of Captured Tumor Cells

To determine whether isolated tumor cells can be re-cultured, 5,000 L3.6pl cells were spiked into whole blood and subjected to the capture and release process as discussed above. The released cells were then seeded into cell culture dishes for propagation in culture. As comparison, 5,000 intact L3.6pl cells (not subjected to the culture and release process) were directly seeded for culture with the same conditions. Results showed that both adhered well and proliferated on the culture dishes, forming large clusters and colonies by day 9 (shown in Figure 4-13 A & B) and growing to confluence with longer time (14 days), although the captured cells took a little longer to reach confluence than intact cells. Then we were able to trypsinize these cells and seed them to other culture dishes, where they grew as adherent monolayers. The isolated cells have successfully undergone multiple (>8) passages without loss of viability or detectable changes in behavior. Flow cytometry tests indicated that the isolated cells maintain binding behavior with anti-EpCAM, as shown in Figure 4-13C. These results clearly demonstrate that tumor cell lines isolated from whole blood retain both their viability and their proliferation ability, which are crucial for CTC cellular analysis.

4.3.6 Isolation of CTCs from Patients with Pancreatic Cancer Using the GEM Chip

Blood samples from patients with metastatic pancreatic cancer (stage IV) were analyzed for CTC enumeration using the above-optimized device and conditions. Since EpCAM has been known to be overexpressed in pancreatic adenocarcinoma, anti-EpCAM was used as the capture agent.\textsuperscript{155} Milliliters of patient blood were pumped through the antibody-coated device. After fixation and permeabilization, three-color
immunocytochemistry was utilized to identify and count CTCs from nonspecifically captured white blood cells, using FITC-labeled anti-Cytokeratin (CK, green), PE-labeled anti-CD45 (red) and DAPI (blue) for staining. As shown in Figure 4-14, CTCs are DAPI+/CK+/CD45- cells, while WBCs are DAPI+/CK-/CD45+ cells. More images of CTCs were shown in Figure 4-16 and WBCs were shown in Figure 4-17. A significant population of “double positive” cells with both hematopoietic and epithelial markers (CK+/CD45+) were found in quite a few patient samples (average ~2 “double positive” cells in 1 mL patient blood). Since the origin and significance of these cells are under debate, we temporarily excluded them from CTC counting. For the 18 pancreatic cancer patient samples processed, CTCs were found in 17 cases (>94%), with an average number of 3 CTCs per mL of blood, as shown in Table 4-1. To examine the possibility of false positives, we investigated capturing CTCs from whole blood of normal healthy individuals. Similar volumes of blood were run through our device using the same protocol. Table 4-2 shows the results from blood samples of nine healthy donors. Zero CTCs were detected from blood samples of all normal healthy individuals studied, thus showing a false positive rate of zero. Additionally, we found much fewer “double positive” cells in healthy donors’ blood than in patient blood, indicating that most of the “double positive” cells could be the heterogeneous CTCs or the nonspecific binding of anti-CD45 to CTCs. Further studies with additional markers are required to understand and explain these “double positive” cells.

For capturing CTCs from patient blood, much more nonspecific capture of white blood cells was observed than the spiking experiments using healthy donor’s blood, which could due to complexity of patient blood conditions. The high purity of the GEM
chip shows more advantage over the traditional mixing chip when enumerating patient CTCs. The GEM chip would have been able to detect an average of ~23 CTCs from 7.5 mL blood, much higher than the cut-off number of CellSearch system. Considering that CellSearch is inefficient for pancreatic cancer, the GEM chip reported here could become a powerful tool for CTC enumeration in pancreatic cancer. In addition, with a flow rate of 1 µL/s (3.6 mL/h), 1 mL blood sample can be processed within 17 min, which gives sufficient throughput for clinical applications.

We also released and attempted to culture the captured patient CTCs in vitro, using the above-mentioned protocol for re-culturing spiked tumor cells. Similar release efficiency was obtained for patient CTCs. However, CTCs were not able to proliferate or propagate in the culture dish, although they were found adhered to the culture dish. We processed 12 pancreatic patient blood samples (with 5-10 mL volume for each sample), but CTCs from them were not proliferating till now. We suspect that during the progression of metastasis, CTCs, shed from a primary tumor and entered into bloodstream, might lose their ability to proliferate.

4.3.7 Monitoring Anti-cancer Treatment Response Using CTCs

To demonstrate the unique clinical potential of our device and system, we evaluated the relation between the CTC number and tumor size in patients with pancreatic cancer undergoing chemotherapy. Three patients with stage IV metastatic pancreatic cancer (deemed unresectable) were included in the analysis. Each patient received identical standard treatments with the identical palliative chemotherapy and with X-ray computed tomography (CT) scans done at the same intervals. Blood samples were collected at baseline and at the first day of each subsequent treatment cycle. CTCs were captured and counted using the device and methods discussed above.
Investigators were blinded to the demographic and clinicopathological characteristics of the patients. The number of CTCs captured at different treatment cycles is plotted in Figure 4-15 A-C. In general, the CTC number decreased with continuation of treatment and modeled the CT scan results (which represent standard clinical response measurements). The CTC number correlated proportionally with CT scan-measured tumor size in each of the three patients. Figure 4-15D & E show that tumor size decreased as treatment progressed for patient #3, which was reflected by the trend of CTC number in Figure 4-15C. CT scan data from patient #1 and patient #2 also indicated either reduced primary tumor size or reduced metastatic tumor burden (data not shown). Together, these results indicate that CTC quantification using our device correlates with clinical response and findings from CT imaging, but causes significantly less harms to patients than standard clinical radiographic measurements. With the noninvasive nature of our approach, it could provide a powerful tool for monitoring early response or failure to cancer treatment and potentially early cancer diagnosis and relapse prediction.

4.4 Conclusion

In this chapter, we demonstrated an efficient CTC capture platform based on a geometrically enhanced mixing (GEM) chip. The device achieved >90% capture efficiency, >84% purity with a throughput of processing 3.6 mL blood in 1 hour. The system was then utilized to isolate CTCs from pancreatic cancer patient blood samples, with CTCs detected in 17 of 18 samples. We also successfully demonstrated positive correlation in monitoring anti-cancer treatment response using the CTC numbers obtained from our device. In addition, the captured cells were released from device with >61% release efficiency, and with >86% viability. Furthermore, we demonstrated
the ability to culture the captured cells, a critical requirement for post-isolation cellular analysis. Although it is extremely challenging to culture the isolated CTCs from patient blood and to develop a new cell line, our system shows the possibility to culture spiked tumor cells, after the sophisticated capture and release process, while maintaining their viability and proliferation capability. Therefore, our CTC capture system shows great potential for efficient CTC enrichment, isolation, and cellular/genetic analysis, leading to now feasible “liquid biopsy” of pancreatic cancer. Our future efforts include further improving CTC capture purity, culturing the captured CTCs from patient, cellular and genetic study of isolated CTCs.
Figure 4-1. Picture and design of the microfluidic geometrically enhanced mixing chip (GEM chip). A) Picture of the 3 in. × 1 in. microfluidic GEM chip, consisting of eight parallelized channels with single inlet and outlet. B) Micrograph (4 × bright field) of the staggered herringbone grooves inside a channel, showing their asymmetry and periodicity, scale bar = 200 µm. C) A narrow groove design based on reported herringbone (HB) chip, with 50-µm groove width, purple dots show cells captured inside channel. D) Cross-sectional view of the wide groove GEM chip, with channel depth of 50-µm and groove depth of 50-µm; the groove pitch is set to be 200 µm, the groove width is chosen to be 120 µm.
Figure 4-2. Picture of the photomask used for fabrication of the two-layer microfluidic mixing device. A) The mask for the main channel layer; B) the mask for the herringbone mixer layer. Alignment makers were designed on the both sides of the mask; Blank region was designed surrounding the markers, which make the alignment easier.
Figure 4-3. Scheme of SU-8 mold fabrication process for the two-layer mixing device. A) With two-development process, the coating of the second layer is uneven; B) Single developing process with multiple coating and exposure, with even coating of the second layer.
Figure 4-4. 3-D view and SEM image of the herringbone micromixer structure inside the microfluidic channel. A) The overall layout of the device. B) Scanning electron microscopy image (SEM) of the herringbone groove structure in PDMS. The groove width here is 80 µm with a groove pitch of 160 µm. C-D) The patterned SU8 structure on silicon mold, with one layer of large channel, and another layer of herringbone ridges.
Figure 4-5. A high aspect ratio micropillar device tested for cell capture. A) Layout of the device, with overall channel design the same as the micromixer device. The inset is the scanning electron microscopy (SEM) image of the array of high aspect ratio PDMS micropillars. B) Microscopy image shows the specific capture of target cells inside the micropillar based device (red), with few control cells (green). The image is an overlay of fluorescence image with bright field image, (scale bar = 50 µm) This micropillar device have more nonspecific cell capture than micromixing device, especially for blood samples, thus mixing device is used for all the subsequent experiments.
Figure 4-6. Schematic illustration of flow and shear stress on cells inside channel. A) The flow inside channel is pressure driven, with parabolic velocity profile; while shear stress varies from channel bottom to top; B) Diagram of the motion of target cells under hydrodynamic flow; cells experienced shear force from flow and binding force from ligands (antibodies or aptamers).
Figure 4-7. Flow cytometry test of anti-EpCAM binding with different types of pancreatic cancer cells. A) L3.6pl cells; B) BxPC-3 cells; C) MIAPaCa-2 cells. Data shows that anti-EpCAM binds well L3.6pl cells or BxPC-3 cells, while does not bind with MIAPaCa-2 cells, indicating that L3.6pl and BxPC-3 cells express EpCAM, while MIAPaCa-2 cells do not express EpCAM. Streptavidin phycoerythrin Cy5 (SA PE-Cy5) was used to label the biotinylated anti-EpCAM.
Figure 4-8. Representative images of cells before and after capture. A) 1:1 mixture of target L3.6pl cells (red) and control MIAPaCa-2 cells (blue) before sorting; B) L3.6pl cells (red) among MIAPaCa-2 cells (blue) after sorting. Scale bar = 50 µm. Target cells were efficiently captured while most control cells were removed by washing.
Figure 4-9. L3.6pl cell capture efficiency as a function of flow rate. A) Reduced capture occurred at a high flow rate because of a larger shear force and the reduced interaction time between cells and antibody-coated surfaces. B) Capture efficiency of L3.6pl cells and BxPC-3 cells at the optimal flow rate of 1 µL/s, with >90% capture efficiency for both types of cells. Error bars represent standard deviations (n=3).

Figure 4-10. Comparisons of capture efficiency and purity of L3.6pl cells with different groove width: 50-µm (conventional narrow groove HB chip), 80-µm, and 120-µm (wide groove GEM chip). Capture purity is defined as the ratio of the number of target cells captured to the number of total cells captured. Error bars represent standard deviations (n=3).
Figure 4-11. Regression analysis of the number of the L3.6pl cells captured by the microfluidic device versus the number of the cells spiked in 1 mL of lysed or whole blood. A) lysed blood; B) whole blood. The x-axis indicates the number of spiked cells, y is the number of captured cells. Error bars represent standard deviations (n=3).
Figure 4-12. Cell release and cell viability testing. A) By high flow rate washing alone, a release efficiency of 34% was obtained; with a combination of trypsinization and high flow rate washing, the release efficiency reached 62% for L3.6pl cells. B) Cell viability before cell capture process (extracted directly from culture) is ~99%. Cell viability immediately after cell capture in device is ~89%; after release the viability is ~86%, without significant difference. The high viability indicates that released cells are suitable for subsequent cell culture. Error bars represent standard deviations (n=3). C) Fluorescence image of the L3.6pl cells after capture and release with PI/AO staining. The orange (red merged with green) color indicates nonviable cells (PI and AO staining), while the green color alone indicates viable cells (AO staining alone). Scale bar = 50 µm. D) Flow cytometry test shows that the captured and then released L3.6pl cells maintain their binding capability with anti-EpCAM, without any differences compared to normal L3.6pl cells.
Figure 4-13. Phase contrast micrograph (10 ×) of re-cultured cells: A) re-cultured BxPC-3 cells; B) re-cultured L3.6pl cells after 9 days of growth. Scale bar = 100 µm. C) Flow cytometry test showing that the captured and then recultured cells maintained their binding capability with anti-EpCAM, without any differences compared to intact cells.

Figure 4-14. Fluorescence microscope images (40 ×) of CTCs captured from patient blood. A) A representative image of CTCs, with DAPI+, Cytokeratin+ and CD45-; B) typical image of white blood cells (WBCs), with DAPI+, CK-, and CD45+. Scale bar = 10 µm.
Figure 4-15. CTC number under different treatment cycle correlates with tumor size by CT scans. A-C) The number of CTCs per mL of blood from pancreatic cancer patients at different treatment cycles for three patients: A) patient #1; B) patient #2; C) patient #3. D-E) CT scan image of patient #3 at D) the beginning of the treatment (cycle 1); E) the latter stage of treatment (cycle 11); the red arrows indicate regression of the primary pancreatic cancer. Each treatment cycle is 14 days.
Figure 4-16. Fluorescence images of white blood cells, with DAPI positive, cytokeratin negative and CD45 positive (scale bar = 10µm).
Figure 4-17. Fluorescence images of circulating tumor cells, with DAPI positive, cytokeratin positive and CD45 negative (scale bar = 10µm).
Table 4-1. Quantification of CTCs per mL of blood among 18 samples from patients with metastatic pancreatic cancer.

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Table 4-2. Quantification of CTCs in healthy donor blood.

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CHAPTER 5
SUMMARY AND FUTURE WORK

5.1 Summary

CTC isolation and enumeration provides an alternative to invasive biopsy for cancer diagnosis and monitoring of cancer progression. The isolation of CTCs involves two challenging steps: 1) maximizing the capture of target cells; 2) minimizing the capture of non-target cells. These two steps correspond to the CTC capture efficiency and purity, or the sensitivity and specificity of CTC detection. This dissertation presents three novel methods for high-performance CTC analysis, including capture, enrichment, release, re-culture, as well as purity and viability testing. Devices, techniques and experimental conditions were studied and optimized for achieving these specific goals.

First, we developed a microfluidic device with unique micropillar geometry, together with high affinity nucleic acid-based ligands, for high-efficiency, high-purity and high-throughput cancer cell capture. The channel geometry and flow rate was systematically studied to find the optimal geometry and flow rate for the best efficiency and purity of cancer cell capture. With the optimized condition, we achieved efficient capture of as few as 10 colorectal tumor cells from 1 mL whole blood. The viability of captured cells was proved to be high, making captured cells suitable for subsequent cellular and genetic study. Efficient release of captured cells was achieved using toehold-mediated DNA hybridization. We also demonstrated cell capture in thermoplastic (COC) device with one-step surface functionalization of COC.

Secondly, gold nanoparticle-incorporated microfluidic devices were developed for high-efficiency and high-throughput cancer cell capture. Instead of engineering complicated microstructures inside channels, we used nanoparticles conjugated with
DNA aptamers for efficient cell capture in a flat channel device. The DNA aptamer-conjugated nanoparticles allowed multivalent binding, offered increased surface area, and packed ligands with high density, thus enhancing the cancer cell capture. With a passive mixing microdevice, the DNA nanospheres enabled rapid cancer cell capture from whole blood with high efficiency and purity.

At last, a geometrically enhanced mixing (GEM) microfluidic chip was developed for capturing CTCs from patient blood. The antibody-coated GEM chip achieved efficient capture of spiked pancreatic tumor cells. We also investigated the release and the re-culture of the captured tumor cells; and captured cells were successfully released and re-cultured. Then the device was applied to the capture of CTCs from patients with metastatic pancreatic cancer. CTCs were precisely verified and enumerated and the CTC number was compared with the therapeutic treatment response. The CTC counts from the GEM chip corresponded well with clinical CT scans of tumor size. Thus, the GEM chip has a great potential for cancer diagnosis, treatment response monitoring and cancer prognosis.

### 5.2 Future Work

#### 5.2.1 Aptamer-Enabled Cancer Cell Isolation

We have successfully demonstrated isolation of cancer cells from whole blood using aptamer and aptamer-conjugated nanoparticles in microfluidic devices. In the future, we can use EpCAM aptamers or gold nanoparticles conjugated with EpCAM aptamers for capturing CTCs from patients with non-hematological cancers. Using aptamers targeting cancer stem cells (CSCs), we can identify CSCs from patient blood. Aptamer-based cell capture will lead to many facile methods for viable release of captured cells, such as synthesis of a disulfide bond on aptamer, and synthesis of a
photocleavable linker or biotin on aptamer. Aptamers can also be incorporated into hydrogels or branched DNA polymers coated on device surfaces for enhanced cell capture. Besides gold nanoparticles, other nanomaterials such as graphene oxides, DNA micelles, and quantum dots can be used for enhanced CTC capture or detection inside microfluidic device.

### 5.2.2 CTC Isolation from Patient Blood

We have demonstrated the efficient isolation of CTCs from patients with metastatic cancer using a geometrically enhanced mixing chip. In the future, we need to further improve the purity of cell capture and reduce the nonspecific capture of non-CTCs, making CTC enumeration more accurate. We can also use negative capture methods to capture blood cells and collect CTCs. We can use different antibodies other than anti-EpCAM for CTC capture, such as anti-HER2, anti-EGFR and anti-PSA. We can also incorporate mesenchymal markers to capture those CTCs that have already undergone EMT. In addition to the traditional CTC definition by CellSearch system (DAPI+, CD45-, and cytokeratin+), new CTC definition and standards should be set with more statistic data.

We need to successfully culture CTCs with cell stimulation and develop new cancer cell lines. On-chip cell culture techniques can be developed. The biology study of CTCs will lead to the finding of the mechanism of metastasis. Certain genes in CTCs can be studied to discover tumor micrometastasis and can potentially be used for gene therapy. CTCs can be collected and lysed and tumor genes can be studied with polymerase chain reaction (PCR) and gene sequencing. Circulating tumor DNA (CTDNA) can be detected and studied with droplet microfluidics-based digital PCR. The combination of CTC and CTDNA analysis will provide a new platform for cancer
diagnosis, monitoring treatment response, cancer prognosis, biology and genetic study of cancer progression and metastasis.

5.2.3 Other Potential Methods for CTC Detection

One method includes the use of microfluidic droplets for partitioning blood into nanoliter aliquots and detecting CTCs from blood aliquots, which will substantially increase the throughput. Activatable aptamer probes\textsuperscript{165} can be added simultaneously with cells into droplets, so that enhanced staining of cancer cells can be achieved within droplets. Signal amplification method can be applied in droplets for sensitive CTC detection.\textsuperscript{166, 167} Another method includes incorporating magnetic beads inside microfluidic device for enhanced CTC isolation with controllable CTC release. Other methods include combining size-based, dielectrophoresis-based and magnetic beads-based methods together inside a microchip for efficient CTC isolation. Ideally, a high-performance microchip should be designed so that blood samples flow into chip and come out with two streams: one is CTCs and the other is blood cells. The microchip should show its simplicity with complicated parts embedded inside chip, and should be easily operated by clinicians and untrained personnel.
APPENDIX A
VELOCITY AND SHEAR STRESS IN MICROCHANNEL

Given the Navier-Stoke’s equation for incompressible flow driven by a pressure gradient between two parallel stationary plates (2-D channel flow, fully developed, Newtonian fluids):

\[- \frac{\partial P}{\partial x} + \mu \frac{\partial^2 u}{\partial y^2} = 0\]  

(1)

where \( \mu \) is the dynamic viscosity of the fluid, \( P \) is the hydrodynamic pressure, and \( u \) is flow velocity in x direction, as shown in Figure 4-6.

Since it is 2-D, fully developed flow, then:

\[\mu \frac{d^2 u}{dy^2} = \frac{dP}{dx}\]  

(2)

Upon integration:

\[u(y) = \frac{1}{2\mu} \frac{dP}{dx} y^2 + c_1 y + c_2\]  

(3)

where \( c_1 \) and \( c_2 \) are constant.

With boundary conditions at the wall and at the center of channel:

\[\frac{du}{dy}(y = 0) = 0, c_1 = 0\]

\[u = 0 @ y = \pm \frac{h}{2}, c_2 = -\frac{1}{2\mu} \frac{dP}{dx} \frac{h^2}{4}\]

(4)

where \( h \) is the channel height.

Thus:

\[u(y) = \frac{1}{2\mu} \frac{dP}{dx} (y^2 - \frac{h^2}{4})\]  

(5)

The velocity profile is parabolic.
Volumetric flow rate per unit width (the width is defined to be the length in the \( z \)-direction) is:

\[
\frac{Q}{W} = \int_{-h/2}^{h/2} u(y) dy = -\frac{h^3}{12\mu} \frac{\partial P}{\partial x}
\]  

(6)

where \( W \) is the channel width.

Then, shear stress:

\[
\tau = \mu \frac{\partial u}{\partial y} = \frac{\partial P}{\partial x} y = -\frac{12\mu Q y}{Wh^3}
\]

(7)

The shear stress is linear across the channel, with zero shear at the channel center \((y=0)\) due to symmetry.

Shear stress at the wall:

\[
\tau_w = \mu \left. \frac{\partial u}{\partial y} \right|_{y=-h/2} = \frac{6\mu Q}{Wh^2}
\]

(8)
APPENDIX B
PROTOCOL OF RBC LYSIS FOR WHOLE BLOOD SAMPLES

1) Centrifuge blood sample at 2500 rpm for 8 min

2) Carefully obtain the buffy coat using a disposable, plastic transfer pipet

3) Add red blood cell (RBC) lysis buffer (buffer to blood ratio = 10:1)

   RBC lysis buffer:
   - 155 mM ammonium chloride
   - 10 mM Tris
   - pH 7.5

4) Incubate in RBC lysis buffer for 15 min

5) Centrifuge at 1200 rpm for 5 min

6) Discard supernatant

7) Repeat step 3-6 till there is minimal presence of RBCs

8) Wash off remaining RBCs with PBS: Add 5 mL of PBS, centrifuge at 1200rpm for
   5min, discard supernatant, re-suspend pellet in 1 mL PBS

9) Perform CTC capture with microfluidic device using the lysed blood
APPENDIX C
BLOOD PROCESSING SAFETY PROTOCOL

1) Safety Training:

Personnel who handle the human blood samples must go through the University of Florida Bloodborne Pathogen (BBP) Training and Biomedical Waste (BMW) Training. An initial training and subsequent annual trainings are required.

2) BBP:

BBPs are pathogenic microorganisms present in blood and other potentially infectious material (OPIM) that can cause disease in humans. The most common BBPs are Hepatitis B virus, Hepatitis C virus and Human immunodeficiency virus (HIV). The primary route of occupational exposure to BBPs is percutaneous exposure (e.g., cut or puncture with contaminated sharp objects, such as needles, scalpels, glass). Personnel should have Hepatitis B vaccine before handling human blood samples; the vaccine is safe and effective for preventing infection.

3) Exposure Prevention:

Always take universal precautions against BBPs. Controls need to be done to protect against BBP exposures, including engineering controls (sharp containers, biosafety cabinet, sharps with safety features); safe work practices (e.g., do not recap needles, hand washing); administrative controls (decontamination and disinfection) and personal protective equipment (gloves, goggles, lab coats).

4) Exposure handling:

Part of this section is adapted from the University of Florida Bloodborne Pathogen (BBP) Training and Biomedical Waste (BMW) Training handout material.
If one gets an exposure, he or she should wash wound with soap and water for 5 minutes and flush mucous membranes for 15 minutes and seek immediate medical attention. Afterwards, he or she should notify supervisor, contact compensation office and allow medical to follow-up with appropriate testing and required written opinion.

5) Biomedical waste processing:

Biomedical waste must be inactivated by bleach or autoclaving and segregated properly (sharp containers, autoclave bags, biological waste box), then transported by a registered BMW transporter (Stericycle, Inc. Eaton Park, FL). Bio-spill kit (bleach, absorbent material, autoclave bags, gloves, safety glasses, dust pan and scoop or tongs for broken glass) must be stored in lab.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Weian Sheng was born in Anhui, China in 1988. He received his bachelor’s degree in mechanical engineering from Huazhong University of Science and Technology, Wuhan, China, in June 2009, with a double bachelor’s degree in optoelectronics. With strong interests in mechanical and electronic engineering, he came to the United States for graduate study in January 2010. He enrolled in the PhD program of mechanical engineering at the University of Florida and worked as a graduate research assistant at the Interdisciplinary Microsystems Group. He got a Master of Science in mechanical engineering in May 2012. He received his Doctor of Philosophy in mechanical engineering from the University of Florida in December 2013.