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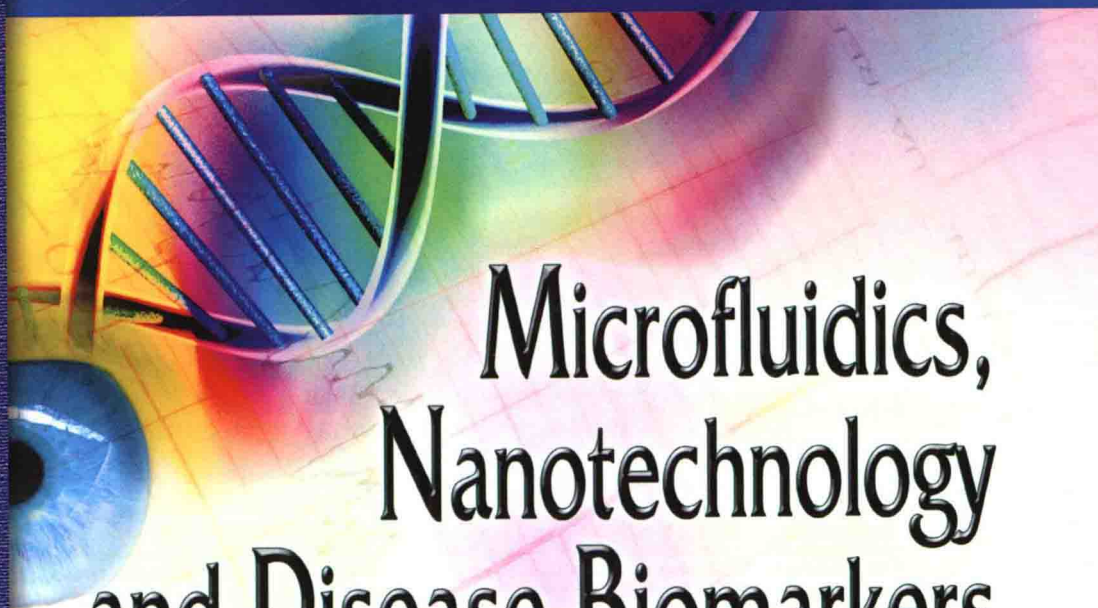

Microfluidics, Nanotechnology and Disease Biomarkers for Personalized Medicine Applications

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NEW DEVELOPMENTS IN MEDICAL RESEARCH

MICROFLUIDICS, NANOTECHNOLOGY AND DISEASE BIOMARKERS FOR PERSONALIZED MEDICINE APPLICATIONS

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PREFACE

We are now beginning to enter an exciting era of personalized medicine. The possibility of highly accurate disease diagnosis, prognosis, and treatment regimes that are specifically tailored for individuals is becoming a reality. Since the completion of the Human Genome Project, we now have thousands of new disease biomarkers for many common diseases including cancer. Never before has humanity had such intimate insights into cancer at the molecular level. Our molecular understanding of cancer is continuously supplemented by new biomarker discoveries emerging everyday through numerous genome wide association studies (GWAS) and other "high throughput" molecular studies in areas such as proteomics. With a plethora of biomarkers now at our disposal, the scientific and engineering challenge in diagnostics is shifting from biomarker discovery to biomarker validation and effective translation of new diagnostic technologies into the complex clinical environment.

Many of the recent biomarker discoveries have been facilitated by ground-breaking technology advances in research methodologies that enable high-throughput biomolecular and biomarker read-out. For instance, most GWAS studies are now enabled by high throughput, Next Generation Sequencing (NGS) technologies. Over the past decade, NGS technologies have seen a spectacular advance which in 2013 has seen the achievement of the $1000 genome, accessible within a few hours of run time. Similarly, while NGS methods have dramatically accelerated new biomarker discoveries in Genomics, technological advancements in other high throughput methods such as Mass Spectrometry have also lead to accelerated discovery of numerous (thousands) of Proteomic and Metabolomic biomarkers for disease.

Although such high throughput technologies have been extremely effective for biomarker discovery in a research environment, many of these are not well suited for routine clinical use. One major limitation of research techniques is the need for large amounts of sample input which often is not usually available in clinical settings. Other disadvantages include expansive infrastructure, high running cost, slow and tedious protocols, and a lack of "plug-and-play" automation. With limited clinical biopsies, and limited recourses within pathology laboratory settings, it is often not necessary to interrogate every known biomarker – with consequent complex bioinformatics analysis. Rather, a more focussed analytical approach is required. New innovations to enable affordable, clinically-focused technologies that can accurately, easily and rapidly detect relevant biomarkers with limited sample input are therefore required to enable many applications in personalized medicine.

In this book, we hand-pick and discuss a selection of emerging technologies that may potentially solve important limitations of current research-focused technologies. For instance, to help remove a major bottle neck for protein-based diagnostics, we discuss the application of yeast-derived single chain Fragment variable (scFv) antibody-like molecules as a potential low cost, and highly flexible alternative to antibody-based diagnostics. For circulating tumour cells (CTCs), which themselves have been gaining interest as potential biomarkers and as a major resource for understanding cancer progression, we explore various strategies combining microfluidics with nanotechnology for capturing these rare cells from blood. DNA methylation is another potential cancer biomarker that has found increased interest. Therefore, an evaluation of some current and emerging technologies for detecting clinical DNA methylation is covered in this book. Personalized medicine may entail tracking a patient's response to treatment, hence in this book; the application of microfluidics to detect metabolites in biological fluids is discussed. Finally, the ultimate goal of personalized medicine is targeted therapy. One promising approach is RNAi technology which uses short nucleotides to disrupt cancer pathways. In this book, nanoparticle approaches to deliver these short nucleotides are also discussed.

In conclusion, we owe thanks to many colleagues who have helped with this book. We are especially grateful to all members of the Trau Laboratory at the Australian Institute for Bioengineering and Nanotechnology (University of Queensland). We thank all contributors for writing high quality chapters. We also thank Nova for publishing this book.

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March 7, 2013
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Chapter 1

BIO-MEMS (MICROFLUIDICS) FOR CTC DETECTION IN CANCER PATIENTS

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ABSTRACT

Micro- and nano-technologies are emerging fields that have been embraced by those in basic medical sciences as well as in clinical medicine. Most notable medical applications have involved micro-sized devices with integrated micro- and/or nano-scale features used for controlled drug delivery or biomolecular analysis. BioMEMS (Biological Micro-Electro-Mechanical Systems) devices have served as conduits for micro and nanotechnologies to enter clinical medicine. Emerging theoretical applications have further proclaimed micro technology as a multifaceted biomedical discipline particularly in cancer detection, treatment, and prognosis. Recent advances have positioned nano-biotechnology such that it can directly impact patient survival and enhance the quality of cancer treatment through personalized therapies thereby reducing time and costs involved.

In the last decade, several technologies have been implemented in both macro and micro systems to isolate and analyze circulating tumor cells (CTCs) from peripheral blood. New exciting technological advances made in the fields of biomedical-engineering such as nanofabrication in micro-/nano-electromechanical systems (MEMS/NEMS), microfluidic devices along with innovations in the toolset of biology (microfluidic chips for DNA array) and medical (microsurgical tools and drug delivery) have significantly improved the state-of-art devices for detection and isolation of CTCs for diagnostic and prognostic purposes. In this chapter we present the overview of recent progress made in developing various microfluidics-based micro-/nano-technologies for isolation, detection and understanding the biological aspects of CTCs. More importantly, strategies based on recent developments in BioMEMS are described that are currently being pursued in

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research and clinical settings. The limitations of the current strategies and the challenges to impart clinical relevance are also discussed.

1. INTRODUCTION

1.1. Circulating Tumor Cells as Driving Force in Cancer Metastasis

Circulating tumor cells (CTCs) are the cells shed by primary tumors into circulation and are known to be key players in metastasis. They can ultimately lodge, invade and proliferate in distant secondary sites initiating metastatic lesions [1]. Tumor metastasis accounts for almost 90% of cancer-related deaths [2].

More than 0.5 million cancer- related deaths occurred in 2011 with 1.6 million people diagnosed with cancers [3]. To cure epithelial-based cancers–such as cancers of the breast, prostate, lung, colon, and pancreas–therapies need to be directed toward those cells that cause metastases including CTCs. Early stage metastasis or tumor progression is often difficult to detect with routine diagnostics which require invasive tissue biopsy or indirect protein quantification. Understanding tumor metastasis is essential for preventing tumor progression and developing effective therapies.

During the process of metastasis, cancer cells continue interacting with the surrounding microenvironment (Figure 1). A small fraction of cancer cells in the primary tumor migrate (after acquiring sufficient aggressive traits) and invade through the basement membrane, extracellular matrix, and surrounding tissue consisting of fibroblasts, endothelial cells, pericytes and bone marrow derived immune cells [4]. For example, prostate cancer metastasizes to bone and lung cancer metastasizes to bone, adrenal gland, liver and brain [5]. It is believed that organ-specific homing might be related to passive trapping as well as active attraction from the distant sites.

There are three states of existence after cancer cells enter into the secondary sites. They can be dormant, form micrometastases, or actively proliferate into macrometastatic lesions depending upon the growth factors and cytokines present in the foreign microenvironment. Mouse metastasis models suggest that primary tumors might release factors before CTC dissemination to prepare the secondary sites for successful translocation [6]. In return, stromal cells in the educated secondary sites might release factors to attract CTCs.

The tumor microenvironment in the primary sites might harbor contextual signals to trigger the epithelial-to-mesenchymal transition (EMT), a cell-biological program that regulates the malignant transition of tumor cells to enter a stem cell-like state. Such cells, termed as cancer stem cells (CSCs), are highly invasive and can survive better in circulation. These circulating-CSCs (CCSCs) constitute a small sub-population of CTCs with the potential of self-renewal in the distant organ sites for colonization [7]. CTCs also exhibit organ-specific homing, known as metastatic organ tropism, when they selectively lodge in metastatic sites. As the majority of metastatic lesions are never biopsied due to anatomic inaccessibility they are unavailable for biological characterization, however, analyzing CTCs through 'liquid biopsy' offer a readily accessible means of studying the biology of metastatic cells.

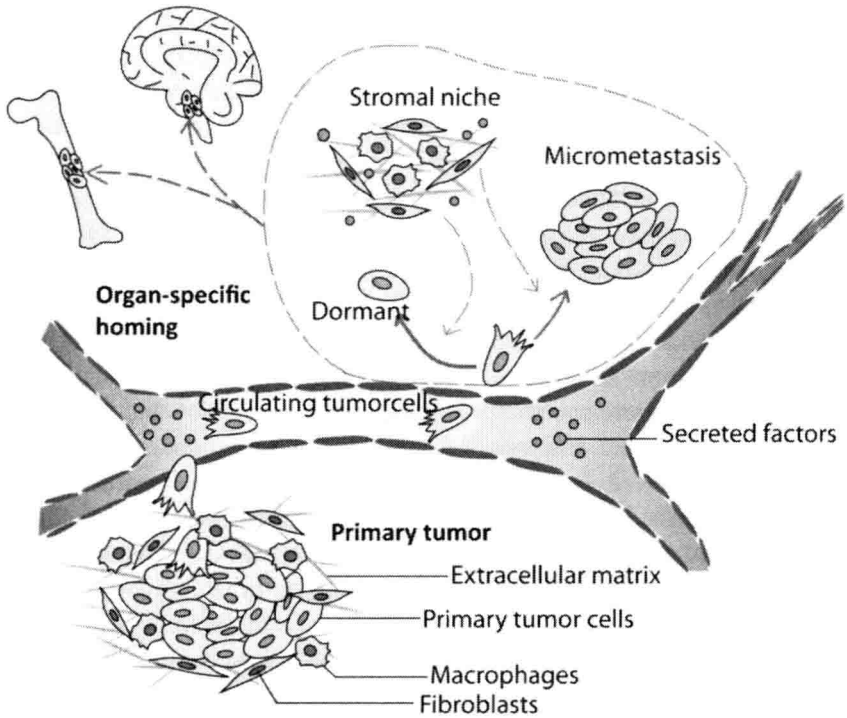

Figure 1. CTCs in tumor metastasis and tumor microenvironment.

1.2. Clinical Implications of CTCs

CTCs are commonly found in the blood of patients with cancer but are rare in the blood of healthy volunteers' and those with benign disease. It was previously thought that metastasis occurred late in disease progression; however, evidence from CTCs has shown that metastasis may be an early event. This is supported by the fact that CTCs are found in patients with early breast cancer, prostate cancer and colon cancer. A study by Huseman et al 2008 demonstrated that dissemination of tumor cells can occur at pre-invasive stages of cancer [8]. Further they also found both in mice and early human breast cancer that the presence of CTCs was independent of tumor size. Biological understanding of tumor progression and metastasis as well as clinical application of tumor derived cells requires the successful isolation of cancer cells from a mixed cell population, including the detection of circulating tumor cells (CTCs) from blood samples and cancer stem cells (CSCs) in tumor tissue.

The role of CSCs in perpetuating cancer metastases is increasingly becoming more evident and there is a growing recognition of the tumor-derived circulating CSCs (CCSCs) in the development of metastases. Recent evidence shows that presence of CSCs in tumor tissue or CTCs in blood represents a reflection of tumor aggressiveness and is believed to be a root cause of overt metastases [9]. Also not all CTCs have the ability to metastasize as only a small fraction of CTCs, presumably the CCSCs, are able to survive the shear stress and environmental changes associated with blood circulation to become seeded at a distant site [10]. We now know that dissemination of CTCs involves a dynamic interplay between cancer

cells, the tumor microenvironment, and numerous freely floating cancer-derived circulatory elements in the blood [7, 9, 10]. However, owing to the rarity of such cancers cells in peripheral blood or body tissues, detecting these cells requires methods with high sensitivity and specificity, which sets tremendous challenges for the implementation of these assays into clinical routine.

A plethora of novel technologies have emerged over recent years for the detection of CTCs [11, 12]. Most techniques exploit physical or biological properties of tumor cells that distinguish them from the overwhelming majority of blood cells. Physical properties include differences in cell size, density, deformability and electrical charge whilst biological properties include the expression of specific surface proteins, invasion capacity, and viability. In general, CTC isolation techniques involve stepwise enrichment of the tumor cell population followed by a more specific procedure for their detection and isolation. The most established application of CTC analysis so far is based on CTC counts (CellSearch System) and has been FDA approved for monitoring patients with breast, prostate and colorectal cancers [13-15]. In metastatic breast cancer an initial and first follow-up CTC count of greater than 5 per 7.5 ml blood is predictive of shorter progression free survival and overall survival [13, 16]. A CTC count greater than 5 per 7.5 ml blood at any time during therapy is indicative of a rapid disease progression and mortality [17]. Again, it is worth noting the CellSearch System is demonstrated well for prognosis of patients' disease rather than as a diagnostic tool. As with many existing technologies, including CellSearch, the challenge remains that the number of CTCs is highly variable among patients. Thus CTC numbers cannot identify tumor burden or disease stage and not all patients have detectable CTCs as defined by CellSearch constraints.

2. Advent of Microfluidics for CTC Isolation and Detection

The knowledge that CTCs exist in blood circulation of cancer patients is not new and evidence probably existed as early as the 1860s [18]. Later studies and developments in medical science have greatly helped in deciphering the biological role of CTCs in various cancers and formed the basis of what Sir James Paget proposed in 1889 as the "seed and soil" hypothesis to explain the mechanism of CTC mediated metastasis [19]. Since then, several attempts have been made both at biomedical and technological levels to develop and optimize methods for successful detection and characterization of CTCs from peripheral blood in cancer patients. More recently, accumulating studies have demonstrated that CTC counts may be used for cancer diagnosis because their level may correlate with the tumor progression [20]. Also, isolation of CTCs from peripheral blood is a relatively non-invasive procedure that offers an easy way to characterize not only tumor progression but tumor specific markers as well.

Among various current technologies that are extensively being applied for the detection and isolation of CTCs, microfluidics-based BioMEMS hold great promise for cancer diagnosis and also are an emerging tool for understanding cancer biology. Microfluidics devices can be highly sensitive to isolate cells within the range of 1-10 CTCs among the background of more than 10^9 blood cells from a minimum of 1ml whole blood. Moreover,

microfluidics-based CTC detection and isolation systems have the advantage of being able to function at the single cell level and can be tailored to specific cell types. The isolation and detection of single CTCs using microfluidics possess a tremendous clinical value, as it would allow genomic profiling of scarce cancer cells that may be resistant to chemotherapy and potentially metastatic. Applying single-cell sequencing techniques to a single CTC not only enhances its diagnostic value but also provides the additional advantages of resolving impure mixtures and analyzing limited amounts of input DNA, [21, 22] thereby producing more reliable data. Most current single-cell genomic profiling techniques such as microarray comparative genomic hybridization (aCGH), next generation sequencing (NGS), and commercially available whole genome amplification (WGA), have been applied to profile gene copy number variations (CNVs) in single cells of various cancer types, including CTCs [23, 24]. CNVs may arise due to the duplication or deletion of the genome regions on certain chromosomes and certain gene copy numbers are known to be elevated in cancers or associated with the development of drug resistance. In a recently published study, NGS was combined with fluorescence-activated cell sorting (FACS) and WGA in a method called single-nucleus sequencing (SNS) to measure high-resolution (approximately 50 kb) copy number profiles of single cells [25]. Although application of whole genome sequencing to single CTCs is still in early stages of development, with future innovations it would be possible to profile multiple samples from patients, such as the primary tumor, metastases, and CTCs to trace an evolutionary lineage and determine the pathways of progression and site of origin. Additionally, in a microfluidics system, the physical laws on micro-scale offer the control of physics, biology, chemistry, and physiology at cellular levels to engineer an integrated system to investigate cancer cell migration, angiogenesis, and tumor microenvironment. Applying these phenomena enables researchers to create better-controlled microenvironments, closely resembling physiological conditions, for the study of cell behavior in response to internal or external cues.

While the previous discussion highlights advantages of microfluidics technology for studying the fundamental biology of cancer, microfluidics also has the potential to benefit patient care and treatment. Microfluidic-based platforms are portable and can be designed for point-of-care diagnostics while also having the advantage of being low-cost to make routine clinical testing feasible. Quick and accurate analysis of patient cells could aid in treatment plan decisions due to identification of specific cancer mutations. Developing and applying state of the art microfluidic CTC detection and isolation technologies to address the unmet challenges in cancer research can expand the horizons of not only fundamental biology but also the management of disease and patient care. Despite the various microfluidic technologies available in the field, few have been tested clinically, which can be attributed to various challenges existing in bridging the gap between technology and real life applications.

2.1. Microfluidic Technologies for Isolation of Circulating Tumor Cells (CTCs)

Microfluidic devices are being developed for many biological applications such as PCR, genomics, proteomics, flow cytometry, biochemical sensing, cell culture, and blood analysis. The small size of microfluidics is apt for cellular analysis using small amounts of reagents.

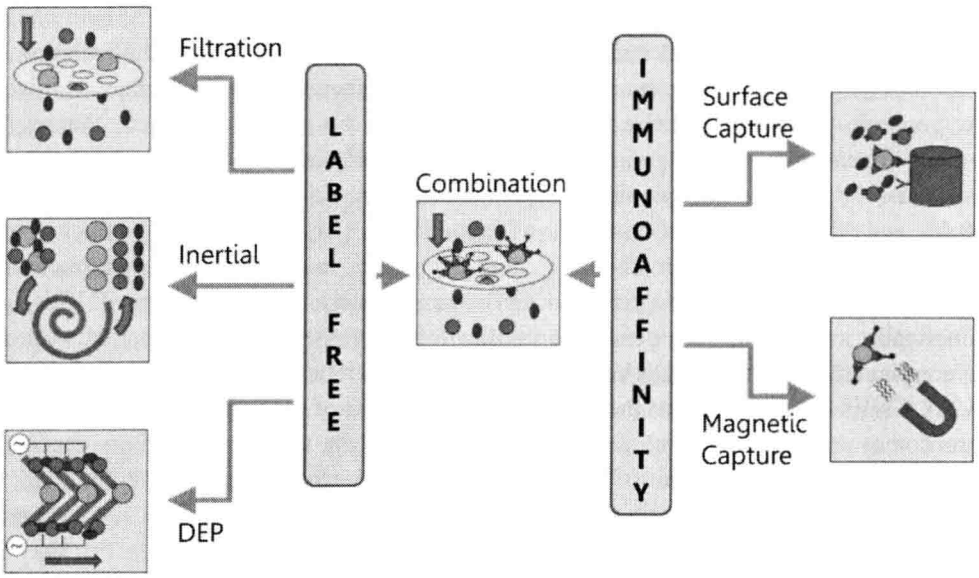

Figure 2. Techniques for CTC isolation in microfluidic devices including methods based on physical cell properties such as size, targeted separation based on surface markers, and combination techniques using both physical (label free) and targeted (immunoaffinity) methods.

Sensitivity and specificity of assays are enhanced by the tight control of samples and reagents within a microfluidic device. Valves, pumps, heating elements, chambers, and channels can be configured within a small space to move and process samples. Ultimately, researchers would like to develop lab-on-a-chip devices to autonomously analyze sample with less time and error than human technicians. Commercially, microfluidic devices have limited functionality. Most microfluidic chips on the market are for use in larger machines and are not completely autonomous systems, often requiring extensive sample preparation or analysis. Scientists and researchers have been designing microfluidic-based devices for cancer cell isolation including downstream analyses because of the inherent advantages listed above. Manipulating channel design, flow profiles, functionalization, and surface area/contact area has led to several methods for the isolation of circulating tumor cells (Figure 2). The emerging microfluidic technologies generally isolate CTCs via their biochemical or physical properties and analyze the CTCs via immunofluorescent staining.

2.1.1. Immunoaffinity-Based Isolation

One widespread CTC isolation method is based on antibody-antigen interactions. The most commonly used surface antigen is Epithelial Cell Adhesion Molecule or EpCAM, first identified in late 1970s [26]. EpCAM is normally expressed on epithelial cells and is not expressed on mesenchymal or blood cells. EpCAM is over-expressed in breast, colon, lung, prostate, gastric, ovarian and renal carcinomas [26-28] and hence widely employed as the target antibody in almost all immunoaffinity-based CTC isolation strategies. The two main methods used for targeting cancer cells in immunoaffinity microfluidic devices are to functionalize device surfaces with antibody or to functionalize magnetic beads with antibody for cell capture using a magnet.

Magnetic beads are commercially available in sizes ranging from 50nm – 5μm. The beads are typically superparamagnetic having either an iron oxide core or a porous material mixed with iron oxide particles. The bead surface is manufactured with different surface functionalities, including biomolecules that can be coupled with antibodies. Superparamagnetic particles have large magnetic moments but magnetic strength per particle is proportional to particle volume [29]. Therefore, smaller beads have less magnetic strength per bead, requiring extensive surface coverage to magnetically capture/deflect a cell within a microfluidic device. Two benefits of smaller beads are better diffusion for more efficient binding and less sedimentation in devices due to their small mass. Larger beads conversely have more strength per bead and less bead coverage is required for cell collection. Bead coverage is limited based on available cell surface receptors and possible steric hindrance of the beads themselves [29]. Larger beads with less coverage could be beneficial for further cell analysis after magnetic sorting, since endocytosis may be reduced due to larger bead size.

Once cells are labeled with magnetic beads, they can be separated in the presence of a magnetic field. Magnets can be permanent, typically neodymium (NdFeB), or electromagnetic. Electromagnetic field strength can be manipulated by varying the applied current while permanent magnets can provide magnetic induction up to 1 Tesla without energy consumption. Electromagnets are useful for stop flow regimes since the particles can be captured while the magnet is on and easily be released by switching the electromagnet off. Magnetic sorting has been done on a macroscale using MACS technology for large batch processing [30]. In this technique, bead packed columns are exposed to permanent magnets and used to positively or negatively collect cells. Then cells are collected for analysis with highly variable yields, losing up to 50% of CTCs [31, 32]. Cell loss can result from low expression of targeted cell surface marker, inefficient cell-bead binding, positively selected cells remaining trapped in a column, cell death, or sample preparation.

Microfluidic devices can provide a more controlled environment for magnetic sorting through spatial positioning of cells, beads, and magnets as well as sample preparation, throughput, and interaction times. Magnets can be placed very close to channels [33] or wires/metal plating can be implemented in specific layouts for optimum magnetic fields [34] (Figure 3). Channel size and layout can be configured to achieve high magnetic field gradients of 10^3-10^4 T/m [29]. High magnetic gradients require close sample proximity to the magnet because the magnetic field will dampen quickly. Microfabrication processes enable appropriate design layouts to achieve the necessary spatial positioning between channels and magnetic elements. Since flow is almost always laminar within microfluidic channels, cells will follow streamlines through a channel unless an opposing force deflects the cell into another streamline. Microfluidic magnetic sorting uses this phenomenon to deflect magnetically labeled cells into different streamlines for separation. In theory, microfluidics should also be beneficial for magnetic labeling of cells because the small scale allows for quick diffusion and many cell-bead interactions in a confined space. However currently, there are no reports of integrated on-chip sample preparation and magnetic sorting, only separate devices for microfluidic mixing or magnetic sorting.

The ability to selectively label cells via antibody interactions has led to microfluidic magnetic designs for CTC capture and analysis. The FDA approved CellSearch (Veridex) system demonstrates separation of CTCs from peripheral blood using EpCAM-coated magnetic beads.

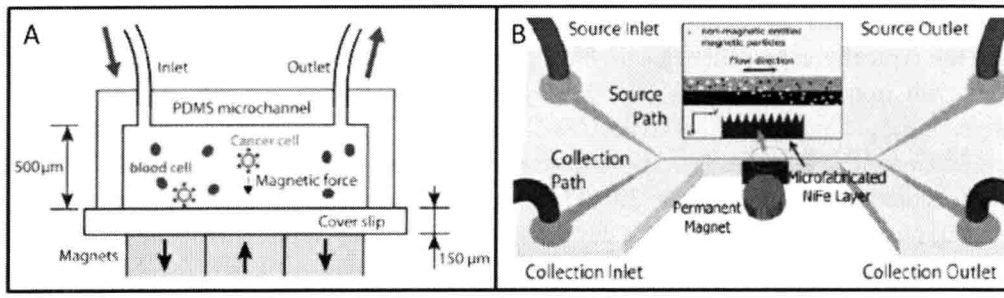

Figure 3. Examples of microfluidic magnetic sorting. A) CTC capture via permanent magnets, reproduced from [33] with permission from the Royal Society of Chemistry. B) CTC deflection *via* microfabricated magnetic layer, reproduced from [34] with permission from Springer.

The CellSearch system captures and separates magnetically tagged tumor cells in a microfluidic cartridge. Further confirmation is achieved through fluorescent immunostaining. Few other microfluidic magnetic devices have been created for CTC isolation. Some examples include magnetic deflection and capture in microchannels with permanent or induced magnets [33, 35, 36] and self-assembled magnetic bead arrays linked with antibodies constructed directly in plain microchannels [37-39]. Most recently, Kang et al presented a novel CTC isolation approach which incorporates microfluidic magnetic separation within a device that permits removal of captured CTCs and culturing *in vitro* [40]. The device consists of a main channel flanked by two rows of dead-end side chambers for magnetically labeled CTC collection. After capture, CTCs can be released by moving the magnet to the opposite side of the device.

Microfluidic immunoaffinity-based magnetic sorting has been limited due to several factors. Often magnetic preparation occurs in large batches in volumes of 5-10ml off-chip and throughput on microfluidic platforms is generally low [41]. Then further post-processing (typically fluorescent immunostaining) is needed to identify and count CTCs, making the overall process labor and time intensive. However there are some key factors that make microfluidic magnetic sorting highly useful for CTC isolation. Magnetic labeling and capture is highly specific and multiple antibodies can be used in a single device for multiple cell type capture [42]. There is also the opportunity to have negative or positive selection or sequential labeling and processing to sort several subpopulations of cells. Fractionation of magnetically labeled samples is also possible as a function of surface marker expression on target cells [43]. Parallel chambers can improve throughput and magnetic field gradients can be precisely controlled [44]. Taking advantage of the high specificity of magnetic sorting and using multiple antibodies for a capturing a wider population of CTCs could lead to improved designs.

The more frequently used method for immune-based microfluidic CTC capture is to coat channels and channel features with antibody to physically capture cells within a chip. Typically the devices are made of polydimethlysiloxane (PDMS) and features are then coated with antibody through GMBS-silane chemistry (Figure 4). There is also an epoxy-functionalized method which uses different chemistry to achieve antibody labeling [45]. Within a microdevice, antibodies can be patterned to allow for multiple cell type capture.

Clearly antibody selection is highly important for specific cell capture, but equally important is the microfluidic channel design features that create multiple cell-antibody interactions for efficient capture of CTCs and avoid capturing unwanted blood cells.

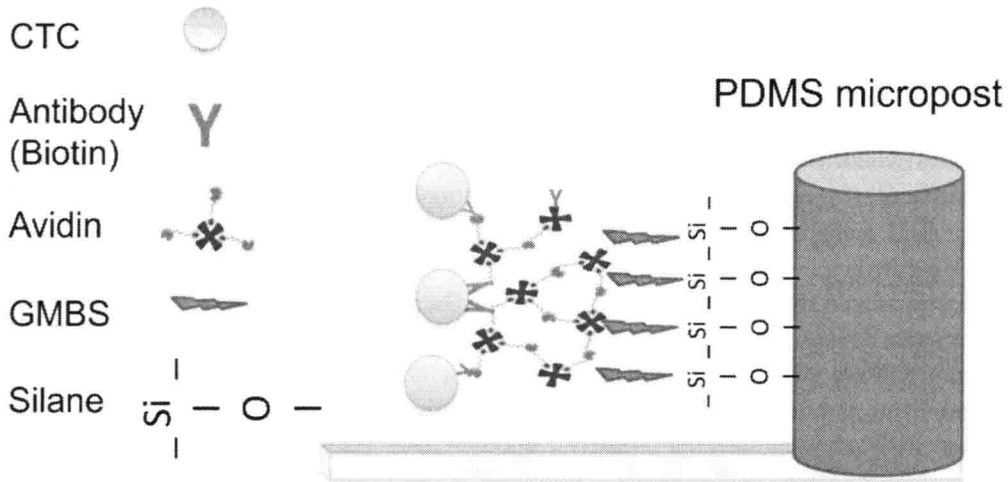

Figure 4. GMBS-silane functionalization of PDMS for antibody-CTC capture.

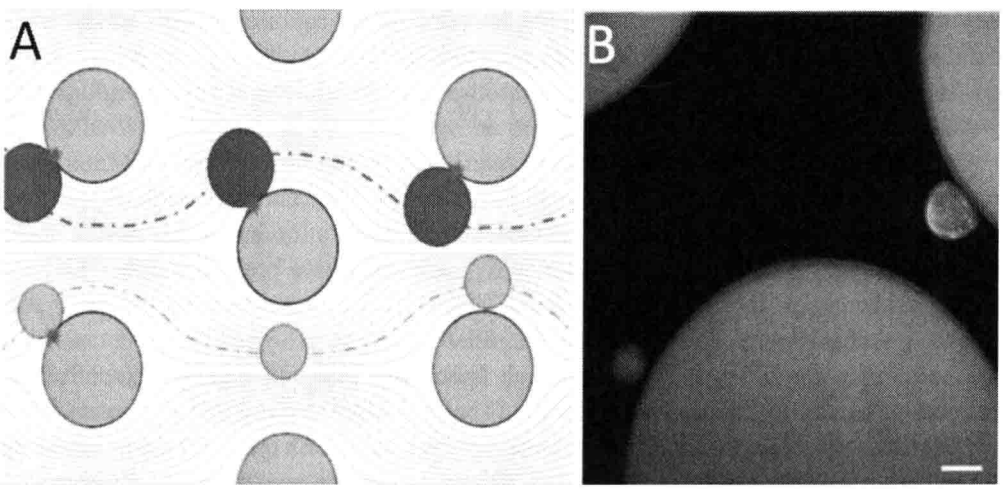

Figure 5. Micropost capture of CTCs. A) Streamlines and effect of micropost positioning on cell capture, reproduced from [49] with permission from the Royal Society of Chemistry. B) Fluorescent image of a CTC (blue and green) captured on a micropost within a microfluidic chip (scale bar 10μm), reproduced from [144] with permission from the Rockefeller University Press.

Many designs accomplish this by incorporating microposts in specific patterns and layouts to achieve multiple cell interactions [46-49] (Figure 5). The Kirby group illustrates how post placement affects cell interactions and movement through their GEDI device [50]. Again, the flow in a microfluidic device will be laminar so the cells will follow streamlines, therefore posts need to be positioned to break up the continuity of the streamlines and bring cells in close proximity with the functionalized surfaces (Figure 5). After CTC capture, washing steps ensure low non-specific binding [51]. In addition, the amounts of available antibody and antigen expression on the CTC surface also play a role in CTC capture [52]. Microposts are not required for surface capture, plain microchannels [51, 53, 54], herringbone patterns [55], and alternate designs such as induced bio-rolling [56], nano-topology [57], and