The incorporation of microfluidics into circulating tumor cell isolation for clinical applications
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Abstract

The second leading cause of death in the United States, cancer is at its most dangerous as it spreads to secondary locations. Cancer cells in the blood stream, or circulating tumor cells (CTCs), present an opportunity to study metastasis provided they may be extracted successfully from blood. Engineers have accelerated the development of technologies that achieve this goal based on exploiting differences between tumor cells and surrounding blood cells such as varying expression patterns of membrane proteins or physical characteristics. Collaboration with biologists and clinicians has allowed additional analysis and will lead to the use of these rare cells to their full potential in the fight against cancer.

Introduction

Complicating the study of cancer is its inherent heterogeneity: a potentially broad spectrum of cells may be present in the primary tumor, but not all of these cells are capable of completing the metastatic cascade, the multistep process by which cancer spreads, causing over 90% of cancer deaths [1]. The traditional invasive tissue biopsy may miss the cells that are most dangerous to the patient. In contrast, the “liquid biopsy,” or blood draw, presents a minimally invasive alternative that could target those cells already traveling in the blood to a distant location. These circulating tumor cells (CTCs) are incredibly rare and may be present at a frequency as low as one CTC per one billion normal blood cells [2]. There have been several macroscale attempts to isolate CTCs based on how they differ from the surrounding blood cells, including the FDA approved CellSearch system [3]. However, these technologies suffered from drawbacks such as the low yield and sensitivity, fixation requirements, and high white blood cell (WBC) contamination [4-7]. The successful sensitive selection of viable cells was greatly advanced through the introduction of the CTC Chip [8], a microfluidic technology that also marked the entry of engineers into this field. Microfluidic systems offer the advantages of low footprint, small sample volume, low reagent usage, pre-established inexpensive rapid prototyping methods, diffusion dominated transport, and a length scale on par with cellular systems [9], making them a natural fit for use in CTC research.

Engineers continue to play an integral role in the further optimization of CTC isolation, aiming for increased sample throughput, target cell sensitivity and purity, and viability to ultimately allow the complete interrogation of this useful cell population. As the interest and publication of CTC technologies continues to increase [10], engineers working with teams of clinical collaborators are using varied principles and techniques within microfluidic capture devices (**Figure 1**). Exploitation of expression of cell surface markers, size variation, and other differences have allowed some success (**Table 1**), and will be covered below, in addition to applications of such devices and potential future directions and challenges.

Immunocapture of CTCs: a biomarker dependent but highly specific technique

Immunocapture, which is used by CellSearch, the CTC Chip, and many subsequent devices (**Figure 2**), takes advantage of the variety in proteins expressed on the cell membrane of

CTCs but not of WBCs, such as the epithelial cellular adhesion molecule (EpCAM), that may be targeted by antibodies against such moieties that are tethered to a surface or feature. The CTC Chip consisted of 780,000 microposts etched in silicon which were then functionalized with antibodies against EpCAM (anti-EpCAM) and was validated with blood samples from lung, prostate, pancreatic, breast and colon cancer patients. This device viably detected cells in a greater percentage of patients and at lower levels and with higher purity than shown by the CellSearch system. This enabled the molecular characterization of CTCs demonstrating tumor specific genetic alterations present in CTCs [11]. However the need for higher throughput, sensitivity, and the ability to further characterize and study these cells beyond enumeration prompted further developments.

Computational fluid dynamics simulation assisted the redesign of micropost shape and layout in the geometrically enhanced differential immunocapture (GEDI) octagonal micropost device that has been functionalized with anti-prostate specific membrane antigen (PSMA) [12] or anti-HER2 to target breast and gastric cancers as validated with patient samples [13]. In contrast to micropost devices, high aspect ratio serpentine thermoplastic microchannels maximize collisions between anti-EpCAM functionalized channel walls and CTCs. By using 51 parallel microchannels embossed in polymethylmethacrylate (PMMA), the high throughput microsampling unit (HTMSU) enabled efficient sample processing followed by immediate enumeration of captured CTCs *via* platinum conductivity sensor [14]. Modifications to the inlet and outlet design as well as the substrate material, now cyclic olefin copolymer (COC), yielded the high-throughput (HT) CTC device [15]. In the NanoVelcro system, cells were released using a thermoresponsive polymer following capture on high surface area silicon nanopillars within a microfluidic chip capped with a chaotic micromixer to increase contact between cells and antibody functionalized surfaces [16].

The chaotic micromixer chamber was first used in the Herringbone (HB) Chip, a follow up to the CTC Chip [17]. Consisting of several parallel functionalized channels in polydimethylsiloxane (PDMS), this device detected CTCs in 14/15 prostate cancer patient samples. Subsequently, the herringbone chamber was integrated with a degradable layer-by-layer (LbL) assembled coating consisting of gelatin and functionalized nanoparticles to increase antibody presentation and allow both single cell and bulk release [18]. Utility was verified with breast and lung patient samples.

Besides multiplexing to increase throughput, a radial flow strategy was used to increase flow rate while decreasing the linear velocity and therefore shear stress exerted on the cells. This OncoBean Chip [19] also featured a redesigned functionalized micropost structure to minimize flow separation, increasing the area on the post utilized in capture.

In contrast to the aforementioned 3D features, the GO Chip incorporated the nanomaterial graphene oxide (GO) for the first time to capture CTCs. GO allowed highly specific and selective capture of CTCs on an effectively 2D surface through a functionalization chemistry that presented the antibody on a high surface area material [20]. The device was verified by capturing CTCs from breast, lung, and pancreatic patient samples.

An alternative to a functionalized surface is magnetic beads functionalized with antibodies that adhere to cells, which may then be separated using an external magnet. In the CTC-iChip, cells in blood samples were magnetically labeled in a preprocessing step, followed by the initial separation of small cells through deterministic lateral displacement (DLD) and alignment by inertial focusing (to be further discussed in “Size based capture”) and ultimate separation through magnetic sorting of the prelabeled cells [21]. In “positive selection” mode, magnetic beads were functionalized to target CTCs, while in “negative selection” mode, magnetic beads were functionalized against WBC markers, allowing those cells to be removed, leaving any remaining cells, including EpCAM negative cells, for further analysis. That the cells are not bound to a surface facilitated further study.

Other immunomagnetic systems prioritize ease-of-use. The VerIFAST (Immiscible Filtration Assisted by Surface Tension) system [22] processed small volumes of peripheral mononuclear blood cells that have been prelabeled with functionalized magnetic beads. Using a handheld magnet, labeled cells are then dragged through successive chambers machined in polystyrene that are gated by oil trapezoids to separate CTCs from non-target cells and guide them into a staining well. This allowed for analysis of both blood and mini-bronchoalveolar lavage samples from lung cancer patients.

Immunoaffinity microfluidic separation has turned to a variety of materials and structures to improve in areas where isolation technologies are still lacking. Each device balances added functionality of various features and materials with the associated necessary expertise or increased fabrication logistics and costs. Downstream analysis is hindered when cells remain attached to the capture substrate, although the nascent field of cell release and immunomagnetic capture may counter this. A commonality among these devices is that a specific population is assumed to be the entirety of the target CTC population based on the choice of capture antibody, missing cells in transformations such as the epithelial to mesenchymal transition (EMT). Some of these drawbacks have been addressed through the incorporation of nanomaterials [23]. Another category of CTC isolation techniques prioritizes label free capture and often has the added advantage of high throughput, although this strategy has its own drawbacks.

Size based capture

CTCs also differ from blood cells in size and deformability, offering molecular marker-independent, high-throughput, and inexpensive options for isolation (**Figure 3**). CTCs are generally larger and stiffer than WBCs, leading to the early use of commercial filters [24, 25]. To solve problems with earlier filters including fixation requirements, non-uniform pore sizes, and low pore density, the separable bilayer (SB) microfilter was microfabricated by etching parylene polymer via reactive ion etching to precisely control pore sizes and density [26]. Parylene is ideal for this application because it is mechanically strong while still malleable, with good biocompatibility and low membrane fouling. The bilayer design consisted of a bottom layer with 8 μm pores and a top layer with 40 μm pores, trapping CTCs between the two layers that could be separated easily, leaving CTCs accessible. A flexible micro spring array (FMSA) was

designed as a high-porosity filter [27] capable of processing 7.5 mL whole blood without clogging while still preserving viability. CTCs were detected in 76% of clinical samples from breast, colorectal, and lung cancer patients. Microclusters and multinucleated CTCs were enriched from patients from all three cancers.

An alternative approach to separation exploited unique properties of particles moving in microchannels. Under laminar flow, deterministic lateral displacement (DLD) within an array of microposts has been used to separate particles of different sizes [28]. Depending on the geometry of the microarrays, particles above and below a certain size follow different and predetermined migration paths. The CTC-iChip (also mentioned in the Immunocapture section) utilized DLD to first separate nucleated cells (CTCs and WBCs) from red blood cells (RBCs) using an array of microposts with 32- μm gaps [21]. When flowing through the device, small cells like RBCs remained along their original streamlines, whereas larger cells like CTCs and most WBCs were fully deflected into the coincident running buffer stream by the end of the array.

Other separation approaches exploited inertial forces. Within microchannels, particles of different sizes can migrate across streamlines to focus at different positions due to the shear induced lift force and the wall induced lift force. In vortex technology, cells of various sizes are aligned by size in different streamlines in straight channels by inertial focusing followed by multiple expansion-contraction reservoirs to create laminar microvortices that can trap large cells within reservoirs [29]. As cells entered the expanding regions, wall lift forces were diminished and cells mainly experienced lateral lift forces, which are proportional to the cell volume. The larger lift forces on CTCs pulled them into the vortices while other blood cells experiencing smaller forces passed through the vortex and remained in the main stream.

Curvature in microchannels causes particles to experience an additional lateral Dean drag force because fluids in curved channels develop a secondary lateral flow. The combination of the inertial lift force and the Dean force leads to particle migration which can result in high resolution separation. Single spiral, double spiral, cascaded spiral, and slanted spiral structures have been designed and optimized to maximize separation efficiency for various parameters including channel length, height, width, radius of curvature, and flow rate [30-33]. Generally, near the outlet of spiral devices, larger CTCs focus near the inner wall due to the combination of the inertial lift force and the Dean drag force. To this end, Hou *et al.* designed a multiplexed spiral device that detected CTCs in clinical samples from breast and lung cancer patients [34].

CTC enrichment by size offers a fast, inexpensive, and label-free way to harvest CTCs. However, as there is a wide range of CTCs sizes, sized based separation often suffers from low purity and the risk of losing smaller CTCs. Additionally, size based separation often requires preprocessing of blood, like RBCs removal and further, dilution or runs the risk of device clogging. To date, size based technologies have shown proof-of-concept clinical validation but have not been applied in large scale clinical or biological investigation.

Other separation methods based on physical properties include the interplay of dielectrophoresis (DEP) forces and inertial forces in microfluidic devices to lead to different patterns of cell migration to viably separate cells [35]. Similarly, acoustophoresis causes particles

within the fluid to move toward regions with minimal acoustic radiation forces in distinct migration patterns [36, 37]. Although promising, approaches like DEP and acoustophoresis have the disadvantages of low throughput, low sensitivity and purity, and additional required steps like RBC lysis and may require further clinical validation.

Applications of CTC isolation technologies

The inclusion of engineers into the field of CTC research has been fruitful as far as the development of new technologies, but their utility will only be fully realized as those technologies are incorporated into medical research and practice, such as use in early detection, serial monitoring of response to treatment and disease progression, and biological study. To assess the potential for early disease detection, the GEDI platform was used to sample for CTCs in patients with precancerous cystic lesions [38]. The HTMSU was used to monitor CTC levels before and after the administration of a new therapeutic in a pancreatic patient-derived xenograft (PDX) mouse model [39]. Response to treatment in one lung cancer patient was monitored using the NanoVelcro technology in addition to whole genome amplification and Sanger sequencing to detect relevant mutations in EGFR at time points before and after treatment with Gefitinib [16]. Consequences of metastasectomy were evaluated through CTC enumeration over multiple time points with the FMSA device in metastatic colorectal cancer patients [40]. Microfluidic technologies have also been used to illuminate biological subtleties of these cells in transit. The HB Chip was used to study levels of RNA transcripts associated with EMT in breast cancer patient CTCs [41], while CTCs isolated from a mouse model of pancreatic cancer using the CTC-iChip have undergone single cell RNA sequencing further implicating the extracellular matrix in the metastatic cascade [42].

An area of emerging investigation is proliferation of CTCs *ex vivo* to allow further biological analysis and personalized medicine applications. Successful expansion of CTCs isolated by a modified CTC Chip was achieved in 14 out of 19 lung cancer patients [43]. Further analysis on cultured CTCs included transwell invasion assays and next-generation sequencing (NGS). CTCs isolated using the CTC-iChip were cultured successfully in 6/36 attempts [44]; NGS was used to develop a targeted drug testing regime. The use of cultured CTCs for functional testing and therapeutic guidance represents the next frontier of CTC.

Conclusion

The study of CTCs is contingent on their effective separation from surrounding blood cells and has been made possible by microfluidic technologies as realized by the incorporation of engineers into this field. While each microfluidic isolation technique features both benefits and drawbacks, they have led to both verification with patient samples and the further study of cancer by tracking disease progression or response to treatment, analyzing genetic material, and expanding captured cells to open further analytical doors. Furthermore, they have established CTCs as a formidable alternative to tissue biopsy, the “liquid biopsy”.

As efforts in this field progress, there are several considerations to which attention should be paid. As evidenced by Table 1, metrics by which the performance of such technologies is reported are highly inconsistent. To best evaluate ongoing research in the field, standardization is required to allow comparison across platforms to elucidate room for improvement as well as beneficial modifications to be adopted. Another challenge facing the field is that sensitivity often comes at the expense of specificity and vice versa. Next generation technologies should seek to optimize with respect to both of these quantities to maximize impact. Additionally, at the heart of this field of research is the fight against cancer, and consequently the design of these technologies must allow the downstream analysis necessary for biological study and clinical management. Given the rarity of CTCs, it would be highly advantageous for engineers to develop downstream analytical assays that are easy to operate with low numbers of cells, including single cell genomic, proteomic, and functional assays. This can revolutionize not only the CTC field but also fulfill the greater need in cancer biology. Through thoughtful design choices and collaboration across disciplines, engineers can contribute their skill set to confront a dire medical issue.

Acknowledgements

This work was supported by the National Institutes of Health (NIH) Director's New Innovator Award (1DP2OD006672-01) and a Department of Defense (DoD) Office of the Congressionally Directed Medical Research Programs (CDMRP) Career Development Award to S. Nagrath. This material is based upon work supported by the National Science Foundation Graduate Research Fellowship Program under Grant No. DGE 1256260 to M. Kozminsky.

Captions

Figure 1 Circulating tumor cell (CTC) isolation technologies. A brief history of CTC isolation technologies beginning with the first FDA approved technique, CellSearch. Microfluidics was introduced in 2007 with the CTC Chip. Subsequent developments have occurred in the areas of immunocapture and size based isolation. Figures used with permission from (left to right): Janssen Diagnostics LLC; [17]; [21] Copyright 2013 American Association for the Advancement of Science; [20] Copyright 2013 Nature Publishing Group; [26]; [19] Copyright 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim; [31] Copyright 2014 AIP Publishing LLC.

Figure 2 Immunocapture methods for microfluidic circulating tumor cell (CTC) isolation. Examples of a number of techniques used to improve metrics such as sensitivity, purity, throughput, and ease of use or fabrication of immunocapture devices include the use of silicon microposts, thermoplastics, micromixers, radial flow, nanomaterials, and immunomagnetic separation. Figures used or adapted with permission from (clockwise from bottom left): [12] Copyright 2010 The Royal Society of Chemistry; [14] Copyright 2008 American Chemical Society; [15] Copyright 2013 Springer-Verlag Berlin Heidelberg; [16] Copyright 2015 American Chemical Society; [17]; [18] Copyright 2015 WILEY-VCH Verlag GmbH & Co. KGaA,

Weinheim; [19] Copyright 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim; Photo credit Joseph Xu; [21] Copyright 2013 American Association for the Advancement of Science; [22] Copyright 2014 The Royal Society of Chemistry.

Figure 3 Size based technologies for circulating tumor cell (CTC) separation. Based on differences in size between CTCs and white blood cells, microfilters and inertial sorting techniques have been incorporated into microfluidic CTC isolation. Figures used or adapted with permission from (clockwise from top right): [29] Copyright 2014 The Royal Society of Chemistry; [31] Copyright 2014 AIP Publishing LLC; [34]; [26].

Table 1 Circulating tumor cell (CTC) isolation technologies. Relevant performance characteristics of the discussed CTC isolation technologies. Capture efficiency refers to the percentage of cells isolated in cell spike experiments with cancer cell lines in whole blood. Purity refers to the captured number of target cells as opposed to captured non-target cells as expressed either as a percentage or log depletion. Blank spaces indicate that this metric was not provided by the reference.

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****CTC culture to the end of developing personalized treatment based on the response of cultured cells to a panel of drugs.**

Figure 1

Figure 2

Figure 3

Table

Table 1

Technology	Year	Capture efficiency	Purity	Throughput	Clinical verification	References
CellSearch	2004	85.50%	low		breast, bladder, colorectal, gastric, lung, ovarian, pancreatic, prostate, renal	3
CTC Chip	2007	>60%	50%	1 mL/hr	breast, colon, lung, pancreatic, prostate	8, 11, 43
GEDI	2009	78-85%	68%	1 mL/hr	breast, gastric, pancreatic, prostate	12, 13, 38
HTMSU	2008	94.50%		1.6 mL/hr	pancreatic (PDX mouse)	14, 39
HT-CTC Chip	2014		86%	1.38 mL/hr	prostate	15
NanoVelcro	2011	95%		0.5 mL/hr	lung	16
Hb Chip	2010	92%	14%	1.2 mL/hr	prostate	17, 41
LbL Hb Chip	2015	96%	high		breast, lung	18
Oncobean	2014	82.7-100%	higher with increased flow rates	up to 10 mL/hr	breast, lung, pancreatic	19
GO Chip	2013	94.20%	high	1 mL/hr	breast, lung, pancreatic	20
CTC-iChip	2013	77.8-98.6%	2.5-3.5 log depletion	8 mL/hr	breast, colorectal, lung, pancreatic, prostate	21, 42, 44
VerIFAST	2014	90%			lung	22
SB microfilter	2014	78-83%	$1.7-2 \times 10^3$	around 5 ml/hr	tested in mouse model	26
FMSA device	2014	92.6%	1.4×10^4	around 45 mL/hr	breast, colorectal, and lung	27, 40
Vortex technology	2014	10-20%	57–95% for clinical samples		lung, breast	29
Multiplex spiral device	2013	>85%	10%	3 ml/hr	lung	34
ApoStream (DEP)	2011	70%	reduction of WBCs 99.33% ± 0.56% (2-3 log depletion)	1 ml/hr	prostate, breast, lung, hepatocellular, bladder	35
taSSAW	2013	>83%	around 90% removal rate of WBCs (1 log depletion)	1.2ml/hr	lung	36, 37