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Microfluidics for Research and Applications in Oncology

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Abstract

Cancer is currently one of the top non-communicable human diseases and continual research and developmental efforts are being made to better understand and manage this disease. More recently, with improved understanding in cancer biology as well as advancement made in microtechnology and rapid prototyping, microfluidics is increasingly being explored and even validated for use in the detection, diagnosis and treatment of cancer. With inherent advantages such as small sample volume, high sensitivity and fast processing time, microfluidics is well-positioned to serve as a promising platform for applications in oncology. In this review, we look at recent advances in the use of microfluidics - from basic research such as understanding cancer cell phenotypes as well as metastatic behaviors to applications such as detection, diagnosis, prognosis and drug screening. We then conclude with a future outlook on this promising technology.

Keywords: Microfluidics; Microfabrication; Oncology; Cancer diagnosis; Cancer cell invasion; Circulating tumor cells (CTCs); Cell sorting; Single cell analysis, Tumor-on-a-Chip

Introduction

Cancer is one of the world's deadliest human diseases [1]. The number of cancer-related deaths is increasing at a rapid rate due to the increasing life expectancy of our aging population as well as unhealthy lifestyles and diets. World Health Organization (WHO) estimates that cancer related deaths will keep on increasing worldwide and will reach to ~ 11 million by 2030 [2]. As such, a better understanding of cancer is extremely important for us to wage a successful war against this dreaded disease.

The past decade has seen substantial growth in the number of breakthrough technologies such as microfluidics, which have been successfully developed and used for both basic and applied research on cancer [3, 4]. With a length scale comparable to that of a cell, the microfluidic-based platform allows extensive manipulation from the cellular to sub-cellular levels. Furthermore, with evident advantages such as small sample volume, high sensitivity, fast processing speed, high spatial resolution, high portability and low cost, microfluidic devices have emerged as promising tools for research and applications in oncology. In fact, numerous applications of microfluidics for cancer research are right now continually being demonstrated [5, 6].

In this review, we will focus on recent advances made in the development of microfluidic devices for both basic and applied research in cancer. For microfluidic applications in basic cancer research, we will first review the use of microfluidics for the characterization of cancer cells such as their physical and structural properties. Next, we will look at the various state-of-the-art microfluidic platforms for the study of cancer cell migration to better understand the invasive steps these cancer cells take during metastasis. For microfluidic applications in applied cancer research, we will start with a discussion on the potential clinical utilization of microfluidics for the separation and isolation of cancer cells from blood

as a possible route for diagnosis and prognosis. We will then conclude with looking at some of the state-of-the-art applications in the use of microfluidics for single cancer cell analysis, development of tumor-on-a-chip system and cancer drug screening. Some of these emerging applications are summarized schematically in **Fig. 1**.

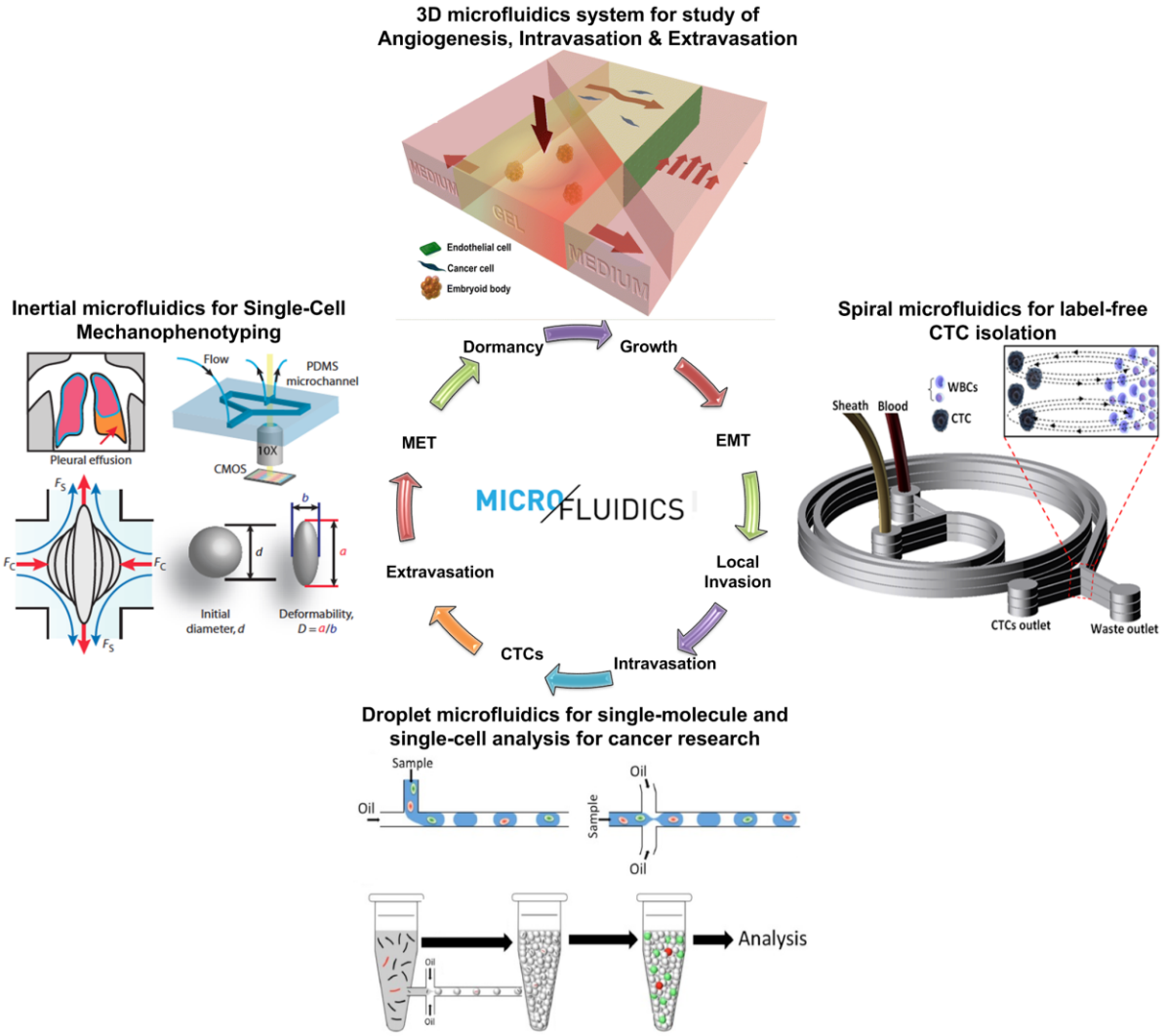

Figure 1. Illustration featuring some applications of microfluidics in cancer research. ***Top***: Schematic representation of a 3D co-culture microfluidic device designed for mimicking tumor microenvironment. Cancer cells (e.g., can be in form of spheroid) are mixed with 3D hydrogel scaffold and placed in vicinity of a thin layer of endothelial cells for investigation of different mechanisms involved in intravasation, extravasation and angiogenesis. In addition, it can be used as a drug-screening platform for therapeutic Epithelial to Mesenchymal Transition (EMT) blocking agents. Reproduced with permission from [7]. ***Right***: Schematic illustration of a spiral microfluidic device developed for high-throughput, label-free isolation of circulating tumor cell (CTCs) from blood. Reproduced with permission from [8]. ***Bottom:*** Diagrammatic representation of droplet microfluidics for single-cell and single-molecule analysis. Rare cancer cells or target molecules can be captured, expanded and investigated within a single droplet. Reproduced with permission from [9]. ***Left***: Schematic illustration showing working principle of a microfluidic system developed for single-cell mechanophenotyping. The deformability of the cells can be measured in real-time using high-speed videos and correlated to their benign or malignancy. Reproduced with permission from [10].

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1. Microfluidics for Basic Cancer Research

1.1 Phenotyping of cancer cells

Diseases such as cancer do induce changes in the structural and biophysical properties of the cells. *In vitro* studies have shown that oncogenic cells are more deformable than healthy cells [11, 12]. Some of the change in the biophysical properties of the cancer cell is due to the conversion of an organized, filamentous and stiff cytoskeletal network to a more disorganized and less filamentous state. Consequently, this change in subcellular structures makes cancer cells more deformable and hence, may actually aid in their metastasis by being able to more easily squeeze through the extracellular matrix (ECM) and intravasating into the blood circulatory system. Therefore, in the context of metastatic spread, there is a need to understand the dynamic and phenotypic changes in terms of the structural and biophysical properties of the cancer cell during different stages of metastasis and how these changes can assist in their invasion.

There are two types of microfluidic devices that have been used extensively to probe the biomechanical properties of cancer cells - microfluidic channels and microfluidic optical stretchers. For example, the deformation of cancer cells in a narrow microchannel mimics the transportation of cancer cells in the circulatory system or circulating tumor cells (CTCs) to distant secondary locations in the body. The cells either pass through or get trapped within the narrow channels, as they would have encountered during metastasis [13]. Using a straight channel microfluidic device, Hou *et al.* were among the first to compare the deformability of benign (MCF-10A) and non-metastatic (MCF-7) breast tumor cells using quantitative parameters such as cell velocity, cell transit time and cell deformation, as shown in **Fig. 2(a)** [14]. The entry of the breast cancer cells into the constricted microchannels was subsequently modeled by Leong *et al.* [15]. Following the work of Hou and co-workers, Byun *et al.* subsequently observed that alongside higher deformability, lower surface friction might also be a crucial biophysical parameter that can contribute to greater invasiveness of cancer cells through narrow capillaries [16]. Later, Adamo *et al.* developed a high-throughput microfluidic device to investigate the relationship between the transit time through the narrow channel and cell stiffness, as shown in **Fig. 2(b)** [17]. Stiffer cells had a longer transit time as compared to less stiffer ones. Using MUlti-staged Serial Invasion Channels (MUSIC device), Mak and colleagues observed that mechanical confinement at the sub-nuclear scale could induce dynamic transition in cell motility and morphometric shapes [18]. More recently, Gossett *et al.* developed a microfluidic based Deformability Cytometry (DC) for single-cell mechanophenotyping (**Fig 2(c)**) [19]. The deformability of the cells can be measured in real-time to diagnose clinical pleural effusion samples as benign or malignant with sensitivity of 91% and specificity of 86% [10].

Microfluidic optical stretcher was also used to examine the deformation of a single cell in suspension [20], as depicted in **Fig. 2(d)**. Here, the cellular mechanical properties were determined by the laser-induced force that was evenly distributed over the entire cell surface [21]. Using this cell stretcher, Guck *et al.* sequentially suspended, trapped and deformed

isolated breast cancer cells through a microfluidic channel [22]. However, a drawback of this device is that the force generated by the laser on the cell surface is not sufficient to induce deformation that a migrating cancer cell encounters *in vivo* [23]. In another study, Lincoln and colleagues developed a novel microfluidic cell stretcher that combined an optical stretcher with a microfluidic cell delivery system. According to their observation, metastatic cells showed a higher relative axial strain as compared to the normal cells [24].

While flowing through the circulatory system to their specific secondary locations, cancer cells may be subjected to various mechanical cues such as fluid shear stress (FSS), physiological confinement from the narrow capillary walls and varied ECM stiffness [25]. The magnitude of FSS that the cancer cells are exposed to can vary from 0.1 Pa in venous circulation to 3 Pa in arterial circulation [26]. Low FSS could promote EMT and metastasis whereas high FSS could lead to dormancy of the cancer cells, which are then more susceptible to the attack of the immune cells [27, 28]. Additionally, the magnitude of the FSS plays a decisive role in the attachment of the cancer cells to the endothelial cells of the blood vessels during extravasation phenomenon [29]. Another way by which the cancer cells could undergo extravasation is by getting trapped in the narrow confinements of the blood capillaries. However, the occluded cells show a low efficiency to colonize may be due to the higher FSS they are exposed to or due to the lack of specific chemical adhesions [30]. To mimic this, Irimia *et al.* designed a series of microfluidic devices that could mechanically constrain migrating cancer cells inside micro channels with various channel dimensions [31]. They demonstrated that cancer cells move rapidly inside the 3D confinement without any external gradients. Das *et al.* developed a microfluidic device to study cellular dynamics during stress adaptive responses under micro-confinement conditions [32]. They observed that cancer cells respond rapidly to FSS when subjected to confinement of less than 70 µm. In another study, the effect of stiffness and confinement on cancer cell migration was observed independently using a microfluidics system [33]. Under confined conditions, cells migrate rapidly as the stiffness increases and this relationship is dependent on the non-muscle myosin II (NMMII)-based contractility.

What's next?

The biomechanical and biophysical properties of cells have been shown to be useful markers of cell state and human diseases [34]. In contrast to the conventional and low-throughput techniques (i.e., static approaches) such as atomic force microscopy (AFM), micropipette aspiration and magnetic twisting cytometry (MTC), microfluidic approaches offer a higher throughput (> 100 cells/sec) and flexibility for automation thus facilitating analysis of large number of cells. In addition, diagnostics using label-free physical biomarkers can significantly reduce the cost of medical care and improve patient outcomes. Until now, most of the proof-of-concept validations have been done using cancer cell lines. We are anticipating with a myriad of technologies that have been developed over the past decade for CTC sorting (see section 2.1), new hybrid technologies with improved capabilities will emerge for analysis of primary cells. Several excellent reviews on cancer mechanobiology need to be highlighted here before we move into next section [2, 34-36].

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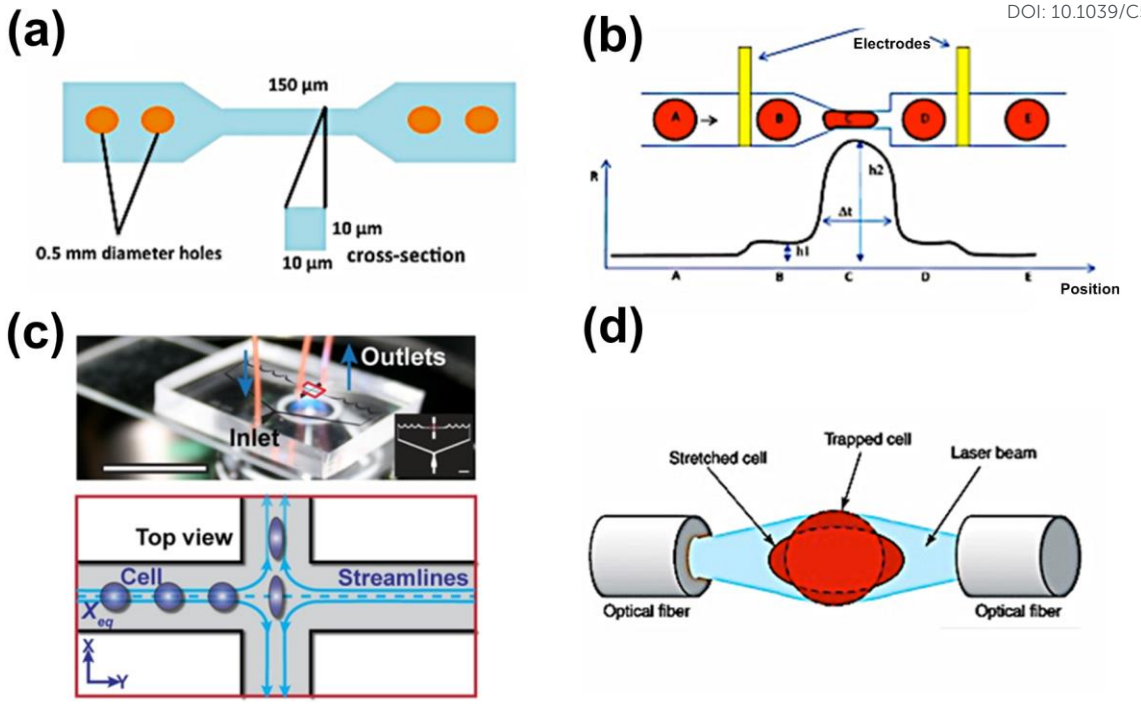

Figure 2. Microfluidic technologies for measuring cell deformability. **(a)** Schematic representation of a straight channel microfluidic device with 150 µm length and 10 by 10 µm cross-sectional area. The cell gets deformed when it passes through the channel and different quantitative parameters like entry time, transit velocity, and elongation index were measured. Reproduced with permission from ref [14]. **(b)** Schematic representation of the suspended cells moving through the narrow constriction of a microfluidic device. As the cell moves, electrical resistance could be measured across the two electrodes placed on either side of the constriction. This resistance increases from point A to a value 'h1' when the cell is positioned before point B. The resistance then peaks to a value 'h2' when the cell is constricted at point C and then drops again to 'h1' as the cell moves out at point D. Finally, the resistance returns to the baseline at point E when the cell crosses the downstream electrode. The time required by the cell to travel through the constriction is measured by the width of the signal (Δt). Reproduced with permission from ref [17]. **(c)** *Top:* Photograph of a microfluidic device with inset showing channel design developed for single-cell mechanophenotyping (the red region highlights deformation junction shown in the bottom figure). *Bottom:* Cells are introduced into the microfluidic device and stretched under continuous flow. The images of the stretched cells are captured using complementary metal-oxide semiconductor (CMOS) camera. Cells are exposed to compressive and shear forces within the device and deformability and diameter of the cells are quantified to diagnose a cell as benign or malignant. Reproduced with permission from ref [19]. **(d)** Diagrammatic representation of the stretching of a cell in the axial direction using an optical stretcher. The optical forces from the two divergent laser beams trap the cell in the middle. When the laser power is increased, a force is generated on both sides of the cell, thereby stretching it along the laser beam axis. Reproduced with permission from ref [20].

1.2 Cancer cell migration studies

Metastasis is the spread of tumor cells from the primary tumor to their secondary niche in which they undergo colonization and subsequently form micrometastases. During this

process, tumor cells undergo invasion through the 3D ECM and remodel it through the action of various matrix metalloproteinases. Genetic analysis of the cancer cells reveals mutations in the genes and signaling pathways involved in cell motility, proliferation and survival [37, 38]. Therefore, in the context of metastatic dispersion, cancer cell migration and invasion need to be further studied.

Cell migration is a sequence of biophysical processes that are spatiotemporally regulated by signaling gradients. In general, there are two types of cell migration: (1) single cell type that includes amoeboid and mesenchymal migrations, and (2) collective cell type in the form of cell sheets or strands. Depending on the microenvironmental cues, cancer cells can interchange between the different modes of migration [39-41]. Recently, Haeger *et al.* investigated the role of ECM density and porosity on the mesenchymal tumor cell invasion [41]. Interestingly, they observed that highly porous ECM leads to single cell migration whereas less porous and highly dense matrix results in collective cell migration. Using a nested collagen assay, Sun *et al.* observed that matrix modifications by adjacent cells could provide a physical barrier to the invading cancer cells [40]. During cancer metastasis, there is a disruption to the healthy physiological gradient that guides cell migration. This leads to abnormal cell motility and hence cancerous outgrowth. Microfluidic systems could efficiently provide an *in vitro* control of the spatiotemporal gradients involved in cell migration. In general, there are two types of gradients generated by microfluidic devices that regulate cancer cell migration: (i) chemical gradients, and (ii) electrical gradients.

1.2.1 Chemical gradients

Movement of cells towards a chemical gradient is known as chemotaxis. An efficient chemo-gradient can be generated in three different ways: flow-based, diffusion-based, and surface micropatterning-based gradients. In the flow-based microfluidic gradients, two concentrations of biomolecules are mixed inside a microfluidic device with designed patterns to form a chemical gradient. The characteristic feature of this flow-based device is the generation of low Reynolds number. An example of this type is the "Christmas tree" device that generated a stair-shaped chemical gradient [42]. Using this device, Wang *et al.* showed that metastatic breast cancer cell lines respond to the polynomial gradient of epidermal growth factor (EGF) concentration but are insensitive to the linear gradient [43]. The crucial benefit of the flow-based system is the greater regulation of spatiotemporal gradient that can be controlled by varying the rate of flow of different solutions. However, the shortcoming of this type of devices is that it requires a greater amount of solutions to maintain the gradient. The cells are also subjected to shear stresses that could affect cellular motility.

In the diffusion-based microfluidic gradients, passive diffusion occurs to establish a soluble gradient between the source reservoir with higher chemical concentration and the sink reservoir that has a lower concentration. Passive diffusion could be established between the source and the sink by either confining microchannels to a narrow diameter or by filling the channels with hydrogel. For example, Zhang and co-workers developed a high-throughput device with 3120 microchannels, the M-Chip, to investigate mesenchymal mode of cancer

cell migration in the presence of a chemotactic gradient, as illustrated in **Fig. 3(a)** [44]. The hydrogel-filled channels are capable of mimicking tumor microenvironment more accurately, in which diffusion of water-soluble biomolecules occurs through the gel pores. In a similar study, Abhyankar *et al.* developed such a device, in which the channels were filled with type-I collagen and a linear gradient of EGF was established through it. They found that, in the presence of EGF gradient, the speed of cancer cell movement was statistically faster [45]. In order to understand how fluid flow in the tumor microenvironment affect cancer cell migration, Haessler *et al.* developed a microfluidic device that enabled them to culture cells in 3D under well-defined interstitial flow conditions [46]. They observed that under interstitial flow, the percentage of migratory cell increased. Nguyen *et al.* developed an impedance sensing based microfluidic device to investigate the migration of single cells embedded in 3D matrices (**Fig. 3(b)**) [47]. They observed a rapid variation in the magnitude of impedance change for highly metastatic cells but not for less metastatic ones. Therefore, the impedance spectra could be used to detect cancer cell migratory properties at the single-cell level. Although longer time is needed for diffusion gradients to develop, the diffusion-based devices have some advantages compared to the flow-based ones: the gradients could be developed with little reagent consumption, and cells are not subjected to any shear stresses.

In the surface micropatterning-based microfluidic gradients, the interaction between the cell and its surrounding ECM serves as a guiding signal for cellular haptotaxis. The surface-bound signals via different pattern shapes and concentration gradients could regulate the integrin-ECM interaction, thereby providing guiding cues for cell migration [48]. Interestingly, when cancer cells were placed in a pattern of two reservoirs that were connected by bridging ratchets, it migrates through the ratchets with a larger lamellopodial protrusion, indicating a strong regulation between cytosolic volume and cytoskeletal organization [49].

1.2.2 Electrical gradients

Cancer cell migration can also be guided by physiological electrical fields that are generated at the boundary between cancerous tissue and surrounding normal tissue [50]. This is known as electrotaxis or galvanotaxis. Microfluidic devices have certain advantages over conventional devices in the study of *in vitro* electrotaxis. The precise microchannel geometry helps in the establishment of a uniform and stable electrical field, reducing the effect of joule heating and increasing the potential for high throughput experiments [51, 52]. The electrotaxis of cancer cell has been studied in a polymethylmethacrylate (PMMA) made microfluidic device [51]. This system could generate electrical fields with three different strengths in a single microfluidic channel. In another study with lung cancer cells, it was observed that the filopodia and EGF receptor were polarized towards the cathode [52]. Therefore, filopodia of cancer cells could sense the electric field in the tumor microenvironment, and this could have a crucial role in metastasis.

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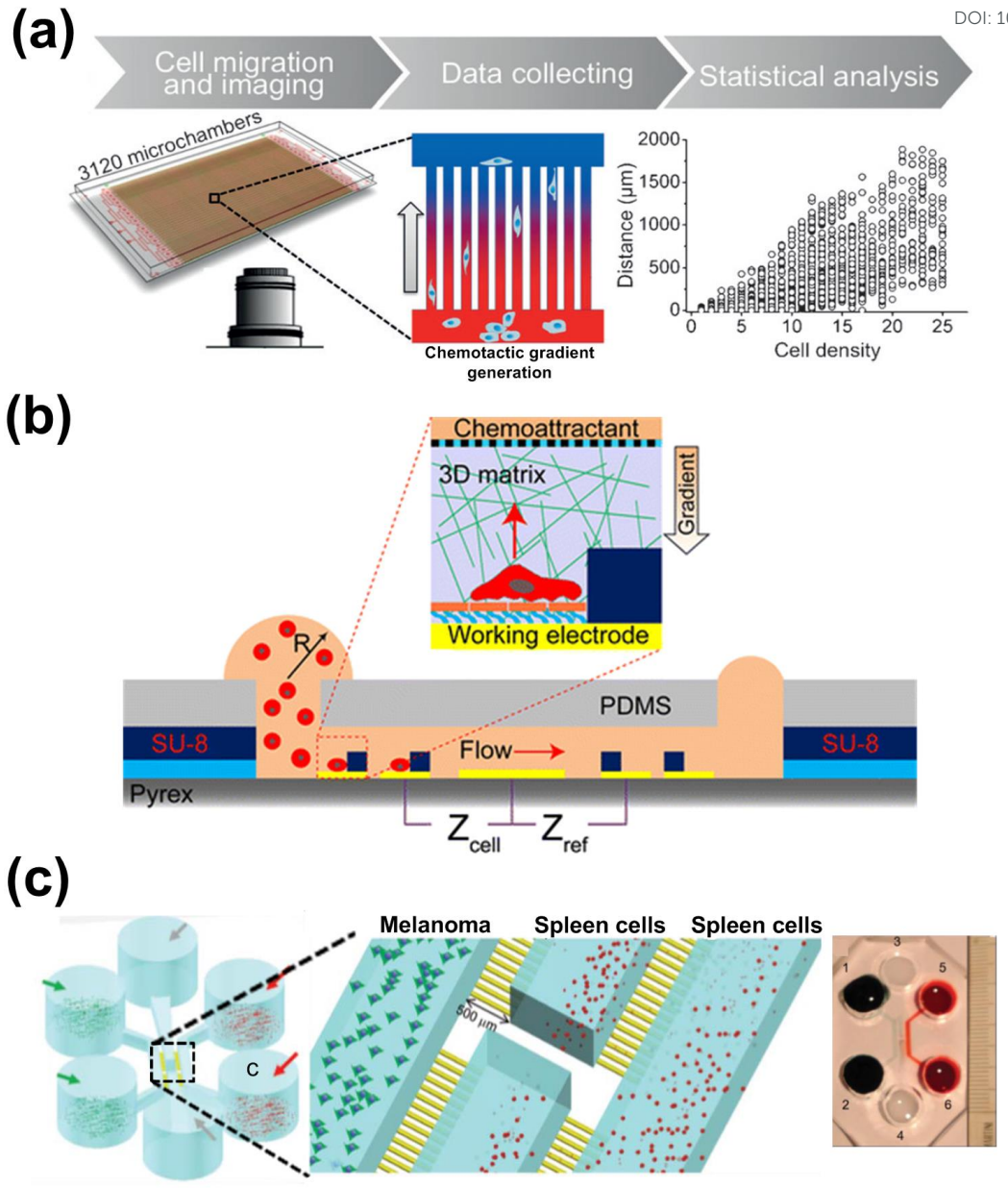

Figure 3. Effects of the gradients in tumor cell migration. **(a)** Schematic representation of the design and experimental workflow of the M-Chip used to investigate the mesenchymal mode of migration. The cancer cells migrated through the narrow channels in presence of chemokine gradients. Cell velocity and percentage of migrating cells were determined for statistical relevance. Reproduced with permission from ref [44]. **(b)** Diagrammatic representations of the design of impedance sensing chip. Single cancer cells could be successfully captured on microelectrode posts and the magnitude of impedance change was monitored during migration in presence of chemoattractant gradient (inset). Reproduced with permission from ref [47] **(c)** Diagrammatic representation of the microfluidic platform used to study the interaction between immune cells and cancer cells. (left) Green arrows indicate melanoma cell reservoir, grey arrow indicates buffer reservoir and red arrow indicates spleen cell reservoir. (middle) Magnified representation of different loading compartments. (right) Melanoma cells are loaded in reservoir 1 and 2 (indicated in black) and spleen cells are loaded in reservoir 5 and 6 (indicated in red). Reservoir 3 and 4 consists of cell culture media. Reproduced with permission from ref [53].

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What's next?

Much progress has been made to use microfluidics to monitor the migratory response of cancer cells in the presence of an efficient gradient comprising biochemical and biophysical factors [42, 45, 51]. However, the tumor microenvironment is far more complex. It would be interesting to understand the effect of various autocrine and paracrine secretions by the stromal cells and the dynamic nature of the ECM on the invasive potential of cancer cells. Microfluidic devices can be fabricated to compartmentalize these different stromal cells like endothelial cells, macrophages in a 3D *in-vivo* like situation and monitor their effect on the migratory behavior of the tumor cells. Furthermore, it would be exciting to observe the migration of the heterogeneous populations of CTCs in 3D. How is the dynamic transition of EMT regulated as the CTCs migrate across the physical and biochemical cues provided by the stromal cells and different ECM conditions in terms of different matrix rigidity and porosity? This may lead to the development of a better system to observe the effect of anti-metastatic drugs with cancer progression. Though a number of established technologies are mentioned here, for a more comprehensive review about cancer cell migration, the reader is referred to these recent reviews in the literature [54, 55].

1.2 Co-culture and cell-cell communication

Tumor microenvironment is a critical component of cancer biology and is responsible for cancer initiation, metastasis and drug resistance [56, 57]. It provides tumor cells with a complex system of non-cancerous cell-cell communication though paracrine secretion and various biophysical and mechanical cues from the ECM [58]. Tumor microenvironment comprises endothelial cells, fibroblasts, inflammatory cells, mesenchymal stem cells (MSCs) and it has been shown that the tumor stromal cells have different genetic profile compared to their normal counterpart [59]. It is difficult and technically challenging to mimic such complex microenvironment *in-vitro*. However, the advancement of microfluidic technology has provided an opportunity to replicate such intricate cell-cell interactions with spatiotemporal biochemical gradients and dynamic biomechanical microenvironments.

The endothelial cells are present in the wall of the blood capillaries and act as a barrier in the process of tumor intravasation, extravasation and angiogenesis. For better understanding of tumor cell adhesion on endothelial cells, Song *et al.* designed a microfluidic device with integrated flow control and endothelial monolayer [60]. They revealed that metastatic breast cancer cell adhesion is enhanced by either basal addition of CXCL12 or by increasing shear stress. Recently, Zhang *et al.* developed a microfluidic device to mimic the process of tumor extravasation *in-vitro* and observed that CXCR4 receptor expressing tumor cells could transmigrate across the endothelial boundary in presence of CXCL12 gradient, thereby indicating the importance of CXCL12/CXCR4 signaling during extravasation [61]. Zervantonakis and colleagues designed a 3D-system with two independent micro-channels for tumor and endothelial cells, and a 3D hydrogel matrix connecting them together [62]. They observed that when the endothelial barrier is disrupted by TNF-α secreted from macrophages, the rate of intravasation increases.

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Tumor stroma consists of a subpopulation of fibroblasts known as cancer associated fibroblasts (CAFs) that are the source of multiple growth factors and ECM remodeling in the tumor microenvironment [63]. Hsu *et al.* studied the molecular mechanism underlying the activation of fibroblast by α-smooth muscle actin (α-SMA) and observed that the activation process could be inhibited by the presence of transforming growth factor beta 1 (TGF-β 1) receptor/ALK5 inhibitor in the fibroblast media, thereby indicating a potential target of cancer therapy [64]. In another study, Sung *et al.* developed a 3D compartmentalized microfluidic device to study the transition to invasive breast cancer phenotype *in-vitro* by co-culturing mammary epithelial cells with fibroblasts [65]. This device provides a low-cost method to screen for the inhibitors of various signaling pathways involved in this transition process. In an intriguing study, Huang and colleagues designed a microfluidic device to pattern different types of cells including macrophages and metastatic breast cancer cells in 3D matrix and observed that macrophages invaded into the adjacent gel containing breast cancer cells but not into the gels without cancer cells [66]. In a recent work, Businaro *et al.* demonstrated the anti-tumor activity of interferon regulatory factor 8 (IRF-8) by co-culturing immune cells and melanoma cells in a microfluidic platform, as demonstrated in **Fig 3(c)** [53]. They observed that melanoma cells acquired a more invasive phenotype and migrated more towards the IRF-8 knock out spleen cells as compared to the wild type cells. Ma *et al.* investigated the role of MSCs in cancer progression using precise cell patterning and stable chemokine gradient in 2D and 3D microenvironments [67]. They observed that MSCs enhanced the invasiveness of cancer cells in the presence of CXCL12 gradient, thereby indicating the importance of MSCs and CXCL12–CXCR4 signaling in cancer progression. The use of bacteria for cancer targeting has emerged as a promising therapeutic tool [68]. However the mechanism and specificity of cancer targeting is not well understood. Recently, Hong *et al.* observed a preferential migration of *Salmonella typhimurium* towards cancer hepatocytes in response to the chemo attractant alpha-fetoprotein (AFP) secreted by the cancer cells [69].

What's next?

In the context of cancer research, it has been shown that there are many types of signals that make up the intercellular communications through physical contact, electrical signal, diffusion of soluble factors and transduction of mechanical cues [70]. We are envisioning that next generation of microfluidics systems will seamlessly identify a specific signal (e.g., via microvesicles) from the vast background of communication information while facilitating the incorporation and analysis of various factors such as cytokines and growth factors within tumor microenvironment. In addition, future advancements will enable live cell imaging of intracellular events such as the activation of signaling pathways within microfluidic systems due to cell-cell interactions.

2. Microfluidics for Applied Cancer Research

2.1 Cancer cell detection and isolation

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In general, a majority of the cancer-related human deaths are due to metastasis in which cancer cells spread to a distant location from the initial site of primary tumor [71]. Primary tumor cells generally intravasate into the circulatory system although some cancer types do this via the lymphatic system. Also, recent studies have suggested that intravasation may also occur at a much earlier stage [72]. These cancer cells in the bloodstream or CTCs are believed to carry relevant information about the main tumor that they come from [73, 74]. In fact, the quantity of CTCs in cancer patients are known to correlate with the stages of cancer as well as the effectiveness of cancer therapies and treatments [75]. As such, the detection of these cancerous cells in the circulatory system of cancer patients is of utmost importance as it allows the detection of cancer at the early stage even before metastasis occurs, and this can lead to more effective treatment of the disease.

One of the eminent applications of microfluidics is cell separation and isolation. In contrast to conventional cell separation methods, microfluidic-based separation techniques possess several advantages including small sample volume, high throughput, sensitivity, and low fabrication cost. In cancer research, microfluidic cell sorting devices have been successfully employed to separate CTCs from cancer patient's blood [5, 76]. In comparison with hematologic cells, CTCs cannot be easily studied because of their rarity (i.e. in the order of 1 to 10's cells per mL of whole blood). Recent studies have shown that CTCs are different from other hematologic cells in terms of their surface molecule expressions [77], size [78], density [79], stiffness [80], adhesion [81], and electrical properties [82]. So microfluidic device can leverage these distinct properties of CTCs to achieve accurate isolation.

2.1.1 CTC isolation using tumor specific markers

Tumor cells originate from an outgrowth of abnormal tissue. Cancer cells from epithelial origin express epithelial cell adhesion molecules (EpCAM) on their surfaces that do not exist on hematologic cells. This enables cancer detection by immunochemical means [77]. For example, the Food and Drug Administraton-approved CellSearch platform uses ferrofluids loaded with EpCAM-coated particles to capture CTCs [83]. In the context of microfluidics, Hoshino *et al.* presented an immunomagnetic CTC detection micro-chip in which cancer cells were captured inside a wide microchannel embedded with high intensity magnetic field more efficiently [84], as illustrated in **Fig 4(a).** A core advantage of magnetophoretic separation over CellSearch platform is its continuous operation and higher throughput. IsoFlux^{TM} is a newly developed microfluidic platform by Fluxion Biosciences that implemented microfluidic channels to expose CTCs tagged with magnetic particles to intensive and highly uniform magnetic fields for efficient cell recovery [85].

A diverse range of microfluidic systems with geometrically enhanced microstructures have been developed recently for efficient CTC sorting. One example is the CTC-chip developed by Nagrath *et al.*, which uses EpCAM coated silicon microposts to capture CTCs from whole blood [86]. Another one is a geometrically enhanced differential immunocapture (GEDI) chip developed by Kirby *et al.*, which specifically improved the collision frequency between target

cells to antibody coated microposts, thereby enhancing CTC enrichment [87]. One commercial example of such systems is the CEE^{TM} microfluidic chip developed by Biocept, which utilizes biotin-streptavidin binding for CTC capture. In this platform, the randomly positioned microposts which are coated with streptavidin are used to capture biotinylated antibodies tagged CTCs [88]. Moreover, nanostructures such as silicon nanowires, nanoclusters and nanobeads have emerged as a promising CTC detection platform [89, 90]. The efficiency of CTC enrichment could be further improved by combining affinity interactions with nanostructured surfaces. Aptamer functionalized nanotextured substrates that mimic nanostructures of the basement membrane *in vivo*, were used for CTC separation from patients' blood [91]. Bichsel *et al.* developed a microfluidic platform to isolate and culture cancer cells from spiked blood in 3D hydrogel matrix [92]. They observed a clonal expansion to 3D spheroids after 6 days of culture *in vitro*.

2.1.2 CTC isolation using biophysical markers

Biophysical marker-based approaches exploit other signatures of CTCs such as cell size, stiffness, density, electrical and magnetic properties to separate them from normal blood cells [93, 94]. Emerging label-free separation methods have the potential to address the key shortcoming of affinity-based approaches (i.e., loss of EpCAM-negative cancer cells and high cost) and present greater flexibility in the subsequent characterizations of these cells such as fluorescence in situ hybridization (FISH) or post-isolation culture [95]. Size-based separation of CTCs has been widely employed by various researchers as an inexpensive and quick method for many years. ISET® (Rarecells, Paris, France) and ScreenCell® are two such commercial systems for the cheap and rapid enrichment of CTCs [96]. The size-based membrane filters are track-etched isoporous membranes with uniform pore sizes (6-10 μm diameter holes) that allow blood components to flow through but not the larger CTCs [97, 98]. For example, Hosokawa *et al.* developed a size-selective microcavity array for separating CTCs based on the differences in size and deformability between the cancer cells and hematologic cells (**Fig 4(b)**) [99]. In addition to EpCAM positive cells, they could successfully isolate EpCAM negative cells that cannot be captured by biochemical marker based techniques.

Size-based isolation of cancer cells can also be performed using a combination of laminar flow in microchannels with precisely controlled microstructures [94]. For example, our group used an array of crescent-shaped pillar traps to isolate CTCs from whole blood [100]. Lim *et al.* employed isopore microfabricated filters to build a lab-chip device for highly efficient and high-throughput detection of CTCs [101]. Chung and co-workers recently designed a microfluidic cell sorter with the weir-shaped barrier to separate CTCs from the whole blood in a continuous manner [102]. In an interesting study, Kim and colleagues employed anti-EpCAM conjugated microbeads to further increase the size of CTCs to enable better discrimination against leukocytes during filtration [91]. Using 5 μm microbeads, they could increase the average diameter of breast cancer cell, MCF-7 from 16.6 μm to 26.5 μm, thus allowing efficient separation with minimum WBCs contamination. Deterministic lateral displacement (DLD) is another promising micropost design to isolate CTCs from blood. Recently, Loutherback *et al.* developed a novel DLD system with triangular microposts,

which can sustain higher flow rate and avoid clogging compared to conventional circular microposts, for efficient CTC separation (~ 85%) at a flow rate of 10 mL/min [103].

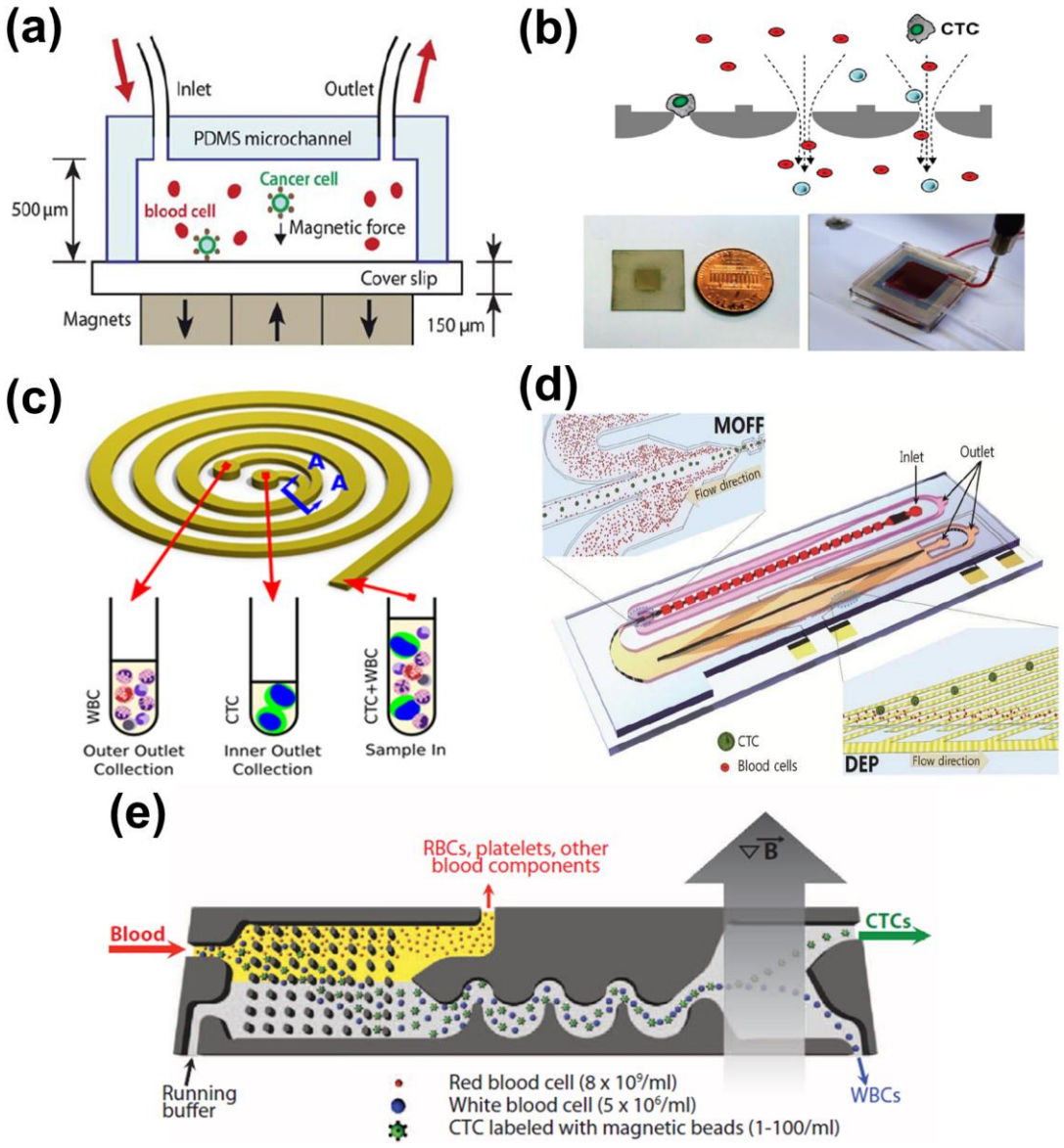

Figure 4. (a) Schematic illustrating the operation of immunomagnetic cancer cell selection where CTCs in blood are attached with immunomagnetic beads and arrested inside the magnetic field as the patient blood flows through the device. Reproduced with permission from ref [84]. **(b)** Diagrammatic representation of the size-selective microcavity array used for separating CTCs from other blood cells. The designs of the micro-cavity are optimized to capture the CTCs while the blood cells can pass through. Reproduced with permission from ref [99]. **(c)** Slanted spiral microfluidic device for high throughput CTC separation. Normal hematologic cells were trapped at the deeper channel region near the outer wall whereas CTCs were focused near the inner wall. Reproduced with permission from ref [104]. **(d)** Schematic of a hybrid microfluidic device combining hydrodynamic separation with DEP. CTCs were partially purified at the concentration/expansion section and then sent to the DEP zone for further purification. Reproduced with permission from ref [105]. **(e)** Device schematics showing the operating principles of CTC-iChip. RBCs and platelets could be removed in the

first stage using DLD size sorting and then immunomagnetic-labeled CTCs or WBCs could be enriched or depleted at the subsequent stage by means of positive or negative selection respectively. Reproduced with permission from [106].

Among label-free approaches, inertial microfluidics has been attracting much interest recently in cell biology for high-throughput, label-free CTC sorting [107, 108]. In 2011, our group introduced a high-throughput separation method using inertial focusing microfluidics [109]. This technique combines cell focusing in high aspect-ratio straight microchannels with pinched flow dynamics for isolating CTCs with throughput of > 10^8 cells/min. Later, Sollier *et al.* and Hur *et al.* combined inertial microfluidics with micro vortex cell trapping to create a high throughput system to entrap CTCs [110, 111]. In addition to the straight microchannel, spiral channel could also be used to perform inertial separation using combination of inertial and Dean drag forces. Recently, we have reported a spiral microfluidic device with rectangular cross section that is capable of processing blood with hematocrit level of ~20-25% and with processing speed of 3 mL/hr for isolating CTCs through a two-stage cascaded system [112]. The larger CTCs focused tightly near the inner wall due to strong inertial lift forces while the smaller blood cells travelled back towards the outer wall. Steric crowding is minimized as the smaller blood cells are not focused, hence enhancing the separation efficiency of the device. Due to the large microchannel dimensions and higher flow rate, clogging was also reduced, making the performance of this device superior to other reported CTC capture microfluidic devices. More recently, we have discovered that by employing trapezoidal cross-section microchannels instead of conventional rectangular one, we can modify the shape of velocity profile and generate stronger Dean vortices near the outer wall, thus enhancing the separation efficiency and throughput [104]. By incorporating a trapezoidal cross-section spiral microfluidic chip (**Fig. 4(c)**), we have reported ultra-high throughput separation of CTCs (7.5 mL in < 10 min) from blood of patients with metastatic breast or lung cancer and minimal effect on the viability of the CTCs.

Dielectrophoresis (DEP) has also been proposed as an alternative approach for isolation of CTCs based on their dielectric properties [113-115]. In a non-uniform electrical field, particles like cells experience different DEP forces according to the particle size and dielectric polarizability. Thus, cells of different physiological states can be separated using this label-free approach [93]. The DEP force is usually classified by force direction as positive DEP referring to the force towards the increasing electrical field and negative DEP referring to the force towards the decreasing electrical field. Wang *et al.* developed a microfluidic device with embedded comb shape electrodes (called dielectrophoretic field-flow-fractionation (DEP-FFF)) and employed it for separation of breast cancer cells from a mixture of cells in a solution [116]. Moon and colleagues recently designed a hybrid microfluidic device combining dielectrophoresis with hydrodynamic separation, as illustrated in **Fig. 4(d)** [105]. Normal blood cells and CTCs flow through a multi-orifice microfluidic structure at the first separation stage in which most of the blood cells were removed due to size difference. The remaining cells then entered the DEP separation region for further purification. For a more comprehensive coverage of other CTC isolation techniques, readers can refer to other recent reviews [117, 118].

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Combination of various techniques could be applied to build up a hybrid system for better separation performance. In a recent report, a hybrid microfluidic chip (CTC-iChip) combining DLD with inertial focusing and magnetophoresis was developed to isolate CTCs as shown in **Fig. 4(e)** [106]. The fabricated device was capable of processing whole blood at a flow rate of 8 ml/hr in antigen-dependent and antigen-independent modes. In another study, Liu *et al.* combined DLD and affinity-based technique to isolate breast cancer cells with 90% efficiency at flow rate of 9.6 mL/min [119]. The DLD chamber consisted of triangular microposts to trap the CTCs while blood cells were carried along the streamlines. The capture chamber was functionalized with EpCAM and overlaid with a fishbone structure to boost CTC isolation.

What's next?

In the last one decade, isolation and characterization of CTCs has received enormous attention and has resulted in a wide range of commercial and laboratory technologies (**Table 1**). Although CTCs are currently used in numerous clinical trials (i.e., over 520 registered clinical trials) [120], a standard protocol for CTC detection is still not available, making direct comparison of the reported results for different methods a challenging task [121]. Therefore, developing a universal protocol to evaluate the performance of the existing and forthcoming techniques is equally important, but is still missing [120]. We are envisioning that development of hybrid systems that take advantages of both affinity and label-free techniques can overcome shortcoming of individual approaches for clinical purposes. Additionally, greater knowledge into the biology of CTCs, which can be obtained through single-cell analysis (see section 2.2), will enhance CTC assay development. In the future, by leveraging on advantages of lab-on-chip systems, clinicians can think of a fully integrated analyser that can detect cancer, its stage and the therapeutic targets via a simple blood test. Comprehensive review articles covering microfluidic-based CTC technologies were recently published [118, 122-124].

Table 1. Summary of various microfluidics approaches reported for CTC enrichment and detection.

Microfluidic technologies		Device features	Isolation efficiency	Isolation purity	Detection limit	Processing speed	Cancer type tested	Ref
Biochemical marker-based methods	Functionalized magneticpartide	Magnetic nanoparticle conjugated with anti-EpCAM coating, PDMS microchip on top of magnets	>85%	-	5 CTCs/mL	10mL/hr	Cancer cell lines COLO205, SKBR3 and PC3, spiked in healthy blood sample	125
		CTC iChip: combination of size-based sorting, inertial focusing and magnetic bead based sorting.	>75%	7.8% (+ mode: 1500 WBCs/mL; - mode: 32000 WBCs/mL)	0.5 CTC/mL	0.9mL/hr	Cancer cell lines SKBR3, PC3, MDA-MB-231, and MCF10A. Blood from melanoma, prostate, pancreatic and breast cancer patients	106
	Functionalized micro/nano structure	GEDI device: micropost coated with J591 monoclonal antibody in PDMS channel	~90%	68%	-	1mL/hr	Cancer cell line PC3, LNCaPB and C4-2. Blood from patients with metastatic prostate cancer	87
		Herringbone chip: PDMS channel with anti-EpCAM antibodies coated herringbones structure	90%	86%	10 CTCs/mL	1.2mL/hr	Cancer cell line PC3 and blood sample from 15 patients with metastatic prostate cancer	126
		Graphene oxide nanosheet functionalized with anti-EpCAM antibodies and adsorbed on patterned gold substrates	>80%	-	-	3mL/hr	Cancer cell line MCF7, HS578T and PC3 and blood sample from breast, lung, pancreatic cancer	127
		Aptamers against over-expressed Epidermal Growth Factor Receptors (EGFR) on nano-textured PDMS channel	-	85%	-	1mL/hr	Human glioblastoma (hGBM) cells	128
Physical maker-based methods	Physical barrier-based	Membranes with 6~8μm large pores	>74%	-	2 CTCs/mL	-	Cancer cell lines H2030, H1975 and HT29	96
		Arrays of crescent-shaped pillar structures with 5μm gap	>80%	>80%	1 CTC/mL	0.7mL/hr	Cancer cell lines MCF7, MDA-MB-231 and HT29.	100
		Size-gradient filter glass chip: polymer beads conjugated with anti-EpCAM antibodies.	90%	Average 350 WBCs/mL	10 CTCs/mL	1.2mL/hr	Cancer cell lines MCF7 and MDA-MB-231	91
		Weir-based barrier with gap 10μm	>90%	-	-	20mL/hr	Cancer cell lines A431 and SKBR3	102
	Hydrodynamic based	Inertial focusing channel followed by expansion-contraction micro vortices reservoir trap	36.80%	84%	-	>20mL/hr	Cancer cell lines MCF7, OVCAR5, M395, PC3 and A549	110
		Inertial focusing channel coupled with pinched flow dynamics	>80%	<850 WBCs/mL	-	>20mL/hr	Cancer cell lines MCF7 and MDA-MB-231.	109
		3-D slanted spiral microchannels	80%	1200 WBCs/mL	-	25mL/hr	Cancer cell lines and patient samples	104
	Dielectrophoresis	Combination of multi-orifice flow fractionation and dielectrophetic cell separation within a PDMS device	75%	0.30%	-	7.5mL/hr	Cancer cell line MCF7	105

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2.2 Microfluidics and single-cell cytomics

Cancer is a heterogeneous disease with variations among the cells in the tumor. Due to this heterogeneity, there is a deviation in the response of tumor cells to anticancer drugs as illustrated in **Fig. 5(a)** [129]. Thus, there is a need to track the characteristics of individual cells within a subpopulation. Single cell analysis (SCA) has the ability to recognize the individualistic character and behavior of these cells and thereby, enabling personalized treatment. Among conventional approaches for single-cell analysis, microscopic imaging is the most common method and has been used extensively in cancer research. However, high-throughput assays on single cancer cells are technically challenging. Flow Cytometry approaches such as Fluorescence Activated Cell Sorting (FACS) has been the "gold standard" in cancer biology for many years; however, as data are acquired at a particular time point, these techniques hinders dynamic monitoring of cellular response [130]. Therefore, development of high throughput single cell analysis technology remains the key challenge to realize this aim. In this context, microfluidic devices has developed as a exciting prospect to address these challenges [130, 131].

2.2.1 Droplet microfluidics

Droplet microfluidics, as a subset of microfluidics, has emerged as a promising approach for single cell encapsulation and monitoring of enzymatic activities at the single-cell level [132]. Compartmentalization of single cells into individual droplets within a continuous phase of oil allows chemical isolation of each cell, thus facilitating the analysis of cells without cross contamination or loss of genetic materials [133]. For example, Brouzes *et al.* developed a high throughput droplet-based assay for cytotoxicity screening of mammalian cells to either assess drug cytotoxicity or to screen cancer cells for therapeutic targets via synthetic lethality [134]. In another study, Linfen and colleagues introduced a microfluidic system for alginate droplet formation and on-chip tumor cell culture [135]. Using this platform, they have shown the dose-dependent cytotoxic effect of doxorubicin on trapped cells where a notable decline in the cells' viability was observed by increasing the concentration of the doxorubicin. Chen *et al.* recently introduced an integrated microfluidic device comprising of a biomolecule concentrator and a micro-droplet generator to detect enzyme activity (e.g., matrix metalloproteinases (MMPs)) with greater sensitivity [136]. In a recent study, our group incorporated inertial and droplet microfluidics to measure the activity of receptor tyrosine kinases (RTKs) in lung cancer cells triggered by cell surface ligand binding (**see Fig. 5(b)**). The results revealed a heterogeneous response in single cells and indicated varied levels of drug resistance when treated with Giftinib, which is a tyrosine kinase inhibitor [137].

2.2.2 Microwells and microtraps

Another innovative way to study single cells in a high-throughput manner is to encase them inside microwells or traps. A great advantage of microwell techniques is that simultaneous analysis of large number of living cells can be done on a single chip [138, 139]. A relatively straightforward solution to trap cells using microwell arrays where cells are seeded and

allowed to settle into individual compartments that can hold a single cell in each location. Using this concept, Rettig and Folch developed a system for high throughput screening of fibroblasts and rat basophilic leukemia (RBL) cells with close to 92% single RBL cell occupancy [140].

Another common approach for trapping cells is to create side channels in a main transport channel, where the side channel dimensions are biased to trap only single cells by suction when a fraction of the total flow is aspirated this way [141]. Using this, Valero and colleagues developed a microfluidic device for immobilization of cancer cells (HL60) and real-time monitoring of the apoptotic process using electric field mediated or chemically induced stimuli [142]. Di Carlo *et al.* [139] also proposed another mechanism for single cell trapping using hydrodynamic approach, where trapping post arrays (U-shaped structures) were arranged in slanted rows in a flow-through chamber with identical size similar to single cells, as shown in **Fig. 5(c)**. They have studied the intracellular carboxylesterase kinetics and concentration in HeLa, Jurkat and T293 cell lines using fluorescence imaging. The same platform was also used to perform an intracellular inhibition assay on the HeLa cancer cells by perfusing the trapped cells with NDGA, resulting in a 60% inhibition of the carboxylesterases [143].

2.2.3 Other techniques

In addition to the aforementioned approaches for single-cell analysis, which are typically high-throughput and low-cost, a myriad of alternative microfluidics techniques employing optical, electrical and other mechanisms has been developed. For example, a diverse set of microfluidics devices with integrated electrodes has been emerged, which utilizes dielectrophoresis (DEP) to create spatially defined cell arrays [141]. Integrating optics with microfluidic platforms, called 'Optofluidics' has been emerged also as an alternative approach for precise single-cell analysis. Surface acoustic wave (SAW) microfluidic devices can be used also for non-contact trapping of cells or microparticles. Despite great advantages that these platforms offers such as a stable immobilization of flowing cells, biocompatibility and accurate cell manipulation; some major obstacles such as cost, complicated optical alignments, tuning of the media conductivity and potential damages to the cell fate prevented their prevalent usage [144]. One can refer to the recent review articles for more detailed information on research in these fields [145-147].

What's next?

Over the past decade, the advent of various microfluidics techniques has enabled scientists to do high throughput screening of cancer cells (i.e., drug discovery) analysis that are challenging by conventional means. Despite great achievements in this field, the realization of effective and automated systems for pretreatment or fractionation of complex samples (e.g., dissociation of tissue samples into cellular components) still remains a challenge. With rapid advancements in the field of Micro Total Analysis Systems (μTAS), we are envisaging future integrated systems with ability to obtain precise biochemical and mechanistic data from individual cells, thus assisting further for the personalized medicine [148].

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2.3 Microfluidics and OMICs

2.3.1 Genomics and transcriptomics

The genome of an organism contains the blueprint of all cellular and physiological activities. In most cancers, the genome is quite unstable, ranging from a few point mutations to extensive aneuploidies [149]. Therefore, single cell genomic and/or transcriptomics studies could provide an idea of the cellular functions and thereby, predicting the state of the disease. In contrast to the traditional approaches for genomic analysis, the recent microfluidic-based platforms provide a promising tool with reduced cost and higher throughput.

The recent development of multilayer soft-lithography by Stephen Quake and other groups [150, 151] has increased control of fluids and cells in the integrated microfluidic devices [152]. The realization of pneumatically controlled microvalves with low dead volumes further enabled the development of fully automated systems such as the C1™ Single-Cell Auto Prep System by Fluidigm [153]. This technology has been used in many applications, such as a recent work on identifying the gene-expression profile of colon cancer stem cells [154]. In a pioneering work, Powell and colleagues performed a microfluidic-based single cell transcriptional analysis of CTCs isolated through an immunomagnetic-based enrichment device [155]. Compared to cancer cell lines, they observed that CTCs have a greater transcript level expression of genes associated with metastasis and EMT phenomenon. In another study using single cell microfluidics-based RT-PCR, Chen *et al.* analyzed the gene expression profile of 84 EMT-related genes in the CTCs from prostate cancer patient [156]. They observed an increased expression of a subgroup of EMT-associated genes (e.g., PTPRN2, ALDH1, ESR2, and WNT5A) in the CTCs of castration-resistant cancer. This unique expression characteristic of a subgroup of EMT-associated genes in the CTCs from prostate cancer could provide an opportunity to monitor the response of chemotherapeutic agents with disease progression.

2.3.2 Proteomics

In comparison to genomic analysis, single cell protein measurement represents another level of difficulty. Proteomic studies allow us to understand the post-translational modification of the proteins that might not be detected by the analysis of gene expression. Fang and colleagues designed an integrated microfluidic system for *in-vitro* kinase activity assay that can measure enzymatic activity from minute amount of clinical samples [157]. This device has potential application to monitor kinase activity from small amount of clinical specimens like blood, bone marrow and biopsy samples. Using a single channel multistage immunoblotting microfluidic device, Hughes *et al.* identified prostate-specific antigen (PSA) in cellular lysates and serum samples obtained from metastatic prostate cancer patients [158]. All the immunoblotting steps could be performed within the device, as shown in **Fig. 5(d)**. For a better understanding of single cell functional heterogeneity, Ma and co-workers developed a single cell barcode chip to quantitatively determine the amount of secreted proteins from single cell [159]. In another study, Fan *et al.* developed an integrated blood barcode chip (IBBC) that could rapidly and quantitatively measure plasma proteins from microliters of blood from

cancer patients [160]. This chip could detect large number of protein biomarkers within 10 minutes time and thus provide a non-invasive, low cost, robust device for clinical diagnostics. A Microfluidic Antibody Capture chip (MAC chip) has been introduced recently which can quantitatively estimate the changes in copy number of proteins from a single cell [161, 162]. Using this chip, Salehi *et al.* reported successful identification of tumor suppressor protein, p53, in single cancer cells with 88% precision [161].

2.3.3 Metabolomics

The most sensitive response of a cell to various changes in the microenvironmental condition occurs at the metabolic level [163]. Recent studies showed that the isogenic population of cells displays a heterogeneous metabolic phenotype because the cells are at different physiological states [164, 165]. Therefore, metabolomics represents a biochemical snapshot of physiological and pathological states by simultaneously observing many macro and micromolecules as well as monitoring the functions of many cellular pathways. PDMS-based microfluidic devices were used for the exposure of breast cancer cells to a concentration gradient of As_2O_3 and N-acetyl-cysteine for glutathione modulation [166]. The gradient-dependent effects of the glutathione-induced activity on the chemotherapeutic sensitivity to Adriamycin were observed. Amantonico and colleagues also recently combined microfluidics with mass spectroscopy for continuous deposition and metabolic analysis of ADP, ATP, GTP, and UDP-Glucose at the single-cell level [167].

What's next?

Single cell analysis can be a powerful tool in developing personalized treatment in clinic. The genetic mutations associated with the primary tumor and the CTCs of a cancer patient can be analyzed in a high-throughput way and chemotherapeutic drugs can be targeted against the particular signaling pathways in a more efficient manner. Microfluidic devices can be developed that integrate the sorting of the cancer cells from the body fluid and subsequently analyze the genetic copy-number variations at a single cell resolution in a cost effective way. Most of the CTCs that successfully arrive in their respective secondary locations enter dormancy in the new microenvironment and do not colonize [168]. Among other factors, the metabolic activity of the individual CTCs will determine whether they will initiate micrometastasis formation. Improved microfluidic devices are required to monitor the metabolic state of individual CTCs and correlate them with the probability of metastasis initiation in patients.

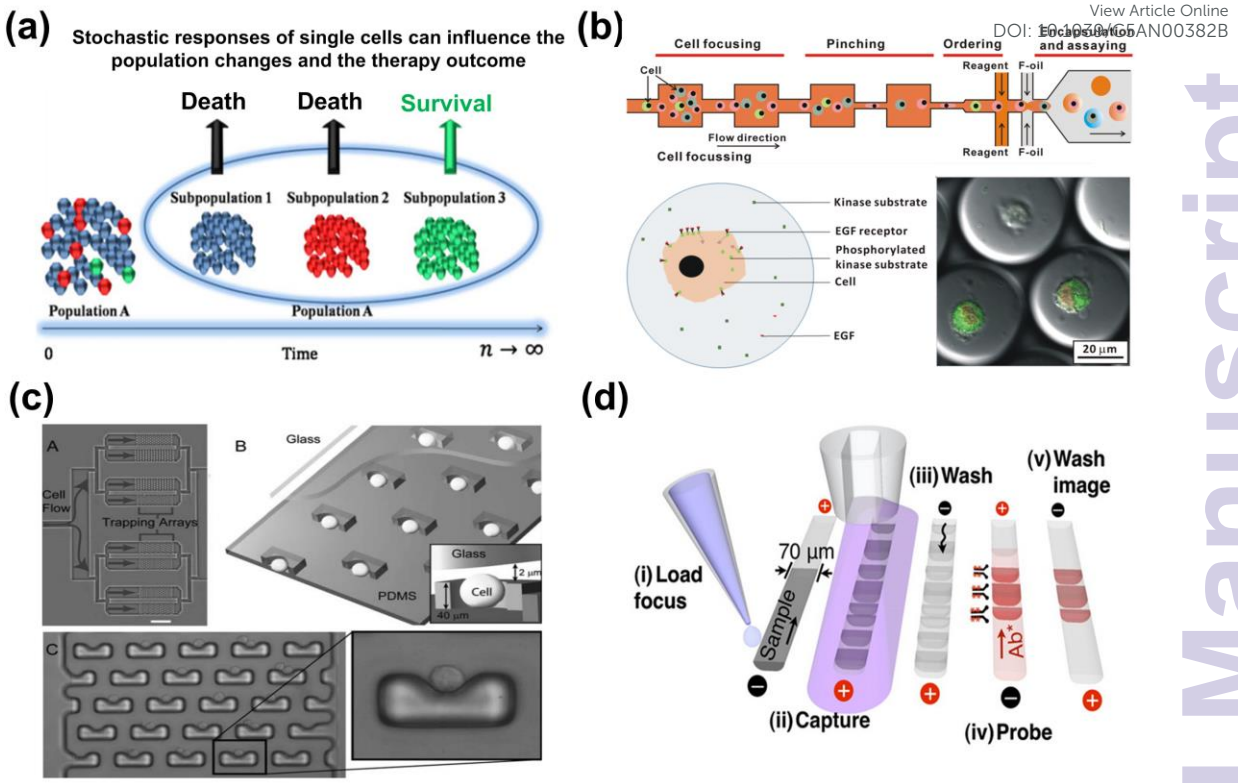

Figure 5. Microfluidics for single cell analysis. **(a)** Responses of cancer cells to therapeutic agents. Colors (blue, red, green) represent three different subpopulations of cells. During test, three different phenotypes were formed. While subpopulation 1 dies immediately in response to the drug and subpopulation 2 dies after some delays. However, subpopulation 3 gains some resistance against the drug that helps them to survive even in the presence of drug. Reproduced with permission from ref. [129]. **(b)** *Top*: Schematic representation of the microfluidic device designed for single cell encapsulation. Cells initially flowing through the cell-focusing channel (90 and 30 µm wide) in which the cellular clumps are separated and then enter to the cell-pinching region (90 and 12 µm wide), which aids in cell ordering before they reach the droplet-forming region. *Bottom*: Schematic representation of single cell kinase signaling and also few PC9 cells in droplets. The cells were labeled with a nuclear stain DRAQ5 (red). The cells are triggered using unlabeled EGF and indicate the phosphorylation of the kinase substrate (green) accumulated inside the cell. Reproduced with permission from ref [137]. **(c)** Hydrodynamic trapping of a individual cell using microfluidic ports that allow changing of solutions. Suspended cells inside the media enter the device and enter the individual chambers where they get trapped. Reproduced with permission from ref [139]. **(d)** Analysis of protein isoform using the microfluidic immunoblotting device. All immunoblotting steps were completed in a single micro channel in 80 mins. Reproduced with permission from ref. [158].

3. Tumor-on-a-Chip: Developing an *in-vitro* disease model using microfluidics

One of the main challenges in current oncology research is the need to develop improved experimental systems that mimics the complexity of the tumor microenvironment in a physiologically relevant manner. Tumor microenvironment is characterized by high levels of spatiotemporal complexity and heterogeneity with dynamically evolving molecular

ecosystem that impact on drug delivery, therapeutic response, and tumor progression [169]. In earlier years, researchers relied on monolayer cell culture and animal models for drug development and screening [170, 171]. However, both these systems have some limitations: monolayer cell culture lacks the tissue complexity as well as controlled flow conditions whereas animal models are expensive and have limited resolution for imaging techniques. Recent developments in tissue engineering, microfluidics and biomaterials along with incorporation of advanced 3D printing technologies have led to growth of micro-scale functional units referred to as 'organ-on-a-chips'[172, 173]. These biomimetic microfluidic systems can precisely mimic physiological conditions and also permit simultaneous regulation of chemical/electrical gradients, shear stresses, and cellular microenvironment within the system [174, 175].

With the recent development of tumor-on-a-chip technologies, it is possible to mimic the complex cellular functions in 3D microenvironment under dynamic flow conditions [169]. Tumor-on-a-chip devices have the potential to develop better *in-vitro* disease model systems with greater significance to human tumor heterogeneity and organ scale complexity. In 2008, Walsh and colleagues developed one of the first microfluidic devices that could mimic the microenvironment gradients present in tumors. They used time-lapse fluorescent microscopy to measure the growth rate of tumor cells within micron-scale chambers and monitor the viable, apoptotic, and acidic regions of the tumor in real-time similar to in vivo conditions [176]. Later on, Sung and Schuler developed more sophisticated microfluidic platform with 3D hydrogel cell cultures to test the cytotoxic effects of anti-cancer drugs while mimicking multi-organ interactions (**See Fig. 6(a)**) [177]. They have employed colon cancer cell line (HCT-116), hepatoma cell line (HepG2/C3A) and Myeloblast cell line (Kasumi-1) to culture the tumour, colon and marrow compartments, respectively. Through a systematic study, the toxicity of Tegafur, an oral an anti-cancer drug, on tumor cells, liver cells, and myeloblasts were assessed. Recently, Albanese *et al.* developed a tumor-on-a-chip microfluidics device to understand the trafficking of synthetic nanoparticles through 3D tissue architecture under controlled flow conditions, as shown in **Figure 6(b)** [178]. They observed that the nanoparticles mostly accumulate in the tissue periphery and the penetration efficiency could be improved by selective receptor targeting. They confirmed their observations in a murine xenograft model and demonstrated that tumor-on-a-chip device could be used for *in-vitro* screening of optimal nanoparticle dimensions before in-vivo studies. Vidi and colleagues in an intriguing study also developed a disease-on-a-chip model which mimics portions of mammary ducts and growth of cancer cells within phenotypically normal breast luminal epithelium [179]. They observed that morphology of grown cells within these hemichannels is different with those cultured on flat surfaces. This platform can eventually provide a framework for the design and test of anticancer therapies.

The research on cancer drugs demands fast, cost-effective, and reliable screening methods to test new drug candidates. Conventional macro-scale screening technologies, however, are usually time consuming, expensive and rely on large volumes of sample and reagents. Recent development in microfluidic technology brings hope to the laborious cancer drug screening process. The miniaturized drug screening systems are capable of handling small volume of

samples and reagents, obtaining faster cellular responses, and high-throughput processing through simple multiplexing of microdevices [180, 181]. The advances of microfluidics for cancer drug screening are particularly suitable for point-of-care (POC) testing and customized cancer therapeutics.

Increasing number of microfluidic devices have been applied to study the responses of cancer cells against different drugs and various dosages. These are promising approaches to optimize the effect of chemotherapy prior to drug prescription. For instance, Siyan *et al.* developed a microfluidic gradient generation system to study the drug resistance of human lung cancer cells [182]. They observed the crucial role of Glucose Regulated Protein-78 (GRP78) in developing chemotherapeutic resistance against anti-cancer drugs. Ye *et al.* introduced an integrated microfluidic device capable of high content drug screening [183]. By developing drug gradient with multiple gradient generators, the responses of cancer cells to different concentrations of anticancer drug could be accurately monitored inside the microfluidic chip. In vitro microfluidic technology could be incorporated with tissue engineering practices to mimic the *in vivo* responses of the cancer cells to various drugs. For example, Yu *et al.* developed a droplet-based microfluidic system to form tumor cell spheroids via an on-chip culture and subsequently tested the cancer drugs [184]. They discovered that tumor spheroids possessed a higher viability as compared to the cells in the conventional monolayer culture.

What's next?

Microfluidic technologies provide an excellent platform to replicate the *in-vivo* complexity of the tumor microenvironment using microliter amounts of chemical agents in the presence of dynamic flow conditions. Microfluidic devices can be compartmentalized to culture different cell types, mimic organs and evaluate complex interactions between the tumor cells and the neighboring stromal cells in a physiological relevant way. It is expected that such tumor-on-a-chip designs can be further developed to analyze patient tumor biopsies and predict therapeutic outcome of chemotherapeutic agents. In near future, we anticipate further development of tumor-on-a-chip platforms to perform basic research, to increase the efficacy and throughput of drug discovery, and to translate the knowledge gained from *in-vitro* studies to clinical settings.

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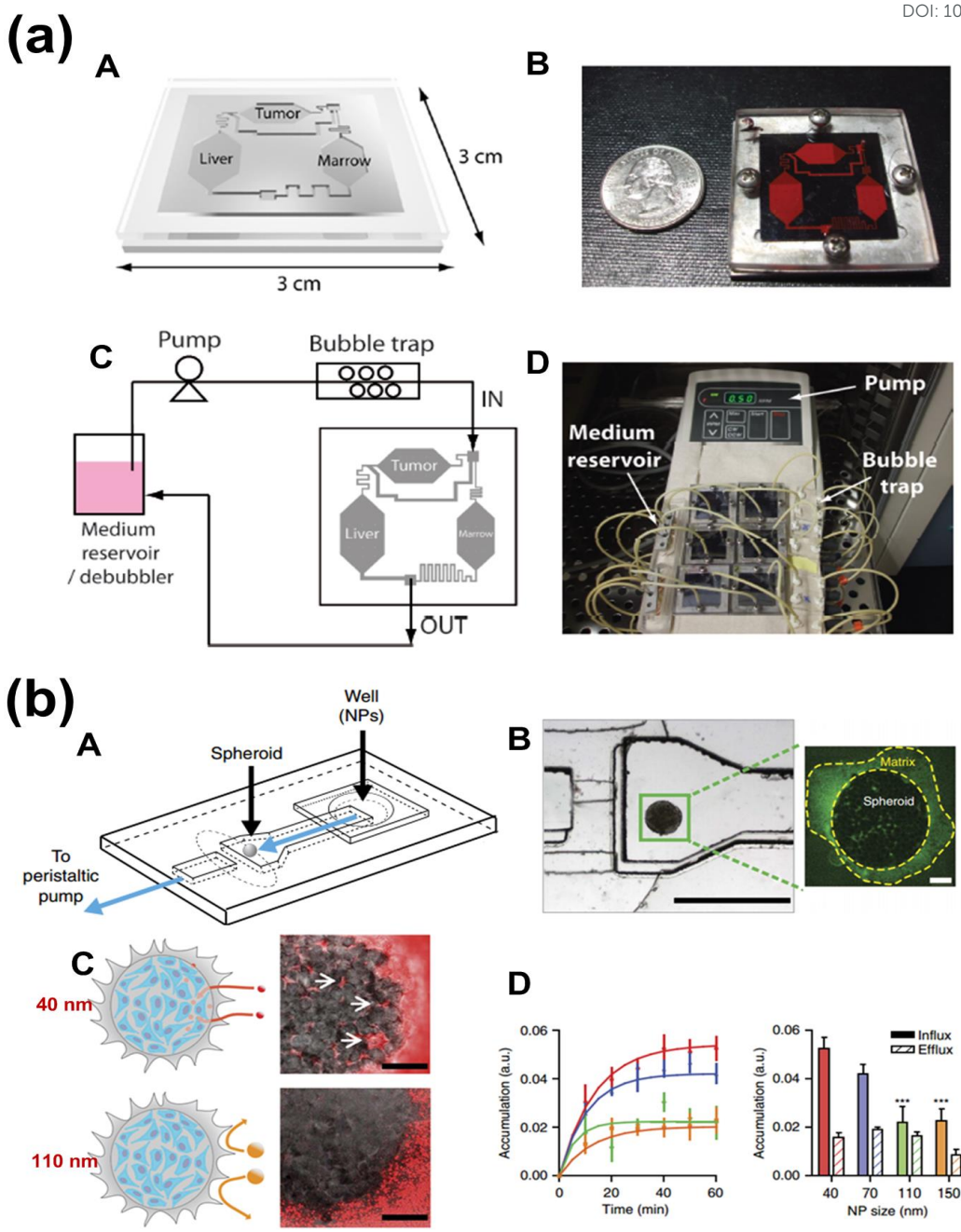

Figure 6. Tumor-on-a-chip systems. **(a)** A microfluidic chip consisting of liver, tumor and marrow compartments, interconnected with channels that mimic the blood flow pattern in the human body (A); the actual fabricated device (B); schematic diagram of operation setup of a single system with medium recirculation (C); and picture of a working prototype with multiple chips (D). Reproduced with permission from [177]. **(b)** Schematic of a microfluidic device designed for growth and analysis of tumor spheroids on a chip (A), optical picture of the spheroid chamber with a spheroid stained with anti-Laminin-FITC (B); schematic and image of the 40 nm fluorescent nanoparticles entering (*top*) the spheroid and accumulating in the interstitial spaces, and schematic and image of the 110 nm (*bottom*) fluorescent nanoparticles being excluded from the spheroid (C); and tissue accumulation of different sized nanoparticles administered to the spheroid (D). Reproduced with permission from [178].

4. Conclusions and Future Perspectives

Here, we reviewed recent progress made in the development of microfluidic-based platforms for both basic and applied research in cancer. As outlined in the review, microfluidic tools are now beginning to be intensively developed and used for cancer research. Nevertheless, despite all the progress made, several challenges still need to be overcome. There is a need for standardized and user-friendly microfluidic-based platforms that can integrate various biophysical cues of the tumor microenvironment (e.g., matrix rigidity, elasticity, and topography) with the biochemical factors secreted from the heterogeneous subpopulation of the stromal cells in a physiologically relevant manner. This can lead to better understanding of the effect of microenvironmental cues on the migratory behavior and mechanical properties of the cancer cells. Efforts need to be stepped up in developing new strategies to mimic the cancer stem cell niche and to provide real-time high-throughput assays for the analysis of multiple parameters at a single cell level. In fact, the use of microfluidic technology for single cell analysis has rapidly gained traction recently [185]. Further developments in this field, such as significant increase in the microfluidic device throughput, sensitivity, and resolution; enhancement of the device capabilities; and integration of the different device functionalities to generate novel single cell-based assays, are expected. At the same time, it is anticipated that the long-term tracking and monitoring of cancerous cellular activities at the single cell resolution can be achieved in the near future provided that the long-term incubation or prolonged growth of cells in microfluidic devices [92, 186].

In the case of CTC enrichment to detect or isolate CTCs from patient's blood, it is still a great challenge given the CTC heterogeneity, variable expression of markers (e.g., downregulation of EpCAM during EMT), and reduced ability for survival through multiple purification steps. There is clearly a lack of an integrative approach for microfluidics to perform more complex functions. For example, one might think of a fully integrated drug screening device which comprises a separation section where cancer cells were initially separated and then transferred to a culture channel to refresh the culture medium and subsequently directed to a multiplexed immunoassays section to perform downstream analysis. Such assays require little materials as compared to the existing laboratory methods that could be a big advantage to preserve the precious primary cells and patient tissues. However, the integration and automation of such systems remain considerable hurdles and require significant advancements in the field of microfluidics.

The intriguing microfluidic-based tissue models can be further improved to mimic the *in vivo* complexity at the micro scale and can be subsequently used for basic cancer research and high-throughput drug screening prior to clinical testing. It is highly desirable to develop microfluidic-based platforms to mimic the vascular architecture with interstitial pressure gradient for a more accurate predictive capability. For example, a recent study has demonstrated the feasibility of embedding microfluidics within ECM to define vascular networks with controllable diffusive gradients [187]. The versatile three-dimensional microfluidic technology and similar approaches are expected to provide a simplified platform for modeling the sophisticated *in vivo* tissues and simultaneously, to assist the screening and

identification of key soluble factors and conditions driving the various cellular events. Furthermore, it is highly anticipated that these approaches will motivate the progressive development and application of novel "organ-on-a-chip" models, such as those integrated with a disease element like tumor, in basic and applied cancer research [188]. In fact, these past few years have seen the rapid development and application of the exciting "tumor-on-a-chip" model for the elucidation of complex *in vivo* aspect of cancer in a controllable *in vitro* environment [169, 178].

Overall, we foresee an exciting prospect for the microfluidic-based platforms in cancer research and applications. With many research and development activities going on in this area, we envision that practical and commercial microfluidic devices for the all-inclusive cancer basic research, clinical diagnosis and even anti-cancer drug screening are very close to realization.

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