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Review

Review: Microfluidics technologies for blood-based cancer liquid biopsies

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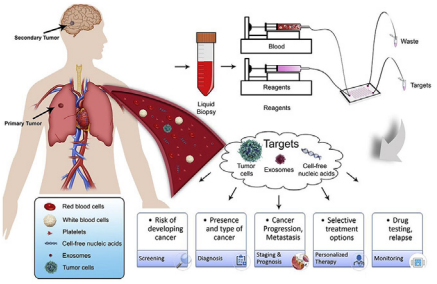
HIGHLIGHTS

- Blood based liquid biopsies provide unique opportunities for management of cancer.
- Circulating tumor cells, exosomes and cell free nucleic acids provide important insights.
- Microfluidics can accomplish efficient isolation of tumor cells and cell-derived products.
- This review highlights blood-based liquid biopsies and their use in clinical decision making.

GRAPHICAL ABSTRACT



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ABSTRACT

Blood-based liquid biopsies provide a minimally invasive alternative to identify cellular and molecular signatures that can be used as biomarkers to detect early-stage cancer, predict disease progression, longitudinally monitor response to chemotherapeutic drugs, and provide personalized treatment options. Specific targets in blood that can be used for detailed molecular analysis to develop highly specific and sensitive biomarkers include circulating tumor cells (CTCs), exosomes shed from tumor cells, cell-free circulating tumor DNA (cfDNA), and circulating RNA. Given the low abundance of CTCs and other tumor-derived products in blood, clinical evaluation of liquid biopsies is extremely challenging. Microfluidics technologies for cellular and molecular separations have great potential to either outperform conventional methods or enable completely new approaches for efficient separation of targets from complex samples like blood. In this article, we provide a comprehensive overview of blood-based targets that can be used for analysis of cancer, review microfluidic technologies that are currently used for isolation of CTCs, tumor derived exosomes, cfDNA, and circulating RNA, and provide a detailed discussion regarding potential opportunities for microfluidics-based approaches in cancer diagnostics.

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Contents

1. Overview

Cancer remains one of the leading causes of death worldwide. In the United States alone, there were ~1.6 million new cases and nearly 500,000 cancer related deaths in 2016 [1,2]. According to the American Cancer Society, solid tumors of the breast, lung, bronchus, prostate, colon, rectum and bladder remain the most common causes of cancer [2]. It is estimated that in the United States, > 15 million people are currently living beyond a cancer diagnosis with this number expected to rise to almost 19 million by 2024 representing an enormous cost burden (>$ 130 billion/year) [1,2]. New technologies have made detection of solid tumors routine process; but to improve prognosis, enhance quality of life, drive down treatment costs, and enable positive outcomes for cancer patients, better technologies are necessary to enable detection of cancers before symptoms appear, monitor disease progression and evaluate patient response to treatment.

Advances in genomics and molecular technologies have created great interest in the use of liquid biopsies as a non-invasive alternative to surgical biopsies to evaluate the wealth of information contained in cancer-associated cells and biomolecules found in bodily fluids [3–6]. Apart from being relatively low-risk to patients, these measurements can be made dynamically, enabling correlation of disease burden and progression to quantifiable biomarkers [5,7]. Liquid biopsies are not limited to blood [4,8] and can be performed using other bodily fluids including urine [9–11], stool [12,13], saliva [14–16], and cerebrospinal fluid [17–19]. However, urine or stool samples only provide insights into specific types of cancers (bladder or colon) whereas blood is more universal and can potentially be used to detect all cancers. The disadvantage of blood samples is the presence of vast amounts of other cellular and molecular content that can greatly complicate detection and evaluation of biomarker targets. This review focuses on blood-based liquid biopsies, the challenges of isolating CTCs and other tumor-related products that are present at an extremely low frequency in blood and the use of microfluidics based technologies to sort through complex samples to identify specific targets. Fig. 1 highlights locations of the primary tumor, metastasis of circulating tumor cells from the primary tumor location to secondary sites, and extravasation and establishment of the secondary tumor. During this process of metastasis, several cells (CTCs) and cell derived products (exosomes, cell-free DNA and RNA) are released and transit via circulating blood and can be sampled to provide valuable screening, prognostic and diagnostic information that can benefit patients via personalized therapeutics and to evaluate response to treatment.

Solid tumors originate in the epithelium of organs like the breasts, lungs, and colon and are the primary cause of malignancies [20,21]. Metastasis from solid tumors accounts for >90% of cancer-related deaths and each stage of this process can result in release of specific cells or cell-derived products into the bloodstream [21,22]. The formation of a primary tumor requires the acquisition of resistance to apoptosis or programmed cell death [23–25]. Metastasis begins with tumor cells from the primary site invading adjacent tissues, migrating into blood circulation, travelling through the circulatory system, extravasation following arrest at a distal organ or tissue and expansion at the new location [6,26]. The primary stages of metastasis involve the detachment of epithelial cells from the extracellular matrix (ECM) and disruption of the actin cytoskeleton resulting in a rounded and unattached phenotype which typically is a trigger for apoptotic cell death via anoikis and amorphosis (due to loss of contact of adherent cells with the ECM) [27–29]. This is followed by some cells migrating and entering circulation [28,30]. Once metastatic cells enter circulation, only a fraction of cells successfully evade the body's immune surveillance and establish metastatic foci at secondary locations [31,32]. After establishment at a secondary locations, tumors expand by recruiting new blood vessels and impacting normal function of tissues and organs [31,32]. Metastasis and tumor growth are intrinsically linked to the host circulation and blood is used to

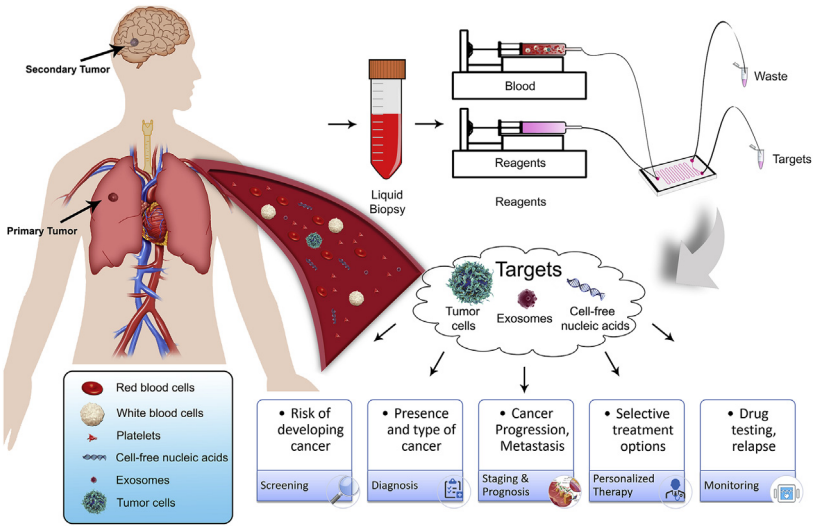

Fig. 1. Schematic highlighting progression of cancer and presence of cancer cells and cancer cell-derived products in blood circulation and the use of microfluidics-based approaches to process liquid biopsies from patient's blood and enable isolation of specific targets which can then be used for a wide array of processes that can impact screening, diagnostics, prognostics and treatment of patients.

transport of cells (CTCs), tumor-derived exosomes, and cell-free ctDNA and RNA. Therefore, liquid biopsies of blood have great potential to identify and evaluate biomarkers to aid in early detection of cancer, monitoring disease progression and evaluating the response to treatment [6,7,33].

Microfluidic devices allow for manipulation of fluids within architectures on the size scale of 10s of micrometers (comparable to the size of a single cell) [34,35]. Miniaturization offers benefits both in terms of speed and costs using small amounts of reagents and buffers at significant faster sample processing rates [36,37]. Devices can be designed to ensure levels of precision not possible with conventional macroscale approaches while ensuring that every cell or biomolecule is evaluated in a homogeneous fashion which is extremely important when probing low abundance cells and biomolecules [38]. Microfluidics can also enable design of entirely new separation techniques where scaling effects can be exploited to ensure laminar flow, amplify secondary forces, and define unique geometries to selectively direct/confine/capture cells and cell-derived products [39,40]. Thus far, there is significant interest in microfluidic technologies for cancer diagnostics to isolate cancer cells and cell-derived products. The vast majority of microfluidics-based approaches have focused on CTC isolation [41,42]. This can be attributed to various factors including the rapidly growing interest in CTCs in cancer diagnostics, the complexity of the problem of isolating CTCs which occur at extremely low frequency in circulating blood, and the inability of conventional macroscale approaches to satisfactorily address this issue. Other cancer cell-derived products like exosomes and circulating cell-free genetic materials have not been pursued with the same level of interest as CTCs possibly due to the fact that they can be isolated with a fair degree of reliability using conventional approaches. Therefore, microfluidic adaptation of these approaches has been relatively slow despite potential advantages that could transform these laboratory scale processes into point-of-care devices with enhancements in speed, purity and efficiency.

Currently, the only FDA approved test for evaluation of CTCs is the CELLSEARCH® Circulating Tumor Cell Kit. CELLSEARCH® is not intended for early diagnosis but is routinely used to monitor and predict cancer progression in metastatic cancer and evaluate response to chemotherapy [43]. CTCs counts using CELLSEARCH® have been shown to be a reliable independent predictor of progression-free survival (PFS) and overall survival (OS) in a percentage of patients with metastatic breast [44,45], colorectal [44], and prostate cancer [44]. While this test is not accepted as a substitute for solid tumor imaging, it is used to supplement imaging in the assessment of disease progression in patients. CELLSEARCH® uses a combination of immuno-magnetic capture along with live-cell imaging to identify CTCs and discriminate them from leukocytes using conventional macroscale approaches. Several companies including Inivata [46], Epic Sciences [13], Guardant [10], Janssen Diagnostics [15], Cellsee [18], Rarecells [22], Biofluidica [21] and SRI International [47] offer tests for CTCs and cfDNA. Some of these approaches utilize microfluidic interfaces to enhance capture efficiency or to provide confined geometries for sorting and isolation. In the research community, there is ongoing interest in microfluidic adaptation of CTC capture using both immuno-affinity based approaches and via exploitation of unique physical properties of these cells. There is also high interest in microfluidics-based approaches for capture of exosomes. However, thus far, these approaches are still in the pre-clinical or early clinical testing phases and will require more time before FDA approval is obtained and offered to patients.

In this review, we provide an extensive overview of different microfluidics approaches for isolation of different targets from blood-based liquid biopsies that can provide prognostic or diagnostic value. We also discuss the origins of each of these targets, their frequency of occurrence in blood, their physical and biochemical properties that can be exploited to enable separations, and the type of predictive information contained within these targets. This review is intended to provide researchers and commercial entities seeking to implement new technologies for cancer monitoring with the background necessary to understand the biological significance and complexities associated cancer biomarkers, identify potential biomarkers, and establish design parameters to successfully translate microfluidics-based technologies into the clinical setting.

2. Circulating tumor cells

2.1. Origins and frequency of occurrence

CTCs in blood were identified in 1869 from blood samples of a

patient suffering from extensive breast cancer [48]. Since then, there have been efforts to identify the origin and gain a broader understanding of the process of CTC migration into blood and extravasation into secondary sites [26]. CTCs originate either from the primary tumor or from a secondary metastatic site and enter blood circulation [49]. The exact mechanisms underlying the migration of CTCs into the blood stream is still not fully understood but various events including the hypoxic tumor environment [50], ECM remodeling [51], active proliferation [51] and epithelial to mesenchymal transition (EMT) [52] are all recognized as possible mediators of increased migratory potential of CTCs. Following entry into the blood stream, the CTCs are present in circulating blood transiently, possibly resulting from immune clearance, apoptosis, or secondary localization which translates to an extremely small number in circulation [53]. CTCs have been found in blood of a small percentage of patients well before clinically detectable metastasis [54,55]. However, consistent identification of CTCs is difficult due to variability in how cancer progresses in different individuals and in part due to the low sensitivity of methods to enumerate them [56]. Relatively, numbers of CTCs in circulation can increase as a function of expansion of the primary tumor or tumor cell proliferation at metastatic sites [57]. Recent work has also shown that the numbers of CTCs in circulation can decrease in response to reduction in tumor burden with effective therapy [57]. In developed metastatic cancer, CTCs are found at a rate of 1–10 CTCs per 10 mL of blood or 1 CTC per billion nucleated cells [49].

2.2. Predictive information in CTCs

The most basic information that can be obtained from CTCs is the confirmation of their presence and the evaluation of their numbers [31,32]. Quantification of numbers of CTCs is now commonly used as a prognostic marker in metastatic cancer to evaluate effectiveness of therapy via correlation of CTC numbers to disease state, with a decrease in CTC count suggesting successful targeting of the tumor [58–60]. Given that CTC numbers have poor correlation with tumor burden and the fact that there is high patient-to-patient variability, the patient's baseline threshold is used as a point of comparison rather than evaluation based on predefined threshold [61]. Intact and viable CTCs have been maintained using cell culture to determine proliferative potential and responsiveness to chemotherapeutic drugs [62,63]. More recently, CTC clusters comprising of 2–100s of cells have been identified in circulation and have been found to possess 100-fold higher metastatic potential [64–66]. The presence of even a single CTC cluster in blood liquid biopsies correlates with significantly reduced progression-free survival rates in patients with various types of cancers [66,67]. CTCs can be profiled as a mixture or via single cell profiling to characterize disease in patients, identify CTC heterogeneity and determine distinct subsets of cells which can provide information that can be used to direct patient-specific therapy [68,69]. Molecular expression signatures of CTCs also provide important information regarding the patient's status. Proteins contained within cells and cell surface markers including cell surface receptors can be evaluated using immunofluorescence microscopy or flow cytometry and used to classify disease phenotype [70,71]. Specific mutations and splice variants can be profiled using genetic screening and also used as measures of disease burden to guide effective treatment [72,73]. While there is little doubt regarding the wealth of information regarding the patient's immediate condition, identifying consistent and universal biomarkers has been the major challenge. Reliable and highly sensitive methods to isolate intact CTCs from blood liquid biopsies will hasten progress in identification of new and reliable biomarkers.

2.3. Physical and biochemical characteristics of CTCs

CTCs are a heterogeneous population and their characterization can play a major role in the selection of technique used for their isolation. They range in size from 4 to 20 μm, stain positive for epithelial cell adhesion molecule (EpCAM) and negative for cluster of differentiation 45 (CD45) [74]. CTCs can also be found in clusters of 2–100 cells and can range in size from 10 to 100s of micrometers in diameter. To avoid bias associated with EpCAM-mediated cell capture, cancer-type specific biomarkers can be exploited using antibodies or aptamers targeted towards specific markers. When isolated using density gradient centrifugation using a Ficoll or Percoll gradient, CTCs fractionate along with peripheral blood mononuclear cells (PBMCs) with an estimated density ~ 1.064–1.065 g/cm^3 [75]. CTCs are more deformable than WBCs which is an aspect that has been exploited for isolation [76]. CTCs are known to express both epithelial and mesenchymal proteins as a consequence of EMT [77]. More specific markers including different cytokeratins and specialized markers like prostate-specific membrane antigen (PSMA) can allow identification of CTCs associated with different organs and metastatic sites [78]. Despite information regarding cancer-type specific labels and biomarkers, it is important to understand that there is no universal biomarker that can be used to classify CTCs and these cells are a heterogeneous population with a percentage of cells expressing a particular marker. Detailed molecular characterization of CTCs at the gene and protein level can provide valuable insights into the biology of the specific type of cancer and potential for metastasis [79]. This information can then be used to classify patients for metastatic risk, select therapeutic options and monitor disease progression and effectiveness of therapy [80].

2.4. Microfluidic devices CTC capture

As previously discussed, CELLSEARCH® is the only FDA approved test for evaluation of CTCs in patients with cancer and is used as a surrogate for imaging to provide additional information as an independent predictor of cancer progression in metastatic cancer and to evaluate response to chemotherapy [43]. Considering the potential for microfluidics to significantly outperform conventional macroscale devices, it is interesting to note that the only FDA approved device is a macroscale device. While microfluidics based approaches have only recently been pursued for cancer monitoring, this is possibly due to the fact that microfluidics-based approaches have yet to demonstrate efficiency and reliability necessary to serve as standalone monitoring tool. Another important factor could be the lengthy nature of the FDA approval process. The complexities associated with CTC detection make reliable isolation of CTCs with conventional macroscale techniques difficult to accomplish and not surprisingly, microfluidics-based approaches have been aggressively explored. Detection and isolation methods exploit physical or biochemical differences between cells to enable discrimination. Microfluidics provides technology to discriminate between cells at the single-cell level thereby enabling separation efficiencies not possible with bulk methods. The CELLSEARCH® system is not a microfluidic system and uses a combination of magnetic nanoparticles to separate target cells and imaging to confirm that the captured cells are indeed CTCs. Critical parameters for evaluation include throughput (mL of blood that can be processed per hour given the low numbers of CTCs), capture efficiency (% of spiked CTCs that can be captured from a known volume of blood) and purity (% of cells captured that are CTCs). The CELLSEARCH® platform can process large volumes of blood (several mL), has a capture efficiency of ~85% but is associated with low purity. This process utilizes several processes including the labeling and capture steps,

that if miniaturized could be significantly improved.

Demonstration of microfluidics-based systems for CTC isolation started with the CTC-chip in 2007 where a microfluidic device with silicon pillars was functionalized with capture antibodies [81]. Following the success of this device in achieving successful identification of CTCs in >99% of patient samples, several other groups and companies began developing microfluidics-based approaches to capture CTCs. While a majority of these efforts focused on exploiting cell surface markers to enable isolation, others focused on unique physical characteristics (label-free) of CTCs such as size, shape, deformability, and behavior under conditions of microscale flow. Thus far, label-free approaches have yet to demonstrate the efficiencies associated with immuno-affinity based methods but with development of new methods and devices, it appears that label-free technologies may provide not only the sensitivity to compete with immuno-affinity based techniques but provide superior performance in terms of throughput. Overall, while there is significant potential for microfluidics to impact CTC isolation, microfluidics is also associated with some limitations. These limitations primarily relate to throughput and the ability to process sufficient amounts of sample to be able to isolate or detect suitable number of CTCs for subsequent analysis. While parallel processing has been suggested as means to address this issue, it still is a major challenge that will need to be addressed. Other challenges involve non-specific capture of cells and dealing with the heterogeneity within CTC populations. A more detailed discussion on the limitations of microfluidics can be found in the 'Future Directions' section. Table 1A and B summarize microfluidic approaches for immuno-affinity and label-free capture of CTCs respectively and are organized based on capture efficiency.

2.4.1. Microfluidic devices for immuno-affinity capture of CTCs

The first microfluidic device for capture of CTCs was demonstrated in 2007 by Nagrath et al. [81] and consists of a silicon microfabricated platform with 78,000 pillar structures (Fig. 2A). The silicon surface was functionalized with a capture antibody targeted towards EpCAM to enable capture of CTCs of epithelial origin. Preliminary studies focused on demonstration of the ability of this device to capture cancer cell-lines associated with prostate, bladder, lung and breast cancer followed by evaluation of blood samples from cancer patients. This device was able to identify CTCs in 115 out of 116 samples processed, demonstrating its utility for detection. Captured CTCs were confirmed using a combination of both positive (DAPI/Cytokeratin) and negative (CD45) immunofluorescence staining. Apart from screening, this platform was also used to monitor patient response to treatment and showed that the CTC numbers of correlated with response to treatment. A follow up study used the same platform to evaluate specific mutations in epidermal growth factor receptor (EGFR) [82]. In terms of performance, this device operated at a flow rate of 1 mL/h at a capture efficiency of ~60% with a purity of ~50%.

The basic concept of immuno-affinity capture with silicon post was further enhanced by using design optimization to determine size and organization of the posts and translated into a 'geometrically enhanced differential immuno-capture' (GEDI) device [83]. This device was used with different capture antibodies to probe

Table 1
Summary of (A) microfluidic immuno-affinity based and (B) label-free approaches for isolation of circulating tumor cells (CTCs). Note: Table is organized based on reported capture efficiency.

Authors	Description of Method	Capture Efficiency	Purity	Downstream Clinical Application
Circulating Tumor Cells (Antibody based approaches)				
Nagrath et al. [81]	EpCam antibody and microposts rocking chip	99%	50%	Epithelially derived cancer detection, diagnosis, and monitoring
Adams et al. [88]	High throughput microsampling unit and EpCAM antigen	97%	>99%	Epithelially derived cancer detection, diagnosis, and monitoring
Stott et al. [85]	Herringbone-Chip microfluidic mixing and EpCAM antigen	93%	14%	Epithelially derived cancer detection, diagnosis, and monitoring
Reátegui et al. [90]	Gelatine nanocoating, microfluidic mixing, EpCam, EGFR, and HER2 antigens	93%	80%	CTC detection, diagnosis, and monitoring
Murlidhar et al. [84]	Radial flow mixing with bean-shaped microposts and EpCAM antigen	93%	–	Epithelially derived cancer detection, diagnosis, and monitoring
Sheng et al. [86]	GEM microfluidic mixing and EpCAM antigen	90%	80%	Epithelially derived cancer detection, diagnosis, and monitoring
Ozkumur et al. [95]	CTC-iChip system. Inertial focusing, antigen coated magnetic bead sorting, cell size sorting	90%	98%	CTC enrichment and quantification
Gleghorn et al. [83]	GEDI chip: Microfluidic mixing and prostate-specific membrane antigen	85%	68%	Diagnosis and monitoring prostate cancer progressing
Allard et al. [251]	CELLSEARCH: ferrofluid antibody enrichment	85%	Low	Outcome diagnostic for epithelial derived cancers
Yoon et al. [89]	Graphene oxide nanosheets and EpCAM antigen	73%	–	Epithelially derived cancer detection, diagnosis, and monitoring
Ke et al. [91]	Microfluidic and thermoresponsive anti-EpCAM adhesion	70%	35%	Epithelially derived cancer detection, diagnosis, and monitoring
Circulating Tumor Cells (Antibody free approaches)				
Warkiani et al. [118]	Centrifugal throughput, Dean's drag, and inertial lift	100%	80%	CTC detection and analysis
Sollier et al. [119]	Micro-scale vortices and inertial focusing	100%	57-94%	CTC detection and analysis
Ding et al. [124]	Acoustic wave focusing	99%	83%	CTC enrichment and quantification
Gupta et al. [126]	Dielectrophoretic field-flow fractionation	99%	73%	CTC detection and analysis
Parichehreh et al. [123]	Inertial focusing	>95%	>99%	CTC enumeration
Hou et al. [114]	Centrifugal throughput, Dean's drag, and inertial lift	93%	85%	CTC detection and analysis
Ozkumur et al. [95]	CTC-iChip system. Inertial focusing, antigen coated magnetic bead sorting, cell size sorting	90%	98%	CTC enrichment and quantification
Harouaka et al. [105]	Micro spring array	90%	$10^{-}4$ enrichment	CTC enrichment and quantification
Sun et al. [115]	Dean's drag and inertial lift forces	89%	–	CTC quantification

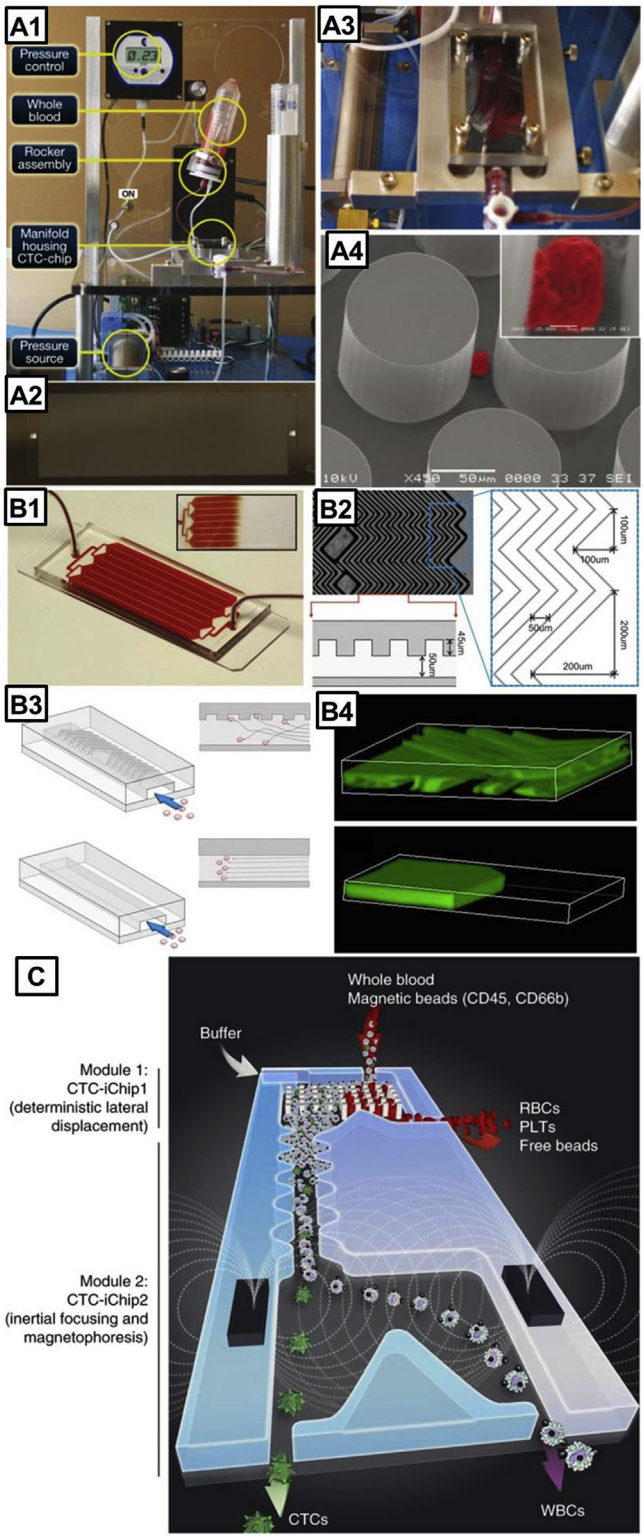

Fig. 2. Examples of microfluidic devices for immuno-affinity based capture of CTCs. (A) CTC chip: the first example of a microfluidic device for isolation of CTCs from blood [81]. (A1) Complete setup for delivery of blood samples to the CTC chip, (A2) Pressure source which drives fluid flow, (A3) CTC chip with blood perfusing the chip, and (A4) Picture of micromachined posts with captured CTC. (B) Herringbone Chip: Demonstration of how efficient mixing can enhance interaction of CTCs with capture surfaces [85]. (B1) Herringbone chip perfused with blood, (B2) Design and placement of herringbone structures within the microfluidic channel, (B3) Schematic demonstrating rotational fluid flow within the microfluidic channels, and (B4) Simulation of fluid flow within the channels. (C) CTC-iChip: Example of how multiple separation mechanisms can be integrated together on a single platform to de-bulk samples and enable efficient separation [94].

prostate cancer using both cell-lines and patient samples. Optimization of post geometry and arrangement resulted in a capture efficiency of ~80% and purity of ~68% which is an appreciable advance over the original CTC chip. Murlidhar et al. [84] further optimized post shape to minimize flow separation around the posts and enhance cell interaction with the post surface, unlike commonly used flow through configurations, the sample flows radially outward and can process samples at flow rates up to 10 mL/h. The reported capture efficiency is between 80 and 100% with lower efficiency at higher flow rate. However, the purity is increased with increasing flow rates.

Other device designs and modifications were incorporated to enhance throughput, capture efficiency while minimizing non-specific capture. Stott et al. [85] developed a herringbone (HB)-chip which incorporates a staggered double herringbone structure on the roof of the microchannel to induce rotational flow that results in chaotic mixing within microfluidic channels (Fig. 2B). Cells travelling through the channel are forced to interact with the capture surface thereby enhancing interactions between the cells and capture antibodies. The HB-chip when operated at a flow rate of 1.2 mL/h resulted in an extremely high capture efficiency of ~90% at ~15% purity. The low purity is presumably due to the low flow rate which is insufficient to dislodge cells that are weakly bound to the capture channel. The concept of the HB chip was combined with the concept of the GEDI chip and resulted in a geometrically enhanced mixing (GEM)-chip [86] which operated at a higher flow rate (3.6 mL/h) and maintained the same capture efficiency. The capture purity was significantly improved to >80% which can be directly attributed to the higher flow rate which is sufficient to dislodge weak non-specifically bound cells.

Apart from design variations, other modifications have included the use of different types of materials for microfluidic device construction; high-aspect ratio structures fabricated via embossing of polymethylmethacrylate (PMMA) was used to construct a high-throughput microsampling unit (HTMSU) [87,88]. Further complexity was incorporated in the form of sensors for on-chip conductivity measurements which correlates to cell counts. Despite its name, the operating flow rate was low, at ~1.2 mL/h with a capture efficiency of ~95%. Yoon et al. [89] combined nanotechnology-based approaches to develop a graphene oxide (GO)-based chip which enhances antibody presentation to the flowing sample. The GO-chip operated at a flow rate of 1 mL/h is associated with a capture efficiency of ~95% with minimal non-specific binding.

CTCs captured within immuno-affinity capture devices are typically stained and evaluated on-chip or lysed to extract proteins and nucleic acids. With the need to isolate intact CTCs for further analysis or cell culture, some groups have focused on developing methods to release CTCs following capture. Using the basic concept of the HB chip, Reategui et al. [90] developed a technique where layer by layer deposition of gelatin and streptavidin were used to immobilize streptavidin coated nanoparticles to ultimately bind biotinylated antibodies for CTC capture. Following capture, the captured cells were released by increasing the temperature to dissolve the gelatin or through acoustic activation. Another group also developed a system based on the HB-chip and developed thermoresponsive polymers that could make immobilized antibodies either available (room temperature) or unavailable (when cooled to below critical solution temperature ~ 4 deg C) [91]. This NanoVelcro system can capture and release cells via targeted temperature changes. Both approaches operate at relatively high flow rates but attain high efficiency capture (>90%) at relatively high purities. In another example, Hou et al. [92] demonstrated that capture and release could be accomplished using polymer grafted silicon structures.

With importance being placed on retrieval of captured cells, there have also been efforts to miniaturize conventional immunomagnetic separation techniques. While every aspect of immunomagnetic separation including sample-bead mixing and incubation, capture, washing and release can benefit from miniaturization, there are examples of where one or more of these approaches have been exploited for CTC isolation. The VeriFAST system utilizes blood samples mixed with paramagnetic beads conjugated with capture antibodies in a microfluidic device with two unmixable fluids [93]. The labeled cells are manually moved from the blood sample containing well across an oil-pinning well using a hand held magnet. In another example, the CTC-iChip exploits size-based separation to de-bulk the sample of blood cells and then accomplished CTC isolation using immuno-magnetic beads (Fig. 2C) [94]. Both these techniques have relatively high throughput (>5 mL/h), high efficiency (>90%) and reportedly high purity [95]. More recently, there appears to be a continuing trend to incorporate two or more approaches to accomplish sample pre-processing or debulking followed by more specific immuno-affinity capture. Ahmed et al. used a pillar array to debulk non-target cells followed by imaging, detection and analysis of captured CTCs with a capture efficiency of 92% and a capture purity of 82% [96]. Shields et al. used a three stage process where cells were first aligned using standing acoustic waves (SAW), separated using antibody-conjugated magnetic beads and isolation of individual CTCs into microwells for single cell analysis [97]. Green et al. used a combination of shear stress and immuno-affinity capture to isolate phenotypically unique CTCs based on EpCAM expression levels [98]. Finally, optical trapping and photoacoustic detection of nanoparticle labeled CTCs has also been developed to obtain high efficiency CTC capture [99,100].

2.4.2. *Microfluidic devices for label-free CTC capture*

Label-free approaches for CTC isolation have focused on three specific properties of CTCs: size, deformability, surface energy/charge. Given the heterogeneous nature of CTCs and the wide distribution in the levels of expression of different biomarkers, label-free approaches may provide the means to avoid biomarker 'bias' and potentially isolate larger numbers of cells with diagnostic/prognostic value. However, at the same time, the disadvantage is the lack of specificity which can lead to lower levels of purity of captured cells. Several companies have commercially available products for enrichment focused on size-based discrimination of CTCs from other blood cells. Size-based sorting does not compare in terms of purity associated with antibody-based approaches, however, these approaches provide significantly higher throughput. The use of size also prevents EpCAM induced screening bias and identification of CTCs of non-epithelial origin including mesenchymal CTCs that are common in metastasis. These types of assays are associated with a quick initial enrichment step followed by staining and analysis; Screencell offers a simple 6.5 μm filter for enrichment of fixed CTCs and 5.5 μm filter for unfixed CTCs [101]. Confirmation of CTCs is then accomplished via imaging of the enriched sample. Several other companies including Parsortix (weir-type filter) [102], Creativ Microtech (precision micromachined filters) [103], Ikonisys (size filtration combined with size based isolation) [104] have all developed similar methods where cells are sorted by size and further evaluated using imaging. Harouaka et al. developed a highly sensitive filter array using flexible springs fabricated using Parylene C [105]. This flexible micro spring array (FMSA) processes whole blood in a very gentle fashion to enable separation of cancer cell lines spiked into whole blood (Fig. 3A). Separation efficiency was ~90% at a throughput of 45 mL/h. The feasibility of this approach was also demonstrated using samples from patients with a variety of cancers and the number of CTCs isolated corresponds well with results from the CELLSEARCH platform [106]. Gogoi et al. exploit both the size and deformability of CTCs to enable trapping and automated staining and screening of CTCs using a microfluidic device reporting 94% sensitivity and 100% specificity [107]. CTC clusters are significantly larger and have been sorted from blood liquid biopsies which contains individual cells in suspension via a process called deterministic lateral displacement (DLD) which causes preferential migration of cells in flow via interaction with obstacles placed at locations along the flow path. Using a two-stage process Au et al. demonstrated separation of small and large clusters of breast cancer cell lines spiked in blood with 99% recovery of large clusters, cell viabilities over 87% and greater than five-log depletion of red blood cells [108].

Cells in flowing fluid can also be fractionated into unique populations based on size differences. In microfluidic channels the balance between inertial lift forces and wall lift forces in high aspect ratio microchannels enables focusing of particles at unique locations close to the wall [109,110]. The larger the particle the closer the focusing location was to the outer wall. This phenomenon has been exploited to separate CTCs or cancer cell lines from red blood cells and white blood cells in a continuous flow fashion at relatively high throughputs. Straight rectangular channels are associated with multiple focusing locations (2 or 4 depending on the aspect ratio) which can be reduced to one focusing location within curved channels. There have been many examples of rectangular cross-sectional channels organized in a spiral fashion to enable focusing and separation of CTCs [111–113]. Examples of these types of devices include, simple spiral (>85% efficiency, 3 mL/h, 3 log depletion) (Fig. 3B) [114], 6-loop double spiral (>85% efficiency, 20 mL/h, enrichment factor of 19) [115] multiplex spiral [116] cascaded spiral (>85% efficiency, 33 mL/h, 98% depletion) [117] and 8-loop slanted spiral where the walls were at different height with the outer wall being taller than the inner wall (>80% efficiency, 55 mL/h, 4 log depletion) [118]. While these flow-based technologies for size-based separation offer reasonable efficiency and high throughput, they cannot process whole blood directly and require some prior pre-processing. Even with sample pre-processing, the contamination with non-specific cells is high, for example a 99.9% depletion of red blood cells still amounts to a RBC:CTC ratio of 1×10^6:1 which is a major obstacle to evaluating CTCs.

Other approaches that exploit inertial effects uses a rectangular flow channel with chambers on either side of the microchannel. The fluid within these small chambers is stagnant for the most part and fluid flow in the rectangular channel results in generation of vortices within the chambers. Particles migrating to the walls of the channels interact with the recirculation associated with these vortices and remain trapped in the chamber and are subsequently released from the chambers at higher flow rates. Sollier et al. [119] demonstrated that such a design can be used to capture CTC but the efficiency was ~20% and the purity just over 50%. The same technology was used for classification of large captured CTCs [120] and for labeling of CTCs with magnetic beads [121]. Parichehreh et al. [122,123] developed a system that exploits both inertial focusing effects and surface energy based migration in a technique called 'inertia enhanced phase partitioning' (Fig. 3C). Cells are introduced into a microfluidic channel as a thin stream flanked on either side by Dextran. For red blood cells, the energetically favorable location is within the Dextran whereas the CTCs and white blood cells prefer saline and do not migrate towards the Dextran. As red blood cells move to the dextran, inertial focusing effects move the red blood cells towards the outer wall whereas CTCs and white blood cells remained in the middle between the two Dextran streams. Operating at a flow rate of 1.2 mL/h, a two-pass system using this device resulted in >99% depletion of red blood cells with 98% recovery of spiked cancer cell line cells.

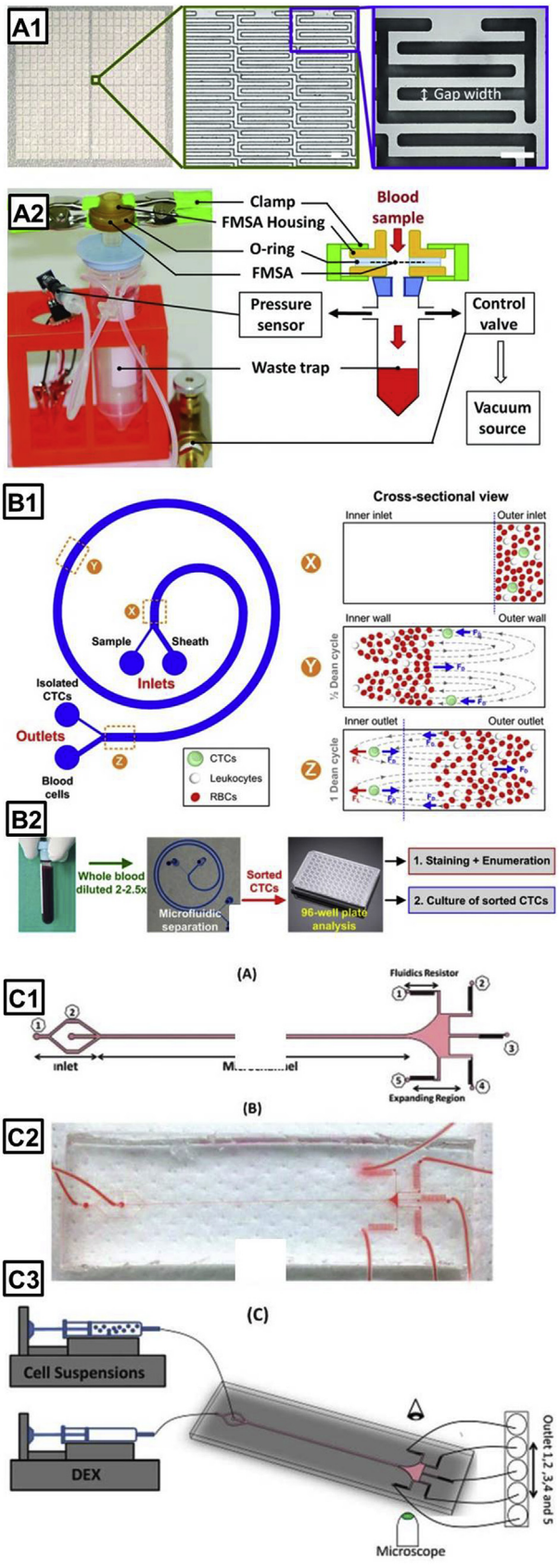

Fig. 3. Examples of microfluidic devices for label-free capture of CTCs. (A) Filtration: Exploiting size differences to ensure separation of CTCs based on differences in size and deformability [105]. (A1) Microscopic images of flexible spring microarrays that are used for exosome capture, and (A2) Actual setup including a schematic of the various components of the capture system. (B) Spiral Devices: Demonstration of the use of inertial focusing within microfluidic channels to enable size based separation of cancer cell lines from other blood cells [114]. (B1) Design and mechanism of separation of cells and particles using spiral microchannels, and (B2) Actual devices and workflow for processing of samples for CTC isolation. (C) Surface Energy: Using differences in cell surface energy to enable initial phase partitioning and the initial separation is enhanced via inertial forces [122,123]. (C1) Schematic of the device for inertia enhanced phase partitioning, (C2) Actual fabricated device and (C3) Setup for operation of the device.

Acoustic waves in the form of tilted angle standing surface acoustic waves (taSSAW) have been used to influence particle migration within microfluidic channels. Ding et al. [124,125] optimized the power and angle of inclination that could efficiently separate cancer cell-lines spiked in red blood cell depleted blood. At a maximal flow rate of 1.2 mL/h, this technique achieved 83% purity with 1 log depletion of white blood cells. Dielectrophoresis (DEP) is used in a commercially available device called ApoStream to sort CTCs from other blood cells [126,127]. A combination of acoustic focusing and DEP was used to pre-concentrate target cells using acoustic focusing and trapping of target cells using DEP was used for CTC isolation with 76% recovery of target cells and only 0.12% contamination with non-target cells [128]. With optimized voltage, frequency, buffer and electrode configurations, this device can operate at a maximum flow rate of 1.5 mL/h with 70% purity and 2–3 log depletion of white blood cells. But the viability of cells was excellent suggesting potential for sub-culturing isolated cells.

Both immuno-affinity and label-free microfluidics approaches have shown great promise for CTC capture. Several factors including throughput, ability to directly process blood samples without significant pre-processing and strategies to deal with the large heterogeneity associated with CTCs need to be addressed prior to widespread clinical adaptation.

3. Tumor cell derived exosomes

3.1. Origins and frequency of occurrence

Exosomes are vesicles that are shed directly from cells via budding of the plasma membrane [129–131]. Exosomes vary in size ~ 25–300 nm and play important roles in transport of signaling molecules to facilitate cell-cell communication [131,132]. Exosome contents include nucleic acids (DNA and RNA), proteins, metabolites, and lipids and facilitate normal physiological processes [133,134]. In disease, exosomes can also mediate pathological signaling [135,136]. Formation of exosomes involves initiation, budding of endocytotic membrane into the cell lumen, formation of multi-vesicular bodies, selection and degradation or secretion of vesicular bodies. Exosomes are secreted in various bodily fluids including blood and are highly stable under physiological conditions and are found at relatively high abundance [131,137]. Typically 1 mL of serum is known to contain between 1×10^9–1×10^{12} exosomes [138]. While there is evidence to suggest that the exosome cell-surface markers and intra-vesicular contents contained within exosomes change in patients with cancer [139–141], there is also evidence to suggest that the numbers of exosomes in serum increases due to cancer [142,143].

3.2. Predictive information in exosomes

An increase in number of circulating exosomes can be used as a simple but non-specific evaluator of cancer [142,143]. Exosome surface markers provide valuable information that can be used to determine their origin and signaling associated with the cells from which they were secreted. These cell surface markers have been

widely used to enable isolation using immuno-affinity capture. Expression of epithelial derived markers like EpCAM can be used to identify tumor cell-derived exosomes [144,145]. Identification of specific organs associated with the tumor can also be identified via examination of specific protein biomarkers to distinguish different types of cancer. Specific protein markers have been identified for breast [146,147], prostate [148,149], pancreatic [150], ovarian [151], and colorectal cancers [152]. The DNA and RNA contained within the exosomes are similar to cell-free DNA and RNA and can be profiled and used as biomarkers for diagnosis and longitudinal disease monitoring [153–156]. A more detailed discussion regarding the predictive information of DNA and RNA is available in sections below.

3.3. Physical and biochemical characteristics of exosomes

Exosomes are variable in size ranging from 25 to 300 nm and their isolation typically requires ultracentrifugation of serum samples [157]. Exosomes also have well-defined surface markers that have been utilized for immuno-affinity separations. Non-specific capture of exosomes uses common exosome markers CD9, CD63, and CD81 [158,159] either immobilized to a surface or coupled to magnetic beads whereas cancer specific exosomes can be isolated using CD24 [144,146], CD37 [160], CD53 [161], CD73 [162], CD82 [139], and EpCAM [127,128]. Exosomes characterization can be accomplished by profiling for specific protein markers based on the endosome biogenesis pathway associated proteins including annexins [163], flotilin [164], Alix [165], Tsg101 [166], tetraspanins [153,154], heat shock proteins 70 and 90 [167], and EpCAM [127,128].

3.4. Microfluidic approaches for capture and analysis of exosomes

Conventional approaches for isolation of exosomes include ultracentrifugation [145,168], ultrafiltration [139,169] or immunomagnetic bead based techniques [170–172]. While all three of these approaches successfully isolate exosomes, each is associated with disadvantages which can have a significant impact on targeted isolation of tumor cell associated exosomes which are observed at low frequency in circulation [173]. Ultracentrifugation is a time consuming (4–5 h) process, associated with low yield (<25% recovery), high impurity (all vesicular components), compromised integrity of the vesicle and need for highly skilled personnel [174,175]. Density gradient ultracentrifugation can enhance purity and fractionate vesicular components but the additional processing further complicates an already tedious process. Ultrafiltration using membranes with small pores has also been used to remove large contaminants via size-exclusion. Since this process relies exclusively on size as a basis for separation, protein contamination is a significant issue. Moreover, nanometer sized pores on the filters combined with small open area for fluid transport provides high fluidic resistance and requires large pressure to move fluid across the membrane resulting in limited throughput. Immuno-magnetic bead-based approaches offer a simpler alternative and are increasingly being used for exosome isolation despite issues with capture efficiency and purity. Finally, a simple and easy to use commercially available product Exoquick accomplishes isolation of exosomes via precipitation; despite its scalable nature, the yield of exosomes is low and variable [138]. Microfluidic options for exosome isolation are similar to those available for CTC isolation and either relies on immuno-affinity mediated capture or label-free approaches. Considering the size of exosomes, exploitation of physical properties to enable separation is limited in comparison to CTCs.

The main advantage of microfluidics based approaches for exosome capture is the adaptability for low abundance exosomes (i.e.) exosomes shed from cancer cells as opposed to exosomes commonly found in circulation. Microfluidic approaches for exosome isolation primarily rely on enhancing immuno-affinity capture via miniaturization and exploiting microarchitectures, high surface-area to volume ratios, and flow phenomena to immobilize a large number of capture probes and enhance and prolong interaction of exosomes with the capture probes. Other label-free microfluidic approaches have also been developed to isolate exosomes using electrophoresis, dielectrophoresis, sieves, physical trapping and optical trapping. Table 2 summarizes microfluidic approaches for both immuno-affinity and label-free capture of exosomes organized based on capture efficiency.

3.4.1. Immuno-affinity based approaches for exosome isolation

A microfluidic device was developed by Chen et al. as an alternative to conventional ultracentrifugation for isolation of exosomes [176]. To accomplish capture of exosomes, the authors covalently immobilized CD63, an antibody that is specific to exosomal vesicles to the surfaces of a microfluidic channel (Fig. 4A). The channel floor contained a herringbone structure to induce fluidic rotation within the channels and enhanced interaction of the exosomes within the channel with the capture antibodies on the walls. This technique was rapid, simple and processing of ~400 μL of serum samples from non-small cell lung carcinoma patients resulted in isolation of sufficient number of exosomes for isolation and analysis of tumor RNA [176]. Kanwar et al. took this concept further by enabling detection capabilities to quantify captured exosomes via staining with a fluorescent carbocyanine dye (DiO) that specifically labels

Table 2
Summary of microfluidic techniques for isolation of exosomes for diagnosis and evaluation of cancer. Note: Table is organized based on reported capture efficiency.

Exosomes				
Authors	Description of Method	Capture Efficiency	Purity	Sample
Shao et al. [183]	Immunomagnetic beads (Exo chip)	90%	–	Exosome isolation from plasma sample
Liang et al. [252]	Filtration	81%	90%	Exosome isolation from urine sample and cell culture medium
Liu et al. [191]	Viscoelastic flow	>80%	>90%	Exosome isolation from cell culture medium
Lee et al. [187]	Acoustic force	>80%	–	Exosome isolation from cell culture medium
Chen et al. [176]	Immunoaffinity	42-94%	–	Exosome isolation from serum sample
Zhao et al. [151]	Immunomagnetic beads	42-97%	–	Exosome isolation from plasma sample
Wang et al. [188]	Filtration through nanowires	45-60%	–	Liposome isolation from water/PBS
Santana et al. [189]	Filtration through microarrays based on the principle of deterministic lateral displacement	39%	99%	Exosome isolation from cell culture medium
He et al. [184]	Immunomagnetic beads	–	–	Exosome isolation from plasma sample
Kanwar et al. [177]	Immunoaffinity	–	–	Exosome isolation from serum sample
Dudani et al. [182]	Immunobead combined with inertial force	–	–	Exosome isolation from cell culture medium

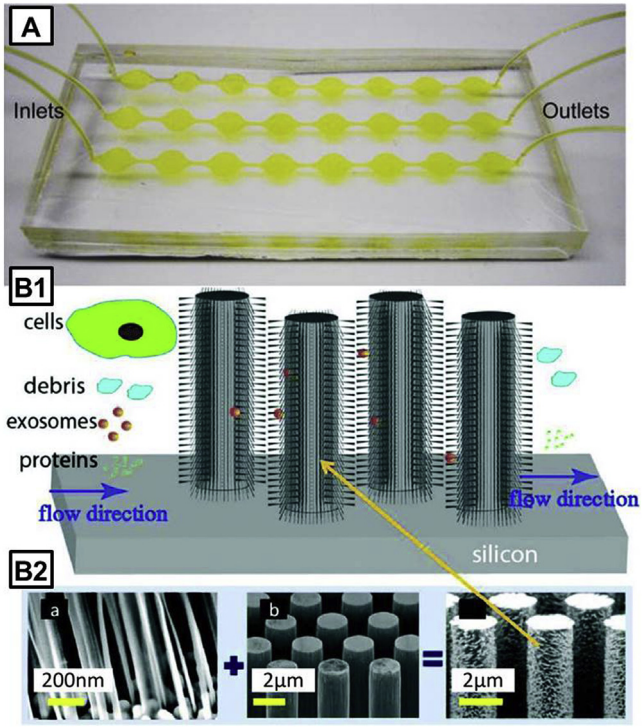

Fig. 4. Examples of microfluidic devices for isolation of exosomes: (A) Herringbone Chip: The herringbone chip was the first demonstration of microfluidics to isolate exosomes. Capture was accomplished using affinity of exosomes to specific antibodies immobilized within the device [176]. (B) Ciliated Structures: Ciliated structures exploit size difference between exosomes and cells in blood to enable selective trapping of exosomes for subsequent isolation [188]. (B1) Schematic demonstrating the process of size exclusion and capture using ciliated structures, and (B2) High magnification images of the posts with ciliated structures including and image of exosomes captured on the structures.

the exosomes and can be quantified using a standard plate reader [177]. Comparison of serum samples from patients with cancer and healthy controls revealed a nearly 2.5 fold increase in captured exosomes in cancer patients with detectable differences in miRNA profiling. After processing 400 μL of serum they were able to isolate nearly 15–20 μg of protein and 10–15 ng of total RNA [177]. Zhang et al. utilized Y-shaped microposts functionalized with CD81 antibody along with a graphene oxide/polydopamine coating to prevent non-specific adhesion and enhance capture of exosomes via an increase in capture surface area and reported a recovery yield of 72% with almost no non-specific binding [178].

Several other groups have combined immuno-affinity capture with different types of detection and quantification techniques. Examples include electrokinetic, force-based, optical, electrochemical, electro-optical and acoustic-based approaches. One specific example is the nano-plasmonic exosome sensor (nPLEX) where immuno-affinity capture within microfluidic channels is coupled with surface plasmon (SPR) resonance based detection [179]. Rather than focus on a single capture ligand, nPLEX contains an array of up to 36 different proteins to enable phenotyping of captured exosomes. Using this setup, ovarian cancer patients were differentiated from healthy controls via differential phenotyping [179]. While immuno-affinity capture on surfaces has been successful in isolating useful amounts of target exosomes, non-specific binding of contaminating species is a cause for concern [180,181]. These species can affect detection techniques and are particularly an issue when intra-vesicular contents of the exosomes need to be retrieved [181].

To enhance sorting efficiency and minimize non-target molecules and vesicles, Dudani et al. developed an approach which combines immuno-affinity capture of exosomes on modified polystyrene beads which were then separated from the original sample solution into a buffer via inertial migration in rectangular cross-section microfluidic channels [182]. Using this setup, exosomes were isolated from blood spiked with supernatant from cancer cell cultures. Following red cell lysis and incubation with the immuno-modified beads, a suitable number of exosomes were captured on the surface of the polystyrene beads [182]. While the separation mechanism exploits microfluidic advantages, a four hour incubation period is necessary for binding of the exosomes to the beads limiting the attractiveness of this approach for clinical applications. Several other groups (Shao et al. [183], He et al. [184] and Zhao et al. [151]) have also used immuno-affinity capture using functionalized microbeads to separate exosomes from bodily fluids using magnetic separation within microfluidic devices, however, only Shao et al. [183] reported capture efficiency of >93%, whereas; others did not evaluate capture efficiency of their devices. Typical samples volumes that can be processed are in the range of 10s–100s of microliters.

3.4.2. Label-free approaches for exosome isolation

The small size of exosomes limits the use of flow phenomena for focusing, trapping or directed migration within microfluidic channels. Microfluidic adaptation of ultrafiltration can potentially be used to fractionate exosomes based on size. One group attempted to achieve microfluidic ultrafiltration as a means to separate exosomes from whole blood [185]. Exosomes were transported across a size-exclusion membrane either using pressure or an electrical field (~80 ng RNA from 100 μL blood). While reported results seem comparable to conventional macroscale ultrafiltration, there was no significant improvement. While the pressure driven approach was faster, the electric field driven separation resulted in higher purity [185]. However, there appears to be several disadvantages associated with purity, throughput and processing speed. More recently, Woo et al. developed a lab-on-a-disc for exosome isolation called the 'Exodisc' which utilized centrifugal force for transport of fluid across a size-exclusion filter and using urine samples reported removal of >95% contaminating proteins and >95% recovery of exosomes in addition to reporting >2 orders of magnitude higher RNA than exosomes isolated using conventional ultracentrifugation [186]. Lee et al. accomplished capture of exosomes from packed red blood cell units via use of acoustic force to enable transport through a filter and demonstrated >80% recovery [187]. Another approach that exploits the size difference between exosomes and other larger cells, vesicles and molecular species in blood consists of an array of posts containing ciliated structures [188] (Fig. 4B). The ciliated structures are silicon nanowires and serve two purposes. First, they act as a barrier to the entry to larger contaminants. Once a vesicle interacted with the posts, it was trapped within the cilia ensuring capture of the target species. Depending on the spacing between the cilia, specific size vesicles can be targeted [188]. While capture is accomplished within 10s of minutes and imaging of vesicles is relatively straightforward, isolation of intact vesicles requires dissolution of the silicon nanowires and can be complicated. This device was tested with a binary mixture of vesicles of two different sizes [188]. It is difficult to speculate how this technology will translate to capture of exosomes from serum or blood in terms of throughput and purity. Santana et al. used micro-pillar architectures within microfluidic channels to separate exosomes from cell culture medium based on vesicle size and demonstrated recovery of >35% of all vesicles [189]. More recently, the use of deterministic lateral displacement (LDL) was used in silicon nanofluidic devices where the precisely machined silicon structures in low Peclet (Pe)

number flows enabled particle movement via diffusion and displacement to compete with each other resulting in separation of exosomes and colloids between 20 and 110 nm [190]. Liu et al. exploited elastic lift forces when samples are suspended in a viscoelastic medium to enable sorting of exosomes from other vesicular bodies and reported a >80% purity and >90% recovery of exosomes in fetal bovine serum [191].

Microfluidic technologies for exosome isolation are still in early stages of development but results confirm that microfluidics may provide simpler and efficient techniques to conventional filtration and centrifugation based approaches. A better understanding of the utility of exosomes in cancer monitoring and a better understanding of the physical and biochemical markers of target exosomes will enable development of more sophisticated microfluidic approaches.

4. Cell-free tumor cell DNA (ctDNA) and circulating RNA

4.1. Origins and frequency of occurrence

DNA: Recent high throughput sequencing studies have identified specific genetic mutations that enable survival and expansion of solid tumors. Virtually all cancers carry somatic DNA mutations which are only present in tumor cell DNA. Identifying tumor DNA provides a highly specific biomarker that can be used to for disease diagnosis and monitoring. While tumor cell DNA is abundant in tumors, their analysis requires invasive biopsies or the capture and analysis of CTCs. Tumor cell associated DNA has been reported in blood samples of patients with malignant and non-malignant cancer [192]. Presence of tumor DNA in blood is a consequence of tumor cells undergoing apoptosis or necrosis causing nucleosomes to be released into blood, circulating freely in plasma [193,194]. Necrosis results in larger fragments of up to 10,000 base pairs (bp) and apoptosis leads to DNA fragmentation resulting in fragments as small as ~ 100 bp. The half-life of cell free ctDNA is very short and is cleared from circulation within a couple of hours. In aggressive cancers, the increased tumor burden results in higher levels of necrosis leading to large amounts of cell free ctDNA [195].

RNA: Tumor cell associated RNAs have been reported in blood liquid biopsies of patients with different types of solid tumors [196]. Considering the role of messenger RNAs in cellular function, RNA from apoptotic or necrotic cancer cells in circulation could potentially provide valuable information regarding intracellular tumor cell phenotype and function. There are several types of circulating RNA including coding RNA or messenger RNA (mRNA), and non-coding RNAs like micro RNA (miRNA), long non-coding RNA (lncRNA), and small interfering RNA (siRNA). The abundance of mRNA in circulation is low as it is unstable and subject to degradation. In contrast, non-coding RNAs are stable and usually found at detectable levels within circulation [197].

4.2. Predictive information in ctDNA and circulating RNA

The identification and relative amount of ctDNA in circulation correlates with tumor burden. Screening for ctDNA within blood can provide a monitoring tool for early diagnosis and monitoring of response to treatment. The size of ctDNA fragments can provide information regarding the mechanism of cell death which can be used to understand tumor regression or remodeling. The most promising utilization of ctDNA may in the identification of specific mutations that are predictive of acquired resistance to chemotherapy via selection of resistant tumor cells during drug treatment. The tumor genome also plays a critical role in how patients respond to chemotherapeutic drugs. Genome sequencing has resulted in identification of somatic genetic mutations and genomic, transcriptomic and epigenetic changes in various tumors have being mapped. This information can be used to develop chemotherapeutic drugs that have the best chance for clinical benefit in cancer patients.

The presence of circulating mRNA in cancer patients has been known for over two decades. Evaluation of circulating mRNA can potentially provide information regarding key intracellular processes in tumor cells and serve as biomarkers for diagnosis and monitoring. Their increased presence in serum or blood has been previously found to be predictive of clinical outcome and disease prognosis [198,199]. Of the non-coding RNAs, miRNA is the most promising target and is actively being evaluated as a cancer biomarker. miRNAs are highly conserved short strands of non-coding RNA and is known to play a role in repressing or activating translation of proteins. miRNAs are known to be dysregulated in cancer enabling crucial processes like proliferation, metastasis, apoptosis and angiogenesis. Profiling and quantification of miRNAs in circulation can provide important information for diagnosis, longitudinal patient monitoring and development of therapeutics [200–202]. Other non-coding RNAs like small interfering (siRNAs) and long non-coding RNA (lncRNAs) are attractive targets, however, there is not much information regarding how they might influence onset and progression cancer.

4.3. Microfluidic approaches for capture and analysis of ctDNA and circulating RNA

The use of microfluidic approaches for DNA and RNA isolation in the context of cancer has not been extensively explored. This can be attributed to the fact that there is limited information available in terms of circulating DNA or RNA in blood or serum samples and their relevance to cancer. While microRNAs are stable and commonly found in circulation, other nucleic acids like DNA and mRNA are usually found within intact cells or exosomes where they are protected from degradation and clearance. In cancer, rapid turnover of cells via either apoptosis or necrosis results in detectable amounts of cell-free ctDNA and mRNA in circulation with the amounts directly correlating to stage of cancer and the size of the tumor [193,194]. While tumor cell DNA and mRNA can provide valuable insights into specific mutations and transcriptional activity, the use of this information for evaluation of cancer requires more extensive investigation.

Microfluidic approaches for isolation of nucleic acids present both in low and high abundance have been widely explored for several non-cancer based applications. These techniques are broadly applicable to any disease where nucleic acids need to be isolated from fluids that contain other molecular and cellular contaminants. Therefore, in this section we will review promising microfluidic-based approaches for nucleic acid isolation from blood or plasma samples that can be readily adapted for cancer diagnostics. Microfluidic approaches for nucleic acid isolation are typically preferred if the sample volume is small and if either the target nucleic acids occur at low frequencies. Microfluidics also offers rapid and high-efficiency separations with the potential to integrate pre- and post-isolation process into a seamless fashion to create point-of-care (POC) technologies that can be deployed in resource limited settings or in the clinic. Microfluidic devices for isolation of nucleic acids can be broadly classified as either solid phase isolation or liquid phase isolation. Solid phase isolation techniques rely on the use of a charged surface or immobilized probes to bind and capture nucleic acids. Liquid phase isolation techniques rely on the mobility of nucleic acids via charge/polarizability or solution chemistry for separation. A summary of possible microfluidic approaches that can be adapted for capture of ctDNA and circulating RNA are listed in Table 3 and organized based

Table 3
Summary of microfluidic approaches for isolation of nucleic acids. Note: Table is organized based on reported capture efficiency of the approach.

Cell-free nucleic acids				
Authors	Description of Method	Capture Efficiency	Purity	Sample
Wu et al. [229]	Photoactivated polycarbonate surface	98%	–	DNA extraction from E.coli
Cao et al. [234]	pH mediated Chitosan-coated beads	75%	–	DNA extraction from lysed whole blood
Wu et al. [216]	Monolithic tetramethyl orthosilicate-based sol-gels	~70%	–	DNA extraction from lysed whole blood
Wen et al. [215]	Capillary-based photopolymerized monolith	69%	–	DNA extraction from whole blood
Duarte et al. [218]	Magnetic silica beads in polyester microfluidic device	>65%	–	Nucleic acid extraction from lysed whole blood
Günal et al. [206]	Monodisperse-porous silica microspheres	50%	–	DNA extracted from lysed whole blood
Nakagawa et al. [239]	Amine-coated surface based on the electrostatic interaction between surface amine groups and DNA	27-40%	–	DNA extraction from lysed whole blood
Sonnenberg et al. [245]	Dielectrophoresis	550 ng/ml from 25 μl	–	Cancer related DNA isolated from whole blood directly
Zhang et al. [247]	Phenol-chloroform extraction of nucleic acid from low copy/single bacteria	10 fold higher	–	DNA isolation from P.aeruginosa
Yang et al. [246]	Electrophoresis	–	–	DNA extracted directly from blood plasma
Persat et al. [244]	Isotachophoresis	–	–	Nucleic acid extraction from lysed whole blood
Root et al. [232]	Oligonucleotide capture matrix	–	–	RNA extraction from 10% serum sample

on reported sensitivity of the approach.

4.3.1. Microfluidic solid phase extraction

The microfluidics field has heavily borrowed techniques and processes from the semiconductor fabrication industry and it is not surprising that a majority of early generation microfluidic devices used silicon as the primary substrate for device fabrication. Silica is negatively charged and when treated with high concentrations of a chaotropic agent like guanidinium or sodium iodide, a salt bridge forms between the negatively charged silica and the negatively charged nucleic acid backbone to enable non-specific binding [203]. To capture cell-free nucleic acids, it is important to debulk the sample of cells and vesicles to ensure that cellular or vesicular nucleic acids are not captured as chaotropic agents can potentially cause lysis and release of intra-cellular or intra-vesicular contents. Captured nucleic acids can be extracted by washing with organic solvents like ethanol or isopropyl alcohol. The most basic version of this device is a rectangular cross-section microfluidic channel which still has significantly larger surface area to volume ratio than available with any macroscale approach. Silica beads packed within microfluidic channels have also been used for capture of nucleic acids to further increase the capture surface area [204–206]. Beads are packed into narrow microfluidic channels or capillaries using a weir structure that enables packing of beads within the channel. Packed silica beads have been successfully used to capture both DNA and RNA which have either been eluted for further processing or subsequent processing including amplification and detection performed on-chip [207]. A major disadvantage of this approach is the extremely high fluidic resistance resulting in high back pressure. An improvement over packed silica bead capillaries is embedding of silica beads in hydrogels [208]. Silica beads were directly incorporated into the hydrogel solution prior to gelation resulting in free standing structures or channels filled with silica bead containing hydrogel. Due to the high porosity of the hydrogel, the back pressure necessary to feed sample through is diminished. Successful DNA isolation has been demonstrated using this approach. Hydrogels have been incorporated into both glass and polymer microfluidic devices with the polymer devices providing cheaper alternatives. Variations of the same approach have been accomplished using construction of silicon microstructures using surface micromachining or bulk micromachining approaches with the goal of increasing surface area available for a given volume for nucleic acid capture [209–213]. Precisely defined microstructures greatly minimize variability typically seen with packed beads or beads in hydrogel but the cost of manufacturing these structures is high. Silicon microstructures have been used for DNA isolation and integrated within micro total analysis systems (μTAS) for seamless integrated processing. There are also several other examples including silica membranes [214], porous silica-based structures [215], etched silica structures [216] which have all been used for nucleic acid isolation The major drawback of silica or silicon based capture is the use of the harsh chaotropic agent which can interfere with downstream processing [207].

An alternative to immobilized beads is the use of paramagnetic beads to enable either immobilization or extraction of captured nucleic acids to minimize the amounts of non-specific contaminants [217–220]. Paramagnetic beads have large surface area to volume ratio, can be temporarily immobilized to interact with sample and then extracted during washing steps from the microfluidic device or moved to another chamber within the microfluidic device for subsequent processing. Other tunable features that can be adjusted depending on the application are: the charge on the surface of the beads, surface chemistry, and immobilization of specific capture moieties. Beads can be introduced in suspension within the sample and efficient mixing can enhance interaction of the magnetic beads with targets thereby minimizing incubation times. Examples of paramagnetic bead-based approaches include silica coated beads that possess the ability to bind nucleic acids non-specifically in the presence of a chaotropic agent. The most common approach using silica coated paramagnetic beads involves immobilization of beads within a microfluidic channel using an external magnetic field with sample solutions and washing buffers flowing over the beads. Once the capture and washing steps are complete, the magnetic field is removed to allow elution of the beads in an elution buffer [218]. In other examples the solution is maintained stationary and the beads directed through the solution via application of a magnetic field. The beads are then exchanged from the sample solution into buffer solutions [221,222]. There are also examples of silica coated paramagnetic bead-based isolation techniques integrated within μTAS [219]. Alternatives to silica coating include the use of electrical charge to selectively capture negatively charged nucleic acids. This is accomplished by coating the beads with a multivalent cationic polymer like polyethyleneimine which is positively charged at low pH. The sample is introduced into the microfluidic device where beads are immobilized in a low pH solution for capture, following capture, the solution is washed and then a higher pH elution buffer is used to collect the captured nucleic acids [223,224]. This approach has been shown to be highly effective for low abundance nucleic acids and has been integrated within μTAS [220]. Finally, isolation of mRNA can also be accomplished using non-

specific probes such as oligo-dT which binds to the poly-adenosine tail. This technique provides a degree of specificity in isolating only mRNA sequences and the combination of this approach with paramagnetic beads can be scaled up as the beads are not saturated with non-specific nucleic acids [225,226]. This approach can potentially be of great value when probing cell-free mRNA released from tumor cells into circulation. If the target nucleic acid sequence is known, then specific oligonucleotide sequences can be generated and functionalized into paramagnetic beads to enable capture of desired targets [217,227,228]. The only drawback of this approach is the fact that there needs to be prior knowledge regarding the target sequences.

Microfluidic devices with functionalized surfaces have also been used for capture of nucleic acids. Depending on the type of material used to construct the microfluidic device, these approaches can vary. Silicon and glass have been commonly used for fabrication of microfluidic devices but are expensive. Cheaper alternatives include various polymeric materials that have already been shown to be effective for nucleic acid isolation including polycarbonate (PC) [229], (poly) dimethyl siloxane (PDMS) and polymethylmethacrylate (PMMA). Surface modifications can either be accomplished via simple adsorption, covalent cross-linking or deposition. Examples of coatings used for nucleic acid capture include oligonucleotides [230–232], chitosan [233,234], aluminum oxide [235,236], activated polycarbonate [237,238] and amines [239].

4.3.2. *Microfluidic liquid phase extraction*

Liquid phase extraction refers to techniques that do not utilize probes to capture nucleic acids, rather, nucleic acids are selectively trapped or migrated based on their response to an applied electric field or specific solution chemistry. Both electrophoresis (EP) and dielectrophoresis (DEP) have been used to selectively migrate or trap negatively charged nucleic acids to separate them from positively charged contaminants. DEP is primarily used for trapping and has been used with the commercially available Nanogen platform that exploits the polarizability of nucleic acids to trap chromatin from lysed cells using an alternating field and then using a direct current field to remove contaminants [240,241]. Gel electrophoresis within microfluidic channels has been used to separate nucleic acids based on their differences in length [242]. Nucleic acids fractionated using this approach can be extracted as it exits the gel using buffer solutions. Another approach that exploits charge is isotachophoresis where a two buffer system containing a leading and trailing electrolyte with electrophoretic mobility higher and lower than the nucleic acids respectively. When an electric field is applied, the nucleic acids migrate to the interface where the field gradient exists away from the impurities. Microfluidic adaptation of this approach has been successfully validated for isolation of DNA from lysed blood samples [243–246]. While charge based approaches are effective and can result in highly pure nucleic acid samples, they are more complex in terms of both device fabrication and device operation. The throughput may also be limited and their integration with μTAS may be more complicated.

Liquid phase extraction using organic solvents like phenol-chloroform extraction is a commonly used macroscale approach to exploit aqueous and organic phases to enable partitioning of biomolecules into energetically favorable locations. Following cellular lysis, biomolecules including cellular debris and proteins fractionate into the organic phase leaving nucleic acids in the aqueous phase. This approach has been adapted within a microfluidic platform which allows automated metering and collection of the two phases which can then be processed on-chip for subsequent amplification and detection [230,247]. The purity of nucleic acids attainable using this approach is very high but there are also issues involving the use of hazardous solvents which make operation and disposal a problem.

Given that circulating cell-free DNA and RNA are not aggressively being pursued as markers to monitor cancer, there have not been many efforts to develop microfluidic devices to isolate and evaluate circulating cell-free nucleic acids. However, recent literature suggests that both cell-free DNA and RNA may contain important prognostic and diagnostic information that can be harnessed to monitor, stage and treat cancer. This section summarizes microfluidic approaches for capture of nucleic acids and any of these approaches can be adopted and modified to process blood liquid biopsies. There may be some sample pre-processing that needs to be accomplished prior to nucleic acid isolation. We also refer you to recent reviews by Ansari et al. [248] and Bruijins et al. [249] that provide greater detail on microfluidic approaches for isolation and analysis of nucleic acids.

5. Future directions

Miniaturization offers advantages over conventional approaches and can enable completely new separation methods not possible using macroscale techniques. Despite these obvious advantages, adoption of microfluidics based approaches for clinical or commercial applications has not been widespread. This can be attributed to the complexities associated with translating laboratory prototypes to pre-clinical or clinical devices and the reluctance of users to adopt new and unfamiliar technologies. Prior to pursuing a microfluidics option, it is important to ask the following questions **(1)** Is there a critical or unmet clinical need? **(2)** Will this problem benefit significantly from miniaturization? **(3)** Can this process be performed reliably and consistently in the clinical setting? **(4)** Can the miniaturized protocol be automated with minimal user intervention? As long as the answer to the first two questions is ‘yes’ and the answer to the third and fourth questions are not ‘no’, it is worth the time and effort to develop microfluidic options to address the particular problem.

For cancer diagnostics, the answer to the first two questions is ‘yes’ and recent progress in CTC and exosome isolation suggest that the answer to both questions three and four is ‘highly probable’. Isolation of CTCs for example has been likened to ‘finding a needle in a haystack’ where the need is to develop technologies to find 1–10 cells in 10 mL of blood, 1 in 1×10^9 RBCs or 1 in 1×10^7 WBCs. This is a problem that conventional macroscale approaches cannot address in a reliable fashion with consistently high levels of purity and efficiency. Another critical issue is the biomarkers used to classify and isolate CTCs and exosomes. CTCs and exosomes are highly heterogeneous populations and expression of various cell surface and intracellular markers are reflective of various factors including the physiology of the individual, organ/tissue of origin, interaction with the local microenvironment, location within the primary tumor, stage of the tumor, mechanisms that enabled release into circulation, their interaction with other cellular and biomolecular components in blood. Therefore it is important to understand that biomarker selection is critical and can dictate the success of the type of separation process in demonstrating clinical benefit. Microfluidic approaches have shown clear superiority where miniaturization and unique flow phenomena have been exploited to isolate CTCs in numbers not possible using conventional methods. This high sensitivity in terms of being able to probe low-abundance targets comes at the cost of throughput and purity. Most CTC platforms have high efficiency (ratio of captured target cells to target cells in the sample) but there is also an increase in non-specific binding of leukocytes. Profiling of cells contained in

small volumes adversely affects throughput and this can be a major issue with CTC profiling as sometimes ~50 mL of blood needs to be processed to obtain a significant number of cancer cells for analysis. To address the issue of throughput, integrating multiple smaller devices in a massively parallel fashion has been proposed. This however is a challenge and care needs to be taken to ensure that there is a high-level of consistency between devices integrated in parallel. Other options include sample pre-processing to de-bulk the initial sample of contaminating cells. Any technique seeking to address CTC isolation must understand and provide solutions that can address issues with processing of 10s of mLs of sample in a relatively quick time. Techniques like density gradient centrifugation or even inertial focusing have been used to separate out red blood cells from the samples to provide highly de-bulked samples that contain CTCs at higher concentrations and are easier to process. The disadvantages include the time and effort to de-bulk the samples and additional opportunities to lose already rare CTCs during processing. The issue of non-specific binding can be partially addressed by using appropriate blocking strategies with surface functionalization using proteins (albumin) or cell repulsive compounds like (polyethylene glycol or pluoronic). Important parameters to be considered include functionalization density and anchorage mechanism which can greatly affect their function and utility. Addressing or overcoming issues related to purity and throughput and increasing numbers of captured intact CTCs is the key to overcoming important gaps in knowledge. Given the multitude of uses of intact CTCs in early diagnosis, longitudinal monitoring of patients, drug testing and developing patient specific therapeutics, there are a large number of companies developing CTC isolation kits. A majority of these companies have adopted microfluidics-based approaches [10,13,15,18,21,22,46,47] and are faced with the challenge of addressing concerns with throughput and purity.

Microfluidic isolation of exosomes faces similar challenges to those associated with CTC isolation, however, the number of exosomes is significantly larger than CTCs and issues concerning throughput are minimal. Given the success of conventional macroscale approaches in isolating intact exosomes, microfluidic approaches may need to focus on isolation and quantification of cancer specific exosomes which are available at lower frequencies and where microfluidic advantages associated with interrogation of samples in small volumes is a significant advantage. While microfluidics may not have significant benefit over conventional approaches when it comes to unbiased evaluation of exosomes in serum, microfluidics certainly has an advantage when it comes to identification cancer cell-associated exosomes with specific surface markers. Microfluidic approaches can be used to accomplish rapid and highly efficient identification with opportunities to integrate further analytical complexity downstream including lysis and extraction of nucleic acids, proteins, metabolites and lipids. An area that needs to be addressed is the sample pre-processing to rapidly remove cellular components and abundant proteins from plasma without loss of exosomes. Currently, the use of exosomes for cancer diagnostics is still in the early stages and significant work needs to be done prior to use of exosomes or exosomal contents for diagnosis or patient monitoring. As a result, there are not many efforts to commercialize platforms for exosome isolation for cancer. Examples discussed in this review clearly demonstrate that there is great potential for microfluidic approaches and with advances in our understanding of exosome cancer biology and advancements in microfluidic separations, it is only a matter of time before commercially available exosome based cancer screening assays become available.

A variety of microfluidic techniques for capture and isolation of nucleic acids were discussed in this review and clearly establish that microfluidic adaptation can accomplish both specific and non-specific capture of target nucleic acids in a highly efficient and rapid fashion. Microfluidics provide two unique advantages: **(1)** integration of nucleic acid capture within μTAS to accomplish cell capture, lysis, nucleic acid isolation, amplification, quantification, and detection all on a single platform for point-of-care applications, and **(2)** The ability to isolate low-abundance nucleic acids found in circulation or in a single cell with high efficiency. While there are several examples of integrated devices, the integration of different components is not always seamless and operation of these devices may also be complicated. Regardless, with advancements in technologies to design and fabricate microfluidic devices will provide unique tools to probe low-abundance nucleic acids efficiently and rapidly. As with exosomes, there is limited knowledge regarding cell-free DNA and RNA and how they can be exploited for cancer diagnostics and monitoring. There are already a handful of companies offering tests to probe cell-free tumor cell DNA and it appears that with new knowledge regarding the relevance of these markers, that the number of applications will expand rapidly and microfluidics-based approaches will become essential for point-of-care translation.

As mentioned earlier, a major concern with microfluidics-based approaches is the poor rate of translation of promising technologies to the research or clinical setting and ultimately commercialization. Particularly in the area of cellular and biomolecular separations, it is rather surprising that microfluidics-based approaches have not supplanted conventional approaches given the advantages of microfluidics-based technologies. In reviewing literature, there appears to be a large disparity between the numbers of new microfluidics technologies being developed versus those actually undergoing commercialization or clinical translation. To achieve commercialization, it may be beneficial to learn prior shortcomings. Shields et al. [250] present a comprehensive review detailing the challenges with translation of microfluidics-based approaches for cell sorting including **(a)** Changing from a mindset where we develop technologies and then search for an application to a mindset where we design new approaches based on a critical unmet need, **(b)** creating user-friendly interfaces that can enable ease of operation of complex microfluidic devices, minimizing the need for initial setup for smooth translation as a commercial product, **(c)** developing modular solutions and standardized manufacturing to address the cost and volume issues necessary for successful commercialization, and **(d)** adopting aggressive strategies to protect intellectual property and efficient marketing new technologies. For other applications, conventional technologies may provide a comparable solution; but in the case of cancer monitoring, the low abundance of target species necessitates successful translation of microfluidics based approaches to accomplish better treatment options and disease management. Therefore, it is critical that these issues be carefully considered prior to development of new technologies to enable smooth and rapid translation to the research and clinical setting.

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