Cancer Biol Med 2018. doi: 10.20892/j.issn.2095-3941.2018.0256

REVIEW

Advances in isolation and detection of circulating tumor cells based on microfluidics

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ABSTRACT Circulating tumor cells (CTCs) are the cancer cells that circulate in the peripheral blood after escaping from the original or metastatic tumors. CTCs could be used as non-invasive source of clinical information in early diagnosis of cancer and evaluation of cancer development. In recent years, CTC research has become a hotspot field wherein many novel CTC detection technologies based on microfluidics have been developed. Great advances have been made that exhibit obvious technical advantages, but cannot yet satisfy the current clinical requirements. In this study, we review the main advances in isolation and detection methods of CTC based on microfluidics research over several years, propose five technical indicators for evaluating these methods, and explore the application prospects. We also discuss the concepts, issues, approaches, advantages, limitations, and challenges with an aim of stimulating a broader interest in developing microfluidics-based CTC detection technology.

KEYWORDS Circulating tumor cells; isolation and detection; microfluidics

Introduction

To date, cancer is one of the leading causes of death in human beings. Statistics show that 90% deaths of cancer patients are related to cancer metastasis[1]. Detection of cancer mainly includes conventional instrumentation methods and non-invasive methods. In general, detection on conventional instrumentation is more complicated, expensive, and harmful to the health of patients. The non-invasive methods refer to judging the stage of cancer development or assessing the effect of cancer treatment by analyzing the changes of certain components in human urine and peripheral blood. Therefore, the non-invasive methods are more convenient, accurate, and harmless to patients, and have become the mainstream of cancer diagnosis in the future.

Studies have shown that the number of circulating tumor cells (CTCs) in peripheral blood is closely related to the degree of cancer cells metastasis and the stages of cancer development. Therefore, the stage of cancer development can be evaluated by detecting the number of CTCs in peripheral blood[2].

In general, detection of CTCs includes four stages: capture, enrichment, detection, and release[3]. Capture mainly refers to physical interaction or antibody/antigen interaction between CTCs and materials (antibodies/aptamers). Enrichment refers to isolation of CTCs from the peripheral blood and detecting them by using various methods including fluorescence microscopy[4], fluorescence spectrophotometery[5], optical microscopy[6], surface-enhanced Raman scattering (SERS)[7], flow cytometry[7,8], and electrochemical methods[9]. In recent years, many novel materials have been proposed for detection of CTCs. Tang et al.[10] reported an immunoassay protocol for the detection of CTCs by using Pt@Ag nanoflowers (Pt@AgNFs) and AuNP/Acetylene black (AuNP/AB) nanomaterials. Such materials are not only biocompatible, but also can amplify the current signals. Li et al.[11] proposed a detection method by using quantum dots and gold nanoparticles as signal probes. The enriched CTCs can also be released for further phenotype identification and molecular analysis[3]. CTCs collection and characterization depend on both technical methods and biological properties of the tumor cells that is being studied[12].

CTC detection includes five technical indicators: capture rate, purity, limit of detection (LOD), throughput, and

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Received August 14, 2018; accepted October 19, 2018.
Available at www.cancerbiomed.org

biocompatibility. The capture rate refers to the proportion of CTCs captured in the total number of CTCs in the blood sample, also known as capture efficiency or recovery rate. The purity is defined as the ratio of CTCs to the total number of cells in the enriched sample. LOD refers to the detection limit of the CTC concentration in the blood[3]. The throughput is defined as the volumetric rate for processing blood samples[13]. Biocompatibility means the cells that can maintain integrity even after sequential processing.

Many technologies and devices have been developed to capture and enrich CTCs, including filtration, centrifugation, magnetic separation, micro/nano substrates, and microchips[14]. Microfluidic chip integrates conventional, chemical or biological laboratories onto a micrometer-scale chip to achieve reaction, separation, detection, cell culture, and other basic operations[15,16]. Therefore, among all the technologies, microfluidics shows significant advantages such as small size, less sample and reagent demand, short analysis time and high efficiency, high sensitivity and precise operation, miniaturization, and portability. Furthermore, the geometrical dimension of the microfluidic channel matches the cell size and shape very well. Hence, various operations like controlling the cellular environment and analyzing cellular behaviors at the single-cell-level can be identified by controlling the flow field or design of microfluidic channel. Therefore, microfluidics is becoming ideal tools for CTC isolation in the future[17-19]. In this review, we focus on the discussion of CTC isolation and detection methods based on microfluidics.

Isolation and detection methods of CTCs based on microfluidics can be divided into two categories: label-free and label-based methods.

The label-free methods are mainly based on cell physical properties including size[20], deformability[21,22], compressibility[23], density[24], dielectric properties[25], and viscosity[9,26]. Many technologies and structures have been developed to realize the label-free isolation and detection of CTCs including micropores[9,27], micropillar arrays[28,29], deterministic lateral displacement[30], spiral channels with Dean flows[31,32], vortex-mediated deformability cytometry (VDC)[21,33], inertial focusing[34], acoustic waves[35-37], dielectrophoresis[38], and optical methods[39,40]. Recently, many CTCs filtration devices have been fabricated using various polymers including parylene, SU8, polydimethylsiloxane (PDMS), polyethylene glycol diacrylate (PEGDA), and palladium[20]. Label-free methods are generally simple to process and do not need expensive biomarkers and long incubation time. However, due to the poor specificity, the isolation purity is relatively low. Leukocyte contamination is the major concern in isolation of CTCs.

The label-based methods are mainly based on the affinity binding between CTCs and molecular recognizers based on unique surface protein expression of CTCs. Molecular recognizers mainly include antibodies, transferrin, sialic acid, and peptides[25]. The label-based methods could be divided into two categories: one uses the nanoparticle-coated microchannels. Nanoparticles could be spin-coated on the channel with certain pattern[41], or the channel could be designed with different structures like herringbone structure[42] to increase the rate of affinity. The other category involves the use of nanoparticle-coated microstructures including electrospun fibers[43], medical wires[44], nylon fibers[45], and surface-modified vein indwelling needles[46]. Furthermore, label-based methods can also be divided into another two categories depending on the type of target cells: negative sorting and positive sorting. In negative sorting, leukocytes are considered as the target cells. The most widely used biomarker, anti-CD45, is used for affinity binding with leukocytes. In recent years, many other biomarkers for leukocytes, such as anti-CD66[47] and CD15[13], have been proposed. Positive sorting considers CTCs as target cells. Most of the positive sorting methods use epithelial cell adhesion molecule (EpCAM) as the specific biomarker. However, once CTCs undergo the epithelial-mesenchymal transition (EMT), the methods based on EpCAM could be ineffective because EMT causes lower expression of EpCAM. Thus, those CTCs with low EpCAM expression or EpCAM-negative could be lost, which will severely decrease the capture rate. Furthermore, most of the positive label-based methods are not conducive to further cell analysis because cells are either dead or immobilized to a surface after positive sorting[48].

Considering the limitations of label-free methods and label-based methods, many researchers have been trying to combine label-free methods and label-based methods to improve the isolation efficiency. One of the successful case is CTC-iChip[30,47]. The chip mainly includes two structures. First, non-nucleated cells and free beads are eliminated by deterministic lateral displacement. The remaining white blood cells (WBCs) and CTCs are then subjected to inertial focusing for precise positioning. Finally, leukocytes and CTCs are separated by magnetophoresis based on anti-CD45 and anti-CD66 beads. Meunier et al.[49] proposed antibody-functionalized microfilters to isolate CTCs based on both size and antigen-antibody affinity binding. Numerous methods based on microfluidics have been proposed in recent years; however, most of them could not be clinically applied because of the complex control and processing, low

throughput, and high costs of microfluidic chips. Until now, CellSearch is the only FDA approved assay for CTC enumeration, which can be used as a prognostic test for certain types of cancer[50]. In this review, we propose five main challenges and corresponding methods for the isolation and detection of CTCs based on microfluidics (**Figure 1**), which emerged from 2015 to 2018.

Current key challenges and corresponding methods

The low abundance of CTCs in peripheral blood

It is accepted that to achieve a reliable information about CTCs at least 3 mL of patient's blood needs to be processed. In general, it is estimated to contain approximately 10^9 non-nucleated red blood cells, 10^7 nucleated blood cells[39] and 10^8 platelets[44] per mL of whole blood. However, there are only 1–10 CTCs per 7.5 mL of whole blood. Therefore, sorting and detecting CTCs in such a huge blood cells is a major challenge.

Han et al.[51] reported a new material DNA nanospheres (DNANSs) produced using the rolling circle amplification technique. Anti-epidermal growth factor receptor antibodies (anti-EGFR Abs) were conjugated to the DNANSs, which could be used to confirm the presence of CTCs. A few of anti-EGFR Ab-DNANSs could be bound to one tumor cell simultaneously, so that the amplified fluorescence signals could be induced. The signals could be used to detect single cell or diverse cancer cell types simultaneously.

Yoon et al.[52] designed a flow-restricted microfluidic trap array for deterministic single-cell capture of CTCs. Every cell capture site had two paths: a trapping channel, and a bypass channel to the next capture site. The flow resistance to the trapping channel was designed lower than that of the bypass channel. Once the capture site was occupied by one cell, the resistance became relatively higher to guide the approaching cells to the next capture site, ensuring single-cell capture for every capture site. The flow-restricted trap array achieved a

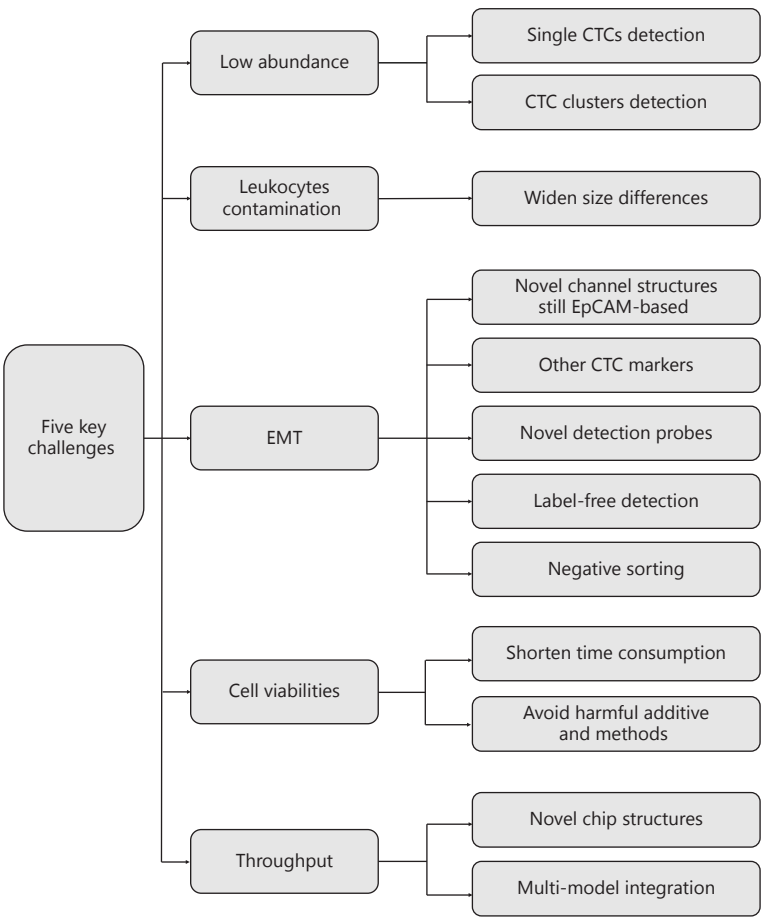

Figure 1 Five key challenges and corresponding methods based on microfluidics of current CTCs detection.

capture efficiency of 71% from whole blood drawn from mice with full-blown cancer metastasis.

Among the CTCs, CTC clusters are multicellular groupings of primary tumor cells, and have 23 to 50-fold increased metastatic potential than single CTCs[14]. There are two reasons to support above conclusion. Firstly, CTCs in the cluster have a better survival rate than single CTCs because of the cell-cell adhesion between CTCs in the CTC cluster that could prevent the cells from anchorage-dependent apoptosis. Secondly, the CTC clusters in a blood circulation system can combine with the surrounding blood cells, mesenchymal cells, epithelial cells, and platelets to allow them to escape from the immune cell attacks, and to promote cancer metastasis. Furthermore, some clinical studies have shown that cancer metastasis is related to the size and concentration of the CTC clusters in the blood[2]. In recent years, many methods have been proposed to isolate and detect circulating tumor single cells and clusters simultaneously.

Bhagwat et al.[53] demonstrated the first pre-clinical demonstration a platform for enriching and isolating single cells and clusters. The platform integrated magnetic separator, acoustic microfluidic platform, and in-line BD influxTM cell sorter (**Figure 2A**). The processing steps include: (i) immunomagnetic depletion of WBCs and red blood cells (RBCs); (ii)microfluidic acoustic washing and debris removal in-line with cell sorting; (iii) a 200 μm nozzle configuration with more than 5-fold decrease in sheath pressure for simultaneous capture of single cells and clusters.

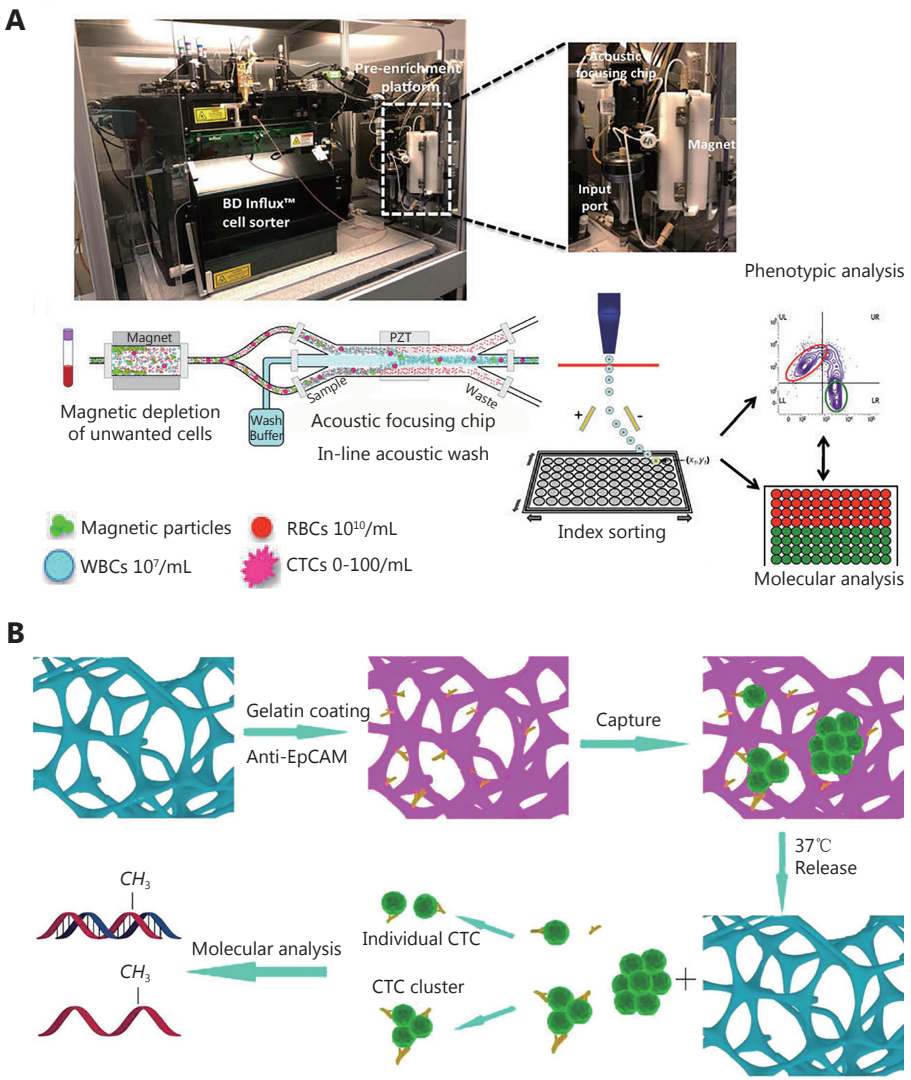

Figure 2 (A) Integrated pre-enrichment platform and workflow from ref. 53 with permission from Nature Scientific reports, copyright 2018. (B) Scheme of capture and release of individual and cluster CTCs from ref. 14 with permission from Analytical Chemistry, copyright 2017.

The average recovery rate was 77% and the possible sources of cell loss were discussed. About 10% of spiked cells were found in the waste from the acoustic focusing chip, 5% of cells were found in the magnetic tubing, and the remaining fraction was probably lost in the dead volume of the tubing. Besides, in 4 out of 7 replicates, the LOD of the platform was 1 cell in 1 million WBCs and CTC clusters >5 cells from whole blood.

Chiu et al.[2] proposed an optically-induced-dielectrophoresis (ODEP)-based microfluidic system for isolating cancer cell clusters based on their size characteristics. The chip was designed with a T-shaped microchannel containing a main and a side microchannel. A dynamic square light image array was implemented at the junction of the main and side microchannels as a virtual cell filter. This virtual cell filter could isolate cancer cell clusters from the leukocytes based on their size difference. The captured CTC clusters were then delivered to the side microchannel for second-step isolation for higher purity. The H209 small cell lung cancer cells and leukocytes were spiked to assess the system. The results demonstrated that the proposed chip could isolate CTC clusters with a purity of (91.5 ± 5.6)% and a recovery rate of (70.5 ± 5.2)% with the sample flow rate at 0.5 μL/min.

Cheng et al.[14] presented a 3D scaffold gelatin-microchip for capturing and reversible releasing of single CTCs and CTC clusters (**Figure 2B**). The 3D PDMS scaffold was coated with gelatin through layer-by-layer assembly. Since gelatin dissolves at physiological temperature (37 °C), it not only decreases the adhesion between captured CTCs and 3D scaffold but also allow the cell-friendly release of CTCs. The experimental results demonstrated that (87 ± 13)%, (89 ± 7)%, and (93 ± 3)% of capture efficiency together with (74 ± 20)%, (73 ± 8)%, and (58 ± 3)% of recovery ratio were achieved for blood samples spiked with 100, 30, and 10 MCF-7 cells, respectively. Single CTCs spiked with 4–168 were captured from whole blood samples of 20 cancer patients. 1–163 CTCs were successfully released and collected from the chip. CTC clusters having 1–7, each containing 2–15 CTCs were found from whole blood samples of 10 out of 20 cancer patients, and 1–5 CTC clusters could be thermally released and collected integrally at 37 °C.

Some studies have shown that the platelets also increase the difficulties of CTC detection due to the following reasons: (i) the direct contact between platelets and CTCs may induce EMT; (ii) the platelets cloak on the CTCs and may shield them from being captured by antibodies; (iii) activated platelets may enhance the combination between CTCs and leukocytes, which results in CTCs loss in negative enrichment methods[54].

Although various methods of CTC isolation and detection have been studied for many years, it is difficult to apply them in the clinic. One of the difficulties is that there are very few CTCs in their whole peripheral blood. To solve the problem of the low abundance of CTCs, the following three research approaches are needed: (i) designing novel structures[54] or particles to capture CTCs simultaneously to amplify the detection signals; (ii) enabling the microchips to capture and detect single CTCs and CTC clusters simultaneously; (iii) studying how to solve the platelet and other blood cell coverage problems.

Leukocyte contamination

In general, the diameter of CTCs, nucleated blood cells, and non-nucleated red blood cells are 14–25, 7–15, and 6–9, respectively[55]. The size and bio-characteristics overlap between CTCs and leukocytes could cause leukocyte contamination, which decreases the purity. The CellSearch system suffers from leukocyte contamination with 1000–5000 WBCs when 7.5 mL of whole blood is processed. CTC-ichip, which obtains 2.5-log depletion of WBCs still contains significant leukocyte contamination of 17264–39172 WBCs/mL. The immunomagnetic negative selection, which depletes leukocytes from RBC-lysed whole blood samples achieves 2.5–3-log depletion of WBCs. It still needs further reduction of leukocytes contamination[50].

Bu et al.[56] combined the conventional size-based CTC isolation with hypo-osmotic swelling for widening the size differences between CTCs and large leukocytes (**Figure 3A**). The capture rates were (71.9 ± 4.2)% and (71.3 ± 4.5)% when ECTCs and MCTCs were spiked into PBS solution, respectively. Likewise, the capture rates were (67.4 ± 0.7)% and (66.6 ± 6.4)% when ECTCs and MCTCs were spiked into human blood samples, respectively. The LOD was at least three CTCs when the blood samples were filtered under hypotonic condition. The average purity of CTCs in hypotonic condition was (2.7 ± 1.3)%.

Cushing et al.[50] demonstrated the first continuous flow-based acoustophoretic negative selection of WBCs (**Figure 3B**) using anti-CD45. The activated negative acoustic contrast elastomeric particles (EPs) that specifically bind to WBCs showed 86-fold (MCF-7) and 52-fold (DU145) reduction of WBCs in the cancer cell fractions.

Sun et al.[20] proposed a low-cost microfluidic chip using conventional polycarbonate membrane. Microbeads grafted with aptamers against epithelial cell adhesion molecule (EpCAM) were employed on the membrane to enlarge the

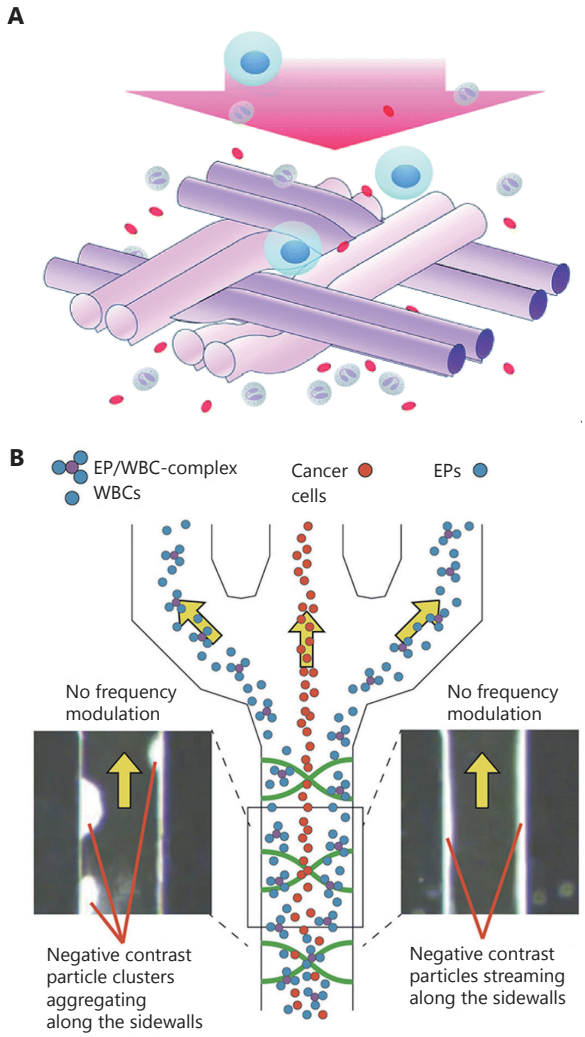

Figure 3 (A) Size-based CTC isolation enhanced by hypo-osmotic swelling from ref. 56 with permission from RSC Advances, copyright 2017. (B) Acoustophoretic separation approach from ref. 50 with permission from Analytica Chimica Acta, copyright 2018.

size of CTCs, thereby increasing the capture efficiency of smaller CTCs in patient blood[54].

To solve the leukocyte contamination problem, there are mainly two directions: label-free and label-based methods. Compared with label-based methods, label-free methods are more conducive to maintain cell viabilities and enable the device and process simpler and is less time-consuming. To widen the size differences between CTCs and large leukocytes, one method is to enlarge the size of CTCs based on cell characteristics. Another way is to widen the size differences by combining CTCs with certain particles based on affinity binding. As per above discussion, the former method is more conducive to the downstream cells analysis.

EMT

Studies have shown that EMT could induce cells to lose their EpCAM expression[54] while epithelial phenotype[57] could meditate cells to possess higher deformability[54]. Therefore, previous positive sorting based on EpCAM may lose CTCs with low EpCAM expression or EpCAM-negative. It will decrease the capture efficiency of CTCs. Meanwhile, Yu et al.[58] pointed that the mesenchymal CTCs are associated with metastasis and disease progression. Wu et al.[59] proved that there are more mesenchymal CTCs than EpCAM-positive CTCs in metastatic stages of cancer. To overcome the challenge of EMT and capture more heterogenic CTCs, different studies have been conducted from the following five different perspectives.

Designing novel channel structures but still EpCAM-based

Jack et al.[5] proposed an immunomagnetic method to separate CTCs based on their different levels of EpCAM expression, which enables extensive studies on tumor cell heterogeneity in a clinically meaningful manner. CTCs with different EpCAM expression levels could combine different amounts of magnetic particles. Hence, by changing the distance between the magnet and flow, these CTCs having different amounts of magnetic particles could be captured at different positions of the channel to separate them.

Kwak et al.[60] proposed a spiral shaped microfluidic channel (**Figure 4**). It could capture EpCAM positive and EpCAM negative CTCs simultaneously by EpCAM specific conjugation of magnetic nanoparticles (MNP), and selectively position heterogenic CTCs based on their EpCAM expression levels by magnetic field gradient. The circular-shape CTCs trapping segment is comprised of a continuous spiral shape channel whose radius gradually decreases towards the permanent magnet at the center. Along the spiral channel, 513 CTC trapping sites, which are capable of capturing cells, are present. Each trapping site was perpendicular to the direction of main fluid flow and had a high length-to-width ratio to prevent the escaping of captured CTCs by secondary fluid flow. MCF-7 and MDA-MB-231 cells as EpCAM positive and EpCAM negative cells, respectively, were used to test the capturing efficiency and selectivity of the platform. The results demonstrated that MCF-7 cells were captured in outer two circles with a capture rate of (96.3 ± 1.5)% and MDA-MB-231 cells were captured in inner two circles with a capture rate of (81.2 ± 3.5)%. The average purities of MCF-7 and MDA-MB-231 cells were 85.9% and 82.8%, respectively. An optimum flow velocity of

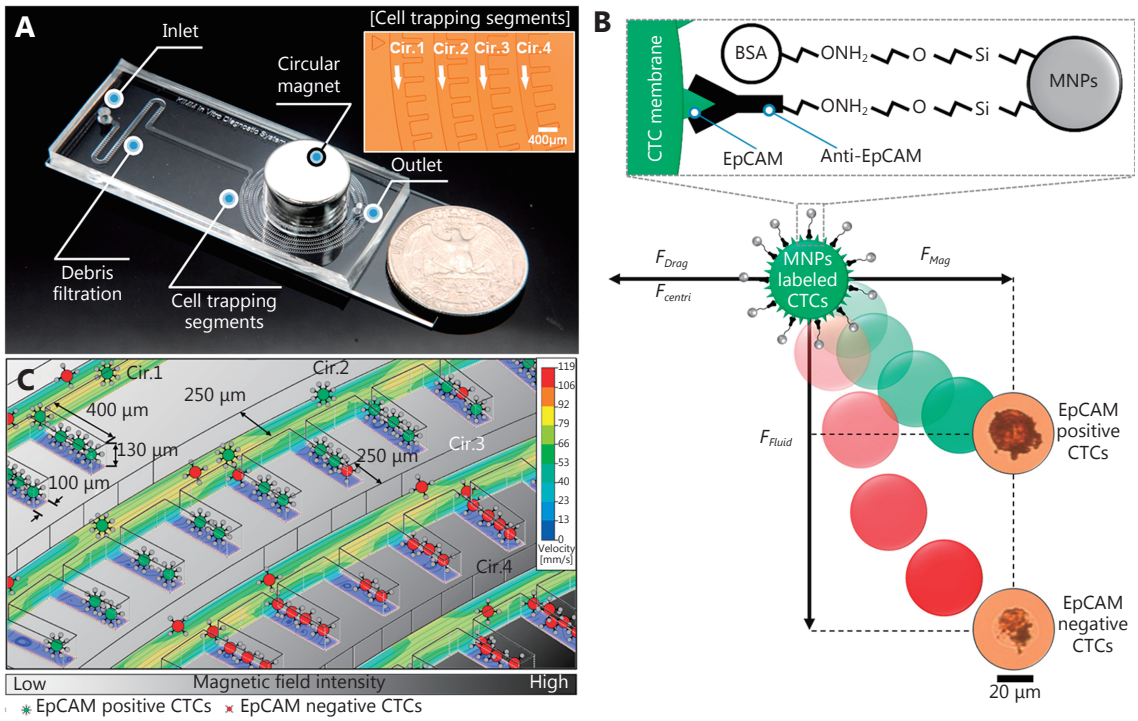

Figure 4 Spiral shape microfluidic channel and trapping segments (inset image) from ref. 60 with permission from Biosensors and Bioelectronics, copyright 2018.

150 μL/min was selected to obtain high capturing efficiency with EpCAM negative or EpCAM positive CTC separation.

The advantage of the above methods is that they can separate CTCs based on their different levels of EpCAM expression. However, they still could not detect and quantify the number of heterogeneous CTCs with low EpCAM expression.

Looking for other CTCs markers

Once CTCs undergo EMT, the positive sorting based on EpCAM expression would be inefficient. Hence, finding supplemental or replacing markers for EpCAM is an alternative approach. A large number of markers, including N-Cadherin, O-Cadherin, VCAM-1, ICAM-1, CEA, CA19-9, CA72-4[61], pepsinogens[62], MUC1[63], EphB4, CD44[6], CD133, CD146, CK18, CK19[5], Ctnnd1[64], PSMA, HER2[45], EGFR[65], TROP-2, and FAPα, have been investigated for CTC detection. However, most of these markers only target certain cancer cells specifically and some markers perform similar to EpCAM in EMT. Thus, they could not be used as supplemental markers for EpCAM[13]. Since there are more mesenchymal CTCs in metastatic stages of cancer, it is also important to find EMT markers for evaluating metastasis and prognosis in cancer patients.

Xu et al.[6] developed a microchip embedded with HA-modified electrospun PLGA nanofibrous membrane to capture cancer cells. Hyaluronic acid (HA) receptors are CD44s and they are over-expressed in many tumor cells including breast cancer, epithelial ovarian cancer, head and neck squamous cell carcinoma, leukemia, non-Hodgkin's lymphoma, and lung cancer. HA as biomarkers for capturing tumor cells has several advantages. Firstly, common blood cells have poor expression level of CD44 and poor adhesion to HA. Secondly, HA is one of the major component of the tumor extracellular matrix, which not only captures CTCs but also maintain a better cell integrity. It is convenient to in situ culture and analyze. Furthermore, HA-modified microstructures are easier to be analyzed by optical microscopy.

Li et al.[66] used folic acid (FA) as probe to detect ovarian cancer CTCs. They decorated bovine serum albumin (BSA) on MNP to form BSA-MNP. FA was then conjugated on the BSA-MNP surface to form FA-BSA-MNP. In simulant experiments, the FAR-positive (FAR^+) ovarian cancer cell, SKOV3, was used to simulate the CTCs. The K562 cells and HL-60 cells suspensions were used to simulate the blood cells. The capture efficiencies for SKOV3 in the cell culture medium were 66.84% and 66.81% in the presence of HL-60

cells and K562 cells, respectively. In clinical experiments, SKOV3 cells were spiked in whole blood of a healthy human. The capture rate ranged from 37.35%–61.3%. The reduction compared to simulant experiments may owe to the difference of FAR expression in each SKOV3 cell. Besides, the viability rate of cells captured by FA-BSA-MNP reached to 92.7%.

An et al.[67]developed a benzoboric acid modified gold-plated polymeric substrate with 3D surface array capable of capturing and releasing tumor cells. The substrate was modified with organic molecules containing a benzoboric acid group instead of antibodies, which can reversely bind with the sialic acid molecule, which is overexpressed in tumor cells. This platform not only performs the capture of CTCs but also demonstrates the release of captured CTCs by using excess sialic acid, which facilitated the sequential analysis of CTCs with better cell integrity.

Hsu et al.[68] proposed a noncatalytic endosialidase as a biomarker, which has high affinity to polysialic acid and is a cell-surface glycan that is highly expressed small cell lung cancer (SCLC) tissue. This enzyme-based system further enhances the capture efficiency *via* multivalent binding compared to their EpCAM counterparts. This platform has the potential to be used as a superior alternative to the EpCAM-based methods, particularly for those tumor cells that overexpress polysialic acid.

Li et al.[69] investigated the expression of EMT markers including vimentin, twist, ZEB1, ZEB2, snail, slug, and E-cadherin in CTCs. Triple-immunofluorescence staining results showed that vimentin and twist expression could be detected in CTCs with 84.8% and 80.4% of the hepatocellular carcinoma (HCC) patients, respectively. Coexpression of twist and vimentin in CTCs could be detected in 69.6% of the HCC patients. The twist and vimentin expression levels in CTCs could serve as promising biomarkers for evaluating metastasis and prognosis in HCC patients. Moreover, recent studies have demonstrated that vimentin is expressed in the CTCs of breast cancer and advanced prostate cancer patients[70,71].

Although extensive research has been done to find biomarkers of CTCs, EpCAM is still the universal CTC biomarker, since most of the novel biomarkers are generally targeted at specific types of cancer cells. Moreover, it is generally difficult and expensive to find novel biomarkers.

Preparation of other types of detection probes

Aptamers are single-stranded nucleic acids with a specific three-dimensional structure. They have high affinity towards molecules ranging from small molecules such as ATP and proteins to intact cells. Aptamers can be designed through a systematic evolution of ligands by exponential enrichment (SELEX) system *in vitro*[1]. Aptamers-based sorting methods could be divided into two categories: aptamer-functionalized nanoparticles and aptamer-functionalized microchannel/microstructures. Since CTCs have relatively higher adhesion towards rough surface than common blood cells, the capture efficiency could be improved by coating more aptamers on the surface of the channel to increase the surface roughness[25].

Ding et al.[72] developed EGFR peptide magnetic nanovesicles (EPMVs) using a peptide derivative. Peptide amphiphile (PA) was used to construct nanoparticles (NPs) that can directly search for CTCs in a peptide-targeted manner. This approach could overcome limitations of traditional peptide nanocarriers such as immune magnetic beads and magnetic liposomes.

Pu et al.[73] developed the A-1 peptide, which could capture both epithelial-like and mesenchymal-like A549 cells (**Figure 5A**). The A-1 peptide was obtained by bacterial surface display method and flow activated cell sorting (FACS) technology. The results demonstrated that capture efficiency of A-1 peptide chip for M-A549 (heterogeneous CTCs) was similar to that of E-A549 (common CTCs) at (58.0 ± 19.7)%, while anti-EpCAM based chip exhibited poor capture efficiency for heterogeneous CTCs.

Bahadur et al.[74] synthesized six kinds of fluorogenic peptides based on EpCAM-binding peptide, Ep114. They replaced the amino acids of Ep114 with aminophenylalanine that was modified with environmentally sensitive 7-nitro-2,1,3-benzoxadiazole. The experimental results, which showed two peptides, Q4X and V6X, not only maintained binding ability of Ep114 but also exhibited the fluorogenic detection of EpCAM-expressing cells.

Li et al.[75] designed a novel DNA-templated magnetic nanoparticle-quantum dot (QD)-aptamer copolymers (MQAPs) for magnetic isolation of CTCs. The MQAPs could amplify magnetic response, extraordinary binding selectivity for target cells, and ultrabright ensemble QD PL for single cell detection. Besides, MQAPs were free from binding with blood cells, which could reduce leukocyte contamination and improve the capture purity of CTCs. The capture rate and purity of MQAPs could reach 80%.

Compared with antibodies, aptamers own the following advantages: relatively smaller size, low immunogenicity[1], *in vitro* rapid chemical synthesis in large quantities, easily modified, more stable, and possess long-term stability[25]. However, the capture efficiency of aptamer-based methods needs further improvement.

Label-free methods

Except for the above label-based methods, label-free methods have been studied to capture more heterogeneous CTCs.

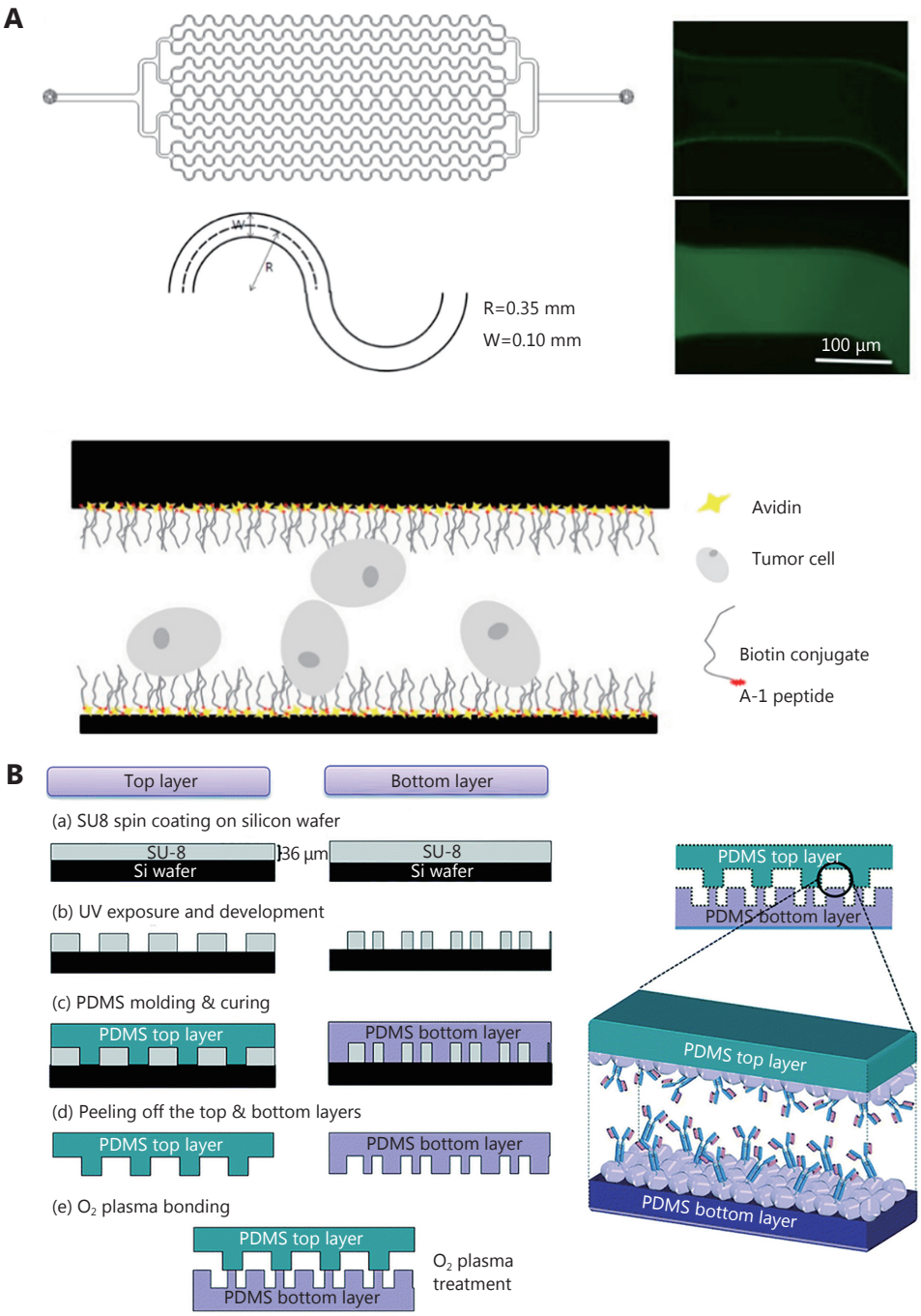

Figure 5 (A) Microfluidic chip modified with NeutrAvidin and subsequently linked with biotin conjugated A-1 peptide from ref. 73 with permission from Biosensors and Bioelectronics, copyright 2017. (B) The dual-patterned chips from ref. 79 with permission from Lab on a chip, copyright 2016.

Che et al.[21] reported a label-free high throughput microfluidic approach to isolate, enumerate, and characterize CTCs by VDC. It consists of an initial vortex region, which enriches large CTCs, followed by release into hydrodynamic stretching region that deforms the cells. Visualization and quantification of cell deformation was revealed by a high-speed camera. The VDC demonstrated a detection rate of putative CTCs above healthy baseline (93.8%) when compared to standard immunofluorescence (71.4%).

Fan et al.[76] proposed a novel size-based isolation method.

They integrated the precise, high-porosity, and low-cost PDMS microfiltration membrane with microfluidic devices similar to a sandwich structure to enable the recovery, staining, washing, and detection of CTCs. To evaluate the detection efficiency of the device, A549, SK-MES-1, and H446 were spiked into the healthy human peripheral blood. The results showed that the microchip could achieve a capture rate (over 90%) at relatively high processing throughput (~10 m/h). This size-based microfiltration chip not only captured more heterogeneous CTCs to get more useful data about the metastatic process, but also offered a cheaper and simpler filtration tool in contrast to existing CTC filtration devices, which rely on low-porosity track-etch filters or expensive lithography-based filters.

Yang et al.[77] designed a novel wedge-shaped microfluidic chip (CTC-ΔChip) fabricated by two pieces of glass through wet etching and thermal bonding technique. This chip could achieve CTCs enrichment by the differences in size between CTCs and blood cells and could identify CTCs with three-color immunocytochemistry method ($CK^+/CD45^-$/$Nucleus^+$). The capture efficiency of CTC-ΔChip was (93.7±3.2)% in DMEM and (91.0 ± 3.0)% in the whole blood sample under optimized conditions. In clinical experiments, CTC-ΔChip could identify 7.30 ± 7.29 CTCs from 2 mL peripheral blood with a positive rate of 75% in gastric cancer (GC) patients.

The advantages of the label-free methods are as follows. Firstly, they can capture nearly 100% CTCs whether they are EpCAM-positive CTCs or heterogeneous CTCs. Secondly, it is conducive to maintain cell viabilities without affinity binding with antibodies or aptamers. However, the purity of label-free methods is lower than label-based methods, which limits its development.

Negative sorting for leukocytes

All of the four methods above used CTCs as the target cells to solve the EMT issues. In fact, negative sorting methods may be a better way to capture the common and heterogeneous CTCs. Since leukocytes are labeled by antibodies instead of CTCs, negative sorting methods not only decreases the loss of heterogeneous CTCs but also maintains the cell integrity to the downstream cell analysis in the whole process. In recent years, numerous methods have been developed to improve the purity, which is the key indicator for negative soring methods.

Liao et al.[78] proposed a 3D spheroid cell culture model, which was used to further purify viable CTCs after conventional negative isolation process. The results demonstrated that the cancer cell purities were increased by 10.6–80.3-fold after spheroid cell culture for 8 days meanwhile maintaining CTC viabilities. The average purity of CTC-related cells was (34.8 ± 14.0)%, which was greatly improved compared with conventional negative isolation methods.

Bu et al.[79] developed a dual-patterned immunofiltration device for the rapid and efficient enrichment of heterogeneous CTCs based on the negative selection (**Figure 5B**). The chip consisted of top and bottom layers, which were functionalized by anti-CD45 antibodies, which significantly enhanced the binding chance between leukocytes and antibodies, which facilitated the high purity of CTCs. The results showed that (97.07 ± 2.79)% of leukocytes were eliminated with less than 10% loss of cancer cells at the flow rate of 1 mL/h. CTCs were collected from the blood of 11 cancer patients and an average of 283.3 CTC-like cells were identified while less than 1 CTC-like cells were found from healthy donors.

Chen et al.[80] presented a cell sorting method based on anti-CD45 antibody-modified five-layer magnetic nanospheres. The leukocyte depletion efficiency was up to 99.9% within 30 min in mimic clinical samples and the capture purity of the spiked HCC cells was increased by 265–317-fold. Meanwhile, the isolated HCC cells remained viable at 92.3%, which could be directly recultured for specific immunocytochemical identification.

Currently, the common biomarker for negative sorting is CD45. The negative sorting might become a hotspot for exploring the design of antibodies modified microstructures and microchannels for further improving WBC capture efficiency in the future.

Cell viability in the sorting process

Cell integrity is expected after a series of isolation and enumeration processing for further cell culture or cell detection. However, there are many factors that may damage the cell viability. Firstly, it is difficult for cells to maintain integrity if the processing time is too long. Secondly, in some sorting methods especially filter microstructures, large shear force induced by fast flow may damage the cell integrity. Furthermore, enzymes that are mainly used to release captured CTCs will also cause great damage to cell viability and even cause death. Therefore, maintaining cell viability while increasing the capture efficiency is also another research hotspot.

Wu et al.[81] proposed a cell-friendly one-step strategy, which integrated incubation, magnetic separation, and fluorescence identification that shows the whole process of

capture and detection in relatively short time. The results demonstrated that 5 tumor cells/mL of whole blood could be only detected *via* 20 min incubation. Besides, (93.8 ± 0.1)% of detected tumor cells maintained cell viabilities and proliferation abilities with negligible changes in cell functions.

Zhao et al.[48] developed a laminar-flow microfluidic forward scattered light (FCS) device to enrich rare CTCs from patient's blood in a biocompatible manner with a high throughput (6 mL/h), a high recovery rate (92.9%), and an average purity (11.7%). Besides, an analytical model was designed to guide the optimization and design of parameters including cell flow rates, magnetic fields and its gradient, ferrofluid concentrations, and compositions to improve the isolation efficiency.

Xiao et al.[82] designed a microfluidic chip integrated with zwitterion-functionalized aligned polyethyleneimine/polyvinyl alcohol (PEI/PVA) nanofibers for efficient capture and rapid release of CTCs (**Figure 6A**). FA-coated nanofibers could efficiently capture cancer cells overexpressing FA receptors. Captured CTCs could be then released in a non-destructive manner by using tris(2-carboxyethyl)phosphine (TCEP) (reducing agent to break disulfide bonds) to break the disulfide bonds between FA and poly [2-methacryloyloxyethyl phosphorylcholine (PMPC)]. FA receptor-expressing cancer cells could be isolated with a high capture efficiency (92.7%) and a considerable purity (43.4%) in 30 min. Besides, 97.7% of the captured cells could be released within 5 min.

Zhu et al.[83] proposed a novel protocol that coat the FA and MNPs on the surface of RBCs by hydrophobic interaction and chemical conjugation. The engineered RBCs could be used to capture CTCs in a magnetic field and treated with RBC lysis buffer to release captured CTCs. This method could avoid the damage to CTC viabilities caused by the direct combination between CTCs and MNPs. Owing to homology effects between RBCs and WBCs, non-specific binding between MNPs and leukocytes could be reduced. The results demonstrated that the capture efficiency was over 90% and the purity was above 75%. Approximately 90% of the released MCF-7 cells retained their viabilities.

Wang et al.[84] developed a thermoresponsive chitosan nanofiber substrate assisted by poly(N-isopropylacrylamide) (PNIPAAm) brushes and DNA hybridization to capture, purify, and release CTCs effectively. The PNIPAAm brushes were designed to enable WBCs to detach from aptamer-PNIPAAm-chitosan-nanofiber (aptamer-P-CNFs) surfaces during the conformational transition, which could improve the CTCs purity. Furthermore, an intact and effective release of CTCs without any added agents could be identified by complementary sequences efficiently hybridizing with

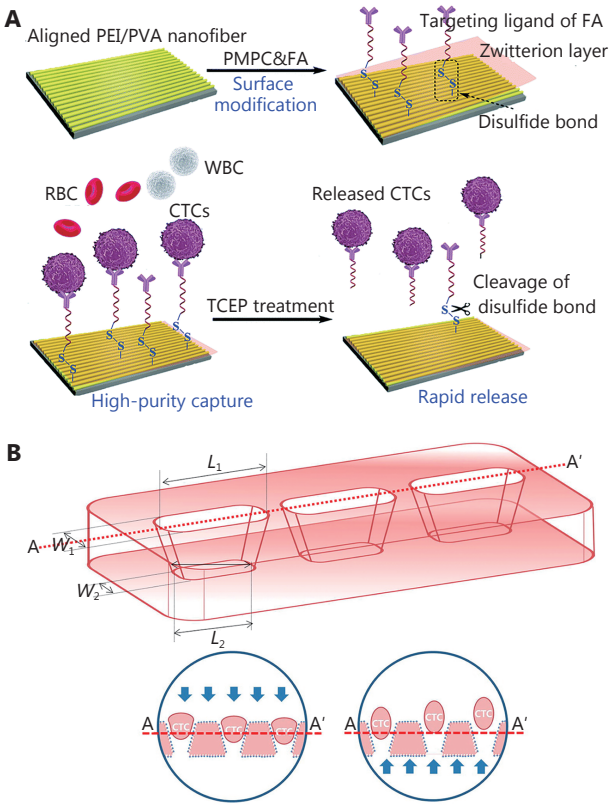

Figure 6 (A) Schematic of the functionalization of the aligned PEI/PVA nanofibrous mat for highly efficient capture and rapid release of CTCs from ref. 82 with permission from Material Chemistry, copyright 2018. (B) PEG-modified tapered-slit SU8 filter from ref. 85 with permission from Analytical Chemistry, copyright 2016.

aptamers, which also made CTCs further purified. To test the platform, 10, 50, 100, and 1000 MCF-7 cells were spiked in 1 mL human whole blood, respectively. The capture rate was 50%–70% and over 90% of detected CTCs could be released from the substrates. The percentage of viable cells was (95 ± 3)% in the release group.

Kim et al.[85] proposed an advanced tapered-slit filter (TSF) by grafting with polyethylene glycol (PEG), which had a wider inlet entrance and gradually narrower slit exit (**Figure 5B**). Compared with conventional CTCs filters having straight holes, TSF generated lower stress concentrations, which could reduce the damage to cells under high flow rate. Furthermore, the uniquely shaped slit facilitated viable CTC isolation, and maintained capture efficiency over 80% and cell viabilities over 70% after 5 days of culture on the filter. This demonstrated approximately 30% improvement of efficiency since PEG could reduce the nonspecific binding of CTCs on the filter surface.

Yan et al.[86] developed an electrochemical microchip by designing a cactus-like, topologically structured conductive array, which consisted of a PDMS micropillar-array core and an electroconductive gold coating layer with a hierarchical structure, which could present a high capture efficiency (85%–100%) for different cell lines in both buffer solution and whole blood. The fast release of captured CTCs could be identified at a low voltage (–1.2 V) originating from an electrochemical cleavage of the Au-S bonds without using other biochemical agents, which facilitated the cell integrity for downstream biomedical analysis.

To maintain the cell viability after sequential processing, the following two points should be attained: less time-consuming and appropriate release methods. Besides, sequential steps like incubation, isolation, and detection could be integrated on one chip, one step process is also used for reducing the time consumed. The next studies will focus on exploring appropriate release methods without the use of additives such as enzymes that are detrimental to cell viabilities. However, existing methods could enable the downstream analysis of released cells without the effect of particles such as magnetic beads, and the released cells still combine with antibodies or aptamers, which will also affect the detection results in some degree.

Low throughput and time-consuming

Microfluidics could exhibit a better capture efficiency and purity than conventional filters, centrifugation, and magnetic separation. But methods based on microfluidics generally have low throughput, and high processing time. There are several reasons for such limitations. Firstly, the size of microfluidic chip is small, which determines the low throughput and less sample. Secondly, in biophysical methods, if the flow is too fast to make the shear force greater than adhesion force, captured CTCs are lost, which could decrease the capture efficiency. Thirdly, in label-based methods, the flow rate should be controlled precisely to enhance the combination of CTCs with biomarker-coated structures. Besides, affinity-based methods also need a longer incubation time. Various methods have been developed to increase throughput and decrease the time consumption. These methods involves the following two categories.

The design of chip structures to increase the throughput

Zhu et al.[83] developed a microchip platform integrated track-etched magnetic micropore capture, size-based capture, and on-chip Turbo fluorescent *in situ* hybridization (FISH). This integrated chip could isolate both single CTCs and CTC clusters simultaneously from whole blood and allow *in situ* RNA profiling, achieving sample-to-answer in less than 1 h for 10 mL of whole blood. Besides, *in situ* RNA profiling could avoid CTCs loss compared to off-chip cell analysis.

Shen et al.[31] presented a single-layer, sheathless, large dimension, and ultra-low-aspect ratio (AR) microfluidic chip (**Figure 7A**) combining a spiral channel with a series of micro-obstacles, which could create Dean flow and induce another hydrodynamic force (Dean drag force). The throughput of this chip is 1.89×10^9 cells/min. Besides, high collection efficiency (> 95%) and enrichment efficiency (> 2.29×10^5-fold) of CTCs could be achieved.

Kim et al.[87] proposed a novel protocol using TEMPO membrane having 8 μm pores with a fluid-assisted separation technology (FAST). Size-selective CTC isolation was performed during the entire filtration process through the liquid filled membrane pores, which were conducive to pass small-size CTCs at a lower pressure, thus solving the clogging problem to some extent. This chip could exhibit a high recovery rate (95.9 ± 3.1)%, selectivity (> 2.5-log depletion of white blood cells), and throughput (> 3 mL/min). Besides, the FAST disc was able to detect 0–540 CTCs/7.5 mL CTCs from 15 of 18 (83.3%) samples taken from breast cancer patients and 2–485 CTCs/7.5 mL taken from 63 of 76 (82.9%) stomach cancer patients.

Integrate multiple processes and reduce processing steps to decrease time-consumption and CTCs loss

Li et al.[88] proposed a simple, rapid, and sensitive platform to detect CTCs based on magnetic separation and nanozyme activity of MNPs. The enzymatic action of MNPs could be used to oxidize the reagents in the tube whose color change proves the existence of CTCs. Besides, CTCs quantification could be achieved by using UV-vis absorbance of TMBox at 652 nm. The whole process could be finished in 50 min with capture efficiency (70.2 ± 6)% and LOD (79 cells/7.5 mL).

Jack et al.[89] proposed a microfluidic chip with multiple function modules including inertial sorter, passive mixer, on-chip magnetic labeling and sorting, and the analysis of CTC miRNA and mRNA (**Figure 7B**). Although the magnetic sorting rate was limited at 50, it could be multiplexed using parallel sorters to increase the entire process with purities greater than 80% and a recovery rate of (91.99 ± 4.94)%.

Chiu et al.[90] proposed a microfluidic system using a capillary tube to deliver a cell suspension sample directly to the inlet of a constriction microchannel. This platform could significantly facilitate the subsequent cellular electrical property characterization and reduce the sample loss

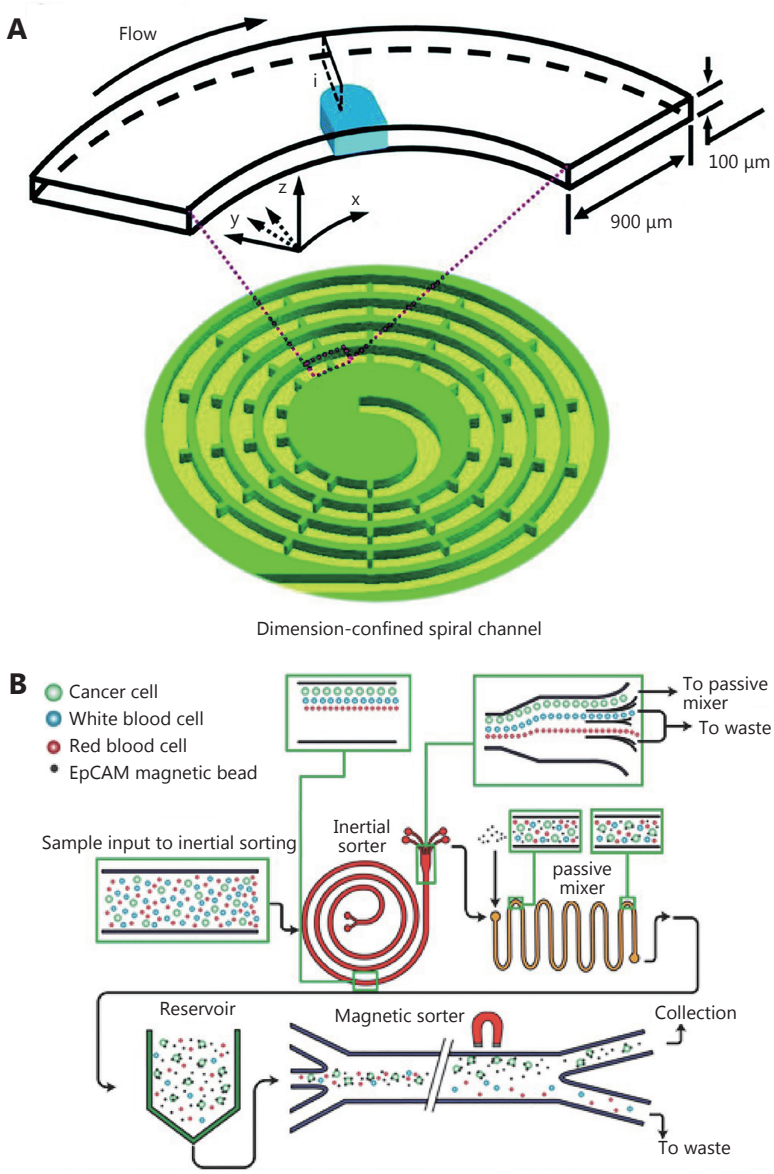

Figure 7 (A) Microfluidic device containing dimension-confined spiral channel from ref. 31 with permission from Lab on a Chip, copyright 2017. (B) CTCs isolation platform integrated inertial sorting, passive mixing with incubation and magnetic sorting from ref. 89 with permission from Advanced Science, copyright 2016.

phenomenon in which cell processing ratio could be raised from 0.2% to 49.3%–60.0% under the flow rate at 100 μL/h.

Shields et al.[57] developed a microfluidic platform, which is comprised of three modules. The first module was designed to align cells using acoustic standing wave. The second module was used to separate magnetically labeled cells using magnetic field gradient. The third module was spatially defined magnetographic array for on-chip staining and analysis, which was made and operated separately. The platform could process 6.0 mL of blood within 120 min after erythrocyte lysis and the isolation efficiency was up to 89%.

Most of the current methods of CTC isolation and detection based on microfluidics are difficult to apply clinically due to the low throughput and time-consuming processing. Label-free methods do not require a long incubation time. However, clogging problems are the main obstacles to limit the throughput of size-based methods. FAST has achieved good results in solving clogging problems; however, more efficient methods are still needed. Apart from increasing the throughput of single module, integrating multiple modules on one chip is a promising method. It not only decreases the loss of CTCs but also shortens the time

consumption, which is important to maintain the cell viability.

Challenges and technological prospects

To date, great advances in CTC isolation and detection technologies based on microfluidics have been made, with obvious advantages. However, these novel advances cannot satisfy the current clinical requirement, and there are some challenges, such as low abundance of CTCs in peripheral blood, leukocyte contamination, EMT, cell viability and the throughput. Various methods emerging from 2015 to 2018, which aimed at solving above problems are summarized, and five key indicators of these methods are listed in **Table 1**.

To promote clinical translation, further studies should be carried out in the following aspects. Firstly, CTC clusters may have more metastatic potential than single CTCs, hence, methods, which could isolate and detect single CTCs and CTC clusters simultaneously should be studied. Secondly,

Table 1 Five key parameters of summarized methods

Capture rate		Purity (leukocytes elimination)	Throughput	Biocompatibility	Clinical studies		Referece
Cell line/medium	Efficiency				Cancer type	LOD	
YFP+ cells/whole blood	77%	(97.3 ± 1.2)%	0.5 mL/h	-	Spiked tumor cells	1 cell/million WBCs	53
H209 lung cancer cells/whole blood	(70.5 ± 5.2)%	(91.5 ± 5.6)%	0.5 μL/min	-	-	-	2
MCF-7 cells/whole blood	(87 ± 13)%~ (93 ± 3)%	-	50 μL/min	(58 ± 3)%~ (74 ± 20)%	EDTA anticoagulated whole blood samples	4–168 CTCs/ 20 patients 1–7 CTC clusters/ 10 patients	14
ECTCs/PBS ECTCs/whole blood	(71.9 ± 4.2)% (67.4 ± 0.7)%	(2.7 ± 1.3)%	1 mL/12 min	(82.1 ± 2.1)% (viability reduction)	Colorectal tumor cells	3 CTCs at all six samples	56
MCTCs/ PBSMCTCs/ whole blood	(71.3 ± 4.5)% (66.6 ± 6.4)%			(80.5 ± 2.5)% (viability reduction)	Healthy donors	None	
MCF-7	(96.3 ± 1.5)%	85.9%	150 μL/min	-	-	-	60
MDA-MB-231	(81.2 ± 3.5)%	82.8%					
SKOV3 cells/HL-60 or K562 cells	66.84% or 66.81%	-	-	92.7%	SKOV3 cells/human whole blood	37.35%–61.3%	66
A549 cells/diluted human blood	(58.0 ± 19.7)%	-	1 μL/min	-	-	-	73
CEM, Ramons and DAPI cells	80%	80%	-	-	-	-	75
MCF7 cells/10 × diluted blood	25%–35%	(35.1 ± 7.3)%	>10 mL/h	56%	Stage IV NSCLC	-	21
BGC823, HCT116, PC3/DMEM medium	(93.7 ± 3.2)%	-	-	-	BGC823/ human blood	7.30 ± 7.29 CTCs/2 mL	77
BGC823, HCT116, PC3/blood	(91.0 ± 3.0)%						
OECM-1 cells/PBMCs	-	(34.8 ± 14.0)% (in clinical tests)	-	-	Head-and-neck cancer	-	78
A549/PBS	-	(97.07 ± 2.79)%	1 mL/h	-	Pancreatic, lung, breast and colon cancer	283.3 CTC-like cells/11 patients	79

Continued

Continued

Capture rate		Purity (leukocytes elimination)	Throughput	Biocompatibility	Clinical studies		Referece
Cell line/medium	Efficiency				Cancer type	LOD	
HCC cells	3%–93%	99.9%	Within 30 min/mL	92.3%	HCC	3 CTC subtypes/ one sample	[80]
MCF-7 cells	(98.1 ± 0.8)%	59.5%	-	(93.8 ± 0.1)%	MCF-7 cells/healthy blood	5 cells/mL	[81]
ATCC, Manassas, VA	92.9%	11.7%	6 mL/h	(80.8 ± 2.4)%	NSCLC	1165 CTCs/ 6.5 mL 369 CTCs/ 5.5 mL	[48]
HeLa cells	92.7%	43.4%	Within 30 min/mL	97.7%	Ovarian, cervical, breast cancer	1–16 CTCs/mL	[82]
HCT116 cells	(93.92 ± 3.92)%	>75%	-	>90%	-	-	[83]
MCF-7 cells	(93.49 ± 3.03)%	80%					
MCF-7 cells/ whole blood	50%–70%	-	-	(95 ± 3)% in the release group	-	-	[84]
H358/blood	(83.6 ± 7.6)%	-	5 mL/h	(89.8 ± 6.4)%	Lung cancer	8.6 CTCs/mL	[85]
SW620/blood	(81.3 ± 5.3)%			(85.2 ± 7.0)%	Colorectal cancer	19.4 CTCs/mL	
PC3, MCF-7 and NCI-H1650 cells	85%–100%	-	1–3 mL/h	high	Lung cancer	-	[86]
HeLa, MCF-7, K562	>85.8%	>86.1%	1.89×10^9 cells/min	-	-	-	[31]
MCF-7, MDA-MB-231, MDA-MB-436, HCC78, AGS	(95.9 ± 3.1)%	-	>3 mL/min	good	Breast cancer patients stomach cancer patients	0–540 CTCs/ 7.5 mL 2–485 CTCs/ 7.5 mL	[87]
Melanoma CTCs, LM-MEL-33 cells	(70.2 ± 6)%	-	Within 50 min	-	LM-MEL-33cells	79 cells/ 7.5 mL	[88]
PANC-1 cells	(91.99 ± 4.94)%	>80%	N*50 μL/min	-	-	-	[89]
A549 cell line	-	(85.6 ± 4.5)%	100 μL/h	-	-	-	[90]
LNCaP	89%	>92%	200 μL/min	-	-	-	[57]

biological and physical methods could be used to widen the size differences between CTCs and leukocytes to avoid the size overlap. Thirdly, to overcome the challenge of EMT, many other biomarkers and biological probes could be studied to improve the isolation efficiency of positive sorting. Negative sorting methods could be used to reduce the loss of heterogeneous CTCs, even though such methods result in lower purity than positive sorting methods. Most importantly, to maintain cell integrity and viability for downstream cell analysis, the whole process should be finished in shorter time. Besides, the reagents and methods used for capture and detection, especially release of CTCs, should avoid the damage to cells. Furthermore, microfluidic chips could be integrated with multiple modules in one chip to reduce the processing time and avoid cells loss. In this manner, the throughput and process efficiency could be improved. Opportunities often come with challenges. A combination of several CTC detection technologies might allow us to overcome some of the above challenges and promote clinical usage of such methods in near future.

Conclusions

To conclude, main advances in isolation and monitoring

methods of CTCs based on microfluidics were reviewed. These methods include two categories: label-free and label-based. Label-free methods are generally based on the physical characteristics including size, deformability, density, dielectric properties, and viscosity. Size-based methods generally suffer from the size overlap between CTCs and leukocytes, which has poor isolation purity. The clogging problems also limit the flow rate and throughput. Dielectrophoresis, whose isolation efficiency depends on the conductivity of the cells and frequency of the electric field, requires a high-intensity electric field. The corresponding thermal effects may damage the cell viability. Acoustic separation is mainly based on bulk acoustic waves (BAW), surface acoustic waves (SAW), and plate acoustic waves (PAW). The isolation efficiency is significantly depending on the position of pressure nodes, which need to be controlled precisely. Optical techniques including Raman spectroscopy are attracting increasing attention in recent years, however, the main limitation is still the throughput issue.

Label-based methods are mainly based on affinity binding between CTCs and ligands. Most of these techniques require pre-enrichment steps to make the sensitivity reach an acceptable level. The pre-enrichment steps, such as immunomagnetic bead enrichment, will make the operation of the detection more complicated and increase the cost of time and expense[67]. Although there existed some challenges and bottleneck in CTC isolation and detection, five technical indicators, including capture rate, purity, LOD, throughput, and biocompatibility, have been proposed to evaluate these isolation methods and current technologies based on microfluidics exhibit greater clinical translation prospects. We believe that these innovative CTC detection technologies will change the strategy of tumor diagnosis and therapeutic monitoring in the near future.

Acknowledgements

This work is supported by National Key Basic Research Program of China (Grant No.2017FYA0205300 and No.2015 CB931802), and National Natural Scientific Foundation of China (No. 81571835).

Conflicts of interest

The authors declare no competing financial interest.

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Cite this article as: Zou D, Cui D. Advances in isolation and detection of circulating tumor cells based on microfluidics. Cancer Biol Med. 2018; 15: 335-53. doi: 10.20892/j.issn.2095-3941.2018.0256