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Microfluidic platform for circulating tumor cells isolation and detection

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Abstract: Circulating tumor cells (CTCs) are essential biomarkers for liquid biopsies, which are important in the early screening, prognosis, and real-time monitoring of cancer. However, CTCs are less abundant in the peripheral blood of patients, therefore, their isolation is necessary. Recently, the use of microfluidics for CTC sorting has become a research hotspot owing to its low cost, ease of integration, low sample consumption, and unique advantages in the manipulation of micron-sized particles. Herein, we review the latest research on microfluidics-based CTC sorting. Specifically, we consider active sorting using external fields (electric, magnetic, acoustic, and optical tweezers) and passive sorting using the flow effects of cells in specific channel structures (microfiltration sorting, deterministic lateral displacement sorting, and inertial sorting). The advantages and limitations of each method and their recent applications are summarized here. To conclude, a forward-looking perspective is presented on future research on the microfluidic sorting of CTCs.

Introduction

Tumor metastasis causes approximately 90% of cancer-related deaths (Dillekas *et al.*, 2019); therefore, detecting the metastatic process in real-time is crucial. In contrast to invasive biopsy techniques, which have been found previously to cause pain to patients (Hirahata *et al.*, 2022), liquid biopsy techniques that are minimally invasive and enable real-time detection have recently gained increased attention (Belotti and Lim, 2021; Cortes-Hernandez *et al.*, 2020; de Rubis *et al.*, 2019). For cancer diagnosis and treatment, early screening, and prognostic analysis, liquid biopsy is performed by extracting effective components, such as circulating tumor cells (CTCs) (Alix-Panabières and Pantel, 2016), circulating tumor DNA (ctDNA) (Gorgannezhad *et al.*, 2018), and exosomes (Contreras-Naranjo *et al.*, 2017), which are present in human body fluids. Among these, CTC and ctDNA are approved by the Food and Drug Administration (FDA) to be utilized as important biomarkers in clinical diagnosis, which has become an important milestone in clinical trials of liquid biopsies (Alix-Panabieres and Pantel, 2021).

CTCs are cells shed by a tumor lesion (primary or metastatic lesion); they break through the tissue matrix and eventually enter the bloodstream (Nowell, 1976). Compared with ctDNA, which is a free gene fragment, CTCs can express the entire genetic information of a tumor from genome to functional protein, thereby making it a hot research topic in the field of liquid biopsy (Alix-Panabieres and Pantel, 2013). Compared with traditional clinical imaging tests, CTC testing has broad application prospects in clinical practice (de Wit *et al.*, 2018), such as assisting in clinical diagnosis and cancer staging, reflecting early disease status of the patients for determining patient prognosis (Shaw *et al.*, 2017), detecting treatment effects in real-time, and providing individualized medical treatment (D'Avola *et al.*, 2018). Meanwhile, the RNA sequencing of CTCs can reveal the biological significance of CTCs in the metastatic process (Baccelli *et al.*, 2013).

Despite the importance of CTCs in cancer diagnosis and detection, the number of CTCs in peripheral blood is extremely low, with only 1–10 CTCs/mL in early-stage cancer patients and a few hundred CTCs/mL in advanced-

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stage patients, compared with the billions of blood cells/mL (Kahn *et al.*, 2004), hence, the efficient enrichment of CTCs in peripheral blood is essential. Early physical crude separation techniques were based on the separation of CTCs by special membrane devices and density gradient centrifugation. Among theseThe typical application is the isolation by the size of epithelial tumor cells (ISET system) developed by Rarecells Diagnostics (Paris, France) (Farace *et al.*, 2011). To improve the purity of CTC enrichment, many pharmaceutical companies have focused on using immunomagnetic beads. For example, Johnson & Johnson (New Jersey, USA) launched the FDA-approved CellSearch system (Rushton *et al.*, 2021). However, this product was discontinued in early 2016 because of its low detection sensitivity and inability to isolate live CTCs. Apart from the CellSearch system, the AdnaTest by Qiagen (Todenhofer *et al.*, 2012), the MACS developed by Miltenyi (Voena *et al.*, 2002), and the MagSweeper system conducted by Illumina (Cann *et al.*, 2012), were also conducted in the past few years. These commercial sorting methods based on immunomagnetic beads are highly specific and highly integrated but still limited in clinical applications due to the high cost of the instruments and supporting consumables and missed inspection. Therefore, miniaturized, low-cost, and efficient sorting systems remain a common focus of scientific and industrial interest.

Microfluidics has rapidly evolved in recent decades from molecular analysis to cell biology because of their ability to control the mechanical, biological, and fluidic environment at the molecular and cellular levels (Autebert *et al.*, 2012). Cell sorting on microchips offers many advantages over conventional methods, as it reduces the size of the required equipment, eliminates potentially biohazardous procedures, and simplifies the complex protocols typically associated with cell sorting (Bhat *et al.*, 2022). In addition, microchip devices are ideally suited for parallelization, allowing complete lab-on-a-chip equipment to be used for cell isolation, analysis, and experimental processing (Shields *et al.*, 2015). Microfluidic technology offers many advantages over conventional non-microfluidic devices, including portability, improved sensitivity, lower operating costs, and higher throughput, making this technology promising in CTC sorting applications. This review focuses on the recent advances and the advantages and disadvantages of two types of microfluidic chip-based CTC sorting: active and passive sorting. The latest methods proposed in the same category (active sorting or passive sorting) are similar in many aspects, so this classification strategy could provide more information and guidance on chip manufacturing (whether to integrate with additional devices), sorting mechanism (whether to involve additional forces), sorting indicators (recovery, purity, throughput, etc.), which could facilitate subsequent scheme comparison and methods selection based on the actual situation.

According to the different cell manipulation methods by microfluidic chips, CTC sorting based on microfluidic platforms can be divided into active and passive sorting techniques (Fig. 1).

Active sorting involves the manipulation of different cells by external field sources (e.g., electric, magnetic, optical, and acoustic fields) to sort CTCs from blood cells. Passive sorting involves using various migration trajectories of different cells based on the hydrodynamic properties of the flow channel with no interference from any external field.

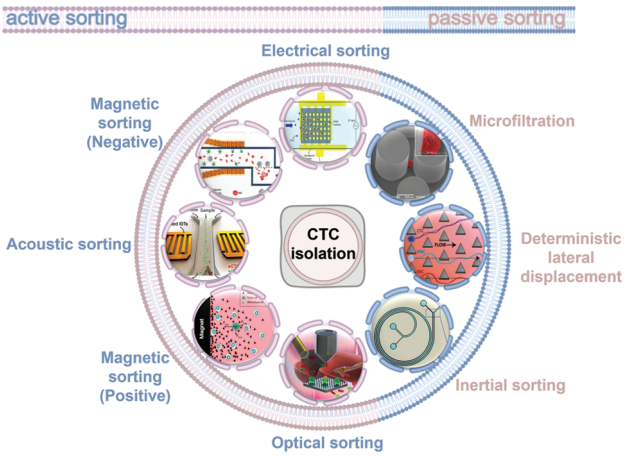

FIGURE 1. Summary of CTC isolation method including active sorting and passive sorting, where active sorting includes electric field sorting, magnetic field sorting, acoustic field sorting, and optical tweezers sorting. Passive sorting includes microfiltration, deterministic lateral displacement, and inertial sorting.

Active sorting

Among the active sorting methods, electrical sorting is currently the most widely used particle control method, primarily in the form of electro-permeation, electrophoresis, and dielectrophoresis (Hajba and Guttman, 2014; Semaan *et al.*, 2021). Electrical sorting is based on the dielectric properties of CTCs (Fig. 2a). Jahangiri *et al.* (2020) introduced a label-free cytological slide chip (CSC) based on the AC electric field stimulation of breast cell lines and blood cells at low frequencies (1–200 kHz). AC-CSC can be used to separate CTCs from leukocytes (1% MDA-MB-231:99% white blood cells (WBC)) with a capture efficiency of 90%. Arslan *et al.* (2022) proposed a continuous-flow, antibody-free, dielectrophoresis-based microfluidic device to separate CTCs from blood cells. CTC recoveries ranged from 74% to 98% at a frequency of 1 MHz and an amplitude of 10–12 Vpp. The main commercial cell sorting systems based on electrophoresis technology are the DEPArray system (Medoro, 2003) (Menarini Silicon Biosystems, Castel Maggiore (BO), Italy) and ApoStream system (Gupta *et al.*, 2012) (ApoCell, Houston, TX, USA). Although such systems could achieve a high sorting accuracy, the additional electrodes and other equipment increase the cost of microfluidic chip fabrication and affect cell activity owing to problems such as cell electroporation caused by the electric field.

Magnetic sorting is primarily based on the immunological properties of cells (Fig. 2b). It involves binding magnetic beads coupled with specific immunological markers to the target cells and their separation from unlabeled nontarget cells through the magnetic field generated by a permanent magnet or electromagnet (Bakhshi *et al.*, 2021; Ghafouri and Badieirostami, 2021). Depending on the coupling target,

magnetic sorting can be positive sorting, which directly captures tumor cells, and negative sorting, which captures WBC. Wang *et al.* (2021) developed an immunomagnetic bead made of triiron tetroxide and calcium carbonate, which enabled the complete sorting of all subpopulations of tumor cells. Kim *et al.* (2013) proposed a CTC separator using immunomagnetic beads bound to the CTC, separated by transverse magnetic electrophoresis to purify 90% of the spiked CTC to 97% at a flow rate of 5 mL/h. Mishra *et al.* (2020) proposed an ultra-high-throughput microfluidic chip, the LPCTC-iChip, which rapidly separates leukocytes from over six billion nucleated cells. This increased the CTC separation capacity by two orders of magnitude, with a recovery rate of 86% at an enrichment of 105. During the capture process, the nonspecific interaction between magnetic beads and leukocytes worsens the purity problem. To address this problem, Zhu *et al.* (2018) proposed the coating of cancer-targeting molecules, such as folic acid and magnetic beads on red blood cells, that would adhere to CTCs to obtain CTC-RBCs. Subsequently, red blood cells could be lysed to obtain CTCs. The CTC purity was >75%. Shi *et al.* (2017) used a microfluidic device with a wavy herringbone structure to separate CTCs using magnetic nanoparticles coated with anti-EpCAM. The magnetic nanoparticles were trapped on the wavy herringbone U-shaped sites on the polydimethylsiloxane (PDMS) surface by an external magnetic field and the magnetic particles were released by removing the magnetic force. Tests were performed on whole blood at low concentrations (down to 100 mL^{-1} of HCT-116 cells) with high capture efficiencies in the range of 81%–95%. Although magnetic field sorting has a high sorting accuracy, it requires an external field and is not easily integrated with low throughput. The positive sorting method does not remove the magnetic beads; moreover, it is harmful to cells, and is unsuitable for subsequent tumor cell culture analysis. The negative sorting method does not ensure the purity of separation and has a long processing time.

Acoustic sorting uses the various directions of motion of different particles under the force of acoustic radiation to achieve sorting (Fig. 2c). Acoustic radiation is generated by piezoelectric crystals and sound surface waves (Augustsson *et al.*, 2012; Karthick *et al.*, 2018). The most popular method was developed by Professor Huang Jun of Duke University, in which multiple pairs of forked-finger transducers were used to generate acoustic surface waves and create standing waves to assist in the aggregation of different cells in the flow channel at locations such as wave peaks and troughs, thereby completing sorting (Wu *et al.*, 2017). Antfolk *et al.* (2015) described a simple acoustic absorption-based cell separator that recovered 86.5% of cancer cells at a sample flow rate of 100 μmL/min. Increasing the acoustic intensity resulted in the 94.8% recovery of cancer cells with 2.2% leukocyte contamination. Wu *et al.* (2019) presented an acoustic device integrated with 36° lithium niobium oxide coated with indium tin oxide to separate CTCs. Their results showed 91.5% ± 4.5% average separation efficiency of cancer cell lines. A method for the acoustic separation of CTCs from leukocytes was investigated by Wu's group (Wu *et al.*, 2018). This method integrates acoustics and microfluidics to isolate rare CTCs from peripheral blood at high throughput with minimal cellular damage, maintaining the structural, biological, and functional integrity of the cells. At a throughput of 7.5 mL/h, the method achieves at least 86% recovery of CTCs from leukocytes while maintaining cell proliferation capacity. This acoustic sorting method can achieve high throughput. However, the integrated acoustic radiation generation device increases the integration difficulty and fabrication cost of the chip, while the cell size-based sorting method limits the purity of sorting and is harmful to cells.

Optical sorting is based on the different deflection angles of the trajectories of particles of different sizes and refractive indices under the action of energy traps formed by laser beams (Polimeno *et al.*, 2018). Hu *et al.* (2019) constructed a light-controlled system to trap tumor cells in the blood (Fig. 2d). The tumor cells were used to target and bind homologous blood cells, which significantly enhanced the difference in optical constants between tumor cells and blood cells and the tumor cells were efficiently sorted (tumor cell recovery of 90% and purity >50%). Chou *et al.* (2017) proposed a cell manipulation and flow rate control technique based on optical-channeled electrophoresis, in which a four-stage cell isolation system using four optical-based virtual cell filters were designed in the optically-induced-dielectrophoresis system to improve the cell separation efficiency. The method resulted in a cell separation purity of 94.9% ± 0.3% and cancer cell recovery of 54% ± 7%. However, the auxiliary optical system is limited in its highly complex setup, extremely low throughput, and the damage to cells is also unacceptable for clinical use.

Passive sorting

Among the passive sorting techniques, microfiltration sorting (Kolostova *et al.*, 2014; Rawal et al., 2016; Sonoda *et al.*, 2020; Zinggeler *et al.*, 2015), such as the use of microcolumns to complete tumor cell screening based on cell particle size, was proposed by Professor Mehmet Toner of Harvard Medical School and the work is published in Nature (Nagrath *et al.*, 2007). Han *et al.* (2022) designed a reusable membrane filtration device that uses a horizontal rotor and fluid assist in passing CTCs through a centrifugal filtration membrane for rapid and efficient cell capture (Fig. 3a). The device achieved an average capture rate of 95.8% for cancer cells, with a survival rate of >90% and a leukocyte removal rate of 99.72% after four treatments. A new method based on biophysical properties was developed by Liu's group (Liu *et al.*, 2018), who designed a pyramid-shaped microchamber for efficient isolation of CTC from breast cancer patients at an outlet height of 6 μm and a flow rate of 200 μL/min, with a CTC capture efficiency of over 85%, and no blockage problems. To date, filter-based sorting solutions no longer use a single cutoff diameter. For example, McMasters has reported a lamellar microporous sieve filter that uses microfabrication processes, such as photolithography, to provide arrays of 10, 15, and 20 μm microporous pores that can effectively achieve the screening goals of simultaneously sorting multiple cell sizes; moreover, it is not harmful to health and does not require any external field. However, the

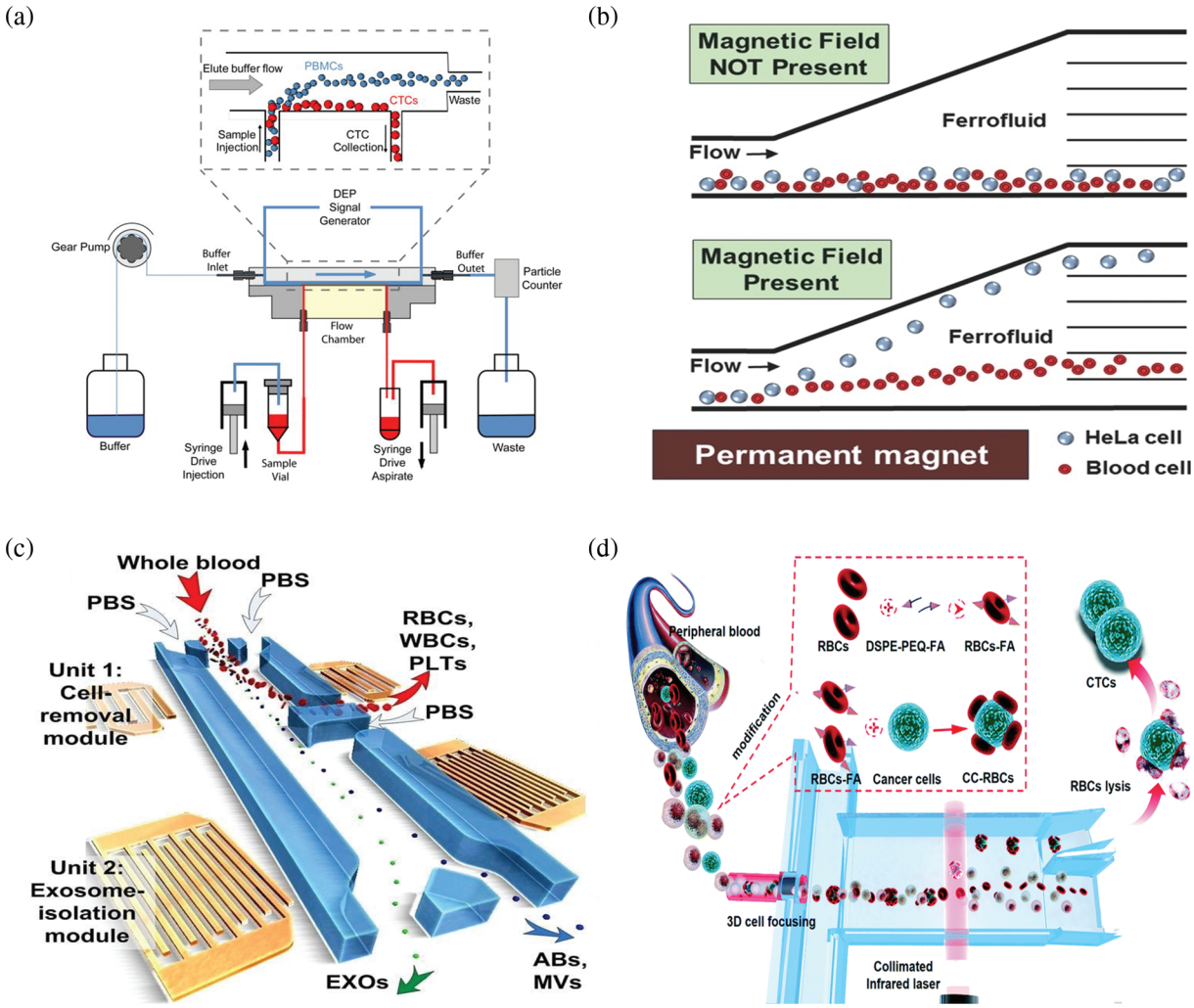

FIGURE 2. Schematic diagram of the microfluidic device for separating circulating tumor cells (CTC) in active sorting. (a) Schematic diagram of the dielectrophoresis apparatus used to separate CTCs (Gupta *et al.*, 2012); (b) Schematic representation of label-free isolation of HeLa cells in a ferromagnetic fluid by magnetic buoyancy under a magnetic field (Zhao *et al.*, 2016); (c) Schematic diagram of the integrated acoustic-fluidic device for the separation of CTC (Wu *et al.*, 2017); (d) Schematic diagram of the optical separation device used to separate CTCs (Hu *et al.*, 2019).

sorting purity of microfiltration sorting is extremely low, making it difficult to meet clinical testing requirements.

The concept of deterministic lateral displacement (DLD) was introduced by Huang of Princeton University in their study published in Science (Huang *et al.*, 2004). The main design idea was to set up a series of inclined microcolumn arrays in the microfluidic channel to achieve particle sorting based on the different angles of deflection of the trajectories of particles of different sizes after perturbation by the columns (Edd *et al.*, 2020; Fachin *et al.*, 2017; Khodaee *et al.*, 2016; Liu *et al.*, 2018; Tang *et al.*, 2022) (Fig. 3b). Liu *et al.* (2013) introduced a microfluidic system integrating a microfluidic DLD array and a cell capture structure. The system achieved a CTC capture rate of 90% and capture purity of >50% at a cell concentration of 10^2 cells/mL. Au *et al.* (2017) developed a two-stage continuous microfluidic chip to isolate and recover of viable CTC clusters from blood. The method used both the size and asymmetric geometric properties to sort clusters via DLD, with a 99% CTC recovery rate and cell survival rate of over 87%. In 2021, Liu *et al.* (2021) proposed the concept of the deterministic lateral displacement of filters by combining filtration concepts with DLD structures for hydrodynamic sorting. This resulted in a separation efficiency of >96%, high cell purity of 99.995% through WBC removal, high cell viability of >98%, and high processing efficiency of 1 mL/min. Bhattacharjee *et al.* (2022) designed an asymmetric microcolumn array that regulated the flow resistance of fluid in the flow channel, which was more conducive to the differentiation of tumor cells from leukocytes. Ahmed *et al.* (2017) developed a size-controlled immunocapture chip with a triangular microarray structure that selectively enhances CTC interactions by deterministic lateral displacement. The microcolumns of this chip successfully captured more than 90% of CTCs (92.2% ± 6.4%). However, the sorting purity of deterministic lateral displacement sorting was lower, and the processing of microcolumn arrays was more difficult, resulting in the low success rate of microarray fabrication.

Currently, inertial sorting is the most popular and widely used sorting scheme in passive sorting (Fig. 3c). The main principle is based on the hydrodynamic properties of

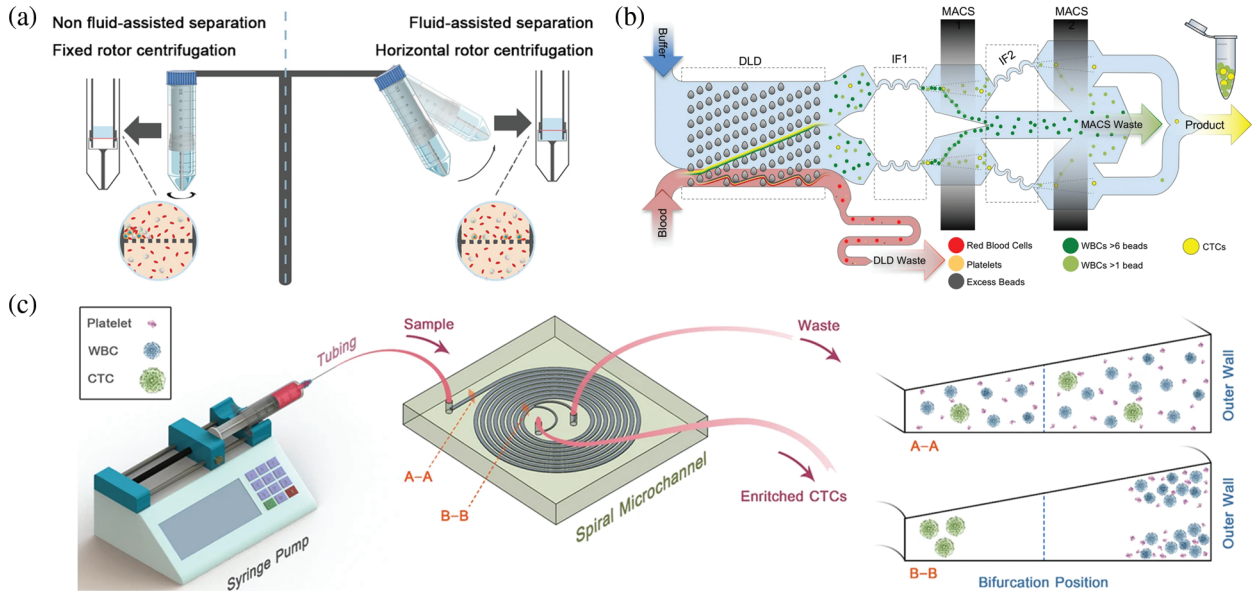

FIGURE 3. Schematic diagram of the microfluidic device for separating circulating tumor cells (CTC) in passive sorting. (a) Schematic diagram of a polydimethylsiloxane microfiltration membrane integrated microfluidic device for CTC capture (Han *et al.*, 2022); (b) Schematic diagram of a deterministic lateral displacement microfluidic chip for CTC isolation (Fachin *et al.*, 2017); (c) Schematic of the inertial sorting microfluidic chip for CTC separation (Kulasinghe *et al.*, 2017).

particles in the flow channel under the joint action of inertial lift, Dean's traction, and other forces to complete the high-throughput separation of particles of different sizes (Mutlu *et al.*, 2020; Oakey *et al.*, 2010; Smith *et al.*, 2021; Zhou *et al.*, 2019). In addition to Toner's group at Harvard University, which pioneered the concept of inertial microfluidics in the Nature Journal, di Carlo *et al.* (2009) at the University of California, Los Angeles, proposed the

TABLE 1

Summary of active and passive methods for circulating tumor cells (CTC) sorting

	Isolation method		Basis	Recovery	Flow rate	Purity	Advantage	Defect	Ref.
Active sorting	Electric field sorting		Dielectric properties	90%	6 μL/min	Not mentioned	High sorting accuracy; no mark	Not easy to integrate	Jahangiri *et al.* (2020)
	Magnetic field sorting	Positive sorting	Immune properties of tumor cells	97%	5 mL/h	Not mentioned	High sorting accuracy	Irremovable magnetic beads	Kim *et al.* (2013)
		Negative sorting	Immune properties of white cells	86%	73 mL/h	Not mentioned	High flux; no mark	Long processing time	Mishra *et al.* (2020)
	Acoustic field sorting		Cell size, cell density	86.5%	100 μL/min	Not mentioned	High sorting accuracy; no mark	Not easy to integrate	Antfolk *et al.* (2015)
	Optical separation		Cell size, refractive index	90%	1.5 μL/h	>50%	High sorting accuracy; no mark	Extremely complex system	Hu *et al.* (2019)
Passive sorting	Microfiltration sorting		Cell size	80%	60 mL/h	Not mentioned	Simple structure; low cost; no mark, no outfield	Low sorting accuracy	Sonoda *et al.* (2020)
	Deterministic lateral displacement		Cell size	90%	1.2 mL/min	<50%	No mark; high throughput; no outfield	Difficult to fabricate	Edd *et al.* (2020)
	Inertial sorting		Cell size	85%	5 mL/min	35.2%	No mark, high flux; no outfield; easy to integrate; low cost	Low sorting accuracy	Zhou *et al.* (2019)

concept of inertial microfluidics. Khoo *et al.* (2018) at the National University of Singapore have been working on developing and commercializing spiral inertial microfluidic chips for tumor cell sorting. Wang *et al.* (2022) at Southeast University have been working on the theory and application of inertial microfluidic chips. A double-spiral microfluidic device with a finely tunable cutoff value of 9 μm and a separation range of 2 μm was proposed and validated by Pena (Rodriguez-Pena *et al.*, 2022). They isolated 17 CTCs/mL from the peripheral blood of a hepatocellular carcinoma patient with leukocyte and erythrocyte removal rates of 96.03% and 99.99%, respectively. Using the inertial lift, Gao *et al.* (2020) developed a label-free separation microfluidic device using multi-stage channels, a chip with mainly a herringbone channel, a rectangular vessel, and inertial focusing microchannels, which can be used to separate CTC. At a flow rate of 9 L/min, the device had a separation efficiency of 90% and a purity of 84.96%. However, this method is based solely on cell size, and the purity of separation is unable to meet clinical requirement.

The above methods were compared in detail and summarized in Table 1.

Conclusions

CTC, as one of the main markers of liquid biopsy, is an effective means of improving our understanding of cancer metastasis. The ability to sort high-purity, high-viability CTCs is, therefore, critical to the development of clinical medicine and cell biology. Researchers have conducted various more efficient microfluidic devices than traditional filtration, centrifugation, and immunomagnetic bead methods. This paper reviewed the latest developments in microfluidic-based CTC sorting via active and passive sorting and compared the advantages and disadvantages of different methods. In general, additional external sources, such as electrodes, signal generators, electromagnets, forked-finger transducers, and light transmission paths, must be integrated into the microchip when building an active sorting system. However, these additional devices increase the complexity of the system and the difficulty of processing. More importantly, the addition of sources is detrimental to cell activity and, consequently, to clinical studies of downstream cancers. In contrast, the passive sorting method does not affect cell activity. However, because the cell particle size is the main sorting criterion, it cannot overcome the problem of the size overlap between CTCs and leukocytes owing to the presence of cell heterogeneity. Therefore, chips with integrated active and passive sorting should be the focus of subsequent research. The multimethod coupled integrated system could solve the current problems of CTC sorting, such as the inability to simultaneously achieve a high recovery rate, high purity, and high throughput. Based on the current research status, we believe the microfluidic device could be more reliable and stable in the future when separating CTCs, and these novel microfluidic technologies for CTC capture and isolation will play a crucial role in the early detection of cancer, real-time monitoring and more.

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