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Review Article

Novel approaches in cancer management with circulating tumor cell clusters



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ABSTRACT

Tumor metastasis is responsible for the vast majority of cancer-associated morbidities and mortalities. Recent studies have disclosed the higher metastatic potential of circulating tumor cell (CTC) clusters than single CTCs. Despite long-term study on metastasis, the characterizations of its most potent cellular drivers, i.e., CTC clusters have only recently been investigated. The analysis of CTC clusters offers new intuitions into the mechanism of tumor metastasis and can lead to the development of cancer diagnosis and prognosis, drug screening, detection of gene mutations, and anti-metastatic therapeutics. In recent years, considerable attention has been dedicated to the development of efficient methods to separate CTC clusters from the patients' blood, mainly through micro technologies based on biological and physical principles. In this review, we summarize recent developments in CTC clusters with a particular emphasis on passive separation methods that specifically have been developed for CTC clusters or have the potential for CTC cluster separation. Methods such as liquid biopsy are of paramount importance for commercialized healthcare settings. Furthermore, the role of CTC clusters in metastasis, their physical and biological characteristics, clinical applications and current challenges of this biomarker are thoroughly discussed. The current review can shed light on the development of more efficient CTC cluster separation method that will enhance the pivotal understanding of the metastatic process and may be practical in contriving new strategies to control and suppress cancer and metastasis.

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1. Introduction

Metastasis is a complicated, multistep process where cancer cells detach from the primary tumor, migrate to adjacent tissues, invade and travel through the bloodstream or the lymphatic system, survive, proliferate, colonize in distant organs and finally establish a new tumor (Fig. 1a) [1–12]. These tumor cells that travel through the bloodstream or the lymphatic system are called circulating tumor cells (CTCs).

After decades of research, our understanding of metastasis is still inconclusive, even though more than a century has been

passed since the first report of Thomas Ashworth in 1869 on the presence of circulating tumor cells (CTCs) in the bloodstream [13–15]. Currently, metastasis is assumed to be responsible for around 90% of cancer-related deceases [16–18]. Despite decades of research and experiments, cancer therapies have not been sufficient yet, and the mortality rate of cancer metastasis has marginally ameliorated. Mechanistic understanding of the metastasis process can lead to the development of anti-metastatic therapies that improve patient mortality [19]. An increasing number of studies have shown the important role of CTCs in cancer metastases. CTCs supply more straightforward and comprehensive information about the tumor [20]. They can be used for various experimental purposes, e.g., examining the response of cancer cells to chemotherapy, predicting the overall survival, noninvasively monitoring the drug susceptibility, metastatic therapy and as early detection and prognostic biomarkers [21–25]. Additionally, US food and drug administration (FDA) approved CTCs clinical applications for

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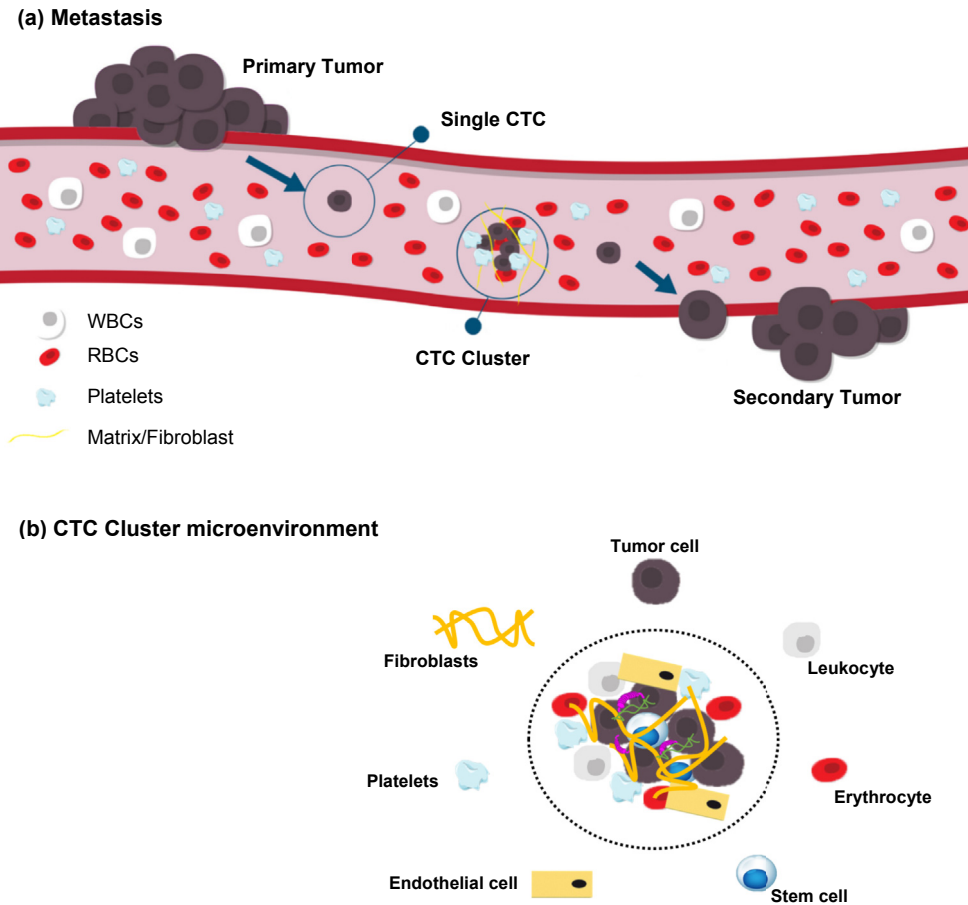


Fig. 1. (a) Circulating Tumor Cells (CTCs) detach from primary tumor as single cells and clusters, shed into the bloodstream, and migrate to colonize in distant organs, known as metastasis. It is assumed 1 ml of blood can comprise 1–10 single CTCs and roughly one CTC cluster, millions of WBCs and billions of RBCs. Copyright © 2017 Vortex Biosciences. **(b)** The microenvironment of CTC cluster comprises immune cells, platelets, dendritic cells, cancer-associated fibroblasts, and tumor stroma. Such microenvironment can protect CTC clusters from blood shear damage and immune attacks that provides CTC cluster metastatic advantages. Reproduced after Vortex Biosciences.

personalized treatment in metastatic colorectal, prostate, and breast cancers.

Conventional hypotheses assume that metastasis is established by the invasion and proliferation of individual CTCs into distant organs after the epithelial–mesenchymal transition (EMT), which increases the invasiveness of the CTCs [26]. However, the discovery of CTC clusters in clinical and animal models [27], the groups of two or more tumor cells with strong cell–cell contacts, has challenged this assumption. Individual CTCs might not be the only cause of metastases; rather, multicellular aggregates of CTCs, CTC clusters, may play a significant role [28,29]. For the first time, in 1954, Watanabe studied metastasis in mouse model and reported the higher potential of CTC clusters in tumor metastases [30]. In the following decades, the 1970s, experimental studies also demonstrated the higher capacity of CTC clusters in metastases compared to that of single CTCs. Fidler et al. found that, if cancer cells were aggregated into clusters before injection, these cells established several-fold more tumors than the equal numbers of individual cancer cells [31]. Other researches later confirmed this finding [32–37].

Based on *in-vitro* quantification methods, it is known that CTC clusters comprise 5–20% of the total CTCs depending on the disease stage in both human and animal models [38–40]. However, a recent study indicated that the proportion of CTC clusters in the late stage of metastatic cancer is much higher than previously assumed [41]. Following studies also demonstrated that CTC clusters, despite

their rarity, are responsible for seeding ~50–97% of metastatic tumors in mouse models [42]. This indicates that CTC clusters have 23 to 100 times higher metastatic potential than individual CTCs [39,42]. Interestingly, single CTCs with the lower metastatic potential could acquire higher metastatic capability when incorporating with other cells in a cluster [43]. This justifies the critical role of CTC clusters in cancer metastases. Experiments also revealed that the detection of only one CTC cluster in blood at any given time point correlated with significantly lower survival rates in the patients with prostate, colorectal, breast and small-cell lung cancers [28,39,44]. Altogether, it is quite likely that CTC clusters play a far more significant role in the metastasis process than previously believed.

CTC clusters are not simply a collection of tumor cells. CTC clusters include some other non-tumor cells such as endothelial cells, erythrocytes, stromal cells, leukocytes, platelets, and cancer-associated fibroblasts [39,45–51]. These non-malignant counterparts were believed to provide advantages for CTC clusters survival. Higher metastatic potential of CTC clusters has been reported to be related to several factors. These factors include the cooperation of heterogeneous cell phenotypes within the clusters [52], strong cell–cell adhesions, which protect the tumor cells against anoikis [53], and physical shielding against the attacks of the immune cells (Fig. 1b) [54].

Despite all the research and hypotheses to date, the rarity of CTCs in blood sample (1–100 CTCs per 10^9 blood cells and even

fewer CTC clusters) and deficiencies of the existing separation methods limit our knowledge about CTC clusters. Many questions about CTC clusters formation, distribution and properties are still to be answered. To address these questions, an efficient separation platform is the first step to capture sufficient viable CTC clusters. Such platform makes subsequent molecular, genetic and biological analyses possible. Over the past several years, rapid progress in CTCs research has resulted in the development of technology that also can separate CTC clusters. However, currently, limited specialized techniques have been developed for the separation of CTC clusters.

In recent years, great attention has been paid to CTC clusters because of their importance in cancer metastases, and the number of the published articles on CTC clusters has exponentially increased (Fig. 2). Despite the recent advances and discoveries, CTC cluster has not yet been reviewed comprehensively. Herein, we collate many interesting publications to provide a comprehensive review about CTC clusters from all the related aspects, including separation methods as well as their clinical applications and provide scopes for the future research direction.

2. Separation techniques and devices

Rarity is a significant challenge for the separation of CTC clusters. A 10 ml of a peripheral blood sample from a metastatic cancer patient typically contains 0–100 single CTCs and roughly 0–5 CTC clusters (only about 5–20% of all CTCs) [39] among approximately 50×10^9 RBCs, 80×10^6 WBCs and 3×10^9 platelets [55]. Another challenge for CTC cluster separation is possible dissociation during the blood sample processing. An efficient platform to isolate CTC clusters would have the capacity to separate intact CTC clusters of different shape, size, and composition, autonomously of cell surface markers with minimum manipulation, fast processing time, and vigorous clinical feasibility and validity. To date, numerous strategies have been developed for isolating single CTCs from blood sample [56–61] based on the physical (e.g., size, density, deformability, electrophoresis, dielectrophoresis), or biological (e.g., antibody expression) differences of CTCs and non-tumor cells. However, only a few platforms have been developed specifically for CTC clusters separation. To date, microfluidic devices appear to be the most encouraging platform for separating CTC clusters, as they have several unique features, such as the ability to process whole blood without preprocessing, which results in less cluster dissociation, fast processing time, and collection of live CTC clusters without manipulation. Up to now, most studies around clusters have relied on the strategies designed for individual CTCs, which

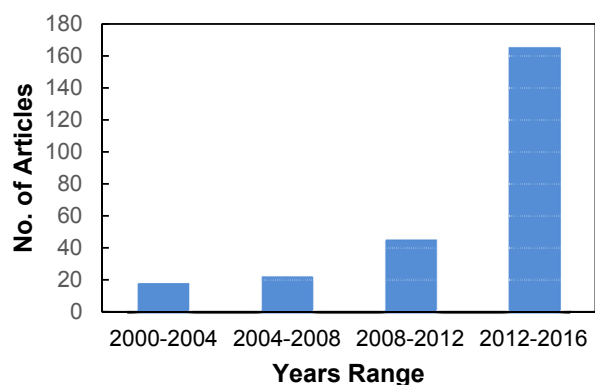


Fig. 2. The number of articles in "CTC Cluster" & "Circulating Tumor Cell Cluster" in 2000–2016 according to PubMed trend shows that the published articles around CTC cluster have been increased in recent years.

have insufficient efficiency to separate clusters. CTC clusters were observed fortuitously, using these platforms, which usually underestimated the number of the CTC clusters due to the limitations of the employed techniques. The platforms with the capability of isolating CTC clusters are summarized in Table 2 and are briefly reviewed in this section. Recent progress in active separation methods can also aid the development of more advanced CTCs-detecting techniques [62]. Investigating active detection techniques is out of the scope of current paper that focuses mainly on passive platforms, which are more feasible and have higher potential to be commercialized.

2.1. Antibody-based devices

Antibody-based methods are the most widely used techniques for CTCs separation. These methods rely on the expression of cellular surface markers and either isolate cancer cells (positive selection) or remove normal blood cells, thereby enriching cancer cells (negative selection). The antibodies mainly pertain to epithelial cell surface markers that are absent from other blood cells [63–66]. The epithelial cell adhesion molecule (EpcAM) Antibody, cytokeratin antibody (anti-CK) and CD45 are the most common antibodies for distinguishing CTCs and other blood cells. However, there are still some limitations in these techniques, such as difficulties in distinguishing between CTCs and non-malignant epithelial cells [67]. Furthermore, capturing CTCs that have undergone the EMT process cannot be appropriately done using antibody expression techniques.

One simple technique for detecting and capturing the presence of CTCs in a blood sample is a high-resolution imaging method. In this method, blood is first lysed, then the remaining nucleated cells are plated on a surface and stained with antiEpcAM-fluorescent antibodies to discriminate cancerous from other cells. However, this technique is incompatible with the applications that require the recovery of viable CTCs because the cells are fixed during processing. CytoTrack™ solve this issue by developing a pre-scanner blood sample at high rates (up to 120 million cells/min) and recorded the potential CTCs targets, and operator can select specific cells to be isolated by CytoPicker™ for further analyses and corroboration [68] (Fig. 3a). RareCyte also developed a similar platform [69]. Commercial Epic CTC Platform (Epic Sciences Inc., USA) as another high-speed automated imaging platform uses anti-CK/CD45/DAPI (4',6-diamidino-2-phenylindole) immunofluorescent staining to detect CTCs. The epic platform was reported to be highly efficient for CTC clusters detection [70]. Ensemble-decision aliquot ranking (eDAR) [71,72] is another imaging platform that uses multi-color line-confocal to identify and enumerate EpcAM labeled cells. In this platform, a switching mechanism steers positive aliquot to slits filtration unit and negative aliquot to waste collection thorough different channels [73] (Fig. 3b). CTC clusters with low EpcAM expression were observed in the patient blood samples, utilizing eDAR [73].

Another technique is CellSearch® [26,74,75] (Veridex, USA), which is a magnetic-activated cell sorting (MACS) method. This technique is the first and only clinically validated and an FDA-cleared blood test for CTCs enumeration and separation. In this method, a 7.5-ml blood sample is centrifuged to separate solid blood components from plasma. Using magnetic nanoparticles coated with antibodies to target EpcAM. The cells that have bound

Table 1
CTCs, Leukocyte and Erythrocyte size range.

Cell type	CTC	Leukocyte	Erythrocyte
Size Range (μm)	12–30	6–20	4–8

Table 2
CTC cluster separation platforms.

Subcategory	Platform	Similar methods	Separation criteria	Key features	Throughput	Capture efficiency
Microfluidics/Antibody	HB-Chip [104]	CTC-chip [246], GEDI [102], GEM [105], OncoBean Chip [247]	EpCAM	Passive micro vortices mix sample to increase CTC-antibody-coated surface	15–80 μ l/min	79% for spiked single cells/~15% CTC cluster from patient blood sample
Microfluidics/Antibody	Modular Sinusoidal Microsystem [108]		EpCAM	Three modules for separation, enumeration, and imaging	~160 μ l/min	86% for spiked cells/71% CTC cluster from patient blood sample
Filtration	ISET [®]	ScreenCell [®] [158]	Size/Deformability	8- μ m pores filters	~3000 μ l/min	43% for single cell in patients sample/5–100% CTC cluster from patient blood sample
Filtration	FMSA [146]	CellSieve [®] [148], Microcavity array [154]	Size/Deformability	flexible micro spring array, process whole blood sample without preprocessing	750 μ l/min	76% % for single cell in patients sample/44% CTC cluster from patient blood sample
Microfluidics	Cluster Chip ^a [190]		cell–cell adhesion	even two-cell clusters can be efficiently captured, only separate CTC clusters	~40 μ l/min	30–40% CTC cluster from patient blood sample
Microfluidics	ClearCell [®] FX [182]	Vortex Chip [248], Double spiral microchannel [249], eDAR [71]	Size/Inertial Focusing	RBC lysis required, easy to manufacture	~1000 μ l/min	100% CTCs in patient samples/ CTC Cluster observed
Antibody/Image processing	CytoTrack [68]	FASTcell [™] [250], EPIC platform [®] , RareCyte [69]	EpCAM	Similar capture efficiencies with CellSearch	Scan 120 M cells/min	~69% for single CTCs in patients sample/Clusters observed
Antibody	CellSearch [®] [75]	Vita-assay [™] , EasySep [®] [84], AdnaTest [®] , MACS [251], MagSweeper [252]	EpCAM	FDA approved		20–80% for single cells in patient samples/CTC clusters observed
Microfluidics	DLD Chip ^a [193]		Size/Asymmetry	Single and cluster CTCs separation with 87% viability	~17 μ l/min	66–99% CTC cluster capturing
Microfluidics/Antibody	3D scaffold chip ^a [195]	CMx platform [253], nanostructure coated chip [254], GO Chip [111]	Size/EpCAM	Single and cluster CTCs separation	50–100 μ l/min	80% single cells & 86% CTC cluster from spiked cells
Antibody	CellCollector [®] [89]		EpCAM	In-vivo CTCs isolation, CE approved, large volumes blood processing	30 min operation time	70% for single CTCs in patients sample/CTC cluster observed
Centrifugation	OncoQuick [®]		Density	Porous membrane for additional separation	~1 h operation time	70–90% single spiked cells/CTC cluster observation potential
Centrifugation/Antibody	RosetteSep [®]		Density/Antibody	Negative selection by repulsion unwanted cells	~1 h operation time	77% single spiked cells/CTC cluster observation potential

^a Specially developed for CTC cluster separation.

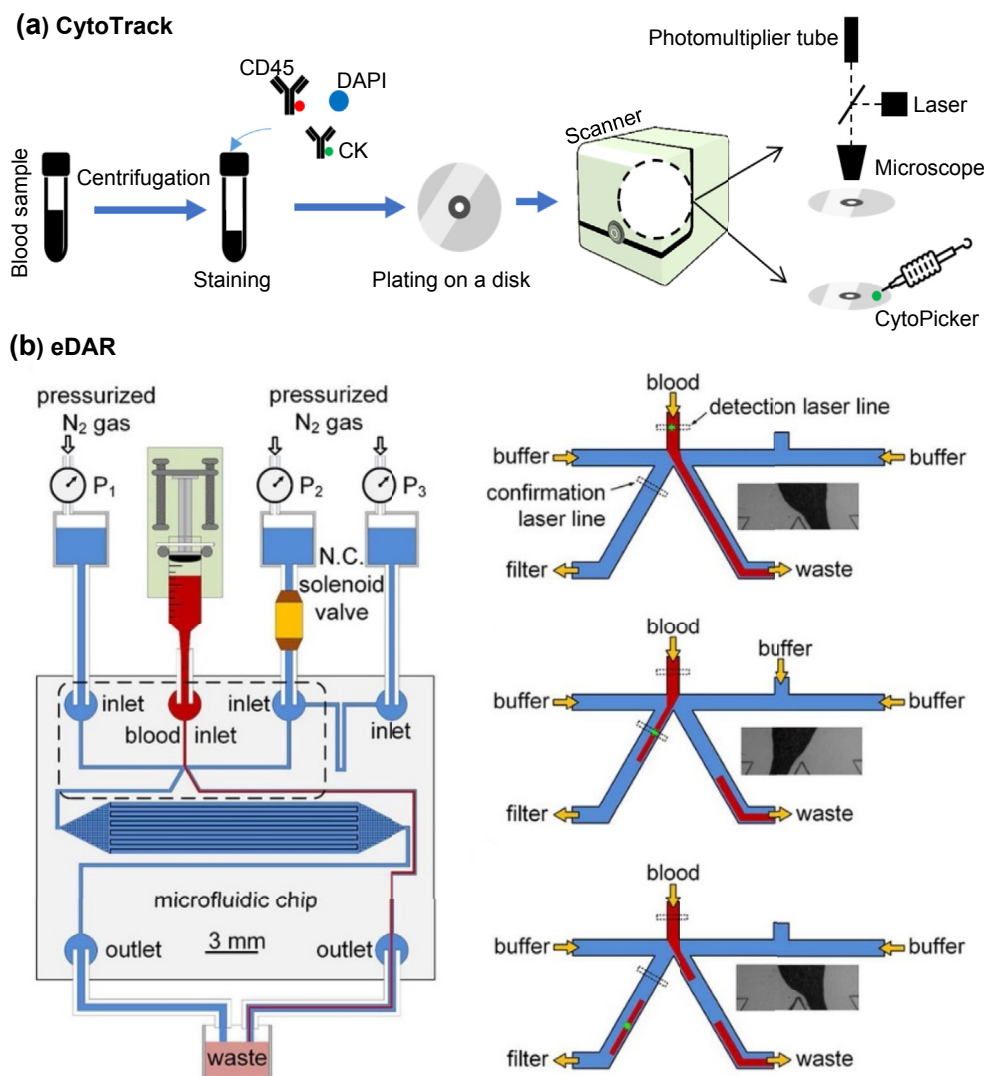


Fig. 3. Imaging antibody-based techniques of CTC cluster separation. **(a)** The CytoTrack schematic workflow. Blood sample centrifuged and RBCs lysed, fluorescent antibodies added, plated on a glass disk. The scanner excited stained cells with a laser at 488 nm and the signals are detected by a photomultiplier tube (PMT). The positions on the disk with possible CTCs are recorded as hotspots. Then the CytoPicker can isolate the intact cells from the disk. **(b)** Microfluidic chip and hydrodynamic switching scheme of eDAR platform. When a CTC was detected by confocal system in blood stream, the blood flow was switched to the CTCs collection channel, in which aliquot is steered to a filtration area with 20,000 microslits. The blood flow was switched back to waste collection channel after the aliquot was sorted. Adapted with permission from ref [73] under the terms of the Creative Commons Attribution License. Copyright© 2013, American Chemical Society.

to the nanoparticle are pulled to the magnets, and the rest of the cells are removed [76]. Therefore the CTCs are magnetically separated from other blood cells and subsequently identified with the use of fluorescently labeled antibodies (Fig. 4a) [75]. In CellSearch method, a CTC cluster is defined as a group, comprising more than two cells expressing EpCAM, cytokeratins (CKs 8, 18, and 19) and DAPI without expression of CD45 [25,53,77–83]. There are also some techniques that use similar CellSearch principle, labeling CTCs with antigen-specific antibodies linked to magnetic beads like Dynal Magnetic Beads® (Invitrogen, USA), AdnaTest (Adnagen AG) (uses a cocktail of antibodies e.g., EpCAM and MUC-1, and AdnaTest Cancer-type cocktail unlike CellSearch anti-EpCAM antibodies), and EasySep® (negative selection) (Stem Cell Technologies, Canada) [84] that CTC clusters have also been observed using them.

Beside CellSearch, Vitax Inc. developed the cell adhesion matrix (CAM) assay [85]. The CAM assay exploits the invasive characteristic of cancer cells in collagen to isolate metastatic

invasive circulating tumor cells (iCTCs). When patient blood samples are applied to the CAM-coated tubes (Vita-Cap™) or culture plates (Vita-Assay™), iCTCs that uptake cell-adhesion matrix preferentially adhere to CAM (Fig. 4b). This technique separates CTCs in metastatic prostate and breast cancer [86,87].

The limited blood sample volumes from cancer patients (5–20 ml) may impose a severe restriction on the separation of rare CTCs. CellCollectors® (GILUPI GmbH, Germany) is a European Conformity (CE) approved *in-vivo* CTCs isolation base on antibody affinity [88]. The system consists of a needle, which is placed directly in the peripheral arm vein of a patient with up to 1.5 L of blood pass via an indwelling catheter for 30 min. The flexible needle is made of stainless steel, a gold coating layer of 2- μ m thickness and a hydrogel coating layer with 2–10 μ m thickness. On the hydrogel layer, antiEpCAM-antibodies are conjugated to identify and isolate the EpCAM-positive CTCs that can be analyzed in downstream analyses (Fig. 4c). GILUPI claims that CellCollectors

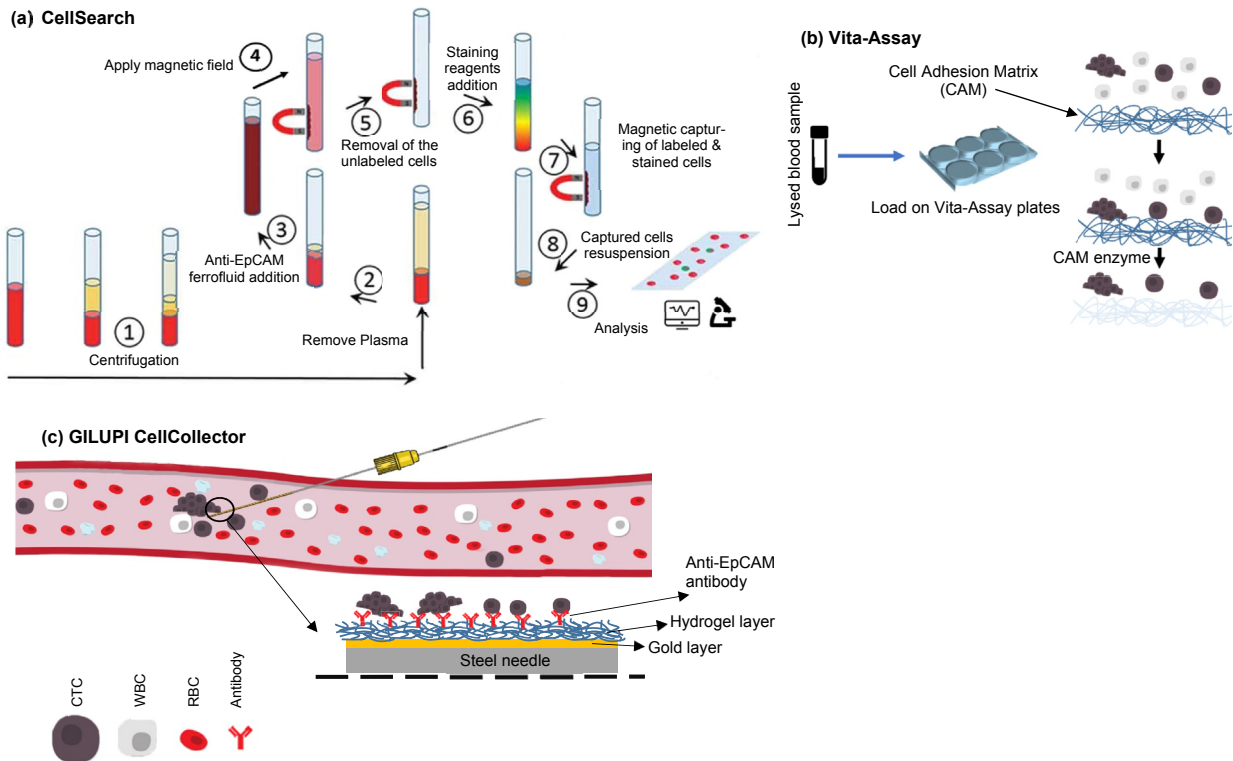


Fig. 4. Antibody-based technique of CTC cluster separation. **(a)** The CellSearch procedure. 7.5 ml of blood sample is centrifuged, plasma removed, added anti-EpCAM-ferrofluid, incubated in presence of magnetic field then captured cells is analyzed. Adapted with permission from ref [76] under the terms of the Creative Commons Attribution License. Copyright© 2016 Swennenhuis, J. F. et al. **(b)** Blood sample lysed then with complete cell culture medium is loaded on Vita-Assay and incubated in CO₂ incubator. CTCs are captured based on their preferential adhesion to CAM. Identification and enumeration of iCTCs can be made by image microscopy and flow cytometry **(c)** The GILUPI CellCollector is placed directly into the bloodstream of arm vein via an indwelling catheter for 30 min, captures CTCs by conjugated antiEpCAM-antibodies coated on golden-hydrogel layer.

can detect 70% of CTCs in lung, breast, colorectal and prostate cancer patients [89–97]. Further studies with other tumors are currently in progress.

Methods based on biochemical properties also could be combined with and strengthened by microfluidic technologies [98]. Adams et al. designed a microfluidic device containing a series of the high-aspect-ratio microchannel (35 μm width \times 150 μm depth) that were replicated in polymethyl methacrylate (PMMA). The microchannel walls were covalently decorated with antibodies directed against cells expressing the EpCAM [99]. Increasing the throughput of the antibody-based methods, Sequist et al. introduced another microfluidics platform called CTC-chip. A standard microscope-slide-sized silicon chip with μm -sized posts array that coated with antiEpCAM-antibodies, to maximize the interaction of the CTCs with the functionalized surface (Fig. 5a) [100]. The CTC-chip was able to capture CTC clusters in lung cancer [101]. Geleg-horn et al. inspired by CTC-chip described geometrically enhanced differential immunocapturing (GEDI), a theoretical framework for the use of staggered obstacle arrays to create size-dependent particle trajectories that maximize prostate circulating tumor cells (PCTC)-wall interactions while minimizing the interactions of other blood cells [102,103].

The abundant number and intricate structure of the CTC-chip microposts presented a challenge for clinical research. Stott et al. developed a microfluidic device, herringbone HB-chip. The HB-chip design utilizes passive mixing of blood cells through the generation of micro vortices created by angled grooves leading to significantly increase the number of interactions between target CTCs and the antibody-coated chip surface (Fig. 5b) [104]. The device was later optimized geometrically [105]. HB-chip was one of the first microfluidic platforms that can captures the clusters from

metastatic patient blood samples [106]. Inspired by the HB-chip, Hyun et al. proposed a geometrically activated surface interaction (GASI) to increase the surface interaction between the leukocytes and the anti-CD45 immobilized surfaces for CTCs enrichment through negative selection [107]. Another group also proposed the same functionalized surface platform for positive selection, modular CTC sinusoidal microsystem [108] (Fig. 5c). This micro-system has a higher recovery rate for CTC clusters and has been commercialized by BioFluidica.

The principle of enhancing antibody–CTCs interaction for CTCs separation has been inspired many similar methods that also have the potential for CTC clusters separation. Crammed 100–200 nm pillars were coated with the relevant antibody (anti-EpCAM) [109,110]. Instead of micropost arrays, some capturing methods inspired by Stott's work use antibody-coated surfaces to increase antibody-CTCs interactions [111,112].

One of the major limitations in the positive selection of antibody-based methods is its inability to target cancer cells with reduced expression of cancer-associated markers. In the EMT process, cells lose their epithelial characteristics and acquire more mesenchymal-like phenotypes. Consequently, EpCAM expression significantly decreases, especially in the cells within the clusters [113]. In addition, EpCAM also can be detected in other diseases such as benign colon disease can be misinterpreted as cancer cells [114]. Therefore, such positive detection relying on EpCAM expression may disregard some critical subpopulations as the precise number of CTCs may be underrated [115–117]. One idea to overcome this limitation was proposed to target the actin-bundling protein platin3, a novel marker that is not downregulated by CTCs during EMT and not expressed in blood cells [118], N-cadherin, O-cadherin, epidermal growth factor receptor (EGFR), the cytoskeletal

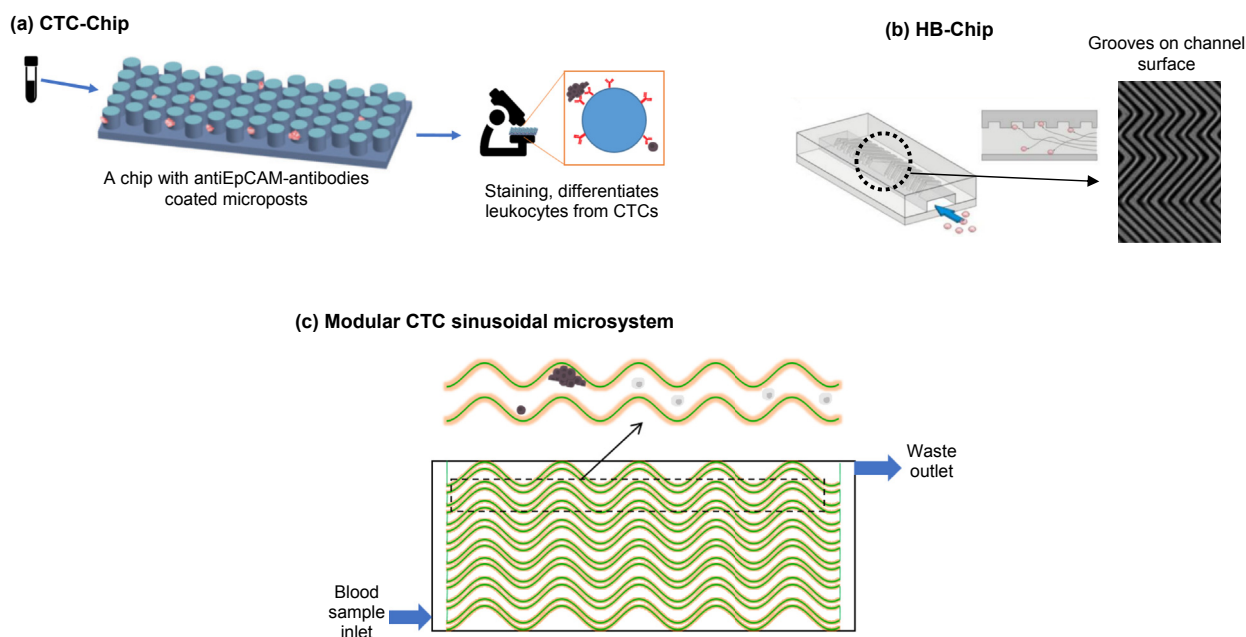


Fig. 5. Enhanced cell–antibodies interaction techniques of CTC cluster separation. **(a)** In CTC-Chip, CTCs are captured against the anti-EpCAM-coated microposts. **(b)** The HB-Chip with its grooves on channel surface generates the mixing effect to enhance cells–surface interaction. Reproduced with permission from ref [104] Copyright© 2018 National Academy of Sciences. **(c)** Modular CTC sinusoidal microsystem. This module consisted of an array of high-aspect ratio sinusoidal microchannels with a nominal width of 30 μm and depth of 150 μm that activated by anti-EpCAM antibodies.

protein vimentin [119,120], and cancer-specific biomarkers [103,121]. However, it was reported inexistence vimentin expression among cells within clusters [122]. In negative selection, leukocytes attachment to CTCs cluster [123] may lead to excluding precious subpopulation of clusters from detection. In addition, circulating endothelial cells are CD45⁻, that can exaggerate the final enumeration of CTC clusters [119]. Another limitation of antibody-based platforms is the lack of a general marker that could be used for a variety of cancer cells. Any marker can distinguish specific tumor cells, but their application is limited by the heterogeneity of tumors and, consequently, the different genetic characteristics of the cell even in the same cancer cells [124]. Most antibody-based separation strategies have generally been employed towards carcinomas as no specific marker targeting other cancer types (e.g. Sarcomas) exists so far.

Lately, a new class of CTC-affinitive agents, viz. aptamers, demonstrated a great potential in the detection of CTCs as an alternative to antibodies [125–128] with some advantages such as high affinity, low cost, simple modification, and simple release mechanisms. Aptamers are synthetic low-molecular-weight single-stranded DNA/RNA which have been engineered to bind to specific targets, such as cancer cells with high affinity and selectivity. Aptamers can bind to cell membrane targets [129,130], and can also be selected against whole cancer cells [131]. Some research groups developed microfluidics-based cell-affinity devices to capture CTCs using aptamers [132–134].

Consequently, in antibody-based methods, the prevalence of CTC clusters was rare [64]. In general, the efficiency of antibody-based methods chiefly depends on two factors: the expression and specificity of the target antigen and the affinity between antigens and antibodies, and the efficiency of labeling process. Compared to single CTCs, CTC clusters have smaller surface-to-volume ratios, which reduce the efficiency of antibody-based platforms to detect clusters that can be more obvious in larger CTC clusters [135]. Although antibody-based methods have been used widely for CTCs separation, there are still some drawbacks,

such as high cost and the need precise procedure, which pose challenges for using them pervasively in CTCs detection for clinical applications.

2.2. Physical property-based devices

Differences in physical properties such as cell density, size, and deformability, can be utilized to separate CTCs and CTC clusters. For instance, CTCs can be separated by filtration due to their larger size compared to other blood cells (Table 1). Most separation platforms based on physical properties use microfluidic technologies. Microfluidic platforms not only provide better efficiency in CTCs separation [136] but also facilitate the integration and the automation of high-throughput low-cost sample processing to achieve a real lab-on-chip solution [137].

Based on the assumption that CTCs especially CTC Clusters are larger than other blood cells (Table 1), microfiltration techniques demonstrate a great potential for attaining high throughput analysis of sample volume. ISET[®] (isolation by size of epithelial tumor cells) (Rarecells diagnostics, France) is developed based on trapping the major epithelial cells (20–30 μm) while passing other cells (6–12 μm) through the pores of predefined size and shape. ISET[®] used a module of filtration (10–12 well) containing polycarbonate track-etch-type membrane, which comprises numerous randomly distributed 8- μm -diameter, cylindrical pores to separate CTCs from blood cells through size and deformability (Fig. 6a) [138]. RareCells claims that ISET[®] sensitivity threshold is one CTC in 10 ml of blood. ISET[®] platform also was demonstrated to be able to separate CTC clusters in different metastatic cancer [139–145]. However, such filtration platforms also retain some larger non-tumor cells, which is why this techniques are considered not very specific. CTC clusters from liver and lung cancers captured by ISET[®] are undetectable by CellSearch, shows more sensitivity of ISET[®] for CTC clusters separation than antibody-based methods [53,115].

Another microfiltration platform, called FMSA (flexible micro spring array), enriches CTCs based on their size and deformability.

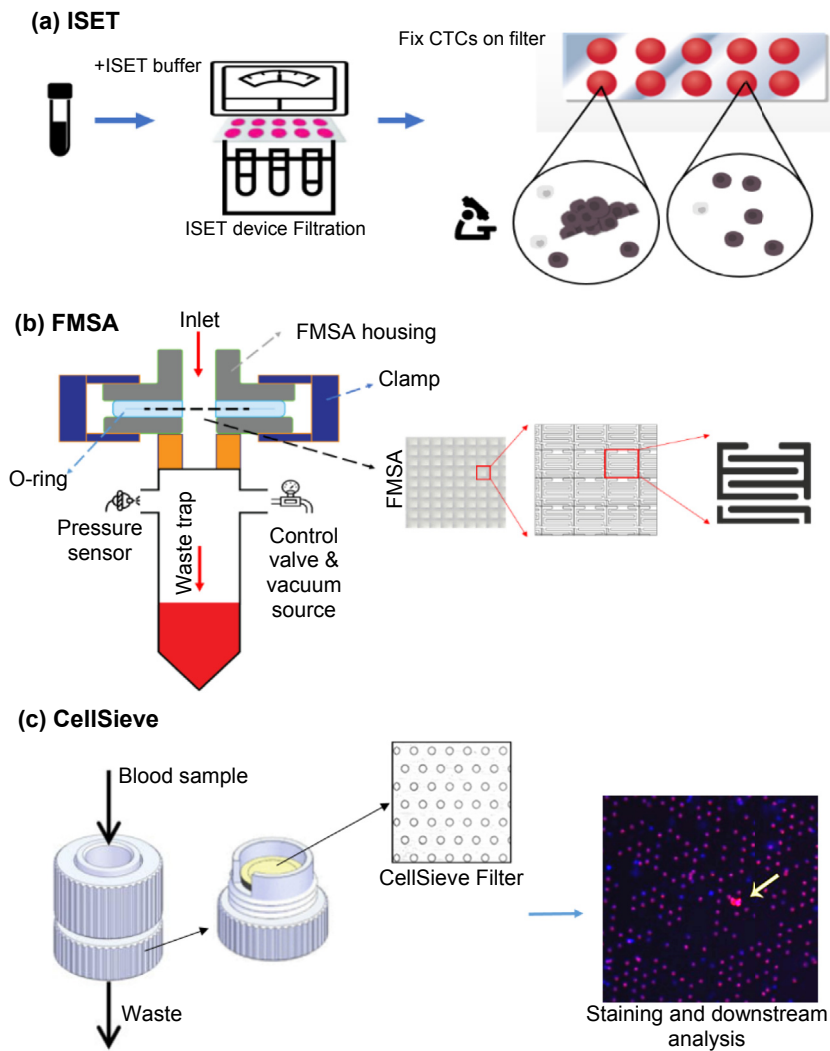


Fig. 6. Filtration techniques of CTCs separation. **(a)** ISET: 10 ml of blood are diluted, treated with the filter. **(b)** FMSA setup schematic: it enriches CTCs based on their size and deformability. **(c)** CellSieve™ microfilters, a 10 μm thick modified SU-8 polymer film with an array patterned filter with 7 μm diameter pores. Reproduced with permission from ref [150] under the terms of the Creative Commons Attribution License. Copyright© 2017 Hayashi et al.

FMSA is a 0.5 cm^2 filtration membrane with a novel micro spring geometry, which was designed to maximize the throughput and allows for prompt CTC enrichment directly from peripheral blood sample without preprocessing [146]. Blood sample passed through the FMSA device under accurately controlled pressures. Cells with a specific size are trapped in FMSA plate. The platform then uses antibodies for immunofluorescent detection. CTC clusters were separated from 44% of 7.5-ml whole blood clinical samples of breast, lung, and colorectal cancer in <10 min (Fig. 6b) [146,147].

CellSieve™ is another filtration platform with ~160,000 5–9 μm pores, spaced at 20 μm intervals [148,149] (Fig. 6c). It was reported detection of CTC clusters in sarcoma patients using CellSieve™ [150]. CTC cluster separation method based on physical properties can be also combined with and strengthened by microfluidic technology, leading to other microfiltration and microcavity platforms [151], as introduced by Mohamed, Tan, Xu and Zheng and others [152–157] that have potential for CTC clusters separation [158]. Among shortcomings associated with filtration platforms, clogging is one of the most critical one [159]. Some groups rectified clogging problem of filtration methods by developing a filter device that could periodically be cleared [160] or by geometrical optimization microcavity [161]. Filtration platforms allow direct filtering

of peripheral blood samples without preprocessing and are more cost-effective compared to antibody-based methods [162]. However, the intense tension stress and a mechanical lesion at the pore edges of the microfiltration techniques could cause deformation and remodeling [163] that affect the viability and integrity of cells, especially in CTC clusters, thus making the majority of them not suitable for further biological analysis [164].

Centrifugation is one of the earliest strategies for CTCs separation [165]. OncoQuick® (Greiner Bio-One, Germany) as a CTCs separation platform is based on density gradient centrifugation [61]. The kit includes a 50 ml tube that is separated into two sections by a porous barrier. The lower section contains the separation medium which prevents blood from mixing with the gradient before centrifugation. The upper section accommodates up to 30 ml of the blood sample for processing. During centrifugation, the cells are separated according to their densities. The denser blood components such as red blood cells and white blood cells migrate through the porous barrier into the lower section. Less dense cells, including the CTCs, settle at the interphase layer between the separation medium and the plasma in the upper section [166]. After washing steps, the captured CTCs can be used for further analyses (Fig. 7a) [167]. OncoQuick results in higher tumor cell enrichment

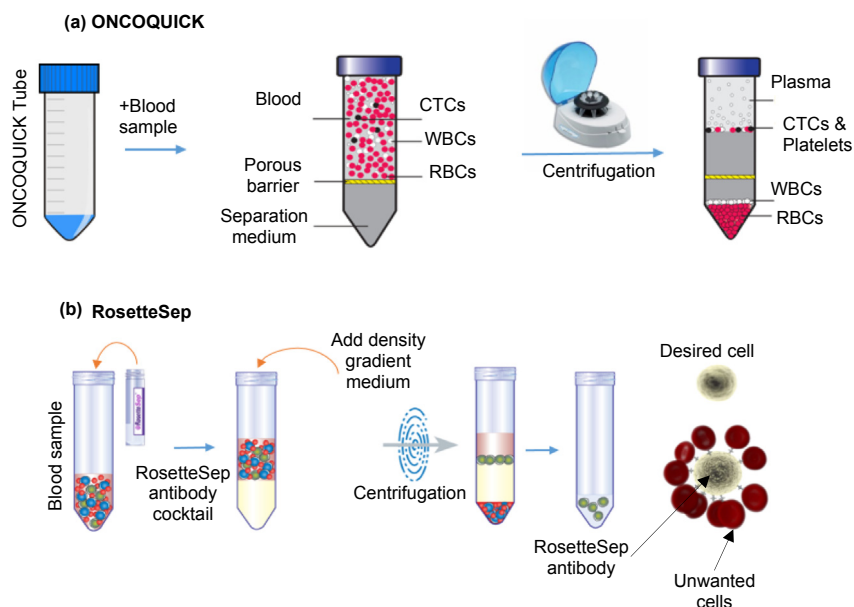


Fig. 7. Centrifugation techniques of CTCs separation. **(a)** OncoQuick workflow schematic: the cells are separated according to their different buoyant densities. Reproduced with permission from ref [166]. Copyright© 2018 Greiner Bio One International GmbH. **(b)** RosetteSep workflow schematic: Unwanted cells are cross-linked to RBCs by specific antibodies, forming dense immunorosettes. Reproduced after StemCell. Copyright© 2018 STEMCELL Technologies Inc.

than in traditional Ficoll density gradient centrifugation [168] but less accurate and less sensitive in CTC enumeration as compared with CellSearch [169]. Some clinical studies have utilized OncoQuick for CTCs enrichment [170–173], CTC clusters were observed in some of their results.

A more advanced density gradient centrifugation technique is the RosetteSep[®] (Stem Cell Technology, Canada), which is based on the negative selection. RosetteSep[®] is a physical-biochemical-based method where antibodies crosslink a variety of unwanted cells, specifically leucocytes and RBCs, forming aggregates termed 'rosettes'. The 'rosettes' sediment in the erythrocyte layer during the centrifugation step using a gelatin density gradient. The CTCs are negatively enriched in the mononuclear layer (Fig. 7b) [174].

Centrifugal spiral microfluidic devices utilize inertia and the Dean flow [175–178] for CTCs separation. These microchannels have been designed with various cross-section geometries such as the rectangular [179,180], trapezoidal [181,182] and the stair-like [183] configurations. Two contrary inertial force (F_L) and shear gradient force (F_W) are dominant on particles with size ratio $a/h > 0.1$ (where a is particle diameter and h is channel height), while the secondary flow (Dean vortex) in curvilinear channels controls the movement of smaller particles. Inertial lift forces confine CTCs to a specific region of the channel cross-section, while smaller blood cells continue to be entrained along the Dean vortices. Using this method, CTCs and blood cells are focused to distinct streams within the microchannel and can be collected through two separate outlets. The throughput of these devices is shown to be reasonably high (as 7.5 ml of blood per 8 min) with high separation efficiency [182]. These devices are also used for cell retention in perfusion culture flask [184], cell fractionation, and filtration [185]. Hou et al. developed a spiral microchannel with intrinsic dean drag and inertial lift forces for size-based separation of CTCs from the blood sample. Dean flow fractionation (DFF) platform facilitates simple coupling with downstream biological assays of cancer cells (Fig. 8) [179].

A year later Warkiani et al. upgraded the DFF platform with a trapezoidal cross-section (ClearCell[®] FX) [182] for ultra-fast label-free CTCs separation from peripheral blood samples using the Dean

drag force coupled with the inertial lift force. This technique utilizes the intrinsic Dean vortex present in a curvilinear microchannel, along with inertial lift forces that focus large cells like CTCs against the inner wall to separate cancer cells based on size. The trapezoidal cross-section, averse to the common rectangular cross-section, can alter the core position of the Dean vortex, to achieve more effective separation (Fig. 8). With upgraded DFF, single CTCs and clusters successfully were isolated. More than 80% of the spiked cancer cells were recovered from 7.5 ml of blood within 8 min [182]. This method is particularly attractive because of its simplicity and the high processing rates, approximately 0.5–1 ml/min for RBC-lysed blood samples. The high throughput makes DFF beneficial for applications that require the isolation of CTCs from large volumes of blood, such as early detection. Recently, using this device, CTC clusters were observed in the head and neck cancer [186].

Clusters are on average larger than individual CTCs and healthy blood cells. However, strategies that rely solely on size-based separation may have limitations when applied to CTC clusters. The majority of clusters consist of 2–4 individual CTC. Individual CTC size varies dramatically, ranging from 12 to 30 μm even within the same patient [187,188]. This overlap size range of large single CTCs and leukocytes (~6–20 μm) with clusters (Table 1) [189] can lead to reducing the size disparities of most clusters with large singles and leukocytes. On the other hand, clusters often assume alignments that mask their most extended axes during size-based separation [55].

Some technique discussed so far have been designed and developed specifically for separation single CTCs. CTC clusters were incidentally observed in many of these single CTC isolation platforms. Addressing the drawbacks of these platforms, Sarioglu et al. fabricated an exclusive platform for separation of intact and viable CTC clusters [190]. The team developed the Cluster-Chip, to capture CTC clusters independently of tumor-specific markers from the unprocessed blood. CTC clusters are isolated through bifurcating triangular pillars as traps under low-shear stress conditions that preserve their integrity. The Cluster-Chip captures CTC clusters by relying on their cell-cell junction (Fig. 9a). This platform is able to capture CTC clusters in 30–40% of patients with metastatic breast,

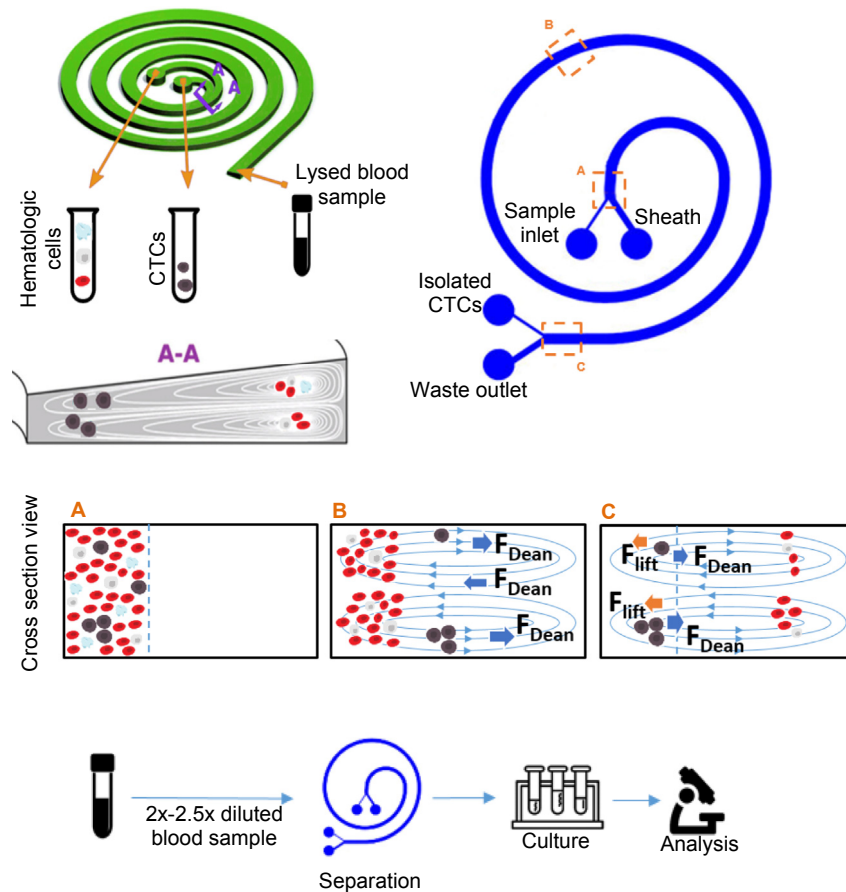


Fig. 8. Inertial focusing technique of CTCs separation. CTCs focused near the inner wall due to the combination of the inertial lift force and the Dean Drag force while white blood cells and platelets are trapped inside the core of the Dean vortex formed closer to the outer wall. Reproduced with permission from ref [179,182] under the terms of the Creative Commons Attribution License. Copyright© 2013 Springer Nature Limited. Copyright© 2018 Royal Society of Chemistry.

prostate, and melanoma cancer at a blood sample flow rate of 2.5 ml/h [190]. The recovery of clusters immobilized on micropillar arrays is challenging due to the requirement of an operation temperature of 4 °C and flow with shear stress greater than physiological one for releasing the CTC clusters [55]. Recently Inspired by Cluster chip Gao et al. amended an earlier size-based CTC separation platform [191], which captured CTC clusters and single CTCs separately [192].

To address this limitation, Au et al. proposed a two-stage continuous microfluidic chip that separates intact CTC clusters from blood samples [193]. This platform designed to utilize deterministic lateral displacement [194] to sort clusters based on geometric properties such as size and asymmetry. The first stage separates larger clusters based solely on their large size; using standard cylindrical DLD micropillar arrays to deflect particles with shortest axial diameters of 30 μm or more. The second stage was designed with asymmetric hybrids of elliptic cylinders and “I”-shaped pillars with the 30 μm ceiling. The second stage imposes the clusters that failed to be captured in the first stage to align their longitudinal axes “flat” in the flow direction (X–Y plane) (Fig. 9b). Therefore, the second stage sorts CTCs by discriminating asymmetric clusters from symmetric single cells. This strategy isolates 99% of clusters containing 9 or more cells and 66% of smaller clusters from whole blood. In a DLD-Chip, CTC clusters experience physiological or even lower shear stress and have short residence times. This platform separates clusters with over 87% viability and unhindered proliferation abilities. However, this strategy is limited by its relatively slow blood flow rate of 1 ml/h.

Another microfluidic device deliberately designed to isolate CTC clusters is “antibody-functionalized 3D scaffold gelatin-microchip”, which can efficiently separate clusters by combining antibody recognition and physical barricade effect of the scaffold structure [195,196]. Improving capture efficiency of marker-dependent strategies by CTCs-antibody interaction increment idea [197], Cheng et al. coated the 3D PDMS scaffold with multiple thermosensitive gelatin layers and functionalized it with anti-EpCAM antibodies. This scaffold with porous structure generates uncontrolled migration of cells that leads to increasing cell–structure interaction. After pumping blood sample into the scaffold chip at a flow rate of 50 $\mu\text{l}/\text{min}$ to capture CTCs, gelatin hydrogel dissolves at physiological temperature (37 °C) and washing with PBS (Phosphate-buffered saline), allowing the cell-friendly release of CTCs for further analysis (Fig. 9c). Using this microchip, free individual and cluster CTCs were successfully obtained from the blood sample of cancer patients. This platform captured more than 88% of MCF-7 single CTCs with 60–70% recovery ratio and 82%–100% of two-to over nine-cell cluster with 50–100% recovery ratio respectively, with the high viability of more than 90% [195].

2.3. Additional CTC clusters separation & detection techniques

Besides all the platforms mentioned above, some additional methods have been developed to detect or separate CTC clusters. Ge et al. proposed a novel strategy integrating subtraction enrichment and immunostaining-FISH (SE-iFISH[®]) (immuno-fluorescence in situ hybridization) [198]. The integrated platform enables

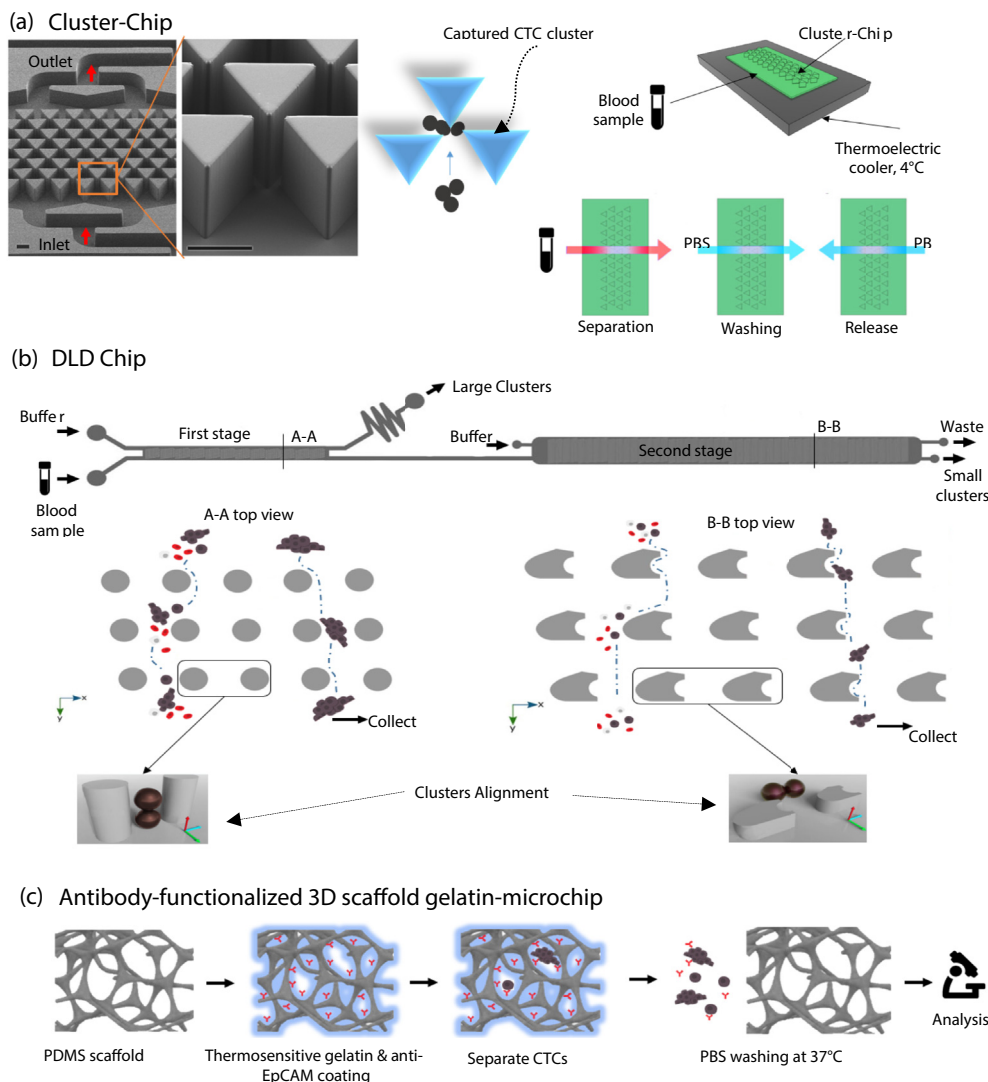


Fig. 9. Exclusive CTC cluster separation methods. **(a)** Operation of the Cluster-Chip. CTC clusters captured, whereas single cells pass through multiple rows of shifted triangular pillars that form consecutive cluster traps. Scale bars is 60 μm . Reproduced with permission from ref [190]. Copyright© 2015, Springer Nature. **(b)** Two-Stage DLD Chip operating Principles; array of cylindrical micropillars in first stage (A–A top view) deflects large clusters; while asymmetric pillars in second stage (B–B top view) deflects small clusters. Reproduced with permission from ref [193] under the terms of the Creative Commons Attribution License. Copyright© 2017 Au et al. **(c)** Scheme of capturing and release of individual and cluster CTCs using “antibody-functionalized 3D scaffold gelatin-microchip”. Reproduced with permission from ref [195]. Copyright© 2017, American Chemical Society.

effective depletion of WBCs RBCs by immunomagnetic and centrifugation, to establish a high-throughput detection of CTCs irrespective chemical markers and physical properties. The SE-iFISH platform was able to efficiently detect CTC clusters from prostate cancer [199].

A photoacoustic technique exploits strong optical absorption of melanin to image and sense melanoma CTCs *in vivo*. This platform utilized linear-array-based photoacoustic tomography (LA-PAT) technique for label-free high-throughput *in-vivo* CTC cluster detection. In addition, LA-PAT can quantify the number of cells in the CTC clusters and study their kinetics in the blood circulation by analyzing the contrast-to-noise ratios of the photoacoustic signals [200].

Jiang et al. utilized both physical and biological properties of CTC clusters to introduce a new isolation technique treats platelets as a marker for the separation of platelet-cloaked CTC clusters. In this method, CTCs were targeted by capturing platelet-covered cells. This platform incorporated a two-step microfluidic strategy. The

first step depletes free platelets by size, using deterministic lateral displacement (DLD). The second step isolates platelet-covered clusters, using the herringbone CTC chip (HB-Chip), which as mentioned induces micro vortices to enhance cell-capture surface interactions. This platform enabled the separation of CTCs from ~60% of epithelial lung and breast cancer, and also 83% of mesenchymal melanoma cancer [201].

Ozkumur et al. developed a three-step strategy that combines microfluidics and magnetic-based in which small CTC clusters were observed [202]. After the magnetic labeling of cells in whole blood, DLD was used to deplete RBCs, platelets, and other small blood cellular debris from the sample. Next, inertial focusing was utilized to align nucleated cells within a microfluidic channel by introducing asymmetrically curved channels. These channels help to extenuate the cellular collisions and ensure cellular displacement only as a function of magnetic force in the next step. At the last step, CTCs (positive selection) or WBCs (negative selection) immunomagnetically deflect into the collection channels.

Table 3
CTC cluster clinical applications.

Subcategory	Clinical significance	Type of cancer	reference
Prognosis and diagnosis	The presence of CTC clusters is associated with worse clinical outcome (PFS & OS)	Breast cancer	[39,206–209,213,239,255]
		Prostate cancer	[39]
		Small cell lung cancer	[28]
		Melanoma cancer	[212]
		Gastric cancer	[205]
		Colorectal cancer	[44]
		Liver cancer	[145]
Molecular & Genomic analysis	The presence of CTC clusters is associated with larger primary tumor and metachronous development of lung metastases Studied on EGFR, KRAS, and PIK3CA gene inhibition with therapeutic purposes Mutations in the KRAS and EGFR genes relevant for treatment decisions Reported EGFRVIII expression on CTCs by molecular analysis Sequence whole exomes of CTCs EML4-ALK gene rearrangement	Pancreatic ductal adenocarcinoma	[211]
		Renal cancer	[256]
		Colorectal cancer	[218]
		Lung cancer	[90]
		Squamous cell carcinoma of head and neck	[41]
		Prostate cancer	[220]
		Lung adenocarcinoma	[222]
Therapeutic and drug	Increment CTC cluster number in non-responder chemotherapy patients Antioxidant genes testing as a method for treatment monitoring Chemotherapy resistance of the circulating tumor cell clusters Developed a microfluidics platform for study tumor cell–drug interactions and drug responsiveness Elevation circulating tumor cell number correlates with macroscopic progression of primary tumor Study CTCs and response to chemotherapy CTC cluster dissociating and metastatic condition Studying enzalutamide, everolimus and BKM-120 In situ drug screening on patient-derived CTC clusters	Colorectal cancer	[235]
		Prostate cancer	[228]
		Colon cancer	[63]
		Breast cancer	[233]
		Colorectal cancer	[235]
		Breast cancer	[237,238]
		Breast cancer	[123]
		Prostate cancer. Renal cell cancer	[245]
Breast cancer	[236]		

3. CTC clusters applications

CTCs can be used for various clinical purposes, e.g., examine the cancer cells respond to therapeutic regimes, predict overall survival, noninvasively drug susceptibility monitoring, metastatic therapy and as early cancer detection and diagnostic biomarkers. Due to the higher metastatic potential, CTC clusters serve as a noninvasive method with high potential for diagnosis, prognosis, and treatment in many academics studies and clinical trials. In the following sections, we discuss experimental attempts of using CTCs in a clinical context (Table 3). According to [ClinicalTrials.gov](https://www.clinicaltrials.gov) there have been more than 400 recruiting clinical trials that utilize CTCs up to January 2019. The extreme effort is required on CTC clusters applications.

3.1. Prognosis and diagnosis

Conventional tumor biopsy poses disadvantages such as sampling bias, sampling difficulty, and harm to patients. Since CTCs are present in the peripheral blood of carcinogenesis cancer patients even in the early stage [203], detection them from blood sample, especially CTC clusters due to their higher metastatic potential, as liquid biopsy could be a great alternative to conventional tumor biopsy [204] for cancers prognosis and diagnosis. Recent experimental studies have revealed a direct and robust association between the presence of CTC clusters recovered from venous patient blood and the significantly reduced survival rate as well as lousy prognosis in some types of cancer [28,38,205–210]. Researchers also demonstrated the correlation of CTC clusters number present in a blood sample with worse progression-free

survival (PFS) and overall survival (OS) [39,152,209,211–213], but any correlation between the number of CTC clusters and tumor type or stage [38,63,214,215]. However, in a recent study, the presence of CTC cluster in a blood sample of the patient was correlated with resistance to therapy in epithelial ovarian cancer (EOC) [216].

3.2. Molecular and genomic analysis

The molecular analysis of CTCs facilitates the identification of the molecular drivers of cancer in the patient body [217]. In a recent work, molecular profiling of epidermal growth factor receptor variant type III (EGFRVIII) was shown to be a good indicator of squamous cell carcinoma of head and neck [41]. After the immunohistochemical analysis, EGFRVIII expression observed in CTCs of a patient, also was detected in the primary and the metastatic tumors of the same patient [41]. In a study of Gasch et al. [218], the mutations of PIK3CA, KRAS, and BRAF genes were analyzed using Sanger sequencing for predicting the resistance against anti-EGFR therapy in five patients. The PIK3CA gene displayed two different mutations in two separate CTCs in a patient, which indicates the CTC analysis capability to inform us about the tumor mutational heterogeneity. Using sensitive deep-sequencing genomic analyses of CTCs in patients with prostate and colorectal cancer demonstrated that mutations in CTCs resemble mutations in both the primary tumor and metastases [119,219–221]. Zhang et al. recently utilized CTCs-derived organoids in genetic analyzing of lung adenocarcinoma CTCs to detect ALK instability and rearrangement [222]. Therefore, relinquish these mutations in therapeutic regimes can affect the efficacy of drugs against mutinied targets [223,224].

3.3. Therapeutic and drug

Recent studies have specified that CTCs could be used in frequent genetic profiling to monitor the evolving mutational outlook and drug sensitivity patterns for individual patients [222,225–227]. Giesing et al. showed antioxidant genes of CTC clusters analyzing as a novel method with superb prognostic and predictive properties for monitoring treatment regime [228]. The authors suggested that the antioxidant genes helps CTC clusters as a survival and defence mechanism in confronting with immune surveillance.

Recently, CTC-derived organoid cultures have emerged as a novel technique in medical research and precision medicine as they preserve tumor tissue heterogeneity and drug-resistance responses, and thus are suitable for high-throughput drug screening [229,230]. CTC-derived organoids could help to identify mutations in CTCs and epigenetic information about tumors, and screen treatment regimes in real time [63,227]. Some groups recently have developed microfluidic platforms to study tumor cell–drug interactions were assessed. Subsequently, the pharmacological efficacy of chemotherapeutic drugs [231,232], tumor cells for drug responsiveness [233] and resistance [234] were monitored. Molnar et al. demonstrated that the CTC clusters number in blood sample reflects the chemotherapeutic sensitivity in colorectal cancer [235]. Recently, Khoo et al. developed a platform to evaluate drug response on CTC clusters using patient-derived CTCs cultures. The team designed tapered microwells on microfluidics platform to allow CTC clusters formation without pre-enrichment and subsequent drug screening in situ [236]. In addition, the elevation of CTCs number in peripheral blood is associated with macroscopic progression of tumor. Another study [237,238], demonstrated that CTCs number (≥ 5 per 7.5 ml blood) with and without CTC cluster [239] after the first chemotherapy could be a biomarker of disease progression and monitoring of treatment strategy. The authors proposed that the patients with unchanged blood levels of CTCs represent cancer cell resistance to the adopted therapy, so they should shift to other treatment regimes.

The improvement of long-term survival is still disappointing. For most of the approved new cancer drug regimens, the survival time is only 1–2 months [240,241]. A major reason for these modest gains is that these drugs were not developed to directly target agents responsible for metastasis [63], especially CTC clusters [242]. A novel strategy for combating metastasis could be to dissociate CTC clusters into less potent individual CTCs in the circulation (e.g., by weakening the adhesion energies between cancer cells within clusters). Choi et al. tried such a strategy practically using urokinase [123,243]. The authors claimed that urokinase could lyse fibrin to dissociate CTC clusters and as a result reduced the prevalence of metastasis in animal models. In addition, they demonstrated a reduction in the number of CTC clusters that incubated with urokinase *in vitro*. *In-vivo* urokinase utilizing in the blood of treated mice showed a decreased number of CTC clusters compared to the control. Therefore, the results suggest that urokinase disintegrates CTC clusters into individual CTCs [123]. However, some researchers do not agree with disaggregation of CTC clusters in the bloodstream as a metastasis treatment. They caution that urokinase treatment may also include the risk of increasing invasiveness of tumor cells and metastatic spreading, resulting in the opposite effect of that, as reported by Choi et al. [244].

In the field of cancer drug development, Gao et al. used CTCs-derived organoids for testing the new version of androgen receptor antagonist (enzalutamide) and PI3K-kinase pathway inhibitors (Everolimus and BKM-120) [245].

Overall, despite of all experimental studies in CTC cluster, currently, the clinical importance of CTC clusters remains elusive.

Further study is requisite to exploit the full potential of CTC clusters in real-world clinical applications.

4. Conclusions and outlook

CTC cluster analysis as a noninvasive liquid biopsy is a new expanding field that can introduce unprecedented horizon in early cancer diagnosis and therapy assessment in clinical trials. Nevertheless, due to inefficient separation platforms and heterogeneous biology, there are still many fundamental unsolved issues about CTC clusters. As such, to date, it is not clear the metastatic potential of included tumor cells in a cluster compared to single CTCs and the effect of CTC cluster size and cell number on its metastatic potential. Whether dissociating CTC clusters into single CTCs can effectively reduce their metastatic risk. How the associated non-tumor cells included in CTC clusters increase their survival and more efficient distant colonization, as well as CTC cluster collective migration are among the outstanding questions in CTC cluster biology.

Despite the significant progress in separation methods, substantial work still needs to be done to achieve a platform to efficiently identify, enumerate, and isolate intact CTC clusters in a reasonable time with minimal manual intervention. Subsequent developments in CTC cluster separation technologies will enhance our knowledge about these multicellular aggregates and their contribution to metastasis progression and can translate laboratory-based concepts to clinical applications in real-world settings. Complementary studies should be undertaken to characterize CTC clusters and to utilize their clinical value.

Monitoring treatment regime is a great potential field of interest toward individual treatment. Therefore, the next step after developing an efficient separating platform for CTC cluster is *ex-vivo* patient-derived CTCs culturing. However, to date, no techniques have been presented for CTC clusters culturing. The future research should focus on developing strategies for long-term culture of patient-derived CTC clusters.

Due to their higher metastatic potential, CTC clusters are expected to be utilized broadly in cancer and metastasis clinical trials in the coming years. We envision that liquid biopsy and qualitative and quantitative monitoring of CTCs, especially CTC clusters, will allow the clinician to establish more effective personalized treatments.

Conflicts of interest

The authors declare no conflicts of interest.

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