

# Advancing Techniques and Insights in Circulating Tumor Cell (CTC) Research

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## 1 Introduction

Cancer is a major cause of mortality worldwide, with a disease burden estimated to grow over the coming decades. Circulating tumor cells (CTCs) are rare cancer cells released from the primary or metastatic tumors and transported through the peripheral circulatory system to their specific secondary locations. The presence of CTCs in a cancer patient's blood has been used as a prognostic biomarker, with lower CTC count correlating with greater overall survival [1]. In spite of its clinical potential, the isolation and detection of CTCs has been a challenging task due to its rare presence amongst other blood cells (as low as 1–10 CTCs per billions of blood cells) and variability in terms of both morphological and biochemical markers. Recent developments of microfluidics technology have paved the way for better isolation and characterization of CTCs due to several advantages such as lower sample volume, higher sensitivity and throughput and lesser production cost [2, 3].

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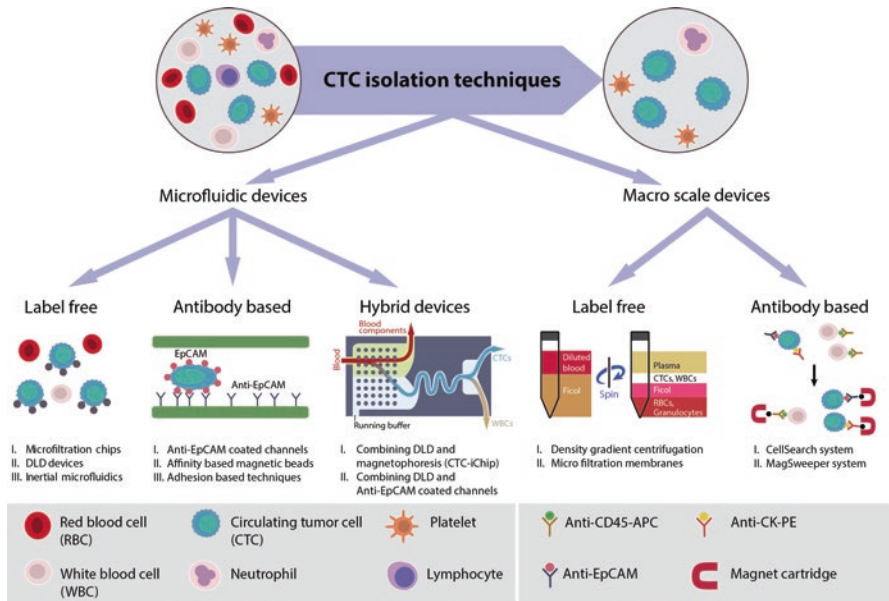
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**Fig. 1** Schematic classifications of various circulating tumor cell (CTC) isolation technologies. CTC isolation platforms can be classified into two major classes: microfluidics and conventional macro scale devices. The microfluidic devices can be further sub-divided into label-free (microfiltration, deterministic lateral displacement (DLD) devices and inertial microfluidics), antibody-based (anti-EpCAM coated channels, affinity-based magnetic beads and adhesion-based techniques) and hybrid techniques (combining DLD with magnetophoresis or anti-EpCAM coated channels) that uses the advantages of both label-free and antibody-based methods. Macro scale techniques can also be further classified into label-free (density gradient centrifugation and micro filtration membranes) and antibody-based (CellSearch system, MagSweeper system) platforms

In this chapter, various CTC isolation devices are classified under two major categories: microfluidics and conventional macro-scale devices, as illustrated in Fig. 1. We will be discussing both label-free methods and antibody-dependent methods for CTC isolation, and will provide discussion and future perspectives on the advantages and drawbacks of both these techniques on potential clinical applications. Advancement in these technologies for CTCs and associated components, such as exosomes, led to an unraveling of tumor variation, ranging histology, molecular, proteomic and functional heterogeneity, which will be discussed in the subsequent sections.

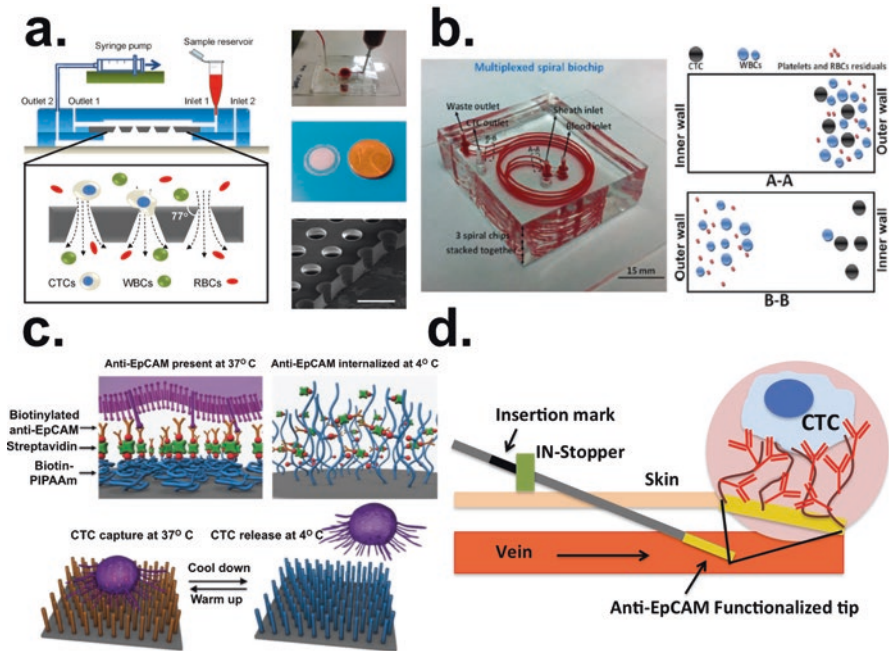
CTCs are heterogeneous in terms of the morphology and surface expression of various biomarkers. Therefore, it is an uphill task to isolate these rare cells from clinical samples in the presence of billions of other hematologic cells. Recent technological advancements observed that CTCs differ from blood cells in various biophysical properties (such as size, adhesion and stiffness) and cell surface receptor expressions (such as Epithelial cell adhesion molecule (EpCAM) and Cytokeratin (CK)) [4, 5]. Current microfluidics techniques and conventional methods can target such distinct properties of the CTCs and achieve high isolation efficiency and throughput along with greater cell viability for downstream single-cell analysis.

## 2 Microfluidics Devices

Over the past decade, microfluidics technologies have been extensively utilized for study of human disease such as cancer. Microfluidic systems normally leverage on the disparities in the intrinsic properties of the different cell populations (i.e., size, deformability, surface charge, density, etc.) to achieve separations. Isolation and characterization of CTCs using microfluidic systems has been a flourishing area of research which can be broadly categorized to label-free and antibody-based approaches (see Fig. 1).

### 2.1 Label-Free Technologies

Differences in the biophysical properties between the CTCs and blood cells, such as size, deformability, magnetic susceptibility and electrical behaviors have been exploited to develop label-free sorting of CTCs. The key advantages of this method compared to the antibody-based devices are the collection of a complete pool of CTC population consisting of both EpCAM positive and negative cells and greater compatibility to a wide range of assays that require viable unlabeled cells. Size and deformability-based sorting of CTCs can be achieved by using microfiltration devices and a membrane pore size of around 8  $\mu\text{m}$  has been proved optimal for CTCs capturing [6]. The size of the CTCs varies between 6 and 30  $\mu\text{m}$  and is usually greater than normal hematologic cells [7]. 3D membrane micro filter consisting of two-layers of membrane has been developed with the upper membrane pore size diameter of 9  $\mu\text{m}$  and the pores are aligned centrally to the smaller pores (8  $\mu\text{m}$ ) on the basal layer [8]. When blood samples are passed through the device, the CTCs are captured in the upper membrane while other blood cells pass through the gap between the two membranes. This device has an isolation efficiency of 86 % and processing throughput of 3.75 ml/min. Recently, a novel design of the 3D membrane micro filter is introduced with 5 times greater upper membrane pore diameter than that of the lower membrane pores (8  $\mu\text{m}$ ) [9]. The CTCs are captured between the gap of the two membranes and the cell can be analyzed by separating the two membranes. Isolation efficiency ranging from 78 to 83 % can be obtained using this device with higher cell viability (greater than 70 %). In order to mitigate the pressure buildup problem during membrane filtration, cross-flow conformation in parallel direction to the filtration membrane was introduced [10]. Membrane microfilters developed using this principle can obtain a capture efficiency of 98 % for MCF-7 cells spiked into blood samples. In another interesting study, weir-shaped structures are used as barrier across the filtration chip to trap most of the CTCs while the blood cells can pass through the narrow opening at the upper portion of the barrier [11]. This device has an isolation efficiency of >95 % and processing speed of 20 ml/h. Microfiltration devices can also be integrated with conical shaped holes to achieve highly efficient cell capture (96 %) at 0.2 ml/min processing rate, as illustrated in



**Fig. 2** Diagrammatic representation of CTC isolation devices. (a) Integration of microfiltration with conical holes for size-based capturing of CTCs. The sample is processed through inlet 1 and it passes through the filter and subsequently gets collected by constant pulling from a syringe pump at outlet 2 (*top right*). Representation of the microfluidics platform processing clinical samples (*middle right*). Image of 9 mm diameter micro-filter (*bottom right*). Scanning electron micrograph (SEM) of conical holes (scale 40  $\mu\text{m}$ ). Reproduced with permission from [10]. (b) Representation of multiplexed spiral biochip with two inlets for clinical samples and sheath fluid respectively. The CTCs are sorted due to the action of inertial lift and Dean drag forces and are segregated towards the inner wall of the microfluidics channel (A–A) whereas the WBCs and platelets are concentrated towards the outer wall (B–B). Reproduced with permission from [12]. (c) Working principle of Thermo-responsive NanoVelcro substrate for capturing CTCs with biotinylated anti-EpCAM antibodies at 37  $^{\circ}\text{C}$  and subsequent release of the captured CTCs at 4  $^{\circ}\text{C}$  due to temperature-dependent conformational changes of the polymer brushes that changes the availability of anti-EpCAM antibodies on the surface. Reproduced with permission from [13]. (d) Schematic of the working principle of Gilupi nanodetector system for capturing CTCs from the in-vivo environment. The gold-plated medical steel wire is coated with hydrogel (indicated in *brown*) and functionalized with anti-EpCAM antibodies (indicated in *red*) and is inserted into the patient vein to capture EpCAM positive CTCs. Reproduced with permission from [14] (Color figure online)

Fig. 2a [10]. Clearbridge Biomedics has commercialized a microfluidics device, the CTChip<sup>®</sup> with an array of crescent-shaped structures composed of three closely spaced micropillars that can trap the CTCs from clinical samples with high throughput and without channel clogging from the cell debris [15, 16]. This device can isolate single and double CTCs and the viable cells can be retrieved and cultured by reversing the flow direction. ISET<sup>®</sup> (Rarecells, Paris, France) and ScreenCell<sup>®</sup> are two commercialized systems for sized-based isolation of CTCs that provide

cost-effective and high-throughput enrichment of fixed and viable CTCs respectively [17, 18]. The major advantages of the microfiltration devices are its simple design and its ability to obtain the CTCs from the whole blood in a single pass while maintaining its cellular integrity for detection and further downstream analysis. Although size based enrichment of CTCs provides a high-throughput, label-free technique, it has some drawbacks; such as the cells are subjected to high mechanical stress that can alter their normal function [19] and there is a size overlap between the CTCs and leukocytes that can increase the probability of isolating more contaminating cells [20].

Dielectrophoresis (DEP) phenomena have also been used for CTC isolation based on the differential motion of the cells when exposed to a non-uniform electric field. Cells can exhibit attractive and repulsive behaviors depending on their size and dielectric polarizability under a non-uniform electric field [21]. Compared to normal cells, CTCs have greater surface area and higher capacitance per unit area that provides them a unique dielectric property and thereby affecting their motion in presence of an electric field. Contactless DEP (cDEP) is developed with greater sensitivity and eliminating the drawbacks of traditional DEP such as high cost, air bubble production, electrode delamination and culture contamination [22]. This device reports an isolation efficiency of 64.5% for carcinoma cells from concentrated RBC solution. ApoCell laboratories have commercialized ApoStream device in 2010 and validation study using breast cancer cells spiked into clinical sample has observed an isolation efficiency of 86.6% with higher viability (97.6%) [23]. The main advantage of DEP devices is that the effect of therapeutic agents could be determined by monitoring the differences in the frequency responses of the cells under the action of various chemotherapeutic agents.

Deterministic lateral displacement (DLD) is another label-free method used for sorting CTCs in microfluidic devices. A novel DLD platform with 58  $\mu\text{m}$  triangular micropost and 42  $\mu\text{m}$  gap is used to capture CTCs at high efficiency (>85%) and throughput (10 ml/min) [24]. Inertial microfluidics is also used for isolating CTCs by combining inertial lift forces and pinched flow dynamics to focus particles in their preferential equilibrium position along a microfluidic channel. Inertial microfluidics can be combined with microvortex particle capturing to isolate CTCs in a high-throughput, clogging free manner [25]. Apart from straight channels, spiral microfluidics can be used to achieve inertial sorting by combining inertial and Dean drag forces. Our group developed a spiral microfluidics chip with rectangular cross section to isolate CTCs (varying from 5 to 88 CTCs/ml of blood) from 20 metastatic lung cancer patients [26]. Strong inertial lift forces focus the bigger CTCs towards the inner wall while the smaller hematologic cells move towards the outer wall. Due to greater channel dimensions (500  $\mu\text{m}$  width and 160  $\mu\text{m}$  height) and increased flow rate (3 ml/h), clogging of the microchannels by the cell debris is also mitigated, thereby increasing the performance and sensitivity of the device. More recently, we further developed our spiral microfluidics chip with trapezoidal cross-section that can separate the CTCs from metastatic breast and lung cancer clinical samples in an ultra-high throughput manner (7.5 ml within 8 min) [27]. Our group subsequently developed multiplexed spiral microfluidics chip for ultra-high throughput sorting of

viable clinical CTC (12–1275 CTCs/ml for breast cancer patients and 10–1535 CTCs/ml for lung cancer patients) for further downstream single-cell characterization such as fluorescence in-situ hybridization (FISH) and proteomics analysis, as represented in Fig. 2b [12]. In another study, contraction-expansion array microfluidic channels is used to enrich cancer cells spiked into blood suspension with 99.5% efficiency [28]. In negative selection methods, the CTCs are untouched; however the RBCs are lysed and the WBCs are magnetically removed using specific markers such as CD45, CD61 [29].

## 2.2 Antibody Based Technologies

Antibody mediated CTC isolation techniques are dependent on the specific binding of the cell surface receptors with the antibody bound matrix. The matrix could be of mainly two types, such as magnetic beads or functionalized microfluidics channel. The two commonly used markers for CTC isolation and detection are EpCAM and different subtype of CK. However, due to the occurrence of epithelial to mesenchymal transition (EMT), all CTCs do not express these markers and therefore these techniques fail to collect some subpopulations of the CTCs [30, 31]. CTCs can be selectively labeled with antibody-tagged magnetic microbeads (diameter: 0.5–5  $\mu\text{m}$ ) or nanoparticles (diameter: 50–250 nm) and sorted in a non-uniform magnetic field, whereby the labeled CTCs migrate to a region of higher magnetic field and get trapped with an isolation efficiency of 86% [32, 33]. A straight microchannel with many square indentation on its sidewalls can be used to pull and trap the EpCAM tagged CTCs towards the sidewalls under the influence of an external magnetic field [34]. Fluxion Biosciences have commercialized a microfluidics device, IsoFlux™, which can trap magnetically labeled CTCs when passed through a microchannel with uniform magnetic field. Other promising strategies to further develop the enrichment of EpCAM-positive CTCs in a highly sensitive and efficient manner includes 3D nanostructured substrate or nano “Fly Paper” Technology [35] and a Velcro-like microfluidics platform with isolation efficiency ranging from 40 to 70%, as depicted in Fig. 2c [13].

Adhesion-based CTC isolation techniques depend on the binding affinity of CTCs to a surface whose biochemical (using antibody coated surface) and structural properties (using nano topographical features) can be modified to favor suitable adhesion. This technique can be performed either in static [36] or dynamic flow modes [37], while the later one is more sensitive due to the greater interaction between the cells and the surface and prevention of non-specific adhesion due to fluid shear forces. Using 3D structures such as microposts inside a microfluidics channel (the CTC-Chip), the effective surface area can be enhanced and the collision frequency between the cell and the EpCAM functionalized surface can be increased [38]. The CTC-Chip has recovery rate of 60% and the recovery efficiency does not depend on the different expression of EpCAM by the CTCs due to the greater collision frequency. Geometrically enhanced differential immunocapture

(GEDI) chip is developed to further increase the effective collision frequency between the CTCs and the antibody-coated microstructure to enhance the isolation efficiency (~90%) [37]. In another study, herringbone-chip was discovered with anti-EpCAM coated herringbone structures to increase the collision between the CTCs and antibody functionalized PDMS channels [39]. This device has an isolation efficiency of 91.8% with 95% cell viability. Recently, geometrically enhanced mixing (GEM) chip is introduced with a further improved herringbone micromixers to enhance the throughput and isolation purity in antibody-dependent devices [40]. CTCs are detected using this device in 17 out of the 18 pancreatic cancer patients studied and the CTC number correlates with the tumor size in three advanced stage patients during the period of the treatment. Adhesion-based CTC isolation techniques using micropost arrays has been commercialized by OnQChip™ (On-Q-ity, MA, USA) and the CEE™ chip (Biocept Laboratories, CA, USA). Graphene oxide sheets can be functionalized with anti-EpCAM antibodies and adsorbed on gold patterned substrates for efficient and sensitive enrichment of CTCs [41]. The increased surface area and biocompatibility of graphene oxide enables greater loading capacity of anti-EpCAM antibodies on its surface and the isolated cells can be cultured on the gold substrates for further downstream characterization such as RT-PCR and drug testing.

Different techniques can be combined to create a better hybrid platform for effective enrichment of CTCs with higher throughput. The CTC-iChip uses both antigen dependent (magnetophoresis) and independent (DLD with inertial focusing) strategies to isolate CTCs with greater sensitive (0.5 CTCs per ml) from clinical samples with lower CTC numbers [42]. Another study combined DLD with EpCAM functionalized isolation chambers to enrich CTCs with greater efficiency (90%) and throughput (9.6 ml/min) [43]. CTCs are initially sorted from other blood cells in the DLD compartment comprising of triangular microposts and subsequently they are captured in an EpCAM functionalized chambers with fishbone conformation to enhance the isolation capacity.

### 3 Conventional Macro Scale Devices

#### 3.1 Label-Free Technologies

Density gradient centrifugation is a label-free technique using centrifugal forces for sorting cells from blood based on the difference in their sedimentation coefficient. When a clinical sample is subjected to density gradient centrifugation, the denser RBC and neutrophils settles at the base of the tube whereas the CTCs, plasma and mononucleated cells are collected above the buffy coat. In another study using silicon-blending oil as a floatation media, cancer cells are identified in 53% and 33% of gastrointestinal tract cancer and breast cancer patients respectively [44]. OncoQuick centrifugation system has been developed with a porous membrane within the centrifugation tube to prevent the mixing of the separation

media with the clinical sample before centrifugation. This system has a greater CTC isolation efficiency compared to the Ficoll density gradient centrifugation [45]. OncoQuick has been used for the enrichment of CTCs from metastatic breast cancer patients and it can successfully detect CTCs in 69.2% of the clinical samples [46]. RBCs and WBCs can be cross-linked to form rosettes using a cocktail of antibodies and RosetteSep™ (STEMCELL Tech., BC, Canada) commercialized this technique. On application of centrifugal forces these rosettes can be efficiently separated from the CTCs due to their greater densities [47]. In another study, the use of sieve material as a filter with pore size of 4.5  $\mu\text{m}$  could separate 100% of the HeLa cells spiked into blood and detect cancer cells in 19 out of the 50 cancer patient specimen [48].

### 3.2 *Antibody Based Technologies*

Notable achievement in the isolation techniques of CTCs has been accomplished by the introduction of FDA (Food and Drug Administration)–approved method, the CellSearch system (Veridex) [49, 50]. CellSearch system consists of a semi-automated platform for capturing EpCAM positive CTCs using immunomagnetic cell sorting and the cells are subsequently identified by antibody staining of various subtypes of cytokeratins. This system was validated for CTC detection in metastatic breast cancer patients and it was reported that CTC assessment could provide critical prognostic information such as overall survival and progression-free survival [51–53]. However, CellSearch system can only successfully isolate EpCAM positive sub-population of the CTCs and the isolated CTCs are permanently labeled with antibodies that limit their downstream characterization. Illumina Inc. (CA, USA) commercialized MagSweeper system, which consists of a robotic arm with a magnetic rod that continuously shifts the region of high magnetic field within a multiwell plate [54]. The speed and trajectory of the rod movement has been standardized to favor the adsorption of EpCAM-labeled CTCs on the rod and prevents non-specific adsorption by other non-target cells. This system can perform at a high throughput (9 ml/h) and higher isolation efficiency (~81%). Flow cytometry technologies such as fluorescence-activated cell sorting (FACS) can be utilized to simultaneously detect several fluorescently labeled antibodies and thereby increasing CTC isolation purity [55]. However, this technique can not provide greater sensitivity for CTC isolation due to the varied expression pattern of different antigens on the CTCs depending on the types and stages of cancer [56]. Anti-EpCAM coated detachable microbeads can be used to enlarge the size of the CTCs before passing the whole blood sample through the microfiltration device (8  $\mu\text{m}$  pore size) [57]. Leukocytes and other blood cells can pass through the filter while the larger CTCs remain trapped on the filter membrane. This device reports a capture efficiency of 89% and isolated 1–31 CTCs/ml of clinical samples. CTCs can also be isolated directly from the in vivo environment using the Gilupi nanodetector®



system, as shown in Fig. 2D [14]. The nanodetector is coated with anti-EpCAM antibodies and inserted in the peripheral arm vein for 30 min to collect larger number of CTCs.

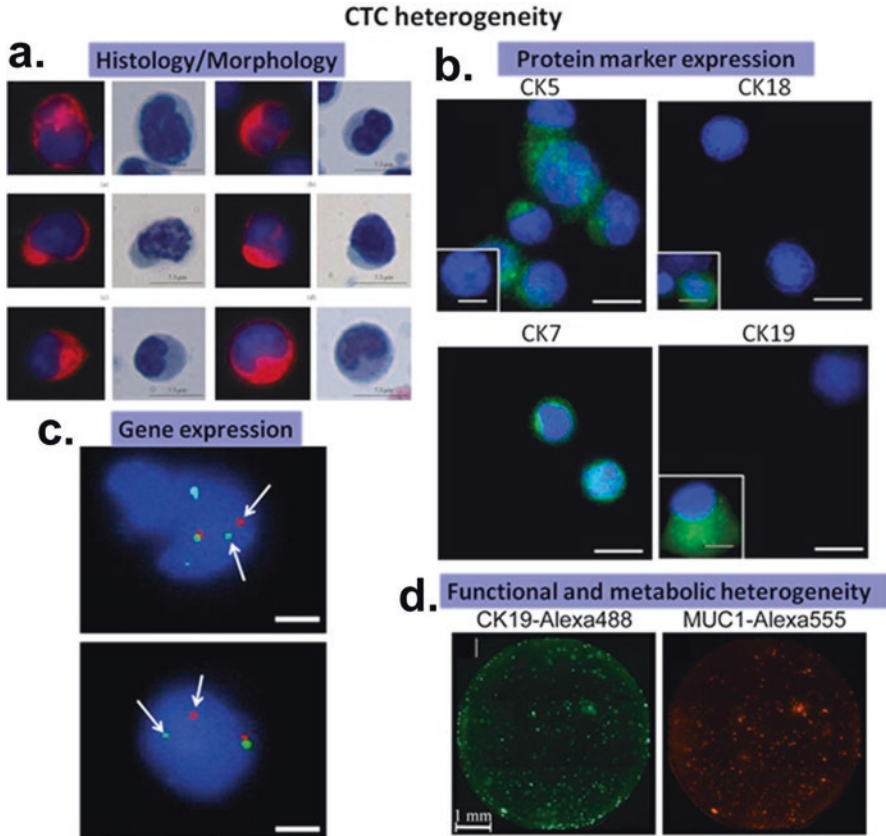
## 4 Characterization of Cancer Cell Heterogeneity

Tumors demonstrate huge heterogeneity in morphology, immuno-phenotype, and genotype [58]. Novel therapeutic approaches for BRAF mutant cutaneous melanoma [59] and ALK rearranged NSCLC [60], in the recent decades have been unable to increase the survival of patients with advanced stage cancer. This is largely attributed to tumor heterogeneity and the rise of drug tolerant or resistant subtypes under the influence of ineffective drug therapy [61]. Similarly, CTCs have been found to demonstrate a similar range of heterogeneity across multiple parameters, including morphology, histology and proteomics (Fig. 3).

### 4.1 Histology and Morphology

Observations of tumor histology are often correlated with disease severity [65], and have been found to exhibit strong correlations to genotype [66]. Generally, malignant cancer cells are identified as cells with a round or oval nucleus, and a high nuclear to cytoplasmic (N/C) ratio [67]. However, cancer subtypes display distinct morphology variations, such as the established case of small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) [68].

Recent analysis with specific cancer subpopulations [62, 69, 70], such as disseminated tumor cells (DTCs) and CTCs revealed extensive intertumor histological variation (within the same tumor type among individuals) [71]. CTCs are derived from the blood samples of cancer patients and selectively isolated by targeting protein expression [49], negative selection [29], physical properties [26, 42], or by means of expansion [8, 63, 72]. CTCs can be isolated on a routine basis via a non-invasive process of blood withdrawal [73], allowing monitoring of therapy-associated changes in protein expression, a process which could illuminate the mechanisms that facilitate drug resistance development. Proteomics profiling of CTCs has revealed some of the extent of heterogeneity present. Results suggest the role of multiple pathways, such as the process of EMT transition [74], which is involved in the metastatic cascade [75, 76]. CTC size and other physical parameters may also vary significantly [77], which limits the sensitivity of CTC enrichment devices. These variations can be observed within the cells obtained from tumors of the same patient. Upcoming high throughput spectral imaging techniques will allow rapid processing of single-cell images, providing dynamic insights to cancer cell morphological changes under stimulus or drug exposure (see Fig. 3a) [78, 79].



**Fig. 3** Varied manifestation of CTC heterogeneity. (a) Pleomorphism of CTC morphology obtained from the same patient demonstrated with immunofluorescence staining (left) and Papanicolaou stain (right) [62]. (b) Immunostaining of various forms of cytokeratin reveals heterogeneity in protein marker expression. Representative images of minority cohort are provided in boxed images (bottom left). Scale bar, 20  $\mu\text{m}$  [63]. (c) Molecular FISH analysis carried out with clinical human CTCs from an NSCLC patient using Vysis ALK Break Apart FISH probe. Varied frequency of separation of the original gene fusion signal (arrows) is detected in cells obtained from the same sample [12]. Scale bar: 16 mm [12]. (d) Antibody coated membranes demonstrates active protein secretion of CK19 (green) and MUC1 (red) from viable CTCs respectively [64] (Color figure online)

## 4.2 Proteomics and Genetic Profiling

Although some histology aspects of cancer cells correspond to genotype, intertumor variation in protein expression has been identified in tumors classified under similar subtypes [80]. Depending on the microenvironment [81], as well as tissue organization and composition [82], cancer cells may be exposed to varying stimulus which induces different rates of oncogene or tumor suppressor gene expressions.

For example, accumulation of mutations in the adenomatous polyposis coli (*APC*) gene may lead to abnormal production of APC protein, an alteration associated with increased colorectal cancer risk [83]. Cancer cells presented with the APC mutation also has increased resistance towards certain drug strategies [84].

The varied protein expression often confers cancer cells with favorable characteristics for growth and survival. For example, the amplified production of human epidermal growth factor receptor 2 (HER2) has been suggested to induce cluster formation in membranes, leading to heightened susceptibility to tumorigenesis [85]. Processing and comparison of large databases have led to resources that provide quantitative values for EMT phenotypes, based on various protein expressions [86]. These dynamics have hindered attempts to completely map the extent of cancer heterogeneity [87]. Our inability to fully comprehend cancer progression often generates conflicting hypotheses and reduces our ability to generate effective treatment strategies.

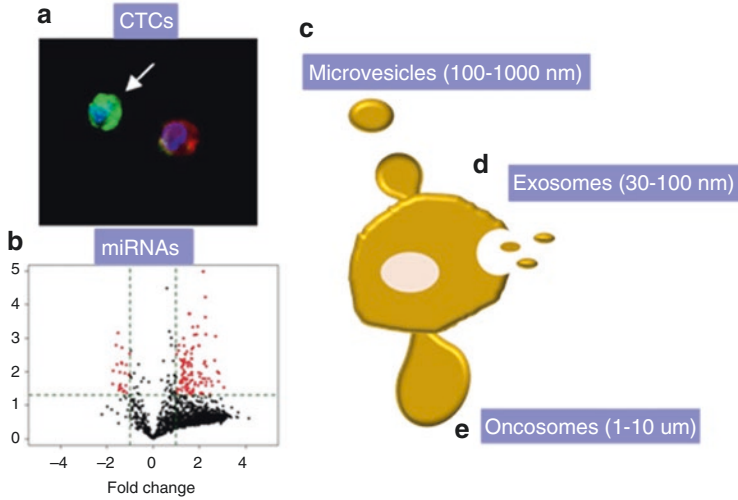
#### 4.2.1 Understanding CTC Proteomics for Diagnostic and Prognostic Relevance

Tumor-associated proteins have been regularly identified to characterize cancer for administering precise therapeutic treatment. The golden standard involves extraction of these proteins from tumor biopsies [88]. However, few proteins demonstrate actual clinical utility due to cancer heterogeneity [89], except for few instances such as the prostate-specific antigen (PSA). CTC has been broadly defined in a similar way as DTCs [90], namely being positive for epithelial markers (e.g. EpCAM and CKs) and negative for leukocyte markers. However, the proteomics of CTCs are extremely varied (Fig. 3b), and may even not reflect the phenotype observed from the tumor(s) of the same patient. Hence, researchers have been looking for unique specific markers.

To better identify the full CTC spectrum, researchers have started to explore tumour-associated proteins detectable in the serum. Although the field of serum proteomics is in its infancy, technological advances are surfacing which will aid in identifying novel markers. The serum proteome of tumors is a rich source for the analysis of cancer cell activity and interaction with the tumor niche [91]. Circulating tumor DNA (ctDNA), which are short fragments of 150–200 bp DNA, are released from apoptotic CTCs and are now a major interest for diagnostic purposes [92]. ctDNA constitutes about 3–93% of the total cell-free DNA (cfDNA) and ctDNA can be used as a liquid biopsy biomarker for cancer treatment by analyzing somatic mutations, chromosomal aberrations and loss of heterozygosity [93]. Recently, de novo multiplexed identification of ctDNA mutation is performed from different types of tumor (SHIVA trial) and 28 of 29 mutations detected in the tissue biopsy can be identified in the ctDNA from 27 cancer patients. This signifies the potential of ctDNA as a biomarker [94]. DEP microelectrodes can be used for the isolation of ctDNA from whole blood; however, this system is unsuitable for separation of small fragments of apoptotic DNA (<0.01  $\mu\text{m}$ ) and the use of DEP microelectrodes also suffers from cross-sample contamination issues and higher fabrication cost [95, 96].

In another study, digital PCR is used to define malignant growth in various types of cancer such as pancreatic, ovarian, colorectal and they can identify ctDNAs in greater than 75% of the patients [97]. Additionally, next generation sequencing (NGS) techniques has also been used for sequencing ctDNA and to monitor the response of patients to anti-cancer treatments [98]. ctDNA retains the spectrum of gene mutations found in tumours, and has the same ability to capture mutation patterns as per tumor biopsies [94]. A supposed advantage of utilizing ctDNA is the heightened abundance of material over CTCs, as well as the ease of detection over capturing CTCs [97]. Most importantly, the technology advancement in DNA profiling has enabled sensitive detection of a small amount of genetic material, in fact less than the amount of genetic material within a single CTC [93].

MicroRNAs (miRs) are small (20–22 bases), single stranded fragments of nucleic acid that can negatively regulate gene expression and many tumors have a characteristic miR-based tissue profiles. The miRs can be released into the microenvironment due to an active process or via passive secretion from a dying cancer cell; however, some miRs are not secreted from tumor cells [99]. Circulating miRs can be identified from serum and plasma of clinical samples and different types of solid tumors such as breast, colon, gastric can be distinguished based on miR profiles [100]. miRs from cancer cells [101] may also be isolated from plasma or found within microvesicles (100–1000 nm) [102], exosomes [103] and oncosomes (1–10  $\mu\text{m}$ ) (Fig. 4). Exosomes are cell-derived nanovesicles (30–100 nm) that are secreted into tumor microenvironment and play a critical role in cell-cell communication. Exosomes contains genetic information and signaling molecules including proteins, lipids, mRNA, miR and dsDNA that help in the formation of the premetastatic niche at the secondary tumor site [104, 105]. Exosomes can be extracted from the body fluids using conventional methods such as ultracentrifugation and combination of sucrose gradient ultracentrifugation with ultrafiltration centrifugation. However, the former method is much simpler in terms of sample preparation (requires 4–5 h) and has greater isolation efficiency (5–25%) [106]. Recently, Exo-Quick™ (System Biosciences) and Exo-spin™ (Cell Guidance System) commercialized the isolation kits for exosomes in a cost effective and user-friendly manner. Exosomes can also be isolated by targeting antibody-immobilized magnetic beads against specific antigens that are expressed on their surface such as Alix, annexin, EpCAM, Rab5, CD63, CD81, CD82, CD9 [107]. This technique has greater isolation efficiency than conventional methods; however, it fails to collect the entire population, as some of the exosomes do not express the targeted protein on its surface. Microfluidics isolation of exosomes has been developed using ExoChip, which uses fluorescent imaging to quantify the exosomes that are captured on anti-CD63 coated chips [108]. Interestingly, this study observed a greater number of exosomes in cancer patients ( $2.34 \pm 0.31$  fold higher) compared to healthy individuals, which indicates the significance of exosomes as a circulating biomarker. Microfiltration devices (with membrane pore size of  $<500$  nm) have also been used for exosome sorting from whole blood in a highly efficient manner [109]. This device can be combined with electrophoresis and pressure driven flow to further increase the enrichment efficiency. Label-free identification of exosomes can be



**Fig. 4** Utilizing CTC components for diagnostic and prognostic relevance. (a) Intact CTCs are the primary targets for isolation and enrichment for further analysis and utility. A representative image of enriched CTCs (*white arrow*) is provided (*Green*: cytokeratin; *Red*: CD45; *Blue*: Nuclei stain, Hoechst). Scale bar: 20 mm [12]. (b) An example of a scatter plot demonstrating the differential signal intensities of miRNAs isolated from serum samples of cancer patients and healthy controls [101]. (c–e) Extracellular vesicles can be classified into three categories based on size—microvesicles, exosomes and large oncosomes. Oncosomes appear to display unique properties and could serve as an important target for cancer characterization (Color figure online)

carried out using surface plasmon resonance (SPR) sensor chip, the nano-plasmonic exosome (nPLEX) [110]. The nPLEX chip can be functionalized with various exosomal antibodies and can potentially identify 12 exosomal markers within 30 min.

Research for novel markers in glioma has been extensive for the recent years, and several new markers such as CXCR4 [111] and haptoglobin [91] has been discovered. However, the heterogeneity of cancer complicates the validation of any new candidate markers, and extensive single-cell analysis will be required. Some clinicians are also keen to utilize the serum markers from CTCs, namely exosomes and ctDNA, as diagnostic markers for cancer. For example, the mutant EGFRvIII protein, found in exosomes [112], has been demonstrated as a potential therapeutic marker. Markers from ctDNA, such as miR181 [113], can also be a source for diagnostic means, if the half-life of ctDNA is established [114]. To overcome the problem of information loss due to DNA degradation, extracellular vesicles (EVs) may also be extracted from blood serum. EVs are vesicles that bud off from membranes or secreted from cells for intercellular communication [115], and the transported components are protected from degradation. In the latter instance, these vesicles form exosomes [116].

Another alternative to overcoming the low genetic material available from rare cancer cell subpopulations, such as CTCs, is via single-cell analysis (SCA). Advanced systems, such as the microfluidic image cytometry (MIC) [117] and

mass cytometry platform (CyTOF) [118], are available. They can simultaneously separate, process and analysis information from large panels of protein expression, such as the Akt kinase [119, 120].

### 4.3 *Molecular Profiling*

Ideally, CTCs can help to reflect the complete tumor spectrum, if the technological limitations impeding enrichment and analysis of rare cells are overcome. Recent advances now allow the simultaneous monitoring of multiple gene expression in parallel. Sensitive transcriptomic tools, such as the single-cell PCR based approach (SINCE-PCR) [121], serve to obtain quantitative and qualitative information which can be beneficial in both scientific and clinical settings. Single-cell analysis can also be carried out with fluorescence in-situ hybridization (FISH) (Fig. 3c).

Thus far, the identification of CTCs has been made complicated due to the lack of specificity of known markers. In fact, most existing molecular subtyping methods do not distinguish cancer cells from normal stroma tissue. Varied histopathological sub-types [122] exhibit different sensitivity of markers as well, and some markers may be common across several cancer types [123, 124]. Variation also exists across tumors from the same patient [125, 126], and some specific cancer types only have traces of molecular similarity with other cancer types [127]. This hinders the accuracy of tumor profiling, since the small amount of clinical samples may be insufficient to reveal rare mutations or intrinsic changes that occurred from response to therapeutic treatment [128]. Such extent of tumor heterogeneity may contribute to differential response to anti-cancer treatment [93], due to incomplete tumor profiling or adaptation to evade treatment.

The rise in advanced technologies, such as the Ensemble Decision Aliquot Ranking (eDAR), is now revealing vast heterogeneity in CTC gene expression, both within and across different patients [129]. An integrative approach to enrich, recover and characterize CTC exomes enabled extensive profiling of prostate CTCs. These profiles revealed a match to a portion of the tumor tissue from the same patient [130]. The spectrum of mutations varied significantly from cell lines, highlighting the issue of translational relevance of immortalized cancer cell phenotypes [131]. Extensive profiling also enables mapping of clonality. Preliminary studies suggest that the cells from the original tumor and metastases are phenotypically distinct, but presence of non-metastatic intermediate phenotypes which are found to persist in both the original tumor and its metastases [132]. Screening of various nuclei at the single-cell level also revealed extensive heterogeneity for KRAS, PIK3CA and other gene expressions [133–135]. These molecular variations may be the reason for a high failure in various anti-cancer regimes, due to an inaccurate diagnosis, or because the drugs are unable to target all cancer subtypes [93]. Detailed single-cell profiling of tumors may reveal novel mechanisms of tumor progression, such as gene amplification (e.g. MET) or chromosomal rearrangements [e.g. ALK (anaplastic lymphoma kinase)] [136].

## 4.4 *Lipidomics Profiling*

Lipidomics is a relatively new area of research, and is now increasingly utilized due to development of novel technologies such as the time-of-flight secondary-ion mass spectrometry (TOF-SIMS) [137]. Detection of key enzymes involved in lipid metabolism, such as stearoyl-CoA desaturase 1, may also provide a signature for monitoring lipid levels, even in single cells [138].

## 4.5 *Functional and Metabolic Heterogeneity*

Generally, the EMT spectrum of CTCs demonstrates both inpatient and interpatient heterogeneity. Researchers have also showed that not all phenotypes within the EMT spectrum may be present; with some being fully epithelial-like or mesenchymal-like [139]. More recently, it has been hypothesized that single-cell metabolomics [140] can be a unique factor for the identification of cancer cells from blood cells. These studies may be aided by the novel integrated systems, such as the nanostructure-initiator mass spectrometry (NIMS), which will serve to reveal cancer cells that may have a higher metastatic potential [141]. However, functional and metabolic studies of CTCs are highly limited by the method of CTC enrichment, which is often stressful to the primary cancer cells and compromise cellular viability.

### 4.5.1 **Heterogeneity in Cultured CTCs**

The functional properties of CTCs can be investigated with viable CTCs. However, most CTC enrichment techniques are laborious and require harsh procedures which compromise CTC viability. Analysis of the spatial-temporal characteristics of CTCs may be possible by the establishment of short-term CTC cultures with in vitro tumorsphere assays [142], structured microwell assays [63], or long-term CTC cell lines spontaneously immortalized after >6 months in culture [72, 143]. Secretion capabilities of CTCs from various cancer types can also be investigated with a membrane-based assay that provides quantification of proteins [64]. The proliferative capabilities of CTCs vary depending on marker expression [142], as well as response to therapeutic treatment [159]. More specifically, upar+/int  $\beta$ 1 – CTC subsets are shown to expand better than other combinational subsets of CTCs, and longer treatment duration correlates with lower proliferative potential. Upar-/int  $\beta$ 1 – CTC subsets also appeared to be more mesenchymal and displayed delayed clustering.

CTC may also demonstrate varied capabilities for adhesion, albeit most studies report low adhesive capabilities of CTCs. These low adhesive properties may reflect their heightened potential for extravasation and migration. Interestingly, it has been

described that low adhesive CTCs demonstrate high plasticity and may eventually adhere to form spheroids under suitable 3D gel conditions [142]. Understandably, the more mesenchymal phenotypes also demonstrate higher motility and invasiveness. Analysis of these factors and other functional phenotypes, such as the formation of invadopodia [142], may reveal valuable information of the metastatic cascade occurring *in vivo*.

#### 4.5.2 Cancer Stem Cells

Technological advances have enabled isolation of rare cancer subtypes with unique properties. Cancer stem cells (CSCs) are cells present in the tumor that demonstrates heightened tumorigenic potential. They are found to demonstrate distinct protein signatures, for example, breast CSCs are recognized as CD44+/CD24− [144] or aldehyde dehydrogenase 1+ (ALDH1+) cells [12, 145]. Recently, other pluripotency factors, such as POU5F1 (OCT4), have been associated with the CSC subtype [146], supporting their potential role in metastasis. CSCs are often speculated to be associated with worsened patient prognosis [147], as they are found with anti-cancer drug resistant or tolerant traits [148]. Despite the distinctive characteristics of CSCs, the clinical relevance of this rare sub-population is still unclear, and further functional analysis may help to resolve this question.

## 5 Discussions and Conclusion

Compared to conventional macro scale devices, microfluidics technologies have provided several advantages in the field of CTC isolation such as higher throughput, greater sensitivity, portability, and ease of operation [149]. Additionally, this technique enables manipulation of fluid and particle motion at a micron-scale that is critical for CTC enrichment considering their rare occurrence in the blood. The antibody based isolation techniques provides some inherent benefits including higher specificity, lesser leukocyte contamination and enriching viable CTCs due to minimum sample handling procedure. However, these devices suffer from low throughput (1–3 ml/h) and are dependent on the expression of specific antigen markers. Due to the occurrence of EMT, some subpopulations of CTCs might not express the epithelial markers such as EpCAM and this might lead to the loss in capture efficiency [150, 151]. Label-free approaches can overcome these drawbacks of antibody-dependent methods by isolating CTCs based on their physical properties including size, deformability and dielectric properties in an unbiased manner independent of EpCAM expression. However, these techniques are limited by the heterogeneity of the cancer cells in terms of their size (CTC size varies from 6 to 30  $\mu\text{m}$ ) and thereby resulting in contamination from other blood cells and decreasing the isolation purity [152].



Overall, CTC isolation devices have made rapid progress over the last decade; however, till date there are few FDA approved methods (the CellSearch system and cobas EGFR Mutation Test) [153] for clinical diagnosis. Therefore, development of universal optimal protocol for examining the performances of these different devices is essential. Recent advancement of microfluidics technology has paved the way for solving some of the initial problems associated with conventional macroscale systems such as lower throughput, purity and sensitivity. The development of hybrid systems such as CTC-iChip that uses the advantages of both antibody-dependent and label-free approaches might overcome the limitations associated with both these systems individually. Additionally, negative selection devices that rely on the depletion of leukocytes may be an alternative unbiased approach for CTC enumeration. In future, we envision the development of an integrated, fully automated CTC enrichment platform that can isolate the CTCs from a simple blood test, characterize their biochemical and biophysical properties and identify the potential targets for chemotherapeutic administration.

Cancer manifests as a heterogeneous disease in several different ways [154], and detailed profiling of this manifestation is essential to develop personalized drug therapeutic strategies [155]. Biopsies of tumors are generally undesirable due to the invasive nature and possibility of dissociation of cancer cells during the surgical process. Hence CTCs represent an alternative source of cancer cells obtained via blood withdrawal, so termed as liquid biopsy [155]. The CTCs may also provide a better profile for the tumors, as they are shed from a range of sites as compared to a single biopsied region.

Several questions remain which impede the clinical utility of CTCs. The relevance of CTCs in influencing therapeutic strategies is in question since it is not known how CTCs persist within the circulation and contribute to metastasis. CTC lifespan has also not been conclusively defined, and it is speculated that this may vary from a few hours to years [156, 157]. The persistence of CTCs in blood fuels the conjecture that these rare cells may play a role in tumor relapse. Overall, the clinical relevance of CTCs will be determined by their resemblance to the original tumor phenotype, their transition in blood and their functional dynamics with time. CTCs and associated markers (e.g. microRNAs) could help to rectify the incomplete tumor profile. The characterization of CTC components may shed light for a single pathway [158] or on the patient conditions at a point of time during treatment, but monitoring of a patient's condition will require serial sampling or culturing of CTCs which could reveal dynamics of an individual's cancer progression.

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