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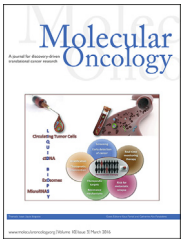

Circulating tumor cell technologies☆

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ABSTRACT

Circulating tumor cells, a component of the "liquid biopsy", hold great potential to transform the current landscape of cancer therapy. A key challenge to unlocking the clinical utility of CTCs lies in the ability to detect and isolate these rare cells using methods amenable to downstream characterization and other applications. In this review, we will provide an overview of current technologies used to detect and capture CTCs with brief insights into the workings of individual technologies. We focus on the strategies employed by different platforms and discuss the advantages of each. As our understanding of CTC biology matures, CTC technologies will need to evolve, and we discuss some of the present challenges facing the field in light of recent data encompassing epithelial-to-mesenchymal transition, tumor-initiating cells, and CTC clusters.

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1. Introduction

The field of research encompassing technologies to detect, isolate, and characterize circulating tumor cells (CTCs) has exploded in recent years; PubMed listed approximately 16,688 articles under the key phrase "circulating tumor cell" as of August 2015, and of those 1248 were published in 2014. While it seems like this burgeoning field has only recently blossomed, its roots date back as far as 1869, with the first reported description of CTCs by Thomas Ashworth (Ashworth, 1869). After a sparse trail of publications, early reports on methods for detecting CTCs via filtration (Salgado et al., 1959) and sedimentation (Alexander and Spriggs, 1960) emerged nearly 100 years later. Perhaps the most commonly used technique for detection today, immunomagnetic separation, was not reported until 1998 (Racila et al., 1998). In addition to introducing a new detection method, Racila et al. also demonstrated that CTCs exist early in disease and correlate with disease progression – providing a new perspective on the potential role of CTCs in modern cancer research and therapy.

Today, researchers look to profiling components, including CTCs, in serial blood draws (liquid biopsy) to transform the current landscape of cancer therapy by i) determining patient prognosis, ii) monitoring tumor recurrence and therapeutic responses in real-time, iii) identifying new therapeutic targets, iv) elucidating drug resistance mechanisms, and v) improving our current understanding of tumor progression and metastatic disease. Multiple studies have demonstrated that elevated CTC counts indicate a poor prognosis for metastatic breast, prostate, colorectal, and lung cancer patients (Cristofanilli et al., 2005; Smerage et al., 2014; de Bono et al., 2008; Cohen et al., 2008; Miller et al., 2010; Krebs et al., 2011). However, U.S. institutions governing cancer care have not adopted any CTC technologies into their existing guidelines for routine clinical care, because, despite their prognostic significance, their use has yet to improve patient survival (Attard and de Bono,

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2011; Harris et al., 2007; Riethdorf and Pantel, 2010; Smerage et al., 2014). Researchers have thus shifted their focus from enumeration to characterization to expand the applications for CTC technologies and enable fruition of their clinical utility.

A key technical challenge to the realization of the aforementioned applications is the current availability of a system capable of efficiently capturing the extremely rare CTC populations from patient blood samples in a manner amenable to downstream processing; approximately one CTC exists among a background of millions to billions of blood cells (Nelson, 2010; Yu et al., 2011). To transition from CTC enumeration to molecular characterization, researchers will need methods that provide greater recovery rates. Current methods for CTC capture utilize a vast array of strategies, which include sorting based on physical characteristics (e.g., size, density, deformability, and electrical charge) as well as more 'specific' biological properties, such as tumor cell surface marker expression (Table 1). Despite the ingenuity of these techniques, the substantial heterogeneity of CTCs makes it difficult to isolate pure and representative CTC subpopulations. Moreover, CTCs have experienced an identity crisis in recent years: the initial definition of CTCs as nucleated cells expressing the cell surface marker, epithelial cell adhesion molecule (EpCAM+), and/or cytokeratin (CK+) in the cytoplasm, and lacking the leukocyte common antigen (CD45−) continues to evolve as new data suggest even greater heterogeneity, including EpCAM− and/or CK- CTCs (Marrinucci et al., 2012; Mikolajczyk et al., 2011; Pecot et al., 2011; Serrano et al., 2014). Therefore, developing enrichment technologies that can effectively capture CTCs of varying phenotypes remains a complex, challenging, and constantly evolving endeavor.

In this review, we will provide an overview of current strategies used to capture CTCs with specific examples from each category. We will discuss the advantages and challenges associated with the different strategies used and how the field has evolved to keep pace with our maturing understanding of CTC biology. Our aim is to inform the reader of the current spectrum of enrichment technologies available and to highlight the advantages and pitfalls of current strategies to enable the development of improved CTC enrichment methods.

2. Metrics for measuring the performance of CTC enrichment platforms

Later in this review, we will introduce multifarious approaches for CTC detection. Similar to any new technology with commercial potential, CTC detection platforms must be repeatable, reliable, rapid, cost-effective, and suited to large-scale production and use. In addition, they need to capture extremely rare cells from clinically relevant blood volumes, usually around 7.5 ml. These requirements have formed the basis for a standard set of performance criteria used to evaluate and compare CTC technologies: capture efficiency, purity, enrichment, and throughput, as discussed in greater detail below. It is important to note that these metrics are assessed using cells from cancer cell lines that are spiked into blood obtained from healthy donors; to perform these tests, the number of target cells must be known, and the actual number of CTCs in a patient sample is always unknown. Thus, while spiked cells from cancer cell lines are not the same as CTCs (Powell et al., 2012), it is not feasible to assess these metrics using clinical samples. Moreover, the use of cell lines overpredicts device performance, as cancer cell lines tend to be more homogenous in both their cell surface markers and physical properties, and more physically distinct from leukocytes, than patient CTCs.

Capture efficiency, or yield, quantifies the ability of the device to capture tumor cells (TCs) in spiked blood samples. This metric is defined as the number of tumor cells captured divided by the total number of tumor cells spiked into a blood sample.

$$\frac{(TCs)_{CAPTURED}}{(TCs)_{ACTUAL}} \tag{1}$$

Enrichment is similar to capture efficiency but instead refers to the factor increase of tumor cells within a volume relative to a background of other cells (primarily leukocytes) before and after running the sample through the device being evaluated.

$$\frac{(TCs)_{CAPTURED}/(WBCs)_{CAPTURED}}{(TCs)_{ACTUAL}/(WBCs)_{IN}} = \text{Capture Efficiency} \times \frac{(WBCs)_{CAPTURED}}{(WBCs)_{IN}} \tag{2}$$

Purity describes the ability of the device under evaluation to specifically capture tumor cells or CTCs within a background of contaminating cells (such as leukocytes). Purity is defined as the number of CTCs captured divided by the total number of nucleated cells captured, and it is the one metric discussed that can be measured from clinical samples.

$$\frac{(CTCs)_{CAPTURED}}{(CTCs + WBCs)_{CAPTURED}} \tag{3}$$

Throughput indicates how quickly the device can process a sample, and it is commonly written as either volume or cells per unit time (Jin et al., 2014).

Recently a greater emphasis has been placed on developing technologies that produce samples compatible with downstream analyses of captured CTCs. Genomic and transcriptomic analyses, for example, may not always require live cells, but they do require high purity because contaminating leukocyte DNA or RNA can confound results. However, some analyses, such as cell deformability measurements and drug response assays, do require the retrieval of viable cells. In response, two additional criteria, cell viability and release efficiency, have been introduced to the CTC lexicon. *Cell viability* is defined as the percentage of CTCs that are still alive following enrichment, and *release efficiency* refers to the percentage of captured CTCs that can be retrieved from the device.

3. CTC enrichment strategies: immunoaffinity

In 1998, Racila et al. reported CTC enrichment by immunoaffinity, and it is still the most widely used and only FDA-approved strategy to date. This method uses specific biomarkers expressed on the cell surface (e.g., EpCAM and CD45) to capture cells. The antibodies used for selection are typically tethered to either the device surface or a magnetic substance (i.e.,

Table 1 – CTC technologies.

Subcategory	Technology	Selection criteria	Key features	References
Immunoaffinity – Positive Enrichment *Antibodies targeting tumor-associated antigens are tethered to magnetic particles (immunomagnetic) or the device surface to capture CTCs.*				
IM	CellSearch®	EpCAM	FDA-Approved	(Cristofanilli et al., 2004; Hayes et al., 2006; Riethdorf et al., 2007)
	AdnaTest	Antibody Cocktail	CTCs captured then multiple cancer markers measured by RT-PCR	(Andreopoulou et al., 2012; Müller et al., 2012; Musella et al., 2015)
	MACS	EpCAM	Pos/Neg enrichment, high surface area to volume	(Miltenyi et al., 1990; Pluim et al., 2012)
	MagSweeper	EpCAM	High purity, can process WB, 9 mL/h throughput	(Deng et al., 2014; Kim et al., 2014; Lohr et al., 2014; Powell et al., 2012; Talasaz et al., 2009)
Microfluidic (μF) Micropost Arrays	CTC-Chip	EpCAM	Micropost array optimized for cell-antibody contacts, 1–2 mL/h	(Nagrath et al., 2007)
	GEDI	PSMA/HER2, Size	Size-based separation minimizes contamination	(Galletti et al., 2014; Kirby et al., 2012)
	OncoCEE	Antibody Cocktail	Staining procedure labels capture antibodies	(Mikolajczyk et al., 2011)
Microfluidic Surface Capture	Herringbone Chip	EpCAM	Microvortices mix sample, clusters observed, 4.8 mL/h	(Stott et al., 2010)
	GEM	EpCAM	Microvortices mix sample, 3.6 mL/h	(Sheng et al., 2014)
	Graphene Oxide Chip	EpCAM	Planar geometry, 1–3 mL/h	(Yoon et al., 2013)
	Modular Sinusoidal Microsystem (commercialized by BioFluidica)	EpCAM	Three modules: selection, impedance, and imaging, clusters observed, >86% purity, >7.5 mL/h	(Kamande et al., 2013)
Microfluidic IM	Ephesia	EpCAM	Self-assembly of magnetic μm-beads into columns, >3 mL/h	(Autebert et al., 2015; Saliba et al., 2010)
	Magnetic Sifter	EpCAM	Vertical flow configuration, 10 mL/h	(Earhart et al., 2013)
	LiquidBiopsy	Antibody Cocktail	Automated, sheath flow minimizes non-specific binding, continuous flow, 5 mL/h	(Winer-Jones et al., 2014)
μF, IM	Isoflux™	EpCAM	Automated, continuous flow	(Harb et al., 2013).
μF, IM	CTC-iChip	EpCAM, Size	Pos/neg enrichment, size-based separation debulks WB, inertial focusing aids in magnetic deflection, 8 mL/h	(Karabacak et al., 2014; Ozkumur et al., 2013)
IM, *in vivo*	GILUPI CellCollector™	EpCAM	Can process large volumes of blood	(Saucedo-Zeni et al., 2012)
Immunoaffinity – Negative Enrichment *Antibodies targeting leukocyte-associated antigens are tethered to magnetic particles or the device surface deplete unwanted background cells.*				
IM	EasySep™ Human CD45 Depletion Kit	CD45	Simple, easy-to-use batch separation	(Liu et al., 2011)
	QMS		Continuous flow, high-throughput	(Lara et al., 2004; Wu et al., 2013)
	MACS			(Giordano et al., 2012; Lara et al., 2006)
μF, IM	CTC-iChip	CD45, CD66b, Size		(Karabacak et al., 2014; Ozkumur et al., 2013)
Biophysical – Density Gradient Centrifugation *Sample placed on top of a separation medium and centrifuged to separate different cell populations based on their relative densities.*				
	Ficoll-Paque®	Density	Inexpensive, easy-to-use	(Weitz et al., 1998)
	OncoQuick	Density, Size	Porous membrane above separation media for additional separation by filtration	(Balic et al., 2005; Clawson et al., 2012; Lagoudianakis et al., 2009; Müller et al., 2005; Obermayr et al., 2010; Rosenberg et al., 2002)

Table 1 (continued)

Subcategory	Technology	Selection criteria	Key features	References
	RosetteSep™ CTC Enrichment Cocktail	Density, Antibody Cocktail	Antibody-labeling alters cell density	(He et al., 2008)
	Accucyte Enrichment and CyteSealer™	Density	Sequential density fractionation enriches target cells. Additional CyteFinder™ and CytePicker™ modules for high-throughput imaging and single-cell recovery	(Campton et al., 2015)
Biophysical – Microfiltration in Two and Three Dimensions				
Size-based cell separation using pores or three-dimensional geometries.				
2D, Track-etched	ISET®	Size, Deforma-bility	Filters fixed samples through 8-μm pores, 10–12 wells can process 1 mL each, clusters observed	(Chinen et al., 2013; Farace et al., 2011; Hofman et al., 2011; Ilie et al., 2014; Krebs et al., 2012; Pailler et al., 2013; Vona et al., 2000)
	ScreenCell®		Hydrophilic surface, fixed/live samples, 7.5/6.5-μm pores	(Desitter et al., 2011; Freidin et al., 2014)
2D, Lithography	CellSieve™		Constructed from a transparent, flexible, non-fluorescent photoresist, 7-μm pores	(Adams et al., 2014a, 2014b, 2015)
	Flexible Micro Spring Array (FMSA)		Constructed from parylene-C, can process WB, captures viable cells, clusters observed	(Harouaka et al., 2014; Kaifi et al., 2015)
3D	FaCTChecker		Constructed from parylene-C, captures viable cells between membrane layers	(Zhou et al., 2014)
	Parsortix		Viable cells released by reversing flow	
	Resettable Cell Trap		Pneumatically-controlled microvalves	(Qin et al., 2015)
	Cluster Chip		Triangular pillars designed for CTC clusters	(Sarioglu et al., 2015)
Biophysical – Inertial Focusing				
Cells are passively separated by size through the application of inertial forces in microfluidic devices that affect positioning within the flow channel.				
	Vortex	Size	No RBC lysis required, captures viable cells, easy to manufacture, clusters observed	(Sollier et al., 2014)
	ClearCell® FX		RBC lysis required, 1–1.5 mL/min, captures viable cells, easy to manufacture	(Khoo et al., 2015, 2014; Warkiani et al., 2014)
Biophysical – Electrophoresis				
Separates cells based on their electrical signature using an applied electric field.				
	ApoStream®	Electrical Signature	DEP-FFF, continuous flow, captures viable cells, >10 mL/h	(Gupta et al., 2012; Shim et al., 2013)
	DEPArray™		Requires pre-enrichment, allows recovery and manipulation of viable, single cells through DEP cages	(Carpenter et al., 2014; Fabbri et al., 2013; Fernandez et al., 2014; Manaresi et al., 2003; Peeters et al., 2013; Polzer et al., 2014)
Biophysical – Acoustophoresis				
Separates cells based on acoustophoretic mobility, which is size dependent, by exposing them to acoustic waves.				
	Acoustophoresis Chip	Size	Acoustic pre-alignment and separation	(Antfolk et al., 2015a, 2015b; Augustsson et al., 2012)

(continued on next page)

Table 1 (continued)

Subcategory	Technology	Selection criteria	Key features	References
Direct Imaging Modalities *Technologies developed to i) improve the efficiency of imaging or ii) replace enrichment through high-speed fluorescent imaging.*				
Pre-Enrichment Required	Microfluidic Cell Concentrator (MCC)	None	Passive pumping concentrates samples ~5x	(Casavant et al., 2013)
	ImageStream®	CK, CD45, DRAQ5	Hybrid of flow cytometry and fluorescence microscopy, 5000 cells/sec	(López-Riquelme et al., 2013; Zuba-Surma and Ratajczak, 2011)
Enrichment-Free	EPIC	CK, CD45, DAPI	Automated digital microscopy, clusters observed	(Marrinucci et al., 2012; Nieva et al., 2012)
	FASTCell™	CK, CD45, DAPI	Fiber-optic array allows larger field-of-view, 25M cells/min, low resolution	(Das et al., 2012; Krivacic et al., 2004; Somlo et al., 2011)
	CytoTrack	CK, CD45, DAPI	Special glass disc scanned as it spins, clusters observed, 100 M cells/min	(Hillig et al., 2015)
In vivo	Photoacoustic flow cytometry (PAFC)	Absorption spectra	Non-invasive label-free interrogation of large blood volumes	(Galanzha and Zharov, 2013)
Functional Assays *Viable CTC enrichment based on bioactivity of cells, such as protein secretion or cell adhesion.*				
	EPISPOT	Protein secretion	Discriminates between viable and apoptotic CTCs using protein secretion	(Alix-Panabières, 2012; Alix-Panabières and Pantel, 2015; Ramirez et al., 2013)
	Vita-Assay™	Cell adhesion matrix (CAM)	Enriches for viable CTCs based on preferential CAM adhesion. Clusters observed.	(Friedlander et al., 2014; Lu et al., 2010)

IM – immunomagnetic, μF – microfludic, WB – whole blood. Companies that have commercialized the technologies above are mentioned in the text.

immunomagnetic capture), allowing the capture of cells via a magnetic field. During positive selection, tumor-associated cell surface antigens, such as EpCAM, are targeted, whereas negative selection removes background cells by targeting antigens not expressed by CTCs, such as CD45. One of the challenges to affinity-based capture methods is the ever-changing definition of CTCs; the heterogeneous array of surface markers expressed by CTCs has, to date, made it impossible to identify a universal CTC-specific antigen.

3.1. *The biological foundation for affinity-based CTC enrichment strategies*

Affinity-based CTC enrichment technologies either capture CTCs by specifically targeting tumor-associated antigens (i.e., positive enrichment), or they deplete hematopoietic cells by targeting CD45 (i.e., negative enrichment). Positive enrichment typically attains high cell purity, which depends on antibody specificity. Negative enrichment technologies evade some of the pitfalls of positive enrichment; for example, CTCs are not tagged with a difficult-to-remove antibody, and antibody selection does not bias the subpopulation of CTCs captured. However, these advantages come at the cost of purity, as negative enrichment strategies typically have a much lower purity than positive enrichment (Baccelli et al., 2013; Lara et al., 2004; Yang et al., 2009).

Hitherto, the majority of positive selection technologies for CTCs derived from epithelial tumors (e.g., breast, prostate, colon, and lung) have targeted the epithelial cell surface marker, EpCAM, with subsequent immunohistologic detection using cytokeratin (CK) and DAPI nuclear staining (Pantel et al., 2008). Until recently, the accepted immunohistologic definition of a CTC was a nucleated EpCAM+/CK+/CD45− cell. Although non-malignant epithelial cells with these same immunohistologic characteristics are generally not found in the blood of healthy individuals (Allard et al., 2004), researchers have found EpCAM+/CK+/CD45− cells in the bloodstream of patients with benign colon diseases (Pantel et al., 2012), pancreatic lesions (Cauley et al., 2015), and benign breast disease (Crisan et al., 2000; Franken et al., 2012). These studies support the need for additional molecular characterization of circulating epithelial cells. Multiple groups have also reported the occurrence of a CK+/CD45+ or 'double positive' cell phenotype in the bloodstream of metastatic cancer patients (Yoon et al., 2013; Lustberg et al., 2014; Riethdorf et al., 2007; Sheng et al., 2014; Stott et al., 2010). Both the identity and the significance of these obfuscating cells have received much debate with no resolution. It is important to be cognizant of both benign circulating epithelial cells and double positive cells as their identity could easily be misconstrued and cause false-positive or false-negative events or denote as yet unknown biological phenomena.

Technologies that have employed epithelial markers to capture and identify CTCs have successfully demonstrated the prognostic significance of EpCAM+/CK+/CD45−. However, the emergence of new data highlighting the importance of

epithelial-to-mesenchymal transition (EMT) and stem cell markers in CTCs has given researchers pause as they struggle to determine the significance of different CTC subpopulations. Many affinity-based enrichment technologies use epithelial markers, which are down-regulated during EMT, to capture CTCs. In response, researchers have expanded the antigen repertoire used in affinity-based capture to include stem cell markers (e.g., CD133), mesenchymal markers (e.g., cell-surface vimentin, CSV) (Satelli et al., 2014), and cancer-specific antigens (e.g., HER2, PSMA) (Galletti et al., 2014; Kirby et al., 2012; Winer-Jones et al., 2014).

3.2. *Positive enrichment technologies*

3.2.1. *Immunomagnetic enrichment technologies*

Despite the drawbacks facing technologies that utilize epithelial markers for positive selection, the biological relevance of EpCAM+/CK+/CD45− cells in the peripheral blood is well-substantiated by the prognostic value of CellSearch® (Cristofanilli et al., 2004; Hayes et al., 2006; Riethdorf et al., 2007). Currently licensed by Janssen Diagnostics, the CellSearch® system is the only CTC technology approved by the FDA to aid in monitoring patients with metastatic breast, prostate, and colorectal cancers. The CellSearch® system uses ferrofluid nanoparticles functionalized with an EpCAM antibody to allow magnetic separation of EpCAM+ cells from solid blood components following centrifugation. The captured cells are then immunostained to confirm the expression of CK 8, 18, 19, and DAPI, as well as the lack of CD45 expression. The successful accreditation of the CellSearch® system by the FDA has led to its current prominence as a type of gold standard within the field; emerging technologies routinely compare their results to CellSearch® to validate their system.

Another commercially available platform for CTC detection is AdnaTest (Adnagen AG). AdnaTest enriches CTCs through the use of magnetic, antibody-coated beads. Unlike CellSearch, which uses the anti-EpCAM antibody exclusively, AdnaTest uses a cocktail of antibodies (e.g., EpCAM and MUC-1, AdnaTest Breast™) specific to the type of cancer (e.g., breast, prostate, ovarian, and colon). Following selection, captured CTCs are lysed and tested for expression patterns of various cancer-associated tumor markers (e.g., GA733-2, MUC-1, and HER2, AdnaTest Breast) using multiplex reverse transcription-polymerase chain reaction (RT-PCR); samples are defined as CTC-positive if the measured quantity of at least one of the tumor markers is above a defined threshold (Adnagen suggests >0.1 ng/μL). A comparison of the ability to detect CTCs using the AdnaTest Breast versus the CellSearch system determined that the two platforms had equivalent sensitivity; out of a total of 55 metastatic breast cancer patients, 20 were identified as CTC positive (≥5 CTCs) using CellSearch, whereas 29 were identified as CTC positive (≥0.15 ng/μL) using AdnaTest methods (Andreopoulou et al., 2012). In contrast, AdnaTest Colon exhibited superior sensitivity, identifying CTCs in 81% of metastatic colorectal cancer patients compared to 21% with CellSearch (Raimondi et al., 2014).

Another enrichment technology based on immunomagnetic separation is the magnetic cell sorter (MACS) (Miltenyi et al., 1990). MACS uses high-gradient magnetic separation to capture cells labeled with magnetic nanoparticles (10–85 nm diameter) conjugated to antibodies for enrichment (anti-EpCAM) (Giordano et al., 2012; Pluim et al., 2012) or depletion (anti-CD45) (Giordano et al., 2012). The sample is passed through a column filled with plastic-coated steel wool that can be magnetized and demagnetized in the presence and absence of a magnetic field, respectively, allowing the capture and subsequent elution of magnetically labeled cells. The unique design of MACS technology, which involves strong magnetic fields generated across materials with a large surface area to volume ratio, allows efficient capture of desired cell populations. Studies have used MACS to capture CTCs from metastatic cancer patients to study p-ERK expression following *ex vivo* EGF stimulation (Pluim et al., 2012). In another clinical study both enrichment and depletion strategies were employed to capture and profile CTCs from HER2-positive breast cancer patients for EMT-related gene expression (Giordano et al., 2012).

Blood contains more than 10^9 RBCs per ml compared to single or double digit quantities of CTCs and can therefore interfere with CTC isolation and/or detection. On the other hand, pre-processing blood prior to capture, such as by centrifugation or red blood cell lysis, can cause a reduction in CTC capture efficiency and result in CTC loss. The MagSweeper, an immunomagnetic enrichment technology, isolates CTCs with relatively high purity and, unlike many CTC isolation technologies, is capable of processing whole blood without centrifugation or red blood cell lysis. A robotically-controlled magnetic rod isolates CTCs by sweeping through wells containing samples pre-mixed with antibody-coated magnetic beads at a rate that accounts for shear force to ensure the detachment of adsorbed, non-magnetically labeled cells (Talasaz et al., 2009). The MagSweeper can process blood at a rate of 9 ml/h, and it can be easily scaled up to process multiple samples in parallel using an array of magnetic rods controlled by a single automated system. The system was validated using clinical samples: CTCs were isolated from 70% of patients with primary and metastatic breast cancer, and no CTCs were found in blood from healthy donors or patients with lymphoma, a non-epithelial cancer (Powell et al., 2012). Additional molecular analysis on cell lines verified that the capture process did not perturb gene expression. The high purity levels obtained by the Magsweeper make it amenable for downstream genomic analysis, which can be easily confounded by leukocyte contamination. The MagSweeper has been used for genetic profiling of CTCs in multiple studies, including the first high-throughput, single-cell transcriptional profiling study in breast cancer (Powell et al., 2012), single-cell detection of PIK3CA mutations in CTCs and breast cancer metastases (Deng et al., 2014), mRNA-Seq (Cann et al., 2012) and single-cell whole exome sequencing in prostate cancer (Lohr et al., 2014), and analyses of stem cells in colorectal cancer (Kim et al., 2014).

3.2.2. *Microfluidic-based positive enrichment technologies*

3.2.2.1. *Micropost arrays*. Microfabrication methods have made it possible to create structures at or below the cellular length scale, which provides a unique advantage for cell separation. In 2007, Nagrath et al. employed these methods to develop the first microfluidic device designed for CTC

enrichment (Nagrath et al., 2007). Microfluidic devices allow precise control of fluid flow, which is important because the efficiency of cell capture depends highly on cell-antibody contacts that can be controlled through fluid flow velocity and direction. The CTC-Chip developed by Nagrath et al. consisted of an array of 78,000 microposts chemically functionalized with the anti-EpCAM antibody; the geometric arrangement of the microposts and the fluid flow velocity were optimized to promote cell attachment to the antibody-coated posts. A fluid flow of 1–2 ml/h was selected for optimal capture, which highlights one of the disadvantages characteristic of microfluidic technologies: the low-throughput rate and inability to handle large sample volumes. One advantage of the CTC-Chip, and many of the subsequent microfluidic chips discussed in this section, is the ability to process whole blood. The CTC-Chip achieved a purity of 50% when used to capture CTCs from the peripheral blood of patients diagnosed with metastatic cancer (lung, prostate, breast, colon, and pancreatic) with sample concentrations ranging from 5–1,281 CTCs/ml. Subsequent studies demonstrated the ability to isolate CTCs from patients with metastatic, non-small-cell lung cancer and perform an EGFR mutational analysis on DNA recovered from the chip (Maheswaran et al., 2008).

The development of the CTC-Chip was followed by a number of different microfluidic-based strategies for CTC enrichment. One of these devices, the geometrically enhanced differential immunocapture (GEDI) device, combines positive enrichment using antibody-coated microposts with hydrodynamic chromatography to minimize non-specific leukocyte adhesion (Galletti et al., 2014; Kirby et al., 2012). Hydrodynamic chromatography separates the cells based on their size. The GEDI chip achieves this by offsetting the antibody-coated microposts, which causes size-dependent collision rates that ultimately influence cell trajectory. The first reported demonstration of the GEDI chip used an anti-prostate specific membrane antigen (PSMA) to capture CTCs (Kirby et al., 2012). The GEDI chip was used to isolate CTCs, defined as PSMA+/CD45− cells, from castrate-resistant prostate cancer (CRPC) patients. They achieved a 2–400-fold increase in CTC counts compared to CellSearch® with a median of 54 (range 0–1,200) cells collected from CRPC patients and 3 (range 0–22) from healthy donors. To demonstrate the ability to perform downstream analyses on cells isolated with the GEDI chip, cDNA sequencing and immunostaining were performed on prostate cancer cell lines to detect androgen receptor mutations. The GEDI device uses a flow rate similar to the CTC-Chip of 1 ml/h. OncoCEE (CEE, cell enrichment and extraction), commercialized by Biocept, is another micropost-based device that has put a twist on the conventional anti-EpCAM approach by using an antibody cocktail for CTC capture (Mikolajczyk et al., 2011). In addition to anti-EpCAM, the OncoCEE antibody cocktail includes antibodies to other tumor-associated (e.g., HER2, MUC-1, EGFR, TROP-2) and mesenchymal markers (e.g., N-Cadherin). Samples processed with antibody mixtures had higher cell capture rates, including EpCAM− cells, than those processed with anti-EpCAM alone. To further enhance CTC detection, the OncoCEE system uses a novel in situ staining protocol that fluorescently labels the capture antibodies bound to CTCs. In a clinical study, the OncoCEE platform detected CK+/CD45− CTCs in 53% (19/36) of samples from patients with metastatic breast cancer (Kalinsky et al., 2015). The study also demonstrated a high concordance (79%) of the estrogen/progesterone receptor (ER/PR) status between CTCs and primary tumor/metastatic biopsy.

3.2.2.2. *Surface-based*. Although promising, there are inherent constraints in large-scale production of complex micropost-based devices that require surface chemistry modifications. Additionally, current techniques for detection and characterization of CTCs rely heavily on immunocytochemistry and other techniques that require high-resolution imaging that is difficult in the presence of nontransparent three-dimensional micropost arrays. These limitations have led to the development of surface-capture microfluidic devices such as the herringbone (HB) chip (Stott et al., 2010), geometrically enhanced mixing (GEM) chip (Sheng et al., 2014), and the graphene oxide (GO) chip (Yoon et al., 2013). Instead of micropost arrays, these devices use antibody-coated surfaces to facilitate CTC capture. The simplified architecture of surface-capture devices is better suited to large-scale production and also allows fabrication of transparent devices more amenable to imaging. In addition, they can often be run at higher flow rates of 1–3 ml/h, 3.6 ml/h, and 4.8 ml/h for the GO, GEM, and HB chips, respectively.

Both the HB and the GEM chip enhance cell-antibody collision events using a method adapted from Stroock et al. to induce mixing within microchannels using transverse flow (Stroock et al., 2002). The HB chip successfully captured CTCs in 14/15 samples collected from metastatic prostate cancer patients, and further downstream analysis included RNA isolation and subsequent RT-PCR analysis to identify the TMPRSS2-ERF fusion transcript. Isolating CTCs from 17/18 samples from pancreatic cancer patients validated the effectiveness of the GEM chip. In contrast to the HB and GEM chip designs, the GO chip uses an EpCAM-functionalized graphene oxide nanosheet adsorbed onto a gold surface with 58,957 flower-shaped patterns to facilitate CTC capture, and it was successfully used to isolate CTCs from metastatic breast, pancreatic and early-stage lung cancer. Another microfluidic platform fabricated using non-standard methods and materials is the modular CTC sinusoidal microsystem (BioFluidica) (Kamande et al., 2013). Instead of soft lithography, the sinusoidal microsystem employs hot embossing of plastic to form the high aspect ratio (30-μm wide, 150-μm deep) sinusoidal channels characteristic of its selection module. The first of three modules comprising the microsystem, the selection module has wide inlet and outlet channels that run perpendicular to the bed of 50–320 parallel sinusoidal channels between them. The sinusoidal channels are coated with antibodies (anti-EpCAM, and/or anti-seprase/FAP alpha) to enable capture of CTCs of different phenotypes, and the 320-channel version of the platform can process a volume of 7.5 ml in <45 min. Following capture, target cells are removed from the device using a trypsin solution and fed into the second module. The second module counts the cells by measuring the electrical signal as the released cells travel through an impedance sensor. In the third module, an array of microchannels form pores that collect the target cells in an organized configuration where they are subsequently stained and imaged. The last module reduces the time required for imaging by concentrating the cells into a smaller

area, bringing the cells into a single focus plane, and organizing them into a configuration that allows cell indexing. The microsystem detected a mean of 53 CTCs/ml (range 9–95) in blood samples from patients with metastatic pancreatic ductal adenocarcinoma (PDAC) and achieved >86% purity. To further demonstrate the sensitivity of the platform, an average of 11 CTCs/ml were detected in samples acquired from patients with local resectable disease.

3.2.2.3. *Immunomagnetic*. One challenge to the use of surface-capture devices is the flexibility for downstream processing; captured CTCs are immobilized on the surface of the device and cannot be easily retrieved for further analysis. Cells captured in these devices may be released after trypsinization (Yoon et al., 2013; Sheng et al., 2014), however trypsin is very likely to cleave many surface receptors of interest for subsequent characterization. Immunomagnetic techniques, such as the macro-scale enrichment strategies discussed above, circumvent this issue by immobilizing CTCs on magnetic beads. One example of a technology that has implemented an immunomagnetic strategy on a micro-scale is the Ephesia chip. The Ephesia chip uses microcontact printing to create magnetic traps that promote the self-assembly of functionalized magnetic beads into an array of 48,000 columns within a microfluidic channel (Autebert et al., 2015; Saliba et al., 2010). The Ephesia design combines aspects of immunomagnetic sorting and micropost-based enrichment designs, while overcoming some of the limitations of previous micropost technologies. For example, the functionalized beads can be prepared in large batches, thereby reducing production costs and making the design more amenable to commercialization. Additionally, the 4.5 μm beads do not obfuscate cell imaging to the same extent as opaque 100 μm posts, and the device can be operated at a relatively high throughput of >3 ml/h. To validate use of the Ephesia chip as a CTC enrichment platform, Autebert et al. isolated CTCs from 6/8 prostate cancer and 4/5 breast cancer patient samples and compared their results to CellSearch; they achieved similar or higher CTC counts in 10/13 samples. Another microfluidic-based immunomagnetic capture technology that features a unique architecture is the Magnetic Sifter (Earhart et al., 2013). In contrast to other microfluidic designs, the Magnetic Sifter uses a vertical flow configuration that sieves the sample through a dense array of 3808 square magnetic pores (40 × 40 μm) arranged in a honeycomb pattern. This unique configuration allows high efficiency capture of CTCs at a flow rate of 10 ml/h. Earhart et al. validated the Magnetic Sifter through the detection of CTCs in 100% (6/6) of samples from non-small cell lung cancer patients with no CTCs detected in samples from healthy donors. Following capture, CTCs were lysed and tested for EGFR mutations off-chip.

Two automated, commercial systems that use microfluidic, immunomagnetic strategies for CTC enrichment include LiquidBiopsy (Cyvenio) and IsoFlux™ (Fluxion Biosciences). LiquidBiopsy uses a multilayer sheath flow to minimize nonspecific binding to magnetized surfaces (Winer-Jones et al., 2014). The fixed sample, labeled with magnetic nanoparticles, enters the microfluidic device through a central channel between two density-adjusted buffer streams. As the sample flows through the capture region of the device, a large magnetic field deflects the labeled cells from the sample layer, through the top buffer layer and to the upper glass surface where they are captured. The multilayer sheath flow approach allows high-throughput processing with sample flow rates of 5 ml/h. Studies with spiked cell lines have demonstrated the ability to capture desired cells using antibodies targeting EpCAM, Trop2, Her2, Muc1, and MelCAM with the LiquidBiopsy platform. Similar to LiquidBiopsy, Isoflux™ also uses a continuous flow process (Harb et al., 2013). The IsoFlux™ platform consists of three fluidic reservoirs (sample well, isolation region, and waste well) interconnected by microfluidic channels. The contents of the sample well are fed into the isolation region using continuous flow at a reduced velocity optimized to produce a desired residence time within the isolation region. The roof of the isolation region consists of a removable, low-adherence polymer disk below a magnet. The high magnetic field attracts cells labeled with anti-EpCAM coated magnetic beads (4.5 μm), while gravitational and flow forces bias unbound cells to continue moving through the microfluidic channel to the waste well. IsoFlux™ exhibited higher sensitivity than CellSearch® in the detection of CTCs from prostate cancer patient samples with detection rates of 95% (21/22) and 36% (8/22), respectively, for samples with >4 CTCs (CK+, CD45−, DAPI+). To further validate the clinical utility of the platform, CTCs isolated from metastatic colorectal patient samples were evaluated using qPCR for point mutations on the KRAS gene.

Finally, Janssen Diagnostics has licensed the CTC-iChip, a microfluidic immunomagnetic-based CTC enrichment technology (Karabacak et al., 2014; Ozkumur et al., 2013). The CTC-iChip allows whole blood processing using, first, a micropillar array that separates mononuclear cells from smaller blood components (i.e., red blood cells, platelets) by hydrodynamic size-based sorting. Then, the larger cells are organized into a near single-file line using inertial focusing in preparation for the third step, magnetophoresis. Here, CTCs are immunomagnetically separated from background cells using positive or negative enrichment. This strategy of using inertial focusing prior to magnetic separation facilitates the precise deflection of either CTCs or white blood cells into collection or waste streams with minimal magnetic force. In contrast to other technologies developed by the same lab (Nagrath et al., 2007; Stott et al., 2010), the CTC-iChip can process samples at a faster rate of 8 ml/h. To demonstrate the widespread utility of the device, validation studies were performed with clinical samples from lung, prostate, pancreas, breast, and melanoma using either anti-EpCAM coated microbeads (1 μm) for enrichment or anti-CD45 coated beads for depletion. Using positive enrichment, ≥0.5 CTCs/ml were detected in 90% (37/41) of samples from patients with castrate-resistant prostate cancer. Higher CTC counts were obtained for samples (metastatic prostate, breast, colorectal, and pancreas cancer) exhibiting lower CTC burdens (≤30 CTCs/7.5 ml) with the CTC-iChip than CellSearch indicating a greater sensitivity of the former. A more recent study has used a combination of anti-CD66b, a granulocyte marker, and anti-CD45 beads for improved negative depletion (Karabacak et al., 2014).

3.2.3. In vivo *positive enrichment technologies*

The GILUPI CellCollector™ (GILUPI Nanomedizin) offers a unique *in vivo* alternative for CTC capture (Saucedo-Zeni

et al., 2012). The CellCollector™ uses a structured medical Seldinger guidewire functionalized with an antibody targeting EpCAM to trap CTCs as they flow by. The wire is placed intravenously, allowing it to screen large blood volumes during the 30-min collection period. The wire successfully captured CTCs in 92% (22/24) of cancer with a median of 5.5 CTCs (range 0–50) and 16 CTCs (range 2–515) detected in breast and non-small cell lung cancer patients, respectively.

3.3. *Negative enrichment technologies*

Negative enrichment uses an indirect method to isolate CTCs: they target and remove background cells, such as leukocytes, to achieve a CTC-enriched sample. While they generally do not achieve the same high purity levels as positive enrichment, depletion methods may be preferred for some studies, as they do not bias the sample according to selection markers or apply difficult-to-remove labels. Two negative enrichment platforms that researchers have used to isolate CTCs from clinical samples include the commercialized EasySep® system (STEMCELL Technologies, Vancouver, Canada) (Liu et al., 2011) and the Quadrupole Magnetic Separator (QMS) (Lara et al., 2004; Wu et al., 2013). The EasySep™ system immunomagnetically depletes unwanted cells by first incubating samples with the EasySep™ Human CD45 Depletion kit, which contains magnetic nanoparticles and tetrameric antibody complexes targeting CD45. Next the magnetically labeled cells are separated from unlabeled cells by placing the sample-containing tube into the EasySep™ magnet, which creates a magnetic field that holds labeled cells in the tube while unlabeled cells are poured out. Using the EasySep™ human CD45 depletion kit described for CTC enrichment, Lui et al. detected CTCs in 56% (47/84) of samples from a variety of epithelial cancers and 53% (17/32) from melanoma after analysis by flow cytometry (Liu et al., 2011). In contrast to the EasySep™ batch process, the QMS is a flow-through, high-throughput magnetic cell sorter. The QMS is analogous to a magnetic flow cytometer; the sample is fed into the cylindrical separation system around a core (stream a), while a separate inlet stream forms a sheath flow between the feed stream and the cylinder wall (stream b). Four magnets surround the separation system, creating a magnetic gradient that deflects immunomagnetically-labeled cells and causes them to move from stream a to stream b. In a laminar flow regime, with no mixing, the two streams will remain separate, and each stream will exit the column independently through a flow splitter at the outlet. Using the QMS for depletion of CD45+ cells, Wu et al. demonstrated an average nucleated cell log depletion of 2.56 with 77% recovery of nucleated cells following red blood cell lysis for 120 whole blood samples collected from 71 metastatic breast cancer patients (Wu et al., 2013).

In addition to the two systems described above, many of the technologies discussed in the positive enrichment section can function as either positive or negative enrichment technologies by applying different antibodies (e.g., replacing anti-EpCAM with anti-CD45). This is particularly true for technologies using immunomagnetic separation with antibody-functionalized beads or particles, as no alterations to the devices themselves are required. Examples of platforms that have isolated CTCs through both enrichment and depletion modes include MACS (Giordano et al., 2012; Lara et al., 2006) and the CTC-iChip (Karabacak et al., 2014; Ozkumur et al., 2013). The flexibility of these devices makes them particularly adept at capturing different CTC subpopulations, especially as the CTC definition continues to evolve.

4. CTC enrichment strategies: biophysical properties

Strategies for CTC enrichment based on biophysical properties, sometimes referred to as “label-free” methods, have gained increasing popularity in the field. Unlike affinity-based methods, CTCs captured using label-free methods are not “tagged” with an antibody, which can aid in downstream processing. Additionally, selection strategies that rely on biophysical properties are not subject to the conundrum of targeting a specific antigen for enrichment. Instead, they rely on the ability to discriminate between CTCs and other cells (e.g., leukocytes) based on physical characteristics such as density, size, deformability, and electric charge.

In this section, we will first discuss the physical traits observed in CTC populations and exploited by researchers to develop label-free enrichment technologies. We will then provide an overview of both clinically validated and recently developed technologies that use these characteristics to separate CTCs from background cells.

4.1. *Size of tumor cells*

Methods predominantly used to quantify cell size include optical microscopy and flow cytometry. Optical microscopy requires that cells are placed on a two-dimensional surface for imaging, and sizes are reported as either a diameter or an area. In contrast, flow cytometry uses light scattering data to determine cross-sectional area as the laser interrogates each cell. The method used to evaluate cell size influences the results, as cells on a two-dimensional surface may have a flatter, more pancake-like, morphology than cells in flow. Thus, optical microscopy may provide more relevant data for two-dimensional microfiltration, whereas data collected using flow cytometry may be more appropriate for three-dimensional, flow-based CTC enrichment methods (e.g., 3D microfiltration, hydrodynamic chromatography) (Harouaka et al., 2013). Other factors that influence size measurements include cell health, cell cycle stage, media composition, and the fixation process.

Size-based CTC enrichment technologies function on the precedent that CTCs generally exhibit a larger morphology than leukocytes. Multiple texts have reported sizes for leukocytes and other blood cells, and Harouaka et al. provide a detailed summary of reported sizes for tumor cells and blood cells in their review (Harouaka et al., 2013). CTCs and white blood cells collected using a label-free microfluidic technology ranged from 12–25 μm and 8–14 μm in diameter, respectively (Sollier et al., 2014). Of course, the size-based collection method may have underrepresented smaller CTCs. Another study used automated digital microscopy to identify CTCs, and they reported that, on average, CTCs had a cytoplasmic area about two-fold larger than white blood cells (Cho et al., 2012). However, these observations were not statistically

significant, perhaps owing to the great variability in size among both leukocytes and CTCs. Allard et al. observed variations in CTC size ranging from 4–30 μm diameters, even among samples collected from the same patient (Allard et al., 2004). Lazar et al. reported significant differences in size for CTCs isolated from prostate cancer specimens classified as androgen receptor positive (56.42 μm^2) versus negative (95.384 μm^2) (Lazar et al., 2012). On average, CTCs originating from prostate cancer had a cell area of 89 μm^2, whereas cells from the LNCaP prostate cancer cell line had a significantly larger area (142.9 μm^2). The discrepancy in size between CTCs and cell lines further illustrates the need to validate CTC technologies with clinical samples. In addition to observing heterogeneity in size, clinical studies have also reported the presence of elongated, irregularly shaped, multinucleated, and aggregated CTCs (Allard et al., 2004). Interestingly, CTCs in aggregates (i.e., clusters, tumor microemboli) had a smaller morphology than individual, non-aggregated CTCs (Cho et al., 2012). CTCs undergoing apoptosis, or in different stages of the cell cycle, may also contribute to the observed variations in size.

4.2. *Deformability of tumor cells*

Data on the deformability of patient-derived CTCs is limited, as deformability measurements require viable cells and the majority of enrichment methods require fixed samples. However, deformability may affect the performance of some microfiltration technologies, as cells may squeeze through pores unless stiffened by chemical fixation. Deformability has been utilized as a distinguishing marker for isolating tumor cells in microfluidic platforms (Hur et al., 2011). Several studies have indicated that tumor cells exhibit greater deformability than nonmalignant cells (Cross et al., 2007; Gossett et al., 2012; Remmerbach et al., 2009), and that deformability may correspond with metastatic potential (Vazquez et al., 2015; Zhang et al., 2012). Measurements of the nuclear to cytoplasmic ratio (N/C), which may allow one to infer relative deformability, indicate that CTCs are less deformable than leukocytes; Meng et al. reported average N/C ratios of 0.8 and 0.55 for CTCs and leukocytes, respectively. Similar to size and other morphological features of CTCs, several studies have reported significant variation in N/C (Marrinucci et al., 2007, 2010). Interestingly, these differences may function as a biomarker to identify more aggressive tumors, as CTCs isolated from castrate-resistant prostate cancer patients were approximately three times more deformable than castrate-sensitive samples as measured by atomic force microscopy (Osmulski et al., 2014).

4.3. *Centrifugation*

Centrifugation is one of the first methods recorded for CTC isolation. In 1959, Seal observed that the specific gravity for red blood cells, leukocytes, and cancer cells were 1.092, 1.065, and 1.056, respectively, and he theorized that he could exploit these differences to separate these cell types using a technique that he coined silicone flotation (Seal, 1959). By blending silicone oils, he was able to reproducibly achieve a separation medium with a specific gravity of 1.075, chosen to exclude polymorphonuclear neutrophil leukocytes, which had specific gravity greater than 1.075, while allowing the lighter cancer cells and lymphocytes to "float" on the silicone surface following centrifugation. Seal isolated CTCs from 45% (39/86) of patient samples derived from various cancer types using this technique followed by a filtration step.

Today, the use of buoyancy to separate different particles based on their relative densities is called density-based gradient or isopycnic density gradient centrifugation. Although not originally developed for CTC isolation, researchers have used Ficoll-Paque® in this application. By using a reverse transcription-PCR (RT-PCR) assay to amplify cytokeratin 20 transcripts, Weitz et al. detected CTCs in 41% of patients undergoing colorectal resections with a sensitivity of 10 CTCs per 10 ml blood (Weitz et al., 1998). Designed for CTC isolation, OncoQuick® (Grenier BioOne, Frickenhausen, Germany) has combined density-based gradient centrifugation and filtration by integrating a porous barrier into the system above the separation media, which captures CTCs while allowing erythrocytes and some leukocytes to pass through. A comparative study of Ficoll-Paque® and OncoQuick® revealed a higher rate of CTC detection using the OncoQuick® system; following centrifugation, OncoQuick® resulted in a 632-fold enrichment ratio against leukocytes compared to 3.8 with Ficoll-Paque® (Rosenberg et al., 2002). While the tumor cell recovery rates for each system were similar, the increased depletion of mononuclear cells resulted in a simplified workflow for sample processing and immunocytochemical detection. Another comparative study, this time between OncoQuick and the immunomagnetic CellSearch enrichment system, concluded that CellSearch provided a more accurate and sensitive method for CTC enumeration; OncoQuick detected CTCs in 23% (14/61) of patients with metastatic cancer compared to 54% (33/61) detected with CellSearch (Balic et al., 2005). In addition to detecting CTCs in more patients, CellSearch® also detected more CTCs per sample (mean 20/7.5 ml blood) than OncoQuick® (mean 3 CTCs/7.5 ml blood). In contrast to OncoQuick, however, CellSearch and many other positive enrichment systems depend on EpCAM expression, which biases the population of captured CTCs towards EpCAM+ cells (Königsberg et al., 2011). Several clinical studies have used OncoQuick for CTC enrichment (Clawson et al., 2012; Lagoudianakis et al., 2009; Müller et al., 2005; Obermayr et al., 2010).

The RosetteSep™ CTC Enrichment Cocktail (STEMCELL Technologies, Vancouver, Canada) offers a unique method for further depletion of unwanted cells by integrating immunoaffinity-based enrichment with density centrifugation. RosetteSep™ targets unwanted cells through tetrameric antibody complexes that target an extensive mixture of antigens specialized for small-cell carcinoma and lung cancer (anti-CD36) and breast cancer samples (anti-CD56). When centrifuged over a density gradient medium, such as Ficoll-Paque or STEMCELL Technologies' Lymphoprep™, the antibody-labeled cells sink to the bottom with the red blood cells. When used in combination with Ficoll-Paque, the RosetteSep™ antibody cocktail had a higher capture efficiency (62.5%) than Ficoll alone (42.3%) (He et al., 2008). In the same study, He et al. successfully detected CTCs in 77% (10/13) peripheral blood samples from prostate cancer patients using the RosetteSep-Ficoll protocol and analysis by flow cytometry.

AccuCyte enrichment (RareCyte) offers another option for density-based CTC enrichment. Accucyte uses sequential density fractionation to isolate target cells (Campton et al., 2015). This technology has additional modules that can generate a seal between different separation layers (CyteSealer™), provide high-throughput imaging (CyteFinder™), and isolate single cells (CytePicker™).

The reliability and inexpensiveness of centrifugation have made it a widely used method for CTC isolation. However, even the most advanced centrifugation systems are limited in their ability to eliminate leukocyte contamination, resulting in purities of less than 1%. As a result, researchers commonly use centrifugation systems as an initial enrichment step prior to additional processing using other strategies.

4.4. *Microfiltration in two- and three-dimensions*

4.4.1. *Two-dimensional microfiltration systems*

Microfiltration appeared as a method for CTC isolation in 1964 when Seal, observing that CTCs exhibit a larger, more rigid phenotype than blood cells, constructed the first microfiltration setup for CTC enrichment (Seal, 1964). Since then, microfabrication methods have provided more sophisticated techniques for generating microfilters, including track-etching and soft lithography. Track-etching generates nano-to micron-sized pores in thin polycarbonate films through a combination of surface bombardment with charged particles (or irradiation) and chemical etching, and it allows decoupled control of pore size and density (Apel, 2001). Two commercial systems, ISET® (Rarecells Diagnostics) and ScreenCell® (ScreenCell), use track-etched membranes for CTC enrichment. ISET®, or 'isolation by size of epithelial tumor cells', filters fixed samples through 8-μm pores in track-etched membranes (Vona et al., 2000). The ISET® module contains 10–12 wells, each containing 0.6-cm diameter membranes, capable of processing a 1-ml volume. Multiple clinical studies have used ISET® for CTC isolation (Chinen et al., 2013; Farace et al., 2011; Hofman et al., 2011; Ilie et al., 2014; Krebs et al., 2012; Pailler et al., 2013). ScreenCell® uses circular track-etched filters with a hydrophilic surface and cylindrical 7.5 or 6.5-μm pores for filtering fixed or live samples, respectively (Desitter et al., 2011). ScreenCell offers microfiltration setups for cytological studies (ScreenCell® Cyto), live cell culture (ScreenCell® CC), and molecular biology assays (ScreenCell® MB).

Microfiltration systems designed for CTC isolation that use photolithography to construct their membranes include CellSieve™ (Creatv MicroTech) and Flexible Micro Spring Array (FMSA) microfilters. CellSieve™ microfilters are generated from a 10-μm thick layer of photoresist patterned with ~160,000 7-μm pores per 9-mm diameter filter (Adams et al., 2014b). Using CellSieve™, Adams et al. detected CTCs in 100% (10/10) of 7.5-ml samples from metastatic cancer patients with a mean of 56 CTCs per sample (range 12–120). In addition to CTC detection, researchers have also used CellSieve™ to detect cancer-associated, macrophage-like cells in the exploration of their use as a novel tumor biomarker (Adams et al., 2014a). In contrast to CellSieve™, the FMSA is constructed from the biocompatible polymer parylene-C. Both platforms are designed to minimize cell damage and preserve the viability of captured cells. The FMSA achieves the latter goal through the use of flexible micro spring structures (Harouaka et al., 2014). These finger-like structures allow CTC enrichment based on size and deformability, while also mitigating the force experienced by cells trapped in the microfilter. In contrast to other microfiltration techniques, the FMSA can filter whole blood without preprocessing (e.g., dilution, erythrocyte lysis, centrifugation) due to its high porosity. Validation of the FMSA's capability included detection of CTCs in 76% (16/21) of samples from breast, colorectal, and lung cancer patients from whole blood (7.5 ml). In comparison, CellSearch detected CTCs in 22% (4/18) of samples. The higher rate of CTC detection using the FMSA compared to CellSearch was corroborated by another recent clinical study (Kaifi et al., 2015).

4.4.2. *Three-dimensional microfiltration systems*

The FaCTChecker (Circulogix) microfilter consists of two porous parylene-C layers; the bottom layer contains hexagonally-arranged 8-μm pores, and the top layer contains larger 40-μm pores that align with the corresponding hexagon patterns on the bottom membrane (Zhou et al., 2014). Although fabricated in a manner similar to the FMSA above, the FaCTChecker microfilter uses a three-dimensional architecture for cell capture. Large pores in the top membrane allow CTCs to easily pass through and become trapped in the 10-μm gap between the top and bottom layers. The two membranes can then be separated to access captured cells. Similar to the FMSA microfilter, the unique design of the FaCTChecker preserves cell viability by reducing the mechanical stress experienced by captured CTCs. The Parsortix system (ANGLE) provides another example of a three-dimensional microfiltration system, but, unlike the FaCTChecker microfilter, it uses a horizontal configuration as opposed to a vertical one. The Parsortix microdevice has a stair-like architecture that gradually decreases the channel width to ≤10 μm. CTCs larger than the channel width become lodged in the gap, while smaller cells pass through. After CTC capture, flow in the opposite direction releases captured CTCs for harvesting and subsequent molecular characterization. The Resettable Cell Trap (RCT) uses a strategy similar to Parsortix, but, instead of decreasing the channel height, the microdevice uses pneumatically-controlled microvalves to alter the aperture of the flow channel and entrap CTCs in 'pockets' with a height greater than the main channel (Qin et al., 2015). Relaxation of the microvalve causes an increase in the aperture and subsequent release of captured CTCs.

Sarioglu et al. recently reported the development of a novel three-dimensional microfiltration system specifically designed to capture CTC clusters called the Cluster-Chip (Sarioglu et al., 2015). Multiple studies using microfiltration for CTC isolation have reported capturing CTC clusters (aka microemboli) (Desitter et al., 2011; Harouaka et al., 2014; Ilie et al., 2014; Vona et al., 2000). These reports indicate that CTC clusters stay intact when isolated with microfiltration methods, whereas other enrichment strategies may either fail to capture clusters or break them apart. The simple, yet sophisticated, design of the Cluster-Chip captures these circulating tumor microemboli using multiple rows of shifted triangular pillars. The most basic subunit, referred to as a 'cluster trap,' of the design consists of three triangular pillars: two side-by-side pillars create a funnel that terminates at the point of the third triangle. The third triangle functions to bifurcate the fluid flow into two 12-μm wide gaps on either

side of its point. While individual CTCs can pass through these gaps, the bifurcating flow retains clusters as small as two-cells by generating a dynamic force balance between the two fluid streams, the third triangular pillar, and the cell–cell junctions holding the cluster together. To ensure that clusters do not dissociate, the device uses flow velocities well below those generated in human capillaries. Similar to many of the microfluidic devices discussed in the positive enrichment section, and in contrast to traditional filtration systems, the Cluster-Chip has a low throughput rate of 2.5 ml/h. To confirm the utility of the Cluster-Chip, CTC clusters were isolated from patient blood samples. Clusters were identified in 41% (11/27) of patients with breast cancer, 30% (6/20) of patients with melanoma, and 31% (4/13) of patients with prostate cancer. Additional immunocytochemical and molecular analyses were performed on the clusters to identify intra-tumor cell heterogeneity and the presence of adherent leukocytes.

In general, microfiltration allows rapid processing of blood for CTC enrichment. However, these systems are prone to clogging and some setups require parallel processing with multiple filters for large volumes (>1.5 ml). The overlap in size distributions between leukocytes and CTCs makes it difficult to achieve high purity levels with microfiltration and typical capture purities are less than 10%.

4.5. *Inertial focusing*

Inertial focusing passively separates CTCs from other blood cells based on size through the application of inertial effects in microfluidic devices using two forces, i) a shear-gradient lift force, arising from the parabolic profile characteristic of laminar flow, that directs particles towards the channel walls, and ii) a wall effect lift force that directs particles away from the wall (Di Carlo, 2009). Magnitude and direction of these lift forces are governed by channel dimensions, channel aspect ratio, flow rate, and particle diameter. The CTC enrichment platform, Vortex, uses inertial focusing to position cells along channel walls upstream of micro vortices designed to stably trap CTCs (Sollier et al., 2014). By using rectangular, high aspect ratio channels, the equilibrium positions are reduced to two positions centered along the long face of the channel. The channel length allows cells from the blood sample to naturally migrate to equilibrium positions along the channel walls before reaching the first of eight expanding reservoirs. The sudden expansion causes the wall lift force to become negligible, as the force decays with distance from the wall, and the shear-gradient lift force dominates. Larger particles, in this case CTCs, experience a larger lateral force than their counterparts, causing them to enter the reservoirs and orbit stably in microvortices, while smaller blood cells pass by them in the main stream. Trapped CTCs remain in the device until flushed out by perfusing the device with buffer at a decreased flow rate. The Vortex Chip, which runs eight of the channels described above in parallel, can process 7.5 ml of whole blood in 20 min, and it achieves comparable capture efficiencies with and without red blood cell lysis. Using Vortex, CTCs were successfully isolated from clinical samples with limited leukocyte contamination (57–94% purity); CTCs were detected in 12/12 samples (4 breast and 8 lung cancer) and ≥5 CTCs from 9/12 samples.

ClearCell® FX (Clearbridge Biomedics) combines the inertial migration of particles with secondary flow from curved channels to isolate CTCs (Hou et al., 2013; Warkiani et al., 2014). In curved channels, a secondary "Dean's" flow arises as a consequence of differences in flow velocity between the center and walls of the channel. The combination of inertial lift forces and Dean's flow allows precise positioning of cells within the channel. The ClearCell® FX spiral microfluidic channel has a trapezoidal cross-section that improves separation resolution; larger CTCs are positioned along the shorter, inner channel wall, while smaller blood cells are positioned along the taller, outer wall. The spiral bifurcates at the end into "inner" and "outer" collection outlets for CTCs and white blood cells, respectively. The ClearCell® FX chip can process a 7.5 ml sample in 8 min, but it requires red blood cell lysis prior to enrichment. To validate the utility of the device, CTCs were isolated from 10/10 patient samples with advanced metastatic breast and lung cancer (range 3–125 CTCs/ml). In a subsequent clinical study, a multiplexed version of the spiral device was used to detect CTCs in patient samples with an improved processing rate of <5 min per 7.5 ml sample (Khoo et al., 2014). The value associated with capturing viable CTCs was further emphasized in a clinical study that used short-term expansion of CTCs isolated using ClearCell® FX to test anti-cancer therapies (Khoo et al., 2015).

Inertial focusing exerts minimal stress on captured cells and allows recovery of viable cells. In addition, these methods do not require complex, high-resolution features or modified surface chemistries – a boon for low-cost, mass-production. Emerging technologies in this area offer exciting opportunities for use in CTC isolation (Zhou et al., 2013).

4.6. *Dielectrophoresis*

An innovative approach to cell separation, dielectrophoresis (DEP) exploits the distinct electrical fingerprints of different cells, which depend on the composition (e.g., cell membrane, nucleus, organelles), morphology (e.g., size, shape), and phenotype of the cell (Becker et al., 1995; Stoy et al., 1982). During DEP, an attractive or repulsive force is exerted on a particle polarized by the presence of a nearby, nonuniform electric field. The electric field can apply a positive (pDEP) or negative (nDEP) force on the particle, causing it to move towards or away from the electrical field source, respectively. The crossover frequency, defined as the frequency where the DEP force transitions from nDEP to pDEP, depends on the conductivity of both the cell and its surrounding medium. DEP can be applied to cell separation via two distinct strategies: DEP migration and retention. In the first strategy, the electrical field pushes cells in opposite directions by applying opposing forces on them, which is achieved by applying a voltage signal at a frequency between the crossover frequencies of the two cell populations. ApoStream® (ApoCell), a commercial system for CTC enrichment, applies the first strategy through the use of dielectrophoretic field-flow fractionation (DEP-FFF) (Gupta et al., 2012). The sample injection port introduces cells at the bottom of the flow chamber upstream of the buffer. Electrodes line the bottom of the flow chamber and generate an electric field that attracts CTCs (pDEP) to the chamber flow and repels leukocytes (nDEP) towards the center of the channel. After passing through the applied electric field, CTCs are collected

through a collection port in the bottom of the chamber, while the leukocytes exit the chamber through an outlet opposite the buffer inlet. Studies have validated the ability to isolate CTCs from clinical samples using the ApoStream® technology (Shim et al., 2013). The continuous flow design allowed processing of 10 ml samples in less than one hour following a pre-processing centrifugation step.

The commercial technology DEPArray™ (Silicon Biosystems) applies the second DEP strategy, retention, by trapping single cells in DEP cages generated via an array of individually controllable electrodes (Manaresi et al., 2003). DEPArray™ uses nDEP forces to levitate cells, thereby reducing cell adhesion to surfaces, and the DEP cages are formed by generating an electric field above associated electrodes that is in counter phase with the electric field of adjacent electrodes. DEPArray™ is designed for single-cell recovery and not bulk enrichment of CTCs. Multiple clinical studies have used DEPArray™ to recover single CTCs for subsequent genetic analyses following enrichment using centrifugation or immunoaffinity (i.e., CellSearch) (Carpenter et al., 2014; Fabbri et al., 2013; Fernandez et al., 2014; Peeters et al., 2013; Polzer et al., 2014). The use of DEPArray™ technology for CTC recovery will likely be limited to samples with a relatively high number of CTCs (e.g., metastatic carcinomas) due to a cell-loss of approximately 40% during sample loading (Peeters et al., 2013).

5. Direct imaging modalities

All of the enrichment technologies discussed above require subsequent verification of the identity of captured cells. In general, this verification is performed through the use of high resolution imaging with DAPI, CK, and CD45 immunostaining, where CTCs are defined as DAPI+/CK+/CD45−. Although enrichment reduces the total number of cells under investigation, sample imaging remains a time-consuming task. For this reason, researchers have begun developing technologies to improve the efficiency of imaging following enrichment, and some commercial systems, such as CellSearch, DEPArray, and the Modular CTC Sinusoidal Microsystem have incorporated automated high-resolution fluorescence imaging into their workflow. A stand-alone technology, the microfluidic cell concentrator (MCC) uses passive pumping between small transport channels and a larger collection ring to concentrate samples by ~ 5x with minimal cell loss (Casavant et al., 2013). The MCC represents one method for reducing imaging time by concentrating the sample and thereby limiting the area screened. Another strategy to reduce the time required for imaging CTC samples post-enrichment is to develop faster imaging technologies. ImageStream® combines classical flow cytometry with fluorescence imaging to allow high throughput, multiparameter cell analyses (Zuba-Surma and Ratajczak, 2011). A comparative study between CellSearch® and ImageStream® (with immunomagnetic enrichment using MACS) revealed no significant differences in tumor cell enumeration between the two imaging platforms based on studies with spiked samples (López-Riquelme et al., 2013). However, a lower level of precision was reported for low cell counts (1–10 tumor cells) when using ImageStream®. Since then, Amnis has released an upgraded version of ImageStream® that can analyze 5000 cells/sec; a rate 5x faster than reported with the previous model. Additional studies will be needed to ascertain if the new model has improved precision enough to detect CTCs at low concentrations.

5.1. *Enrichment-free imaging methods*

Several imaging platforms have foregone the enrichment step all together through advancements in high-speed, multiparameter, fluorescence imaging. Epic Sciences' enrichment-free CTC detection platform uses HD-CTC imaging, in combination with custom computer algorithms, to screen a monolayer of three million nucleated cells spread on custom glass slides (Nieva et al., 2012). In one clinical study, the platform detected CTCs in 68% (45/66) of blood samples from patients with non-small cell lung cancer. In a separate study, ≥5 CTCs/ml were detected in 80% (24/30), 70% (14/20), and 50% (9/18) of blood specimens from patients with metastatic prostate, breast, and pancreatic cancer, respectively (Marrinucci et al., 2012). The study also compared detection rates with the CellSearch® assay in samples from 15 metastatic cancer patients. Epic's system detected significantly higher CTC numbers and exhibited greater sensitivity than CellSearch; Epic identified ≥2 CTCs/7.5 ml sample in 14/15 samples compared to 5/15 with CellSearch®.

Two additional CTC detection platforms that rely solely on imaging include FASTcell™ (SRI International) and CytoTrack. The fiber-optic array scanning technology (FAST™) cytometer central to the FASTcell™ platform uses an array of optical fibers to form a wide collection aperture that provides a much larger field-of-view than traditional optical systems (Krivacic et al., 2004). In addition, the use of a laser light source and a sensitive photomultiplier detector reduce the exposure time necessary for imaging. The FAST™ cytometer can scan a sample-containing glass slide at a rate of 25M cells/min (Das et al., 2012). However, the trade-off for the increased field of view, which allows such high-speed imaging, is a decrease in image resolution, and subsequent verification of potential CTCs must be performed using high resolution imaging with an automated digital microscope after the initial screening. Clinical studies applying FAST™ have successfully identified ≥1 CTCs in 82% (18/22) of breast cancer specimens and ≥2 CTCs in 42% (24/57) of non-small cell lung cancer specimens, and they have used additional immunostaining to measure HER2 and/or ERCC1 expression (Das et al., 2012; Somlo et al., 2011).

Similar to FASTcell™, the CytoTrack system pre-screens samples at high rates and records potential CTC targets for additional image analysis and verification. CytoTrack uses a special glass disc (CytoDisc™) with a much larger area than typical microscope slides, which allows it to accommodate a monolayer of 100 million cells. The CytoDisc™ is placed in a scanner that spins the disc at high velocities, similar to CD/DVD players, and uses a laser system to scan the entire disc surface. The innovative design of CytoTrack allows screening of 100M cells/min. To complement their technology, CytoTrack has incorporated a pipette system, the CytoPicker™, to allow the retrieval of single cells for subsequent analyses into their scanner. Spiking studies performed with the EpCAM+/CK+ breast cancer cell line MCF-7 demonstrated similar capture efficiencies between CytoTrack (69%) and

CellSearch® (71%) (Hillig et al., 2015). To date, no clinical studies applying CytoTrack's technology have been published.

Another unique, label-free method to detect CTCs is photoacoustic flow cytometry (PAFC) (Galanzha and Zharov, 2013). PAFC uses laser-based technology for real-time detection of CTCs in veins by interrogating the blood flow through the skin. Cells within the blood flow absorb the laser radiation, which causes an increase in temperature and subsequent generation of acoustic waves detected using ultrasound placed near the vein. PAFC can be performed *in vivo*, thus allowing the interrogation of large blood volumes not accessible using *ex vivo* techniques.

5.2. *Functional assays*

Functional assays exploit aspects of live cellular activity for CTC enrichment and isolation. Technologies that utilize functional aspects of CTCs such as the Epithelial ImmunoSPOT Assay (EPISPOT), which captures CTCs based on specific secreted/released/shed tumor-associated proteins, has been validated in several different cancers (Alix-Panabières, 2012; Alix-Panabières and Pantel, 2015; Cayrefourcq et al., 2015; Ramirez et al., 2013). Another functional assay, Vita-Assay™ (Vitatex) that exploits the preferential adhesion of invasive CTCs to specialized matrix has also been tested in metastatic prostate (Friedlander et al., 2014) and breast cancer (Lu et al., 2010). These assays are discussed in greater detail in another review in this series.

5.2.1. *Perspectives*

The recent explosion in the field of CTC biology is reflected in the myriad of CTC technologies developed within the last two decades. New technologies have arisen to address new challenges as our understanding of CTC biology evolves. Even now, entirely new approaches to CTC isolation are being developed; for example, a novel microfluidic approach that uses acoustic waves for size-based separation of CTCs has recently been reported (Antfolk et al., 2015a, 2015b; Li et al., 2015). There is no one-size-fits-all technology for CTC studies, and the appropriateness of any given technology should take into consideration both the type of cancer being studied and the desired downstream analyses. For example, immunofluorescence and FISH will require a greater capture efficiency, whereas genetic analyses will need to emphasize purity, and drug efficacy testing will necessitate retrieval of viable CTCs. Differences in sensitivity, specificity, and detection limits will have a major impact on the results of CTC detection studies. Therefore, it is important to develop performance metrics that can allow researchers to evaluate and compare different technologies. The significance of these metrics, however, is diminished by the lack of an appropriate model system, as the use of cancer cell lines overestimate device performance. Thus, it is also important to validate systems using clinical samples.

Understanding the inherent biases of different enrichment methods, which are not captured by performance metrics, will become more important as the significance of different CTC subpopulations emerge. Despite the success of CellSearch® and other affinity-based technologies, antigen-free approaches are finding more favor as researchers continue to discover even greater heterogeneity in the immunohistologic profile of CTCs. Namely, researchers have identified CTCs with down-regulation of epithelial marker expression characteristic of cells undergoing EMT (Bednarz et al., 2010; Joosse et al., 2012; Lawson et al., 2015; Polyak and Weinberg, 2009). Although normally required for embryogenesis and wound healing, EMT facilitates metastasis when hijacked by disseminating cancer cells (Liu et al., 2014; Thiery, 2003). EMT imparts capabilities typically reserved for tissue remodeling on cancer cells, thus allowing them to overcome cell–cell adhesions promoted by epithelial proteins and stimulating the motility and invasiveness characteristic of mesenchymal cells. The subsequent down regulation of epithelial proteins, such as EpCAM and CK, allows CTCs to escape capture by positive enrichment systems that target these proteins (Gorges et al., 2012; Königsberg et al., 2011). The successful formation of metastases from a primary tumor site implies a need for both migratory and self-renewing capabilities in the pioneering cells. A study by Mani et al. indicates that cells undergoing EMT acquire many of the properties of self-renewing stem cells characteristic of tumor-initiating cells (TIC, aka cancer stem cells, CSCs) (Mani et al., 2008), and studies have found enrichment of TIC populations among CTCs that exhibit mesenchymal characteristics (Aktas et al., 2009). The importance of EMT and TIC subpopulations in the development of metastatic disease indicates that targeting CTCs shrouded by a mesenchymal phenotype could have significant clinical impact. This importance is elevated by studies demonstrating a higher resistance to chemo- and targeted therapy in both mesenchymal CTCs (Aktas et al., 2009; Liu et al., 2014; Mitra et al., 2015; Polyak and Weinberg, 2009) and TICs (Dean et al., 2005). These findings have motivated the development of novel technologies that do not rely on epithelial markers for CTC capture. However, despite the ability to recognize and isolate CTCs exhibiting the attributes of EMT, final visual verification of CTC identity still relies on the conventional CK+/DAPI+/CD45− definition. Interestingly, other epithelial cell types such as cancer-associated fibroblasts (CAFs) have also been found in the blood of patients with metastatic breast cancer (Ao et al., 2015).

The presence of CTC clusters, or microemboli, in clinical samples is another biological observation that has motivated new enrichment strategies, including the CTC-Cluster Chip. The cells comprising clusters often co-express epithelial and mesenchymal markers, indicating a hybrid or partial EMT status that may aid in cluster formation (Jolly et al., 2015; Yu et al., 2013). Observed as early as 1959, CTC clusters have recently gotten the attention of researchers due to their ability to resist apoptosis and form metastases at significantly higher rates than individual CTCs (Aceto et al., 2014). CTC technologies that use high flow rates or tightly-packed structures may fail to capture or break apart clusters. For instance, Stott et al. discussed the finding that the Herringbone Chip isolated clusters of CTCs in contrast to previous observations using the lab's earlier CTC-Chip technology (Stott et al., 2010).

While clinical studies using CellSearch® and other CTC technologies have affirmed that CTC enumeration provides relevant prognostic information, the potential for CTC biology to transform cancer management remains largely untapped. While a study by Heitzer et al. initially found genetic mutations exclusive to CTCs, additional deep sequencing revealed that subclonal populations harbored the same mutations in primary tumors and metastases (Heitzer et al., 2013). Several

other studies have reported discordances in the expression of cancer-specific antigens, such as HER2, between CTCs and the primary tumor (Galletti et al., 2014; Kalinsky et al., 2015; Pestrin et al., 2009). Together, these findings indicate that liquid biopsies may exhibit a much broader spectrum of genetic permutations, which could aid in therapy selection, than conventional primary tumor biopsies.

Another exciting clinical application for CTC technologies is early detection. Currently, the majority of published studies use samples from metastatic or late-stage patients as they have a higher CTC burden. However, Illie et al. laid the foundation for early detection with CTCs in a recent study (Ilie et al., 2014), where samples collected from patients with chronic obstructive pulmonary disorder (COPD), a known risk factor for lung cancer, were tested for the presence of CTCs. They discovered CTCs in five patients who were screened yearly for lung cancer using CT-scans. Within 1–4 years after CTC detection all five patients developed lung nodules and underwent prompt surgical resection. Follow-up studies conducted one year post-surgery showed no tumor recurrence. Current medical procedures can often cure early-stage disease, thus using CTCs as a sentinel of tumor development could save patient lives – especially in asymptomatic cancers for which no routine screening methods are available.

The clinical utility of liquid biopsies will rely on CTC technologies to reach fruition. The next generation of technologies will continue to rise to the challenges presented by our ever-changing understanding of CTCs and their role in transforming clinical care for cancer patients.

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