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# Circulating Tumor Cell Isolation, Culture, and Downstream Molecular Analysis

**Sandhya Sharma**[1,2], **Rachel Zhuang**[2,3], **Marisa Long**[2], **Mirjana Pavlovic**[1], **Yunqing Kang**[4,5], **Azhar Ilyas**[6], and **Waseem Asghar**[1,2,7,*]

[1]Department of Computer & Electrical Engineering and Computer Science, Florida Atlantic University, Boca Raton, FL 33431

[2]Asghar-Lab, Micro and Nanotechnology for Medicine, College of Engineering and Computer Science, Boca Raton, FL 33431

[4]Department of Ocean & Mechanical Engineering, College of Engineering & Computer Science, Florida Atlantic University, Boca Raton, FL 33431, USA

[5]Department of Biomedical Science, College of Medicine, Florida Atlantic University, Boca Raton, FL 33431, USA

[6]Department of Electrical & Computer Engineering, New York Institute of Technology, Old Westbury, New York, USA

[7]Department of Biological Sciences, Florida Atlantic University, Boca Raton, FL 33431

## Abstract

Circulating tumor cells (CTCs) are a major contributor of cancer metastases and hold a promising prognostic significance in cancer detection. Performing functional and molecular characterization of CTCs provides in-depth knowledge about this lethal disease. Researchers are making efforts to design devices and develop assays for enumeration of CTCs with a high capture and detection efficiency from whole blood of cancer patients. The existing and on-going research on CTC isolation methods has revealed cell characteristics which are helpful in cancer monitoring and designing of targeted cancer treatments. In this review paper, a brief summary of existing CTC isolation methods is presented. We also discuss methods of detaching CTC from functionalized surfaces (functional assays/devices) and their further use for *ex-vivo* culturing that aid in studies regarding molecular properties that encourage metastatic seeding. In the clinical applications section, we discuss a number of cases that CTCs can play a key role for monitoring metastases, drug treatment response, and heterogeneity profiling regarding biomarkers and gene expression studies that bring treatment design further towards personalized medicine.

*Corresponding Author wasghar@fau.edu.

[3]School of Medicine, Case Western Reserve University, Cleveland, OH, USA 44106-7078 (present address)



**Conflict of Interest Statement**

The authors declare no conflict of interests.

**Keywords**

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## 1. Introduction

According to the Centers for Disease and Control Prevention (CDC), cancer records the second highest reason of death worldwide, with more than 90% of deaths being caused by cancer cell metastasis (Hong and Zu, 2013; Wicha and Hayes, 2011). This is largely due to the fact that malignant tumors have the ability to shed tumor cells that invade surrounding tissue and enter the lymphatic and circulatory systems (Asghar et al., 2012b). These cells, known as circulating tumor cells (CTCs), ultimately establish new metastasis at other tissue and organ sites throughout the body (Chaffer and Weinberg, 2011; Harouaka et al., 2014; Maheswaran and Haber, 2010; Wan et al., 2011).

CTCs were first discovered more than hundred years ago by Thomas Asworth (Ashworth, 1869). Since then, many studies have focused on discovering efficient CTC detection and isolation techniques, with the prospects of using CTCs as a 'liquid biopsy' for peripheral blood analyses and an early biomarker for response to systemic therapies (Alix-Panabières and Pantel, 2013; Diaz and Bardelli, 2014; Lianidou, Evi S., 2014; Wan et al., 2012; Wang et al., 2013). However, a number of challenges are associated with CTC isolation, detection, and downstream analysis. CTCs are sparse, approximately 1–100 cells can be found per milliliter of blood, along with $10^6$–$10^7$ red blood cells (Asghar et al., 2013; Hafeez et al., 2012; Ilyas et al., 2014a; Ilyas et al., 2014b; Miller et al., 2009). Increasing blood sample volumes is a possible resolution that provides more accurate measurements, but comes with its own time constraints and patient care challenges. CTC heterogeneity is another major obstacle, as various groups of CTCs have significant variations in surface expression of biomarkers (Attard and de Bono, 2011; Ignatiadis and Dawson, 2014). Currently, CellSearch, the exclusively US-FDA (Food and Drug Administration) cleared device for CTC detection is a prognostic indicator for breast, prostate and colorectal cancer (Krebs et al., 2011; Sieuwerts et al., 2009). A great clinical need still exists for low-cost, non-invasive, and efficient CTC detection and isolation devices. Herein, we review the most promising CTC isolation methods and apply more focus on future directions of CTC technology (Figure 1). The different isolation methods are categorized on the basis of specific CTC characteristics such as physical properties (size, elasticity, surface charge) (Asghar et al., 2012b; Gascoyne et al., 2009; Moon et al., 2011; Müller et al., 2005; Vona et al., 2000; Zheng et al., 2011), biological characteristics such as cellular function (Alix-Panabières, 2012; Lu et al., 2010) and the expression of tumor-specific surface proteins (Allard et al., 2004; Helzer et al., 2009; Lu et al., 2013b; McKeown and Sarosi, 2013; Riethdorf et al., 2007; Stott et al., 2010; Talasaz et al., 2009). After isolating CTCs from patient samples, releasing CTCs from the capturing substrate presents with challenges. The successful detachment of CTCs is an important step for establishing *ex-vivo* CTC cultures and obtaining morphological information. In this paper, we review downstream processing steps, describing CTC release from substrate with the use of various enzymatic actions, aptamers

and polymers. Protocols and success rates for culturing CTCs from cancer patients demonstrating heterogeneous CTC morphological properties are also discussed, and a description of *ex-vivo* CTC culturing under various cell culture conditions for disease model development is provided. Moreover, the clinical aspects of CTCs are described, and examples of how CTCs can participate in monitoring metastasis and drug therapy responses are discussed.

## 2. CTC Isolation Methods

Since the discovery of CTCs, several isolation techniques have been developed. However, these techniques are often limited by the presence of extremely low number of CTCs in patient blood (1–100 cells per mL), as well as their fragile and heterogeneous nature (Alix-Panabières and Pantel, 2013; Zheng et al., 2013). CTC fragility becomes a concern when the cells need to be detached from the various chips and membranes that are used to isolate them. We discuss detachment after introducing the major CTC isolation methods developed thus far. Most of the existing technologies consist of a two-step process of cell enrichment and subsequent detection. Cell enrichment involves capturing CTCs based on their physical properties, including size, elasticity, density, and charge (Gascoyne et al., 2009; Moon et al., 2011; Müller et al., 2005; Vona et al., 2000; Zheng et al., 2011), and various biological characteristics, such as cellular functions (Alix-Panabières, 2012) and tumor-specific surface proteins (Allard et al., 2004; Helzer et al., 2009; Lu et al., 2013b; McKeown and Sarosi, 2013; Riethdorf et al., 2007; Stott et al., 2010; Talasaz et al., 2009). Detection methods then allow for single-cell level specificity when counting CTCs and further separating them from normal blood cells. These detection methods include visual microscopy, immunostaining, biomechanical discrimination and polymerase chain reaction (PCR) (Alix-Panabières and Pantel, 2013).

### 2.1 Physical Property-Based Assays

Enrichment via physical properties, such as size and membrane capacitance, allows one to isolate CTCs quickly without labeling (Kim et al., 2016). Unfortunately, these techniques present certain limitations, as current technologies lack specificity and yield less pure results than functional assays due to cell heterogeneity (Hong and Zu, 2013; Wang et al., 2013). Dielectrophoretic field-flow fractionation (DEP-FFF) employs separation by size and polarizability using membrane capacitance and can process 30 million cells within 30 min with high recovery rates. However, it requires very specific parameters such as cell type and electric field frequency (Gascoyne et al., 2009; Zieglschmid et al., 2005). Metacell filtraction device, isolation by size of epithelial tumor cells (ISET), ScreenCellCyto, and dead flow fractionation techniques all use size to select for CTCs (De Giorgi et al., 2010; Dolfus et al., 2015; Hou et al., 2013; Vona et al., 2004; Wang et al., 2013). With the exception of Metacell, these size-based techniques quickly isolate CTCs, which are usually larger in size than other blood cells, but fail to enrich smaller CTCs and those with similar deformability to leukocytes (Dolfus et al., 2015; Joosse et al., 2015; Zheng et al., 2011). It is also difficult to release the captured CTCs from porous membranes for downstream analyses. To overcome this challenge, a Parsotrix method is developed which is a size-based selection method that involves a cassette device for collecting CTCs that are readily available for

subsequent studies, overcoming the detachment limitation (Joosse et al., 2015). In summary, size-based CTC isolation methods provide high throughput, however these methods find limited applicability in clinical settings due to heterogeneity of CTCs in term of their size.

### 2.2 Functional Assays

Functional assays to detect only viable CTCs may overcome some of the limitations of physical heterogeneity. However, current CTC methods based on cell functional properties face issues regarding product purity. These include analyzing CD45 protein levels and collagen adhesion matrix (CAM) removal and uptake, using the EPISPOT assay (Epithelial Immunospot) (Alix-Panabières, 2012) and CAM assay (Vita Assay) (Lu et al., 2010), respectively. The CAM assay measures CTC invasiveness via CAM protein uptake. It produces results with high sensitivity and specificity, but requires over 12 hours for isolation and may fail to isolate more heterogeneous cells due to its biomarker dependence (Monteiro-Riviere et al., 2009). CD45 is a common leukocyte antigen used during the EPISPOT assay to remove leukocytes via CD45 depletion. The assay then detects proteins released by or shed from CTCs during short-term cultures (Alix-Panabières, 2012). Though a very promising technique, problems in EPISPOT detection arise when antigen levels are lower or binding efficiency is reduced (Kalyuzhny, 2005).

Elevated telomerase activity has been associated with malignancy in most cancer types (Blackburn, 2000) and can be used to detect CTCs via a telomerase-specific replication selective adenovirus in a method known as TelomeScan (Kojima et al., 2009). The virus replicates in cancer cells only and marks them with green fluorescence protein (GFP). However, in a breast cancer clinical trials, there are significant discrepancies between CellSearch detection and TelomeScan detection. A probable cause of these differences may be that TelomeScan may more effectively detect epithelial to mesenchymal transition (EMT) or EpCAM negative tumor cells, but also detect hematopoietic stem cells for false-positive results (Kim et al., 2011).

Another method that reportedly overcomes limitations of cell size heterogeneity involves generating nanoroughess on glass surfaces via reactive ion etching (RIE). RIE is then combined with photolithography to make specific patterns on the glass surface that favor CTC attachment to normal cell attachment on the basis of adhesion preferences (Chen et al., 2012b). This is primarily due to focal adhesion or Fanconi Anemia (FA) density. FA protein is responsible for DNA damage repair (Nalepa et al., 2013). Although nanorough glass surfaces show adhesion towards the cancer cells, these surfaces provide low CTC capture purity due to significant nonspecific binding of other blood cells.

### 2.3 Immunobead Assays

The most common methods of CTC enrichment involve immunobead assays and microdevices. Immunobead assays use either positive selection to target tumor-associated biomarkers or negative selection to remove blood cells with common leukocyte biomarkers. Epithelial cell adhesion molecules (EpCAM) are often used in positive selection, and CD45 for negative selection. Magnetic beads functionalized with antibodies specifically attach to these antigens, and cells are subsequently removed by applying a magnetic field. CellSearch,

an immunobead assay that uses anti-EpCAM antibodies, is currently the only US FDA approved device for CTC detection in breast, prostate and colorectal cancer (Harouaka et al., 2014; Kim et al., 2012). For this reason, it is commonly used as a standard for other detection and enrichment methods (Beije et al., 2015). Other popular EpCAM targeting assays include MagSweeper (Talasaz et al., 2009), AdnaTest (Zieglschmid et al., 2005), and IsoFlux (Harb et al., 2013). These immunobead assays provide more pure yields, but require longer sample processing times than size-based enrichment methods (Hong and Zu, 2013). Furthermore, CTCs that undergo EMT are EpCAM negative and are not isolated by anti-EpCAM assays. Negative selection is a one possible solution. For negative selection, CD45 depletion is most commonly used and often in-conjunction with other label-independent techniques such as red blood cell lysis. However, these processes may also result in the unintended removal of less conventional CTCs (Lustberg et al., 2012) that express CD45, resulting in underestimates of CTC numbers (Joosse et al., 2015; Lustberg et al., 2012).

### 2.4 Microdevices and Microfluidic Platforms

Microdevices and microfluidic platforms have revolutionized the healthcare system and found tremendous applications including biological and chemical analysis, fertility analysis, cell sorting, point-of-care diagnosis, infectious disease diagnostics, DNA sequencing, and tissue engineering (Asghar et al., 2015; Asghar et al., 2011a; Asghar et al., 2011b; Asghar et al., 2012a; Asghar et al., 2010; Asghar et al., 2016a; Asghar et al., 2014; Asghar et al., 2016b; Billo et al., 2011; Coarsey et al., 2017; Fennell and Asghar, 2017; Ilyas et al., 2013; Islam et al., 2014; Kanakasabapathy et al., 2017; Rappa et al., 2016; Safavieh et al., 2017; Shafiee et al., 2015; Sher et al., 2017; Vidyala et al., 2011; Yu et al., 2017). These microdevices can be functionalized with various antibody “cocktails” in addition to standard anti-EpCAM proteins, enabling more precise isolation of CTCs. Common biomarkers targeted in CTC isolation include epithelial cell membrane antigens that are relevant to cancer therapy, epithelial growth factor receptor (EGFR) and human epidermal folate binding protein receptor, mucin-1 (MUCI), TROP-2, growth factor receptor 2 (HER2 or EGFR/ERBB2), and mesenchymal stem cell antigens (CD318, N-cadherin and c-MET) (Pecot et al., 2011).

One of the microdevices developed, the CTC-Chip, consists of microposts coated with anti-EpCAM antibodies (McKeown and Sarosi, 2013; Stott et al., 2010). The original chip has now been developed into one with herringbone groove patterns known as the Herringbone (HB) CTC-Chip. With the new design, blood-flow within the channels becomes less streamlined and capture efficiency increases as more CTCs contact antibodies lining the chip’s inner walls. Though cell viability after CTC-Chip and HB-Chip is often compromised, the HB-Chip provides more comprehensive data, primarily through its capture of CTC clusters, and may become important tool for studying tumor metastasis (Nagrath et al., 2007; Sarioglu et al., 2015). A new Ephesia CTC-Chip combines immunobead technology with microfluidics. The beads self-assemble onto a series of magnetic traps for rapid isolation and enumeration of CTCs at 90–94% capture efficiencies and non-specific capture rates below 0.4%. Current challenges involve increasing the processing capacity of larger sample volumes (Alix-Panabières et al., 2012; Karabacak et al., 2014; Saliba et al., 2010).

The CTC iChip is another example of successful isolation technology combinations. It integrates size-based enrichment with either EpCAM-based positive enrichment or CD45 negative depletion with 97% capture yield and 8 mL/h processing rates (Karabacak et al., 2014; Ozkumur et al., 2013). The size-based enrichment process removes unnucleated cells using a micropost array, and antibody-mediated white blood cell (WBC) removal ultimately yields label free, viable CTCs (Karabacak et al., 2014; Ozkumur et al., 2013). This technology is limited to isolation of single cells and 2–4 cell clusters, not tumor microemboli. A device specific to CTC cluster capture, known as Cluster Chip, has recently been developed to account for this issue (Sarioglu et al., 2015). CTC clusters enter microfluidic pores and are trapped by stacked rows of triangular pillars, while single cells flow freely. The clusters are then released via flow reversal. The device's flow rates are extremely slow compared to size-based filters and help to protect cell viability. However, capture yield is low compared to the CTC-Chip (Sarioglu et al., 2015).

Another approach uses standing acoustic-wave fields inside microchannels to capture CTCs based on various physical properties, including size, density, and compressibility. In this particular method, an array of pressure nodes and antinodes is maintained inside a microfluidic channel at a tilted angel to the direction in which fluid flows. In this way, all cells experience different acoustic radiation forces that result in varying movement trajectories, ultimately separating the cells. This method efficiently isolates CTCs (with the recovery rate of 83–90% which is obtained with high input and attachment of polydimethylsiloxane (PDMS) microfluidic channel to the substrate for diminishing the removal of WBCs) without damaging the integrity, functionality and viability of the cells (Li, P. et al., 2015). Magnetic levitation is also investigated to isolate cancer cells based on their specific densities compared to other blood cells (Durmus et al., 2015). MagDense platform is developed which levitates lung, breast, colorectal, and esophageal carcinoma cells at different heights compared to other blood cells as cancers cells shows lower densities (Durmus et al., 2015). Further studies are needed to investigate the CTC capture efficiency and specificity of this approach from clinical samples.

All the aforementioned technologies have been designed for cell capture *ex vivo*. However, a new technology, known as the GILUPI GmbH CellCollector, applies an anti-EpCAM wire directly into the peripheral arm vein and captures CTCs with remarkable efficiency, processing approximately 1.5 L of blood in 30 minutes (Figure 2) (Saucedo-Zeni et al., 2012). For testing 24 cancer patients (non-small cell lung cancer and breast cancer) were examined, in which 22 out of 24 were detected with CTC with the detection rate of 91.6% (Saucedo-Zeni et al., 2012). Though more studies need to be done to verify its clinical applicability, the nanodetector has shown no evident side effects in past applications (Alix-Panabières and Pantel, 2013). The ability to analyze such large blood volumes increases the device's sensitivity and makes it a promising candidate for future CTC studies. Additional uses include for detection of fetal trophoblast cells in pregnant women (Krebs et al., 2014; Luecke et al., 2015). Advantages and limitations of various CTC isolation and detection technologies are summarized in Table 1.

## 3. CTC Detachment from Surfaces

Another important challenge that still has not been overcome for many isolation techniques is to effectively release CTCs from the substrate without impacting the cells. Detachment from filters, immunoaffinity chips, and other substrates require the removal of receptor-ligand interactions and/or focal adhesions (Zheng et al., 2013). Release methods using sheer stress may reduce viability and influence function of these fragile cells (Albuquerque et al., 2000; Born et al., 1992; Chowdhury et al., 2010). Therefore, less invasive methods of detachment have been developed for cancer cells, using temperature, light, electrodes, and aptamers. In this paper, we will discuss the three common methods of detachment technology specific to CTCs, using enzymatic digestion, aptamers, and pH-responsive polymers.

### 3.1 Enzymatic digestion

Employing enzymes to digest the extracellular matrix and detach cells is the standard method of cell release (Zheng et al., 2013). However, this method is known to degrade other cells' membrane proteins and cell-to-cell junction proteins (Keizer et al., 1988). Still, there are several accounts of successful enzymatic release of CTCs (Adams et al., 2008; Dharmasiri et al., 2009). CTC release efficiency of approximately 100% is found in antibody-functionalized microfluidic devices using 0.25% w/w trypsin, though effects on cellular function have yet to be thoroughly analyzed in these studies (Adams et al., 2008; Dharmasiri et al., 2009). Another nanofilm developed for use in microdevices provides 95% release efficiency with 90% cell viability. Briefly, layer-by-layer (LbL) assembly was used to form layers of anionic and cationic polymers, and alginate lyase for enzymatic degradation of the nanofilms. The released cells were then successfully cultured for 5 days afterwards, providing functional evidence of viability (Li, W. et al., 2015). Similarly, nucleases can be used to detach cells from aptamer-functionalized substrates. Endonuclease treatment using BamHI at 37°C for 30 min detached CTCs from polyacrylamide hydrogels at approximately 99% efficiency and at a faster rate than trypsin, with little damage to cell receptors (Li et al., 2013). Nevertheless, further studies must be done analyzing cell viability and function to investigate if enzymatic degradation is a suitable method for downstream CTC cultures.

### 3.2 Aptamers for CTC Detachment

Aptamers are newly emerged powerful tool to study CTCs (Dickey and Giangrande, 2016). These are single stranded oligonucleotides (RNA or DNA) of small MW (8–15 KDa) with some characteristics typically not found in antibodies (Chen et al., 2016). The advantages to using aptamers for CTC isolation is that they provide a high stability resistance to a spectrum of harsh conditions (urea, organic solvents, denaturation), have negligible toxicity and immunogenicity and oriented surface immobilization, thus offering a very efficient and non-invasive detachment method. These features allow for tumor cell penetration and blood clearance, not typical for antibodies. Furthermore, aptamers can be developed against the binding targets in the range between small compounds to large cell membrane or transmembrane proteins on the CTCs. However, there is an evidence that aptamers are unqiue in that changes in environmental conditions such as temperature or pH can alter their

three dimensional confirmations and cause them to lose their affinity to the target molecule (Zheng et al., 2013). Precautions must be taken, however, when using temperature to alter affinity binding, as temperatures around 45–53°C can harm tumor cell functions *in vitro* (Walsh et al., 2007).

As an alternative to temperature and pH-mediated aptamer release, complementary oligonucleotide sequences can be designed to preferentially bind to aptamers and produce changes in aptamer conformation (Figure 3). In one study, researchers effectively released CTCs from anti-EGFR aptamers attached to glass beads using an oligonucleotide known as RELease (Figure 3B). Cells, when bind with aptamers in the presence of RELease molecules, produces fluorescence because of the selective binding. This opens the hairpin structure and release 76% of the aptamers from cellular surface. About 92% of cells were released using this technique, compared to only 69% of cells via soft resuspension (Wan et al., 2012). In another report, researchers were able to recover about 95% of cells in 10 min using this method, while maintaining 99% cell viability (Zhang et al., 2012) (Figure 3A).

### 3.3 Polymers for CTC Detachment

Similar to aptamers, polymers can be designed to respond to changes in external conditions by reversibly changing their conformation via dissolution or deformation (de las Heras Alarcón et al., 2005). The pH-responsive polymers are synthesized by linking structures with weakly acidic and basic functional groups to a hydrophobic base. Once the groups become ionized, groups of similar charges cause repulsions and the polymer expands. Conversely, groups can lose their charges and cause the polymer to contract. Ionization of polymers can also directly affect affinity to extracellular matrix (ECM) proteins, as these are negatively charged under physiological conditions (Bajpai et al., 2008). For example, chitosan is a weak base and its functional groups are deprotonated under strongly basic environments (pH > 7.4), resulting in a negatively charged surface from which ECM proteins dissociate (Yeh and Lin, 2008). Using this method and a pH of 7.65, 90% of CTCs were detached in approximately 1 hour, while viability was preserved at 95% (Chen et al., 2012b). When designing pH-responsive polymers, pH values must be regulated carefully, since proton or hydroxyl group localization may pose a damage to cell viability or influence cell functions. These polymers should also allow high binding specificity and efficiency during preliminary detection and capture techniques (Phillips et al., 2008).

A nanostructured coating was also introduced that can be released from Herringbone CTC Chips via temperature, and can be used for bulk detachment, mechanical stimulation, and for single cell detachment. Gelatin and streptavidin are deposited in alternate layers using LbL assembly, with polystyrene nanoparticles coated with streptavidin covering 22% of the gelatin outer layer. The final product can respond to stimuli at even 135 nm thicknesses, allowing the nanocoating to be used in microdevices. Disturbing cell function is not a concern when using temperature stimulation because the nanocoating dissolves at only 37°C. Cell viability and recovery were 88.3% and 93.2%, respectively, using this method. Alternatively, a frequency-controlled microtip can dissolve 145–215 μm regions of the nanocoating using 15–30 Hz vibrations, releasing cells with 91.5% viability. All cells were

then cultured and grew in confluent colonies after 7 days (Reátegui et al., 2015). However, the effects of mechanical vibrations on cancer cells are not yet clear.

In summary, current technologies that hold the great potential for CTC detachment include oligonucleotide-mediated aptamer release, and stimuli-responsive polymers. Further considerations for developing new release methods should involve biocompatibility, point-of-care integration, detachment efficiency, cell viability, and compatibility with microdevices.

## 4. Culturing CTCs

For therapies that target tumor metastasis and can be tailored to patient-specific tumor characteristics, the culturing of CTCs is an essential step in drug discovery. MetaCell filtration device is a capillary action driven, size-based separation method that isolates and enriches viable CTCs from peripheral blood samples using a porous polycarbonate membrane. CTCs isolated using this filtration method are available for downstream analysis because the cells remain healthy and unharmed, as this method does not require any lysing solution or targeting antibodies for CTC isolation (Cegan et al., 2014; Kolostova et al., 2014a; Kolostova et al., 2015a; Kolostova et al., 2015b; Kolostova et al., 2014b). In a study performed on prostate cancer patients, CTCs were isolated and proliferated *in vitro* at a 64.3% success rate (18 out of 28 CTC-positive peripheral blood samples) (Kolostova et al., 2014a). CTCs were kept viable for 14–28 days by transferring the membrane filter to a cultivation plate and culturing cells directly on the membrane using FBS-enriched RPMI medium (10%), under normal cancer cell incubation environments (37°C, 5% $CO_2$). CTCs were also cultured directly on the well surface after 14 days of membrane culture. It was observed that CTCs under culture conditions become larger, paler, and more elongated. The morphologies of those CTCs growing on the membrane resembled epithelial cells and conglomerating stem cells, while the more prominent nucleoli of the CTCs growing on the well bottom suggested greater plasticity and invasiveness than those growing on the membrane (Kolostova et al., 2014a). Similar methods were used to successfully culture CTCs isolated from lung cancer orthotopic mouse models (Kolostova et al., 2014b), ovarian, cervical, and endometrial cancer (Kolostova et al., 2015b), gastric cancer (Kolostova et al., 2015a), urinary bladder cancer (Cegan et al., 2014), and esophageal cancer (Kolostova et al., 2014b).

It has been reported that other CTC isolation methods such as conventional CTC-Chip, CTC-iChip, MagSweeper and EPISPOT can also serve as effective isolation methods for subsequent CTC culture (Alix-Panabières, 2012; Alix-Panabières et al., 2007; Ameri et al., 2010; Helzer et al., 2009; Ozkumur et al., 2013; Yu et al., 2014). Using orthotopic murine models derived from prostate cancer cell lines, CTCs were collected and cultured on a plastic CTC-chip. After 5 days, cells began forming individual colonies, and by the 12th day of culture, colonies were expanding with less than 1% cell death (Helzer et al., 2009). Current efforts are being made to investigate extracellular matrix microarrays that enable testing of cell lines against a multitude of conditions. Studies have already used these microarrays for isolation and culturing of CTCs captured from mice engrafted with primary human pancreatic tumors (Gach et al., 2014).

Though successfully done by very few studies, *ex vivo* cultures of CTCs may produce more accurate and sustainable disease models than the previously introduced adherent membrane cultures and can be used to form CTC-derived tumors. In one study, researchers conducted xenograft mouse models studies with injected human cancer cell lines. Isolated CTCs were then successfully cultured *ex vivo* under hypoxic conditions and injected into other mice to form CTC-derived tumors. Hypoxic atmosphere is known to cause increase in tumor metastasis and even larger and more aggressive xenografts (Ameri et al., 2010; Gilkes et al., 2014; Spill et al., 2016). The resulting CTC-derived tumors were larger and metastasized more aggressively than the original cell line tumors, ultimately demonstrating the stem-cell like properties of CTCs (Ameri et al., 2010). However, careful considerations must be taken when using xenograft mouse models established with human cancer cell lines. Metastasis may occur late or even not at all. Isolation of human patient CTCs for direct *ex-vivo* culture may instead provide more accurate timelines for metastasis (Yu et al., 2011). In a study, investigators have demonstrated to form xenograft mouse models by implanting patient-derived CTC cell lines (Figure 4) (Yu et al., 2014). Scientists used various techniques to optimize CTC culture proliferation *ex vivo*, including hypoxic conditions and nonadherant cultures. CTCs captured from estrogen receptor-positive (ER+) breast cancer samples, using the CTC-iChip, were cultured and yielded a proliferative index of approximately 30% (range 24 to 32%). After testing four culture protocols, cells were found to grow at best as tumor spheres under 4% $O_2$, with serum-fee media, basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF). CTC cultures grouped together while separating themselves from the cancer cell lines and individual, uncultured CTCs (Yu et al., 2014). Scientists also observed that in adherent monolayer culture, the CTCs were unable to survive after several cell divisions and therefore stressed the importance of nonadherent cultures (Yu et al., 2014). This is likely due to their similarity to undifferentiated human mammary epithelial cells (HMECs), which proliferate as nonadherent mammospheres (Dontu et al., 2003).

When preparing CTC cell lines using peripheral blood samples, it is also important to consider the patients' treatment status [9]. It is reported that there is a higher likelihood of culturing success with increased tumor burden. CTC cell lines were created successfully from 6 out of the 36 patients, and 9 of the failed attempts were responding to breast cancer treatment. Cell lines from several different treatment time points were also generated for three patients, and in those cases, CTC cell line generation was unsuccessful during the early stages of treatment [9]. Downstream cytological analysis introduced many possibilities for CTC cell line studies. Cultured CTCs were very similar to captured CTCs and were used for various drug screenings, incorporating functional testing and genotyping into the drug susceptibility predictions. The CTC-derived tumors were immunohistochemically and histologically similar to the primary tumor. The study did not, however, detect increased expression of pathways related to stem cell activity in CTC cultures when compared with cancer cell lines.

Future studies should be done characterizing the effects of various isolation techniques on CTC morphology and function before these techniques can be confidently used for clinical analyses and CTC culture. There is a great need to optimize conditions for CTC culture and better understand differences between nonadherent CTC cell lines and adherent cell lines, CTC cultures and patient samples or murine models. With a better understanding, all of

these technologies have potential for patient-specific drug susceptibility testing and mutational profiles for cancer patients in the future.

## 5. Detection and Downstream Analysis

CTCs can be detected after enrichment via fluorescence in situ hybridization (FISH) for genome amplification, immunocytochemistry (ICC) for protein markers, and RT-PCR/qRT-PCR for quantifying specific RNA and DNA sequences. ICC is used in majority of the enrichment techniques, and there are a variety of ways that cells are characterized as CTCs. CellSearch requires the cell to be greater than 4μm in diameter, negative for CD45 markers, and positive for cytokeratin (CK) protein marker (form intermediate filaments in epithelial cells) and 4,6-diamino-2-phenylindole (DAPI) nuclear dye (Joosse et al., 2015). Additional labels can be used for therapy-relevant markers, such as prostate-specific antigen (PSA), androgen receptor (AR) (Gasch et al., 2013; Riethdorf et al., 2007), asCK, E-cadherin, vimentin (VIM), N-cadherin (CDH2), and CD133 (a stem cell marker) EMT-associated biomarkers (Armstrong et al., 2011). ICC can also be coupled with size-based and microfluidic isolation techniques, as well as the GILUPI GmbH CellCollector (Maheswaran et al., 2008; Nagrath et al., 2007; Saucedo-Zeni et al., 2012).

Targeting mRNA transcription factors with qRT-PCR offers a highly specific detection method that can be coupled with various isolation techniques, given properly designed primers and target gene selection (Joosse et al., 2015). However, challenges are faced when quantifying gene expression in unpurified samples, leading to false-negatives and false-positives (Pantel et al., 2008; Paterlini-Brechot and Benali, 2007). This is also a hurdle for FISH analyses (Attard et al., 2009). Cells captured using CellSearch appear to be more apoptotic and less likely to generate FISH signals (Attard et al., 2009; Swennenhuis et al., 2009). However, combinations of target genes, biomarkers, and protocols show promise in overcoming these numerous obstacles (Attard et al., 2009; Swennenhuis et al., 2009).

Other molecular profiling methods focus on the genomic identities of single CTCs. Singe cell molecular profiling requires highly pure samples and whole genome amplification (WGA) (Krebs et al., 2014; Punnoose et al., 2012). After amplification, cells can be genotyped using comparative genome hybridization (CGH), Sanger sequencing or next-generation sequencing (NGS) for each single cell (Voet et al., 2013). These techniques have already been performed to analyze genetic changes over an entire cell cycle and for comparing point mutations in CTCs to primary tumors (De Roock et al., 2010; Heitzer et al., 2013; Voet et al., 2013). However, these techniques should be further refined. The number of cells needed for accurate representations of the cancer should be determined (Parkinson et al., 2012). WGA can cause genetic alterations that may be accounted for by using other CTC samples and leukocytes as controls for detecting mutation patterns in singular CTCs (Dean et al., 2002; Krebs et al., 2014).

In addition to sequencing studies, gene-expression analysis must be performed to quantify the phenotypic effects of various genetic and epigenetic modifications. However, the delicate nature of RNA samples compared to DNA create significant challenges, and determining the relevant changes in expression is difficult among the signals produced by other cell

processes and experimental procedures (Asare et al., 2008; Peeters et al., 2013). Techniques like smart-seq, next generation sequencing have been developed to better detect highly expressed RNA transcripts in CTCs derived from melanoma and prostate cancer patients (Cann et al., 2012; Ramsköld et al., 2012; Tang et al., 2009). Most CTC analysis techniques are still at a rudimental stage and are very costly, the area, which needs further development.

## 6. Metastasis and Treatment Response

### 6.1 Diagnosis and Monitoring

As motioned earlier, CTC as noninvasive “Liquid biopsy” is a real-time possibility to monitoring malignancies. It has significant advantage over the conventional solid biopsy (Lianidou, Evi S, 2014). In liquid biology, certain amount of blood (~7.5 ml, in the case of CellSearch method) is required to detect CTCs whereas solid biopsy involves invasion inside the tissues. Moreover, the sample collection in cancers like osteoblastic metastasis in prostate cancer is challenging for solid biopsy and does not provide information about the tumor genome evolution over the time and its spatially heterogeneous nature (Chen et al., 2016; Lianidou, Evi S., 2014). CTCs as liquid biopsy can revolutionize the cancer diagnosis and monitoring in the patients (Alix-Panabières and Pantel, 2013). A study carried out on pancreatic ductal adenocarcinoma (PDAC) patient revealed that CTCs helped in diagnosis and staging of the disease progression. CTCs were detected in 54 out of 72 patients with the use of 1st gen NanoVelcro CTC chip, which confirmed the presence of PDAC. In addition, investigators reported that the presence of >3 CTCs in 4ml of blood helped in discriminating between local/regional and metastatic stages in the patients (Ankeny et al., 2016).

The cellular morphology and nuclear size of CTCs can reveal disease progression. A team collected the blood samples from patients at the different stages of prostate cancer from localized to advance metastatic castration-resistant disease. They identified 3 different subsets of the CTCs ranging from size < 8.54 μm- vsnCTCs (very small nuclear CTCs), 8.54 μm- snCTCs (small nuclear CTCs) and >14.99 μm - lnCTCs (large nuclear CTCs). Both vsnCTCs and snCTCs were present in the patients at the metastatic stage of cancer and vsnCTCs occurred higher in number in visceral organs like lungs or liver. Further in this investigation, 28 prostate cancer patients who had progressed through next generation hormonal maneuvers were examined. It was shown that 15 out of 28 were found with visceral lesions and 13 had bone disease, 6 (non-visceral metastatic patients) out of these 13 developed visceral lesions during their follow-up. Within 86–196 days prior to radiographic detection, 4 of the patients were detected with vsnCTC with the absence of visceral lesions at the time of analysis. Investigators observed the reduction of number of vsnCTC after the initiation of the anti-cancer therapy. This is supporting evidence that the CTC morphology reveals disease progression and can also assist in therapeutic inventions (Chen et al., 2015).

### 6.2 Survival Rate Prediction

CTCs could be an alternative for predicting the feedback of the treatment in cancer patients. Studies have been done to reveal that the quantification of CTCs is useful in predicting the response of the patients towards the drug and overall survival (OS) rate (Miyamoto et al., 2012). To monitor chemotherapy treatment, imaging techniques are often coupled with

blood tumor marker levels, but these indicators may take up to several months to show various drug responses (Boss et al., 2008; Mohler et al., 2012). CTC levels in the blood, however, has been shown to signify changes within a few weeks (Hayes and Smerage, 2008; Saad and Abraham, 2008), before the onset of symptoms (Rao et al., 2012), and seem to yield greater accuracy (Budd et al., 2006). Most of these studies focused on CTC enumeration that has proven a strong correlations with OS rate in patients with melanoma (Mocellin et al., 2004), metastatic lung (Hou et al., 2012; Krebs et al., 2011), colorectal (Cohen et al., 2008), prostate (de Bono, Johann S. et al., 2008), and breast cancer (Budd et al., 2006; Cristofanilli et al., 2004; Riethdorf et al., 2007). The CTC count was monitored in patients suffering from mCRPC (metastatic castrate-resistant prostate cancer), revealed that the patient having more than 5 CTCs/7.5 mL in their blood has poor OS rate in comparison with patient having less than 5 CTCs per mL (de Bono, Johann S et al., 2008). Similar pattern was also observed in breast cancer patients which showed that the patient with persistently elevated CTC counts (>5 CTCs/7.5 mL at baseline and during investigation) exhibited significantly worse OS rate (Krebs et al., 2010). In another study, it has been demonstrated that CTC count is directly related to disease severity. A total count of 177 patients were examined with an average age of 58.0±13.4 years (median 58). From the count studies- before the investigation starts, 10 patients died as they had extremely large level of CTCs in their blood (9, 11, 15, 24, 111, 126, 301, 1143, 4648, and 23,618 CTCs/7.5 ml of blood). 49 patients out of 169 patients, had ≥5 CTCs /7.5 ml of blood, posses free survival rate with lower median progress of about 2.1 months and lower median OS of about 8.2 months. Remaining 114 patients had <5 CTCs/ 7.5 ml of blood and had progress free survival rate of about 7 months and overall survival rate of >18 months. In this study, elevated level of CTCs even after treatment investigation represented that the patients received feeble treatment. In summary, CTC count can be helpful in disease prognosis and management, hence patients might get benefit from alternative therapies during treatment (Cristofanilli et al., 2004).

### 6.3 Therapy Response and Surgery Prediction

CTCs could be useful in analyzing effects of anti-cancer drugs on their therapeutic targets and the analysis of these CTCs can identify the early detection of the resistance to therapy. A study conducted on metastatic breast cancer (MBC), showed CTCs prognostic importance in patients. The patient with the reduced CTCs number after 21 days of therapy was kept on the same therapy whereas the patient with the increase in the number of CTCs after 21 days was subjected to different chemotherapy (Smerage et al., 2014) Another report that used CellSearch to demonstrate the patient outcome, based on CTC count, has shown some interesting results. The trial (S0500 reported in 2014) failed after one cycle of first-line chemotherapy as it showed lack in overall survival in MBC patients. It showed persistant increase in the CTC count after 21 days of first-line chemotherapy (Smerage et al., 2014). This increase in the number of CTCs might be due to ineffective treatment for the patient and a need for change the treatment options or therapeutic drugs based on CTC count during the chemotherapy. These studies reveal that CTCs can provide more accurate prognosis to allow oncologists to get a clear picture of the treatment outcomes. Another study revealed that CTCs could be employed to follow therapy responses and monitoring disease progression (Lu et al., 2013a). A clinical study showed positive response of the therapy on

the metastatic prostate cancer patients. 32 metastatic and 8 localized cancer patients diagnosed with CTCs with the help of 1st generation NanoVelcro Assay, were recruited for this study. As the therapy continued, the CTC quantification was performed with serial enumeration, and within 4–10 weeks of treatment statistical reduction in the CTC count was observed. However, in another case, this assay helped in revealing a negative response of the therapy (Lu et al., 2013a).

CTCs can provide new biomarkers that can help to view insight evolution of signaling responses. A study was designed in prostate cancer patients to depict “Androgen receptor (AR) signaling pathways” as a new biomarker to track castration-resistant disease. Androgen deprivation therapy (ADT) is used for the patients with metastatic prostate cancer as an initial response. However, progression of the disease was observed in the patients showing that the tumor cells resume proliferation despite of the continuous treatment (termed castration-resistant prostate cancer or CRPC) (Stott et al., 2010). In most of the cases, prostate metastatic cancer spread to bone that limits the sample collection to monitor the mechanism of drug resistance (Yuan and Balk, 2009). The “second generation” microfluidic chip (Stott et al., 2010) coated against PSA (prostate specific antigen) and PSMA (cell surface protein) (Lee et al., 2002) antibodies was adopted for broad capture of CTCs, followed by CTC characterization for AR signaling status. Results revealed that 4 out of 5 patients were detected with CTCs in metastatic prostate cancer patient before starting the androgen deprivation treatment. In the end of the treatment, it was observed that majority of CTCs have shown transformation “AR-on” (PSA+/PSMA-) to the “AR-off” phenotype within one month, followed by the complete disappearance of CTCs by 3 months after initiation of therapy. These results showed that monitoring of CTCs derived from the cancer patients could be a dynamic biomarker showing the effect of cancer drugs on their therapeutic targets by tracking the activity of the signaling pathway (AR pathway) (Miyamoto et al., 2012).

CTCs could also help in solving critical issue for the men with prostate cancer. It could assist oncologists to gain more information about making the decision for the treatments or surgeries in these patients. The patients with prostate cancer have to face a complicated decision of whether to go for surgery to eradicate prostate, reason being if the cancer has spread beyond the prostate gland, then the chances of potential surgical failure are more. However, scientists have observed that CTC test could be beneficial for surgery prediction. In a study, researchers examined 138 men with prostate cancer who went through surgery to remove prostate (Olsson et al., 1996). These patients were subjected to tests for CTCs presence. The results revealed that the patients with CTC positive were more likely to experience surgical failure compared with CTCs negative patients (Nemeroff, 2010). This shows that prediction and monitoring of CTC levels in cancer patients holds important information about how efficient and effective the treatment or surgery would be.

## 7. CTCs and Development of Targeted Therapeutics

### 7.1 CTCs Gene Profiling and Targeted Treatment

Currently, almost all the cancer therapies are based on analysis of primary tumor but these therapies fail to encounter metastatic cells, which is the main reason of metastatic relapse

years after primary tumor diagnosis and surgical resection (Heitzer et al., 2013; Lohr et al., 2014; Uhr and Pantel, 2011). Molecular characterization of the CTCs from the metastatic cancer patients could be favorable in evaluating the potential therapeutic targeting and finding the fundamental cause for the abnormal functioning of these cells (Meng et al., 2004; Wan et al., 2013). An extensive study has been carried out in genotypic and phenotypic characterization profiling of CTCs (Fehm et al., 2010; Ignatiadis and Dawson, 2014; Meng et al., 2004). These studies can provide better understanding in context of treatment choice and the discovery of personalized medicine. Presently, the characterization of CTCs has been carried out on different tumor types, that does not limit to breast cancer but also includes prostate and lung cancer types (Jiang et al., 2010; Maheswaran et al., 2008; Miyamoto et al., 2012). A team working on gene profiling of breast cancer patients, aimed to observe heterogeneity and expression pattern of genes in CTCs (Powell et al., 2012). Cell lines (breast cancer) T47D, MCF7, MDA-MB-231, and SKBR3, and both primary as well as metastatic breast cancer samples were obtained for gene analysis. In the circulating tumor gene profiling, gene expression of 510 patients was studied and the cells were isolated by the MagSweeper method. This study was designed to analyze cells of 1700 cases (breast cancer) that indicate three standard genes i.e. GAPDH, ACTB and UBB to establish gene expression and statistical analysis of the results. The total of 31 of the 87 investigated genes were frequently noticeable in 15% of the cells (CTCs) scrutinized. Apart from 3 standard genes, the other 28 overexpressed genes along with few more genes from different studies, listed in Table 2 according to their functional categories. This study revealed that the molecular gene profiling of tumor cells provides a heterogeneous framework of the disease.

It was analyzed that, not all the CTCs were responsible for seeding metastasis in the patients. Only few CTCs have the capacity to do so. CTCs with missing ER, HER2 or PR expression are more aggressive and have a higher potential of forming metastasis in less time and these tumors have limited targeted treatment options (Sørlie et al., 2001) (Bosch et al., 2010; Korsching et al., 2008). This is the reason for the failure of most of the therapies that target these biomarkers. Since these CTCs lose the expression of ER/PR/HER2 biomarkers in them; hence the therapies targeting these biomarkers fail to encounter these cells. These findings also acknowledged that few genes such as S100A9, S100A4, and NPTN were associated to metastasis and had a striking expression. The genes like ZEB2, TGFβ1, VIM, CXCR4 and FOXC1, are correlated to initiation and conservation of EMT, a process which leads towards the hikes of cell invasion by changing the epithelial cells to mesenchymal cells, biologically and morphologically (Barcellos-Hoff and Akhurst, 2009; Bertran et al., 2009; Boye and Mælandsmo, 2010; Chua et al., 2007; Kalluri and Weinberg, 2009; Padua and Massagué, 2009; Polyak and Weinberg, 2009; Rodriguez-Pinto et al., 2009; Thiery, 2002).

In a different study carried out on colorectal cancer (CRC) patients, the CTC DNA was amplified followed by sanger sequencing revealed that PIK3CA, KRAS and BRAF are the mutant genes which may effect the response of anti-EGFR therapy (Zhang et al., 2014). In another study, pancreatic CTCs from the mouse were subjected to RNA sequencing. It was found that CTCs were enriched with WNT-2 gene. In pancreatic cancer cells, WNT-2 genes suppress anoikis, increase anchorage independence sphere formation and upturn the metastatic propensity. Similarly, in humans' pancreatic cancer cells, the WNT gene leads to

the formation of non-adherent tumors. Thus, the molecular profiling of CTCs may act as a candidate therapeutic target as the pancreatic CTCs revealed the presence of WNT signaling in some human cases (Yu et al., 2012).

CTCs could play an important role in drug trials (Fehm et al., 2010; Ignatiadis and Dawson, 2014). The amplification of HER2- Human epidermal growth factor receptor 2 is a main cause of 20% of breast cancers in women. It is considered as one of the biomarkers for MBC patients. The patients with an over-expression of HER2 status are usually subjected to HER2–targeted treatment. In one study, 254 patients with MBC were tested to check HER2 status with the help of CellSearch (Fehm et al., 2010). It was observed that the women having original tumor with HER2 +ve genes also had HER2 +ve CTCs, but there were certain number of cases in which it was observed that the women with HER2 -ve original tumor possess HER2 +ve CTCs (Riethdorf et al., 2010). Formerly evaluated with lapatinib-1500 mg/day (is a EGFR coupled with HER2 tyrosine kinase inhibitor used in combination for HER2 +ve breast cancer patient treatment) in first line of therapy, population was subjected for CTC isolation. The result showed that out of 139 (HER2-negative) patients, 96 were CTC positive, out of which 7 were HER2 positive. When these 7 patients were treated with lapatinib, lack of objective response against lapatinib was observed in one of the patient and disease prevailed longer despite of lower HER2 shift. It was related to the failure of the phase II trial, that was unable to explain any significant interest of the lapatinib therapy in patients with HER2 -ve MBC and having overexpression CTCs with HER2-protein (Baselga et al., 2012; Chen, Y.-J. et al., 2013; Pestrin et al., 2012). However, further detailed studies of CTC characterization can overcome the obstacle of having less information about the biomarkers and it might be possible in future that the CTCs could be evaluated not only for personalized therapies but also for molecular screening of the tissues (Wan et al., 2013), as the attempts have been conducted to study CTCs with mutational outline from different cancer types.

## 8. Summary

The discovery of CTCs as a liquid biopsy has revolutionized the prognosis of cancer. CTCs hold great possibilities for early disease detection, metastasis monitoring and personalizing cancer therapies. Despite these advantages, the clinical use of CTCs as a “liquid biopsy” comes with significant challenges related to CTC heterogeneity, fragility, and incomplete gene expression knowledge. Methods for isolating, detaching, and detecting these cells continue to present with needs for increased specificity, sensitivity, and throughput. Culturing and analysis techniques need to be optimized to better understand the morphological and functional properties of CTC cell lines derived from patient samples. Further studies can then be performed regarding optimal treatment plans using CTCs derived from patient samples, regarding molecular and gene analysis for effective disease monitoring and discovery of new drug targets.

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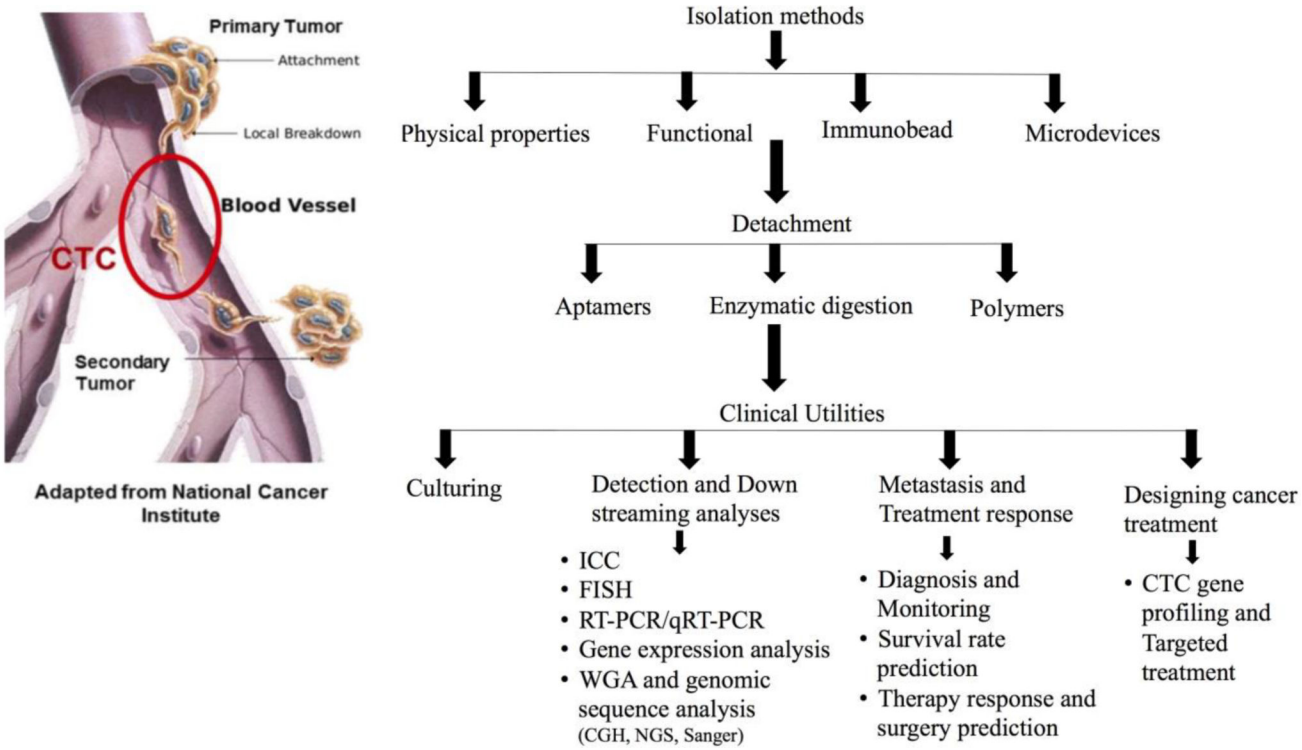


**Figure 1.**

Outline of existing isolation, detection and characterization techniques and promising future clinical utilities.

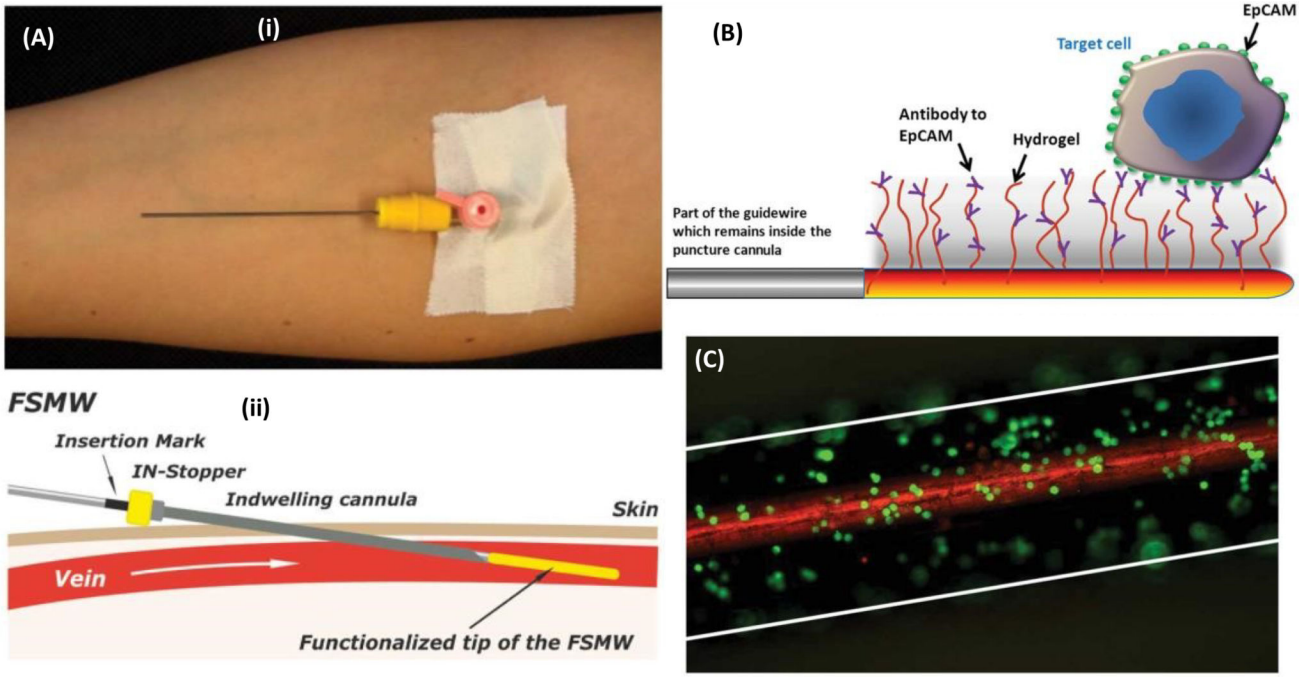


**Figure 2.**

(A) (i) Insertion of the functionalized structured medical Seldinger guidewire (FSMW) into the vein through a conventional cylindrical (instrument is also known as GILUPI GmbH CellCollector). (ii) Wire (anti-EpCAM-antibody-functionalized) is slowly inserted inside the cannula till surface is in contact with the blood flow in vein lumen. (B) Seldinger guidewire-Gold-plated with a size of 200 nm- coated with polycarboxylate hydrogel (functionalized with anti-EpCAM-antibodies) which capture circulating cells expressing EpCAM antigen on surface. (C) Image illustrating breast cancer cells (SK-BR-3) captured by functionalized guidewire. Reprinted with permissions from (Saucedo-Zeni et al., 2012).

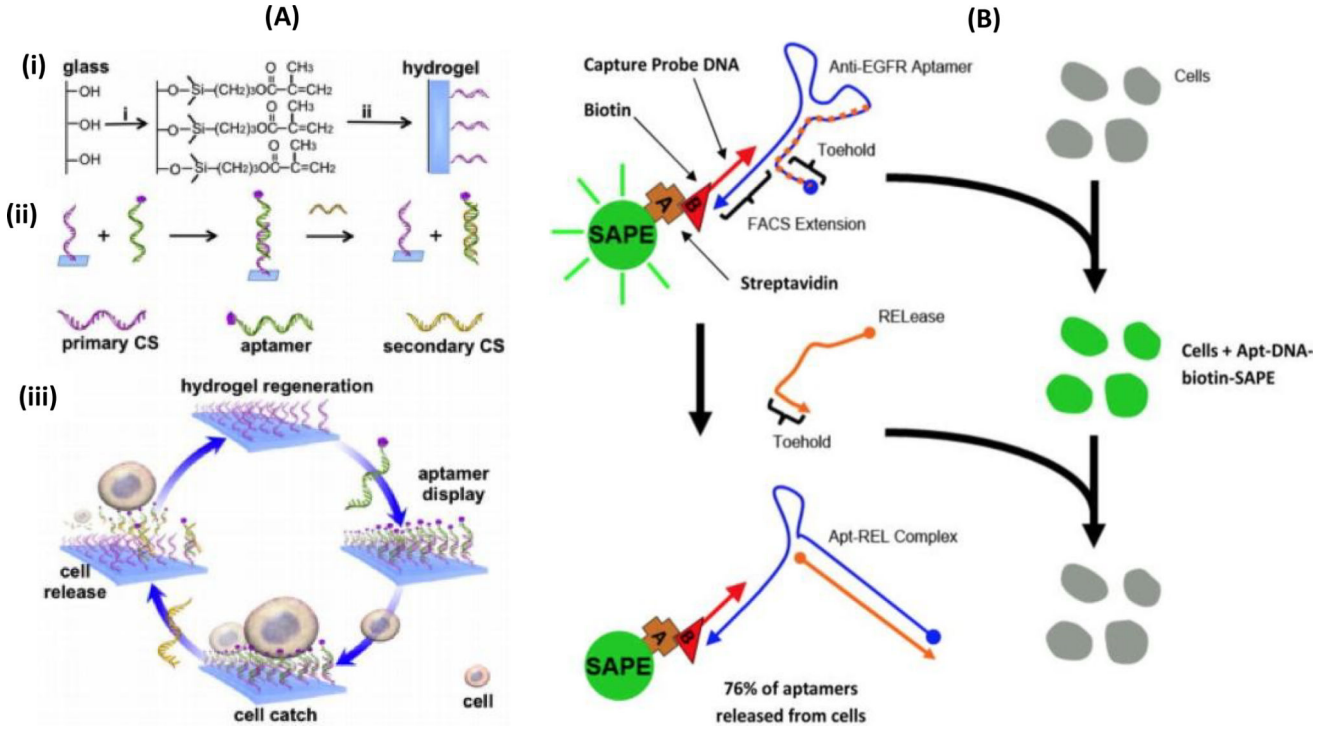


**Figure 3.**

(A) Aptamer-mediated CTC capture and release via complementary oligonucleotide sequences (CS= complementary sequences) (Zhang et al., 2012). (i) explain about the basic structure of the of the hydrogel immobilized on the glass slide (ii) illustration of the aptamer sequence hybridized with complementary sequences (CSs) (iii) represents the process of cell release. The stable aptamer hybridized with triggering CSs, forming a new hybridized state that leads to rapid dissociation of the aptamer from the hydrogel hence releasing the cells from the hydrogel. (B) Illustrating the cell binding and aptamer formed by SAPE of anti-EGFR aptamer – DNA and biotin, and addition of RELease particles. After washing, the fluorescence cells (caused by selective binding with aptamer) completely hybridizes with anti-EGFR aptamer because of the presences of RELease RNA. This leads to opening up its herpin sturucture which releases 76% of anti-EGFR aptamer from the cellular surface. (Wan et al., 2012)

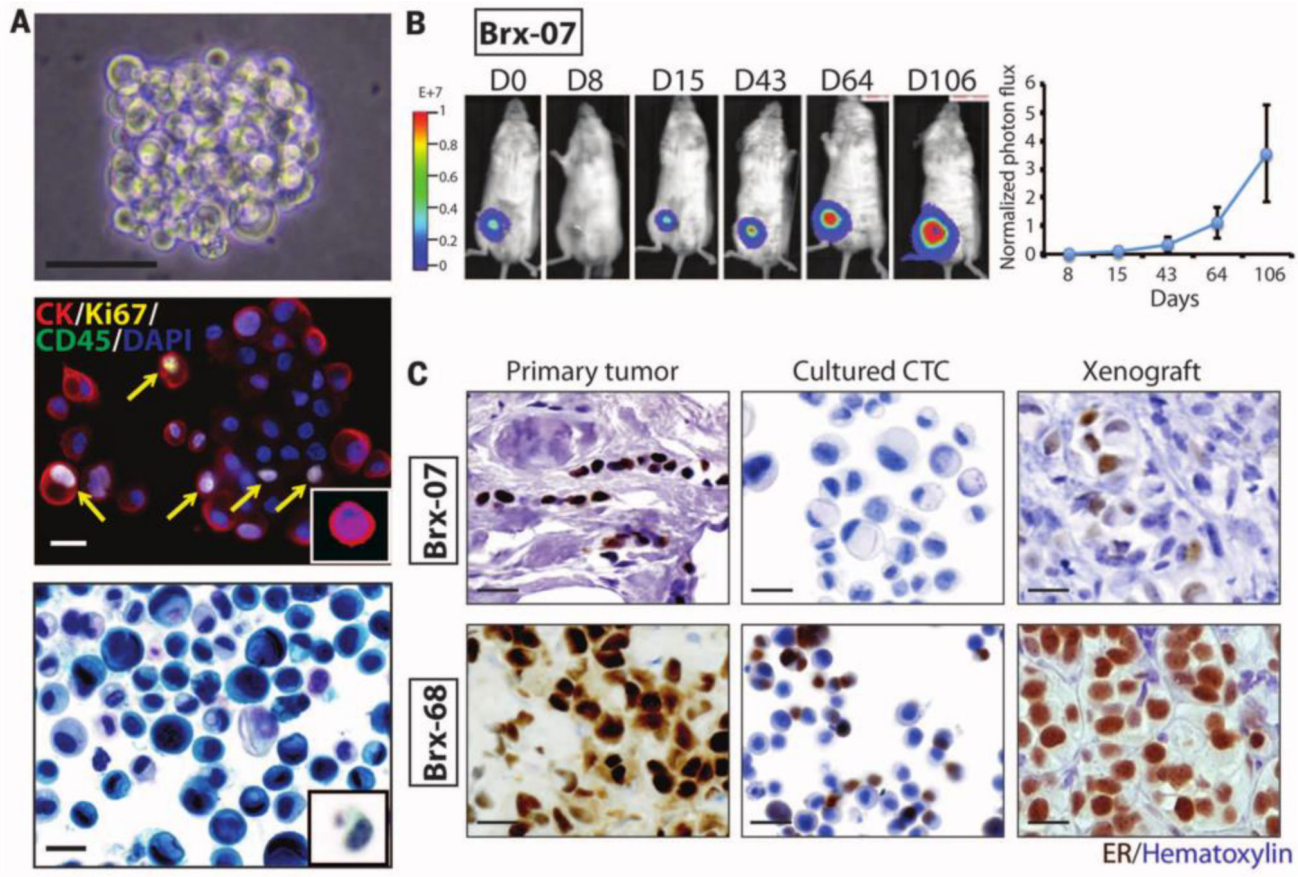


**Figure 4.**

Ex-vivo CTC (breast cancer) culture (A) Nonadherent CTC cultures (B) mouse xenografts derived from cultured CTCs implanted in mammary fat pad. (C) Orignal breast tumors and corresponding CTCs culture and two different CTC lines derived from mouse xenografts. (Yu et al., 2014)

**Table 1**

List of CTC isolation methods and techniques with their advantages and limitations.

| Name | Basic Properties | Advantages | Limitations |
|---|---|---|---|
| Acoustophoresis (Deshmukh et al., 2014; Gossett et al., 2010; Li, P. et al., 2015). | Cells are suspended in fluid and are exposed to ultrasound waves and pressure amplitude<br>This results in an acoustic radiation force<br>The radiation force will then push particles towards the pressure anode or pressure anti-anode, ultimately separating the particles | The strength of the acoustic radiation force is dependent upon the volume of the particle, the density of both the particle and the fluid<br>Isolation factors include the size of the particle and its compressibility | With direct pressure source, any instability in the pressure source can cause deviation in the intended flow lines |
| AdnaTest (Andreopoulou et al., 2012; Müller et al., 2012; Zieglschmid et al., 2005) | Separation by way of anti-EpCAM and anti-MUC1 antibody-targeting immunomagnetic beads<br>CTCs are then detected via RT-PCR assay for tumor-associated transcripts | The variety of selection markers (antibodies) allows for the possibility of characterizing cells for multiple markers, all simultaneously<br>Antibody cocktails are specific to a certain cancer type | Possible false-positive finding due to expression of a selection marker being present in other cells other than CTCs, such as with nucleic acid contamination.<br>CTCs that undergo EMT are EpCAM negative<br>Cells are not viable after detection |
| CAM assay (Lu et al., 2010; Monteiro-Riviere et al., 2009) | Used as a functional cell separation method based on CTC invasiveness compared to other cells.<br>CTCs can be identified using the CAM uptake criteria. | High sensitivity and specificity, leading to effective enrichment and identification based on CTC invasiveness.<br>Downstream analysis is possible. | Isolation step requires more than 12 hours<br>Biomarker dependent |
| CellSearch (Attard et al., 2009; Beije et al., 2015; Harouaka et al., 2014; Hong and Zu, 2013; Kim et al., 2012; Swennenhuis et al., 2009) | Separation by anti-EpCAM targeting immunomagnetic beads | The only FDA-approved blood test for the use in patient care diagnostics, used for metastatic breast, prostate, are colorectal cancer | CTCs expressing low levels of EpCAM are unlikely to be captured<br>Cells captured appear to be more apoptotic |
| CTC-Chip (McKeown and Sarosi, 2013; Nagrath et al., 2007) | Unique microfluidic approach<br>Use of antibody (anti-EpCAM) microposts<br>Controlled laminar flow conditions | High sensitivity and specificity<br>99% success rate in identifying CTCs in the peripheral blood of metastatic pancreatic, prostate, colon cancer, lung, and breast patients | Long blood processing time (1.67mL/hr)<br>Cells no longer viable |
| CTC Cluster-Chip (Sarioglu et al., 2015) | Utilizes bifurcating traps to capture CTCs, release via flow reversal | CTC clusters can be isolated<br>Sensitive enough to capture CTC clusters as small as 2 cells<br>Does not require the use of tumor specific markers for isolation<br>Low flow rates preserve cell viability | CTC clusters were identified in only 30–40% of patients suffering from metastatic breast, prostate, and melanoma cancers<br>Identification of CTCs is lower versus the CTC-Chip |
| CTC-iChip (Karabacak et al., 2014; Ozkumur et al., | Size-based enrichment with either EpCAM-based positive enrichment or CD45 negative depletion | High throughput | Limited to single or small 2–4 cell clusters, not tumor microemboli |

| Name | Basic Properties | Advantages | Limitations |
|---|---|---|---|
| 2013) (Bioengineering, 2014) | Microfluidic design for rapid sorting<br>Magnetically labeled target cells are sorted out<br>Inertial based capture platform | Fast processing time<br>More sensitive in detecting low levels of CTCs, versus commercial technology<br>Capacity to isolate CTCs by methods either dependent or independent of tumor membrane epitopes, making this method applicable to all cancers | |
| Dielectrophoresis (DEP) (Gascoyne et al., 2009; Gossett et al., 2010; Zieglschmid et al., 2005) | Separation can be positive or negative, which will affect the position of the cell in the field<br>Cells are placed in a non-uniform electric field, the field then imparts a net force on the cell due to an induced or permanent dipole<br>Isolation based on polarizability and size | Can be used for selective isolation of cells<br>Either AC or DC currents may be used | Requires specific parameters such as cell type and electric field frequency.<br>DC current can result in a electrochemical reaction on the electrode surface, causing a reduction in convective flow. This reduction can cause cell isolation to be inhibited, as well as free radical generation that results in cellular damage. |
| DynaBeads (Hardingham et al., 1993) | Magnetic separation and isolation based on binding to desired target and beads responding to magnetic field | Can be pre-coupled with biomolecules with an affinity for a desired target, such as: antibodies, proteins, antigens, or DNA/RNA probes | Only 3 types of DynaBeads are available for human tumor cell isolation |
| Ephesia CTC Chip (Alix-Panabières et al., 2012; Karabacak et al., 2014; Saliba et al., 2010) | Isolation by way of immunomagnetic sorting coupled with microfluidics<br>Sample flows across immobilized magnetic beads | Capture efficiency range of 90–94%<br>Ephesia technology can allow for a more flexible platform to perform advanced cell biology testing on cancer cells<br>Required sample volumes are one-tenth those of flow cytometry | Currently has not been formatted to accommodate large volumes of blood |
| EPISPOT (Alix-Panabières, 2012; Kalyuzhny, 2005; Millner et al., 2013) | Removes leukocytes via CD45 depletion and during short-term cell cultures, detects specific marker proteins shed/ secreted/ released from single epithelial cancer cells<br>An adaptation of the enzyme-linked immunospot (ELISPOT) technology | This assay creates a new way to detect viable CTCs and DTCs (disseminated tumor cells)<br>With an extension of a multi-parameter analysis, this could reveal the CTC/DTC protein fingerprint | Requires efficient antigen binding and specific epitope presentation<br>Demands high antigen levels<br>Transition into in vitro cultures may decrease cell viability and reduce detection rates |
| GILUPI CellCollector Nanodetector (Alix-Panabières and Pantel, 2013; Krebs et al., 2014; Luecke et al., 2015; Maheswaran et al., 2008; Nagrath et al., 2007; Saucedo-Zeni et al., 2012) | Ex vivo (FSMW) Functionalized Structured Medical Wire is antibody (anti-EpCAM) coated and applied into peripheral arm vein<br>Isolation occurs in vivo | Overcomes sample blood volume limitations<br>Increases diagnostic sensitivity of CTC isolation<br>No evident side effects | Only used for extraction of CTCs directly from patient's bloodstream, not for use of extraction in blood sample<br>More studies need to be done to verify clinical applicability |
| Herringbone CTC-Chip (Sarioglu et al., 2015; Stott et al., 2010; Wang et al., 2016) | An advanced CTC-chip utilizing a strategy of including herringbones or surface ridges in the walls of the device to disrupt streamlines and | Future goal is to be used for large scale clinical applications Successfully isolated 93% CTCs from patients with prostate cancer<br>Captures CTC clusters | It does not consider the inherent particulate properties of cells.<br>Cells are no longer viable |

| Name | Basic Properties | Advantages | Limitations |
|---|---|---|---|
| | encourage collisions between antibody-coated walls and CTCs | | |
| ISET (De Giorgi et al., 2010; Joosse et al., 2015; Vona et al., 2004; Zheng et al., 2011) | Utilizes a filter-based, size exclusion approach to isolate epithelial cells | High throughput<br><br>Downstream morphological studies can be performed | CTCs can be damaged or fragmented due to multi-step cell processes<br><br>CTC heterogeneity regarding morphology and size |
| IsoFlux Rare Cell Access System (Harb et al., 2013) | Uses next generation sequencing (NGS), with quantitative polymerase chain reactions (qPCR), fluorescence in situ hybridization (FISH), and immunofluorescence<br><br>Uses flow control and immunomagnetic capture to increase CTC isolation | High sensitivity of CTCs from many tumor types<br><br>The variety of selection markers (antibodies) allows for the possibility of characterizing cells for multiple markers, all simultaneously<br><br>Multiple kits for lab usage are available, both for cell enrichment, and downstream analysis | Maximum daily analysis is 12 samples<br><br>CTC may have biomarker heterogeneity Those that undergo EMT are EpCAM negative |
| MagSweeper (Ao et al., 2016; Powell et al., 2012; Talasaz et al., 2009) | Separation by way of anti-EpCAM antibody-targeting immunomagnetic beads | Isolates CTCs without contaminating leukocytes<br><br>The variety of selection markers (antibodies) allows for the possibility of characterizing cells for multiple markers, all simultaneously | The CTCs possessing low or no EpCAM expression will be over-looked (CTCs that undergo EMT are EpCAM negative) |
| MagDense (Durmus et al., 2015) | Magnetic levitation based on specific densities of CTCs | Platform enables single-cell density measurements and imaging | Further studies are required for capture efficiency and specificity for CTCs |
| Metacell Filtration (Dolfus et al., 2015) | Size based separation technique driven by capillary-action<br><br>Commercial device used for isolation of CTCs, allowing for cytological identification | Filtration techniques are sensitive enough to allow cytomorphological and immunocytochemical analysis of CTCs | Filters have a larger pore size (8μm) versus ScreenCellCyto (6.5μm) |
| Microfluidic Silicon Chip (Sequist et al., 2009) | Antibody based | Antibody coated microposts maximize CTC-antibody interactions | Further CTC separation from microfluidic device is challenging |
| NanoVelcro Microfluidic Device (Zhe et al., 2011) | Microfluidic device uses tiny rods coated with antibodies<br><br>After blood passes through, a laser is used to extract CTCs<br><br>Uses 3 color immunofluorescence for detection of CTCs | This is a more advanced prototype that adds a new transparent substrate for isolating CTCs | Only EpCAM-positive CTCs are detected |
| Negative Enrichment QMS (Ignatiadis and Reinholz, 2011; Jing et al., 2007) | A negative selection technique for cell enrichment followed by separation by Quadrupole Magnetic Flow Sorter (QMS) | Improved sample yield and purity<br><br>High throughput and cost effective | CTC expressing CD-45 maybe inadvertently remove from the sample<br><br>Contamination with WBC's might result in unintentional loss of CTCs |
| OncoQuick® (Rosenberg, R et al., 2002; Rosenberg, R. et al., 2002) | Polypropylene tube is inserted above the separation medium | High throughput, inexpensive | Loss of sample while depleting mononuclear cells |

| Name | Basic Properties | Advantages | Limitations |
|---|---|---|---|
| | The medium allows for elimination of erythrocytes, granulocytes, lymphocytes, and mononuclear cells | Increases sensitivity and specificity for tumor cells<br>Significant reduction in co-enriched number of mononuclear cells, with a high CTC recovery rate | Detection depends upon only cytokeratine-20 biomarker |
| Parsortrix (Joosse et al., 2015; Xu et al., 2015) | Isolation of CTCs based on size and compressibility | Purity of CTCs harvested is 3.1%<br>Ability to capture CTC clusters<br>Harvests CTCs with both epithelial and mesenchymal features<br>Uses cassette device to for easy detachment | CTC heterogeneity regarding size |
| pluriSelect (Nadezhda Frolova, 2012) | Antibody based CTC separation pluriSelect uses pluriBead® carrying a tumor-associated anti-EpCAM antibody | Non-magnetic cell separation, can be added directly to a whole blood sample | Currently limited for use in colon carcinoma diagnostics |
| Reactive Ion Etching (RIE) (Chen et al., 2012a; Nalepa et al., 2013) | Adhesion of the CTCs on the nano-roughsurface regardless of size and without using any capture antibody. | The capture efficiency is up to 80% within one hour of cell incubation<br>Does not require EpCAM expression for the cell capture | Suboptima l capture purity |
| RoboSep/EasySep (Wang et al., 2013; Wilcox et al., 2009) | Magnetically label and separate cells by positive or negative selection | High throughput<br>Fully automated instrument, reducing possibilities of cross contamination<br>Design for a customized cell separation protocol can be created by stem cell technologies | Rely on biomarkers for capturing the cells |
| ScreenCellCyto (Dolfus et al., 2015; Wang et al., 2013) | Size-exclusion based isolation method<br>Peripheral blood sample is mixed with filtration buffer<br>Diluted sample is then filtered by aspiration created by a vacuum tube collector<br>Disposable | In addition to ScreenCell devices being used for isolation of CTCs, they are also used for isolation of Circulating Fetal Cells (CFCs) from maternal peripheral blood for prenatal diagnostics<br>Filters in only 3 minutes<br>Not dependent upon EpCAM | Poor specificity<br>Unable to capture CTCs smaller than WBCs |
| TelomeScan (Kim et al., 2011; Kojima et al., 2009) | Detects elevated telomerase activity via a telomerase-specific replication selective adenovirus | May more effectively detect epithelial to mesenchymal transition (EMT) or EpCAM negative tumor cells | May also detect hematopoietic stem cells for false-positive results |

**Table 2**

Gene categorization/grouping on the basis of functional categories after the evaluation of CTCs

| Functional categories | Associated genes |
|---|---|
| Epithelial phenotype | *KRT8* (Powell et al., 2012; Ting et al., 2014), *KRT18* (Powell et al., 2012; Ting et al., 2014), *KRT19* (Powell et al., 2012; Smirnov et al., 2005; Ting et al., 2014), *CTNNB1* (Powell et al., 2012), *Krt7* (Ting et al., 2014), *Epcam* (Lu et al., 2010; Smirnov et al., 2005; Sun et al., 2013; Ting et al., 2014), *EGFR* (Chen, C.L. et al., 2013; Kallergi et al., 2008; Lu et al., 2010; Ting et al., 2014), *CDH1* (Armstrong et al., 2011; Ting et al., 2014), *MUC1*(Lu et al., 2010; Pierga et al., 2007; Zhu et al., 2014), *HER2* (Kolostova et al., 2015b), *ERBB2* (Kolostova et al., 2015b; Lu et al., 2010) |
| Epithelial mesenchymal transition (EMT) | *TGFβ1*(Powell et al., 2012), *FOXC1* (Powell et al., 2012), *CXCR4*, (Powell et al., 2012), *NFKB1*(Powell et al., 2012), *VIM* (Armstrong et al., 2011; Lu et al., 2010; Powell et al., 2012; Ting et al., 2014), *ZEB2* (Powell et al., 2012), *CDH11* (Ting et al., 2014), *PTPRN2* (Chen, C.L. et al., 2013), *ALDH1* (Chen, C.L. et al., 2013), *ESR2* (Chen, C.L. et al., 2013), *WNT5A* (Chen, C.L. et al., 2013), *Twist1* (Aktas et al., 2009; Giordano et al., 2012; Lu et al., 2010), *Akt2* (Giordano et al., 2012; Kolostova et al., 2015b), *PI3Kα* (Aktas et al., 2009; Kolostova et al., 2015b), *SNAIL1* (Giordano et al., 2012), *ZEB1* (Giordano et al., 2012), *TG2* (Giordano et al., 2012) |
| Metastasis | *S100A4* (Powell et al., 2012), *S100A9* (Powell et al., 2012), *S100A14* (Smirnov et al., 2005), *S100A16* (Smirnov et al., 2005), *NPTN* (Powell et al., 2012), *KLK3* (Smirnov et al., 2005), *CEACAM5* (Smirnov et al., 2005), *KRT 20* (Smirnov et al., 2005), *FABP1* (Smirnov et al., 2005) |
| PI3K/AKT/mTOR pathway | *AKT1, AKT2, PIK3R1, PTEN* (Powell et al., 2012) |
| Apoptosis | *BAX, CASP3, CD53, CD59* (Powell et al., 2012) |
| Stem cell phenotype | *CD24* (Giordano et al., 2012; Lu et al., 2010; Powell et al., 2012), *CD44* (Giordano et al., 2012; Lu et al., 2010; Powell et al., 2012), *CD133* (Armstrong et al., 2011; Giordano et al., 2012; Sun et al., 2013), *ALDH1* (Aktas et al., 2009; Giordano et al., 2012; Kolostova et al., 2015b) |
| DNA repair | *PARP1* (Powell et al., 2012) |
| Cell metabolism | *SLC2A1, TFRC* (Powell et al., 2012) |
| Cell proliferation | *RRM1, MAPK14* (Powell et al., 2012) |