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Identification and Quantitation of Circulating Tumor Cells

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Annu. Rev. Anal. Chem. 2017. 10:321–43

First published as a Review in Advance on March 6, 2017

The *Annual Review of Analytical Chemistry* is online at anchem.annualreviews.org

<https://doi.org/10.1146/annurev-anchem-061516-045405>

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Keywords

CTC isolation, live-cell capture, precision medicine, microfluidics, nanotechnology, biosensors

Abstract

Circulating tumor cells (CTCs) are shed from the primary tumor into the circulatory system and act as seeds that initiate cancer metastasis to distant sites. CTC enumeration has been shown to have a significant prognostic value as a surrogate marker in various cancers. The widespread clinical utility of CTC tests, however, is still limited due to the inherent rarity and heterogeneity of CTCs, which necessitate robust techniques for their efficient enrichment and detection. Significant recent advances have resulted in technologies with the ability to improve yield and purity of CTC enrichment as well as detection sensitivity. Current efforts are largely focused on the translation and standardization of assays to fully realize the clinical utility of CTCs. In this review, we aim to provide a comprehensive overview of CTC enrichment and detection techniques with an emphasis on novel approaches for rapid quantification of CTCs.

INTRODUCTION

It has long been accepted that the spread of cancer to multiple organs occurs as a result of cancer cells intravasating from the primary site into the circulatory system and then extravasating and propagating at distant sites, eventually leading to metastatic disease. This notion was first verified by Thomas Ashworth in 1869 during an autopsy of a metastatic patient, in which microscopically observed cells found in the blood resembled cancer cells (1). Detailed investigation into circulating tumor cells (CTCs) remains exceptionally challenging because they are present in extremely rare numbers, ranging from one to a few hundred in a 7.5-mL tube of blood draw (against a background of billions of blood cells). Multiple methods have been adopted to effectively capture and isolate CTCs. These techniques often take advantage of the distinct molecular biomarker profile and the physical traits of CTCs. Although numerous technologies have shown the effective isolation of CTCs, many require preprocessing of the blood samples with reagents that fix the cell, thus preventing the ability to capture viable CTCs for culture and functional characterization.

From a clinical standpoint, the interest in this field arises from the potential utility of CTCs for diagnosis and predicting prognosis, overall survival, evaluation of the risk of recurrence, and tracking the efficacy of chemotherapeutics. Multiple studies in metastatic disease of the breast (2, 3), prostate (4, 5), lung (6, 7), and colorectal system (8, 9) have thus far demonstrated that elevated CTC counts are an indicator of poor prognosis. Although these serial blood draws aimed at enumerating CTCs have proven their prognostic significance, researchers have realized that detailed molecular and functional characterization of CTCs can be powerful tools in precision oncology. CTC characterization will help monitor therapeutic response, drug efficacy, and the metastatic process, which is the underlying mechanism involved in tumor progression, as well as the mechanism leading to drug resistance.

As better fabrication techniques and tools have evolved, numerous developments have helped this field advance into the forefront of cancer research. In this review, we highlight the different methods that have been reported for the enrichment and quantitation of CTCs, with a special focus on translation of these assays into commercially available products and services.

CIRCULATING TUMOR CELL ENRICHMENT TECHNOLOGIES

CTC enrichment methods can be broadly divided into two major categories: label free and immunoaffinity based. Typically, one CTC exists among a background of millions to billions of blood cells, thus presenting a needle in a haystack scenario (10, 11). Hence, the key technical challenge is to efficiently capture CTCs in a manner amenable to downstream processing and analysis. The technologies discussed here represent the significant advances that have been made in this field and showcase the ongoing efforts to develop robust clinical utilities that can potentially aid clinicians in the management of cancer patients.

Label-Free Enrichment of Circulating Tumor Cells

CTC enrichment achieved through the exploitation of biophysical properties is often referred to as label free, as these methods are biomarker agnostic and can capture cells independently of the expression profile exhibited by the CTCs. In other words, this strategy relies on exploiting the density, size, deformability, and electric charge of CTCs. The capabilities often extend beyond CTCs and involve the isolation and enrichment of cells that have undergone epithelial-to-mesenchymal transition (EMT), cells that display cancer stem cell-like features (12), and possibly cancer-associated fibroblasts as well (13).

Density gradient–based circulating tumor cell enrichment. This method employs centrifugation for cell separation, as first demonstrated in 1959 by S.H. Seal. On the basis of his observations that different cell types within whole blood displayed different specific gravities, a blend of silicone oils was used to achieve the optimal separation medium (14).

Essentially, centrifugation results in the separation of the following layers (from bottom to top): erythrocytes, granulocytes, density gradient, buffy coat (the fraction containing mononuclear cells, including CTCs), and plasma. Currently, some of the commercially available separation gradients are Ficoll-Paque (GE Healthcare), Lymphoprep (STEMCELL Technologies), and OncoQuick (Greiner Bio-One). Whereas Ficoll-Paque and Lymphoprep are simply density-gradient media, OncoQuick has incorporated a porous barrier that rests above the separation media to capture CTCs while allowing the erythrocytes and leukocytes to pass through. The rate of CTC capture for both OncoQuick and Ficoll-Paque is approximately 70–90% (15, 16). However, OncoQuick reduced the number of coenriched mononuclear cells (MNCs) by 632-fold (9.5×10^4 the mean number of MNCs) in comparison to Ficoll-Paque, which has a 3.8 depletion factor (1.6×10^7 MNCs). This greatly benefits the end user's task for immunocytochemical tumor detection (15, 16). A pitfall to this technique is the inability to precisely extract all the plasma after centrifugation; thus, it experiences the loss of potential CTCs. In addition, there is possible loss of CTCs that may have migrated into the plasma fraction as well as the loss of aggregate/cluster formations of CTCs that may have been displaced to the bottom of the gradient.

STEMCELL Technologies has developed the RosetteSep CTC Enrichment Cocktail to be used in combination with either their own Lymphoprep or Ficoll-Paque. The method integrates the use of immunoaffinity-based enrichment along with centrifugation and negative selection to remove unwanted cells. When mixed into peripheral whole blood, this antibody cocktail binds to bispecific tetrameric antibody complexes that are directed against cell surface antigens found on human hematopoietic cells (CD2, CD16, CD19, CD36, CD38, CD45, and CD66b) and glycophorin A on erythrocytes. This results in the hematopoietic cells forming rosettes by cross-linking to multiple erythrocytes and thus precipitating at the bottom when centrifuged on a density-gradient medium, while the CTCs are left in the plasma fraction above the density-gradient medium.

RareCyte has gone a step further by developing AccuCyte, which is fundamentally still a density-gradient separation technology that integrates a separation tube and collector device. This allows the buffy coat to be collected into a small volume for application onto a microscopic slide without cell lysis or wash steps, which are regarded as the potential sources of CTC loss during enrichment. For downstream analysis, their CyteFinder system is an automated scanning digital microscope and image analysis system that presents high-resolution images of candidate cells stained for CTC markers.

Size–based enrichment of circulating tumor cells. Filtration techniques for the enrichment of CTC date back to the 1950s and 1960s, as reported by Seal (who also reported the aforementioned density-gradient separation) for testing glass, polyethylene films, and a metal-based filter (17). As fabrication techniques have improved along with material science discoveries, the filtration techniques now emerge as a front-runner for CTC enrichment.

Circulogix. The microfilter technology commercialized by Circulogix, along with their automated fluid handler, is one of the early technologies used for the size-based enrichment of CTCs. Through different architectures of microfilters, this technology allows capture of both fixed cells for enumeration and molecular characterization (round pore filter) (18–20) and viable cells for culture and functional characterization (slot pore filter) (21). The filters are fabricated using

Parylene C material through photolithography to achieve the precise dimensions and geometry for each filter type. The round pore filter provides a matrix of 40,000 pores in a 200×200 -pore matrix array with 8- μm pores for the capture of CTC post fixation with 1% formalin. The captured CTCs are amenable to immunofluorescence for enumeration, interrogation for expression of biomarkers, or fluorescence in situ hybridization (FISH) to detect chromosomal abnormalities. This size-based and biomarker-agnostic technology is able to enrich CTCs from solid tumors, sarcomas, and melanomas. Although the round microfilter technology is a potentially powerful tool for CTC enrichment, enumeration, and immunophenotypic molecular characterization, it requires that the blood samples be partially fixed in 1% formalin prior to filtration to preserve cell integrity and morphology. Cross-linking of proteins introduced by formalin fixation eliminates the possibility to perform functional studies on CTCs, such as subsequent cell culture or protein extraction and analysis.

The slot pore microfilter allows whole blood to be filtered without the need for prior fixation and is reported to have more than 90% capture efficiency and viability. Furthermore, recent developments have led to the ability to release viable CTCs from the filter surface after capture with high release efficiency. To enable maximal release of the cells, a thermoresponsive polymer coating is applied to the filter surface before capture. A transition in temperature after capture results in >90% cell release and the harvest of viable CTCs (22). Both fixed-cell and live-cell samples can be processed using the round pore or slot pore filters through the Circulogix automated FaCTChecker filtration system.

ScreenCell and ISET (Rarecells Diagnostics). In contrast to the Circulogix microfilters created through photolithography, these techniques employ track-etch membranes through either chemical etching or bombarding the membrane with charged particles (23). ISET (isolation by size of epithelial tumor cells) has a 12-well system in which each well uses a 0.6-cm membrane with 8- μm pores that is capable of filtering 1 mL of diluted blood (blood diluted 1:10 with filtration buffer) (24). ScreenCell membranes have slightly smaller pore sizes measured at 6.5–7.5 μm , and samples are processed using their custom ScreenCell Cyto device and color correction filtration apparatus for cytology and live-cell capture, respectively (25).

Parsortix (ANGLE). Parsortix leverages microfluidic channels in contrast to filter membranes. The channels are developed with a stair-like architecture that causes a gradual decrease in width until it reaches approximately <10 μm . Following enrichment, the direction of flow is reversed to harvest the lodged cells (26).

Inertial focusing. This technique leverages inertial effects that are experienced in microfluidic devices comprising two forces: a shear-gradient lift force (directing particles toward the channel wall due to the parabolic profile characteristics of laminar flow) and a wall-effect lift force (directing particles away from the wall). Characteristics such as the channel dimensions, channel aspect ratio, flow rate, and particle diameter directly impact the magnitude and direction of these lift forces (27). Vortex Biosciences has developed the Vortex Chip that uses a high aspect ratio rectangular channel to create two equilibrium positions alongside the channels. This causes the blood sample to gravitate toward the equilibrium positions and flow along the channel walls. Along the channel's length are eight walls opening into reservoirs designed to create microvortices to capture and trap CTCs. As the cells approach these openings, the wall-effect lift force tends to decrease. The shear-gradient force causes the larger cells to get pulled into the reservoirs, where they remain trapped and orbit in the microvortices while the smaller cells remain in the main stream (28). The CTCs are later harvested by using a slower flow rate.

Dielectrophoresis. Dielectrophoresis (DEP) exploits the electrical signature of cells based on their morphology, phenotype, and composition (29). DEP is able to move cells within a nonuniform electric field due to their polarizability (30). While the cells are polarized, applying a positive or negative DEP results in the particle moving toward or away from the electric field source, respectively. In the ApoStream (ApoCell) system, CTCs are attracted by the positive DEP separating them from the rest of the sample that is directed away through negative DEP. This is accomplished through a DEP crossover frequency where the DEP forces make a transition from a negative force to a positive force and thus result in cell separation (31, 32). Whereas ApoStream employs a migratory method for the separation of cells, DEPArray (Silicon Biosystems) uses a retention method through trapping cells in DEP cages generated using electrodes (33, 34). The system is coupled to a high-resolution imaging device and allows for single-cell isolation (35).

Affinity-Based Capture of Circulating Tumor Cells

Traditional immunoaffinity-based enrichment has been the most widely used strategy, with CellSearch representing the sole entity cleared by the US Food and Drug Administration (FDA) to date. The method relies on using biomarkers expressed on the cell surface for either positive selection of tumor cells through an antiepithelial-cell-adhesion-molecule (EpCAM) or negative depletion by removing the background of white blood cells through targeting the antigen CD45, which is expressed by CTCs. In an effort to improve CTC capture efficiency, some technologies opt for an antibody cocktail that contains surface cancer-specific antigens; examples include human epidermal growth factor receptor 2 (HER2) for breast cancer or prostate-specific membrane antigen (PSMA) for prostate cancer, in addition to EpCAM. Typically, the antibodies are conjugated to magnetic beads, and as the antibodies bind to the cells of interest, a magnetic field can be utilized to capture the cell. Alternatively, antibodies are immobilized on a surface, and as the sample flows over this surface, the antibodies bind the cells of interest. The cells can then undergo downstream *in situ* analyses, or the surface bound antibody is cleaved, which allows for the retrieval of captured cells. The pitfall of this system, however, lies in the fact that a universal CTC-specific antigen has not been identified to date. In addition, CTC antigen expression is heterogeneous and can range from a high to a low or negative expression of the marker(s), which makes it difficult to effectively capture these rare cells. It is noteworthy that over that last few years, researchers have recognized that CTCs undergo an EMT and exhibit stem cell markers, thus rendering their expression profiles even more complex.

Immunomagnetic affinity-based positive enrichment. Immunomagnetic affinity-based enrichment can be performed either through positive enrichment or negative enrichment. In positive enrichment, CTCs are targeted using antibodies conjugated with magnetic beads against cell surface markers such as EpCAM. Negative selection allows for the depletion of hematopoietic cells through targeting the surface marker CD45. Both methods have advantages and disadvantages. Although positive selection specifically targets CTCs, thus providing a higher purity of the captured cells, it also fails to capture cells with low expression or negative expression of the marker. Negative selection also allows for the enrichment of the CTC subpopulation, but this results in lower purity. We focus primarily on the positive enrichment approaches because they are far more numerous as commercial entities in this space; however, there are also commercially available negative enrichment platforms on the market. The aforementioned STEMCELL Technologies has commercialized the EasySep Human CD45 Depletion kit. Another platform is the Quadrupole Magnetic Separator, which is also a magnetic-based system but one coupled with a flow-through system that allows for cell sorting.

CellSearch. In recent years, a number of new technologies have tried to further push the envelope on utilizing affinity-based capture of CTCs. This drive can be largely attributed to the prognostic value that has been demonstrated by CellSearch from the enumeration of EpCAM⁺/CK⁺/CD45⁻ cells captured from patient blood samples (2, 36, 37). The CellSearch system uses magnetic capture with EpCAM antibodies conjugated to nanomagnetic particles. Downstream immunostaining for positive identification of CTCs is dependent on positive expression of cytokeratin (CK4, CK 5, CK6, CK8, CK10, CK13, CK18, and CK19), negative expression of CD45, and positive DAPI (4',6-diamidino-2-phenylindole) fluorescence, which form the core identification criteria. A limitation of the CellSearch technique is its reliance on the expression of EpCAM, which can vary. Therefore, these EpCAM-negative or low-expressing EpCAM cells are missed during capture and isolation and excluded from morphological evaluation (35–41). However, as CellSearch is the only FDA-approved system to aid in monitoring patients with metastatic prostate, breast, and colorectal cancers, it has become the primary benchmark for validating new technologies.

AdnaTest (AdnaGen). Whereas CellSearch approaches the isolation of CTCs through a singular antibody directed at capture through EpCAM, AdnaTest involves a cocktail of antibodies (e.g., for breast cancer, EpCAM plus MUC1) directed toward the cancer of interest. CellSearch presently has tests targeting breast, prostate, ovarian, and colorectal cancers. Both systems utilize antibodies linked to magnetic particles for capture, but their downstream analysis methods differ. CellSearch identifies and enumerates CTCs through microscopy, whereas AdnaTest's downstream analysis is achieved through lysis of the isolated cells and the measurement of mRNA transcripts by reverse transcriptase-polymerase chain reaction (RT-PCR) for tumor-associated gene expression (e.g., for breast cancer, GA733-2, MUC1, and HER2). Samples are considered CTC positive in the event that at least one or more of the three markers showing the expression signal intensity is equal to or greater than 0.15 ng/ μ L. Although AdnaTest Breast has an equivalent sensitivity to the CellSearch platform (42), AdnaTest Colon performed with superior sensitivity compared to the CellSearch method (43).

The magnetic-activated cell sorting system (Miltenyi Biotec). The magnetic-activated cell sorting system (MACS) technology is a system that uses a specialized separation column in addition to immune-magnetic labeling with microbeads. The magnetic nanobeads, typically 10–85 nm in diameter, are linked to EpCAM for CTC enrichment (44, 45). Following incubation with the immunonanobeads, the sample is passed through a specialized column that can be magnetized to allow capture of the magnetically labeled cells and thereafter demagnetized to allow release and collection of these cells. Additionally, they have breast cancer-specific microbeads linked with HER2 (44), and if negative depletion of peripheral blood mononuclear cells is desired, CD45-linked microbeads can be utilized.

MagSweeper. The MagSweeper technology isolates CTCs by sweeping a magnetically controlled magnetic rod through wells containing samples that have been premixed with antibody-coated magnetic beads (46). The magnetic rod is covered with an ultrathin (25 μ m) nonadherent plastic sheath. Once the rod has swept the well and captured cells, it moves to a wash station where fresh buffer washes away contaminating unlabeled cells that are nonspecifically bound to the rod. Next, the plastic sheath is disengaged into a release well, and an external magnetic field is applied under the well to facilitate the release of labeled cells, excess magnetic particles, and unlabeled cells trapped along aggregated magnetic particles. Thereafter, the rod reengages with the plastic sheath, and the labeled cells are recaptured, leaving behind the unlabeled cells and excess magnetic

particles. Although this workflow substantially decreases the number of background cells, it also results in a decrease in capture efficiency (46).

Microfluidic affinity-based enrichment. Whereas the previous technologies hinged upon magnetic-based immunoaffinity capture, the technologies in this section describe the utilization of immunoaffinity coupled to microfluidics and the hydrodynamic-based enrichment of CTCs. Microfluidic devices have had success in manipulating microliter amounts of simple liquids in microscale channels (47–49), but thus far they have shown limited capability to deal with the cellular and fluid complexity of large volumes (milliliters) of whole blood samples (50–53).

CTC-Chip. The CTC-Chip was the first to employ the use of microfabrication to create a microfluidic device coupled with antibodies for the capture of CTCs. The design consists of an array of 78,000 microposts that are chemically functionalized with EpCAM antibodies to capture CTCs as they flow through the microfluidic channel. Though the CTC-Chip can process whole blood, the flow rate is limited to 1–2 mL/h, and as flow rates are increased to above 2.5 mL/h, the capture efficiency significantly decreases (53).

CTC-iChip. The second generation CTC-iChip technology first separates the smaller blood components, such as red blood cells and platelets, from larger nucleated cells, such as white blood cells (WBCs) and CTCs, by using microposts; it then uses inertial focusing to align the larger cells into a single file. Lastly, using magnetic fields, the CTCs are separated from the WBCs through either positive or negative enrichment (54).

GEDI Chip. The geometrically enhanced differential immunocapture (GEDI) chip uses a geometry configuration that reduces capture of contaminating leukocytes by generating size-dependent cell-wall collisions. The principle is based on the geometry of microposts' offset from row to row (55, 56). In addition, cancer-specific markers, such as PSMA (56) for prostate cancer and HER2 for breast cancer (55), are coated onto microposts to provide affinity-based capture. The increased CTC capture rate is attributed to the increased collision rate of CTCs into the microposts and the facilitation of affinity-based capture by the respective antibodies. The GEDI chip is able to process 1 mL/h of blood sample (56).

OncoCEE. Whereas the previous two technologies, CTC-Chip and GEDI, have opted to use either EpCAM or cancer-specific marker (PMSA or HER2) antibodies, OncoCEE (CEE: cell enrichment and extraction) has expanded its system to include an antibody cocktail consisting of antibody tumor-associated biomarkers (e.g., HER2, MUC1, EGFR, TROP2) and mesenchymal markers (e.g., N-cadherin) (57). OncoCEE's chip consists of 9,000 variable diameter posts (75–150 μm lumen in diameter) that are randomly placed to disrupt laminar flow and thereby minimize straight-line, regularized streamline flow. This maximizes the probability of contact collision between target cells and the antibody-coated posts, resulting in CTC capture (58).

Isoflux (Fluxion Biosciences). This system is based on microfluidically connected reservoirs in which the sample well flows into an isolation compartment at an optimized velocity to control the incubation time. Prior to the enrichment step, the sample is initially processed using the density gradient-based separation followed by incubation of the buffy coat fraction with anti-EpCAM magnetic beads. In the separation chamber, the prelabeled cells are attracted to the top by a high magnetic field. Meanwhile, the continuous flow carries the unbound cell to the waste reservoir (59).

LiquidBiopsy (Cynvenio). LiquidBiopsy's system uses a multilayer sheath flow to prevent non-specific binding of the cells to the devices' magnetized surfaces. The sample is prelabeled with anti-EpCAM magnetic beads and flows into the device through a central channel with a density-adjusted buffer stream on either side. At the capture region, a magnetic field attracts the labeled cells and directs them out from the central stream to the top upper glass surface. To enhance specific capture, antibodies to specific biomarkers, such as HER2, MUC1, TROP2, and MelCAM, have been utilized with the system (60).

CytoTrapNano (CytoLumina Technologies). CytoTrapNano is based on a polydimethylsiloxane (PDMS) chaotic mixer that is coupled to a microfluidic chip with a patterned silicon nanowire substrate linked to EpCAM. The goal of better capture efficacy through this method is to mimic the concept of Velcro, and cells are captured due to the enhanced local topographic interactions. In addition, the EpCAM antibody is present to further increase the capture efficiency; examples report an efficiency of >80% (61, 62).

Surface-based affinity enrichment. In contrast to micropost arrays, these devices have antibodies coated directly to the surfaces that capture the CTCs as they flow through the device. The advantages of this approach are the easier fabrication and ability to scale up production. In addition, this form of architecture permits faster flow rates while making it easier for imaging. This is in contrast to the microposts' three-dimensional structures, which are nontransparent and thus add an additional complexity to the imaging process. Some of the well-known devices in the category are herringbone (HB)-chip (63), geometrically enhanced mixing (GEM)-chip (64), and the graphene oxide (GO)-chip (65). The HB-Chip and GEM-Chip share similarities, as both are fabricated from PDMS and designed to disrupt laminar flow through the herringbone-induced microvortices; this results in increased cell interactions with the antibody-coated surfaces and thus facilitates CTC capture (63, 64). In contrast, the GO-Chip is fabricated using graphene oxide nanosheets that are adsorbed on a 58,957-flower-shaped patterned gold surface onto which EpCAM antibodies are immobilized (65). BioFluidica has taken a different approach by taking hot-embossed and laser-ablated thermoplastic to create a chip consisting of up to 320 sinusoidal microchannels. The channels are coated with anti-EpCAM and/or antiseprase/fibroblast activation protein alpha to capture different phenotypes of CTCs. These captured CTCs are later released through trypsinization and fed into a secondary module that enumerates the cells via an impedance sensor. Lastly, the cells are transferred to the staining and imaging module (66).

In vivo affinity-based enrichment. Whereas all of the technologies discussed thus far have achieved in vitro enrichment of CTCs, the GILUPI CellCollector offers an in vivo option. A Seldinger guidewire functionalized with anti-EpCAM antibodies is placed intravenously, which captures CTCs as they pass by in the bloodstream. This method allows for larger sample volume, as the wire is left in place for 30 min during the collection period (67).

Imaging-based enrichment-free CTC detection. All of the aforementioned technologies involve some method to enrich CTCs from whole blood prior to analysis. Several technologies have adopted a methodology that skips the enrichment step and focuses on analyzing the whole sample using advanced high-speed fluorescence imaging. The Epic Sciences method involves creating monolayer preparations of nucleated cells from whole blood that has undergone red blood cell lysis (35, 68). The monolayer preparations are split over a number of custom glass substrates (68). Following anticytokeratin and anti-CD45 staining, the slides undergo imaging and analysis using a custom algorithm (35). An alternative system commercialized by MiCareo is called

ensemble-decision aliquot ranking. This method shares some similarities with flow cytometry and involves labeling cells with surface markers such as EpCAM and HER2 for positive identification and cell sorting using their automated system (69).

NOVEL ASSAYS FOR CIRCULATING TUMOR CELL DETECTION

CTC enumeration provides valuable data for cancer diagnosis, prognosis, and disease progression as well as prediction of treatment response. Although CTCs have been concentrated through the enrichment process, there are still substantial amounts of contaminating blood cells accompanying the enriched sample, a situation that requires methods to distinguish one cell type from another. Among current detection methods, immunocytochemistry is the most commonly used to distinguish CTCs from contaminating blood cells (53). CTCs can be identified by using antibodies to recognize cytokeratin, which is specifically expressed in epithelial cells, whereas leukocytes can be excluded by showing anti-CD45 positivity, and nucleated cells are confirmed through DAPI for nuclear staining. Even with significant advances in techniques for CTC isolation that enable improved yield and purity of captured CTCs, an immunostaining approach following an enrichment step remains the gold standard for quantification of CTCs. However, a multiple-step process is the major drawback of immunocytochemistry, which is labor intensive and time consuming, hindering the clinical utility of CTC-based point-of-care tests. Other widely used approaches for identification of CTCs are nucleic acid-based methods, such as real-time quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR), which distinguishes CTCs based on gene expression of epithelial-specific markers, such as EpCAM and cytokeratins (70). However, RT-qPCR requires an RNase-free environment. Additionally, the amount of epithelial-specific transcripts may vary among the CTC population. The recent advent of the digital PCR enables direct analysis and detection of individual CTCs without the need for RNA extraction (71).

With recent advances in the field of nanomaterials, emerging new approaches enable a substantially shorter processing time for CTC detection with high sensitivity as well as the feasibility for detecting CTCs *in vivo* in the comparison of traditional methods, such as immunocytochemistry and RT-qPCR (72–74). In this section, we focus on the latest technologies for CTC identification and quantification based on classification in two different categories. First, we introduce three promising nanomaterials [gold nanoparticles (AuNPs), magnetic nanoparticles (MNPs), and carbon materials] and their unique properties for CTC detection. Second, we discuss several detection techniques based on different sensing principles and the progression of each technique in CTC applications. In each category, we provide a comprehensive overview of the theory behind the techniques and a discussion of their advantages and limitations.

Gold Nanoparticle-Based Techniques

AuNPs have emerged as a promising tool in detecting CTCs due to their unique optical and physical properties (75, 76). Similar to other metal particles, AuNPs have freely moving electrons around the particle surface. When electromagnetic radiation interacts with particles, it induces a collective coherent oscillation of the free electrons across the nanoparticles. This oscillation causes surface plasmon resonance (SPR), resulting in strong light absorption and scattering. The SPR frequency can be tuned based on the particle size, shape, material properties, and surrounding medium. Another advantage of using AuNPs to detect CTCs is that the surface of AuNPs can be easily tailored by surface functionalization due to strong binding with molecules through thiol or amine groups (77).

AuNPs have been widely used as the effective substrates for surface-enhanced Raman scattering (SERS), which provides a unique fingerprint-like spectral pattern and allows researchers to distinguish from other interferences in the complex blood milieu (78). Wang et al. (79) reported that AuNPs conjugated with Raman reporter molecules and epidermal growth factors (EGFs), the ligands of EGF receptor (EGFR), can rapidly detect CTCs in peripheral blood from patients with squamous cell carcinoma of the head and neck; these CTCs have high expression of EGFR. In this study, polyethylene glycol (PEG) is used to form a layer to prevent the binding of blood cells. However, the thick PEG layer attenuates SERS intensity. To overcome this drawback, Wu et al. (80) employed a thin layer of reductive bovine serum albumin instead of using PEG to eliminate non-specific interaction with blood cells. Techniques to identify and quantify different subpopulations of CTCs, especially mesenchymal and stem-like CTCs, are valuable because those types of CTCs are more likely to play an important role in cancer metastasis (81, 82). Nima et al. (83) developed tunable silver and gold nanorods with a narrow SERS spectrum. These SERS nanoparticles were coated with four different Raman-active molecules and tumor-specific antibodies, respectively, which allow multiplexed detection of individual tumor cells in blood (as shown in **Figure 1a**).

Developing techniques that allow detection of CTCs *in vivo* is highly desired because they provide *in situ* monitoring of CTCs in real time and avoid a cumbersome procedure of sample preparation and CTC isolation. However, *in vivo* approaches for CTC detection require highly sensitive deep-tissue detection of CTCs and safe operation in humans in terms of toxicity and immune responses. Photoacoustic (PA) imaging has shown promising potential for *in vivo* imaging application due to its high spatial resolution and a deeper imaging depth (84). Furthermore, there is no requirement of ionizing radiation, which makes PA imaging safer than radionuclide-based imaging techniques, such as positron emission tomography and single-photon emission computed tomography. Recently, AuNPs were widely used as PA contrast agents for *in vivo* tumor imaging due to their strong absorption of light and tunable SPR (85). Another advantage of using AuNPs for *in vivo* applications is that they are highly biocompatible nanomaterials compared to other nanomaterials, such as quantum dots (QDs) (86). To achieve high sensitivity of CTC detection in the bloodstream, a combination of MNPs and AuNPs for magnetic enrichment and PA imaging is employed (87). By using a photoacoustic/photothermal flow cytometry technique combined with the magnetic enrichment method, CTCs were detected 10 min after nanoparticle injection in the bloodstream of the ear vein of a nude mouse with breast cancer xenografts (**Figure 1b**). Later, Hu et al. (88) combined two modules into a single formulation as the multifunctional composite particles. In this study, particles consisted of silica-coated AuNPs, multiple iron oxide-based MNPs, and a PEG outer layer with a targeting molecule. The result shows that detection sensitivity of 1 cell/mL can be achieved.

Magnetic Nanoparticle-Based Techniques

MNPs are commonly used in isolation of analytes, including nucleic acids, proteins, and cells under a magnetic field. However, they can also be utilized as biosensing probes where highly sensitive detection is achieved. Issadore et al. (89) developed a microfluidic chip-based micro-Hall detector for CTC detection based on measurement of magnetic moments of magnetically labeled cells in the presence of an external magnetic field. As a consequence, the signal intensity is proportional to the number of magnetically labeled cells. In addition, using MNPs with different sizes allows multiplexed detection of different CTC subpopulations, which enhances detection accuracy in clinical samples due to tumor heterogeneity. The results showed that this technique can directly detect CTCs in whole blood without any enrichment steps, and it found a higher number of CTCs in patients with ovarian cancer than did the CellSearch system (**Figure 1c**).

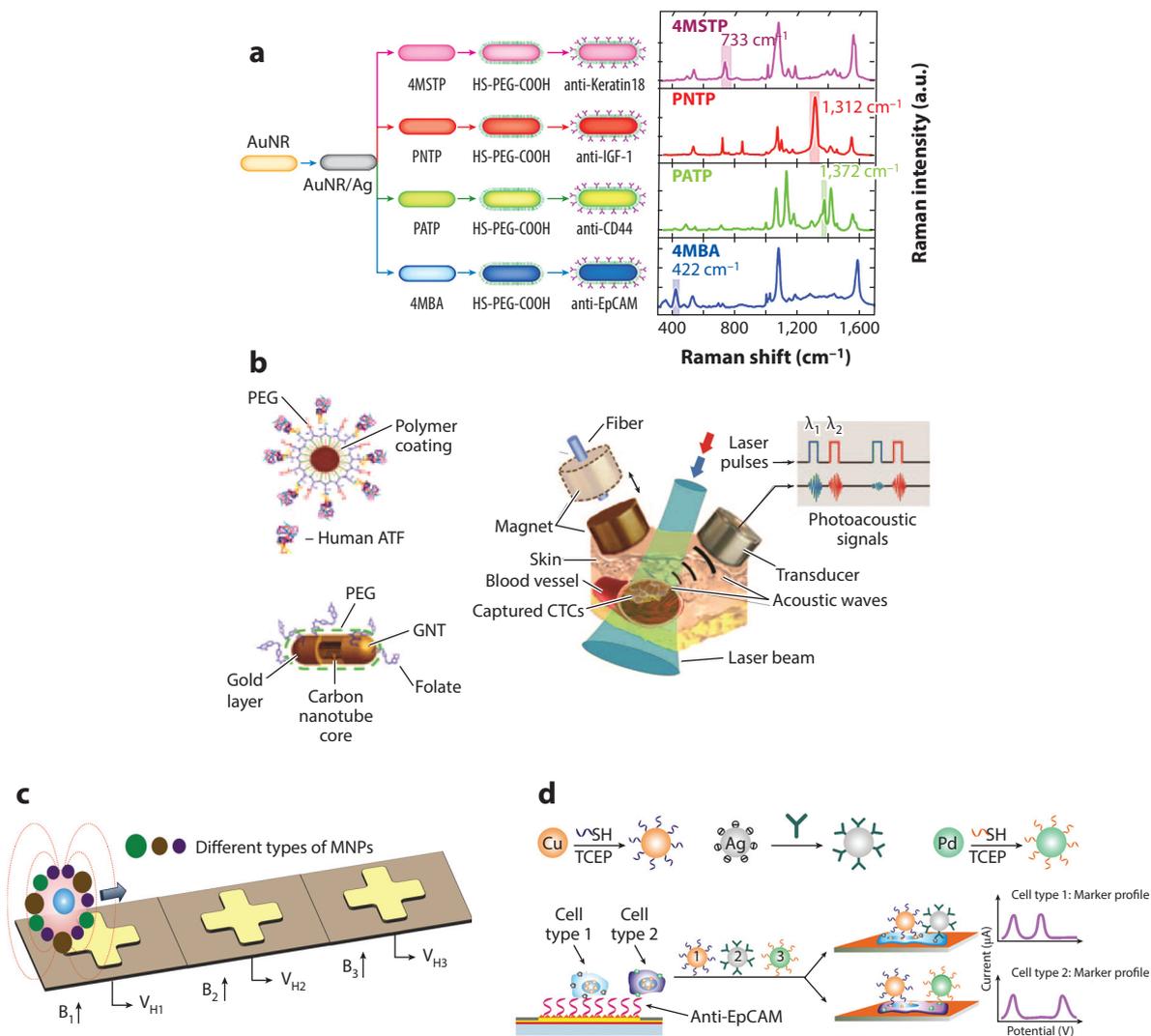


Figure 1

Nanoparticle-based detection methods for CTCs. (a) Multiplex surface-enhanced SERS-based CTC detection using tunable silver-coated AuNRs functionalized with different SERS contrasts and targeting antibodies. Reproduced with permission from Reference 83. Copyright 2014, Nature Publishing Group. (b) Dual-functional platform of magnetic enrichment and gold nanoparticle-mediated photoacoustic CTC detection in vivo. (Top left) ATF of urokinase plasminogen activator-labeled MNPs for CTC enrichment. (Bottom left) Gold-coated carbon nanotubes conjugated with PEG and folate for photoacoustic detection of CTCs. Reproduced with permission from Reference 87. Copyright 2009, Nature Publishing Group. (c) Multiplex detection of CTCs using a microfluidic chip-based micro-Hall detector based on measuring the magnetic movement of cells labeled with different sized MNPs under different magnetic fields. Reproduced with permission from Reference 89. Copyright 2012, AAAS. (d) Multiplexed electrochemical detection of CTCs by labeling cells with different types of metallic nanoparticles after capturing them on SiO₂ electrodes. Reproduced with permission from Reference 111. Copyright 2014, John Wiley & Sons. Abbreviations: ATF, amino-terminal fragment; AuNR, gold nanorod; CTC, circulating tumor cell; EpCAM, antiepithelial-cell-adhesion-molecule; GNT, gold nanotube; MNP, magnetic nanoparticle; PEG, polyethylene glycol; SERS, surface-enhanced Raman scattering; SiO₂, silicon dioxide.

Nuclear magnetic resonance is another technique in which MNPs can be used as proximity sensors to modulate the transverse relaxation time (T₂) of surrounding water molecules (90). Ghazani et al. (91) developed a micronuclear magnetic resonance–based technique for detection and quantification of CTCs in human whole blood. When CTCs are labeled with MNPs in the blood sample, the MNPs create local dipole fields with strong spatial dependence, which accelerates the transverse relaxation of water protons and results in shorter T₂ than in nonlabeled cells. This approach shows that it detects a higher number of CTCs in patients with ovarian cancer in comparison with the CellSearch system. Moreover, the combination of four markers (EpCAM, EGFR, HER2, and MUC1) enables the stratification of 99.2% of samples from various cancers as malignant and is used to increase the sensitivity of CTC detection.

Carbon Material–Based Techniques

Carbon materials possess remarkable electronic properties that make them ideal for applications in electronic devices (92). Carbon nanotubes (CNTs) are promising semiconducting materials due to their high current-carrying capacity, high carrier velocity, one-dimensional electrostatics, and use as active conductive channels of field-effect transistors (FETs) (93). CNT-FETs have the potential for label-free and ultrasensitive detection of chemical and biological molecules due to strong variations of conductance induced by binding of molecules on the surface of CNTs. Shao et al. (94) generated a single-wall CNT-FET array functionalized with antibodies that specifically recognizes surface antigens of tumor cells in blood. The binding of tumor cells to the CNT-FETs diminished 60% of electrical conductivity, and the signal change in conductivity of a single CNT-FET array was produced by the binding of only one cell. Although this technique achieves single-cell detection, determination of the absolute number of CTCs in a sample becomes complicated if the number is more than that of CNT-FET arrays or if CTC binding takes place at locations other than the CNTs. Moreover, assays can only be performed in extremely small sample volumes (<10 μ L). In addition to FET applications, CNT-based impedance sensors have also been developed for real-time CTC detection (95). The multilayer CNTs were immobilized on a surface of an indium tin oxide electrode, and EpCAM antibodies were attached on the top layer for capturing tumor cells. The binding of tumor cells on the device led to an increase of the electron-transfer resistance, and electrical responses were linearly associated with the concentration of the cancer cells spiked in whole blood.

Graphene is another attractive carbon-based electrochemical sensor material with its high electron mobility and conductivity as well as low intrinsic electrical noise (96). Feng et al. (97) developed a label-free electrochemical impedance–based aptasensor for CTC detection by immobilizing AS1411 aptamer on the graphene electrode. AS1411 forms a stable G–quadruplex structure through the treatment of potassium ion solution, showing high binding affinity to nucleolin antigen, which is overexpressed on the surface of tumor cells. Once tumor cells are captured by AS1411, it induces the increase of the electron-transfer resistance; changes in electrical current are measured by using electrochemical impedance spectroscopy and cyclic voltammetry. Moreover, captured viable cells can be released from the electrode via the treatment with complementary DNA of AS1411 (**Figure 2a**). The electrode also can be regenerated by washing with water to remove complementary DNA. Nanographene materials, such as graphene quantum dots (GQDs) and carbon dots, possess bright photoluminescence, and their optical properties can be easily tuned in the visible light region by modulating the size and surface of functional groups (98–100). In recent years, nanographene materials have emerged as superior bright fluorescent probes for biological imaging due to their excellent photostability and biocompatibility with cells and animals (101). Ray and colleagues (102) developed multifunctional biocompatible graphene QDs coated

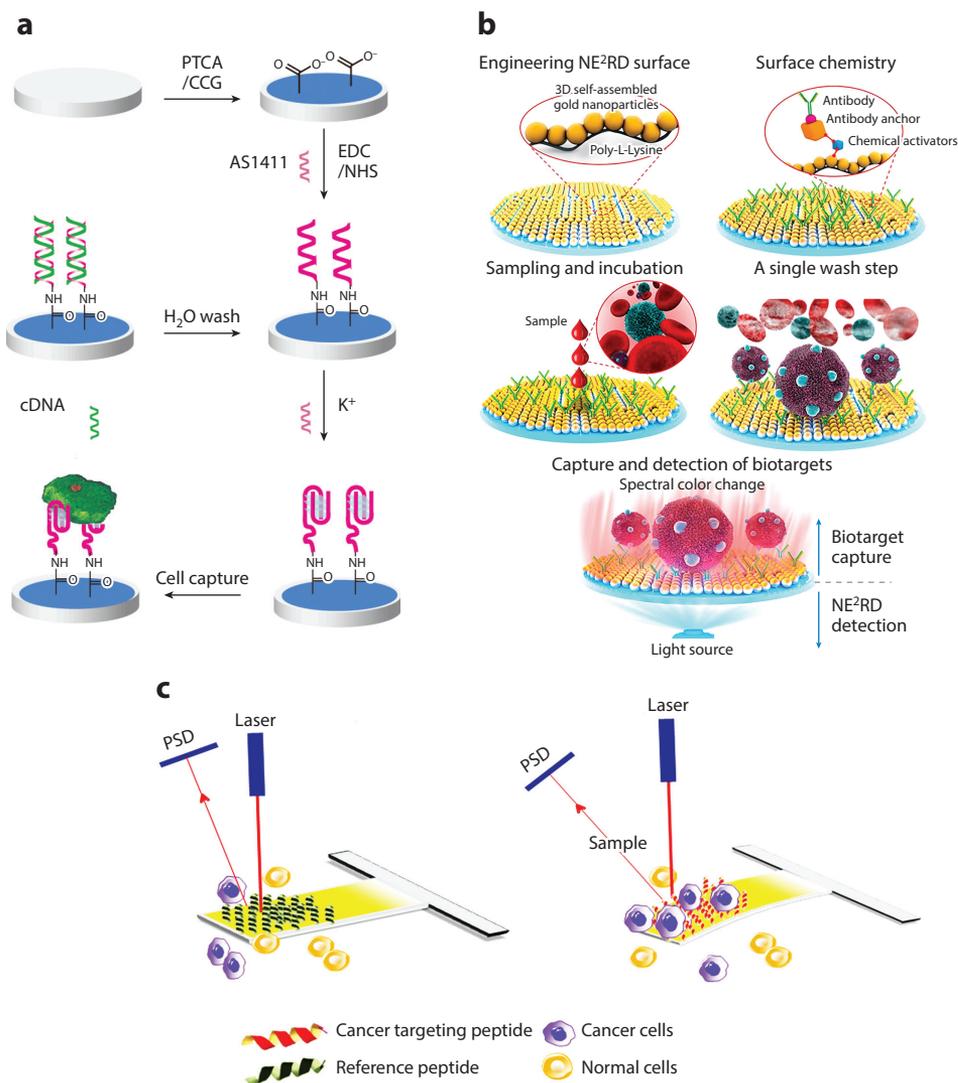


Figure 2

Label-free detection methods for CTCs. (a) Graphene-based label-free CTC sensors functionalized with tumor-specific aptamers for capture, detection, and release of CTCs. Reproduced with permission from Reference 97. Copyright 2011, Elsevier. (b) A NE²RD for label-free detection of CTCs based on measuring the wavelength shifts of incident light. Reproduced with permission from Reference 132. (c) A microcantilever-based device for real-time label-free detection of CTCs based on measuring the deflection of a light beam. Reproduced with permission from Reference 144 under the terms of the Creative Commons Attribution 4.0 International License, <http://creativecommons.org/licenses/by/4.0>. Abbreviations: CTC, circulating tumor cell; NE²RD, nanoplasmonic electrical field-enhanced resonating device; PSD, position sensitive detector.

on the surface of magnetic nanoparticles for the selective isolation and quantification of CTCs. Via attachment of capture antibodies on the GQD-coated MNPs for specifically separating tumor cells from blood under magnetic field, the GQD-labeled tumor cells were quantified using two-photon imaging (103).

Electrochemical-Based Detection Techniques

Electrochemical-based assays that convert a biological event into electronic signals have received significant attention due to their ease of use, low cost of manufacture, and excellent detection limits, even within a small sample volume (104, 105). A well-known example is the glucose meter that has been commercialized as a routine sensor for monitoring blood glucose levels. Based on these advantages, researchers have developed various types of electrochemical-based methods for detection and quantification of CTCs. For example, measuring the transfer of electrons from redox mediators to an electrode can be used to quantify the number of CTCs (106, 107). Qu et al. (107) developed an ultrasensitive CTC detection method by functionalizing tumor-specific aptamers on the surface of a glassy carbon electrode. Once tumor cells are captured by aptamers on the electrode surface, the electron transfer from the redox mediator is hindered and the electrical current decreases. This is correlated with the number of tumor cells on the glassy carbon electrode. The differential pulse voltammetry experiment dramatically shows single-cell detection in the presence of 10^9 WBCs. However, this assay cannot be performed directly with blood samples due to the strong background signals induced by plasma proteins. In addition, the electrode must be pretreated with WBCs to reduce the background signals before starting the CTC detection process. Those drawbacks limit its clinical utility (107).

Electrochemical immunoassays have been investigated for use in CTC detection and integrated with microfluidic techniques in a single platform to perform cell capture, the quantification of capture cells, and the release of capture cells for further downstream analysis (108–110). For example, Hong et al. (109) developed an integrated multifunctional device by immobilizing conductive polypyrrole polymer on the electrode with anti-EpCAM antibodies for capturing tumor cells. After labeling with horseradish peroxidase (HRP)-conjugated nanoparticles on the captured tumor cells, the electrochemical current signals can be generated by the catalytic activity of HRP toward the reduction of hydrogen peroxide. Based on the amount of current generated, the number of cells can be quantified. After electrochemical detection, the captured tumor cells can be released by electrical simulation or glutathione treatment for further analysis (109). Additionally, multimarker electrochemical detection of CTCs can also be achieved by using a panel of redox-active probes to specifically label different target antigens. Wan et al. (111) have demonstrated the feasibility of this approach by utilization of functionalized metal nanoparticles for the specific recognition of different biomarkers on cancer cells. This enables the distinction of different subpopulations of cancer cells based on the electrochemical oxidation potentials of metal nanoparticles (**Figure 1d**).

Electrochemical impedance spectroscopy is another promising approach for detecting CTCs based on the increase of the electron-transfer resistance upon cell binding. Its main advantage over other types of electrochemical-based assays is a label-free method, which avoids the sophisticated protocols during electrochemical detection. The combination of microfluidic techniques into integrated systems has shown significant potential for the quantification of CTCs (112, 113). It is also valuable for distinguishing both epithelial and mesenchymal types of CTCs without the need for any postlabeling due to the different membrane capacitance between the two cell types (114). Recently, Shen et al. (115) developed a label-free and reusable electrochemical device showing the ultrasensitive detection of CTCs and the capability to retrieve captured CTCs by the enzymatic excision of uracil residues in captured aptamers after electrochemical detection.

Optical-Based Detection Techniques

Fluorescence-based techniques are the most widely used detection methods in biological applications. QDs are bright and photostable inorganic nanoparticles with a broader excitation spectrum and a narrow symmetric photoluminescence emission, which allow for multiplex detection using a single excitation laser source without signal overlap (116–118). Researchers have developed several QD-based platforms with a combination of capture modalities for CTC detection *in vitro* and *in vivo* (119–123). However, QD cytotoxicity could potentially cause cell damage, resulting in the negative impact on further downstream analysis (124). Due to recent advances in nanomaterials, several types of photoluminescent nanoparticles have been developed, such as carbon dots and graphene QDs. These have shown high quantum yield and low cytotoxicity in living cells and could eventually replace QDs for CTC detection (102, 103, 125–127).

Label-free optical-based techniques have been used widely over the past decades for monitoring biomolecular interactions, such as protein–protein or protein–small-molecule interactions (128, 129). Several types of the commonly used label-free optical sensors are commercially available, such as SPR, resonant mirror, resonance waveguide grating, and dual polarization interferometry. Those sensors have shown their high sensitivity in biomolecule detection as well as the ability for real-time monitoring, which may provide a route for CTC detection (130). Mousavi et al. (131) developed an SPR-integrated microfluidic chip for capture and detection of CTCs in a single platform. When the cells are captured on the gold nanoslit that causes a wavelength shift of the SPR spectrum, those changes are associated with the number of captured cells. Recently, metal nanoparticles were utilized in the sensitivity enhancement of optical-based devices due to the localized surface plasmon resonance (LSPR). This unique property contributes significantly to enhancing the electric field on the surface of the particle as well as to photon absorption and photon scattering. Additionally, the resonance wavelength of the LSPR can be easily tuned by changing the size, shape, and materials of nanoparticles. Inci et al. (132) developed the nanoplasmonic electrical field-enhanced resonating device for detection of multiple biotargets in various clinical specimens without prior sample labeling or preprocessing. In the study, 10-nm AuNPs are immobilized on polystyrene substrate using poly-L-lysine. Recognition molecules are functionalized on the surface of AuNPs for capturing biotargets. Upon biotarget binding, the changes in wavelength shifts in nanoparticles can be measured (**Figure 2b**).

Bioluminescence-based assays are widely used in a variety of *in vitro* and *in vivo* biomedical applications due to their sensitivity and extremely low background (133, 134). In contrast to the fluorescence that requires external light excitation and results in high levels of autofluorescence background, bioluminescence is produced by a chemical reaction, making it suitable in the application of CTC detection. Bioluminescence-based assays are safe for use with viable cells (135), which enables further downstream analysis of CTCs. Our laboratories developed a bioluminescence-based assay by genetically fusing antibody mimetics with *Gussia luciferase* for rapid detection of CTCs without observing toxicity on the cells so that they can grow and expand in culture (Y.-P. Yang, R. Datar, S. Daunert, and R. Cote, unpublished data).

Mechanical-Based Detection Techniques

Because of the advances in micro- and nanofabrication technologies that can produce smaller and more sensitive mechanical transducers, mechanical-based biosensors have shown their feasibility in detection of biomolecules based on their physical and mechanical properties, such as mass, surface stress, and viscoelasticity (136, 137). Quartz crystal microbalance (QCM) technology is capable of measuring mass variations of bound analytes at the surface of QCM electrodes, and the mass change is related to the change in oscillation frequency of the quartz crystal resonator (138).

Pan et al. (139) developed the magnet-QCM system for the selective isolation and detection of leukemia cells. Surface acoustic wave (SAW) biosensors generate and measure acoustic waves using interdigital transducers on the surface of a piezoelectric material (140). Recently, Senveli et al. (141) developed the microcavity-coupled SAW biosensor that enables the capture and interrogation of single tumor cells. This device may show the future potential in the detection and quantification of CTCs.

Among mechanical biosensors, nanomechanical cantilever biosensors are the most promising devices for biological detection due to their high mass resolution and low mechanical compliances (142, 143). As biomolecules are captured, the binding induces changes in the surface stress of the binding side with respect to the lower side that is mechanically relaxed by the cantilever bending. This phenomenon makes the cantilever vibrate at its resonant frequency. The vibration can be measured using the optical beam deflection method, and the shift in resonance frequency is related to the binding molecule's mass. Etayash et al. (144) developed the peptide-modified microcantilever device for detection of cancer cells in real time (**Figure 2c**). To achieve clinical utility of the cantilever-based device for CTC detection, labeling the captured cancer cells with AuNPs to increase the mass on the surface may improve the detection sensitivity (145).

CONCLUSIONS AND PERSPECTIVES

CTCs have attracted considerable attention and become one of the most active areas of translational cancer research because of their vital role in bloodborne metastasis. However, the inherent rarity and heterogeneity of CTCs present substantial challenges to their clinical isolation, detection, and analysis. The recent emergence of new devices and materials has significantly improved the isolation efficiency and detection sensitivity of CTCs from blood. Among current techniques, microchip-based devices have shown promise in the development of CTC assays for the clinical setting owing to their capability of integrating microfluidic-based isolation techniques with nanomaterial-based detection systems into a single automatic platform. This decreases the processing time and prevents the errors or mistakes in the cumbersome multistep procedures.

Given the heterogeneous nature of CTCs and the increasing evidence showing that only certain subsets of CTCs are capable of progressing into metastases (81, 82), CTC detection techniques not only aim to quantify the total population of CTCs but also place emphasis on identifying subpopulations of CTCs. Another important direction of CTC research is the molecular and biological characterization of CTCs that can guide clinical decision-making. Importantly, these techniques cannot alter the molecular profiles of CTCs during processing, and they still require extensive clinical validation. Current techniques have also enabled the release of those captured CTCs that might allow culture expansion and single-cell analysis of those cells. Other liquid biopsy materials, such as cell-free circulating tumor DNA and circulating extracellular vesicles (exosomes and microvesicles), also have significant potential as surrogate markers in the monitoring and diagnosis of cancer (146–149).

DISCLOSURE STATEMENT

Richard Cote and Siddarth Rawal are shareholders of Circulogix Inc., which commercially produces the filters, filter cartridges, and an automated fluid handler for CTC capture.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health (R01:5R01CA141077-04, R21:5R21CA182050-02, R33:1R21CA123027-01/03) to Richard Cote.

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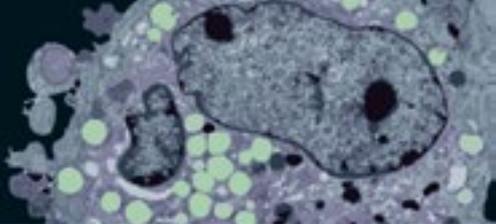
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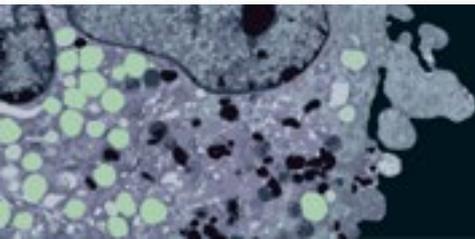
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