Current Trends in Microfluidics for Single Cell Isolation in Cancer Diagnostics Enabling Downstream Proteomics Applications

Abstract
Recent advances in Cancer diagnostics are adding newer insights every day. An extensive review of existing technologies enabling the non-invasive sampling of circulating tumor cells (CTCs) in peripheral blood aptly named as liquid biopsy is presented in this article. The significance of detecting the presence of CTCs which is one of the main objectives of liquid biopsy and their biological implications are described. Various approaches of liquid biopsy such as CTC characterization methods, technical challenges, molecular characterization of captured cancer cells, mechanisms to study these cells and downstream proteomic applications are explored with an emphasis on single cell proteome applications. Examples from recently published works are discussed to highlight the advantages and limitations of the different techniques. We summarize the vast scope and value of the wider implementation of CTCs as a cancer theranostic tool especially to monitor treatment response to targeted cancer therapeutic agents. Finally, a perspective on the future trends and promising research directions in this field are proposed.

Keywords: Cancer diagnostics; Liquid biopsy; Circulating tumor cells; Microfluidics; downstream applications; Single cell proteome analysis; Targeted therapies; Personalized medicine

Introduction
Cancer Diagnosis technologies are changing the healthcare scenario worldwide with huge socioeconomic impact. As we move into an era of individual personalized treatment the need for more sophisticated cancer diagnostics has emerged [1,2]. To date, cancer diagnosis and metastasis monitoring is mainly carried out through tissue biopsy and/or re-biopsy, a very invasive procedure limited only to certain locations and not always feasible in clinical practice [3]. Tissue biopsy only yields information about a very small area of tumor at the time of extraction and in some cases it is difficult or impossible to obtain the tissue sample [4]. The invasive nature of a biopsy poses a risk to patients and can have a significant cost [5]. The mere analysis of primary tumor alone may not yield sufficient information for making critical treatment decisions. There are several reports that many cases of NSCLC do not yield a accessible tissue [6]. Additionally the limitations of tissue procurement for genetic testing in certain cancers such as non small cell lung cancer (NSCLC) have necessitated the development of non-invasive techniques to study the primary tumor [7].

Tumor heterogeneity is another major issue where biopsies often suffer from sample bias leading to false diagnosis [8]. Inability to capture the heterogeneity during tumor development is one of the major reasons responsible for failure of cancer treatment. However it is well known that tumors are dynamic and change their mutation pattern often becoming resistant to non specific treatment. This poses problem to patients undergoing targeted therapies. It is in this context researchers are paving the way for a new diagnostic concept popularly known as liquid biopsy. Basically the objective is to analyze therapeutic targets and drug resistance-conferring gene mutations on circulating tumor cells (CTC) and cell-free circulating tumor DNA (ctDNA or cfDNA) released into the peripheral blood during metastasis [9].

Such detection of circulating tumor derived cells and their components released into the blood stream for cancer diagnosis has created immense interest in both academia and industry trying to translate these findings from bench to bedside. The primary area of interest is to use liquid biopsy as a cancer diagnostic tool that allows for patient screening, monitoring, and treatment response and recurrence detection after surgery [10]. In order to improve disease monitoring over time and to avoid painful procedures such as conventional tissue biopsy and/or radiological assessment, liquid biopsy undoubtedly represents a valuable addition to the oncologist's toolkit.

In this review we present an overview of the current existing technologies of liquid biopsy in various cancers and future challenges. Within the field of oncology, liquid biopsy can potentially be used to monitor tumor burden in the blood and for early detection of emerging resistance in the course of targeted cancer therapies.
Liquid biopsy approaches

Various technologies and devices have been developed for liquid biopsy in recent times using the key components available in blood. Main components in liquid biopsy being used are: circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), and tumor derived exosomes. However, circulating tumor cells (CTCs) constitute the main focus of most research studies. Several researchers have used the circulating tumor cells in peripheral blood to optimize the various technologies for cancer diagnosis and clinical validation.

Circulating tumor cells (CTCs) and biological implications

CTCs are defined as cells with an intact viable nucleus, cytokeratin positive, epithelial cell adhesion molecule (EpCAM) positive and with the absence of CD45. Cells that express cytokeratins but lack the expression of CD45, and those that have the cyto-morphologic characteristics of tumor cells (appropriate size, presence of a nucleus and appropriate nuclear to cytoplasmic ratio), are counted as tumor cells. Unfortunately EpCAM and other markers are not always expressed on CTCs and are down-regulated by processes such as epithelial to mesenchymal transition (EMT) [11].

Circulating tumor cells, a component of the “liquid biopsy”, holds great potential to transform the current landscape of cancer therapy. The detection of CTCs in the peripheral blood of patients with solid epithelial tumors (e.g., breast, prostate, lung, and colon cancer) holds great promise, and many exciting technologies have been developed over the past few years [12]. Detection of CTCs has been widely reported in various metastatic cancers such as breast, prostate, lung and colorectal cancers [13,14]. Recent studies also show a significant correlation between the CTC counts (enumeration) and prognosis of patients with cancer, suggesting that CTCs are either surrogates of metastatic activity or causally involved in the metastatic process [15-17].

Technologies for CTC detection and enrichment methods

The greatest challenge in detection of CTCs is their rarity in the blood. Consequently CTCs have to be enriched prior to any attempt at detection or characterization. A variety of technologies have been used for enrichment and detection of CTCs. Most CTC enrichment and detection technologies are based on their biological/physical properties that distinguish them from other normal blood cells. Most commonly employed technology is affinity-based enrichment. We have performed extensive literature survey to gather information related to various key technologies that have been developed during the last few years that have shown some degree of success in dealing with circulating tumor cells in various cancers. We present these unique and innovative technologies and their cell capture efficiencies in this review.

DFIR imaging technology: Discrete Frequency Infrared Imaging Technology (DFIR) was used by [18]. Involving a series of samples from multiple patients presenting with different cancers. In this approach human blood serum liquid biopsies were prepared by spotting onto a single slide and subsequently air-dried and then samples were imaged as one DFR image mosaic. For each patient, a single representative spectrum comprising of just 14 discrete frequencies was obtained. The spectra were then tested for cancer detection using a radial-basis-function support vector machine (SVM) model. Each cancer spectrum in its class was tested against a model built on a population of remaining cancer patient spectra and all control spectra. The final conclusion of cancer or non-cancer nature of samples depends on a classification test.

Nanotechnology Based Approaches: Studies reported using surfaces patterned with nanostructures can facilitate cell binding by increasing the surface area and disturbing the flow. Wang, et al. [19] patterned the surface with silicon nanopillars and showed improved cell capture as compared to a flat surface. Halloysite nanotubes, multivalent DNA nanospheres, and TiO2 nanoparticles have been studied and demonstrated their abilities to enhance cell capture on the surface [20-22].

Ghadani, et al. [23] used targeted magnetic nanoparticles and a point-of-care micro-NMR system, for the analysis of cancer targets of selected biomarkers (EpCAM, EGFR, HER-2 and Vimentin) in both CTCs and fine needle biopsies of solid epithelial tumors. The study involved preparation of tetrazine (Tz)-modified nanoparticles, sample processing and CTC detection using quad-labeling method in which primary antibodies against EpCAM, MUC-1, HER-2 / EGFR were added as a cocktail. Molecular characterization and the heterogeneity of the captured CTCs were detected by cellular expression levels. Cheng, et al. [24] reported a transparent, bio-compatible nanostructured surface for cancer cell capture and culture of CTCs. Multifunctional materials made of hydroxyapatite/chitosan (HA/CTS) are efficient in CTC detection and proven to be useful for further analysis of CTCs. Recently, Yoon, et al. [25] reported the isolation of CTCs on functionalized graphene oxide nanosheets.

Vortex technologies: Sollier, et al. [26] combined the use of micro-scale vortices and inertial focusing for the high-purity extraction of CTCs from blood samples. Parameters such as channel dimensions and flow rates are considered to arrive at an optimal device for maximum trapping efficiency and purity. Factors such as effect of blood dilution, red blood cell lysis and cell deformability were considered for clinical validation while demonstrating cell viability and independence on EpCAM expression. They were successful in extracting CTCs and enumeration from the blood of patients with breast and lung cancer patients.

Affinity based systems: Most commonly employed technology is affinity-based enrichment to separate CTCs from blood cells. This approach uses the property of distinctive antigens expressed either by CTCs but not blood cells (e.g., EpCAM), or by blood cells but not CTCs (e.g. CD45). Immuno-magnetic separation is the most common strategy which uses magnetic beads coated with specific antibodies that bind to CTCs (positive enrichment) and such devices come in various formats such as columns, beads and cartridges etc.

The CellSearch™ system is currently the only US-FDA approved method for detection of CTCs in breast, colon and prostate cancer. CellSearch™ is a fully automated system for detection of CTCs and has been shown to be reproducible across different independent testing sites. It uses immuno-magnetic beads based enrichment.
technique. Positive selection is done by EpCAM-labeled iron oxide nanoparticles, and subsequent detection of cytokeratin-positive CTCs. The system possesses the capability of additional detection channels for HER2/neu in breast cancers, using magnetic beads coated with a monoclonal antibody-targeting epithelial cell marker, such as EpCAM [27,28] expressed by the CTCs.

**Microfabricated devices**: Current CTC detection techniques mostly rely on epithelial markers such as EpCAM but accumulating evidence suggests that CTCs show heterogeneous EpCAM expression due to the epithelial-to-mesenchymal transition (EMT). Masashi, et al. [29] used a Micro Cavity Array system (MCA) integrated with miniature device for the isolation and detection of CTCs in lung cancer patients. Similarly, Kim, et al. [30] reported a microchip filter device incorporating slit arrays and 3-dimensional flow that can separate heterogeneous population of cells with marker for CTCs.

Enumeration of circulating tumor cells is used for cancer diagnosis and prognosis, while DNA analysis or enumeration of nucleated red blood cells is useful for prenatal diagnosis or hypoxic anemia, and that of circulating stem cells to diagnose cancer metastasis. Isolation of these cells and their downstream analyses can provide significant information such as the origin and characteristics of a disease. Novel microchip platforms: Microfluidics is a technology that enables transport and manipulation of fluids and particles such as cells in the microscale. A typical microfluidic device consists of a microchannel network integrated with various sensors and actuators. Common microchannels have dimensions on the order of hundreds of microns, while the size of cells ranges from several nanometers to tens of micrometers. Therefore, strong interactions between cells, the fluid flow and the microchannels are expected because of the similar length scales. The small size of microfluidic devices also allows for the implementation of new protocols such as single-cell analysis or on-chip cell culture [31]. Such microfluidics platforms are ideal for isolation, enrichment and analysis of rare cells [32,33].

Several microfluidic devices, with a focus on affinity-based isolation (e.g. antigen-antibody reaction) have been proposed in recent papers. Each one of these micro-devices use different sorting mechanisms such as immuno-affinity, physical separation, dielectrophoresis, or magnetic and fluorescence activated sorting [32]. Label-free cell separation techniques have been proposed to get around the limitations of using antibody based methods and to address the issue of EMT where the expression of epithelium-specific biomarkers is highly variable. Examples include DEP and hydrophoresis that have shown promising results for the isolation and detection of CTCs [34]. Recently, DEP-based separation of rare cells, including oral cancer cells, colorectal cancer cells, prostate tumor initiating cells, and melanomas, has been reported [35-39]. In this approach, Cells become polarized in a non-uniform electric field and can be manipulated by the induced dielectrophoresis (DEP) force [34,40].

Shim, et al. [41] recently reported a continuous flow-based dielectrophoretic field-flow-fractionation (DEPFFF) technique. This technique is capable of processing 10 mL of blood sample in less than 1 hour. As cells passed over the electrodes, the target cells were attracted towards the bottom of the channel because of a positive DEP force, while non-target cells were forced away from the bottom because of the negative DEP force. Thus, the two cell types were brought to different heights in the channel, and eventually were separated due to the balance between sedimentation. Microfluidic chips based on immune-affinity principle rely on the differential adhesion between cells can be created either through immobilizing immuno-affinity-binding molecules or implementing specific surface patterns. Compared to other approaches using immunochemical signature, surface-based cell separation requires fewer or no sample preparation steps [42-46]. One of the first reported microchips was an affinity-based microfluidic chip developed for CTC enrichment by creating an array of microposts coated with EpCAM antibodies [47,48]. CTC capture efficiencies were reported to be > 60%. Sheng, et al. [49] also reported the use of micropost structures for capturing CTCs using an aptamer coated surface.

Gleghorn, et al. [50] also used a micropost array to create size dependent enhancement of cell-surface interactions. Their study used the microchips in clinical samples and CTCs from blood samples of cancer patients with metastatic lung, prostate, pancreatic, breast and colon cancers. With these methods monitoring of the CTCs can be real time in metastatic cancer patients with correlation of CTC count with tumor response. In these affinity based microchip methods the blood flow which is slow in initial development of chips needs to be optimized leading to successful capture of CTCs in a given sample (7.5-10ml) of blood. Stott, et al. [51] implemented herringbone structures in a microfluidic channel to enhance cell-surface interaction. The herringbone structures generate chaotic advection current enabling rapid fluid mixing.

Another class of microdevices involves flow through micro channels containing micro pillars, nanowires, or patterned grooves, aimed at increasing the interaction between cells and antibody-functionalized surfaces. A miniature microfluidic chip with a magnetic sifter with dense array of magnetic pores was reportedly developed by Christopher Earhart, et al. [52] for the capture of CTCs in NSCLC patients. In this study a high efficiency of capture of tumor cells labeled with magnetic nanoparticles was reported. One major limitation for surface-based isolation of rare cells is the difficulty in releasing the viable cells after capture.

**Microfluidics for single cell isolation**

Microfluidic lab-on-a-chip technologies for single-cell applications demonstrate potential research areas such as cancer research [31,53,54]. Microfluidics play a crucial role in the protocols for separation, isolation and analysis of singe cell. Currently in hundreds of clinical trials ongoing, assays are lacking for comprehensive molecular characterization of CTCs with diagnostic precision. Alberter, et al. [55] described the importance of single cell CTC capture significance in personalized medicine in view of genomic disparity of potentially high relevance between primary tumors and CTCs. Peker, et al. [56] combined a workflow for enrichment and isolation of pure CTCs with a non-random whole genome amplification method for single cells and applied it to 510 single CTCs and 189 leukocytes of 66 CTC-positive breast cancer patients. Though it is evident that real commercially
available systems of these technologies for single cell are very few, the applications of microfluidics in the area of cancer diagnostics are dynamic.

Size based microchips: The size of tumor cells has been increasingly used as a property for their enrichment. Tumor cells derived from solid tumors are larger in size and this property is used for their separation from peripheral blood [57-59]. Recently, micro fabricated devices for size-based separation of tumor cells have been widely developed to enable precise and efficient enrichment of CTCs from whole blood [60-63]. Masahito, et al. [64] described a size based CTC isolation in NSCLC patients and developed a micro cavity array (MCA) system integrated with a miniaturized device without relying on EpCAM expression and tested its efficacy in NSCLC patients. Very recently the Screencell® device (Paris, France) has been developed, employing similar principles as the isolation by size of epithelial tumor cell platform, where CTCs are enriched in a size-based fashion. Although commercially available, no significant clinical data has yet been reported using this technology.

Alternative microfluidic chip methods: Other alternative microfluidic devices based on hydro-dynamic flow and size-based separations were also developed for the detection of CTCs [65-67]. Dielectrophoresis technique for the separation of target CTCs has also been reported [68-69]. In this approach application of dielectric forces through micro-electrode arrays allows the cells to get separated on the basis of similar properties [70,71]. The capture efficiency of >90% was reported in this technique. Further in this technique blood flow is to be slow and needs to be in isotonic medium with low conductivity. Recent studies also reported using the technique of dielectrophoresis for CTC isolation [72].

Acoustofluidics technologies: For the past few years a new way for particle/cell separation- acoustofluidics has been reported using Acoustic waves to separate cancer cell. Adams, et al. [73] described an ultra-high-throughput (1 L/h) Acoustophoretic microdevice, which was able to remove RBCs from human whole blood with an efficiency rate of 95%. Further, acoustofluidic technology was also utilized for separation of prostate cancer cells from WBCs in blood [74]. Yang & Soh [75] applied acoustic wave technology for sorting of viable MCF7 breast cancer cells from nonviable cells. However, acoustic-wave-based rare cell separation is still in early research stages and needs further development and optimization.

Technical challenges

Detecting CTCs remain technically challenging in view of their very low concentrations of one tumor cell in background of millions of blood cells. Thus their characterization and identification requires extremely specific and sensitive analytical methods [76,77]. This is particularly more tedious task in early stage cancer patients in view of very low concentrations of CTCs and requires more sensitive assays [78].

EMT is another major challenge to detection of CTCs. This mechanism which naturally occurs during the organogenesis is associated with aggressiveness of metastasis and is assumed to leave its impact on the CTCs in blood [79,80]. Some reports suggest that CTCs that have undergone mesenchymal transition may possibly revert back to an epithelial phenotype [81]. If this is also true in patients with cancer, CTCs with the highest EMT/mesenchymal–epithelial transition (MET) plasticity might be the most aggressive ones, supporting both tumor cell dissemination and initiation of metastatic outgrowth [12]. Although the relevance of the EMT to cancer patients is hypothetical it is imperative to optimize the current detection methods for CTCs that enable to distinguish CTCs from other blood cells.

Tumor heterogeneity is undoubtedly a major challenge in CTC analysis. Current targeted therapies for a cancer patient are recommended only after performing a comprehensive genomic analysis of the primary tumor to identify the clinically actionable molecular targets. However, the heterogeneity of the individual tumor cells may limit the purpose of CTCs in evaluating targeted therapy options. The metastatic cells may gain unique and additional genomic characteristics over time not detectable in bulk of primary cells. It is further complicated by the fact that CTCs are known to be circulating in cluster of cells instead of singles. This feature is a major limitation and has several implications in therapeutic decision making process. Hence direct analysis of metastatic cells will provide additional information.

A key challenge to unlocking the clinical utility of CTCs lies in the ability to detect and isolate these rare cells using methods amenable to downstream characterization and other molecular applications [82]. The status of genes that express therapeutic targets together with downstream analysis of molecular targets in specific cancers in CTCs of individual patients of similar cancer cases, might provide new insights and additional information. Implementing CTC analyses as major component of liquid biopsy also gives insights into anti-cancer drug mechanisms.

CTC characterization and Downstream Applications

The role of CTC detection and characterization may become increasingly relevant in order to manage patients being treated in the adjuvant therapies or for metastatic disease. Advances in molecular technology are enabling the molecular characterization of isolated CTCs at single cell level [83,84]. Recent studies on KRAS mutation status in patients with colorectal status successfully validated the molecular characterization of CTCs [85,86]. These studies with concrete data suggest that the molecular characterization of CTCs, in addition to the enumeration of CTCs, can contribute to improvements in cancer patient management. In another study on patients in NSCLC, CTC clusters were isolated and it is hypothesized that forming in clusters provides CTC with advantages over remaining solitary in terms of survival, proliferative capacity, and ability to form micro metastases [87]. This feature makes them amenable for downstream analysis either by genomics or proteomics approaches.

Downstream proteomics applications:

A key challenge to unlocking the clinical utility of CTCs lies in the ability to detect and isolate these rare cells using methods amenable to downstream characterization and other applications [82]. The status of genes that express therapeutic targets together

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with downstream analysis of molecular targets using genomics or proteomics approaches in specific cancers in CTCs of individual patients will give insight. However, the heterogeneity of the individual tumor cells may pose problems using downstream genomics applications for the captured CTCs. Hence direct analysis of metastatic cells will provide additional information. In this context downstream proteomics applications have additional advantages.

Numerous analytical methods have been developed to analyze proteins such as gel electrophoresis, immunoassays, chromatography and mass spectrometry. However, these methods require a large number of cells for analysis, resulting in a population-averaged measurement. Isolation of circulating tumor cells after microfluidic technologies, for diagnostic purposes may require single cell proteomics technologies [88]. However, recent advances in multi-parameter flow cytometry, microfluidics and other techniques have made it possible to measure wide variety of proteins in single cells. Often identical cells behave differently in responding to cancer therapy i.e. effects of drug are varied from cell to cell. Single cell-level measurement of proteins will answer this question providing new insights and more information. In a clinical context, single-cell level examination of isolated cells (CTCs) may provide valuable information about possible drug targets amenable to therapeutic treatment and subsequently to drug therapy.

Challenges in single cell proteomic analysis

The biggest challenges to measuring proteins in single cells are very small amounts of proteins in single cells and the enormous complexity. Proteomic measurements can assumedly be more complex in terms of protein abundance or expression levels, post-translational modifications, protein translocation, interactions with other proteins, DNA and protein activity etc. [88]. It is however, difficult to apply a single analytical method that can measure all of these protein parameters in a single cell and hence a combination of methods needs to be applied.

Single cell analysis by flow cytometry: The most established technique for single cell protein analysis is flow cytometry. Flow cytometry is an effective technique to quantify the localized protein concentrations in an intact cell. The profiling of entire pathways in a single cell is possible by designing suitable flow cytometry panels in intact cells. This has been enabled because of the availability of highly specific antibodies [89,90]. Perez & Nolan [91] are the successful pioneers in using flow cytometry for multi-parameter analysis using multicolor flow cytometry measuring as many as 15 proteins in signaling pathways of single cells. The ability to perform multi parameter analysis established flow cytometry as powerful tool [92,93]. Krutzik & Nolan [94] developed a dye based barcoding method allowing Multi parameter flow cytometry analysis with high throughput screening measurements for drug screening (example multiple kinase measurements for drug screening). Hence flow cytometry can be efficiently used for measurements of variety of cellular proteins such as kinases, phosphatases and glycosylation levels and cytokine production can also be measured in a single intact cell.

Microfluidic flow cytometry: In general flow cytometry requires a sample of large number of cells and sample preparation is done manually. However, it is hard to process samples from a biopsy, tissue specimens, or from blood etc in view of limited number of cells. Of late microfluidic platforms that integrate sample handling with flow cytometry and cell sorting [95,96]. An integrated microfluidic device that can perform imaging and microfluidic flow cytometry has been developed by Srivatsava, et al. [97]. This techniques can perform multiple tasks with in an experiment, i.e. cell culture, stimulation and sample preparation thus drastically reducing the sample amount and reagents etc.

Mass cytometry

A new promising technique called mass cytometry where in the throughput feature of flow cytometry is combined with ultra sensitivity of mass spectrometry [98]. In this approach the cells are stained with 20-30 antibodies conjugated to different metal isotope containing polymers. After injecting the labeled cells the metal tags are quantified using the inductively coupled plasma TOF MS (time of flight mass spectrometry). Bendall, et al. [99] measured 34 parameters including binding of antibodies, viability, and DNA content using this mass cytometry technique.

Affinity arrays for single cell protein analysis: In this approach antibody platforms that are immobilized with surface antigens are used. The antibody-coated PVDF membrane to bind target protein followed by detection with a second antibody was modified to use fluorescence based detection of cytokines from single cells [100]. However this technique is time consuming takes many hours. Ma, et al. [101] developed clinical microchip for the high throughput analysis of single cell cytokines levels from tumor antigen-specific cytotoxic T cells and could be useful for profiling of other immune pathways. Improved affinity array method has been developed where in cytokines released from single cells were detected by antibodies with covalently attached fluorescent oligomers that can be amplified to increase detection sensitivity up to 200-fold [102].

Mass spectrometry based single cell protein analysis: Mass spectrometry is a unique powerful tool for achieving the quantitative analysis entire proteome of single cell including proteins, peptides and PTMs (post translational modifications). In this approach no need for molecular labels and a routine sensitivity femtomolar levels is achieved. For single cell analysis Electrospray MS, LDI MS (laser desorption MS), SIMS (secondary ion MS). For neuropeptides analysis in neurons, MALDI [matrix assisted Laser desorption/ionization] MS is successfully utilized [103]. The biggest limitation of MS is the lack of sensitivity [low signal/noise] to detect low amount of proteins typically found in single cells. Integrating microfluidic cell lysis and capillary electrophoretic separation with electrospray mass spectrometry was used for high throughput detection of hemoglobin in individual erythrocytes was reported by Mellors [104]. Urban, et al. [105] combined microarray with MS analysis and this approach holds promise for increasing the throughput of single cell protein analysis.
Separation based single-cell protein analysis: There are thousands of expressed proteins in a single cell. High resolution separations of these proteins by multidimensional separations enable entire proteome analysis. Conventional analytical separation techniques such as HPLC or slab gel Electrophoresis are non-feasible techniques for single cells. Capillary electrophoresis is a more promising approach and has demonstrated the ability to separate and analyze proteome of single cell in particular in mammalian cells [106]. Two dimensional electrophoresis further adds efficiency achieving total separation of proteome [107]. Huang, et al. [108] reported microfluidic device based approach that has integrating capacities for cell capture, lysis and electrophoresis etc. finally reporting analysis of rare protein species. Despite these advances separation based methods are not routinely used due to the fact analysis of low abundant proteins in single cells is difficult using these methods.

Molecular probes based single cell proteome analysis: Molecular probes enhance the accuracy and sensitivity of protein measurement by their binding to target molecules [proteins of interest]. Antibodies are largely used molecules for labelling purposes. But they do not enter live cells. In studies of new antigens new antibodies need to be generated. Moreover antibodies are too expensive. Hence an alternative strategy of using the GFP [green fluorescent protein] family probes that can be genetically fused with protein of interest that can be detected and quantified in for large scale genome analysis [109]. However, limitations of using GFP probes are: they need recombinant approach of the cells and thus limiting their application to cultured cells only. To overcome this bottleneck a new class of compounds known as Bio-orthogonal fluorescent probes have been developed. These compounds act as smaller probes to tag with proteins. These probes use bisarsenical fluorescent dye which specifically reacts with target proteins [110].

Discussion

Future perspective of single cell proteome analysis

Future of single cell proteome analysis technologies depend on several factors. Top priority is high affinity probes needed for detection of low abundance of proteins, post-translational modifications and protein interactions etc. Development of informatics tools that are customized with single cells and can integrate with other omics technologies [genomics, metabolomics, and transcriptomics] will lead to wide use of single cell technologies.

Another fact is that the current targeted therapy for a cancer patient is made after genomic analysis of primary tumor using molecular target. However, the heterogeneity of the individual tumor cells may limit the purpose of CTC count in therapy efficacy. The metastatic cells may gain unique and additional genomic characteristics over time not detectable in bulk of primary cells. This feature is severe limitation and have implications in therapy decisions. Hence direct analysis of metastatic cells will provide new insights and additional information. Thus it can be concluded that implementing CTC analyses as liquid biopsy also gives insights into anti-cancer drug mechanisms.

Conclusion

Although a number of CTC detection techniques for isolation and enrichment have been developed in the recent years, they need to be continually improved. The concept of liquid biopsy originally meant for the analysis of CTCs is also being applied to ctDNA and exosome analysis. Although published data in metastatic cancer patients with high CTC numbers exists [111-114] larger prospective clinical trials are needed for demonstrated clinical use. Further the new approaches/studies must be validated in clinical-intervention trials with end points such as disease free or overall survival of the cancer patients leading to enabling the outcome of successful individualized therapy. The identification of most aggressive CTCs in metastatic cancers and their tissue of origin would help to localize small, occult metastatic lesions and guide further diagnostic and therapeutic strategies. Future studies are needed to show whether the CTCs in blood are representative of relevant metastatic clones and also needs to be explored in clinical trials. CTCs and ctDNA are complementary technologies and their parallel use in large prospective clinical trials might be able to explain the current resistance by solid tumors to targeted therapies. Prospective multicenter clinical trials should be initiated for treatment decisions of specific cancer patients.

Overall the increasing evidence offered by CTC counts is reflecting cancer progression in real time and in future the CTC detection and characterization should be amazingly promising in developing further targeted therapies to cancer specific Patients. Novel technologies will increasingly enable the molecular characterization of CTCs and definition of biomarkers for therapeutic strategies. Technical progress promises an exciting era of new biologic insights into the process of cancer metastasis. Thus it can be concluded that implementing CTC analyses as a liquid biopsy using microfluidic lab-on-a-chip medical devices can unravel patient status and give new insights into anti-cancer drug mechanisms. In the near future every oncologist will recommend a liquid biopsy test before, during and after a therapeutic intervention. This is possible only when simple affordable point-of-care diagnostic devices are developed for real time circulating tumor cell detection and enumeration, treatment monitoring and surveillance for cancer recurrence.

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Conflict of Interest

The authors disclose that there is no conflict of interest. The authors would like to disclose that the review article covers topics related to their core business model.
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