Review Article
Open Access

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

Volume 3 Issue 4 - 2016

NBRK Venugopal, Shibichakravarthy Kannan
Theranosis Life Sciences, Hyderabad, India

Correspondence: Shibichakravarthy Kannan, Founder & CEO, Theranosis Life Sciences Pvt Ltd, T-Hub, IIIT Campus, Hyderabad, India, Tel +918886233344, Email skannan@theranosis.com

Received: April 07, 2016 | **Published:** May 12, 2016

Abbreviations: CTC, circulating tumor cells; ctDNA, circulating tumor DNA; NSCLC, non-small cell lung cancer; EMT, epithelial mesenchymal transition

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 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 Hughes et al.[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 DFIR 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]

Ghazani 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, biocompatible 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). Masahito 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 microdevices 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 immunoaffinity-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. Polzer 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 (1L/h) Acoustophorestic 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 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 genomic 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 assumingly 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 HPC or slab gel Electrophoresis are non-feasible techniques for single cells. Capillary electrophoresis is a more promising approach and has demonstrated 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 probes have been developed. These compounds act as smaller probes to tag with proteins. These probes use bi-arsenical 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 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 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. 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 muse 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 cfDNA 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.

Acknowledgements

The authors (NBRK and SK) acknowledge the seed grant received from Center for Innovation Incubation & Entrepreneurship (CIIE) affiliated with the Indian Institute of Management (IIM, Ahmedabad) for supporting our endeavor in putting together this review article.

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.

References

1. Lewis JM, Heineck DP, Heller MJ. Detecting cancer biomarkers in blood: challenges for new molecular diagnostic and point–of–care tests using cell–free nucleic acids. *Expert Rev Mol Diagn*. 2015;15(9):11871–200.
2. Ryska A. Molecular pathology in real time. *Cancer Metastasis Rev*. 2016;35(1):129–140.
3. Massihnia D, Perez A, Bazan V, et al. A headlight on liquid biopsies: a challenging tool for breast cancer management. *Tumour Biol*. 2016;37(4):4263–4273.
4. Ondroušková E, Hrstka R. Circulating tumor DNA in blood and its utilization as a potential Biomarker for cancer. *Klin Oncol*. 2015;28 Suppl 2:2S69–2S74.
5. Markuszewski MJ, Kaliszan R. Using bioanalysis for cancer diagnosis and prognosis. *Bioanalysis*. 2014;6(7):907–909.
6. Wan JW, Gao MZ, Hu RJ, et al. A preliminary study on the relationship between circulating tumor cells count and clinical features in patients with non–small cell lung cancer. *Ann Transl Med*. 2015;3(22):352.
7. Marchetti A, Del Grammastro M, Felicioni L, et al. Assessment of EGFR mutations in circulating tumor cell preparations from NSCLC patients by next generation sequencing: toward a real–time liquid biopsy for treatment. *PLoS One*. 2014;9(8):e103883.
8. Bedard PL, Hansen AR, Ratain MJ, et al. Tumour heterogeneity in the clinic. *Nature*. 2013;501(7467):355–364.
9. Pantel K, Alix–Panabieres C. Real–time Liquid Biopsy in Cancer Patients: Fact or Fiction? *Cancer Res*. 2013;73(21):6384–6388.
10. Bayarri–Lara C, Ortega FG, CuetoLadrón de Guevara A, et al. Circulating Tumor Cells Identify Early Recurrence in Patients with Non–Small Cell Lung Cancer Undergoing Radical Resection. *PLoS One*. 2016;11(2):e0148659.
11. Grover PK, Cummins AG, Price TJ, et al. Circulating tumour cells: the evolving concept and the inadequacy of their enrichment by EpCAM–based methodology for basic and clinical cancer research. *Ann Oncol*. 2014;25(8):1506–1516.
12. Alix–Panabieres C, Pantel K. Circulating tumor cells: liquid biopsy of cancer. *Clin Chem*. 2013;59(1):110–118.
13. Mavroudis D. Circulating cancer cells. *Ann Oncol*. 2010;21 Suppl 7:vii95–vii100.
14. Pantel K, Alix–Panabieres C. Circulating tumour cells in cancer patients: challenges and perspectives. *Trends Mol Med*. 2010;16(9):398–406.
15. Zhang L, Riethdorf S, Wu G, et al. Meta analysis of the prognostic value of circulating tumor cells in breast cancer. *Clin Cancer Res*. 2010;18(20):5701–5710.
16. Lucci A, Hall CS, Lodhi AK, et al. Circulating tumour cells in non–metastatic breast cancer: a prospective study. *Lancet Oncol*. 2012;13(7):688–695.
17. Rink M, Chun FK, Dahlem R, et al. Prognostic role and HER2 expression of circulating tumor cells in peripheral blood of patients prior to radical cystectomy: a prospective study. *Eur Urol*. 2012;61(4):810–807.
18. Hughes C, Clemens G, Bird B, et al. Introducing Discrete Frequency Infrared Technology for High–Throughput Biofluid Screening. *Sci Rep*. 2016;6:20173.
19. Wang S, Wang H, Jiao J, et al. Three–dimensional nanostructured substrates toward efficient capture of circulating tumor cells. *Angew Chem Int Ed*. 2009;48(47):8970–8973.
20. Hughes AD, King MR. Use of naturally occurring halloysite nanotubes for enhanced capture of flowing cells. *Langmuir*. 2010;26(14):12155–12164.
21. Han WQ, Su D, Wu L, et al. Tri– and quadri–metallic ultrathin nanowires synthesized by one–step phase–transfer approach. *Nanotechnology*. 2009;20(49):495605.
22. He R, Zhao L, Liu Y, et al. Biocompatible TiO2 nanoparticle–based cell immunoassay for circulating tumor cells capture and identification from cancer patients. *Biomed Microdevices*. 2013;15(4):617–626.
23. Ghazani AA, McDermott S, Pectasides M, et al. Comparison of select cancer biomarkers in human circulating and bulk tumor cells using magnetic nanoparticles and a miniaturized micro–NMR system. *Nanomedicine*. 2013;9(7):1009–1017.
24. Cheng XH, Irimia D, Dixon M, et al. A microfluidic device for practical label–free CD4(+) T cell counting of HIV–infected subjects. *Lab Chip*. 2007;7(2):170–178.
25. Yoon HJ, Kim TH, Zhang Z, et al. Sensitive capture of circulating tumour cells by functionalized graphene oxide nanosheets. *Nat Nano*. 2013;8(10):735–741.
26. Sollier E, Go DE, Che J, et al. Size–selective collection of circulating tumor cells using Vortex technology. *Lab Chip*. 2014;14(1):63–77.

27. Allard WJ, Matera J, Miller MC, et al. Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. *Clin Cancer Res*. 2004;10(20):6897–6904.

28. Riethdorf S, Fritsche H, Muller V, et al. Detection of circulating tumor cells in peripheral blood of patients with metastatic breast cancer:a validation study of the Cell Search system. *Clin Cancer Res*. 2007;13(3):920–928.

29. Hosokawa M, Kenmotsu H, Koh Y, et al. Size–Based Isolation of Circulating Tumor Cells in Lung Cancer Patients Using a Microcavity Array System. *PLoS One*. 2013;8(6):e67466.

30. Kim YJ, Koo GB, Lee JY, et al. A microchip filter device incorporating slit arrays and 3D flow for detection of circulating tumor cells using CAV1–EpCAM conjugated microbeads. *Biomaterials*. 2014;35(26):7501–7510.

31. Chen Y, Li P, Huang PH, et al. Rare cell isolation and analysis in microfluidics. *Lab Chip*. 2014;14(4):626–645.

32. Dharmasiri U, Witek MA, Adams AA, et al. Microsystems for the capture of low–abundance cells. *Annu Rev Anal Chem (Palo Alto Calif)*. 2010;3:409–431.

33. Smith JP, Barbati AC, Santana SM, et al. Microfluidic transport in microdevices for rare cell capture. *Electrophoresis*. 2012;33(21):3133–3142.

34. Hyun KA, Jung HI. Microfluidic devices for the isolation of circulating rare cells: a focus on affinity–based, dielectrophoresis, and hydrophoresis. *Electrophoresis*. 2013;34(7):1028–1041.

35. Broche LM, Bhadal N, Lewis MP, et al. Early detection of oral cancer – Is dielectrophoresis the answer? *Oral Oncol*. 2007;43(2):199–203.

36. Mulhall HJ, Labeed FH, Kazmi B, et al. Cancer, pre–cancer and normal oral cells distinguished by dielectrophoresis. *Anal Bioanal Chem*. 2011;401(8):2455–2463.

37. Yang F, Yang XM, Jiang H, et al. Dielectrophoretic separation of colorectal cancer cells. *Biomicrofluidics*. 2010;4(1):13204.

38. Salmanzadeh A, Romero L, Shafiee H, et al. Isolation of prostate tumor initiating cells (TICs) through their dielectrophoretic signature. *Lab Chip*. 2012;12(1):182–189.

39. Sabuncu AC, Liu JA, Beebe SJ, et al. Dielectrophoretic separation of mouse melanoma clones. *Biomicrofluidics*. 2010;4(2):021101.

40. Saliba AE, Saias L, Psychari E, et al. Microfluidic sorting and multimodal typing of cancer cells in self–assembled magnetic arrays. *Proc Natl Acad Sci USA*. 2010;107(33):14524–14529.

41. Shim S, Stemke–Hal K, Tsimberidou AM, et al. Antibody–independent isolation of circulating tumor cells by continuous–flow dielectrophoresis. *Biomicrofludics*. 2013;7(1):011807.

42. Cheng XH, Irimia D, Dixon M, et al. A microfluidic device for practical label–free CD4 (+) T cell counting of HIV–infected subjects. *Lab Chip*. 2007;7(2):170–178.

43. Li P, Gao Y, Pappas D. Multiparameter cell affinity chromatography: separation and analysis in a single microfluidic channel. *Anal Chem*. 2012;84(19):8140–8148.

44. Didar TF, Tabrizian M. Adhesion based detection, sorting and enrichment of cells in microfluidic Lab–on–Chip devices. *Lab Chip*. 2010;10(22):3043–3053.

45. Li P, Gao Y, Pappas D. Negative enrichment of target cells by microfluidic affinity chromatography. *Anal Chem*. 2011;83(20):7863–7869.

46. Hatch A, Pesko DM, Murthy SK. Tag–free microfluidic separation of cells against multiple markers. *Anal Chem*. 2012;84(10):4618–4621.

47. Maheswaran S, Sequist LV, Nagrath S, et al. Detection of mutations in EGFR in circulating lung cancer cells. *N Engl J Med*. 2008;359(4):366–377.

48. Nagrath S, Sequist LV, Maheswaran S, et al. Isolation of rare circulating tumour cells in cancer patients by microchip technology. *Nature*. 2007;450(7173):1235–1239.

49. Sheng W, Chen T, Kamath R, et al. Aptamer–enabled efficient isolation of cancer cells from whole blood using a microfluidic device. *Anal Chem*. 2012;84(9):4199–4206.

50. Gleghorn JP, Pratt ED, Denning D, et al. Capture of circulating tumor cells from whole blood of prostate cancer patients using geometrically enhanced differential immunocapture (GEDI) and a prostate–specific antibody. *Lab Chip*. 2010;10(1):27–29.

51. Stott SL, Hsu CH, Tsukrov DI, et al. Isolation of circulating tumor cells using a microvortex–generating herringbone–chip. *Proc Natl Acad Sci USA*. 2010;107(43):18392–18397.

52. Earhart CM, Hughes CE, Gaster RS, et al. Isolation and mutational analysis of circulating tumor cells from lung cancer patients with magnetic sifters and biochips. *Lab Chip*. 2014;14(1):78–88.

53. Sims CE, Allbritton NL. Analysis of single mammalian cells on–chip. *Lab Chip*. 2007;7(4):423–440.

54. Lecault V, White AK, Singhal A, et al. Microfluidic single cell analysis: from promise to practice. *Curr Opin Chem Biol*. 2012;16(3–4):381–390.

55. Alberter B, Klein CA, Polzer B. Single–cell analysis of CTCs with diagnostic precision: opportunities and challenges for personalized medicine. *Expert Rev Mol Diagn*. 2016;16(1):25–38.

56. Polzer B, Medoro G, Pasch S, et al. Molecular profiling of single circulating tumor cells with diagnostic intention. *EMBO Mol Med*. 2014;6(11):137113–86.

57. Fleischer R. Cancer filter deja vu. *Science*. 2007;318(5858):1864.

58. Paterlini–Brechot P, Benali NL. Circulating tumor cells (CTC) detection: clinical impact and future directions. *Cancer Lett*. 2007;253(2):180–204.

59. Vona G, Estepa L, Beroud C, et al. Impact of cytomorphological detection of circulating tumorcells in patients with liver cancer. *Hepatolog*. 2004;39(3):792–797.

60. Bhagat AA, Hou HW, Li LD, et al. Pinched flow coupledshear–modulated inertial microfluidics for high–throughput rare blood cell separation. *Lab Chip*. 2011;11(11):1870–1878.

61. McFaul SM, Lin BK, Ma H. Cell separation based on size and deformability using microfluidic funnel ratchets. *Lab Chip*. 2012;12(13):2369–2376.

62. Tan SJ, Lakshmi RL, Chen P, et al. Versatile label free biochip for the detection of circulating tumor cells from peripheral blood in cancer patients. *Biosens Bioelectron*. 2010;26(4):1701–1705.

63. Zheng S, Lin HK, Lu B, et al. 3D microfilter device for viable circulating tumor cell (CTC) enrichment from blood. *Biomed Microdevices*. 2011;13(1):203–213.

64. Hosokawa M1, Kenmotsu H, Koh Y, et al. Size–Based Isolation of Circulating Tumor Cells in Lung Cancer Patients Using a Microcavity Array System. *PLoS One*. 2013;8(6):e67466.

65. Davis JA, Inglis DW, Morton KJ, et al. Deterministic hydrodynamics: taking blood apart. *Proc Natl Acad Sci USA*. 2006;103(40):14779–14784.

66. Huang LR, Cox EC, Austin RH, et al. Continuous particle separation through deterministic lateral displacement. *Science*. 2004;304(5673):987–990.

67. Inglis DW, Davis JA, Austin RH, et al. Critical particle size for fractionation by deterministic lateral displacement. *Lab Chip*. 2006;6(5):655–658.

68. Wang XB, Yang J, Huang Y, et al. Cell separation by dielectrophoretic field–flow–fractionation. *Anal Chem*. 2000;72(4):832–839.

69. Moon HS, Kwon K, Kim SI, et al. Continuous separation of breast cancer cells from blood samples using multi–orifice flow fractionation (MOFF) and dielectrophoresis (DEP). *Lab Chip*. 2011;11(6):1118–1125.

Citation: Venugopal NBRK, Kannan S. Current trends in microfluidics for single cell isolation in cancer diagnostics enabling downstream proteomics applications. *MOJ Proteomics Bioinform.* 2016;3(4):98–106. DOI: 10.15406/mojpb.2016.03.00094

70. Becker FF, Wang XB, Huang Y, et al. Separation of human breast cancer cells from blood by differential dielectric affinity. *Proc Natl Acad Sci USA*. 1995;92(3):860–864.

71. Alazzam A, Stiharu I, Bhat R, et al. Interdigitated comb–like electrodes for continuous separation of malignant cells from blood using dielectrophoresis. *Electrophoresis*. 2011;32(11):1327–1336.

72. Gascoyne PR, Noshari J, Anderson TJ, et al. Isolation of rare cells from cell mixtures by dielectrophoresis. *Electrophoresis*. 2009;30(8):1388–1398.

73. Adams JD, Ebbesen CL, Barnkob R, et al. Micromech. *Microeng*. 2012;22:075017.

74. Augustsson P, Magnusson C, Nordin M, et al. Microfluidic, label–free enrichment of prostate cancer cells in blood based on acoustophoresis. *Anal Chem*. 2012;84(18):7954–7962.

75. Yang AH, Soh HT. Acoustophoretic sorting of viable mammalian cells in a microfluidic device. *Anal Chem*. 2012;84(24):10756–10762.

76. Pantel K, Brakenhoff RH, Brandt B. Detection, clinical relevance and specific biological properties of disseminating tumour cells. *Nat Rev Cancer*. 2008;8(5):329–340.

77. Lianidou ES. Circulating tumor cells–new challenges ahead. *Clin Chem*. 2012;58(5):805–807.

78. Shaw JA, Brown J, Coombes RC, et al. Circulating tumor cells and plasma DNA analysis in patients with indeterminate early or metastatic breast cancer. *Biomark Med*. 2011;5(1):87–91.

79. Yu M, Bardia A, Wittner BS, et al. Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. *Science*. 2013;339(6119):580–584.

80. Pinto C, Widodo E, Waltham M, et al. Breast cancer stem cells and epithelial mesenchymal plasticity—implications for chemoresistance. *Cancer Lett*. 2013;341(1):56–62.

81. Kang Y, Pantel K. Tumor cell dissemination: emerging biological insights from animal models and cancer patients. *Cancer Cell*. 2013;23(5):573–581.

82. Ferreira CR, Yannell KE, Jarmusch AK, et al. Ambient Ionization Mass Spectrometry for Point–of–Care Diagnostics and Other Clinical Measurements. *Clin Chem*. 2016;62(1):99–110.

83. Geigl JB, Speicher MR. Single–cell isolation from cell suspensions and whole genome amplification from single cells to provide templates for CGH analysis. *Nat Protoc*. 2007;2(12):3173–3184.

84. Mathiesen RR, Fjelldal R, Liestol K, et al. High resolution cost–effectives of copy number changes in disseminated tumor cells of patients with breast cancer. *Int J Cancer*. 2012;131(4):E405–E415.

85. Dharmasiri U, Njoroge SK, Witek MA, et al. High–throughput selection, enumeration, electrokinetic manipulation, and molecular profiling of low–abundance circulating tumor cells using a microfluidic system. *Anal Chem*. 2011;83(6):2301–2309.

86. Yang MJ, Chiu HH, Wang HM, et al. Enhancing detection of circulating tumor cells with activating KRAS oncogene in patients with colorectal cancer by weighted chemiluminescent membrane array method. *Ann Surg Oncol*. 2010;17(2):624–633.

87. Hou JM, Krebs MG, Lancashire L, et al. Clinical significance and molecular characteristics of circulating tumor cells and circulating tumor microemboli in patients with small–cell lung cancer. *J Clin Oncol*. 2012;30(5):525–532.

88. Wu M, Singh AK. Single–cell protein analysis. *Curr Opin Biotechnol*. 2012;23(1):83–88.

89. De Rosa SC, Herzenberg LA, Roederer M. 11–color, 13–parameter flow cytometry: identification of human naive T cells by phenotype, function, and T–cell receptor diversity. *Nat Med*. 2001;7(2):245–248.

90. Perfetto SP, Chattopadhyay PK, Roederer M. Seventeen–colour flow cytometry: unravelling the immune system. *Nat Rev Immunol*. 2004;4(8):648–655.

91. Perez OD, Nolan GP. Simultaneous measurement of multiple active kinase states using polychromatic flow cytometry. *Nat Biotechnol*. 2002;20(2):155–162.

92. Sachs K, Perez O, Pe'er D, et al. Causal protein–signaling networks derived from multiparameter single–cell data. *Science*. 2005;308(5721):523–529.

93. Irish JM, Hovland R, Krutzik PO, et al. Single cell profiling of potentiated phospho–protein networks in cancer cells. *Cell*. 2004;118(2):217–228.

94. Krutzik PO, Nolan GP. Fluorescent cell barcoding in flow cytometry allows high–throughput drug screening and signaling profiling. *Nat Methods*. 2006;5(3):361–368.

95. Lindstrom S, Andersson–Svahn H. Overview of single–cell analyses: microdevices and applications. *Lab Chip*. 2010;10(24):3363–3372.

96. Perroud TD, Kaiser JN, Sy JC, et al. Microfluidic–based cell sorting of Francisella tularensis infected macrophages using optical forces. *Anal Chem*. 2008;80(16):6365–6372.

97. Srivastava N, Brennan JS, Renzi RF, et al. Fully integrated microfluidic platform enabling automated phosphoprofiling of macrophage response. *Anal Chem*. 2009;81(9):3261–3269.

98. Bandura DR, Baranov VI, Ornatsky OI, et al. Mass cytometry: technique for real time single cell multitarget immunoassay based on inductively coupled plasma time–of–flight mass spectrometry. *Anal Chem*. 2009;81(16):6813–6822.

99. Bendall SC, Simonds EF, Qiu P, et al. Single–cell mass cytometry of differential immune and drug responses across a human hematopoietic continuum. *Science*. 2011;332(6030):687–696.

100. Casey R, Blumenkrantz D, Millington K, et al. Enumeration of functional T–cell subsets by fluorescence–immunospot defines signatures of pathogen burden in tuberculosis. *PLoS One*. 2010;5(12):e15619.

101. Ma C, Fan R, Ahmad H, et al. A clinical microchip for evaluation of single immune cells reveals high functional heterogeneity in phenotypically similar T cells. *Nat Med*. 2011;17(16):738–743.

102. Choi J, Love KR, Gong Y, et al. Immuno–hybridization chain reaction for enhancing detection of individual cytokine–secreting human peripheral mononuclear cells. *Anal Chem*. 2011;83(18):6890–6895.

103. Rubakhin SS, Sweedler JV. Quantitative measurements of cell–cell signaling peptides with single–cell MALDI MS. *Anal Chem*. 2008;80(18):7128–7136.

104. Mellors JS, Jorabchi K, Smith LM, et al. Integrated microfluidic device for automated single cell analysis using electrophoretic separation and electrospray ionization mass spectrometry. *Anal Chem*. 2010;82(3):967–973.

105. Urban PL, Jefimovs K, Amantonico A, et al. High–density micro–arrays for mass spectrometry. *Lab Chip*. 2010;10(23):3206–3209.

106. Hu S, Le Z, Newitt R, et al. Identification of proteins in single–cell capillary electrophoresis fingerprints based on comigration with standard proteins. *Anal Chem*. 2003;75(14):3502–3505.

107. Sobhani K, Fink SL, Cookson BT, et al. Repeatability of chemical cytometry: 2–DE analysis of single RAW 264. 7 macrophage cells. *Electrophoresis*. 2007;28(13):2308–2313.

108. Huang B, Wu H, Bhaya D, et al. Counting low–copy number proteins in a single cell. *Science*. 2007;315(5808):81–84.

109. Taniguchi Y, Choi PJ, Li GW, et al. Quantifying E. coli proteome and transcriptome with single–molecule sensitivity in single cells. *Science*. 2010;329(5991):533–538.

Citation: Venugopal NBRK, Kannan S. Current trends in microfluidics for single cell isolation in cancer diagnostics enabling downstream proteomics applications. *MOJ Proteomics Bioinform.* 2016;3(4):98–106. DOI: 10.15406/mojpb.2016.03.00094

110. Griffin BA, Adams SR, Tsien RY. Specific covalent labeling of recombinant protein molecules inside live cells. *Science*. 1998;281(5374):269–272.

111. Heitzer E, Auer M, Gasch C, et al. Complex tumor genomes inferred from single circulating tumor cells by array–CGH and next–generation sequencing. *Cancer Res*. 2013;73(10):2965–2975.

112. Dawson SJ, Tsui DW, Murtaza M, et al. Analysis of circulating tumor DNA to monitor metastatic breast cancer. *N Engl J Med*. 2013;368(13):1199–209.

113. Diaz LA Jr, Williams RT, Wu J, et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. *Nature*. 2012;486(7404):537–540.

114. Gasch C, Bauernhofer T, Pichler M, et al. Heterogeneity of epidermal growth factor receptorstatus and mutations of KRAS/PIK3CA in circulating tumor cells of patients with colorectal cancer. *Clin Chem*. 2013;59(1):252–260.

Citation: Venugopal NBRK, Kannan S. Current trends in microfluidics for single cell isolation in cancer diagnostics enabling downstream proteomics applications. *MOJ Proteomics Bioinform.* 2016;3(4):98–106. DOI: 10.15406/mojpb.2016.03.00094