Label-free isolation of circulating tumor cells in microfluidic devices: Current research and perspectives

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(Received 25 October 2012; accepted 17 December 2012; published online 24 January 2013)

This review will cover the recent advances in label-free approaches to isolate and manipulate circulating tumor cells (CTCs). In essence, label-free approaches do not rely on antibodies or biological markers for labeling the cells of interest, but enrich them using the differential physical properties intrinsic to cancer and blood cells. We will discuss technologies that isolate cells based on their biomechanical and electrical properties. Label-free approaches to analyze CTCs have been recently invoked as a valid alternative to “marker-based” techniques, because classical epithelial and tumor markers are lost on some CTC populations and there is no comprehensive phenotypic definition for CTCs. We will highlight the advantages and drawbacks of these technologies and the status on their implementation in the clinics. © 2013 American Institute of Physics. [<http://dx.doi.org/10.1063/1.4780062>]

I. INTRODUCTION

Isolation of cellular subpopulations is a fundamental method for the study of several biomedical disciplines. Identification and separation can be achieved using specific biological markers such as antibodies or by taking advantage of differential mechanical and electrical properties between subpopulations of cells in a “label-free” modus. In this line of consideration, several label-free technologies have recently been published, in particular applied to the identification and analysis of circulating tumor cells (CTCs). The presence of CTCs in the blood is an obligate step in the spread of solid cancers at distant organs and is thus a major event for its malignant progression. CTCs have been demonstrated in the most common solid malignancies,^{1–9} and their analysis holds the promise for the better understanding of the key events underlying the progression of this deadly disease. However, there is not yet a comprehensive definition of CTCs based on biological markers. This complicates the current efforts in understanding the biology and clinical relevance of these cells and calls for the integration of alternative label-free technologies. The earliest microscopic accounts of cancer cells in the blood and the theorization of hematogenous metastases date back around 1840.¹⁰ CTCs are, potentially, the most easily observable event during malignancy, because of the non-invasiveness of venipuncture. On the other hand, their detection and analysis are extremely difficult because of their rarity in blood (usually lower than 10E-5% of total cells) and as mentioned earlier, the lack of a formal phenotypic definition. Various methods for the detection and isolation of CTCs have been established during the last few years, with strong links demonstrated between CTC count and survival or progression after therapy.^{11,12} CTC can be as well used to monitor immediate response to therapy¹³ or the presence of residual tumor activity after surgery. These encouraging results are paving the way for a more widespread study of CTCs in the clinics and in basic

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science.¹⁴ The current development of devices for the analysis of CTCs is increasingly focusing on sensitivity, affordability, and the capability to manipulate tumor cells for the analysis of their genetic makeup, gene transcription, or biological behavior.¹⁵ Several reviews covers in detail various aspects relevant to the analysis of CTCs.^{16–20} We envision the widespread application of CTC analysis as a general diagnostic tool for solid cancers, affordable monitoring of therapy and disease progression and a more precise prediction of chemotherapeutic drug responses. At the same time, devices for CTC analysis will allow a better definition for the biological role of CTCs during cancer progression. Label-free technologies, as we will highlight in the following text demonstrated the potential to solve some of these issues and are thus gaining stronger support for their clinical use.

II. LABEL-FREE METHODS FOR CTCs ENRICHMENT

Several techniques for the label-free isolation of CTCs have been developed, many of them rely on microfluidic approaches (Table I). We can divide them into two major groups, based on their methods for detection and/or enrichment:

- CTC enrichment based on differences between cellular biomechanical properties (e.g., cell size, density, deformability)
- CTC enrichment based on differences in cellular electrical properties

Finally, we present considerations regarding the magnetophoretic separation of red blood cells (RBCs), which is mainly used as a CTC pre-enrichment method for subsequent applications.

A. Separation based on mechanical properties of CTCs

The isolation of CTCs from normal blood cells, based on their differential biomechanical properties, is an early approach that is still of distinctive value. Different biochemical properties of CTCs have been studied and comprise density, size, deformability, and elasticity. The methods based on mechanical properties of CTCs will be presented in a chronological order.

1. Density gradient centrifugation (Figure 1(a))

Density gradient centrifugation method is commonly used as an accessory method for the removal of red blood cells or plasma for CTC enrichment. It is an accessible and economical method for the pre-concentration of CTCs. CTCs can be enriched in the top layer (containing mainly mononuclear cells) and can be thus separated from red blood cells and granulocytes.²¹ Different liquid kits are commercially available (e.g., Ficoll^{22,23}). An improved method, the OncoQuick assay (Greiner BioOne,) uses a porous barrier for the lower phase separation prior centrifugation.²⁴ The CTCs recovery rate with this method has been 70–90%²⁵ using experimental cell lines. Overall, the considerable loss of CTCs and the relatively low enrichment achieved limit the application of this method²⁶ and require subsequent steps.

2. Microfluidic filter (Figure 1(b))

Size-based microfiltration of CTCs in a microfluidic channel was first developed by Mohamed *et al.*²⁷ in 2004. The authors described a filtering device consisting of four segments. Each segment presented an array of columns with gaps of 20 μm , 15 μm , 10 μm , and 5 μm . The depth of the microfluidic channel has been optimized to 20 μm . The testing was performed using whole human blood diluted in EMEM medium and spiked with cultured neuroblastoma cells, but the author did not report on trapping efficiency. In 2006, Chen *et al.*,²⁸ proposed a microfluidic “pool-dam” structure. A series of pools were connected in series with a dam structure, and larger cells were captured in the pools as soon as the cell suspension flowed through the filter. The experiments were performed using SPC-A-1 (lung adenocarcinoma) cells spiked into diluted whole blood. A very efficient separation of the tumor cells was observed for a 5 μm gap. A microfluidic system using multi-obstacle architecture for size-dependent separation of CTCs has also been developed,²⁹ but in this case polymer microbeads conjugated with

TABLE I. Label-free isolation techniques of CTCs using microfluidic approaches.

Reference	Year published	Physical property used for separation	Experimental media	Isolation (efficiency, sensitivity, purity)	Retrieval (efficiency, sensitivity, purity)	Processing time	Clinical sample	CTC definition
33	2000	Size	Diluted whole blood	X(e:NA, s:NA, p:NA)	X(e:NA, s:1cell/ml, p:NA)	<30 min (estimated)	Yes	Cancer specific markers
38	2007	Size			X(e:NA, s:10cell/ml, p:NA)		No	
27	2009	Size/stiffness		X(e:NA, s:NA, p:NA)	X(e:NA, s:NA, p:NA)		No	
116	2009	DEP	Histopague isolated cells	X(e > 90%, s:NA, p:NA)	X(e:NA, s:NA, p:NA)		No	
51 and 52	2009	Size/stiffness	Diluted blood PBS	X(e > 80%, s:1cell/ml, p > 80%)	X(e > 80%, s:NA, p:NA)	2.5 h/5 ml blood	Yes	Nucleated, CKpos, CD45neg
40	2011	Size/stiffness	Diluted whole blood	X(e > 85%, s:NA, p:NA)			No	
46	2010	Size/stiffness	whole blood	X(e > 90%, s:NA, p:NA)		5 min/mL whole blood	Yes	Telomerase positive
41	2010	Size/stiffness		X(e > 90%, s:NA, p:NA)		45 min (estimated without staining)	No	
62	2011	Size	Diluted whole blood	X(e > 80%, s:NA, p > 10 ⁴ (RBC), > 10 ⁵ (PBL))	X(e > 80%, s:NA, p:NA)	15 min/mL whole blood	No	
37	2011	Size	Diluted whole blood	X(e = 100%, s:NA, p:NA)	X(e = 100%, s:2cells/mL, p:NA)		Yes	Morphology, cytokeratin pos
118	2011	Size, electrical properties	RBC+PBL in isotonic PBS	X(e > 75%, s:NA, p:NA160x enrichment)	X(e:NA, s:NA, p:NA)		No	
61	2011	size	Diluted whole blood	X(e:NA, s:NA, p:NA)	X(e > 95%, s:NA, p:NA)		No	
63 and 64	2012	size	RBC in isotonic solution	X(e:NA, s:NA, p:NA)	X(e > 85%, s:NA, p:NA)		No	
30	2012	size	PMBC+Cell lines	X(e > 98%, s:NA, p > 98%)			No	
139	2012	Size/electrical	Diluted whole blood	X(e > 70%, s:100cells/ml)			No	
65	2012	Size, stiffness	Diluted whole blood		X(e > 90%, s:NA, p:NA)		Yes	CK pos, CD45neg
56	2011	size	Diluted whole blood	X(e:NA, s:NA, p:NA)	X(e > 90%, s:NA, p:NA)		No	

anti-EpCAM were used to “enlarge” “MCF-7” breast cancer cells. Recently, McFaul *et al.* proposed a microfluidic filter using a matrix of funnel constrictions.³⁰

Membrane-based filtration is one of the first methods used for CTC enrichment and represents a relatively straightforward and low cost technique. The technique is based on the principle that malignant cells are larger than other blood cell populations.³¹ Most of the reported membranes have pore sizes around 7–8 μm in diameter, with few reporting on membranes with pore size diameters up to 11 μm . Membrane-based filtration allows the isolation of intact cells without modification of their plasma membrane with antibodies, a very important aspect for further purification or analysis of their morphological or biological properties.²⁵ In their early work, Rostango *et al.*³² proposed a “filtration cytometry” method for detection of rare circulating breast cancer cells based on size. Vona *et al.*³³ proposed ISET (Isolation by Size of Epithelial Tumor Cells), which consisted of flowing the blood through a porous (8 μm -diameter) polycarbonate membrane-filter. In this experiment, the blood sample was diluted in the ratio of 1:10 before filtration. The proposed system can run 12 samples in parallel and allows CTCs identification and characterization. Pinzani *et al.*³⁴ reported a clinical study using ISET with blood from breast cancer patients. In this study, they performed qRT-PCR of CTCs (after laser microdissection of the filters. More recently, Hou and co-workers³⁵ used the ISET platform for isolation of CTCs and CTMs (Circulating Tumor Microemboli—a cluster of CTCs) from patients with lung cancer. DeGiorgi *et al.*³⁶ used the ISET microfiltration technique for CTCs collection from patients with cutaneous melanoma. Polycarbonate membranes packaged in a kit (Screen-Cell)³⁷—which uses the ISET technique—allowed isolation of live spiked cancer cells (circular pores calibrated at $6.5 \pm 0.33 \mu\text{m}$ in diameter) or paraformaldehyde-fixed CTCs (with pores diameters of $7.5 \pm 0.36 \mu\text{m}$). After filtration, the membrane can be easily removed, and cells can be accessed for downstream applications for identification and characterization of CTCs. The living CTCs isolated using ISET can be potentially cultured, even though we could not find reports from the literature that demonstrate the successful culture of CTCs from the blood of cancer patients. The reported recovery rate was 82–88% with later studies reporting improved recovery rates and sensitivity. One disadvantage of the polycarbonate membranes is that pores are randomly distributed which may lead to large variability of CTCs capture efficiency (from 50 to 99%) and frequent sample clogging on filter. This inconvenience can be avoided using micro-patentable materials for membrane fabrication. In fact, different materials have been tested and may be used for membrane fabrication: polycarbonate,^{32–37} parylene C^{38–40} nickel⁴¹ and most recently, silicon.⁴²

Parylene C microfiltration membranes were proposed for CTC enrichment in 2007 by Zheng *et al.*³⁸ The material (known in microfabrication for its application in microfluidic devices⁴³ as well as in cell culture⁴⁴), presented the advantage of ready micropatterning. It is a transparent and chemical inert polymer used extensively as a coating for implantable biomedical devices.⁴⁵ As a result, configurable membranes with very well controlled thickness, pore's size and distribution or even pore shape³⁸ can be achieved. In the above-mentioned application, CrAu electrodes were patterned using the microfabrication process on the surface of the Parylene C membrane. After the CTC capturing process, the cells were lysed using 5 V peak-to-peak /10 kHz electrical signal for further PCR amplification. The reported recovery of the prostate cancer cell line LNCaP was reported to be 90%. The same team⁴⁰ recently proposed a membrane with a 3D pore structure. The structure consists of two 10 μm -thick Parylene C membranes separated by a 6.5 μm gap. The microholes fabricated on the first membrane are misaligned with the microholes from the bottom membrane, in such way that the large CTC cells are trapped in the filter structure. In a comparative study between the 2D filter and 3D filters, the authors show that 2D filters might indeed damage or even lyse the cancer cells during the microfiltration process.⁴⁰ Using fluorescence microscopy, the authors further show an increased passage of the cells through the pores of 2D parylene membranes, compared with their 3D counterparts. In addition, the 3D membranes captured viable and unfixed tumour cells, indicating thus various advantages using 3D versus 2D parylene C membranes. The use of parylene C membrane was also reported by Xu *et al.*⁴⁶ The authors showed the detection of telomerase activity from live CTCs captured on a microfilter.

A nickel microfilter has been also reported⁴¹ previously. In this study, the filter presented 100×100 holes with the diameters between 8 and $11 \mu\text{m}$ and was integrated between two PDMS funnels. The top chamber was connected with the reservoir, while the bottom one was connected to a peristaltic pump. Staining and washing processes were performed within the integrated device. Verification of performance was done using spiked cell lines, suggesting an efficiency of greater than 80% with a viability of 98% of the captured cells.

Silicon microsieves for the isolation of CTCs have been recently proposed by Lim *et al.*⁴² The reported microsieve has high pore density ($10^5/\text{device}$) and can be used to separate cells based on the size and deformability of CTCs. In the same report, the authors describe a dedicated microfluidic device that allows an important number of steps such as: CTCs isolation, antibody staining, removal of contaminants, and immunofluorescence imaging. The recovery rate was verified spiking MCF-7 and HepG2 cells in the human blood and has been shown to be greater than 80% for a flow rate of 1 ml/min. The main advantage of using silicon as a material for microsieve fabrication is the excellent definition of pore size using deep reactive ion etching (RIE).⁴³ Because of the high density of pores on the membrane, the device allows the use of undiluted whole blood.

3. Devices based on relative CTC deformability (Figure 1(d))

Isolation of CTCs based on size from clinical samples has been proven in multiple studies. However, these devices might miss more deformable CTC populations. Cross and co-workers^{47,48} characterized single-cell stiffness using AFM (atomic force microscopy) for different cancer cells (lung, breast and pancreatic) and concluded that malignancy induces biomechanical changes at the single-cell level. Stiffness of metastatic cancer cells was reported to be 70% lower than benign cells. Using a microfluidic device, Zhang *et al.*⁴⁹ demonstrated that tumor-initiating cells have increased deformability compared to more differentiated cells. High deformability is an important biomechanical characteristic for cells to circulate in the peripheral blood. CTCs larger than WBCs are predicted to be highly deformable in order to reach distant organs and successfully metastasize, often through capillaries with diameters as small as $6\text{--}8 \mu\text{m}$. Control over deformability of CTCs (e.g., by biochemical means prior processing) in devices that differentially isolate CTCs based on size, might thus increase CTC recovery of populations with a more metastatic or stem cell phenotype. Differential deformability has been used to separate cancer and white blood cells using inertial microfluidics⁵⁰ and will be discussed in Sec. II A 4.

Tan *et al.*^{51,52} proposed a microfluidic chip equipped with an array of traps for cancer cell isolation. Each trap is composed of three pillars (with a diameter of $3\text{--}4 \mu\text{m}$) disposed as arc shape with $5 \mu\text{m}$ -distance between pillars. CTC isolation is achieved similarly to the Japanese game patchinko (the trapping method is illustrated in Figure 1(d)). In this case, the authors report a device based on high deformability of WBCs which allows them to transit through the $5 \mu\text{m}$ gaps. The larger cells are stuck in the arc-shaped traps. A pre-filter ($20 \mu\text{m}$ -gap) prevents larger clumps and debris to access the microwells area. Highly purified cancer cells can be retrieved from the device for downstream applications. In these studies, the authors describe cancer cells as being less deformable than WBCs, but we did not find conclusive information on this aspect and differential biomechanical properties such as deformability and viscoelasticity of cancer cells versus blood cells remains an intriguing field of study.

4. Microfluidic separation based on inertial forces (Figures 1(e) and 1(f))

The flow in microfluidic channels is generally characterized by low Reynold's number (Re) (where the Re describes the ratio between inertial and viscous forces). In most microfluidic applications, the inertial phenomenon was niggled considering a Stokes flow ($Re = 0$). Recently, several inertial effects have been studied in microfluidics particles.

Di Carlo⁵³ describes the most important effects that can be exploited in microfluidic applications: inertial migration of particles (straight channels) and secondary flows in curved channels. Both phenomena can be applied to isolate CTCs.

FIG. 1. Evolution of the CTC enrichment methods based on differences between cellular biomechanical properties: a) density gradient centrifugation in the presence of ficol,¹⁶ b) CTC enrichment using microfluidic filter, c) CTCs enrichment using Si membranes. We filtered 1 ml of whole blood from a colorectal cancer patient using the microsieve described in Lim *et al.* Stainings using cytokeratin (CK) and CD45 reveals CK positive, CD45 negative cellular clusters trapped on the microsieve. Scale bar = $10\mu\text{m}$ d) device based on CTCs size and deformability proposed by Tan *et al.*^{51,52} (Reprinted with kind permission from G. E. Loeb, A. E. Walker, S. Uematsu, and B. W. Konigsmark, J. Biomed. Mater. Res. **11**(2), 195-210 (1977). Copyright 1977 Springer Science and Business Media) e) CTCs enrichment using inertial migration (Reprinted with permission from S. C. Hur, A. J. Mach, and D. Di Carlo, Biomicrofluidics **5**(2) (2011). Copyright 2011, American Institute of Physics) f) Size separation of CTCs in spiral microfluidic channels (Reprinted with permission from J. Sun, M. Li, C. Liu, Y. Zhang, D. Liu, W. Liu, G. Hu, and X. Jiang, Lab Chip (2012). Copyright 2012 Royal Society of Chemistry).

In a straight channel, fluid shear generates lateral forces, which cause transverse inertial migration of particles (“tubular pitch effect”). There are three forces that move the flowing particle to equilibrium position according to its density: (1) shear induced, (2) stresslet-velocity-field-induced, and (3) wall lift.⁵⁴ In a curved channel, centrifugal forces overlap with the above

mentioned inertial forces and generate a double recirculation in the transversal section of the channel (Dean vortex). The strength of this recirculation is described by the Dean number:⁵⁵

$$De = 2(d/R)^{1/2}Re, \quad (1)$$

Where d is the hydraulic channel radius, R is the radius of the curvature. Due to these forces, in the spiral channel, we can observe a transverse particle migration according to its size/mass. At an equilibrium stage, these particles are positioned at a defined distance along the channel cross-section.

The secondary flow effect in a microfluidic channel was used for size separation of particles using a spiral microfluidic channel.^{54–58} Separation of two cell populations in a spiral channel was first reported by Lee *et al.*⁵⁹ and recently used for CTC isolation by Sun *et al.*⁶⁰ In the latter report, the authors used a double spiral channel for separating tumor cells (MCF-7 and Hela) spiked into the blood. The results showed a recovery rate over 90%. The design of the chip, with double spiral, assured a good “packaging” and a better positioning of the inlet/outlets holes.

Inertial migration in a straight channel was used for size-based CTCs separation. Hur *et al.*⁶¹ proposed a method for isolation of larger target cells (tested using spiked cultured cancer cells in blood). First, the blood sample passed through a straight microfluidic channel of 1.5 cm length. At the end of this channel, a series of square reservoirs ($400\ \mu\text{m} \times 400\ \mu\text{m}$) were placed. The cells, which are flowing through the channel, reached equilibrium at a well-defined distance between the center of the particle and the wall due to the previously mentioned inertial lateral forces (wall effect lift forces and shear-gradient lift forces). Once the cells reached the reservoir’s area, larger cells were pushed toward a vortex generated in the reservoir and trapped within, while the small cells were flushed towards the outlet.

The same lab showed that inertial lateral forces act on deformable particles by causing them to migrate towards the center of a straight channel, when in equilibrium with inertial lift forces. This balance leads to focusing positions that can be harnessed to separate cells with differential deformability such as cancer cells and white blood cells. The authors demonstrated this system in a microfluidic device by showing increased enrichment of cancer cell lines spiked in diluted whole blood by means of viscoelasticity-induced forces.⁵⁰

Another spiral device was presented by Bhagat *et al.*⁶² The microfluidic channel, designed with a contractions-expansions array presented three distinct zones: “cell-focusing” areas where the cells aligned at a certain distance from the channel walls due to the inertial forces, “cell-pinching” regions where the larger cells were pushed through the center of the channel; “collection area” where the microfluidic channel became the larger and rare/large cells were collected in the center outlet.

Tanaka *et al.*^{63,64} studied the separation of CTCs from RBCs using inertial migration of cells in a microfluidic device. The device was designed based on the observation that the cancer cells migrated to an equilibrium position of $0.6 \times 1/2$ channel-width measured from the center of cells to the center of the channel. The device is designed to contain a channel of defined width to achieve equilibrium position of CTCs and RBCs, followed by an enlargement of the channel’s width and by a bifurcation in order to collect the cells at different outlets. Hyun *et al.*⁶⁵ reported as well the use of inertial migration in straight channels for the CTC separation using clinical samples.

In some applications, filtration-based techniques were associated with chemical functionalization of the surface (using anti epithelial cell adhesion molecule -EpCAM)—for CTCs capturing. These methods may be considered “marker based” methods we just mention the most relevant work:

- “CTC-chip”⁶⁶ where an array of pillars were used as trapping structure,
- “Herringbone-chip”⁶⁷ where microfluidic mixing was used for high-throughput capturing
- The microfluidic chip proposed by Zheng *et al.*⁶⁸ that combines the inertial migration with antibody-functionalization of the microchannel.

5. Separation using acoustic waves (acoustophoresis)

Acoustophoresis has been used to separate tumor cells from leukocytes in this case by acoustic standing wave forces. Both CTCs and leukocytes were separated by subjecting them to free flow acoustophoresis in a microfluidic device. The CTCs were focused in the center of the channel and further collected in the central outlet. The leukocyte discrimination could be tuned by the system flow rate. The recovery of the CTCs spiked in the blood sample was about 50% at flow rate of 800 $\mu\text{l}/\text{min}$. In a recent work,⁶⁹ the same team proposed an improved design of the microfluidic channel with mechanical traps for cancer cells. The study was performed using three prostate cancer cell-lines (DU145, PC3, LNCaP) and WBC for fixed (with paraformaldehyde) and nonfixed samples. For fixed cells the recovery rate ranged from 93.6% to 97.9% with purity ranging from 97.4% to 98.4% while for non-fixed cell recovery ranged from 72.5% to 93.9% with purity ranging from 79.6% to 99.7%. Overall acoustophoresis seems to be a low throughput method and further research and clinical studies are required to confirm its potential.

B. Separation based on electrical properties of CTCs

Two methods to isolate CTCs have been developed based on electrical properties of cancer cells.

- Dielectrophoresis (DEP)
- Electrical Impedance Spectroscopy (EIS)

1. Dielectrophoresis

In the recent years, dielectrophoresis captured researchers' attention (Figure 2(a)).^{70–79} Dielectrophoresis is defined as the movement of a neutral but polarizable particle in a non-uniform electric field due to the interaction of the particle's dipole and spatial gradient of the electric field. DEP can be generated using alternative current (AC), direct current (DC)^{80,81} or DC-biased alternative current (AC) electric fields.⁸² The main applications in biology are related to cell trapping,⁸³ focusing,⁸⁴ identification,⁸⁵ separation of cell populations,^{86,87} or even cell patterning.^{88–90} According to the electrical properties of the particles, medium and frequency of the electric field, the particles can move to high electric field strength (positive DEP) or to the low electric field strength (negative DEP). This aspect allows separating and collecting populations of cells in different locations of the microfluidic device. Dielectrophoresis can distinguish cells based on their dielectric properties (complex permittivity); it can differentiate cells based on the cell structure, such as a change in cytoplasmic conductivity or the differential content of cellular organelles. The magnitude and direction of the dielectrophoretic force are related to the intrinsic cell's properties, making the collection of the homogeneous groups possible either using an external force (i.e., hydrodynamic force induced by the flow in the microfluidic channel- separation under continuous flow⁹¹) or using, the motion induced by DEP force⁹² only. Analytical modeling of separation of particle populations using DEP is presented by Gascoyne and Vykoukal in Ref. 75, while a concise synthesis of the main separation methods used is covered in Ref. 93.

For a better understanding of the phenomenology, we must consider the expression of DEP force:⁹⁴

$$F = 2\pi r^3 \varepsilon_m R_e[K] \nabla E^2, \quad (2)$$

where ε_m is the absolute permittivity of the suspending medium, ∇E is the local electric field (rms) intensity. $R_e[k]$ is the real part of the dipolar Clausius-Mossotti factor, is giving the sign of the force (positive or negative) being defined as:

$$K = \frac{\varepsilon_p^* - \varepsilon_m^*}{\varepsilon_p^* + 2\varepsilon_m^*}, \quad \varepsilon^* = \varepsilon - j\frac{\sigma}{\omega}, \quad (3)$$

FIG. 2. (a) Working principle of dielectrophoretic separation under continuous flow: one cell population that exhibit negative DEP is trapped in the wells while the other one (that express positive DEP) is flushed to the outlet (Reprinted with permission from C. Iliescu, G. Tresset, and G. L. Xu, Appl. Phys. Lett. **90**(23) (2007). Copyright 2007 American Institute of Physics). (b) Working principle of EIS: two electrodes are placed in a microfluidic channel (Reprinted from C. Iliescu, D. P. Poenar, M. Carp, and F. C. Loe, Sens. Actuators B **123**(1), 168-176 (2007). Copyright 2007 with permission from Elsevier). (c) Magnetophoretic separation of RBCs under continuous flow as presented by Jung and Han (Reprinted with permission from J. Jung and K.-H. Han, Appl. Phys. Lett. **93**(22), 223902-223902-223903 (2008). Copyright 2008 American Institute of Physics).

where ϵ_p^* and ϵ_m^* are the complex permittivity of the particle and medium, respectively. The complex permittivity for a dielectric material can be described by its permittivity ϵ and conductivity σ , where ω is the angle frequency the applied electric field, E . From the expression of the DEP force, two aspects are relevant for separation of two dissimilar cell populations in microfluidic devices:

- DEP force is strongly dependent on the volume of the particle, so theoretically DEP force can be used for size separation of particles.⁹⁵
- DEP force is strongly dependent on the gradient of the electric field.

There are five major DEP techniques, essentially classified based on the method for electric field gradient generation. (1) In conventional DEP, the gradient of the electric field is generated by micrometric-size electrodes having different geometries. (2) Isolating DEP (iDEP) is a method where the electric field gradient is generated by a non-homogenous dielectric medium between the electrodes.^{96,97} (3) Travel wave DEP is based on phase changing of the applied electric field).^{98,99} (4) In optical DEP, an optical image projected onto a photodiode surface generates the gradient of the electric field.¹⁰⁰ (5) medium conductivity gradient is another dielectrophoretic method¹⁰¹ based on the variation of the electrical conductivity of the medium between two parallel electrodes. For each of these methods, different separation techniques were elaborated.

In summary, microfluidic DEP devices confer large opportunities for CTCs separation. The specificity of electrical properties of cancer cells was remarked long time ago by Gascoyne *et al.*¹⁰² and Becker *et al.*^{103,104} An *et al.*¹⁰⁵ demonstrated the specific dielectrophoretic

properties of cancer cells using a DEP activated cell sorter. In their study, malignant human breast cancer epithelial cells (MCF 7) and breast cells (MCF 10 A) were separated based on cell's electrical properties. In a similar study Broche *et al.*¹⁰⁶ showed the difference in electrical properties between oral squamous cell carcinoma (OSCS) and normal keratinocyte cell populations. A low-cost DEP microfluidic device was used for purification of colorectal cancer HCT116 cells from the mixture with human embryonic kidney 293 (HEK 293) and *E. coli* cells.¹⁰⁷ Kang *et al.*⁸¹ used DC DEP to separate WBC and breast cancer cells based on size. In their application, the DC electric field was used to generate iDEP effect and for electrokinetic transport of the particles. The iDEP presents its limitations as the method requires high voltage and generates increased temperature (Joule heating effect) with possible deleterious effects on cell viability. Preconcentration of HeLa cells (metastatic human cervical carcinoma) in a device with concentric circular electrodes has been demonstrated in Ref. 108 where the trapping efficiency of the device was 76%. iDEP was used by Salmanzadeh *et al.*¹⁰⁹ to observe the DEP response of different stages of ovarian cancer cells. Sabuncu *et al.*¹¹⁰ report the use of DEP for differentiation between two malignant cell populations. Wu *et al.*¹¹¹ studied the dielectric properties of colon cancer cells (HT-29) measured using the dielectrophoretic capture voltage spectrum under various medium conductivities.

Alazzam *et al.*¹¹² proposed separation under continuous flow of MDA231 breast cancer cells spiked into blood. The device is a structure comprising interdigitated comb-like electrodes, and the electrode pairs are positioned divergent and convergent with respect to the flow. This method effectively moves the target cells near the edge of the channel, while the blood cells are collected in the center. Cheng *et al.*¹¹³ isolated HeLa cells from human peripheral blood using DEP. Cen *et al.*¹¹⁴ combined travel wave DEP with electrorotation for the manipulation and characterization of human malignant cells.

A commercial microfluidic system based on dielectrophoretic separation, ApoStream™ was recently launched¹¹⁵. The validation study was conducted using two different cell lines, each expressing a different levels of EpCAM: SKOV3 (high level) and MDA-MB-231 (low level). The average recovery of the cells was 75%. Of particular note is the observation that the viability of the MDA-MB-231 cells captured with the DEP device was 97% after culture for seven days. The microfluidic device is a field flow separator, presenting interdigitated electrodes where the mixed cell population flows over the electrode structure. The CTC population expresses positive DEP and is pulled along the floor, flowing near the electrode plane, while the other cells (exhibiting negative DEP) are levitated and transverse the electrode. The separation method is derived from DEP field-flow-fractionation method (depFFF). The depFFF method utilizes the DEP force to position the cell at a defined level in a fluid velocity gradient. The cells having similar properties will travel with the same speed. The depFFF method, using a similar design, was used by Gascoyne *et al.*¹¹⁶ for separation of three different cancer cell lines (MDA-MB-435, MDA-MB-468 and MDA-MB-231) from a mixture with peripheral blood mononuclear cells, with a reported recovery rate exceeding 90%. In another report,¹¹⁷ the depFFF method was used to characterize the membrane capacitance, density, and hydrodynamic properties in correlation with the morphology and size of cultured tumor cells.

Moon *et al.*¹¹⁸ proposed a two-steps process for the separation of CTCs combining inertial separation in a microfluidic device with dielectrophoresis. The hydrodynamic separation presents the advantage of a high-throughput method (10 to 100 $\mu\text{l}/\text{min}$) but is less selective. In a second step, a DEP sorter improves the selectivity of the process. As a result, an efficiency of 99% was reported using MCF-7 malignant human breast cancer epithelial cells spiked in blood. The DEP separator consists of two zones: in the first zone, all the cells (CTCs, WBC and RBCs) are pushed through the channel walls, while in the second zone, the CTCs migrate to the center of the microfluidic channel.

DEP separation techniques may certainly achieve a good purification. However, this is a time-consuming process, where low fluid velocity on the order of DEP force is required. There are several other considerations. The parameters requiring optimization in a DEP sorter are: frequency, voltage, flow rate, and buffer composition. Optimization of the buffer solution is critical for a good separation process.¹¹⁵ The DEP buffer should ideally satisfy several conditions:

- Maintain the cell viability during the separation process⁸⁵
- pH around 7 and osmolarity around 200–400 mOsm/kg to maintain physiological conditions,¹¹⁶
- Preferably low conductivity for a reduced Joule thermal effect.¹¹⁹

Joule thermal effect is one of the main problems that must be controlled in DEP devices. As previously mentioned, the conductivity of the buffer solution, combined with the large power density generated around the electrode can raise the temperature of the buffer affecting the viability of the cells. This problem has been studied in Refs. 87, 120, and 121. From this perspective, iDEP method is not recommended, as it requires high voltages.^{81,96} Separation methods under continuous flow^{91,118} are more advantageous in comparison with sequential methods, as heat is released due to convection.^{92,116} With the use of 3D electrodes or materials with a good thermal conductivity such as silicon, the Joule thermal effect can be reduced.^{83,99} Trapping the CTCs using nDEP may avoid exposure of the CTCs to strong electric fields and temperature gradients around the electrode. The applied voltage may affect the cell physiology,¹²² interaction between the electric field and cell being at the membrane level.^{123,124} In summary, dielectrophoresis may achieve a high purification,¹¹⁸ though at a low throughput.

2. Electrical impedance spectroscopy (Figure 2(b))

The cell size and dielectric properties can be characterized using electrical impedance spectroscopy (EIS). These devices are relatively simple, having a microfluidic channel and two electrodes placed on the bottom of the channel, on the channel walls,¹²⁵ embedded in the walls¹²⁶ or one above the other.¹²⁷ EIS measures the electrical properties of the cells in suspension.^{128,129} The development of microfabrication techniques allowed single cell analysis on microfluidic devices using impedance spectroscopy,¹³⁰ thus conferring new opportunities for CTCs characterization. Tan *et al.*¹³¹ proposed a microfluidic device for quantification of specific membrane capacitance of single cell (acute myeloid leukemia) using EIS. According to Sun and Morgan¹³⁰ the size of the cell can be accurately measured around 500 kHz, increasing the frequency range, the cell membrane and intracellular properties of the cell can be characterized. Kang *et al.*¹³² proposed a microfluidic device with interdigitated electrodes that covers the walls and the bottom of microfluidic channel. An interesting aspect of the design is that the height of the microfluidic channel is smaller than the anticipated cell diameter. Consequently, the cell engages in direct contact with the electrodes, thus increasing the sensitivity of the measurement. A comparative study was performed on normal human breast cells (MCF-10 A) and human breast cancer cells (MCF-7) showing difference for the real part of the impedance and the phase. Holmes *et al.*¹³³ proved that lymphocytes, monocytes, and neutrophils can be identified and counted using this approach. Han *et al.*¹³⁴ used EIS for characterization of several types of cells, including MCF-10 A, early-stage breast cancer cell line MCF-7, invasive human breast cancer cell line MDA-MB-231, and MDA-MB-435 (a melanoma cell line, previously thought to represent breast cancer). The results suggest that each cell line has its own “impedance signature,” in terms of both magnitude and phase of the electrical impedance. The microfluidic device uses negative pressure for the cell trapping in special-design cavities where the cells undergo measurement. The same group (Han *et al.*¹³⁵) also proposed an interesting two-step method for the separation of CTCs from blood. In the first step, a magnetophoretic separation of red blood cells (RBC) platelets, and protein fraction from peripheral blood is performed under continuous flow. In the second step, EIS is used for discriminating between CTCs and WBC.

A recent work by Arya *et al.*¹³⁶ demonstrated breast cancer cell detection using EIS in microfluidic device. In their application, anti-EpCAM was immobilized on the surface of the gold electrode (25 μm -diameter). The capture of MCF7 cell on the surface of the electrode was monitored using EIS. An average modification of $2.2 \cdot 10^7 \Omega$ in the impedance value was noticed once the cell was trapped on the electrode surface. Application of EIS method in cancer cell study is also reported in Ref. 137. Electrical “signature” of the cell was used for cancer cell detection by Asghar *et al.* in Ref. 138. The cells are pushed through a micropore, while electrical signal is collected using Ag/AgCl electrodes. Each cell population gives a distinct pulse behavior.

These studies underline the increasing awareness that EIS can be a useful tool especially for single cell characterization of CTCs. In this light, the identification of an “impedance signature” (Han *et al.*¹³⁴) is an important achievement for development of “diagnostics-on-a chip” and might be used instead of conventional biological markers such as antibodies to detect CTCs. It is important to note that EIS is not a high throughput method and association with other separation techniques is required. Unlike DEP, the measurements are performed at low voltages (maximal value is 1 V) and for these reasons, high-voltage related problems such as Joule heating effect or due to interaction between the electric field and cells are minimized. More clinical trials are required to confirm the potential of EIS for CTC characterization.

Magnetophoretic separation of RBCs from blood can be an interesting tool for enrichment of CTCs. The method is based on magnetic properties of the RBC and the use of high gradients of the magnetic field. The magnetic separation method of RBC from blood method was proposed in 1975 by Melville *et al.*¹³⁹ and has been applied in microfluidic devices over the last decade.^{140–144} The separation of RBC is possible due to the presence of hemoglobin, which gives to the cell a substantial paramagnetic moment. Opposite to RBCs, WBCs and CTCs have diamagnetic properties. This aspect is the starting point for the trapping of RBCs using high gradient magnetic separation. For a better understanding, we can consider the expression of the magnetic force that act on a paramagnetic particle in a non-uniform magnetic field:¹⁴⁵

$$F_m = \frac{1}{2} \frac{\Delta\chi V}{\mu_0} \nabla B^2 \quad (4)$$

Where $\Delta\chi$ is the relative magnetic susceptibility of a RBC relative to the buffer solution; V is the volume of the RBC, μ_0 is magnetic constant (vacuum permeability), B is the magnetic flux density. Equation (4) underlines the importance of generating a magnetic field gradient especially when the size of the particle is in micrometer range. Based on this principle different microfluidic devices for RBC separation were reported. Qu *et al.*¹⁴⁶ trapped RBCs in a microfluidic device using a Ni wire (of 69 μm -diameter), placed in a uniform magnetic field (0.3 T). Jung and Han¹⁴⁷ used an array of ferromagnetic wires, oblique arranged on the bottom of a microfluidic channel in order to generate a lateral magnetophoretic force and redirect the RBCs to a separate outlet. The separation efficiency of RBCs was 93.9% for a flow rate of 20 $\mu\text{l/h}$ using a magnetic flux of 0.3 T.

III. CONCLUSIONS AND PERSPECTIVE

As a general remark, the mechanical-based techniques (filtration and inertial separation) are high-throughput methods, while electrical methods (DEP and EIS) can be used for achieving a high purification of CTCs. The combination of these two directions (the method suggested by Moon *et al.*¹¹⁸) as well as the combination of magnetophoretic separation of RBC with EIS (Han *et al.*¹³⁵) can be not only an interesting approach but a direction to be followed.

Nevertheless, new techniques for evaluation of cell size and deformability can be used for identification and/or characterization of CTCs. In this direction, a recent work of Adamo *et al.*¹⁴⁸ used an microfluidic approach for probing the cell deformability (at single cell level). The cells are “pushed” to a “funnel-shape” constriction, the traveling time being depending on the cell diameter and cell stiffness.

Due to the low number of CTCs in the blood circulation and the technical challenges of downstream manipulation of these cells, enumeration of CTC is currently the only clinically validated analysis. Label-free strategies promise to have greater yield in comparison to currently utilized marker-based approaches which have inherent bias to isolation. Label-free¹⁴⁹ or hybrid technologies⁶⁶ have been show increase the sensitivity of these devices. On the other end, label-free separation might introduce false positives by isolating cellular populations that do not directly derive from the original cancer lesion. Stringent definition of CTCs (such as EpCam positive, Cytokeratin positive, CD45 negative cells), while potentially missing CTC populations, is currently one of the best definition for CTC and is only achievable by using biological

markers. EIS in this sense might improve the definition of CTCs without the use of biologic markers. Other label-free technologies still need to rely on biological markers to identify the correct target population.

CTCs contain further genetic and biologic information that may help to understand the patient's disease and eventually allow individualized therapeutic strategies. The goal for next-generation CTC isolation devices is to permit such analyses, ideally on the same chip or platform. In this line, cellular and molecular on-chip diagnostics¹⁵⁰ is an important direction in CTC research. Of note, CTC cell culture in microfluidic devices upon CTC enrichment is an interesting and extremely challenging approach.¹⁵¹ In this direction, on-chip analysis of cancer related phenotypes such as proliferation rate or protein and gene expression upon experimental drug treatments can be interesting research goals.

In general, and a part from few exceptions, label-free devices lack of testing with clinical specimen, and more collaboration with the clinics should be pursued while designing these devices. When clinical samples are used, usually real-world details in specimen processing need to be considered. In fact, for example, time from blood collection to processing and temperature may play a major part in assay performance. Many devices have been tested using ratios of cancer cells to white blood cells or RBCs that do not correspond to the range of concentrations seen in normal blood or clinical samples, and thus cannot be directly compared in term of retrieval efficiency, purity and sensitivity. Depending on the application, the isolation efficiency needs to be accompanied with data on purity of CTCs population achieved. The sensitivity of the device is an important characteristic and needs to be established, as CTCs are present at extremely low concentration in clinical samples. Size-based separation, especially using filtration devices represent so far the best-tested label-free methods in the clinics, offering the advantage of fast and affordable separation of CTCs from the blood sample. The processing time of whole blood (7.5 ml) for membrane-based filtration is usually quick and often less than 1 h. The recovery rate using spiked cell models is within the acceptable range: >80%. Purity and sensitivities of various devices presented here are comparable or even better than well-established marker-based approaches. Clinical validation of label-free CTC technologies represents thus a very exciting next step in CTC research.

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