Review

Challenging the current paradigm of liquid biopsy through dielectrophoresis (DEP) in prostate cancer

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Simple Summary: Dielectrophoresis (DEP) is a label-free cell-manipulation technique, based on electrical, rather than physical differences, that can be applied for liquid biopsy in the context of cancer prognosis and early diagnosis. In fact, it can overcome current limitation of other platfmorm like low number of CTCs detected (less than one CTC/mL of peripheral blood, thus no sufficient statistics in the case of 10mL of blood samples), genotypic and phenotypic heterogeneity, high-cost and time-consuming means for detection, quantification, isolation and characterization. The aim of this review is to give deep insights of current biotechnologies in order to improve CTC collection in cancer patients.

Abstract: Liquid biopsy via isolation of circulating tumour cells (CTCs) represents a promising diagnostic tool capable of supplementing state-of-the-art for prostate cancer (PC) prognosis. Unfortunately, most of CTC technologies, such as AdnaTest or Cellsearch, critically rely on the Epithelial-Cell-Adhesion-Molecule (EpCAM) marker, limiting the possibility of detecting stem-like cells (CSCs) and mesenchymal-like cells (EMT-CTCs) that are present during PC progression. In this tontext, dielectrophoresis (DEP) is an epCAM independent, label-free, enrichment system, separating rare cells simply on the basis of their specific electrical properties. As compared to other technollgies, DEP represents a superior technique in terms of running costs, cells yield and specificity, but due to its higher complexity, requires still further technical as well as clinical development. Interestingly, DEP can be improved by the use of microfluid, nanostructured materials and fluoroimaging in order to increase its potential applications. In the context of PC, the utility of DEP can be translated in its capacity to detect CTC in the bloodstream in their epithelial, mesenchymal, or epithelial-mesenchymal phenotypes, which should be taken into account when choosing CTC enrichment and analysis methods for PC prognosis and early diagnosis.

Keywords: circulating tumor cells; dielectrophoresis; prostate cancer; detection; prognosis

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1. Introduction

Collection and analysis of tumour cells and tumour-derived products present in body fluids is referred to as liquid biopsy (LB). LBs are becoming an important tool to complement conventional tissue biopsies for therapeutic decision-making in personalized treatment strategies. Whereas several current strategies focus on detection of circulating tumour DNA (ctDNA) in peripheral blood, the analysis of circulating tumour cells (CTCs) as cell-based LBs would provide complementary and clinically relevant information not only on DNA but also on proteins, RNA and cellular functions such as drug responsiveness. The incorporation of CTCs-based LBs into standard diagnostic and treatment guidelines suffers from three fundamental limitations: (i) low number of CTCs (less than one CTC/mL of peripheral blood, thus no sufficient statistics in the case of 10mL of blood samples), (ii) genotypic and phenotypic heterogeneity, (iii) high-cost and time-consuming means for detection, quantification, isolation and characterization.

To surpass these limitations a promising, but highly challenging approach, is to massively augment the analysed blood volume. In this context, diagnostic leukapheresis (DLA) has been established and subsequently validated [1], as a safe procedure to potentially screen 2,5 L of blood and to increase CTCs yield up to 100-fold compared to the volume of a normal blood draw of approximately 10 mL. Via DLA, CTCs are not only detected on larger numbers in metastatic patients but can also be identified more frequently [2] and reliably in non-metastatic patients without significant side effects. The bottleneck of DLA is the high background of co-isolated white blood cells (WBCs), which limits the use of complete DLA-products.

With regard to CTCs, currently available strategies consider their biological and/or physical properties, whereas functionality assays make it possible the identification of these. It is worth mentioning that there is no a gold standard right now, but each technique presents both advantages and disadvantages, while their combination may allow a more comprehensive and exhaustive characterization [3]. Most of the time the approaches related to the biological properties of CTC cells take into consideration the expression of specific markers at cellular surface level [4]. The most widely used marker for the selection of CTCs is the epithelial cells adhesion molecule (EpCAM) [5]. A limitation of the approaches EpCAM-based is represented by the fact that in the case of epithelial-mesenchymal transitions (EMTs), CTCs will not express EpCAM anymore, leading to an underestimation of CTCs number [6]. Alternative markers are represented by mucin 1(MUC1) and epidermal growth factor receptor (EGFR) [7]. Additional approaches include the use of microfluidic platforms (discussed in more details later) as well as devices allowing for the on line in vivo capturing of CTCs and the subsequent analysis by highly sensitive multiplex RT-qPCR assays [8]. Size-based approaches for the isolation of CTCs, including filtration methods, belong to group of methods based on the physical properties of these cells [9].

Among the strategies aiming at both detection and enrichment of CTCs, microfluidic and dielectrophoresis (DEP)-based technologies represent two of the most promising and attracting considerable attention.

DEP is a label-free cell-manipulation technique, based on electrical, rather than physical, differences and may offer promising opportunities to improve CTC detection by separating rare cells simply on the basis of their specific electrical properties [10].

Interestingly, these technologies via isolation of CTCs are a promising diagnostic tools capable of supplementing state-of-the-art PCa diagnostics [11]. Various studies have shown that the blood of patients with prostate cancer may contain CTCs derived from the primary tumour and different metastatic sites [12]. However, the presence of CTCs in patients with PCa is mainly dependent on the platform used for CTC detection. To date, various techniques have been established for CTC enrichment or detection, including techniques that are based on immunomagnetic enrichment and microscopy, such as the CellSearch assay (Janssen Diagnostics, Raritan, NJ, USA), the only platform that has achieved USA Food and Drug Administration approval [13] or the AdnaTest Prostate Cancer (Qiagen, Hilden, Germany) [12].

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Although previous innovative data, these techniques suffer from low CTC yield, especially in the case of the localized forms of cancer, implying a requirement for providing a large volume of blood for PCa detection.

Based on all these premises, the aim of the current review is to give deepen insight on the role and future perspectives of DEP in the context of CTC detection in cancer.

2. Dielectrophoresis: from physic to biological application

DEP is the movement of dielectric particles in an inhomogeneous electric field. DEP occurs when a polarizable particle is suspended in a non-uniform electric field. The electric field polarizes the particle, and the poles then experience a force along the field lines, which can be either attractive or repulsive according to the orientation on the dipole. Since the field is non-uniform, the pole experiencing the greatest electric field will dominate over the other, and the particle will move.

Consider a spherical noncharged particle of radius R in a medium. If the particle is polarized and if there is a field gradient ∇Erms, then, the time averaged force <Fdep> on the particle [14] is given by:

$$\langle F_{DEP} \rangle = 2\pi R \varepsilon_m f_{CM} \nabla |E_{rms}|^2$$

Where ε_m the complex permittivity of the suspending medium and f_{cm} is the Clausius-Mossotti function [14–16] which contains all the frequency dependence of the DEP force. This equation is the basic equation for DEP and shows that, depending on the relative polarizability of the particle with respect to the medium, i.e. the sign and module of f_{cm}, the particle will move either in the direction of the field gradient (positive DEP, pDEP) or in the opposite direction (negative DEP, nDEP) (see **Figure 1**).

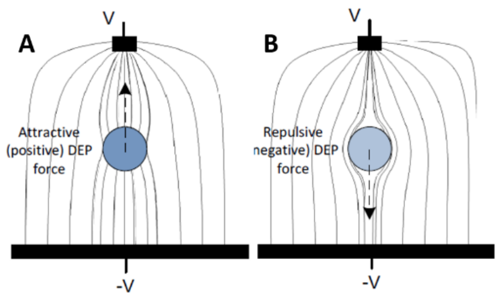

Figure 1. movement of particles under non-uniform electric field. A) attraction towards higher electric field region (pDEP) and B) attraction towards lower electric field regions (nDEP).

f_{cm} is a dimensionless number defined as:

$$f_{CM} = \mathrm{Re}\left[\frac{\varepsilon_p^* - \varepsilon_m^*}{\varepsilon_p^* + 2\varepsilon_m^*}\right]$$

where the terms ε_p^* and ε_m^* are the complex permittivities of the suspended object and the medium, respectively, generally defined as $\varepsilon_{p,m}^* = \varepsilon_{p,m} + \sigma_{p,m}/j\omega$, with $\varepsilon_{p,m}$ and $\sigma_{p,m}$ representing the particles or medium permittivities and conductivities and ω is the angular frequency of the applied signal. From this equation it can be seen that, varying the frequency will allow changing the f_{cm} value and thus the DEP response. Typical DEP chambers use an array of interdigitated electrodes, such as in **Figure 2**, which, depending frequency and thus f_{cm} term, can lead to repulsion from electrodes (*nDEP*) or attraction towards them (*pDEP*) (**Figure 2**, bottom). Since the direction of the force dependents on field gradient rather than field direction, DEP will occur in AC as well as DC electric fields.

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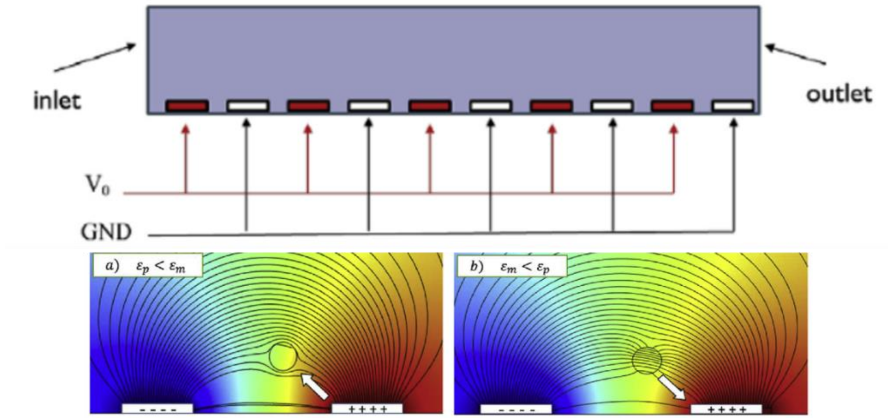

Figure 2. top) side view of interdigitated electrodes channel. bottom) rappresentation of voltage and electric field lines for particle experiencing nDEP (left) and pDEP (right) for the interdigitated electrode geometry.

DEP allows for the controlled manipulation of micro- and nano-sized particles dispersed in colloidal solutions. Application fields include: cell partitioning and isolation [15,17], bio-structure assembling [18], nanostructure (e.g. carbon nanotube) deposition [19] or even filtration systems for oils purification [19]. Dielectrophoresis can thus be used to manipulate, transport, separate and sort different types of particles. Particularly, since biological cells have cell-specific dielectric properties [14], dielectrophoresis has many medical applications and several experiments have been successfully made to separate cancer cells from healthy ones in peripheral blood samples.

In order to separate different cells, their complex permittivities, ε_p^*, need to be identified. For this, single or multi-layered shell models are employed. The simplest case, sufficient for many applications, is the single-shell spherical model, which simplifies cells as a spheric objects enclosed by an extremely thin cell-membranes [14]:

$$\varepsilon_p^* = \varepsilon_{mem}^* \frac{\left(\frac{r}{r-\delta}\right)^3 + 2\left(\frac{\varepsilon_{cyt}^* - \varepsilon_{mem}^*}{\varepsilon_{cyt}^* + 2\varepsilon_{mem}^*}\right)}{\left(\frac{r}{r-\delta}\right)^3 - \left(\frac{\varepsilon_{cyt}^* - \varepsilon_{mem}^*}{\varepsilon_{cyt}^* + 2\varepsilon_{mem}^*}\right)}$$

where r is the cell radius and δ is the membrane thickness, ε_{cyt}^* is the complex permittivity of cell cytoplasm, and ε_{mem}^* represents the complex permittivity of the membrane.

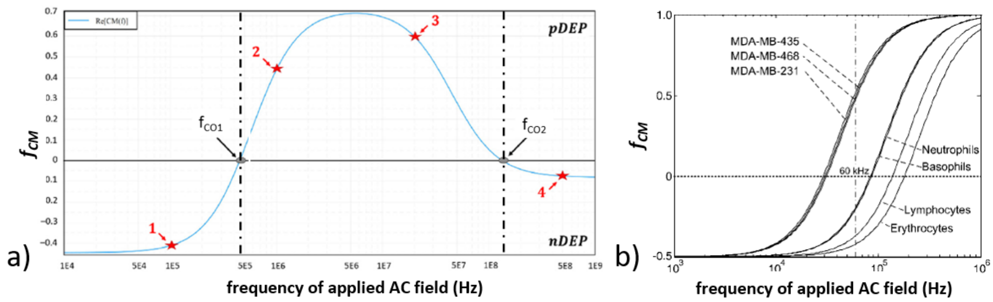

Figure 3. Clauss Mosotti function for typical cells showing the low frequency (tens of kiloherz) and high frequency (tens of Megaherz) crossover frequencies, associated to inversion of cells dipole movement.

Figure 3 shows typical f_{cm} as function of applied electric field frequency: for very low and very high frequencies f_{cm} is negative, indicating a movement towards lower electric field regions, i.e. away from the electrodes; whereas for medium frequencies f_{cm} is positive. As the frequency traverses well-defined crossover frequencies, f_{CO}, f_{cm} and thus the DEP force passes through zero and changes direction. Different cell types will have different f_{CO} so that it is possible to choose an electric field frequency that lies in between the

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crossover frequencies of the different cell types. In this case, cells with the lower crossover frequency will be attracted towards high field regions (e.g., electrode edges or pinched field regions) while cells of higher crossover frequency will be repelled towards low field regions (see **Figure 3b**). In this way, DEP may be used to discriminate between different cell types. The DEP crossover frequency is thus the essential parameter exploited for separating cells. In the case of medium with conductivities much lower than cells one, f_{CO1} can be written as [20]:

$$f_{CO1} = \frac{\sigma_m}{R\varphi C_0}$$

where σ_m is the medium conductivity, R the outer cell radius and $\varphi C_0 = C_{mem}$ is capacitance per unit area of the cell membrane. C_0 is the capacitance per unit area of smooth plasma membrane, determined as $C_0 \approx 0.009$ F/m [21] and φ is the folding factor characterizing the different membrane features, such as ruffles, folds, and microvilli. $R\varphi$ can be considered as the "*dielectric phenotype*" of a given cell type determining its response to DEP manipulation. This sensitive dependency of the dielectric phenotype on membrane folding, in addition to cell size, distinguishes DEP methods from approaches to cell isolation that depend on size alone, such as size filtration.

Over a number of years, a large number of cancer cell types of both cultured and primary origin have been examined and a consistent trend of cancer cells having larger folding factors and radii than both normal cells of comparable origin and blood cells has emerged specifically demonstrating that all of the cell lines derived from solid tumors have crossover frequencies that should allow their efficient isolation from normal blood cell types [22] (see **Figure 4**).

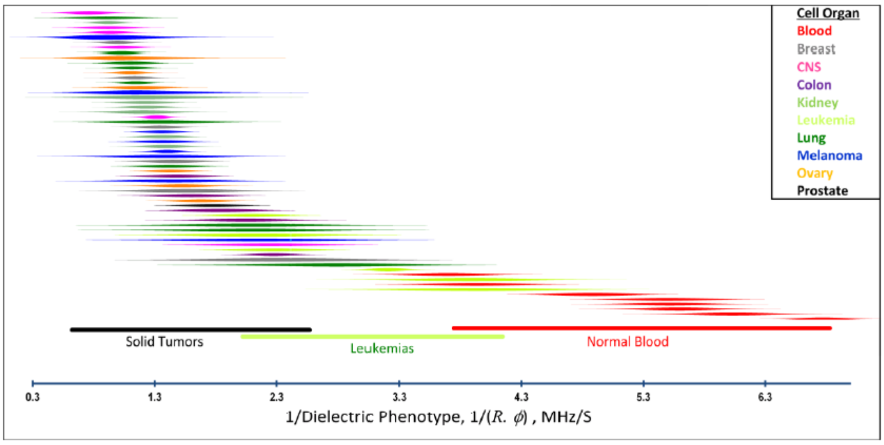

Figure 4. The DEP responses of cancer and normal blood cells expressed in terms of the reciprocal cell dielectric phenotype 1/ $R\varphi$, proportional to the DEP crossover frequency f_{CO1} that determines the behavior of the cells in DEP manipulation and isolation applications. (Adapted from ref [23])

As mentioned, f_{CO1} is sensitive to membrane features. This is because, at low frequency, the electromagnetic field is shielded by the low-conductivity membrane, making it more sensitive to cell shape, its morphology and plasma membrane properties. The more the frequency of the applied signal increases, the more the electric field can penetrate inside the cell and starts to interact with and so probe the cellular content. Consequently, at high frequency, the electromagnetic wave can be deeply sensitive to the intracellular content. second crossover frequency f_{CO2}, in the case of a low conductive medium (e.g. 30 mS/m) can be approximated as [2425]:

$$f_{CO2} = \frac{\sigma_{cyt}}{2\pi}\sqrt{\frac{1}{2\varepsilon_{mem}^2 - \varepsilon_{mem}\varepsilon_{cyt} - \varepsilon_{cyt}^2}}$$

For this reason, f_{CO2} can be used to discriminate cells based on cytoplasm features [26], thought, due to the technical difficulties associated to the high frequency actuation and to

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the high sensitivity of the DEP response to small cells variations within cells subpopulation, the separation of cells based on f_{CO2}, generally referred as ultra-high-frequency, UHF DEP, has not been extensively explored as compared to the low-frequency, f_{CO1}-based, DEP.

Although DEP has the potential capacity of discriminating all of the cell lines derived from solid tumors from normal blood cell types, DEP is a microscale phenomenon and significant technical challenges arise in applying it in a manner capable of processing large cell numbers with sufficient discrimination to process clinical specimens in a reasonable times. Because F_{DEP} increases with the square of the electric field, DEP is normally operated at as high a voltage as possible, to increase the manipulation force and thus decrease separation time. However, in the higher electric fields induced by a larger DEP voltage, the cells will not only experience a greater trapping force but may also exhibit field-enhanced ion leakage (or at even higher voltages electroporation [27,28]) or degradation due to thermal heat generated by Joule heating. Another important factor that can impact the efficiency and purity of DEP separation is the cell loading concentration. As illustrated in **Figure 2**, bottom, the DEP force experienced by a cell depends on the extent to which the cell deflects the electric field. If another cell is nearby, mutual field perturbations will modify the force experienced by both cells. This effect, which is generally referred to as an electric dipole-dipole interaction [10,29], can result in the clustering of cells and the entrapment of both similar and dissimilar cell types [15]. Excessive cell loading was shown to lead to reductions in cell discrimination and separation efficiency in DEP. or example, on an interdigitated array of 50 µm electrodes with 50 µm gaps, the isolation efficiency was 90% at a loading density of 500 peripheral blood cells per mm^3 but only 20% at a loading density of 10,000 peripheral blood cells per mm^3 **Error! Bookmark not defined.**. Another complication arising from deflection of the electric field is the particle–wall dipole interaction, which can result on a-specific electrical adhesion of cells to the microfluidic channel walls. This problem can be mitigated by the use of carefully designed electrodes geometries [30].

Currently, the most advanced DEP based CTC separation system is the *Apostream*™ *(Precision Medicine Group, LLC)*, based on a complex continuous field-flow DEP fractionation schema (FFF-DEP) [31].

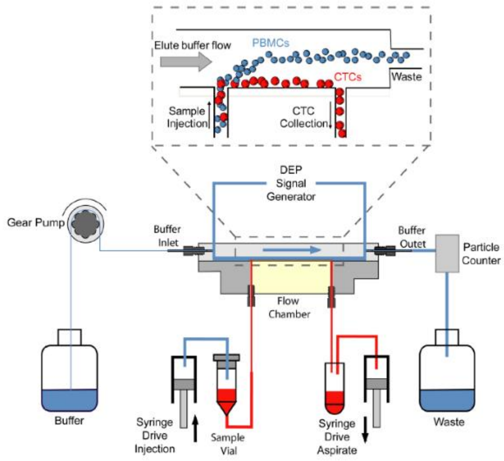

Figure 5. Schematic, side-view, of the Apostream device, showing the complex multi-inlet, multi-outlet, microfluidic system. (Adapted from [31])

The system has been successfully tested in different solid tumors to capture epithelial-like CTCs as well as mesenchymal- and stem-like CTCs[32]. The main limit of ApoStream™, as for similar DEP-based sorting systems, is the low cell throughput, lower than 200 million (2^8) cells/h. Furthermore, the technology is characterized by a complex multi-injection system (see **Figure 5**) which decreases its discriminating power. Nevertheless, the DEP crossover frequencies of cancer cells and blood cell subpopulations are sufficiently different that this results in only a small loss of cancer cell isolation efficiency. The limitation of low throughputs is common to all current CTC separating technologies, using physical, electrical or molecular separation schemes (see **Table 1**). This bottleneck limits the complete analysis of high-volume samples resulting in lower sensitivities and ultimately impacting the capability of these technologies in assessing Minimal Residual Diseases (MRS).

Table 1. Details of extracted devices characteristics can be found in ref [33,34]. **Throughputs have been extrapolated based on processing time*.

	CellSearch®	ISET®	LPCTC-iChip [35]	Cytofluorometers	Apostream
Throughput [Cells/h]	**$1.6x10^{6}$***	**<$1x10^{7}$***	**≈$1.5x10^{9}$***	**10^{8}**	**<$2x10^{8}$**
***epCAM* + independent**	**NO**	**YES**	**YES**	**DEPENDS**	**YES**
antibody independent	**NO**	**YES**	**NO**	**NO**	**YES**
Device specificity	**HIGH**	**MEDIUM** [36]	**HIGH**	**HIGH**	**HIGH** [37]
Downstream analysis	**NO**	**YES**	**YES**	**YES**	**YES**
Enumeration capability	**YES**	**NO**	**NO**	**YES**	**YES**

3. The role of DEP for CTC detection in prostate cancer

Prostate cancer (PC) represents the most common incident cancer in men in developed countries in 2021 [38]. Incident cases increased more for PC than any other malignancy globally, irrespective of development status.

In this context, liquid biopsy via isolation of CTCs represents a promising diagnostic tool capable of supplementing state-of-the-art PC diagnostics [39] and prognosis [40–44].

Various studies have shown that the blood of patients with prostate cancer may CTCs derived from the primary tumour and different metastatic sites [12]. The presence of CTCs in patients with prostate cancer is mainly dependent on the platform used for CTC detection.

To date, various techniques have been established for CTC enrichment or detection, including techniques that are based on immunomagnetic enrichment and microscopy, such as the CellSearch assay (Janssen Diagnostics, Raritan, NJ, USA), the only platform that has achieved USA Food and Drug Administration approval [13]. Among other techniques, The AdnaTest Prostate Cancer (Qiagen, Hilden, Germany) combines immunomagnetic enrichment of epithelial cells with PCR for tumour-associated transcripts [12]. The AdnaTest ProstateCancer (Qiagen, Hilden, Germany) combines immunomagnetic enrichment of epithelial cells with PCR for tumour-associated transcripts.

Another platform is the cytology-based filtration method that has the possibility to detect any tumour-surface-markers on cancer cells, epithelial and non-epithelial, and distinguish between single-CTC and CTC-clusters [45] whereas most of other CTC technologies, such as AdnaTest or Cellsearch, critically rely on the Epithelial-Cell-Adhesion-Molecule (*EpCAM*) marker, limiting their applicability for early-stage PCs where CTCs may have not still developed epithelial characteristics.

DEP is another *epCAM* independent, label-free, enrichment system, separating rare cells simply on the basis of their specific electrical properties [10]. The technique os capable of isolating all type of solid tumour-related immortalized cells from the NCI-60 panel [23], and more recently to co-collect, together with CTCs, also stem-like cells (CSCs) and mesenchymal-like cells (EMT-CTCs) [46].

Depending on the frequency of the externally-applied electric fields, the DEP cell sorting (DACS) will be mainly dependent on membrane morphology (medium frequencies, 10-200kHz) or cytoplasm composition (high frequencies, 5-400MHz) rather than on a cell's phenotype as for fluorescence cell sorting (FACS) or magnetic cell sorting (MACS). The most advanced DACS system, Apostream™(Precision Medicine Group, LLC), is based on a complex continuous field-flow DEP fractionation schema (FFF-DEP) [47] . The system was successfully tested in different solid tumours to capture epithelial-like CTCs as well as mesenchymal- and stem-like CTCs [46,47]. The main limit of ApoStream™, as for similar DEP-based sorting systems, is the use of planar, interdigitated, electrodes configurations causing exponentially decaying DEP forces, this limits the size of the sorting volume, on which the DEP forces can act efficiently, and reduces cell throughput. In the case of ApoStream™ less than 200 million (2^8) cells/h can be processed, so, a DLA sample would be processed in >10 h.

Interestingly, Le Du et al [46] were able to demonstrated that ApoStream was successful in detecting EMT-CTCs among patients after neoadjuvant chemotherapy in breast cancer.

All patients who had at least one CTC had epithelial and/or EMT-CTCs; no patient had only CSC-CTCs. The detection rates of CSC-CTCs were 9% (4 of 47 samples), 22% (8 of 37 samples), and 19% (6 of 31 samples) at time points T0 (before chemotherapy), T1 (after chemotherapy but before surgery), and T2 (after surgery).

Although it has not been investigates in PC patients, ApoStream(®)was able to detect CTC using laser capture cytometry in blood samples from cancer patients (NSCLC adenocarcinoma, breast cancer, ovarian cancer and squamous lung cancer patients) [47].

Based on all these premises, DACS advantages could be also translated in early phase of cancer, when CTC can be potentially available.

In fact, although it is difficult to detect EpCAM marker in early prostate cancer, we have previously published our experience on the Adnatest platform, identifying CTCs even in patients with low-risk clinically localized prostate cancer (22.2%) and high-risk clinically localized prostate cancer (30.9%), showing that EpCAM CTCs, already with epithelial characteristics, can be detected in the context of early stage of PC [48].

In a study performed by Ried et al on 2020 [49], combining blood filtration and microscopic analysis using standard cyto-pathological criteria, authors investigated the role of CTC in a cohort of 47 patients with suspected PCa.

Authors demonstrated that in the 27 men in the early detection group (group ED), 25 men had CTC, while 2 men had no CTC. Twenty out of the 25 men with CTC had ICC-PSA-positive markers, and all of these 20 (100%) were diagnosed with prostate cancer. The high accuracy of the ISET-CTC test combined with the 97% sensitivity and 99% specificity of the PSA-marker presence on prostate cancer cells, suggests an estimated positive predictive value (PPV) of 99% and a negative predictive value (NPV) of 97% for this novel screening test. The average CTC count in the tested samples of men aged 30–83 years (mean = 58 years) was 1–13 CTC/ml (mean = 3.1 CTC/ml), and two patient samples had higher counts of 39 and 65 CTC/ml.

However, it is important to underline that the evolution of CTC proceeds via multiclonal expansion which causes the tumor to be composed of multiple cell subpopulations. The metastasis process consists of several sequential steps: local invasion of the primary tumor cells, intravasation, extravasation, and the establishment of distant metastasis (**Figure 6**).

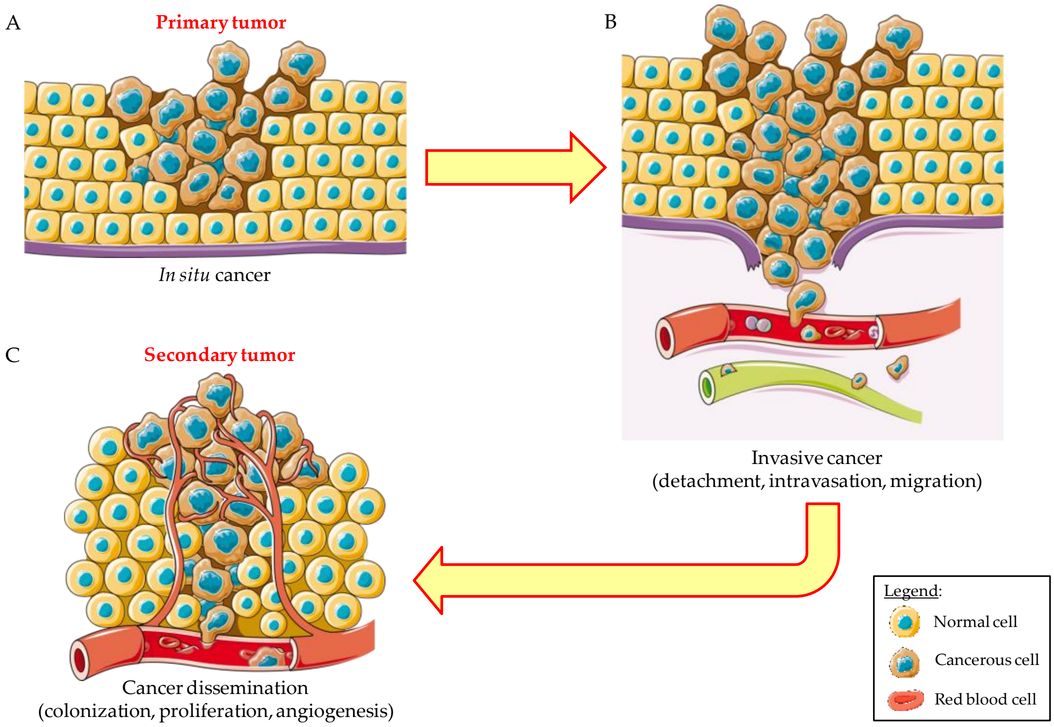

Figure 6. Schematic representation of metastatic process. Metastasis is a multi-stage process starting with the formation and growth in situ of the primary tumor (A). Some tumors can become invasive due to the detachment of cells (CTCs) that are able to enter blood or lymph vessels, a process known as intravasation, and migrate in sites far from the starting point (B). The following step is represented by the dissemination of cancer occurring when "traveling cells" extravasate (exit blood or lymph vessels) and colonize new sites forming secondary tumors (C). Once on the new sites, cancerous cells are able to proliferate and recruit the blood vessels (angiogenesis) needed for trophic support. (Created with https://smart.servier.com)

During the phase of initial local invasion, substantial changes in the morphology of tumor cells occur. In the single-cell invasion pathway, epithelial cells undergo epithelial-mesenchymal transition (EMT), that is, the loss of epithelial characteristics and gain of mesenchymal characteristics. Tumor cell size, including CTC, ranges from 9 to 30 μm. Blood capillaries have a diameter ranging 3–8 μm and thus CTC can become trapped in their lumen [50].

Its application has found interesting results in the context of androgen-directed therapies via AR-V7 (androgen receptor-V7) signaling. Davis et al evaluated ApoStream technology to capture AR-V7 expressing CTCs from blood originating from primary tumor cells (epithelial) and those that undergo epithelial-mesenchymal transition (EMT) of CRPC patients. They found that ApoStream enriched cancer cells from cell lines expressed AR-V7 in both epithelial CTCs (detected in 6/10 patients in CD45- [median: 9.5] and in 7/10 in CD45+ [median: 1] cells and mesenchymal CTCs (detected in 5/10 patients for both CD45- [median: 0.5] and CD45+ [median: 0.5] cells) [51].

Finally, the utility of DEP can be translated in its capacity to detect CTC in the bloodstream in their epithelial, mesenchymal, or epithelial-mesenchymal phenotypes, which should be taken into account when choosing CTC enrichment and analysis methods.

4. Microfluidic technologies for CTCs isolation and analysis

One of the main aim of biological and biochemical studies is to understand the mechanisms characterizing a single cell, which represents the minimal functional unit of life. In this context the application of microfluidic-based devices offers numerous advantages including the rapid and simultaneous separation and quantification of multiple chemical species, the high sensitivity and reproducibility, and the possibility to study the heterogeneity of cell populations [52], the latter being an extremely important factor to be considered for the analysis of CTCs. The use of these devices is also accompanied by a very short analysis time and gives the opportunity to use different detection platforms, such as fluorescence and electrochemical detection just to name a few, making it useful for high throughput single-cell analysis. All these features make microfluidic-based technologies a powerful analytical tool for biomarker detection in personalized therapy and precision medicine.

Over the past two decades, the enormous potential of microfluidic-based technologies for isolating and detecting CTCs from whole blood has emerged. As described in a very recent review written by Cheng and co-workers [53], thanks to the advancement of microfabrication and nanomaterials, different approaches have been developed for isolation (capture + release) and analysis (morpholic, genomic, and protein)/ profiling (transcriptomic and functional) of CTCs on microfluidic platforms; these new approaches are characterized by numerous benefits, especially in terms of cell capture efficiency, purity, detection sensitivity, and specificity, allowing to better explore cancer mechanisms and address increasingly complex biological questions. Using this as a very good example of microfluidics applied to CTC analysis, a fast and efficient microfluidic cell filter for label-free isolation of CTCs from unprocessed peripheral blood obtained by colorectal cancer patients has been developed by Ribeiro-Samy et al. [54]. This device (named CROSS chip) made it possible the capture of CTCs contained in 7.5 ml of whole blood based on their size and deformability in a short time with high purity and efficiency. Of note, CTC enumeration by CROSS chip allowed the stratification of patients with different prognosis. The cells isolated by using this microfluidic device were lysed and further subjected to molecular analysis by employing droplet digital PCR, revealing a mutation in the adenomatous polyposis coli gene for most patient samples considered, confirming their colorectal origin and underlying the adaptability of this technology for downstream applications. Among the different technologies that can be coupled to microfluidics, DEP, being label-free, fast, and accurate represents a very promising one. In fact, as proven by numerous and often recent scientific publications, DEP is becoming a commonly used technique in microfluidics (DEP-on-a-Chip) for particles or cell separation and has been widely applied for bio-molecular diagnostics as well as for medical and polymer research.

5. DEP applied to microfluidic platforms: focus on cell separation and analysis

As previously mentioned, DEP is becoming one of the most promising separation techniques for micro- and nano-scale systems because of its low running cost, the rapid sample processing, and the possibility to easily integrate it into microfluidic devices [55]. These features along with the high efficiency, sensitivity, and selectivity that characterize DEP, make it really attractive for the study of cell behavior, especially at single-cell level. DEP has been used in combination with lateral field flow fractionation (LFFF) to improve the isolation of spiked breast cancer cells from "healthy" blood cells [56]. In this research study, Waheed et al. have developed a continuous-flow, DPE-LFFF microdevice able to isolate green fluorescent protein-labelled MDA-MB-231 breast cancer cells from regular blood cells. In a different study carried out by Piacentini et al., a device able to separate platelets from other blood cells by DEP-FFF was developed [57]. This innovative device uses the so-called "liquid electrodes" design (planar electrodes patterned at the bottom of dead-end chambers positioned perpendicularly to the main channel) and can be employed with low applied voltages, giving a very efficient separation coupled to a very high purity of platelets (~99%) with almost absent cell loss (<2%). Of note, this device is already set up for the integration with an on-chip cell counter allowing the

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measurement of platelet concentration in the blood. More recently, De Luca and co-workers developed a microfluidics platform combining DEP and imaging (DEPArray) that has been used to accurately select single breast cancer cells [58]. A hydrodynamic and direct-current insulator-based DEP (H-DC-iDEP) microfluidic device made of polydimethylsiloxane (PDMS) covered with a glass lid allowing the separation of plasma from fresh blood has also been developed [59]. This represents the first device making it possible for a real-time monitoring of the plasma components without pre- or post-processing steps. As recently demonstrated, DEP microfluidic devices can be used for the separation of different types of cells such as leukocytes (floating cells) and spermatozoa (moving cells) [60]. In particular, the differences in size and membrane properties were considered for the separation of leukocytes, while membrane charge and cytoplasm conductivity represented the two key factors allowing the separation of X and Y spermatozoa. Lastly, DEP coupled to microfluidics has also been employed for the high-throughput selective capture of single CTCs [61].

6. The use of nanostructured materials in DEP for CTC detection

The nanostructured materials due to their peculiar optical, electronic, photothermal, magnetic and chemical properties largely influenced by their quantum size effect, have attracted large interest in the various area of scientific community. Despite nanomaterials such as nanoparticles, nanorods, nanotubes, nanowalls and nanostructured coating are perceived as the most promising nanotechnologies in the vast field of biomolecules detection, including nucleic acid, protein and cells recognition [62,63], their use in DEP for CTCs detection is still limited and leaves plenty of room for improvements.

Recent works reported the use of nanostructured materials for CTCs detection, employing various nanotechnologies such as magnetic-separation, nanofiltration and NanoVelcro. In the magnetic-separation approach the CTCs interacts with specific probe anchored on magnetic nanoparticles surface and recognized on a microarray format [64]. The NanoVelcro is a new technology recently developed by research team of UCLA. It is based of silicon nanowires substrates coated with polymeric nanostructured such as polydimethylsiloxane[65], thermo-responsive Poly(N-isopropylacrylamide)[66] and conductive boronic acid-grafted nanocoating [67]. These nanostructures are properly designed and tested for an efficiently CTCs isolation, purification and enumeration in a miniaturized NanoVelcro chip [68]. Nanostructured materials have been largely proposed as promising agents to increase the specificity for CTCs recognition, through chemically functionalization with molecules or biomolecules probes properly designed to interact with specific targets in CTCs cell membrane [69]. In this scenario, EpCAM antigen is one of the most utilized biomolecule targets widely expressed on the CTCs surface [70], this approach is used in the CellSearch system, a commercially available platform for CTC detection [71]. Moreover, carbon-based nanostructures such as graphene oxide, carbon dots and carbon nanotubes were proposed as promising agent for the CTCs detection, upon functionalization with EpCAM antibodies for direct detection of cancer cells in whole blood by electrical impedance sensing [72]. Moreover, nanostructured coatings have been reported to enhance interactions between substrates and targeted cell surfaces, with a net increasing of cell affinity compared with flat substrates [73]. All these data have encouraged researchers' team to focus their effort on the development of nanostructured materials for the detection of CTCs by DEP technology [74]. Recently Wu and coworker report the fabrication and testing of an Optical-DEP device based on nanostructured PDMS coating, demonstrating an increasing of CTCs recognition performance [75]. Barik and coworkers et al. developed a graphene-based DEP platform produced by nanofabrication process, capable of reversibly trapping nanosized particles and biomolecules with nanoscale precision [73]. Swardy and coworkers have developed a DEP microfluidic device based on silica beads modified with antigen probe capable of binding single cells [76]. Cao et al. developed an iDEP platform composed of a structure with SiO_2 microelectrodes coated with nanosized (100nm) Ag-nanorods, their demonstrated an increasing of cells enrichment factor nearly ten times greater than the naked electrodes [77].

The above mentioned nanomaterials together with additional i nanotechnologies will be further developed to obtain a highly sensitivity and specific CTCs detection method based on DEP approach.

7. Monitoring cell integrity during DEP by fluorescence imaging

An important issue in cell-based DEP is whether the integrity of the cells is preserved during the process. Ideally, biological, biochemical and biophysical properties of the cells must remain unaltered during DEP. In practice, cells are subjected to forces, and cell damage can occur for several reasons, including excessive charging of the cell membrane in the electric field, suspension in non-physiological medium, flow-induced shear stress [78]. The extent of cellular damage will be dependent on the conditions of operation of the device (e.g. applied voltage, buffer, flow rate) and biological parameters (e.g. cell type). Thus, for any specific application, a careful optimization of the DEP protocols is required to preserve the cellular integrity.

Cell viability is the most straightforward parameter to monitor to quantify the extent of cellular damage induced by DEP. Cell viability during the exposure to DEP electric field (1 MHz, 10 V) has been quantified using the fluorescence of Propidium Iodide (PI), a cell-impermeant fluorophore that stains only dead or dying cells due to their loss of membrane integrity [79]. Cell viability of Jurkat cells was dependent on the exposure time and size of the electrode [79]. However, at the conditions of the experiment, cell death was below 10%.

A more subtle question is which biochemical and biophysical alterations are induced on the cells during the DEP exposure time and if they are relevant for any subsequent analysis performed on the cells. In this respect, we believe that an important role can be played by the application of fluorescence imaging approaches to characterize the biochemical and biophysical changes, if any, occurring during DEP.

Fluorescence labeling provides molecular specificity and allows mapping directly the biochemical content of a cell. The resolution of currently available fluorescence imaging techniques range from the size of small organelles to that of single molecules. Imaging flow cytometry is an established technique that combine imaging with a typical resolution of 500 nm with the processing of thousands of cells per second[80] and is the technique of choice for rare cell detection. Confocal microscopy provides a higher spatial resolution, typically 200 nm in the lateral direction and 500 nm in the axial direction [81], sufficient to visualize clearly most of the subcellular components, but the typical number of cells analyzed is much lower, in the order of one cell per second. The recently developed super-resolution microscopy techniques provide spatial resolution down to 20 nm [81], enabling visualization of the finest molecular details. Finally, Forster Resonance Energy Transfer (FRET) can detect biochemical interactions and molecular distances at a spatial scale below 10 nm [82].

Biophysical properties that can be monitored by fluorescence imaging include cellular and organelle shape, viscosity, macromolecular architecture. A fundamental advantage of fluorescence is the capability of labeling multiple species with different colors. This allows performing colocalization analysis and measuring distances between molecular components [83]. For instance, multicolor imaging could be performed to simultaneously visualize different subcellular components and monitor their integrity during DEP. Another interesting property of fluorescence is the sensitivity of specific probes to the molecular environment. Environment-sensitive membrane probes have been used to monitor biophysical changes of the cellular membranes [84]. These techniques could be applied to monitor biophysical changes of the plasma membrane occurring during DEP, eventually leading to more optimized DEP protocols.

8. The role of CTCs in precision medicine

Cancer evolution and recurrence depend on synergic interaction of molecular features (namely genomic mutations, single nucleotide polymorphism, accumulation to CpG island methylation etc.) and phenotypic features of individual clones which prolif-

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erate out of physiological constraints, destroying tissue barriers to spread to other organs and promote immune evasion [85]. Spatial and temporal cancer heterogeneity arise from subclonal evolution, driven by the simultaneous presence of different mutational patterns, consequence of complex and altered molecular pathways which are potentially different between each individual patient, and even between the same patient and at different moments in the development of the disease [85].

A potential strategy to prevent cancer metastasis and warrant clinical benefits to patients is the early detection of potential metastatic clones, carrying driver mutations, that are capable of leading to development and guidance of the tumor phenotype conferring a selective growth advantage to the cell. Driver mutations should be distinguished by passenger mutations which are accessory, and do not play an active role in conferring clonal advantage [86]. The accumulation of driver and passenger mutations is not constant in all tumor cells, leading to a different growth rate of different subclones within the same tumor, characterized by different gene expression patterns, associated to different prognosis [85,86]. During biopsy or surgical resection (both late and invasive techniques), it is almost impossible to select only cancer cells because is present a mixed cellular representation between normal and tumor cells. There is an increasing evidence of the intra-tumour heterogeneity in cancer due to spatial [87] and temporal heterogeneity [88], with a plethora of sub-clonal mutations carried only by a fraction of the tumor cells [89]. For this reason, liquid biopsy, based on detection in the bloodstream of circulating tumor DNA (ctDNA, tumor-derived fragmented DNA not associated with cells) and broader circulating free DNA (cfDNA, degraded DNA fragments released by apoptotic cells and necrotic cells, not necessarily of tumor origin) are emerging means for a not-invasive investigation of the tumor molecular structure [90,91].

Next Generation Sequencing (NGS) allows the identification of mutations even with low representation (up to 3%) by means of very high coverage sequencing, but additional bioinformatic and machine learning tools are required to identify clinically relevant mutations in a background of errors, noise, and random mutations [92], challenging the contribution of passenger mutations that can still be used to improve cancer subclassification [91,93]. Because of genome plasticity, cfDNA could not be fully informative of cancer evolution, representing an average of multiple subclones present in the individual patient, providing the rationale for the search of alternative not-invasive and not-expensive platforms. In this context, microfluidic single-cell manipulation [94] for enumeration and isolation of CTCs are by far the best biological matrix to move from single gene analysis to single cell profiling required for the new era of precision medicine [94–96].

CTCs are released in body fluids from primary or metastatic tumour sites for several, not always recognized reasons, such independence from adhesion to the supportive niche [97,98]. There are some technical issues to overcome to use CTCs isolated by as screening tool for early detection of solid cancer or its recurrence after treatment, including:

- The sample source. Based on the CTCs source (peripheral blood, urine, saliva, or other biological fluids), the spatial clonal heterogeneity could lead to false negative results, by underestimating the disease burden due to the presence of remaining tumor cells in not accessible sites, that could be monitored with coupled imaging techniques, such as PET or MRI or circulating free DNA (cfDNA) by NGS. Recently, several means to isolate and manipulate CTCs have emerged, ranging from using microfluidic to dielectrophoresis techniques [99,100], starting from different kind of biological fluids [101] other than peripheral blood. For prostate cancer there is an emerging interest for seminal plasma, since the electrophoresis of seminal plasma cfDNA enabled the discrimination between subjects carrying tumor or benign proliferation [101]. Alternatively, tumor isolation via liquid biopsy of the urine lacks limitation in the sample volume. For prostate cancer, the first stream of urine (about 30 mL) is sufficient to collect most cells of interest using a spiral microfluidic chip. This approach requires urine filtration or a specific pipeline of preanalytical enrichment to discard large waste elements like urine crystals [102].

- The absolute number of CTCs recovery. On average, 5-50 CTCs can be recovered from every 7.5 mL of peripheral blood from a patient with metastatic cancer, meaning that a 10-5-10-6 sensitivity is required, which is the threshold commonly accepted in the evaluation of minimal residual disease in hematological cancers. CTCs enumeration is clinically relevant, since it is associated to high tumor burden, aggressive disease, and inferior progression free survival [97,98,103]. However, tag-based techniques for CTCs enumeration and isolation can underestimate the amount of CTCs due to the loss of cells without epithelial markers [104].

- The post-CTCs recovery processing to detect either phenotype (e.g. next generation multidimensional flow cytometry, imaging, transcriptomics, metabolomics and proteomics) or genotype aberrations (e.g. ASO-RQ PCR, digital droplet PCR, NGS that can reach 10-6 sensitivity) and their standardization among different laboratories, to investigate, at single-cell level, novel biological mechanisms associated to cancer metastasis and tissue homing [105–109]. The limited number of recovered cells can make impossible post isolation manipulation, requiring large amounts of biological fluids.

- How to improve precision medicine. since CTCs could show a unique morphology and profile of drug sensitivity different from the in-site tumor, that could be challenged to prevent tumor recurrence, arising the question if a comparison with tumor in the primary sites or interactions with the immune system should be further investigated to predict the cancer evolution dynamics. For example in multiple myeloma, compared to primary tissue, CTCs are mostly quiescent (arrested in the subG0-G1 phase of the cell cycle), with proliferation index (percentage of cells in S-phase) significantly lower, though with a peculiar clonogenic potential, arising the question about how to detect unique subsets of patient-paired subclones belonging to different sites [98,107], by mirroring the entire heterogeneity of the tumor. Due to the temporal heterogeneity of cancer, the optimal time points of CTCs detection have not been yet standardized. Few data are available on systematic sequential analysis, it is so far not clear how long patients should be followed during the disease course.

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