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Microfluidic systems for cancer diagnostics

Jose L Garcia-Cordero[1] and Sebastian J Maerkl[2]

Although not employed in the clinic as of yet, microfluidic systems are likely to become a key technology for cancer diagnostics and prognosis. Microfluidic devices have been developed for the analysis of various biomarkers including circulating tumor cells, cell-free DNA, exosomes, and proteins, primarily in liquid biopsies such as serum, plasma, and whole blood, avoiding the need for tumor tissue biopsies. Here, we summarize microfluidic technological advances that are used in cancer diagnosis, prognosis, and to monitor its progression and recurrence, that will likely lead to personalized therapies. In some cases, integrated microfluidic technologies, coupled with biosensors, are proving to be more sensitive and precise in the detection of cancer biomarkers than conventional assays. Based on the current state-of-the-art and the rapid progress over the past decade, we also briefly discuss the next evolutionary steps that these technologies are likely to take.

Addresses
[1] Unidad Monterrey, Centro de Investigación y de Estudios Avanzados del IPN (Cinvestav-IPN), Nuevo León, Mexico
[2] Institute of Bioengineering, School of Engineering, Ecole Polytechnique Federale de Lausanne, Lausanne, Switzerland

Corresponding author: Garcia-Cordero, Jose L (jlgarciac@cinvestav.mx)

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Introduction

Tissue biopsies are the gold standard source for tumor molecular analysis used to confirm, diagnose and classify tumor types, as well as to guide therapies [1]. However, performing a biopsy in a patient is an invasive procedure, and often challenging. In some cases, the size of the tumor and the amount of sample extracted from it, is not sufficient to perform the various molecular tests needed for appropriate diagnosis. The spatial and temporal tumor heterogeneity, in addition to the low accessibility of fresh tissue biopsies, hinders its use to monitor cancer progression and to evaluate the response to cancer therapy [2].

Tumors shed several components that travel in the bloodstream throughout the body: cells breaking off the tumor, DNA, RNA, and proteins released by apoptotic or necrotic tumor cells, or proteins and exosomes secreted by tumor cells. These blood-based tumor biomarkers can provide similar information as a tissue biopsy, possibly pinpoint the identity of the organ of cancer origin, and be used to routinely monitor cancer progression or evaluate therapy efficacy [3]. Performing 'liquid' biopsies has other advantages: drawing blood is less invasive, less expensive and can be collected at different time points during the course of a therapy. Evidence of the clinical utility of liquid biopsies to detect cancer is coming of age and could find widespread use for cancer diagnosis and treatment monitoring in the future [2,4].

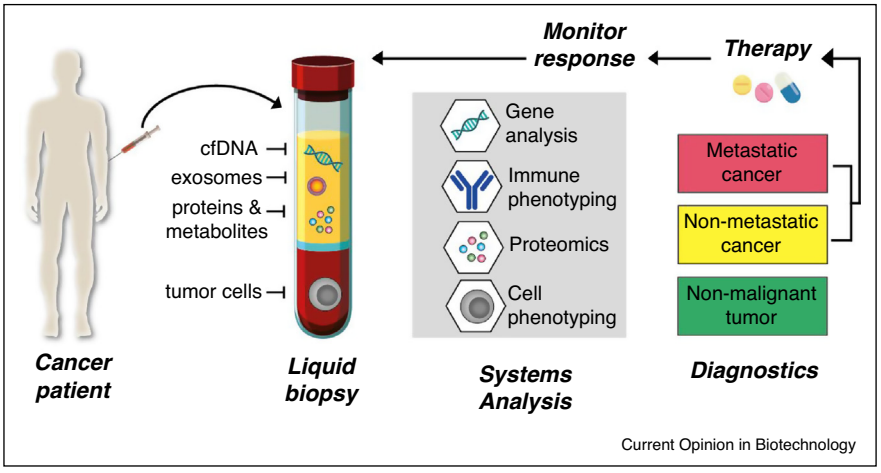
Developing sensitive platforms to routinely quantitate levels of multiple biomarkers directly from small volumes of whole blood at low cost could enable personalized medicine for cancer patients (Figure 1). Improvement in biosensor sensitivity and the development of integrated microfluidic techniques is enabling this type of approach. In this review, we highlight basic biological or clinical aspects of cancer biomarkers and assess the most recent microfluidic technologies used in their detection, quantitation, and analysis. We also discuss future directions in light of what these technologies have already accomplished.

Circulating Tumor Cells (CTCs)

Tumor cells are shed from primary and metastatic tumor sites and travel through the bloodstream as single cells or clusters of tumor cells [5]. Most patients with metastatic cancer have fewer than 10 CTCs in one mL of blood which contains $\sim 1 \times 10^9$ blood cells. The goal of a CTC technology is to isolate and retrieve single CTCs or clusters of CTCs in sufficient numbers with high purity from large volumes of whole blood (>5 mL) and at low shear stress to minimize cell damage. Additional desirable features include the processing of whole blood in a short time (approximately hours) while minimizing the number of manual steps. Once isolated, CTCs must be enumerated and recovered for downstream molecular and functional analysis. These analyses may include immunostaining, obtaining a gene expression profile or single-cell sequencing, culturing and expanding CTCs, xenograft assays, or evaluating cell migration via chemotaxis [6,7].

Most CTCs express epithelial surface markers absent from blood cells, most commonly epithelial cell adhesion molecule (EpCAM). Not surprisingly, the first reported

Figure 1



A few mL of blood are drawn from patients with cancer to quantitate levels of cell-free DNA (cfDNA), exosomes, proteins, and tumor cells. Several analyses are performed on these biomarkers; the resulting data is evaluated to diagnose whether a patient has cancer or not. If positive, the patient undergoes treatment and their biomarker levels are monitored routinely.

microfluidic technology to capture CTCs from whole blood, the 'CTC-chip', used microposts coated with antibodies against EpCAM [8•]. To increase capture efficiency, the collisions between CTC cells and antibody-coated surfaces can be enhanced by adapting microstructures or nano-structures to one of the channel walls [9–13]. Alternatively, CTCs can be tagged with magnetic nanoparticles conjugated with EpCAM antibodies and captured with a magnetic microsifter [14] or sorted in an integrated microfluidic device [15••]. Once captured, cells can be either immunostained on-chip, their surface and intra-cellular signaling proteins analyzed by western blots [16], or released for off-chip analysis.

However, not all cancers have an epithelial origin (e.g. melanoma) and some CTCs may acquire a migratory epithelial-to-mesenchymal transition (EMT) phenotype by downregulating the expression of EpCAM [17]. Although other surface epitopes can be used, such as HER2 or EGFR, the expression of these cancer surface markers is highly heterogenous, even within cells from the same patient [18••]. This expression heterogeneity of CTCs implies that microfluidic technologies based on an immunoaffinity strategy may miss an important number of CTC cells. Panels of aptamers can be used instead of antibodies, but these may also lack some specificity to detect CTCs as they were obtained for cancer cell lines [19,20].

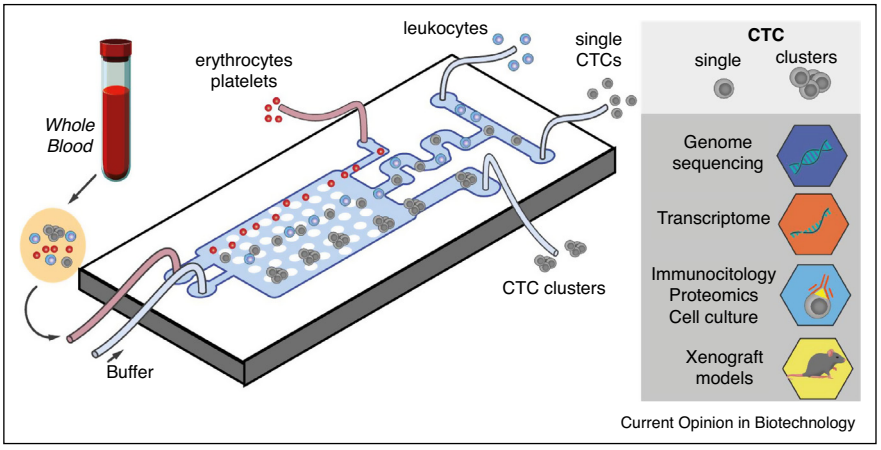
To overcome the limitations of positive or affinity-based selection, CTCs can be sorted based on their physical properties such as size, density, compressibility, deformability or electrical impedance [21,22]. Size differences have been exploited to isolate single CTCs from whole blood using acoustic radiation forces [21], a combination of inertial focusing and Dean vortex flow [23,24], or microscale vortices [17]. More recent, clusters of 2–100 CTCs have been separated from whole blood using lateral deterministic displacement [25•,26]. Reports indicate that, although some CTCs are larger than leukocytes, the vast majority of tumor cells (at least from breast, prostate, and lung cancer patients) have similar sizes as leukocytes [18••]. As with positive selection, these methods can also miss an important number of CTCs that are similar in size to leukocytes.

The heterogeneity of cell size and EpCAM expression levels have led to the development of a microfluidic device, the CTC-iChip, for isolation of CTCs independent of their size (Figure 2) [18••]. Compared to other devices, the CTC-iChip depleted whole blood components in a first stage to arrive at a label-free population of CTCs. This device is one of the most ingenious recent examples of microfluidic engineering: it integrated several microfluidic techniques, provided new biological and clinical insights on CTCs, and was fabricated in a thermoplastic using mass-manufacturing techniques.

Circulating nucleic acids

Cell-free DNA (cfDNA) and other nucleic acid fragments are released from dying cells and possibly by active secretion [27]. Tumor cells shed mutated cfDNA, also known as circulating tumor DNA (ctDNA), that is now regarded as a highly specific marker and used as prognostic marker for some cancers [28]. The short half-life of cfDNA (6 min–2.5 hours) has proven ideal to monitor response to drug treatments. Concentration of cfDNA in blood for healthy individuals ranges from 1–10 ng/mL, while for some cancer patients these levels can increase up to 1000 ng/mL, equivalent to 3000 to 360 000 target

Figure 2



Schematic of an integrated microfluidic device for CTC enrichment. Whole blood is introduced into the device together with a focusing buffer. The device depletes all the blood cells and sorts single and clusters of CTCs using the lateral deterministic displacement method. CTCs can be identified, on-chip or off-chip, by immunostaining or by molecular assays such as DNA sequencing, RNA-based assays, or proteomics analysis. Additionally, CTCs can be expanded in culture plates or investigated *in-vivo* by injecting them in immunodeficient mice.

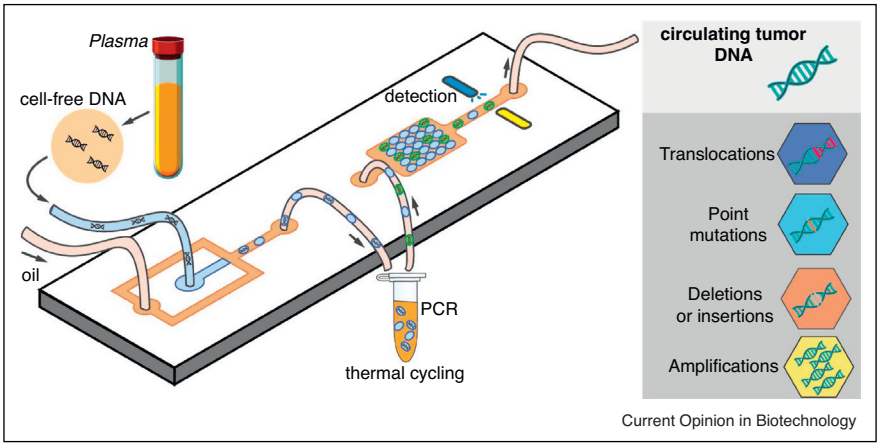
genes per mL of plasma [29,30,31•]. However, high levels of cfDNA can also be indicative of benign tumors, inflammation, or tissue damage [30]. Although levels of cfDNA have been shown to correlate with tumor size and stage (information useful for prognosis and diagnosis), in most cases cfDNA concentrations overlap with those found in healthy individuals [29]. Thus, it becomes necessary not only to quantitate concentrations of cfDNA but also to analyze the ratio of mutated to wild-type genes (ctDNA to cfDNA) [31•], which further complicates the level of detection because of the low frequency of some of these mutations (one mutant template per mL of plasma [3]). Although most work has been focused on cfDNA given its stability, cell-free mRNA, microRNA, nucleosome, and viral DNA are also being investigated [27]. The conventional toolbox for analyzing cfDNA includes different modalities of PCR, DNA sequencing, and microarrays [32,33].

In contrast to CTC microtechnologies, there are not yet reports of integrated microfluidic devices for quantitating cfDNA from whole blood, presumably because of the challenge that is posed by the extraction of DNA from several milliliters of whole blood and the series of preparation steps involved. Such a microfluidic device would need to integrate plasma separation, followed by cfDNA extraction using magnetic beads, affinity columns, filtration or solvent-based methods, which would deliver the purified cfDNA for downstream analysis either by PCR or sequencing. Indeed, several microfluidic components have been developed for other applications that independently perform some of these steps that could potentially be translated to ctDNA analysis [34]. So far, most work has been done on the analytical front. For example, detection of DNA mutations have been achieved in microfluidic digital PCR carried out in picolitre droplets [31•,35•] or on a nanofluidic array [36]. Using one of the former technologies [31•], it was possible to detect a single mutated gene in a background of 200 000 non-mutated genes, sufficient to detect early-stage tumors (Figure 3) [31•]. In this proof-of-concept study, cancer cell lines were analyzed and no human clinical samples have been analyzed. An interesting recent approach has been the use of electrochemical sensors containing nanostructured microelectrodes to detect mutated cfDNA directly from human serum samples without the need of amplification [32,37••].

Exosomes

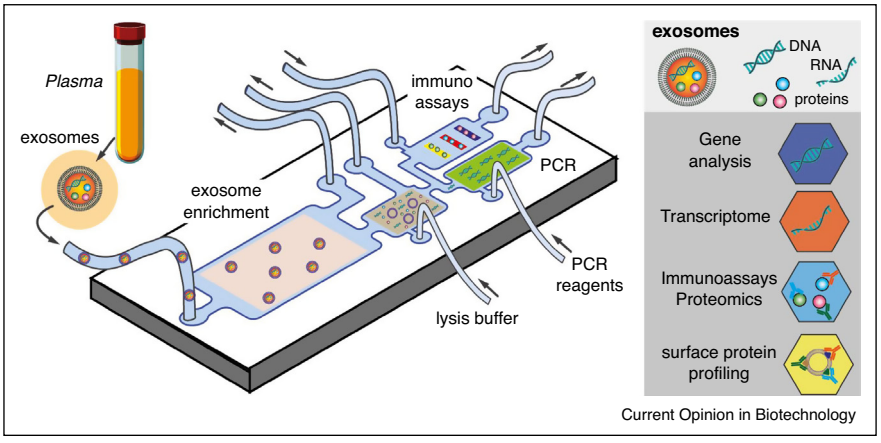
Exosomes are a subset of extracellular vesicles released by tumor cells and non-malignant cells [38,39]. The contents of exosomes include mitochondrial DNA, proteins, mRNA, microRNA, lipids, and metabolites, which can provide information on cellular identity or tissue origin [39,40•]. Exosomes can be found in most body fluids [39], are more stable and abundant than ctDNA, and are present in circulation at early stages of cancers, features that have made them potential cancer biomarker candidates and garnered them considerable attention in recent years [41]. The exosome analysis workflow includes their isolation and quantitation followed by the characterization of their intra-vesicular and extra-vesicular contents, size, and morphology [40•,42]. Current techniques for isolating exosomes (e.g. ultracentrifugation, precipitation, filtration) require extensive purification steps, are laborious, and do not yield high-purities [39]. Exosomes' molecular contents can be analyzed by western blotting, immunoassays, qRT-PCR, sequencing, flow cytometry, mass spectrometry, among others.

Figure 3



Schematic of a droplet digital-PCR device for cell-free DNA detection. cfDNA is isolated from plasma, enriched, and mixed with PCR reagents outside the device. This aqueous phase and oil are connected to the microfluidic device. The sample is partitioned into thousands of 1-nL droplets such that there is on average less than one DNA molecule per droplet. Droplets are collected in a tube or well-plate for PCR amplification. The emulsion is reinjected into the device for fluorescence detection of amplified mutant DNA. The analysis of cfDNA can range from detecting single-point mutations to whole-genome sequencing. Other genetic aberrations such as translocations, deletions, insertions and amplifications can be identified by DNA techniques such as digital-PCR, beads-emulsion-amplification-magnetics (BEAMing) or different types of sequencing.

Figure 4



Schematic of a microfluidic device for exosomes analysis. Serum or plasma is flowed through a chamber containing antibodies that recognize surface proteins of exosomes. Exosomes are captured in this chamber while waste is collected in one of the outlets. Retained exosomes can be stained with different antibodies for surface protein profiling. The exosomes can then be transported to another chamber to be lysed and release their cargo into different chambers. Proteins can be detected by sandwich immunoassays while DNA and RNA can be analyzed using PCR or DNA microarrays. Additionally, exosomes cargo can be analyzed off-chip for further molecular profiling.

Most technologies for capturing and detecting exomes are based on affinity chromatography that target tetraspanins (characteristic surface protein markers of extracellular vesicles, such as CD63) or tumor surface markers (e.g. EpCAM, EGFR, HER2) [43]. However, it is early to define a generic marker that can be used for exosome capture, as some tetraspanins are expressed in low levels and expression levels are dependent on the type of tumor. Significant progress has been achieved in developing stand-alone biosensors for both capturing and quantitation of exosomes [40•,43–50]. Although highly sensitive, these biosensors still require a significant level of manual preparation either for serum isolation or exosome enrichment [44,45,47] and only a few of them have been integrated into microfluidic platforms [40•,51].

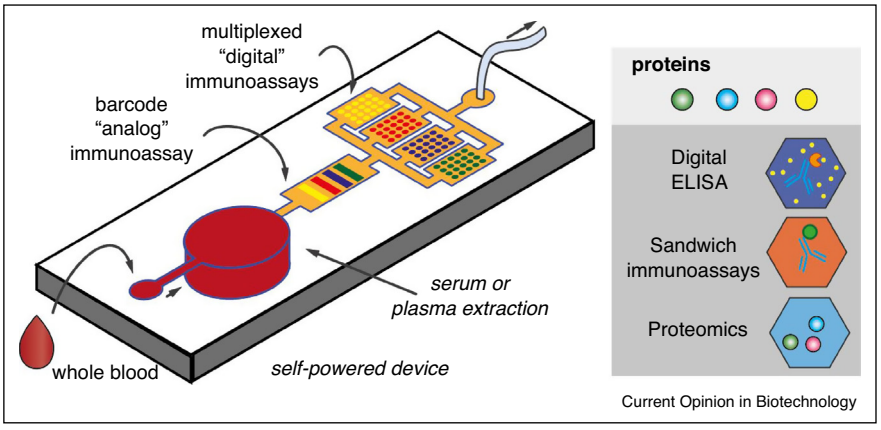
Microfluidic methods for exosome capture and analysis are based mostly on immune-affinity [42,52,53••,54] but there

are reports of size-based separation [55,56]. The most popular approach is to coat a chip surface with specific antibodies against exosome surface markers, while the opposite surface is patterned with micro-structures or nano-structures to enhance capture efficiency [41,57–60]. Among them, the herringbone chip (^{EV}HB-Chip) stands out because it can process several mL of serum and capture extracellular vesicles of different sizes on its nanostructured surface with superior performance than ultracentrifugation and magnetic beads [59]. An important feature of this device and other strategies [61] is that extracellular vesicles can be released from the surface for off-chip analysis (Figure 4). Some of these devices allow surface phenotyping of exosomes by staining them with different antibodies, which is important to find associations between tumor and exosome markers. Reports of on-chip analysis of exosome contents are emerging [42,50,53••,62••]; however, once a consensus panel of exosome generic markers is obtained, we expect integration of on-chip bioanalytical approaches such as PCR, digital PCR, biosensors, and digital ELISA for their analysis.

Protein biomarkers

Tumor cells secrete abnormal levels of peptide growth factors, cytokines, and hormones that can be used as cancer biomarkers [63]. Thus, it is critical to measure panels of proteins in parallel from a few μL of whole blood. The detection of proteins and metabolites in microfluidics is one of the most developed and mature technologies of the field, reaching successful commercialization [34,64]. Significant key advances have been achieved in integration, multiplexing, sensitivity, throughput, and sample volume (Figure 5). For example, integrated microfluidic devices [65] can extract plasma from a droplet of blood and quantitate several proteins in parallel, with identical limits of detection as ELISAs [66••,67•,68]; subfemtomolar concentrations can be detected by implementing digital ELISAs [69,70••,71•]; and several biomarkers can be quantitated in serum volumes as low as 5 nL from thousands of samples in parallel [72•]. However, there are still several technical challenges facing microfluidic immunoassay platforms that need to be addressed before they can be widely employed in clinical settings for the quantitation of cancer biomarkers. These include assay reproducibility and variability, antibody cross-reactivity and immobilization, reagent storage, functionalization, cost, surface passivation, assay temperature, manufacturing, material selection, and so on [34,64,68]. However, in our purview, two critical aspects have mostly remained overlooked in the design of microfluidic immunoassays. Firstly, is the generation of on-chip standard curves for the precise quantitation of protein levels, and secondly, is the detection of a wide range of concentration levels. With a few exceptions [68,72•], most papers report measurements in arbitrary units or concentration values that are estimated from on-chip calibration curves obtained with different devices that are either run in parallel or obtained on previous days. As with ELISAs, technical blunders or quality variations between antibody batches introduce artifacts that lead to assay variability or to reporting of inaccurate concentration levels [72•]. On the other hand, some cancer biomarkers are secreted over a broad dynamic range, spanning several orders of magnitude. This would require the implementation of hybrid 'digital-analog' immunoassays that can provide a linear response in the sub-fM to nM range [70••,71•]. Thus, to be useful in cancer management, future microfluidic immunoassay platforms should consider the integration of calibration curves and different detection modalities to measure a wide range of protein concentrations.

Figure 5



Schematic of a self-powered microfluidic device for protein profiling. A few μL of whole blood are placed in the inlet. Blood cells sediment in a trench where they remain captured. Plasma overflows downstream, and proteins are captured in a chamber containing an antibody array where fluorescent 'analog' sandwich immunoassays are performed. Proteins at extremely low-concentration can be quantitated using 'digital' ELISAs. Unknown proteins can be identified by proteomics techniques.

Conclusions and perspectives

Microfluidic technologies have made impressive strides in the detection and analysis of CTCs, ctDNA, exosomes, and protein biomarkers, in some cases, directly from whole blood. Microtechnologies for CTCs and protein detection have reached a tipping point on the path to commercialization, but we envision the integration of downstream analysis in the same device rather than performing off-chip analysis. We also expect more streamlined microfluidic platforms for the detection of ctDNA from whole blood and anticipate the development of platforms for analyzing exosome contents. Blood, and its derivatives such as serum and plasma, are the most used biological fluid for cancer biomarker discovery and diagnostics [73]. Thus, the predilection is understandable to develop microfluidic systems for blood analysis. However, as briefly noted here, biomarkers can also be found in other biofluids (e.g. urine, saliva, ascites, cerebrospinal fluid), sometimes at higher concentration than in blood, although sample preparation is more challenging it is expected that microfluidic devices targeting other body fluids will emerge.

Mounting evidence indicates that detecting a combination of different cancer biomarkers can lead to a more sensitive and precise detection of cancer and the determination of tumor type [3,73,74]. For example, by assessing the levels of 8 proteins and mutations in cfDNA, several tumor types could be identified, albeit with different sensitivities [3]. Thus, with current state of the art microtechnologies it is not far-fetched to think of implementing a multi-analyte blood test in a single microfluidic device. If commercialized at reasonably low cost and with sufficient sensitivity, microfluidic devices could be used as routine analysis tools in cancer management and shape the future of clinical practice. Equally important to note is that the technologies being developed for cancer biomarker analysis can be translated to help diagnose and detect other pathologies as well.

Conflict of interest statement

Nothing declared.

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