Microfluidic systems for cancer diagnostics

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Abstract

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.

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.

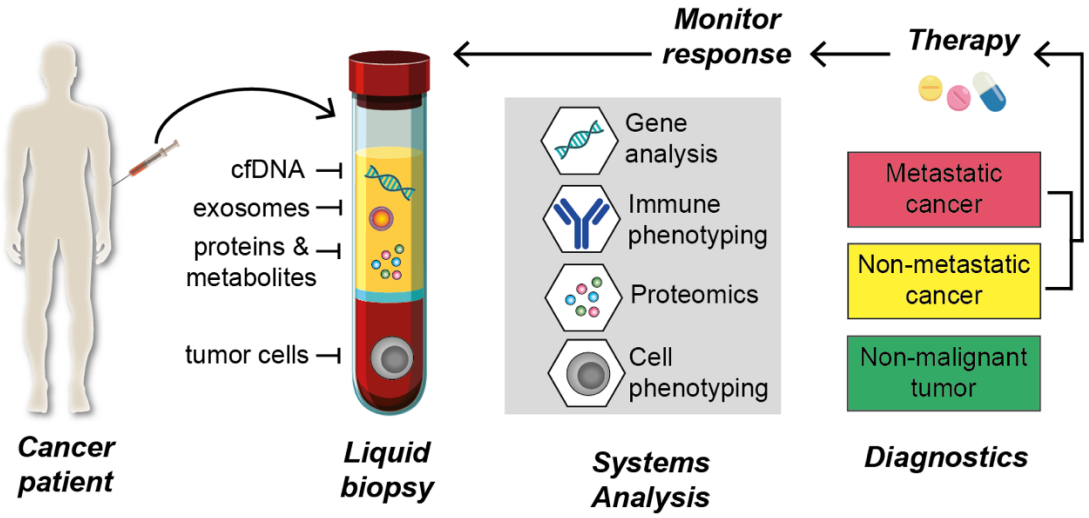

Figure 1. A few mL of blood are drawn from patients with cancer to quantitate levels of cell-free DNA (cfDNA), exosomes, proteins, and tumor cells. Several analyses are performed on these biomarkers; the resulting data is evaluated to diagnose whether a patient has cancer or not. If positive, the patient undergoes treatment and their biomarker levels are monitored routinely.

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 ~$1x10^9$ 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 (>5mL) and at low shear stress to minimize cell damage. Additional desirable features include the processing of whole blood in a short time (~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 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 micro- 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 down-regulating 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 be 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.

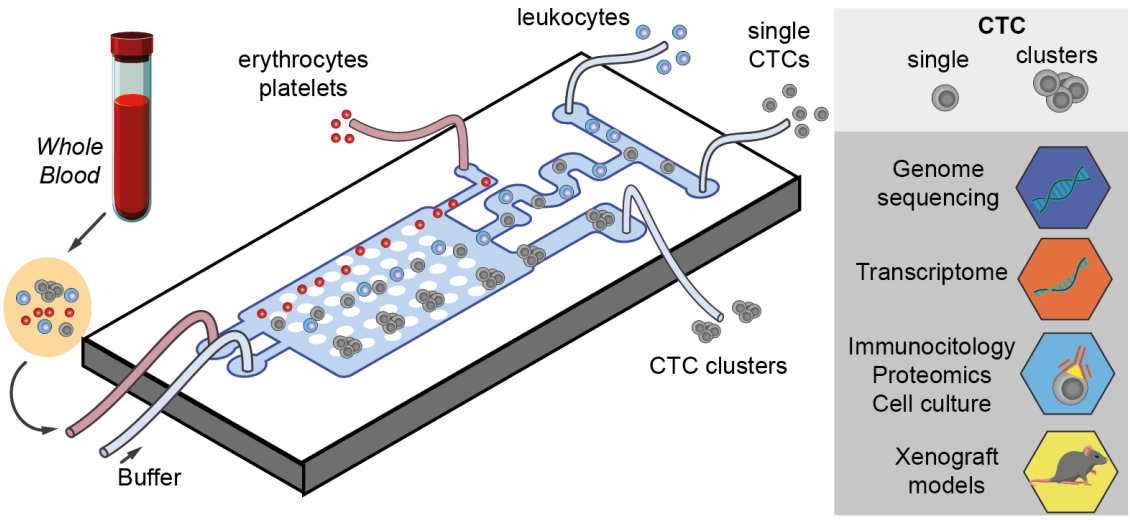

Figure 2. **Schematic of an integrated microfluidic device for CTC enrichment**. Whole blood is introduced into the device together with a focusing buffer. The device depletes all the blood cells and sorts single and clusters of CTCs using the lateral deterministic displacement method. CTCs can be identified, 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.

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 in cancer patients these levels can increase up to 1000 ng/mL, equivalent to 3,000 to 360,000 target genes per mL of plasma [29–31]. Although levels of ctDNA 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 milliliter 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].

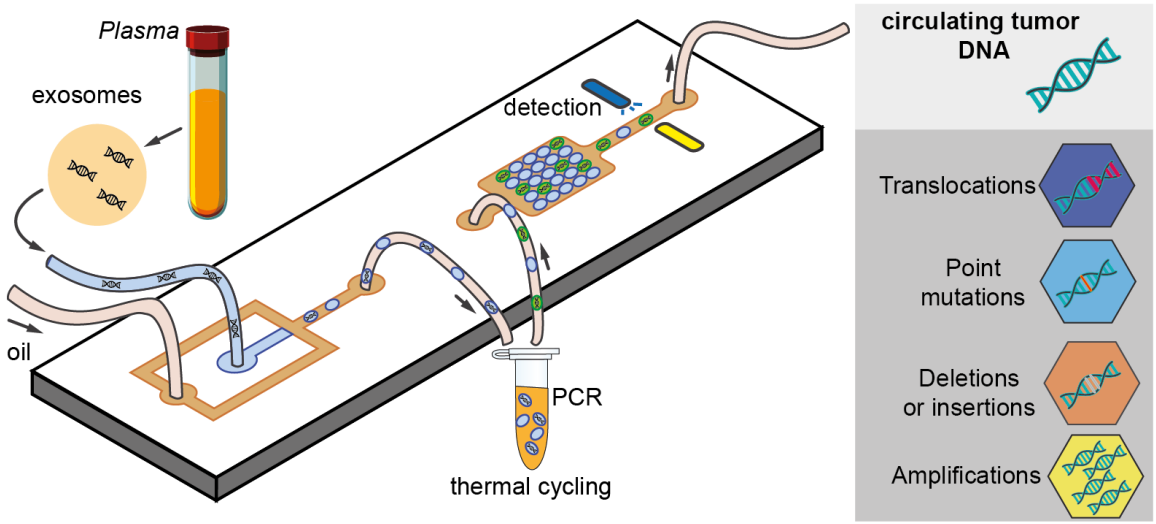

Figure 3. **Schematic of a droplet digital-PCR device for cell-free DNA detection**. cfDNA is isolated from plasma, enriched, and mixed with PCR reagents outside the device. This aqueous phase and oil are connected to the microfluidic device. The sample is partitioned into thousands of 1-nL droplets 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.

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- 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]. An exosomes' molecular contents can be analyzed by western blotting, immunoassays, qRT-PCR, sequencing, flow cytometry, mass spectrometry, among others.

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 [42,52–54] 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- 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.

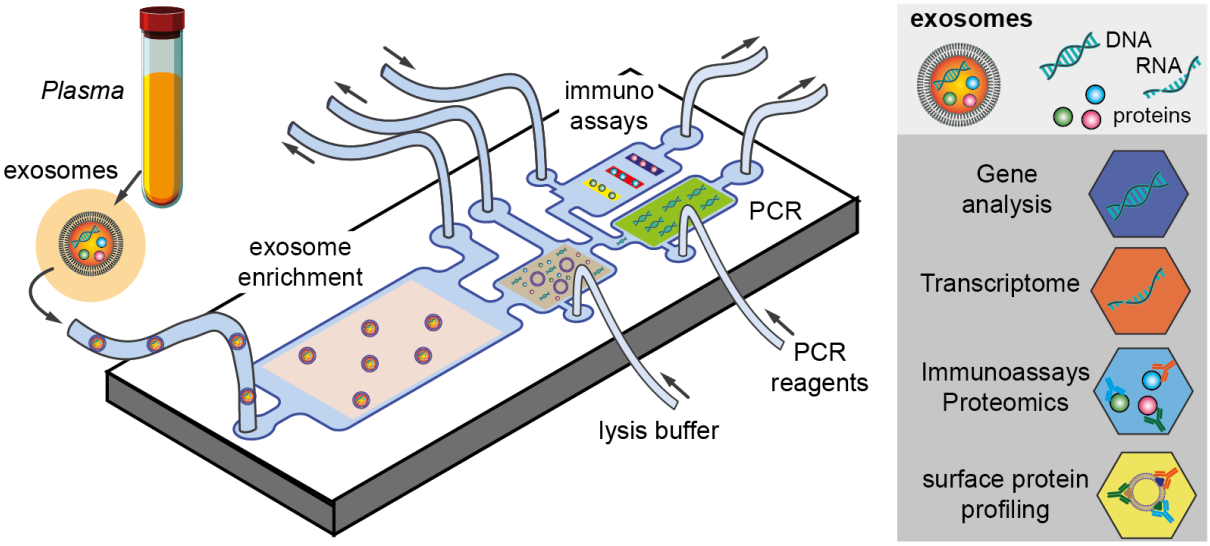

Figure 4. **Schematic of a microfluidic device for exosomes analysis**. Serum or plasma is flowed through a chamber containing antibodies that recognize surface proteins of exosomes. Exosomes are captured in this chamber while waste is collected in one of the outlets. Retained exosomes can be stained 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.

Protein biomarkers. Tumor cells secrete abnormal levels of peptide growth factors, cytokines, an 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 [66–68]; sub-femtomolar concentrations can be detected by implementing digital ELISAs [69–71]; and several biomarkers can be quantitated in serum volumes as low as 5nL 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, etc. [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.

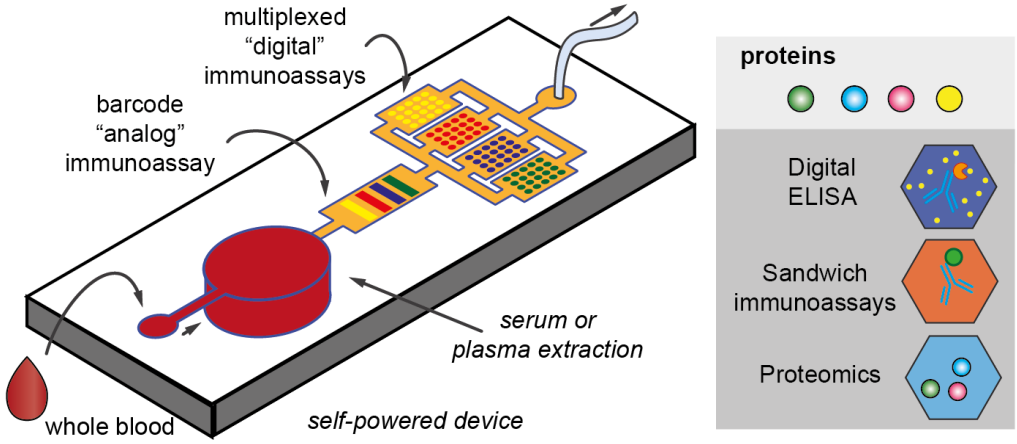

Figure 5. **Schematic of a self-powered microfluidic device for protein profiling**. A few µL of whole blood are placed in the inlet. Blood cells sediment in a trench where they remain captured. Plasma overflows downstream, and proteins are captured in a chamber containing an antibody array where fluorescent "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

None declared.

Acknowledgements

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References

1. Ilié M, Hofman P: **Pros: Can tissue biopsy be replaced by liquid biopsy?** *Transl Lung Cancer Res* 2016, **5**:420–423.

2. Parikh AR, Leshchiner I, Elagina L, Goyal L, Levovitz C, Siravegna G, Livitz D, Rhrissorrakrai K, Martin EE, Van Seventer EE, et al.: **Liquid versus tissue biopsy for detecting acquired resistance and tumor heterogeneity in gastrointestinal cancers.** *Nat Med* 2019, **25**:1415–1421.

3. Cohen JD, Wang Y, Douville C, Wong F, Mattox A, Ptak J, Dobbyn L, Schaefer J, Silliman N, Popoli M, et al.: **Detection and localization of surgically resectable cancers with a multi-analyte blood test**. *Science (80-)* 2018, **359**:926–930.

4. Rothwell DG, Ayub M, Cook N, Thistlethwaite F, Carter L, Dean E, Smith N, Villa S, Dransfield J, Clipson A, et al.: **Utility of ctDNA to support patient selection for early phase clinical trials: the TARGET study**. *Nat Med* 2019, **25**:738–743.

5. Haber DA, Velculescu VE: **Blood-based analyses of cancer: circulating tumor cells and circulating tumor DNA.** *Cancer Discov* 2014, **4**:650–61.

6. Poudineh M, Labib M, Ahmed S, Nguyen LNM, Kermanshah L, Mohamadi RM, Sargent EH, Kelley SO: **Profiling Functional and Biochemical Phenotypes of Circulating**

Tumor Cells Using a Two-Dimensional Sorting Device. *Angew Chemie - Int Ed* 2017, **56**:163–168.

7. Pantel K, Alix-Panabières C: **Liquid biopsy and minimal residual disease — latest advances and implications for cure**. *Nat Rev Clin Oncol* 2019, **16**:409–424.

8. Nagrath S, Sequist L V., Maheswaran S, Bell DW, Irimia D, Ulkus L, Smith MR, Kwak EL, Digumarthy S, Muzikansky A, et al.: **Isolation of rare circulating tumour cells in cancer patients by microchip technology**. *Nature* 2007, **450**:1235–1239.

First paper to report the capture of CTCs using EpCAM antibodies.

9. Stott SL, Hsu CH, Tsukrov DI, Yu M, Miyamoto DT, Waltman BA, Michael Rothenberg S, Shah AM, Smas ME, Korir GK, et al.: **Isolation of circulating tumor cells using a microvortex-generating herringbone-chip**. *Proc Natl Acad Sci U S A* 2010, **107**:18392–18397.

10. Sheng W, Ogunwobi OO, Chen T, Zhang J, George TJ, Liu C, Fan ZH: **Capture, release and culture of circulating tumor cells from pancreatic cancer patients using an enhanced mixing chip**. *Lab Chip* 2014, **14**:89–98.

11. Wang S, Liu K, Liu J, Yu ZTF, Xu X, Zhao L, Lee T, Lee EK, Reiss J, Lee YK, et al.: **Highly efficient capture of circulating tumor cells by using nanostructured silicon substrates with integrated chaotic micromixers**. *Angew Chemie - Int Ed* 2011, **50**:3084–3088.

12. Yoon HJ, Kim TH, Zhang Z, Azizi E, Pham TM, Paoletti C, Lin J, Ramnath N, Wicha MS, Hayes DF, et al.: **Sensitive capture of circulating tumour cells by functionalized graphene oxide nanosheets**. *Nat Nanotechnol* 2013, **8**:735–741.

13. Wang S, Wang H, Jiao J, Chen KJ, Owens GE, Kamei KI, Sun J, Sherman DJ, Behrenbruch CP, Wu H, et al.: **Three-dimensional nanostructured substrates toward efficient capture of circulating tumor cells**. *Angew Chemie - Int Ed* 2009, **48**:8970–8973.

14. Park S, Wong DJ, Ooi CC, Kurtz DM, Vermesh O, Aalipour A, Suh S, Pian KL, Chabon JJ, Lee SH, et al.: **Molecular profiling of single circulating tumor cells from lung cancer patients**. *Proc Natl Acad Sci* 2016, **113**:E8379–E8386.

15. Ozkumur E, Ozkumur E, Shah AM, Shah AM, Ciciliano JC, Ciciliano JC, Emmink BL, Emmink BL, Miyamoto DT, Miyamoto DT, et al.: **Inertial Focusing for Tumor Antigen-Dependent and -Independent Sorting of Rare Circulating Tumor Cells**. *Sci Transl Med* 2013, **5**:179ra47-179ra47.

This paper reports a device to isolate CTCs independent of tumor membrane epitopes

16. Sinkala E, Sollier-Christen E, Renier C, Rosàs-Canyelles E, Che J, Heirich K, Duncombe TA, Vlassakis J, Yamauchi KA, Huang H, et al.: **Profiling protein expression in circulating tumour cells using microfluidic western blotting**. *Nat Commun* 2017, **8**.

17. Sollier E, Go DE, Che J, Gossett DR, O'Byrne S, Weaver WM, Kummer N, Rettig M, Goldman J, Nickols N, et al.: **Size-selective collection of circulating tumor cells using Vortex technology**. *Lab Chip* 2014, **14**:63–77.

18. Fachin F, Spuhler P, Martel-Foley JM, Edd JF, Barber TA, Walsh J, Karabacak M, Pai V, Yu M, Smith K, et al.: **Monolithic Chip for High-throughput Blood Cell Depletion to**

Sort Rare Circulating Tumor Cells. *Sci Rep* 2017, **7**:1–11.

This paper reports an integrated microfluidic device to debulk blood cells and isolate label-free CTCs

19. Zhao L, Tang C, Xu L, Zhang Z, Li X, Hu H, Cheng S, Zhou W, Huang M, Fong A, et al.: **Enhanced and Differential Capture of Circulating Tumor Cells from Lung Cancer Patients by Microfluidic Assays Using Aptamer Cocktail**. *Small* 2016, **12**:1072–1081.

20. Sheng W, Chen T, Kamath R, Xiong X, Tan W, Fan ZH: **Aptamer-enabled efficient isolation of cancer cells from whole blood using a microfluidic device**. *Anal Chem* 2012, **84**:4199–4206.

21. Li P, Mao Z, Peng Z, Zhou L, Chen Y, Huang P-H, Truica CI, Drabick JJ, El-Deiry WS, Dao M, et al.: **Acoustic separation of circulating tumor cells**. *Proc Natl Acad Sci* 2015, **112**:4970–4975.

22. Park ES, Jin C, Guo Q, Ang RR, Duffy SP, Matthews K, Azad A, Abdi H, Todenhöfer T, Bazov J, et al.: **Continuous Flow Deformability-Based Separation of Circulating Tumor Cells Using Microfluidic Ratchets**. *Small* 2016, **12**:1909–1919.

23. Warkiani ME, Guan G, Luan KB, Lee WC, Bhagat AAS, Kant Chaudhuri P, Tan DSW, Lim WT, Lee SC, Chen PCY, et al.: **Slanted spiral microfluidics for the ultra-fast, label-free isolation of circulating tumor cells**. *Lab Chip* 2014, **14**:128–137.

24. Hou HW, Warkiani ME, Khoo BL, Li ZR, Soo RA, Tan DSW, Lim WT, Han J, Bhagat AAS, Lim CT: **Isolation and retrieval of circulating tumor cells using centrifugal forces**. *Sci Rep* 2013, **3**:1–8.

25. Au SH, Edd J, Stoddard AE, Wong KHK, Fachin F, Maheswaran S, Haber DA, Stott SL, Kapur R, Toner M: **Microfluidic isolation of circulating tumor cell clusters by size and asymmetry**. *Sci Rep* 2017, **7**:1–10.

Describes a device to isolate and recover CTC clusters ranging from 2-100 cells

26. Sarioglu AF, Aceto N, Kojic N, Donaldson MC, Zeinali M, Hamza B, Engstrom A, Zhu H, Sundaresan TK, Miyamoto DT, et al.: **A microfluidic device for label-free, physical capture of circulating tumor cell clusters**. *Nat Methods* 2015, **12**:685–691.

27. Mader S, Pantel K: **Liquid biopsy: Current status and future perspectives**. *Oncol Res Treat* 2017, **40**:404–408.

28. Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, Bartlett BR, Wang H, Luber B, Alani RM, et al.: **Detection of circulating tumor DNA in early- and late-stage human malignancies**. *Sci Transl Med* 2014, **6**:224ra24-224ra24.

29. Wan JCM, Massie C, Garcia-Corbacho J, Mouliere F, Brenton JD, Caldas C, Pacey S, Baird R, Rosenfeld N: **Liquid biopsies come of age: Towards implementation of circulating tumour DNA**. *Nat Rev Cancer* 2017, **17**:223–238.

30. Schwarzenbach H, Hoon DSB, Pantel K: **Cell-free nucleic acids as biomarkers in cancer patients**. *Nat Rev Cancer* 2011, **11**:426–437.

31. Pekin D, Skhiri Y, Baret JC, Le Corre D, Mazutis L, Ben Salem C, Millot F, El Harrak A, Hutchison JB, Larson JW, et al.: **Quantitative and sensitive detection of rare mutations using droplet-based microfluidics**. *Lab Chip* 2011, **11**:2156–2166.

One of the first devices to report detection of mutated DNA using droplet digital PCR

32. Das J, Ivanov I, Montermini L, Rak J, Sargent EH, Kelley SO: **An electrochemical clamp assay for direct, rapid analysis of circulating nucleic acids in serum**. *Nat Chem* 2015, **7**:569–575.

33. Lohr JG, Adalsteinsson VA, Cibulskis K, Choudhury AD, Rosenberg M, Cruz-Gordillo P, Francis JM, Zhang CZ, Shalek AK, Satija R, et al.: **Whole-exome sequencing of circulating tumor cells provides a window into metastatic prostate cancer**. *Nat Biotechnol* 2014, **32**:479–484.

34. Gubala V, Harris LF, Ricco AJ, Tan MX, Williams DE: **Point of care diagnostics: Status and future**. *Anal Chem* 2012, **84**:487–515.

35. Hindson BJ, Ness KD, Masquelier DA, Belgrader P, Heredia NJ, Makarewicz AJ, Bright IJ, Lucero MY, Hiddessen AL, Legler TC, et al.: **High-throughput droplet digital PCR system for absolute quantitation of DNA copy number**. *Anal Chem* 2011, **83**:8604–8610.

Together with ref. 31, one of the first devices to report detection of mutated DNA using droplet digital PCR

36. Azuara D, Ginesta MM, Gausachs M, Rodriguez-Moranta F, Fabregat J, Busquets J, Pelaez N, Boadas J, Galter S, Moreno V, et al.: **Nanofluidic digital PCR for KRAS mutation detection and quantification in gastrointestinal cancer**. *Clin Chem* 2012, **58**:1332–1341.

37. Das J, Ivanov I, Sargent EH, Kelley SO: **DNA Clutch Probes for Circulating Tumor DNA Analysis**. *J Am Chem Soc* 2016, **138**:11009–11016.

Stand-alone biosensor for the sensitive detection of mutated ctDNA

38. Poudineh M, Sargent EH, Pantel K, Kelley SO: **Profiling circulating tumour cells and other biomarkers of invasive cancers**. *Nat Biomed Eng* 2018, **2**:72–84.

39. Simpson RJ, Lim JWE, Moritz RL, Mathivanan S: **Exosomes: Proteomic insights and diagnostic potential**. *Expert Rev Proteomics* 2009, **6**:267–283.

40. Im H, Shao H, Park Y Il, Peterson VM, Castro CM, Weissleder R, Lee H: **Label-free detection and molecular profiling of exosomes with a nano-plasmonic sensor**. *Nat Biotechnol* 2014, **32**:490–495.

Paper reports the multiplexed detection of exosomes using nanosensors integrated in a microfluidic device

41. Dong J, Zhang RY, Sun N, Smalley M, Wu Z, Zhou A, Chou SJ, Jan YJ, Yang P, Bao L, et al.: **Bio-Inspired NanoVilli Chips for Enhanced Capture of Tumor-Derived Extracellular Vesicles: Toward Non-Invasive Detection of Gene Alterations in Non-Small Cell Lung Cancer**. *ACS Appl Mater Interfaces* 2019, **11**:13973–13983.

42. Zhang P, Crow J, Lella D, Zhou X, Samuel G, Godwin AK, Zeng Y: **Ultrasensitive quantification of tumor mRNAs in extracellular vesicles with an integrated microfluidic digital analysis chip**. *Lab Chip* 2018, **18**:3790–3801.

43. Liang K, Liu F, Fan J, Sun D, Liu C, Lyon CJ, Bernard DW, Li Y, Yokoi K, Katz MH, et al.:

Nanoplasmonic quantification of tumour-derived extracellular vesicles in plasma microsamples for diagnosis and treatment monitoring. *Nat Biomed Eng* 2017, **1**:1–11.

44. Jin D, Yang F, Zhang Y, Liu L, Zhou Y, Wang F, Zhang GJ: **ExoAPP: Exosome-Oriented, Aptamer Nanoprobe-Enabled Surface Proteins Profiling and Detection**. *Anal Chem* 2018, **90**:14402–14411.

45. Zhou YG, Mohamadi RM, Poudineh M, Kermanshah L, Ahmed S, Safaei TS, Stojcic J, Nam RK, Sargent EH, Kelley SO: **Interrogating Circulating Microsomes and Exosomes Using Metal Nanoparticles**. *Small* 2016, **12**:727–732.

46. Cavallaro S, Horak J, HÅÅg P, Gupta D, Stiller C, Sahu SS, Görgens A, Gatty HK, Viktorsson K, El Andaloussi S, et al.: **Label-Free Surface Protein Profiling of Extracellular Vesicles by an Electrokinetic Sensor**. *ACS Sensors* 2019, **4**:1399–1408.

47. Yoshioka Y, Kosaka N, Konishi Y, Ohta H, Okamoto H, Sonoda H, Nonaka R, Yamamoto H, Ishii H, Mori M, et al.: **Ultra-sensitive liquid biopsy of circulating extracellular vesicles using ExoScreen**. *Nat Commun* 2014, **5**:3591.

48. Jeong S, Park J, Pathania D, Castro CM, Weissleder R, Lee H: **Integrated Magneto-Electrochemical Sensor for Exosome Analysis**. *ACS Nano* 2016, **10**:1802–1809.

49. Yu Y, Li Y-T, Jin D, Yang F, Wu D, Xiao M-M, Zhang H, Zhang Z-Y, Zhang G-J: **Electrical and Label-Free Quantification of Exosomes with a Reduced Graphene Oxide Field Effect Transistor Biosensor**. *Anal Chem* 2019, **91**:10679–10686.

50. Li Q, Tofaris GK, Davis JJ: **Concentration-Normalized Electroanalytical Assaying of Exosomal Markers**. *Anal Chem* 2017, **89**:3184–3190.

51. Shao H, Chung J, Balaj L, Charest A, Bigner DD, Carter BS, Hochberg FH, Breakefield XO, Weissleder R, Lee H: **Protein typing of circulating microvesicles allows real-time monitoring of glioblastoma therapy**. *Nat Med* 2012, **18**:1835–1840.

52. Zhao Z, Yang Y, Zeng Y, He M: **A microfluidic ExoSearch chip for multiplexed exosome detection towards blood-based ovarian cancer diagnosis**. *Lab Chip* 2016, **16**:489–496.

53. He M, Crow J, Roth M, Zeng Y, Godwin AK: **Integrated immunoisolation and protein analysis of circulating exosomes using microfluidic technology**. *Lab Chip* 2014, **14**:3773–3780.

One of the first microfluidic devices to capture exosomes and measure intravesicular proteins

54. Liu C, Xu X, Li B, Situ B, Pan W, Hu Y, An T, Yao S, Zheng L: **Single-Exosome-Counting Immunoassays for Cancer Diagnostics**. *Nano Lett* 2018, **18**:4226–4232.

55. Wunsch BH, Smith JT, Gifford SM, Wang C, Brink M, Bruce RL, Austin RH, Stolovitzky G, Astier Y: **Nanoscale lateral displacement arrays for the separation of exosomes and colloids down to 20nm**. *Nat Nanotechnol* 2016, **11**:936–940.

56. Davies RT, Kim J, Jang SC, Choi EJ, Gho YS, Park J: **Microfluidic filtration system to isolate extracellular vesicles from blood**. *Lab Chip* 2012, **12**:5202–5210.

57. Zhang P, He M, Zeng Y: **Ultrasensitive microfluidic analysis of circulating exosomes using a nanostructured graphene oxide/polydopamine coating**. *Lab Chip* 2016,

16:3033–3042.

58. Kanwar SS, Dunlay CJ, Simeone DM, Nagrath S: **Microfluidic device (ExoChip) for on-chip isolation, quantification and characterization of circulating exosomes**. *Lab Chip* 2014, **14**:1891–1900.

59. Reátegui E, Van Der Vos KE, Lai CP, Zeinali M, Atai NA, Aldikacti B, Floyd FP, Khankhel A, Thapar V, Hochberg FH, et al.: **Engineered nanointerfaces for microfluidic isolation and molecular profiling of tumor-specific extracellular vesicles**. *Nat Commun* 2018, **9**.

60. Chen C, Skog J, Hsu CH, Lessard RT, Balaj L, Wurdinger T, Carter BS, Breakefield XO, Toner M, Irimia D: **Microfluidic isolation and transcriptome analysis of serum microvesicles**. *Lab Chip* 2010, **10**:505–511.

61. Kang YT, Kim YJ, Bu J, Cho YH, Han SW, Moon BI: **High-purity capture and release of circulating exosomes using an exosome-specific dual-patterned immunofiltration (ExoDIF) device**. *Nanoscale* 2017, **9**:13495–13505.

62. Shao H, Chung J, Lee K, Balaj L, Min C, Carter BS, Hochberg FH, Breakefield XO, Lee H, Weissleder R: **Chip-based analysis of exosomal mRNA mediating drug resistance in glioblastoma**. *Nat Commun* 2015, **6**:1–9.

Reports a highly integrated microfluidic devices to capture exosomes from blood and quantiate intravesicular mRNA levels

63. Welsh JB, Sapinoso LM, Kern SG, Brown DA, Liu T, Bauskin AR, Ward RL, Hawkins NJ, Quinn DI, Russell PJ, et al.: **Large-scale delineation of secreted protein biomarkers overexpressed in cancer tissue and serum**. *Proc Natl Acad Sci U S A* 2003, **100**:3410–3415.

64. Barbosa AI, Reis NM: **A critical insight into the development pipeline of microfluidic immunoassay devices for the sensitive quantitation of protein biomarkers at the point of care**. *Analyst* 2017, **142**:858–882.

65. Thorsen T, Maerkl SJ, Quake SR: **Microfluidic large-scale integration.** *Science (80-)* 2002, **298**:580–4.

66. Fan R, Vermesh O, Srivastava A, Yen BKH, Qin L, Ahmad H, Kwong GA, Liu CC, Gould J, Hood L, et al.: **Integrated barcode chips for rapid, multiplexed analysis of proteins in microliter quantities of blood**. *Nat Biotechnol* 2008, **26**:1373–1378.

First device to show on-chip plasma isolation from whole blood and detection of 12 proteins in parallel

67. Dimov IK, Basabe-Desmonts L, Garcia-Cordero JL, Ross BM, Ricco AJ, Lee LP: **Stand-alone self-powered integrated microfluidic blood analysis system (SIMBAS)**. *Lab Chip* 2011, **11**:845–850.

First report to demonstrate the self-powered isolation of plasma from whole blood

68. Aldo P, Marusov G, Svancara D, David J, Mor G: **Simple plextm™: A novel multi-analyte, automated microfluidic immunoassay platform for the detection of human and mouse cytokines and chemokines**. *Am J Reprod Immunol* 2016, **75**:678–693.

69. Kan CW, Rivnak AJ, Campbell TG, Piech T, Rissin DM, Mösl M, Petera A, Niederberger HP, Minnehan KA, Patel PP, et al.: **Isolation and detection of single molecules on paramagnetic beads using sequential fluid flows in microfabricated polymer array assemblies**. *Lab Chip* 2012, **12**:977–985.

70. Piraino F, Volpetti F, Watson C, Maerkl SJ: **A digital-Analog microfluidic platform for patient-centric multiplexed biomarker diagnostics of ultralow volume samples**. *ACS Nano* 2016, **10**:1699–1710.

Authors demonstrated the combination of analog immunoassays and digital ELISA.

71. Rissin DM, Fournier DR, Piech T, Kan CW, Campbell TG, Song L, Chang L, Rivnak AJ, Patel PP, Provuncher GK, et al.: **Simultaneous detection of single molecules and singulated ensembles of molecules enables immunoassays with broad dynamic range**. *Anal Chem* 2011, **83**:2279–2285.

One of the first reports of a digital ELISA implemented in a microfluidic device.

72. Garcia-Cordero JL, Maerkl SJ: **A 1024-sample serum analyzer chip for cancer diagnostics**. *Lab Chip* 2014, **14**:2642–2650.

Authors demonstrate quantitation of protein levels from 5-nL volumes of serum samples.

73. Kulasingam V, Pavlou MP, Diamandis EP: **Integrating high-throughput technologies in the quest for effective biomarkers for ovarian cancer**. *Nat Rev Cancer* 2010, **10**:371–378.

74. Patz EF, Campa MJ, Gottlin EB, Kusmartseva I, Xiang RG, Herndon JE: **Panel of serum biomarkers for the diagnosis of lung cancer**. *J Clin Oncol* 2007, **25**:5578–5583.