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Integrated "Lab-on-a-Chip" Microfluidic Systems for Isolation, Enrichment, and Analysis of Cancer Biomarkers

Sushruta Surappa[1], **Priyanka Multani**[1], **Ugur Parlatan**[1], **Prima Dewi Sinawang**[1,2], **Jussuf Kaifi**[4,5], **Demir Akin**[1,3], **Utkan Demirci**[1]

[1] Canary Center at Stanford for Cancer Early Detection, Department of Radiology, School of Medicine, Stanford University, Palo Alto, CA 94304, USA

[2] Department of Chemical Engineering, Stanford University, Stanford, CA 94305, USA

[3] Center for Cancer Nanotechnology Excellence for Translational Diagnostics (CCNE-TD), School of Medicine, Stanford University, Stanford, CA 94305, USA

[4] Department of Surgery, School of Medicine, University of Missouri, Columbia, MO 65212, USA

[5] Harry S. Truman Memorial Veterans' Hospital, Columbia, MO 65201, USA

Abstract

The liquid biopsy has garnered considerable attention as a complementary clinical tool for the early detection, molecular characterization and monitoring of cancer over the past decade. In contrast to traditional solid biopsy techniques, liquid biopsy offers a less invasive and safer alternative for routine cancer screening. Recent advances in microfluidic technologies have enabled handling of liquid biopsy-derived biomarkers with high sensitivity, throughput, and convenience. The integration of these multi-functional microfluidic technologies into a 'lab-on-a-chip' offers a powerful solution for processing and analyzing samples on a single platform, thereby reducing the complexity, bio-analyte loss and cross-contamination associated with multiple handling and transfer steps in more conventional benchtop workflows. This review critically addresses recent developments in integrated microfluidic technologies for cancer detection, highlighting isolation, enrichment, and analysis strategies for three important sub-types of cancer biomarkers: circulating tumor cells, circulating tumor DNA and exosomes. We first discuss the unique characteristics and advantages of the various lab-on-a-chip technologies developed to operate on each biomarker subtype. This is then followed by a discussion on the challenges and opportunities in the field of integrated systems for cancer detection. Ultimately, integrated microfluidic platforms form the core of a new class of point-of-care diagnostic tools by virtue of their ease-of-operation, portability and high sensitivity. Widespread availability of such

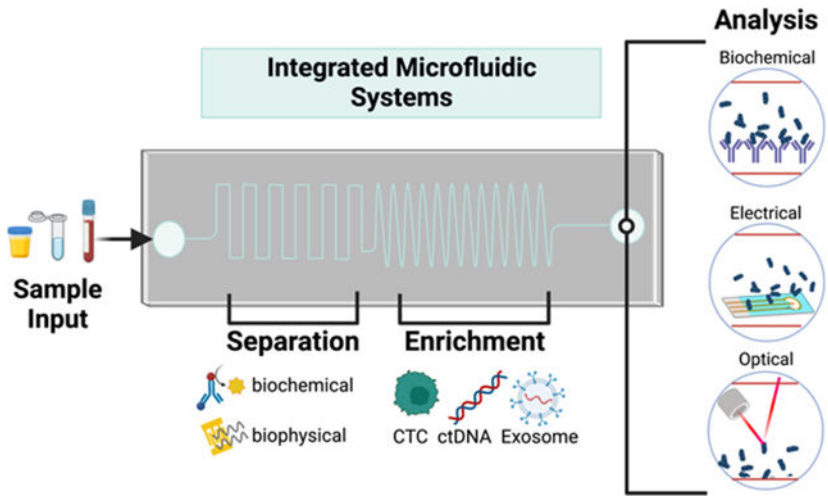
*Corresponding author. utkan@stanford.edu.

Conflict of Interest

Prof. Utkan Demirci (UD) is a founder of and has an equity interest in: (i) DxNow Inc., a company that is developing microfluidic IVF tools and imaging technologies, (ii) Koek Biotech, a company that is developing microfluidic technologies for clinical solutions, (iii) Levitas Inc., a company focusing on developing microfluidic sorters using magnetic levitation, (iv) Hillel Inc., and Hillal Biotech, companies bringing microfluidic cell phone tools for spermograms to home settings, and (v) Mercury Biosciences, a company developing vesicle isolation technologies. UD's interests were viewed and managed in accordance with the conflict-of-interest policies.

tools could potentially result in more frequent and convenient screening for early signs of cancer at clinical labs or primary care offices.

Graphical Abstract



This critical review addresses recent developments in integrated microfluidic technologies for cancer detection with an emphasis on three common subtypes of cancer biomarkers: circulating tumor cells, circulating tumor DNA and exosomes.

Keywords

Liquid biopsy; early cancer detection; lab-on-a-chip; cancer biomarkers

1 Introduction

Cancer is projected to impact nearly 2 million people in 2022 according to the American Cancer Society, making it the second most common cause of death in the United States[1]. The disease is characterized by the abnormal and uncontrolled growth of cells that tends to spread to various parts of the body over time through a process known as metastasis[2]. As such, a tremendous amount of time, effort and resources has been dedicated to the detection, treatment, clinical management and monitoring of cancer all around the world.

Detecting cancer at an early stage can greatly improve patient survival rates and lead to more desirable outcomes[3,4]. Advances in -omics, e.g., proteomics and genomics, have improved our ability to characterize, evaluate and screen various types of cancers in the human body. However, traditional diagnostics still rely on invasive sample collection techniques such as solid organ biopsy that are associated with medical risks and costs, rendering them non-ideal for routine cancer screening. Alternately, tumors are known to shed biomarkers[5,6] (a substance or a marker that is indicative of normal or abnormal process, condition or disease) such as circulating tumor cells (CTCs)[7], extracellular vesicles (EVs)[8], nucleic acids[9], and proteins[10] into circulation. These biomarkers can be collected and analyzed regularly to monitor disease progression and therapy response[11]. The non-invasive collection

and analysis of these biomarkers from various body fluids, such as blood, saliva and urine, is known as liquid biopsy and has recently garnered considerable attention as a diagnostic tool for the early detection of cancer[12–15].

Despite early promise, there are still several technical and clinical challenges in the field of liquid biopsy that need to be overcome to further push it forward as a potential mainstream diagnostic tool. For instance, concentration of CTCs can be approximately seven orders of magnitude[16] lower than that of white blood cells in blood, especially during early cancer. Hence, the biomarkers should first be isolated and enriched from the collected sample before they can be further analyzed[17]. Novel techniques based on a variety of conventional and microfluidic technological approaches have been developed to isolate and enrich biomarkers, and these can be broadly classified into biochemical and biophysical methods[18]. Biochemical techniques make use of affinity-based capture, using molecules such as enzymes, antibodies and aptamers that recognize and bind directly to the biomarker of interest with high specificity and sensitivity. The biomarkers can be captured and isolated from the biological fluid by either creating an affinity matrix using capturing agents/binders[19], or making use of binder-functionalized magnetic beads[20–22]. Alternately, biophysical techniques that differentiate based on physical properties of the target analytes such as size, density, deformability and electric charge, can also be used to separate the biomarkers of interest from the fluid sample[23–25]. These label-free approaches include techniques such as filter-based separation[26–28], external forcefields, including ultrasound[29,30], magnetism[31,32] and electricity[33–35], as well as inertial forces[36–38]. Often, a combination approach of both chemical and physical techniques results in highly efficient isolation and enrichment of analytes[39,40]. It is important to note that isolation and enrichment are often done in conjunction. For example, affinity-based capturing methods both isolate and concentrate the target analyte, thereby achieving isolation and enrichment simultaneously. In some physical isolation techniques, enrichment may constitute an additional step with the goal of increasing biomarker concentration relative to the total collected volume, post-isolation. In any case, both efficient isolation and enrichment is required to provide sufficient biomarker purity and concentration to enable accurate downstream analysis. Once isolated, biomarkers are typically analyzed to determine phenotype, proteomic and genetic expression, drug response and other clinically relevant parameters[41–43]. However, the multiple handling and transfer steps required to process and analyze the collected sample can lead to increased likelihood of cross-contamination, analyte degradation due to prolonged workflow, and increased complexity and cost of analysis. Further, the numerous processing steps are labor intensive and may lead to batch-to-batch inconsistencies and reduced sample yield.

Microfluidic platforms have been developed to complement conventional benchtop techniques as they offer multiplexing capabilities, portability, and lower reagent consumption. Critically, cross-functional platforms can be integrated to form a standalone ‘lab-on-a-chip’ system, capable of executing sequential workflows. Such integrated systems can perform biomarker isolation, enrichment and downstream analysis on a single device. In this review, we cover the recent advances in integrated microfluidic technologies for cancer detection. While numerous reviews have previously focused on singular aspects of biomarker processing such as isolation or analysis, we discuss platforms that integrate

multiple functionalities on a single chip, thereby advancing technological development for clinical lab and point-of-care diagnostics. We categorize liquid biopsy-based cancer detection biomarkers into three commonly targeted subtypes: circulating tumor cells (CTC), circulating tumor DNA (ctDNA) and exosomes. CTCs are cells shed by primary tumors into blood circulation and their presence is associated with development of cancer metastases and poor prognosis[44–46]. Similarly, ctDNA is released from primary and metastatic tumors into the bloodstream and contain tumor-specific mutations, which can help further understand disease progression and treatment efficacy[47,48]. Exosomes on the other hand are of emerging interest as they are highly stable, membrane-bound extracellular vesicles ranging from 50 nm – 150 nm[49] and are key to intercellular communication[50]. They contain proteins and nucleic acid fragments specific to the host cell and can thus offer insights into a cell's gene and protein expression and metabolic processes[51,52]. These biomarkers range from a few nanometers to tens of microns in size, therefore requiring distinct microfluidic isolation and analysis techniques. For each liquid biopsy subtype, we discuss the latest integrated systems capable of processing these markers, with an emphasis on downstream biomarker analysis. We conclude by discussing the major challenges and opportunities in using integrated microfluidic technologies for the early detection of cancer.

2 Circulating Tumor Cells (CTCs)

Circulating tumor cells (CTCs) are rare tumor cells that are released into the blood circulation by leaky primary or metastatic tumors. Once in circulation, they are known to spread to other parts of the body and are responsible for tumor metastases at distant organs[53]. CTCs are attractive targets for non-invasive liquid biopsy, as they can be directly isolated from peripheral blood samples and further analyzed to recover useful clinical information for diagnosis, prognosis, response to treatment and disease progression[46,54–56]. For instance, numerous studies have indicated that CTC concentration levels in blood are directly related to cancer progression and poor prognosis of the patient[57] and as such, CTC count has been used as a marker to predict the response to ongoing treatment[58]. Additionally, the proteomic and genetic analysis of the isolated CTCs can provide further insight into the mechanisms of disease progression[59], causes of drug resistance in certain phenotypes and help guide personalized therapies and treatment strategies[60,61]. In a few cases, viable CTCs could be expanded from the patient's blood *ex vivo* to serve as patient avatars to determine drug responses matching the responses observed in the patient[62,63]. However, despite its tremendous potential for clinical value, CTC based liquid biopsies face challenges while breaking into mainstream clinical practice primarily due to handling and processing challenges. CTCs in circulation are rare and typically only 1–10 cells are present per 1 ml of blood, making it challenging to isolate sufficient CTCs for downstream analysis or expansion[16]. Microfluidic chips provide many advantages over conventional laboratory systems as they can process, manipulate and analyze low-volume samples more efficiently. As such, various integrated microfluidic devices have been developed to efficiently isolate CTCs from small volumes of blood and to perform downstream analysis on the same chip thereby providing greater flexibility and functionality for a wide range of clinical applications.

CTC-specific integrated microfluidic platforms commonly consist of two or more individual modules, where the first module is typically designed to isolate the CTCs from the biological sample and the other module is used for the analysis. Isolation and enrichment of CTCs can be achieved by both physical and immunoaffinity-based techniques[64,65]. CTCs are typically larger than erythrocytes and most leukocytes making it possible to enrich them from the rest of the components in blood using size and density-selective separation[66–69]. Physical separation techniques are label-free, consist of a shorter enrichment time without needing surface chemical modifications, and do not require post-enrichment processing steps which allows them to be used for subsequent downstream analysis. However, achieving high purity with this method is challenging due to the similarity in size between CTCs and other blood cells, such as leukocytes. The issue is compounded by the fact that the size of CTCs can vary depending on the origin of the tumor and that CTCs from clinical patient samples can be smaller than cancer cell line tumor cells that are often used to develop CTC detection platforms [70]. This variability in size and morphology of CTCs presents a challenge when developing size-based isolation and enrichment techniques that are geared toward effective application across different cancer types.

Alternatively, immunoaffinity-based capture techniques can be used to isolate CTCs from the other components in blood with high purity and specificity via positive enrichment[71–74]. For example, it has been shown that most CTCs express the epithelial cell adhesion molecule (EpCAM) surface marker[75]. The microfluidic isolation module can be functionalized with the corresponding EpCAM antibody to capture and isolate CTCs[76]. Antibodies targeting other surface markers, such as prostate-specific membrane antigen (PSMA) and epidermal growth factor receptor 2 (HER2), have also been used in attempts to isolate tumor-specific CTCs[77,78]. However, different subtypes of CTCs can express various surface markers in differing levels of expression linked with their phenotype[79]. Hence, making use of a single antibody-based capture technique can lead to loss of heterogeneous CTC subtypes in samples. Instead, a cocktail of antibodies is likely to be effective in targeting a heterogenous population of CTCs when aiming to isolate over a range of phenotypes. As an alternative immunoaffinity-based approach, negative enrichment, which relies on white blood cell (WBC) depletion has also been employed. Erythrocytes are first lysed, followed by the introduction of antibody-coated magnetic beads that bind to the CD45 antigen expressed by leukocytes[80,81]. This method aims to eliminate WBCs from the sample and allow for the isolation of CTCs for further downstream analysis. Negative enrichment provides the benefit of isolating CTCs independent of their surface marker expression in a label-free manner but can result in lower sample purity. Some WBCs may express low levels of CD45 and therefore cannot be reliably removed from the sample[82]. Additionally, rare CTCs that are surrounded by a massive cluster of WBCs can be lost during negative depletion due to a non-specific bulk effect[83]. A combination of physical methods to first remove RBCs, followed by negative enrichment to eliminate WBCs has been employed in isolating CTCs from whole blood samples with relatively higher efficiency[84] as compared to standalone physical or chemical enrichment techniques. A summary of the advantages and disadvantages of various isolation techniques is presented in table 1.

Once isolated, CTCs are subsequently directed to a second module on the same integrated microfluidic system, where they can then be processed and analyzed based on the

clinical requirements. The downstream analysis of CTCs can be separated into three main categories: physical characterization/enumeration, protein analysis and omics analysis. The physical characteristics of the CTC, such as stiffness, deformability and shape, can be analyzed to determine phenotype whereas CTC count has been shown to be directly correlated to the severity of the disease[85,86]. An integrated microfluidic chip which used a deterministic lateral displacement (DLD) module to isolate CTCs and a second DLD module to sort cells by deformability has been reported[87]. It was found that the deformability of CTCs collected from eight different colon cancer patients was highly heterogeneous, indicating the need to further investigate phenotypic variability. Additionally, CTCs isolated through inertial and antibody-capture based methods have been enumerated using optical, electrical and pH-based methods[88–93]. One such example is shown in figure 2a, where an optofluidic flow cytometer (OFCM) integrating a spiral microfluidic isolation module and fluorescence detection system is used for single cell phenotypic analysis and cell counting[94]. In spiking experiments, the system was able to recover CTCs at over 95% efficiency while processing 1.2 mL of whole blood/hr. The OFCM was also used to count the CTCs based on phenotype by making use of fluorescence labeling of different surface markers. The clinical application of the system was demonstrated by analyzing the blood of 15 patients with stage 4 (metastatic) breast cancer. The OFCM detected CTCs in all 15 patient samples whereas the FDA-approved CellSearch®[95] system that targets EpCAM-expressing phenotypes only detected CTCs in 9 patients, suggesting that the integrated OFCM could potentially be more sensitive than the commercially available and gold standard CTC detection system.

Proteins are the key drivers of cellular function and are hence a critical downstream cell-based analysis targets following isolation of CTCs. By using a cocktail of antibody-based capture agents, various CTC subpopulations or phenotypes expressing different surface markers can first be isolated and barcoded, and then analyzed downstream using fluorescence microscopy and immunostaining techniques[96–100]. In one such example, size-isolated CTCs were first tagged using three different spectrally orthogonal Surface Enhanced Raman Spectroscopy (SERS) aptamer nanovectors based on their surface protein expression[101] (figure 2b). The complex SERS signatures of the individually tagged cells were then analyzed and decoded to obtain phenotypical information related to the cell membrane proteins and identify cancer subpopulations. Although the integrated SERS system was used to isolate and analyze blood samples spiked with CTCs from three different cancer cell lines, the system is yet to be tested on clinical blood samples and user-blinded cell identification. Alternately, the metabolic analysis and protein secretion at a single cell level can be studied by first capturing the CTCs and analyzing the released contents in a confined setting[102–104]. Figure 2c shows a single cell immunoblotting microfluidic system (ieSCI-chip) that demonstrates label-free sorting, cell lysis and electrophoresis-based western blotting[103]. By monitoring protein expression at the single-cell level, the ieSCI-chip was able to successfully identify a subgroup of apoptosis-negative (Bax-negative) cells from cisplatin-treated cells. The system was also used to analyze clinical blood samples where it was found that the ieSCI chip could monitor EpCAM expression levels at the single-cell level. Unlike the commercial CellSearch® system, this allows the ieSCI chip to track epithelial to mesenchymal transition (EMT), enabling it to monitor therapeutic response to anticancer drug treatment. More recently, researchers have developed powerful integrated

microfluidic systems that combine CTC isolation with label-free analysis techniques such as on-chip mass spectrometry and tandem label-free proteomics[105,106]. These systems can identify thousands of proteins in a small sample volume thereby overcoming the challenges faced by conventional label-free proteomics analysis techniques.

Genomic profiling and RNA sequencing of CTCs is of great interest as it can reveal information related to cancer metastasis and drug resistance mechanisms[107–109]. As such, various microfluidic systems integrated with PCR and RNA sequencing capabilities can be used for gene mutation detection[110–114]. A high-throughput microfluidic chip consisting of an inertial CTC isolation module and a multiple miRNA analysis capability is shown in figure 2d. The system utilizes a droplet microfluidic technique to encapsulate single CTCs along with various reagents for miRNA analysis and then subjects the droplet to quadratic isothermal amplification[110]. The small volume of the microdroplets allows for significantly faster and efficient amplification before the fluorescence signals of multiple miRNAs are collected by a detector. An alternative method of quantitatively analyzing single-cell mRNA without any PCR amplification has also been proposed[111]. In this method, magnetic particles are used to selectively hybridize with the targeted mRNA, before forming larger clusters that can be sorted based on their magnetic susceptibility inside the chip. The individual cells can then be visualized via immunostaining to determine their RNA levels. While this method provides an amplification free method of genomic characterization, the system is still limited in throughput. More recently, a high throughput single-cell RNA sequencing microfluidic system capable of massively parallel analysis has been reported[114], however, the number of integrated microfluidic systems capable of genetic sequencing is still limited and is an area of growing research and translational interest.

CTCs have shown tremendous value for early cancer detection[54] and prognosis[57]. Similarly, reappearance of CTCs post treatment can signify a recurrence, making it potentially a powerful treatment monitoring tool[46,55]. Isolated CTCs can also be analyzed downstream to obtain further information about tumor-specific phenotypic and genomic content[59]. Due to the diagnostic potential CTCs, several potential isolation and counting systems have been developed, including but not limited to CellSearch®, Parsortix®, and ClearCell®[95,115,116]. However, there are remaining technological and biological challenges to overcome. Isolating CTCs from blood poses a significant challenge due to their low abundance. Current physical and chemical isolation techniques present a trade-off between purity and efficiency. CTCs carrying heterogeneous surface markers can be isolated from blood using size-based separation, but that would also introduce similar sized WBCs as impurities in the sample. Alternatively, immunoaffinity-based techniques can isolate CTCs with high purity, but risk missing on heterogenous phenotypes. Development of integrated systems that can sample larger volumes of blood or analyze blood *in situ* is one potential approach to overcome the low concentration of CTCs. The field could also potentially benefit from the development of new isolation techniques that can efficiently isolate CTCs, improving on sample purity and allowing access to a larger subpopulation of CTCs with minimal effects on cells[117]. With the development of robust separation techniques compatible with microfluidic systems and maximizing CTC recovery rates in between processing steps, integrated analysis systems have the potential to efficiently isolate CTCs and utilize them for diagnosis, personalized therapy planning and treatment monitoring.

3 Circulating tumor DNA (ctDNA)

Circulating tumor DNA (ctDNA) is a biomarker released from cancerous cells that freely circulates in the bloodstream and other bodily fluids[47]. ctDNA falls under the larger category of cell-free DNA (cfDNA)[118], which are short fragments of non-encapsulated DNA shed into the bloodstream after cell death necrosis or apoptosis[119]. The number of ctDNA differs depending on tumor type, location, and tumor cascade and is released from both primary and metastatic tumor regions[48]. Its analysis can provide valuable insight into tumor heterogeneity and clonal evolution[120], as well as enable identification of tumor-specific genetic mutations[48,121]. With this, ctDNA can be used as a tool for cancer diagnosis, monitoring signs of disease recurrence and help guide treatment in patients with advanced cancers[118,122–124]. Thus, the tremendous clinical potential of ctDNA as a cancer biomarker can be further leveraged by advancing the development of more sensitive and specific lab-on-a-chip technologies.

Current isolation and analysis of ctDNA is a multi-step laboratory-based process. Presently, there is relatively little academic literature discussing ctDNA detection using microfluidics. This is particularly due to the low abundance of the ctDNA in the subset of non-mutated cfDNA (ctDNA can be as low as 0.01% of total cfDNA)[125,126], making it challenging to accurately detect rare mutant targets[127]. In addition, on account of its short half-life (16–150 min)[126] and fragmented nature, cfDNA requires a rapid isolation process to avoid sample degradation. Therefore, efficiently recovering cfDNA in the extraction process will influence the precision of the subsequent processes by reducing the risk of misdiagnosis and improving detection sensitivity[120].

Traditional bench-top isolation techniques rely on a silica-based spin column to extract nucleic acids in laboratories[128,129]. In the presence of a high salt buffer, DNA will be adsorbed by the silica surface because the salt reduces the electrostatic repulsion between the negatively charged silica surface and the negatively charged DNA. Meanwhile, other compounds such as proteins will pass through the column. To ensure purified DNA product, ethanol is used to remove salt and non-specific protein contaminants to avoid inhibition for DNA amplification[130]. As the last step, the captured DNA is eluted in a low-salt buffer (e.g., nuclease-free water). Even though this process is widely used, it is tedious and consists of multiple manual steps, which could lead to batch-to-batch inconsistencies. In order to increase the consistency and avoid cross-contamination, automated systems for nucleic acid extraction are now commercially available (e.g., Qiagen[120], ThermoFisher, Perkin Elmer, Promega, etc.). However, these systems are costly in setup and consume large quantities of reagents[120].

Microfluidic platforms offer a complementary approach to addressing the needs of manipulating various analyses of DNA, proteins, and cells in a versatile manner. Recently, an integrated microfluidic system, called PIBEX, was shown to perform on par with a commonly used commercial product (QIAamp kit) in terms of DNA sample quality and extraction efficiency[126]. The PIBEX consists of multiple reservoirs housing samples and buffers (figure 3a). This allows a continuous process within a chip, eliminating multiple

discrete steps, thereby decreasing the risk of cross-contamination and reducing the workflow complexity and burden on the operators.

CtDNA can be analyzed to detect mutations that carry critical genetic information for cancer diagnosis[131]. Two conventional methods used to analyze ctDNA are: 1) genome-wide sequencing (GWS) and/or next-generation sequencing (NGS), 2) specific gene targeting using polymerase chain reaction (PCR) or droplet-digital PCR (ddPCR)[127]. GWS or NGS can detect various gene mutations (ctDNA) from cfDNAs. However, this option is expensive and has a long turnaround time due to the need to generate and analyze large datasets[132]. ddPCR is lower in cost and quicker, but the analysis is focused only on a specific target[133]. Microfluidic platforms with multiplexing capabilities offer simultaneous analysis of multiple genetic targets[134], thereby reducing processing time. A 2006 study demonstrated a fully integrated microfluidic system for genetic analysis in less than 30 minutes[135]. A syringe pump was used to deliver the sample and necessary reagents to the chip for nucleic acid purification (figure 3b). Another study reported an integrated microfluidic platform that selectively isolated DNA or RNA from low-copy bacterial cells followed by a direct on-chip PCR[136]. The setup allowed an on-chip quantitative PCR assay in a one-step manner whereby all processes were vacuum-driven and carried out in the same microwells within which bacterial nucleic acids were isolated. This integration could avoid sample loss during liquid transfer (Figure 3c).

A commercially available microfluidic system that performs multiplexed PCR sequencing (MMP-Seq) integrated a preamplification (PreAmp) step into its workflow for detection of ctDNA. This integration has enhanced analysis sensitivity despite low-input ctDNA (Figure 3d)[137]. A preamplification step in molecular diagnostics is essential to warrant reliable and reproducible quantification of multiple targets from a small sample size[138]. This step was introduced to the MMP-Seq workflow to increase the concentration of targets for downstream sequencing analysis. The multiplex PreAmp PCR protocol resulted in a sufficient number of DNA per chamber to improve the robustness of the assay. The study also showed that the PreAmp step introduced an unbiased amplification ensuring the same coverage and uniformity of the amplicons from those without amplification. The integrated system required 2 mL of plasma and costs less than $100 per sample. In the clinical validation study, the detection of ctDNA mutation has shown 92% sensitivity and 100% specificity when benchmarked to the matched tissues[139]. The MMP-Seq system could profile 88 cancer genes in 48 samples rapidly and detect variants at frequencies as low as 0.4%. Thus, microfluidic platforms for multiplexed identification of biomarkers have been shown to tackle some of the challenges of ctDNA analysis, from isolation to detection. Moreover, in a follow-up study, the MMP-Seq was recently used to generate transcriptomic data from a larger cohort of 170 ovarian tumor tissues to be coupled with pathology images from ICON7 trial[140]. The integrated analysis of digital pathology and tissue transcriptomes demonstrated a classification and characterization of tumor-immune phenotype. Therefore, innovations in integrated microfluidic systems that could provide isolation and analysis of multiple ctDNAs while ensuring a high sample throughput could advance the broader field of ctDNA in cancer detection.

4 Exosomes

Exosomes are double-membraned nanovesicles secreted into the extracellular matrix and circulatory system from the plasma membrane of the cells[50]. They are a member of the broader family of extracellular vesicles (EVs)[141] and typically have a diameter between 30–150nm[49]. Exosomes contain cellular materials, such as nucleic acids (RNA, DNA), cytokines, and transmembrane proteins which are critical for epigenetic regulation processes[141]. These vesicles also encapsulate lipids and metabolites that play a role in maintaining cellular homeostasis and regulating signaling pathways, respectively[51,142]. Analysis of exosomal cargo via multi-omics methods (e.g., genomics, proteomics, lipidomics, metabolomics) could provide highly valuable insights into disease states[51,52,143]. Exosomes have gained considerable research interest since they possess cell-to-cell communication properties and therapeutic functions[144,145]. The presence of various transmembrane proteins on their surface allows exosomes to be selectively uptaken by the recipient cell to transfer signaling materials[146–148]. As a result, exosomes are widely researched for early detection of cancer and monitoring metastasis in various cancers, such as prostate, pancreas, lung, and breast[149–151].

Since exosome processing typically begins with isolation and enrichment, a standardized, high-yield, and precise isolation technique is necessary to ensure reproducibility and reliability of results from the various downstream analyses. It is challenging to apply traditional centrifugation methods to isolate nano-sized particles, such as exosomes, since they require ultra-high rotational velocities to be separated from larger cells and debris[147]. Some studies address the challenge of isolation by using ultracentrifugation[147], membrane filter isolation (ultrafiltration)[26,148,152], and size exclusion chromatography[153–155]. Ultracentrifugation can generate forces greater than 100,000 g that are necessary for the effective separation of exosomes. However, compared to other methods (e.g., ultrafiltration), ultracentrifuges are bulky, costly and they have poor exosome yield[156]. Physical techniques that can integrate with microfluidics have been used for exosome isolation. Techniques such as acoustophoresis[157–161], dielectrophoresis[33,162–166], and deterministic lateral displacement (DLD)[167–170] have been used for isolation of exosomes. Among these techniques, dielectrophoresis has been widely applied as it is able to provide rapid isolation using a small sample volume (100–200 μL). However, some dielectrophoretic isolation designs may potentially cause damage to the exosome membrane structure due to contact with the electrode[33]. Biochemical approaches such as antibody (Ab)-based filtering methods and functionalized magnetic beads offer a more specific approach to isolating exosomes[171], but subsequent steps may be required to release the captured exosomes from the functionalized beads which may lead to analyte loss[172–174]. Additionally, the heterogeneity of surface markers across exosomes, samples and cell lines, allows only a smaller subgroup of vesicles to be captured when attempting to isolate using binder-based approaches. Membrane-based methods are surface marker agnostic and tend to isolate exosomes purely based on size. They are simple, compact, and straightforward to operate[26,175] and yet, they have the shortcomings of membrane clogging and the co-presence of similar-sized non-EV nanoparticles[176,177]. As a result, a combination of membrane-based size separation followed by further selective enrichment

step with Ab-based capture has shown promising results in recently developed isolation technologies[178].

Integrated systems commonly target three biophysical/ biochemical properties of exosomes - concentration, content, and membrane protein profile, following the isolation process. These processes are needed to confirm the presence of exosomes and characterize the cargo they carry. Measuring the number and size distribution of the nanosized particles is often the initial step to evaluating the isolation process[179,180]. One of the most common methods for exosome counting involves integrating on-chip separation with fluorescence intensity quantification of EVs[33,162,166,181–184]. A representation of a fluorescence-based quantification approach is shown in Figure 4a, where a digital counting application is demonstrated on an integrated lab-on-a-chip platform[178]. The exosomes were filtered from a plasma sample using a 0.22 μm polycarbonate membrane. Then they were stained with CD 63, anti-rabbit antibody-HRP, and tyramide-tetramethylrhodamine markers. The stained exosomes were then observed via luminescence measurements from the attached CD63 tetraspanin markers. Using only 2μL of blood, a limit of detection of 10^5 particles/mL was achieved, indicating the potential to integrate this platform for low sample volume applications such as a fingerstick test. An on-chip technology called EXID presented (less than two hours) quantification of the druggable transmembrane protein, PD-L1, that is expressed by tumor cells to suppress T-cell activation[183]. The platform combined serpentine-shaped microchannels to isolate exosomes using magnetic bead attachment via CD-9 antibodies. The captured exosomes were quantified by measuring the fluorescence signal acquired by the PD-L1 specific probes via an inverted fluorescence microscope with a magnification of 20x. This work presents on-chip quantification of exosomes and can potentially be further extended to profile multiple proteins in a single assay. A unique droplet-based method called ExoELISA has demonstrated absolute exosome counting, with a reported sensitivity of approximately 10 exosomes per microliter[185]. This microfluidic chip first captures exosomes using antibody-attached magnetic beads and then creates oil droplets to capture the enzyme-labelled exosome-bead complexes. While this approach might prove useful when handling samples with low exosome concentration, it needs a careful washing step for optimal sensitivity and is limited by its lower throughput as compared to traditional fluorescence counting techniques. A separate study highlighted the use of a graphene oxide/polydopamine 3D nano-porous structure to capture exosomes (Figure 4b), followed by an ELISA assay[186]. This combination resulted in a high exosomal recovery rate, allowing for an ultrasensitive ELISA assay. According to the study, the platform was tested on colon cancer cell lines and was found to have the potential to be used with multiple fluorescent probes. This suggests that the platform may be used as a diagnostic assay for point-of-care screening of clinical samples.

Profiling of RNAs carried within exosomes can help determine differential gene expression to further understand disease progression[187–190]. Integrated systems have been able to successfully capture and perform exosome lysis on-chip for nucleic acid quantification. For example, researchers developed integrated systems to perform RNA quantification after isolating the body fluid (plasma, urine, or saliva) to analyze the exosomal molecular cargo[172]. In one of these studies, cargo levels of several mRNAs were shown to be different for glioblastoma patients when compared to control groups, using an on-chip system[172,186].

A proof-of-concept quantification of exosomal RNAs using integrated isolation and a digital droplet polymerase chain reactor (ddPCR) has also been demonstrated and was applied for cell culture supernatant-derived exosomes. Then, RNA profiles of lung cancer exosomes were quantified using an on-chip ddPCR[191].

Surface-enhanced Raman spectroscopy (SERS) has established itself as a powerful optical tool for molecular characterization of exosomal content[192]. SERS can be used to analyze exosomal chemical composition, with each peak corresponding to a specific bond vibration. This approach is helpful in identifying biomaterial characteristics. As exosomes carry cargo materials that are heterogenous in quantity and composition, SERS analysis of exosomes has the potential to provide diagnostic information[193]. A recent study (Figure 4c) demonstrates that SERS, when combined with exosome isolation on a single chip, enabled molecular analysis of exosomes[193]. Blood serum samples were obtained from 21 healthy and 20 melanoma patients, and exosomes were captured using anti-MCSP/MCAM/CD61 co-functionalized on EPAC-II (EV phenotype analyzer chip). The chemical analysis of the captured exosomes was performed by calculating their SERS maps, which resulted in the accurate differentiation of melanoma patients from healthy controls. In addition to molecular characterization, SERS can also quantify the amount of miRNA inside a lysed exosome with high sensitivity. In one such example, a study utilizing a microfluidic chip integrated with a SERS substrate, initially enriched the exosomes with a magnetic enrichment chamber before profiling the miRNA structure[194].

Emerging technologies such as optical metasurfaces and plasmonic sensors are promising candidates for integration with microfluidics to miniaturize conventional benchtop isolation and quantification methods[195]. These technologies, such as metasurface sensors, have the potential to serve as highly sensitive, rapid, and accessible diagnostic platforms. A metasurface-based transmission measurement technology was used to profile exosomes using aided imaging (Figure 4d)[196]. An algorithmic signal improvement method called ‘robust spectral shift’ was developed and applied for all dielectric metasurfaces. This algorithm removed the need for expensive and bulky sensors. In a separate study, it was demonstrated that the combination of photonic crystals and transmembrane-specific markers could be utilized to detect parasite exosomes that have the potential to be a biomarker for infectious diseases[197]. The authors functionalized the photonic crystal (PC) surface with CD63 transmembrane proteins and embedded these PC surfaces inside a microfluidic channel. Surface plasmon resonance wavelength was measured with a spectroscopic method and shifts in resonance wavelengths were recorded. It was found that the host and parasite exosomes could be differentiated using the resonance wavelength shift characteristics with a high sensitivity. Furthermore, high-resolution inkjet printing technology was used to develop a three dimensional nanopatterned microfluidic chip capable of analyzing cancer-specific cargo of plasma-derived exosomes, such as MMPs[198]. The same technology was also used to monitor ovarian cancer progression, by analyzing a low volume of exosome preparation derived from clinical plasma samples[199]. These new technologies combine traditional staining methods with sophisticated surface chemistry and material science techniques, thereby integrating sequential isolation and analysis methods in one chip to perform rapid tests.

The potential applications of exosomes in cancer detection are significant. Their abundance in bodily fluids and stability as lipid-bound vesicles with their versatile cargo, make them a valuable source of information for disease diagnosis and monitoring. In addition, downstream analysis of exosomal cargo can provide insights into cellular proliferation and reveal information about a cell's genotypic and phenotypic content. Despite their tremendous potential, it is important to note that the use of exosomes as an early cancer biomarker is still an emerging field of research with many open questions. For instance, there is still a lack of a standardized, reproduceable and rigorous approach for the isolation and enrichment of exosomes[49,200]. The inherent heterogeneity of exosomes in terms of size, cargo and surface markers makes it challenging to reliably isolate a particular subtype and differentiate it from other subtypes in a complex patient sample. Further evolution of integrated microfluidic systems could potentially reduce the variability in sample preparation and improve the reliability of downstream exosome analysis. These systems would also benefit from the development of new label-free isolation[159,160] that can precisely separate these nanosized particles without damaging their structure and biological activity. Moreover, exosomes are involved in intercellular communication and can be found in bodily fluids even in the absence of a disease. Therefore, the proportion of disease-specific vesicles in a clinical sample, such as saliva, plasma or urine, may relatively vary in the total exosome population. Hence, downstream -omic analysis is needed to characterize the cargo[201]. Development of detection platforms with either high sensitivity, or the ability to process large sample volumes could potentially overcome this limitation. Finally, a deeper understanding of the correlation between exosome properties such as its size and cargo to the host's disease state including EV analysis at a single exosome level, could further expand the utility of exosomes as clinically applicable cancer biomarkers.

5 Outlook

The development of an integrated 'lab-on-a-chip' microfluidic system with a sample-in-result-out mechanism has received growing interest, and clinical translation of such technologies might offer new opportunities in disease detection, diagnosis, therapy and treatment monitoring. A unique aspect of these systems is that their designs offer rapid and repeatable operation that could result in reproducible and clinically usable data and insights into the cancer disease. With these benefits, integrated microfluidic systems have the potential to enable low-cost and widely accessible technological solutions for early cancer detection.

Integrated microfluidic systems are a rapidly evolving field of research offering a variety of challenges and opportunities. An advantage of conventional benchtop workflows is the ability to validate results in between processing steps. Still, integrated system can overcome this limitation by including real-time feedback into the overall workflow, providing the user with critical process-related information. This would require the incorporation of robust sensor technology into each individual module of the integrated platform. Another challenge and strength of microfluidics is low volumes. The low volume and low throughput have been addressed by leveraging microfluidic principles to design channels with larger dimensions and increased flow rates, improving the throughput of these fluidic systems. It is also important to note that the ability to multiplex samples and perform multiple analyses in a

parallel manner, is touted as a major advantage of lab-on-a-chip systems and can further increase throughput. However, multiplexing leads to the further reduction of bioanalyte available for downstream analysis, depending on the number of parallel channels, making it challenging to perform multiple meaningful measurements on a single chip. To fully harness the potential of integrated microfluidic systems, it is necessary to improve the sensitivity of existing analysis techniques to deal with low analyte availability, while simultaneously developing new biophysical and biochemical sensors that can break the current ceiling in terms of sensing and precision.

Existing integrated microfluidic systems have benefited from employing innovative ways to isolate and analyze biomarkers, ranging from physical methods such as inertial, magnetic-, acoustic-, and optic-based techniques to adapting biochemical approaches for microfluidics. Currently, this broad array of methods is often limited to specific biomarker subtypes. For example, a workflow beginning with size-based separation may be compatible for CTC or exosome isolation but would be ineffective for the enrichment of ctDNA. In addition, the large size range of various biomolecules makes it challenging to find a single isolation method that can be broadly applied across different biomarkers. Therefore, specific microfluidic techniques or technologies usually need to be developed for each biomarker or size range of targets. This limited adaptability may also be true from a technological standpoint. For instance, processes that involve on-chip PCR or ultracentrifugation steps will require at least a portion of the microfluidic system to withstand high temperatures and velocities. Any downstream steps should be compatible with such conditions. The materials used to make microfluidic devices should also be well-suited for multiple operating conditions. In addition to Polydimethylsiloxane (PDMS), glass, and acrylic which amongst the most popular materials for microfluidics today[202], further identifying new materials that are broadly compatible is an important step in creating fully integrated workflows. While there exist systems that can reliably perform the discrete steps of isolation and analysis, simply combining them into one continuous workflow may be challenging if each process has its own flow rate, temperature, and geometry specifications. Thus, the ability to integrate different standalone modules to form a lab-on-a-chip microfluidic system provides us with versatility in terms of targets and analyses on a single platform. A critical challenge in this field will be to ensure that this versatility is backed by robust, reliable and repeatable operation, similar to what is currently available in dedicated isolation or detection platforms.

Achieving a high level of integration wherein one system can operate on multiple analyte types, such as performing complementary analyses on both CTCs and ctDNA, would require cross-disciplinary collaboration. As integrated microfluidic systems are inherently interdisciplinary in nature, with biological constraints from sample inputs and technical constraints in sample processing, collaboration is key to developing a better understanding of these interdependent challenges. For example, when considering deployment of such systems into clinical practice, expertise from medical professionals can offer valuable insights into the practical considerations involved in clinical translation of point-of-care technologies. Additionally, as many of the systems discussed in this review are lab prototypes, future clinical testing demonstrating clinical utility and improved patient outcomes is essential for successful clinical implementations. As a result, multidisciplinary

collaboration is an important factor in designing adaptable integrated microfluidic systems that leverage novel approaches for biomarker analysis and fill the gap from bench to bedside.

To summarize, although integrated 'lab-on-a-chip' systems have their challenges, technological advances and multidisciplinary collaboration can significantly advance their development. The promising results of existing detection systems are evidence that clinically meaningful liquid biopsies are attainable. The further advancement of such integrated microfluidic systems could potentially improve access to reliable diagnostic tools for early cancer detection in research labs and clinics around the world.

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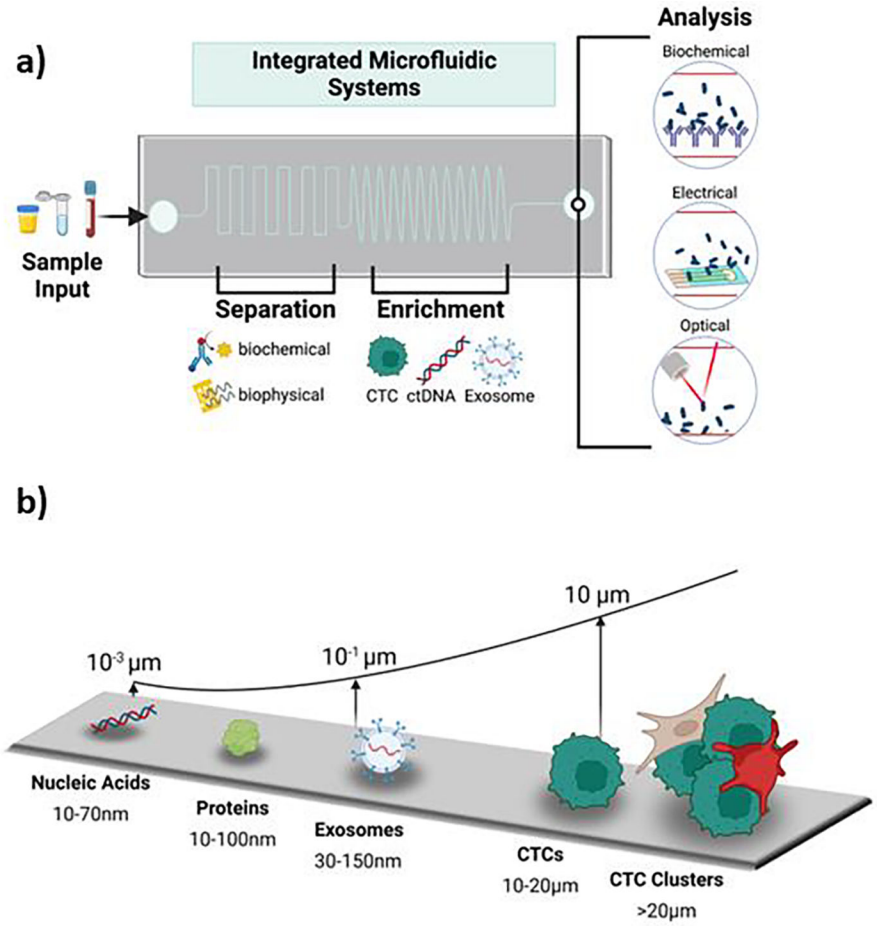

Figure 1: Integrated microfluidic system workflow for liquid biopsies and biomarker size scale.

a) Common input samples include urine, saliva, or blood. Using either biochemical or biophysical techniques, or a combination of the two, the target biomarkers are first separated from the heterogenous input sample. Common biomarkers targeted during the isolation and enrichment processes include CTCs, ctDNA and exosomes. Subsequent analysis often consists of biochemical, electrical, and optical-based techniques. b) Sizes of commonly targeted cancer biomarkers span from tens of nanometers to over twenty microns.

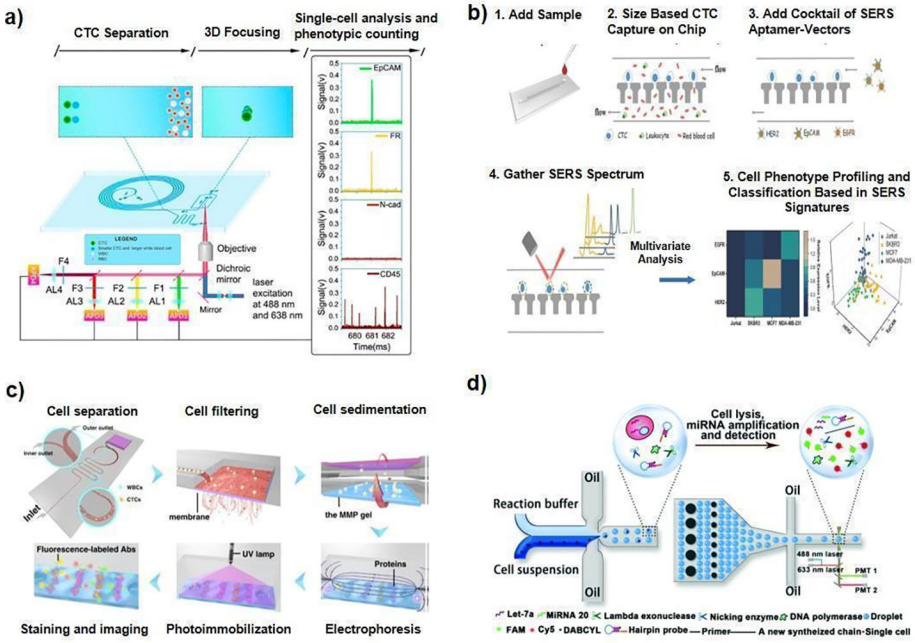

Figure. 2: Various integrated microfluidic systems for isolation and analysis of CTCs.

a) An optofluidic flow cytometer for CTC counting (Reprinted with permission from ref. 94. Copyright 2019. American Chemical Society). b) A SERS-based microfluidic system for proteomic analysis (Reprinted with permission from ref. 101. Copyright 2018. Wiley). c) Single cell immunoblotting microfluidic system for on-chip western blot analysis (ref. 103). d) Droplet microfluidic technique for miRNA (ref.110).

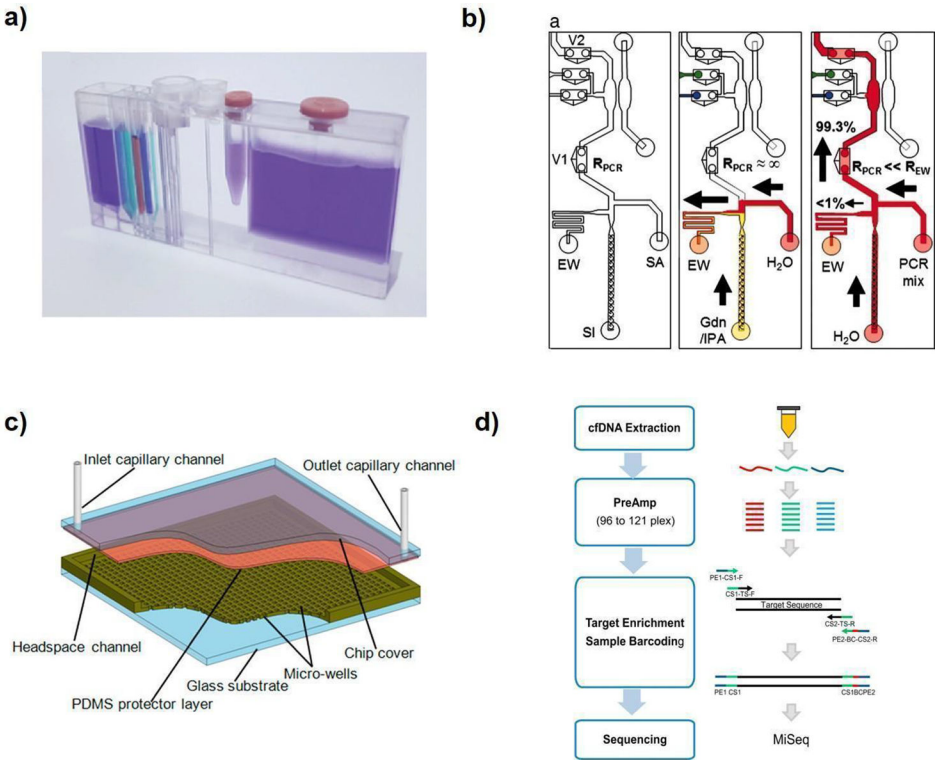

Figure. 3: Circulating tumor DNA (ctDNA) isolation and analysis.

a) Photo of an integrated microfluidic PIBEX chip for cfDNA extraction (ref. 126). b) A fully integrated microfluidic system for genetic analysis (ref. 135). c) Schematic of a 2D liquid phase nucleic acid purification chip (Reprinted (adapted) with permission from (ref. 136). Copyright 2013 American Chemical Society). d) Schematic of a PreAmp MMP-Seq workflow for multiplexing of ctDNA analysis used in next-generation sequencing (Reprinted from ref. 137 with permission from Elsevier).

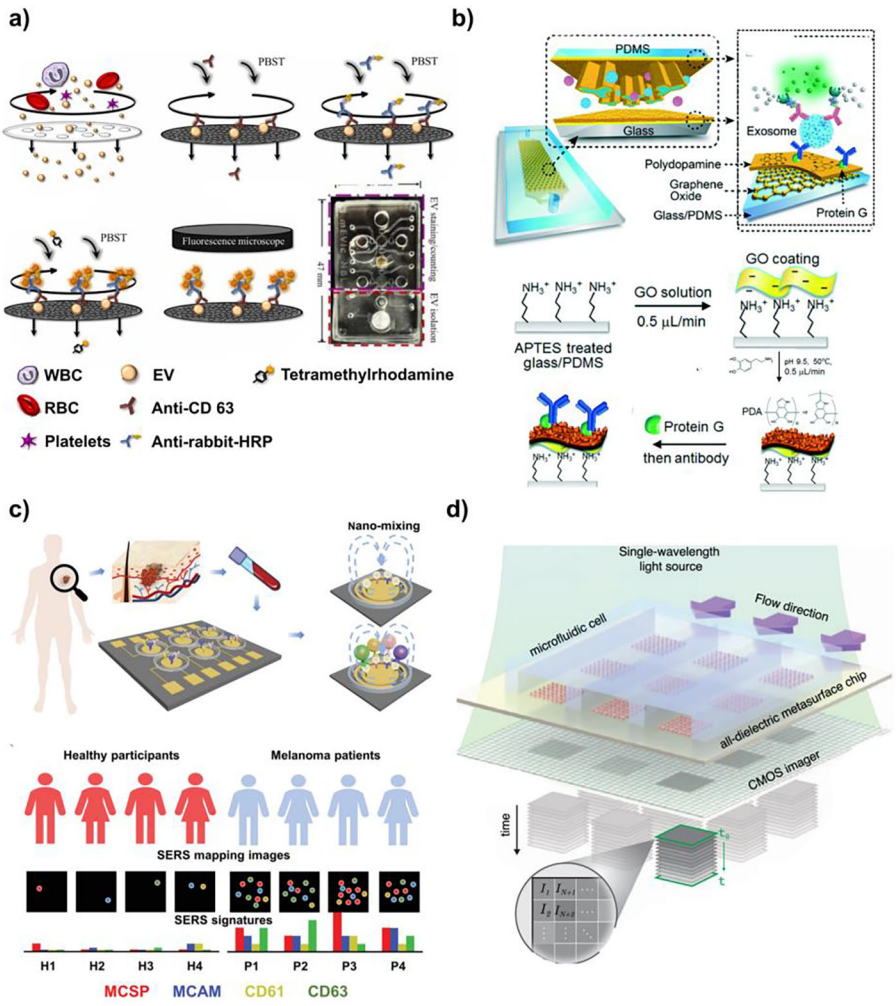

Figure 4: Integrated microfluidic systems for isolation and analysis of exosomes

a) Digital exosome counting platform combined with a membrane-based isolation step (Reprinted from ref. 178 with the permission of Elsevier). b) Fluorescence-based exosome content analysis (Reproduced from ref. 186 with permission from the Royal Society of Chemistry) c) A SERS probe is used for molecular structure interrogation after a magnetic enrichment sequence (Reproduced from ref. 193. © 2021 Wiley-VCH GmbH). d) A microfluidic metasurface bionsensor that utilizes aided imaging to sense extracellular vesicles (ref.196).

Table 1.

Summary comparing biophysical and biochemical CTC separation techniques used in integrated microfluidic systems.

Isolation Method	Advantages	Disadvantages
Size/Deformability based physical separation	Shorter processing time; label free; independent of CTC surface markers	Leukocytes of similar size to CTCs are difficult to separate and remove; the chip can get blocked easily
Immunoaffinity (Positive selection)	High purity; high recovery rates	Loss of low EpCAM expressing or EpCAM negative CTCs; postprocessing required to cleave attached beads; might miss some subpopulations and phenotypes in heterogenous CTC population
Immunoaffinity (Negative selection)	High cell viability; independent of CTC surface marker	Incomplete removal of normal cells (non-CTCs)