Received: 9 June 2023 | Revised: 31 July 2023 | Accepted: 10 August 2023

DOI: 10.1002/dro2.92

REVIEW ARTICLE

Droplet WILEY

Recent advances in droplet-based microfluidics in liquid biopsy for cancer diagnosis

Jingyu Shi[1] | Yu Zhang[2] | Yadi Fan[1] | Yi Liu[1] | Mo Yang[1]

[1]Department of Biomedical Engineering, The Hong Kong Polytechnic University, Hong Kong, China

[2]Department of Mechanical and Automotive Engineering, Royal Melbourne Institute of Technology, Melbourne, Australia

Correspondence
Mo Yang, Department of Biomedical Engineering, The Hong Kong Polytechnic University, Hong Kong 999077, China.
Email: Mo.Yang@polyu.edu.hk

Funding information
Shenzhen Science and Technology Program-Basic Research Scheme, Grant/Award Number: JCYJ20220531090808020; Hong Kong Research Grants Council (RGC)-General Research Fund, Grant/Award Numbers: PolyU 15216622, PolyU 15217621; Guangdong-Hong Kong Technology Cooperation Funding Scheme, Grant/Award Numbers: GHP/032/20SZ, SGDX20201103095404018; Hong Kong Polytechnic University Shenzhen Institute Bai Cheng Bai Yuan Fund, Grant/Award Number: I2022A002; the Research Grants Council (RGC) Postdoctoral Fellowship Fund, Grant/Award Number: PDFS2223-5S07; Hong Kong Polytechnic University Internal Fund, Grant/Award Numbers: 1-ZVVQ, 1-CD6J, 1-CD8M

Abstract

Liquid biopsy, a noninvasive technique to obtain tumor information from body fluids, is an emerging technology for cancer diagnosis, prognosis, and monitoring, providing crucial support for the realization of precision medicine. The main biomarkers of liquid biopsy include circulating tumor cells, circulating tumor DNA, microRNA, and circulating tumor exosomes. Traditional liquid biopsy detection methods include flow cytometry, immunoassay, polymerase chain reaction (PCR)-based methods, and next-generation sequencing (NGS)-based methods, which are time-consuming, labor-intensive, and cannot reflect cell heterogeneity. Droplet-based microfluidics with high throughput, low contamination, high sensitivity, and single-cell/single-molecule/single-exosome analysis capabilities have shown great potential in the field of liquid biopsy. This review aims to summarize the recent development in droplet-based microfluidics in liquid biopsy for cancer diagnosis.

INTRODUCTION

Cancer, known as a malignant tumor, has been a serious threat to human health for a long time. According to WHO, cancer accounted for nearly 10 million deaths in 2020, or nearly one in six deaths.[1] To fight cancer, human beings have never stopped looking for new diagnostic and therapeutic methods. Early screening and accurate diagnosis and treatment of cancer are crucial to improve the quality of life and survival rate of cancer patients. Clinically, tissue biopsy has been considered the gold standard for cancer diagnosis, but it is limited by highly invasive sampling procedures, incomplete representation, and a high risk of complications. In recent years, liquid biopsy, such as the detection of cancer biomarkers in body fluids (e.g., blood, urine, and saliva), including circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), microRNAs (miRNAs), and exosomes, has been regarded as an important development direction for noninvasive cancer detection.[2] Compared with traditional tissue biopsy, liquid biopsy has obvious advantages, such as noninvasive and repeatable sampling, real-time and comprehensive tissue profiling, less risk, and low cost. Moreover, in view of the development of molecular biotechnology, the specific screening and identification of cancer biomarkers through genomics, transcriptomics, or proteomics can accurately find the root cause and therapeutic target of the disease, thereby realizing precise and individualized treatment.[3] Unfortunately, for many diseases, the low abundance and low sample number of biomarkers and the high interference from body fluids prevent reliable screening or detection. For example, CTCs are tumor

cells that are shed from solid tumor tissues and enter the circulatory system.[4] As the most promising targets for liquid biopsy, they not only carry the complete biological information of tumor cells but also exist in the peripheral blood circulation in the early stage of cancer. However, due to the rarity of CTCs (only 1–10 CTCs per 1 mL whole blood), it is a great challenge to isolate and purify CTCs. ctDNA is a free DNA fragment released into the circulatory system by tumor cell apoptosis, carrying tumor-related genetic mutations, and is an important monitoring indicator for early screening, prognosis evaluation, and recurrence monitoring.[5] ctDNA is currently the most widely used liquid biopsy marker in clinical practice. However, the proportion of ctDNA in cell-free DNA (cfDNA) fluctuates between 1% and 93% depending on the stage of cancer progression, which implies that high levels of cfDNA in the blood may not only originate from tumor cells but also from benign tumors, inflammation, or tissue damage, thus making diagnostic analysis difficult.[6] miRNAs are stable endogenous small noncoding RNAs (21–25 nt), which can reflect the presence of early tumors and detect tumor development, recurrence, and drug sensitivity.[7] Unlike ctDNA, miRNA has tissue-specific expression and can more actively enter the extracellular environment through carriers such as exosomes, providing more functional insights.[8] However, there is no reliable miRNA detection method or practical application in clinical practice. Circulating tumor exosomes refer to biologically active vesicles secreted by tumor cells with a diameter of 30–200 nm. The small size of exosomes facilitates their passage through the gaps between endothelial cells into circulation and reaches distant organs. Circulating tumor exosomes, as "cargo" for proteins and nucleic acids, play a crucial role in intracellular communication and are heavily involved in cancer development and metastasis.[9] Current exosome isolation techniques, such as ultra-centrifugation and density gradient centrifugation, require extensive purification steps that are time-consuming and labor-intensive, which may not be available in clinical settings.[10] It can be seen that biomarkers in liquid biopsy cannot be detected directly from clinical patient samples in most cases due to problems, such as small numbers, high background, and difficult separation, hence complex multistep sample preparation methods are required. Additionally, the traditional detection methods for cancer biomarkers, such as immunoassays, polymerase chain reaction (PCR), and sequencing, are often time-consuming, labor-intensive, and require laboratory facilities Therefore, there is an urgency to develop a sensitive and rapid "sample-to-answer" device for early screening and detection of cancer biomarkers.

Microfluidics is a technology that manipulates fluidics at a microscale, which can integrate multiple functions, such as mixers, actuators, reactors, separators, and sensors into a single chip to optimize the detection process.[11,12] Due to the highly integrated system and compact size, microfluidic devices have the advantages of reducing sample consumption, shortening reaction time, improving detection sensitivity, and reducing cost.[13] Droplet-based microfluidics is an important subcategory of microfluidics that utilizes two immiscible liquid phases within microchannels to generate monodisperse water-in-oil (w/o) micro- or nano-droplets.[14] Droplet-based microfluidics not only inherit most of the advantages of traditional microfluidics but also provide many attractive properties over them. First, microdroplets are uniform, controllable, and ultra-small volumes (femtoliter [fL] to nanoliter [nL]), which can dramatically reduce sample and reagent consumptions, enhance mixing and mass transfer inside the droplets, and improve reaction efficiency. Second, microdroplets can be generated and manipulated at very fast rates up to several MHz, which offers great potential for high-speed and high-throughput analysis of droplet-based microfluidics.[15] Third, each droplet is independent of the other and can be used as a closed single microreactor without cross-contamination. Target samples can be individually confined in these interference-free microdroplets with a high signal-to-noise ratio, enabling precise detection with exceptional sensitivity even at the single-cell, single-molecule, or single-exosome level.[16] At the same time, a large number of studies have shown that the molecular and cellular heterogeneity of malignant tumors becomes apparent only at the single-cell level.[17] Accurate detection of multiple cancer biomarkers at the single-cell/single-molecule/single-exosome level contributes to understanding cancer progression and metastasis, which largely affects the diagnosis, prognosis, and monitoring of cancer patients. In this case, droplet-based microfluidics with high throughput, low contamination, high sensitivity, and single-cell/-molecule/-exosome analysis capabilities has become an ideal choice for liquid biopsy. The droplets can be loaded with the CTCs, nucleic acids, or exosomes with less effort, and further manipulated to merge,[18] split,[19] incubate,[20] sort,[21] and mix[22] according to different assay needs. Moreover, droplets are often stable because of the usage of surfactants (usually existent in continuous phase), which prevents coalescences and improves the biocompatibility of the system, facilitating their application in biological fields.

Herein, the purpose of this review is to provide an overview of the recent development in droplet-based microfluidics for liquid biopsy. We will first introduce the field of droplet-based microfluidics and their key characteristics in bioanalysis. Second, the aspects of multiple biomarkers and their recent research in droplet-based microfluidics are summarized. The multiple biomarkers include CTCs, ctDNA, miRNA, and circulating tumor exosomes. Last, the challenges and future perspectives of droplet-based microfluidics for liquid biopsy are illustrated. The aim of this review article is to promote technological innovations in droplet-based microfluidics, enabling them to realize their great potential in liquid biopsy.

DROPLET-BASED MICROFLUIDICS

Droplet microfluidics technology is a diverse and practical tool to realize high-speed flow control of small-volume droplets. In addition to the advantages of reduced sample consumption, shortened reaction time, and improved sensitivity, it also has the potential for high-throughput, single-cell/-molecule/-exosome bioanalysis, which is expected to revolutionize liquid biopsy technology. Before we introduce the advantages of droplet-based microfluidics in liquid biopsy, it is essential to understand how droplets are generated and manipulated because these robust and high-throughput droplet generation methods and diverse droplet manipulation techniques are fundamental for high-throughput and high-sensitivity analysis.

Droplet generation and manipulation

Droplet generation requires two immiscible liquid phases, one of which is called the dispersed phase (droplet phase), and the other is called the continuous phase. Due to changes in flow shear or surface tension, the continuous phase can wrap the dispersed phase at geometric interfaces, resulting in continuous and stable droplet formation. Currently, there are two types of microfluidic-based droplet generation methods, including the active method and the passive method. The active method uses external forces, such as thermal energy, electric field, magnetic field, and so on to drive and control the generation of droplets. Although precise control of droplets can be achieved, the active method is relatively complex to operate and has high equipment requirements. The passive method does not require external force and mainly uses the geometric structure of the microchannel to generate droplets, which has the characteristics of fast speed, high throughput, and simple operation. Therefore, most studies are still based on the passive method to generate droplets. In the passive method, the droplet size and generation frequency of droplets can be further flexibly tuned by the geometric configuration of the chip channels, surface chemical properties, and fluid flow rates. Typically, there are three main device geometries that can generate droplets, including T-junctions, flow-focusing, and co-flowing (Figure 1).[23] Briefly, a T-junction involves the vertical introduction of a dispersed phase (usually the water phase) into a continuous phase (usually the oil or gas phase). When the oil/water interfacial tension is insufficient to maintain the shear force of the oil phase, the water phase ruptures to form droplets.[24] The T-junctions are characterized by simple microchannel structures, high production rate, and low coefficient of variation (CV) of monodisperse droplets as low as 2%,[25] but the generated droplets have insufficient stability and a narrow range of size control.[26] Flow-focusing is the most widely used geometry for droplet generation, which focuses on three flow paths in one channel.[27] The dispersed phase in the central channel can be vertically sheared by the continuous phase from two side channels to form droplets. Compared with the T-junction, the droplets generated by flow-focusing are more stable and have a wider range of size controllability. It is easier to generate droplets that are much smaller than the channel size. However, the flow-focusing method has high requirements on the symmetry of the chip, which needs high processing technology.[28] In co-flowing, the dispersed phase inside the tube flows in parallel with the continuous phase outside the tube, producing droplets at the tips of the internal channels.[29] The co-flowing method has greater advantages than the previous two methods in terms of stable droplet generation and size control, and the droplet generation rate can be controlled in a wide range, from hundreds of droplets per second to tens of thousands of droplets per second.[30] But its disadvantage is that the manufacturing steps are complicated, which are not as good as the T-junction and flow-focusing.[31]

In addition to pressure-based droplet formation in microchannels, it is now possible to generate droplets on flat surfaces using surface tension, which is known as digital microfluidics (DMF).[32,33] DMF is an emerging technology developed in recent years to enable sophisticated laboratory analysis by precise manipulation of microliter to nanoliter droplets simultaneously and individually through insulated electrodes. Droplets generated on the chip surface can be programmatically moved, dispensed, merged, and split according to a preset path. Generally, droplet actuation methods include electrowetting on dielectric (EWOD) actuation,[34] thermal capillary actuation,[35] light-induced actuation,[36] surface acoustic wave actuation,[37] and magnetic actuation.[38] Among them, EWOD actuation is the mainstream actuation technology for DMF because of its fast response, simple system, and high precision.[34] As compared to channel-based microfluidics, DMF shows numerous advantages, including noncontact and addressable droplet manipulation, flexible and versatile chip design, and nondestructive sample handling.

Whether in microchannels or on a flat surface, cells, nucleic acids, or exosomes dispersed in the aqueous phase can be easily loaded into droplets through both passive and active ways. For example, the simplest passive encapsulation method is to randomly obtain single-cell loaded droplets by using a dilute cell suspension as the aqueous phase. However, the number of cells wrapped in the droplets follows a Poisson distribution, which does not guarantee a controlled distribution.[39] To overcome the intrinsic Poisson statistic, deterministic methods such as inertial ordering[40] and hydrodynamic microvortices[41] have been used to passively modulate the cell encapsulation efficiency. Alternatively, the implementation of additional forces (e.g., electric field,[42] magnetic field,[43] optical laser,[44] and so on) can also actively regulate cell encapsulation by manipulating cell displacement and droplet generation rate. Single-cell encapsulation technology has been extensively reviewed and will not be further addressed here.[45–47] Furthermore, once droplets with

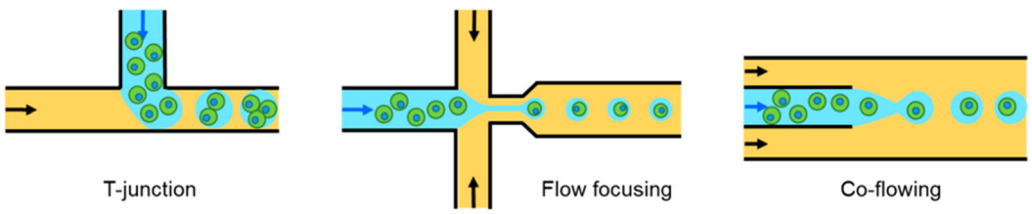

FIGURE 1 Three main microfluidic geometries for droplet production, including T-junctions, flow focus and co-flow. Reproduced under terms of the CC-BY license.[23] Copyright 2018, Choe et al.[23]

encapsulated targets are formed, they can be further merged, sorted, trapped, mixed, and split to execute desired protocols. As the basis for microfluidic chips, these droplet manipulations can be performed by passive hydrodynamic methods as well as active methods using external force (e.g., electric,[48] light,[49] thermal,[50] magnetic,[51] acoustic wave control,[52] etc.). Overall, microfluidics-based droplet generation and manipulation technologies have been developed to an advanced level over the past few decades. Droplets, as a fast-generated, high-throughput, small-volume, and self-contained reactor, have become promising experimental tools for a wide range of applications in biological research. Droplet-based microfluidics with high throughput, high biocompatibility, and simple operability has shown incomparable merits over other methods, especially for single-cell/single-molecule/single-exosome research. In the next section, we will review the strengths of droplet-based microfluidics in bioanalysis.

Advantages of droplet-based microfluidics in bioanalysis

First, the individual droplets generated in a rapid and robust manner can be regarded as single reactors, which provides a high-throughput parallel experimental platform for droplet-based microfluidics in bioanalysis. By adjusting the droplet generation rate and sample concentration, the droplet can be automatically produced and simultaneously loaded with liquid biopsy biomarkers at the single-cell level, enabling tens of thousands of independent sample segmentations. This rapid generation of a large number of independent reaction units is not available in other technologies and has strong practicality in high-throughput experiments. For example, high-throughput next-generation sequencing (NGS), which obtains long and accurate sequence reads by reading the fluorescent signals generated by millions of parallel short-strand DNA synthesis reactions, often cannot effectively determine the source of the measured DNA, limiting the assays at single-cell resolution. However, due to the heterogeneity of tumors, it is particularly important to study the genetic characteristics of cancer at the single-cell level. Single-cell analysis can not only reveal tumor heterogeneity but also detect drug resistance caused by clonal evolution, so as to develop targeted and individualized treatment plans for specific patients. Therefore, the emergence of droplet microfluidics provides a huge platform for bioanalysis at single-cell resolution. Droplet-based microfluidics can easily separate biomarkers down to the single-cell, single-molecule, or single-exosome level with high throughput. The most representative technique is digital droplet PCR (ddPCR), which has been widely used in the field of liquid biopsy. However, ddPCR can only detect known mutations and has low system integration, which needs to be optimized.

Second, droplets are microspheres of very small size, usually only nL or pL in volume. The ultra-small volume brings advantages to droplet microfluidics-based bioanalysis, for example, the biomarkers encapsulated in the droplet have concentrated sample concentration and efficient experimental reaction, which improves the detection sensitivity of low-abundance biomarkers. The microenvironment inside the droplet is controllable, without cross-contamination, which can reduce the background noise of other cells. Sample consumption in the droplet is greatly reduced, resulting in cost savings for bioanalysis.

Third, droplet-based microfluidics can be easily combined with various types of downstream analysis, such as DNA or RNA detection or immunostaining. That is to say, liquid biopsy biomarkers from clinical blood samples, such as CTCs and exosomes, can be enriched and detected upstream on microfluidic chips, and their genomic, transcriptomic and proteomic information can be analyzed downstream, providing a deeper understanding of cancer origin, development, and metastasis. For example, droplet-based microfluidics can link genotype with phenotype through downstream genetic analysis. Droplet-based microfluidics first enables efficient confinement of CTCs, molecules secreted by cells, or exosomes into droplets and facilitates sensitive phenotypic detection (e.g., proteins and metabolites) due to the enhanced local concentrations. Afterward, the captured cells or exosomes can be lysed and subjected to genetic analysis. In addition, droplet-based microfluidics is highly flexible and can be combined with various detection methods, such as imaging, immunological assay, PCR-based detection, and NGS-based detection, to provide convenience for bioanalysis. In conclusion, the comprehensive overview of cancer biomarkers with high flexibility indicates the broad application prospects of droplet-based microfluidics for bioanalysis.

DROPLET-BASED MICROFLUIDICS FOR LIQUID BIOPSY

Over the past decade, liquid biopsy as a noninvasive technique has revolutionized the field of clinical oncology. Liquid biopsy refers to the technology of detecting and diagnosing cancer through body fluids, such as blood, saliva, and urine. Liquid biopsy technologies mainly include the detection of cancer biomarkers, such as CTCs, ctDNA, miRNA, and circulating tumor exosomes. Table 1 shows a comparison of liquid biopsy components and their advantages and disadvantages. Among them, the size of CTC is 12–30 μm; the size of ctDNA is 50–150 bp; the size of miRNA is 21–25 nt; the size of exosome is 30–200 nm. The size and concentration of liquid biopsy components can affect the occupancy efficiency of single cells, single molecules, and single exosomes in droplets, as this is mainly determined by dilution factor and Poisson distribution. This section mainly reviews the current technology of liquid biopsy and the latest research on the application of droplet microfluidics in liquid biopsy.

CTCs

CTCs play a crucial role in liquid biopsy as they are alive and intact tumor cells shed from solid tumor tissue, entering peripheral blood at an early stage and containing all the biological information of the

TABLE 1 Liquid biopsy components comparison.

	Circulating tumor cells (CTCs)	Circulating tumor DNA (ctDNA)	MicroRNA (miRNA)	Circulating tumor exosomes
Size	12–30 μm	50–150 bp	21–25 nt	30–200 nm
Source	Tumor cells that shed from solid tumor tissues and released in blood circulation	Free DNA fragments released by tumor cell apoptosis and found in body fluidics, particularly plasma	Endogenous small noncoding RNAs found in circulation	Extracellular vesicles secreted by tumor cells and found in circulation
Stability	2 h half-life	15 min to 2 h half-life	Stable	Stable
Advantages	Alive; intact; whole information of tumor (e.g., morphology, DNA, RNA, protein); reflect tumor heterogeneity; cell culture capability for functional study	Relatively higher concentration compared with CTCs; easy to isolate and store; comprehensive genetic information (e.g., mutations, epigenetic changes, etc.); tumor-specific; real-time reflection of tumor status	Released by several structures (e.g., exosome); regulate gene expressions	High abundance; rich tumor information (e.g., DNA, RNA, protein); miRNA analysis
Disadvantages	Rarity; fragility; low abundance; difficult to isolate; heterogeneity	Low abundance; high background signal; short half-life period; lack of standard detection methods	Difficult to extract; less tumor-specific; lack of standard detection methods	Difficult to isolate; lack of standard isolation and detection methods
Detection technologies	CTC enumeration; CTC isolation; imaging; PCR-based methods; NGS-based methods; immunoassay	PCR-based methods; NGS-based methods	Northern blot; microarray; in situ hybridization; PCR-based methods; NGS-based methods	PCR-based methods; NGS-based methods; immunoassay
Applications	Early screen; diagnosis; prognosis; monitoring recurrence and metastasis	Early screen; diagnosis; prognosis; monitoring treatment response and recurrence; guide personalized medicine	Diagnosis; prognosis; therapeutic targets	Early screen; diagnosis prognosis; prediction of response to treatment

tumor. According to the famous "seed and soil hypothesis,"[53] CTCs are closely related to the progression, metastasis, and recurrence of cancer, thus CTC-based liquid biopsy has extremely high clinical application value. First, tumor cells can enter the bloodstream before forming visible solid tumors.[54] When imaging or serological tests cannot diagnose early tumors, CTC detection can be used as a favorable means for early cancer screening. Second, CTC-based liquid biopsy can be used for the prognosis of cancer patients undergoing chemotherapy and targeted therapy.[55] If the number of CTCs decreases, it means that the treatment method may be effective, and a precise treatment plan can be formulated in a more timely manner.[56] Last, CTC-based liquid biopsy is convenient, reproducible, and noninvasive for postoperative monitoring.[57] However, the number of CTCs is very small, usually only 1–10 CTCs in 1 mL of peripheral blood, while the number of blood cells is as high as hundreds of millions, which makes the detection of CTCs like searching for a needle in a haystack.

Conventional detection methods for CTC-based liquid biopsy

Currently, there are three main types of CTC isolation methods, including physical isolation, biological isolation, and the combination of physical and biological isolation.[58] In short, physical isolation (e.g., density gradient centrifugation, filtration, and microfluidic chips) is to enrich CTCs based on physical properties, such as size, density, deformability, and dielectric properties. This method has high throughput but low specificity. They can overcome the limitation of incomplete surface protein expression CTCs, but cannot distinguish cells with similar physical properties to CTCs, such as white blood cells (WBCs) that are similar in size to CTCs.[59] Biological isolation is to capture CTCs by relying on their surface biomarkers (positive enrichment method) or removing blood cells to leave tumor cells (negative enrichment method). Comparing the two methods, the negative enrichment method has higher capture efficiency, but the specificity is significantly lower. The positive enrichment method has higher specificity but fails to detect CTCs with low or no biomarker expression. The combination of physical and biological isolation has great advantages as it can compensate for the drawbacks of each technique. Typically, the combined technique starts by filtering out smaller red blood cells and platelets based on size, leaving behind relatively large WBCs and cancer cells. CTCs are then captured by bead-conjugated antibodies that recognize biomarkers expressed on the surface of CTCs and collect CTCs on the chip.[60] In this way, both the high throughput of physical isolation and the high specificity of biological isolation are maintained. The most commonly used biomarker on the surface of CTCs is epithelial cell adhesion molecule (EpCAM). Over the past few decades, immunomagnetic separation techniques based on specific biomarkers recognition of EpCAM have been extensively studied for CTC isolation. The most representative product of this technology is CellSearch®, which has been used clinically as the only FDA-approved CTC detection system.[61] However, The CellSearch® system cannot detect EpCAM low-/nonexpressing CTCs, which are considered to be an aggressive subset of CTCs undergoing epithelial-to-mesenchymal transition (EMT).[62]

These phenotypic or genetic differences in CTCs that arise during tumor growth are referred to as tumor heterogeneity. Tumor heterogeneity can exist among different tumor cells in the same tumor tissue, which is a major obstacle to tumor treatment. However, the traditional analysis method (e.g., PCR-based methods, NGS-based methods, Western blot analysis, etc.) analyzes the cell states through the average value of the population cells, which masks the differences between individual cells, resulting in the loss of a large amount of individual cell information. Therefore, single-cell-based high-throughput analysis is particularly important, which can reveal population heterogeneity, identify a small number of cell subpopulations, and discover the uniqueness of individual cells. Traditional methods for single-cell isolation include micromanipulation,[63] fluorescence-activated cell sorting,[64] and laser-capture microdissection.[65] Briefly, micromanipulation is an easy and inexpensive method using simple mouth pipetting or serial dilution, but it is performed manually with low throughput. Fluorescence-active cell sorting is an accurate and high-throughput modality involving cell-tyle-specific markers for biased samples. However, they are hampered by the need for large cell suspension and are not suitable for low-abundance cell subpopulations. Laser-capture microdissection is a manual method that can preserve information about the spatial location of the sampled cells within a tissue, but it suffers from the incomplete capture of the cytoplasm and low-throughput manner. Moreover, all of these methods require the transfer of isolated CTCs to tubes or microwells for lysis and subsequent genetic analysis, which are prone to contamination and result in low amplification efficiency. Therefore, there is still a need to develop accurate, pollution-free, and high-throughput methods at single-cell isolation for subsequent bioassays.

Droplet-based microfluidics for CTC-based liquid biopsy

Nowadays, the emergence of droplet microfluidics has provided great convenience for the isolation of single cells. As we described earlier, the droplet generation process based on microfluidics is simple, automatic, and high-throughput. Single cells can be easily encapsulated into droplets for independent and undisturbed rapid detection. Droplets not only have a controllable and cross-contamination-free microenvironment but are also produced at extremely high speeds with concentrated contents, paving the way for high-throughput and high-sensitivity liquid biopsy. Typically, the single-cell encapsulated droplets enable further downstream analysis, such as imaging, immunological assay, PCR-based detection, NGS-based detection, and so on. In this section, we will summarize the recent studies on the application of droplet-based microfluidics for genomic, transcriptomic, proteomic, and metabolic analysis of CTCs (Table 2).

TABLE 2 Droplet-based microfluidics for circulating tumor cells (CTCs)-based liquid biopsy.

Target	Droplet generator	CTC enrichment method	Detection method	Biomarker	Disease	Sensitivity	References
DNA	Bio-Rad QX100 digital droplet PCR (ddPCR) system	Size-based ScreenCell® filtration	ddPCR	KRAS mutations	Pancreatic cancer	/	[66]
	Bio-Rad QX 200 ddPCR system	Size-based CROSS chip	ddPCR	APC gene mutation	Colorectal cancer	LoD 0.065 ng/μL	[67]
	Serpentine channel; flow-focusing	WBC extracted Ficoll-Paque TM Plus	dPCR	EGFR L858R mutation	Non-small-cell lung cancer	91% efficiency at 4 cells/μL	[68]
	Acoustic droplet positioning	Anti-EpCAM antibody-modified GNP chip	Sanger sequencing	PIK3CA mutations	Breast cancer	/	[69]
	Digital microfluidics (DMF)	DMF with butterfly structure	Whole genome sequencing	Copy number variants (CNVs) and single nucleotide variants (SNVs) in K562 cells	K562 cancer cell	CNVs with 150 kb; SNVs with ADO of 5.2%	[70]
	Digital microfluidics (DMF)	DynarFace-Chip MB	Mass spectrometry	KRAS mutation	Colorectal cancer	Sensitivity 100% ($n = 5$)	[71]
RNA	Bio-Rad QX 200 ddPCR system	Size-based TRIZOL-LS	RT-ddPCR	PD-L1 and HPRT transcripts	Head and neck cancer	Sensitivity 0.64 copies/μL	[72]
	Bio-Rad QX 200 ddPCR system	Parsortix and ClearCell® FX1 system	RT-PCR; RT-ddPCR	19 transcripts (MCSP)	Melanoma	Sensitivity 53%	[73]
	Bio-Rad QX 200 ddPCR system	Anti-EpCAM-based NanoVelcro Chips	RT-ddPCR	EGFR T790M mutation	Non-small-cell lung cancer	Sensitivity 100% ($n = 27$ out of 46)	[74]
	Bio-Rad QX 200 ddPCR system	Spiral microfluidic device	RT-ddPCR	KRT-19, CEACAM5, AGR2, FDZ7, and LGR5	Colorectal cancer	KRT-19: LoD three cells/well; CEACAM5: LoD 12 cells/well	[75]
	Bio-Rad QX 200 ddPCR system	Anti-EpCAM-based IsoFlux CTCs enrichment kit	RT-ddPCR	AR-V7 transcripts	Prostate cancer	Sensitivity 30.8%	[76]
	DMF	Passive dispensing method	RNA-seq	Rare transcripts	/	Sensitivity 21.53%	[77]
	Double spiral channel chip; flow-focusing	Inertial focusing	Real-time PCR (RT-PCR); RNA-seq	VCAM-1 transcripts; mRNA signatures	Breast cancer	/	[78]

(Continues)

TABLE 2 (Continued)

Target	Droplet generator	CTC enrichment method	Detection method	Biomarker	Disease	Sensitivity	References
Proteins	Microstructure with reversely injected air	/	ELISA	TNF-α; interleukin (IL)-8; MCP-1; MIP-1b; IL-10	Brain tumor; oral squamous cell carcinoma	1 ng/mL	[79]
	Parallel step-emulsion	Size-based microvortices	FRET-based Matrix metalloproteinase (MMP)-cleavable substrate	MMPs	Prostate cancer	Microphage: 1.7- to 200-fold above baseline; cancer cell: 2.6-fold above baseline	[80]
	Microchamber	Size-based microchamber	FRET-based MMP-cleavable substrate; RNA-seq	MMP9; mRNA	Breast cancer	Detection range 0–100 nM MMP9	[81]
	Microchamber	Size-based trapping structure	Immunoassay	Granulocyte growth stimulating factor	Breast cancer	LoD 1.5 ng/mL	[82]
	Flow-focusing	/	Immunostaining	Cytokeratin (surface protein)	Colorectal cancer	/	[83]
	Flow-focusing	/	Fluorescent droplet cytometry (FDC)	CD44; CD104 (surface proteins)	Prostate cancer; breast cancer	/	[84]
	Paired microstructure with reversely injected air	Size-based trapping array	Immunoassay	Prostate-specific antigen (intracellular protein)	Prostate cancer	0.3 nM	[85]
Metabolism	T-junction	/	pH-sensitive dye: Snarf-5F	Lactate	Ovarian cancer; breast cancer; glioblastoma cancer; colorectal cancer	10 CTCs among 200,000 white blood cells (WBCs)	[86]
	T-junction	Anti-CD45 antibody-modified microbeads to remove WBCs	pH-sensitive dye: Snarf-5F	Lactate	Prostate cancer	11 hm-cells per 7.5 mL of blood	[87]
	Droplet-based printing technique	Anti-CD45 antibody-modified microbeads to remove WBCs	pH-sensitive dye: Snarf-5F, mass spectrometry	Lactate	Osteosarcoma; lung cancer	1 CTC among 100 health cells	[88]

Abbreviation: LoD, limit of detection.

Genomic analysis in CTCs

Genetic variations, including deletions, mutations, and rearrangements, are important factors for the unlimited proliferation, invasion, and metastasis of tumor cells. However, genetic mutations are often rare and hidden in a large excess of nonmutated backgrounds, making detection extremely challenging. Therefore, to realize the detection of mutations, not only the sensitivity of detection technology needs to be improved but also the signal-to-noise ratio needs to be increased by methods such as single-cell isolation. The emergence of droplet microfluidics perfectly solves this problem. Due to size constraints, individual cells encapsulated in droplets lead to increased local concentrations of target samples and decreased background signal of other cells/solutions, resulting in improved signal-to-noise ratio and detection sensitivity.

After CTC isolation, the genomes of CTCs can be extracted by cell lysis and further analyzed by PCR amplification, DNA sequencing, or mass spectrometry (MS). ddPCR has been widely used nowadays as a powerful downstream analysis method for the sensitive detection of known mutations at single-cell or single-molecule levels. For example, Kulemann et al. isolated CTCs using a commercially available filtration-based technique and used ddPCR to detect the KRAS mutations in pancreatic CTCs and corresponding primary tumors, showing a matching mutation rate of 58% and a discordant mutation rate of 42% have been achieved.[66] Ribeiro-Samy et al. fabricated a high-throughput CROSS chip (Figure 2a) to isolate CTCs from whole blood samples based on size and deformability with an efficiency of 70%.[67] Moreover, a 7.5 mL whole blood sample can be processed within 47 min using two CROSS chips at a purity of 7.2% without significant cell damage. The isolated CTCs were lysed and characterized by ddPCR to reveal APC gene mutations in colorectal cancer patients with a limit of detection (LoD) as low as 0.065 ng/μL. Although ddPCR is a very sensitive method, it requires complex equipment and operations. To simplify operation, Qiu et al. developed a more integrated single-cell mutation detection platform based on one-step operation, combining multiple processes, such as single-cell encapsulation, cell lysis, dPCR amplification, and signal detection (Figure 2b).[68] In their study, CTCs were negatively enriched by removing WBCs from human blood samples. Then, the single-cell/lysis buffer-loaded droplets were generated by flow-focusing at a rate of over 6000 per minute with an encapsulation rate of 10%. The generated droplets were stored in a glass chamber for dPCR amplification. As a result, point mutations in four cell types can be successfully distinguished using the developed platform under real-time fluorescence observation. Among them, the EFFR L858R mutation of H1975 cells was detected with an efficiency of over 91% at a low abundance of 4 cells/μL.

In addition to the PCR-based method, sequencing is another important method to detect unknown mutations. Wei et al. fabricated an acoustic droplet-induced enzyme-responsive platform for the capture and on-demand release of single CTCs for downstream cell-based genetic mutation detection.[69] CTCs were first captured by an anti-EpCAM antibody-modified GNP chip and encapsulated with calcium alginate hydrogel at the single-cell level, and then efficiently released on-demand using acoustic droplet positioning technology, and finally recovered by microcapillary for Sanger sequencing (Figure 2c). The capture efficiency of this GNP chip for MCF-7 cells spiked in peripheral blood mononuclear cells was 81.4%, but for MCF-7 cells spiked in the whole blood sample was 51.2%. As a proof-of-concept, the developed platform was used to capture CTCs from the clinical blood of breast cancer patients, showing detectable downstream mutations. Ruan et al. designed a highly integrated DMF-based single-cell processing platform for whole genome sequencing (WGS) of single cells.[70] As shown in Figure 2d, the single cells can be physically isolated into nanoliter-sized droplets by a butterfly trap with 100% capture efficiency and individually controlled by EWOD actuation for cell lysis, whole-genome amplification, and sequencing. Using this DMF-based platform, copy number variants (CNVs) and single nucleotide variants (SNVs) can be detected at the smallest bin of 150 kb and an allele dropout rate (ADO) of 5.2%, respectively. However, the sample analysis throughput of this platform is not high due to the limited number of single-cell capture structures. To address these challenges, they further developed another single-cell DMF MS analysis method for multiplex mutational profiling of single CTCs.[71] CTCs were first enriched by immunomagnetic beads-based isolation. Then, by combining wettability-based trapping and hydrodynamic adjustment of cell distribution, single CTCs can be encapsulated into droplets on a DMF chip (capture efficiency ~97%), followed by subsequent cell lysis and genome-wide amplification. After primer extension, the multiplex mutations in single CTCs can be simultaneously detected by MALDI-TOF MS based on inherent mass differences in different DNA sequences. This DMF-scMS platform was able to detect KRAS mutations of heterogeneous CTCs from five colorectal cancer patients with a detection rate of 100%.

Transcriptomic analysis in CTCs

CTC assessment at the RNA level has important prognostic power in clinical practice.[89] Currently, RNA detection techniques include RT-PCR and RNAseq.[90] Among them, RT-PCR is the gold standard method, which adds an additional reverse transcription step to PCR. When combined with droplets, RT-ddPCR showed improved sensitivity and precision over RT-PCR. For example, Strati et al. developed a duplex RT-ddPCR assay to simultaneously qualify PD-L1 and HPRT (a reference gene) transcripts in cDNA derived from CTCs isolated by size-dependent microfluidic chips.[72] The analytical sensitivity of this duplex RT-ddPCR assay was 0.64 copies/μL. The detection performance was further evaluated with CTCs obtained from NHSCC cancer patients, and the diagnostic sensitivity using this assay was 46.5%, which was higher than that using RT-qPCR (23.7%). Aya-Bonilla et al. isolated the melanoma CTCs by two commercial microfluidic devices, Parsortix and ClearCell© FX1 system, and combined immunostaining and transcript analysis (e.g., RT-PCR and RT-ddPCR) for heterogeneous CTC detection.[73] According to their comprehensive study, different methods have different CTC detection rates, the positive rate was 28% using immunostaining, 42% using RT-PCR assay for five genes, and 53% using ddPCR assay for 19 genes. Wang et al. utilized RT-ddPCR to quantify the T790M transcripts in pulmonary venous

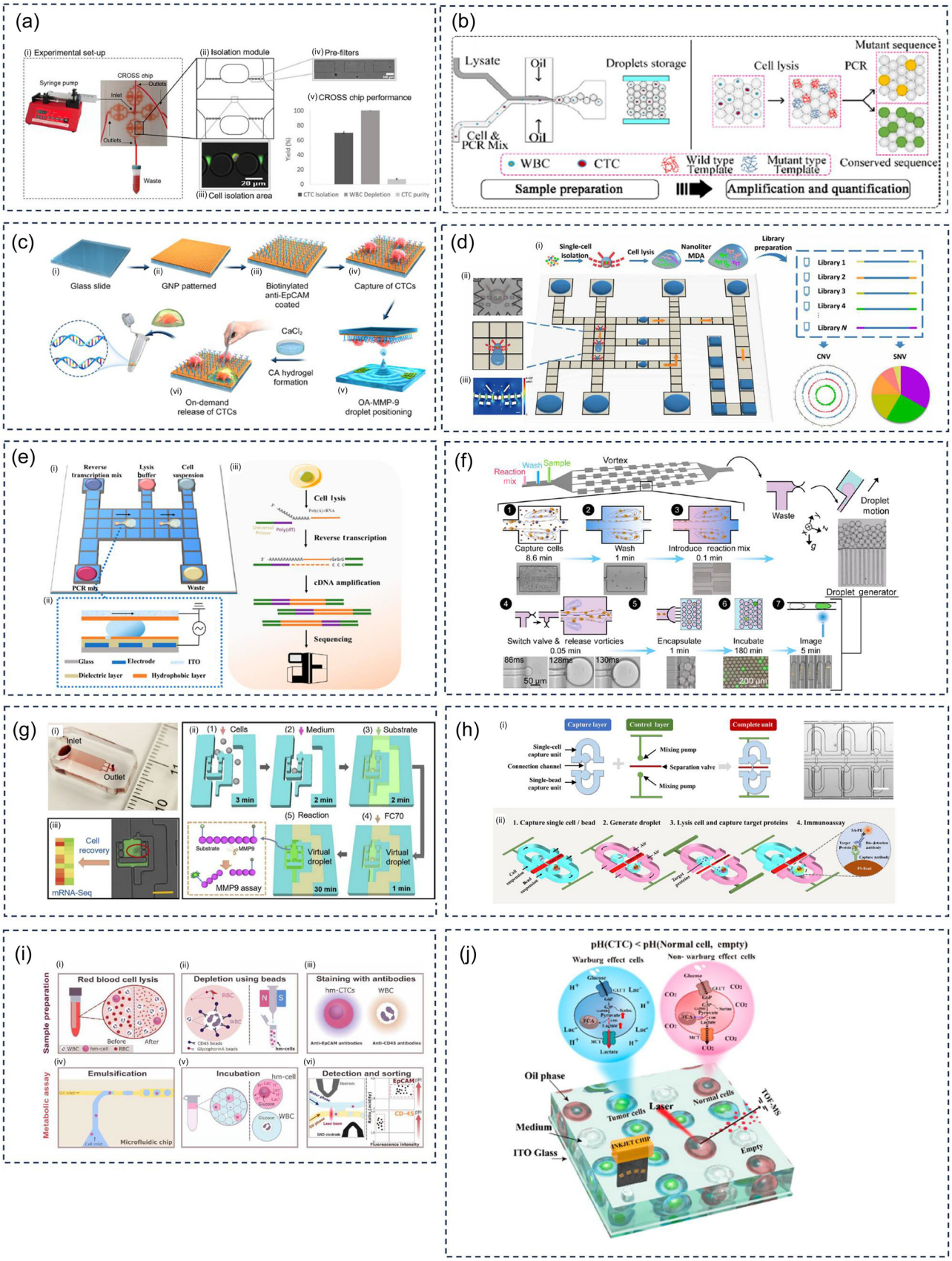

FIGURE 2 (See caption on next page).

CTCs enriched by a NanoVelcro chip through a biotinylated anti-EpCAM method (85.3 ± 5% capture efficiency).[74] By analysis of the paired peripheral arterial and venous blood samples from advanced non-small-cell lung cancer (NSCLC) patients, the developed Nano-Velcro CTC-digital assay has a 100% positive CTC-derived T790M transcript readout. Besides, Winter et al. describe a label-free spiral microfluidic device combined with ddPCR to measure the RNA expression in CTCs with and without RNA preamplification.[75] Ma et al. accurately screened the AR-V7 messenger RNA (mRNA) expression in EpCAM-based immunomagnetic beads-isolated prostate cancer cells through ddPCR.[76] In conclusion, RT-ddPCR is one of the most widely used methods for downstream molecular quantification of CTC-derived RNA transcripts.[91,92]

Except for ddPCR, droplet techniques can also be combined with RNA sequencing. Xu et al. reported a single-cell sequencing method based on DMF for simple, efficient, and low-cost mRNA detection.[77] In Figure 2e, discrete single-cell sub-droplets were first generated on a DMF chip relying on hydrophilic-hydrophobic microfeatures and surface tension. Then, the single-cell droplets were programmable-driven to merge with the lysis mix, RT mix, and PCR mix, respectively. The final droplets with nanoliter volume were achieved on the hydrophobic reaction interface for subsequent digital-RNA-sequencing. With extremely low reagent consumption, high sensitivity, and high precision, this DMF-based RNA-seq platform showed promising applications in rare gene transcript identification, cell type differentiation, and essential genes. However, the analytical throughput in this work was limited by the number of electrodes and needed to be improved. Besides, CTCs also exist in cluster forms (e.g., homotypic CTC clusters and heterotypic CTC clusters), which have a higher metastatic and extravasation potential than individual CTCs due to their physical properties (e.g., large size). Park et al. reported a double spiral channel-induced deterministic encapsulation method based on droplet microfluidic to mimic CTC-neutrophil clusters with pairing ratios ranging from 1:1 to 1:3.[78] The molecular features of CTC-neutrophil clusters were further characterized by different methods, for example, E-cadherin was observed by immunofluorescence staining, VCAM mRNA was determined by RT-PCR, and mRNA transcripts were examined by sequencing. They demonstrated that the CTC-neutrophil clusters have enhanced metastatic capacity. However, CTC clusters have an even rarer population (~0–5 CTC clusters per 10 mL of blood) compared with CTCs. Most studies are still focusing on single CTCs detection.

Proteomic analysis in CTCs

Previously, a large number of studies have focused on genome and transcriptome analysis at the single-cell level, but proteome analysis at single-cell resolution remains challenging. However, single-cell proteomics is just as important as genomics and transcriptomics. This is because proteins may be the functional result of gene expression or mutations, and they are heavily involved in tumor cell processes such as metastasis, differentiation, and proliferation.[93] The most commonly used high-throughput methods for protein biomarker analysis are flow cytometry and fluorescence-active cell sorting,[94] but they are not suitable for analysis of protein released from or secreted by cells. Besides, the main difficulties in single-cell proteome analysis are the low protein abundance and high background signal noise due to the extremely small number of CTCs. Unlike DNA or RNA detection, proteins cannot be directly amplified to improve the signal-to-noise ratio. In this case, high-throughput droplet-based microfluidic with the capability to confine single cells in ultra-small droplets can overcome the major limitation of flow cytometry in analyzing secreted protein from single cells and enhance the signal-to-noise ratio, thereby improving the performance in single-cell protein assays.

Usually, proteins from CTCs can be classified as secreted proteins, surface markers, and intracellular proteins. Among them, secreted proteins include cytokines, protease, and hormones, playing an essential role in mediating cell communications and regulating physiological functions, such as invasion and metastasis.[95,96] Khajvand et al. developed an integrated droplet microfluidic device that interfaced with spatially patterned antibody barcodes for multiplexed single-cell-secreted cytokine protein detection.[79] This platform can isolate single cells into 100 individual droplets (180 pL) with a capture efficiency of over 80%. After 4 h incubation, the presence of 4-plexed cytokines secreted by human macrophages, such as interleukin (IL)-8, MCP-1, MIP-1b, and Tumor necrosis factor alpha (TNF-α)/IL-10, can be sensitively detected using a sandwich ELISA at a detection range from 1 ng/mL to 10 pg/mL. Using this platform, protein secretions and secretion heterogeneity among tumor cells were successfully profiled in clinical samples from human

FIGURE 2 Droplet-based microfluidics for circulating tumor cell (CTC)-based liquid biopsy. (a) Experimental setup for CTC isolation using the CROSS chip. Reproduced under terms of the CC-BY license.[67] Copyright 2019, Ribeiro-Samy et al.[67] (b) The flow of point mutation analysis on the single-cell level. Reproduced with permission.[68] Copyright 2021, Elsevier. (c) Scheme illustration showing the multifunctional enzyme-responsive GNP chip for capture and on-demand release of single CTCs based on an acoustic droplet positioning technique. Reproduced with permission.[69] Copyright 2019, American Chemical Society. (d) Design and operation of the digital-WGS platform. Reproduced under terms of the CC-BY-NC license.[70] Copyright 2019, Ruan et al.[70] (e) Schematic illustration of digital-RNA-seq. Reproduced with permission.[77] Copyright 2020, American Chemical Society. (f) Size-based purification and encapsulation of cells (SPEC) followed by fluorescence analysis of enzyme secretion. Reproduced under terms of the PANS license.[80] Copyright 2018, Dhar et al.[80] (g) Overall working principle of the virtual-droplet-based SCPS platform. Reproduced with permission.[81] Copyright 2020, Elsevier. (h) Working principle of single-cell immunoassay based on the paired chip. Reproduced with permission.[85] Copyright 2022, American Chemical Society. (i) Experimental workflow for isolation of metabolically active cells from patient liquid biopsies using the MA-Chip. Reproduced under terms of the CC-BY-NC license.[87] Copyright 2020, Rivello et al.[87] (j) Illustration for metabolism-based capture and analysis of tumor cells in an open-space platform using a drop-on-demand inkjet printing technique and MALDI MS. Reproduced with permission.[88] Copyright 2021, American Chemical Society.

tumor cell lines and primary/metastatic cancer cells. Matrix metalloproteinases (MMPs) are secreted proteins that can break down extracellular matrix proteins (ECM) to support the metastasis of cancer. Currently, many studies have targeted the activity of MMP as a biomarker for the detection of CTCs. Dhar et al. developed a high-throughput and highly integrated device for the detection of CTC secretions at single-cell resolution, in which CTCs are first separated according to size in a microvortex and then mixed with a FRET-based MMP-cleavable peptide substrate, and finally encapsulated into individual droplets by parallel step emulsification (Figure 2f).[80] The entire process took only 15 min to extract and transfer rare cancer cells from 1 mL of whole blood into individual droplets with a volume of 2 nL. After incubation for 180 min, the proteases secreted by CTCs were detected in droplets by measuring the change of fluorescent signal. Results showed MMP activity ranging from 1.7- to 200-fold higher than baseline levels. When tested with clinical samples from metastatic castration-resistant prostate cancer patients, six out of seven patient samples had CTCs, 87% of which secreted MMP with an average 2.6-fold higher than WBCs, demonstrating great potential for companion diagnostics in liquid biopsy. However, cells suspended in droplets cannot simulate the physiological conditions of tumor cells well due to the lack of cell attachment mechanisms, thereby affecting the number of secreted proteins. Furthermore, it is difficult to implement the washing steps into the droplets. To address these problems, Li et al. fabricated a virtual-droplet array for dynamic single-cell protease detection (Figure 2g).[81] In their study, the physical volume (50 nm) was used as typical droplets to support cell adhesion, provide complete buffer exchange, and achieve sufficient sensitivity. Six hundred and forty-eight CTCs could be isolated and purified by a microfluidic chip with repeating trapping regions and then encapsulated with the FRET-MMP9-substrates by isolation oil to form virtual droplets within 1 min (capture efficiency 90%). The encapsulated single cell can secret and release MMP9 to specific cleave FRET substrates, leading to fluorescence recovery. The total detection process only took 40 min. The significant heterogeneity of CTCs in MMP9 production was demonstrated by dynamic tracking of MMP9 concentration and mRNA-Seq analysis. In addition, the adhered cells were found to produce more MMP9 than suspended cells. Similarly, Armbrecht et al. reported another microfluidic chamber integrated with a bead-based immunoassay for quantification of secreted granulocyte growth stimulating factor from 1152 individual CTCs with a LoD of 1.5 ng/mL.[82]

For surface marker detection, Cai et al. successfully encapsulated single CTCs into calcium alginate droplets and then solidified the droplets into disk-like hydrogel beads.[83] The encapsulated CTCs were further identified and visualized by immunofluorescence staining of surface cytokeratin protein. As a result, CTCs can be easily distinguished from WBCs under the microscope. In their study, droplets functioned as mechanically and chemically stable shells for CTC encapsulation, manipulation, and observation at the single-cell level. Yang et al. developed a fluorescent droplet cytometry (FDC) to profile multiple surface proteins (CD44 and CD104) at single-cell resolution using DNA-functionalized antibodies in droplets.[84] Antibody-DNA conjugates, live cancer cells, and reporter reagents can be encapsulated into single droplets and undergo enzymatic amplification reaction for the maximum fluorescent signals. By reading out the fluorescence signal, droplets containing PC3 cells and MCF-7 cells can be sensitively detected.

Intracellular protein can also be a biomarker for CTC detection. Liu et al. developed a droplet-based microfluidic platform for quantification of intracellular proteins in single cells.[85] In Figure 2h, single CTCs and single detection beads were respectively captured into separate chambers with a high efficiency of 75% when the two chambers were disconnected. The paired droplets (160 pL) containing single cells and single beads were generated by a reversed air input. After that, single cells were lysed and the released prostate-specific antigen (PSA) was recognized by detection beads when the two chambers were connected. By measuring the fluorescent signal change, the single-cell PSA can be sensitively detected by sandwich-based immunoassay with an LoD of 0.3 nM.

Metabolism in CTCs

Metabolism-based assays can largely circumvent the obstacle of tumor heterogeneities; this is because cancer cells exhibit a "Warburg effect" with high glycolytic capacity. High levels of glucose uptake and lactate secretion are two of the most prominent metabolic behaviors shared by all cancer cells. Ben et al. successfully detected CTCs based on the measurement of single-cell metabolism in droplet-based microfluidics.[86] As individual cells are encapsulated into monodisperse droplets, it increases the local concentration of lactate and allows the detection of changes in lactate concentration without further labeling. In their study, lung A549 cells were individually encapsulated into droplets (35 pL) and analyzed by laser-induced fluorescence under an inverted microscope. Through pH-sensitive-induced fluorescence change, A549 tumor cells can be detected as few as 10 in the background of 200,000 WBCs, with an average detection rate of 60%. Based on this work, Rivello et al. developed another metabolic assay chip (MA-Chip) based on single-cell extracellular pH measurement to detect and isolate highly metabolically active cells (hm-cells) in prostate cancer.[87] As shown in Figure 2i, CTCs were first negatively enriched by removing RBCs and WBCs. The isolated CTCs were subsequently encapsulated into droplets with pH-sensitive dye. After incubation at 37°C, the droplets were re-injected into the co-flow chip, read by a fluorescent microscope, and sorted by electrocoalescence. The sorted cells were further collected for mRNA-seq. The results showed that patients with upregulated prostate-related genes had 11 hm cells, compared with 3 hm cells in healthy donors. Patients with more than 5 hm cells had a poor survival rate. However, these designs all used end-point detection, which lost the function of real-time monitoring. In this case, Zhang et al. developed another open-space droplet array with fluid walls for real-time single CTC monitoring and analysis based on a similar metabolism approach and inkjet printing technology.[88] As shown in Figure 2j, the single-cell encapsulated droplet array was generated with high throughput and a high encapsulation rate of 43%

and stuck on the indium tin oxide glass substrate under the domination of interfacial tension. Then the single-cell containing droplet array was coated with homogeneous matrix spots by inkjet ejection technology. By combining both microscopic imaging and mass data, CTCs can be easily distinguished from normal cells.

ctDNA

ctDNA is tumor-derived free DNA fragments that are found in body fluidics, particularly plasma. ctDNA is also a small fraction of cfDNA in circulation. In liquid biopsy, ctDNA as an emerging biomarker can provide comprehensive tumor genetic information, such as single-nucleotide mutations, epigenetics changes, copy number variations, methylation, and so on. Currently, ctDNA is the most widely used liquid biopsy marker in clinical practice. Compared with other tumor markers, ctDNA has a good application prospect due to its relative ease of extraction and storage. Moreover, ctDNA detection is to detect tumor gene mutations from all cfDNA, which has high specificity. The applications of ctDNA detection in clinical practice mainly include an early screening of cancer, auxiliary diagnosis of cancer, monitoring of tumor metastasis, and prognosis evaluation. Due to the short half-life of ctDNA, usually only 15 min to 2 h, the composition of ctDNA can change with the development of tumors, presenting real-time tumor status. Therefore, ctDNA detection can also provide powerful assistance in the prediction of the efficiency of targeted drugs and the selection of treatment options, especially for patients with advanced tumors.

Conventional detection methods for ctDNA-based liquid biopsy

However, the proportion of ctDNA in cell-free DNA depends on the stage of cancer progression. In the early stages of cancer, the detection of ctDNA remains challenging due to the extremely low amount of ctDNA in the blood and the high amount of cell-free DNA in the background. The most commonly used approaches for ctDNA detection can be divided into two categories: PCR-based methods and NGS-based methods. PCR-based methods mainly include real-time quantitative PCR (qPCR),[97] digital PCR (dPCR),[98] BEAMing,[99] and amplification refractory mutation system (ARMS-PCR),[100] which have high sensitivity but low throughput and cannot detect unknown mutations. On the other hand, NGS-based methods, including tagged-amplicon deep sequencing (Tam-Seq)[101] and NGS,[102] have high throughput and broad genomic screening, which enable the detection of unknown mutations. However, due to the interference from cfDNA, cancer-specific mutations are difficult to detect unless sequenced at very high depths, which is very expensive. Therefore, the detection of ctDNA mainly relies on PCR-based technologies. In the next section, we will mainly explain the use of droplet-based PCR technology for ctDNA-based liquid biopsy.

ddPCR for ctDNA-based liquid biopsy

ddPCR is a newly emerged tool for the absolute quantification of nucleic acids that do not rely on a calibration curve.[103] Typically, ddPCR uses tens of thousands of droplets to dilute the reaction templates to achieve single-cell or single-molecule isolation, followed by PCR amplification. After that, the fluorescent signal of each droplet is read one by one by a droplet analyzer. Due to the compartmentalization of target DNA and data acquisition from the end-point reaction, ddPCR has shown higher sensitivity and specificity than qPCR[104] and NGS,[105] which allows the detection and absolute quantification of ultra-low-abundance sequences.[106]

In liquid biopsy, ddPCR is the main method for detecting ctDNA. It provides a sensitive and robust platform for quantifying low-abondance mutations, copy number variations, and methylation in ctDNA at the single-molecule level.[107–110] For example, Zmrzljak et al. used ddPCR to detect KRAS and BRAF mutations in isolated ctDNA from the serum of colorectal cancer patients at an average fractional abundance of 0.23%.[107] Ginkel et al. used ddPCR to detect TP53 mutations in low-level ctDNA from the plasma of head and neck cancer patients at an abundance ratio down to 0.01%.[108] Shoda et al. utilized ddPCR to detect HER2 copy number status in ctDNA collected from the plasma of gastric cancer patients with a sensitivity and specificity of 0.733 and 0.933, respectively.[111] Huang et al. employed ddPCR to detect hotspot mutations (TP53 and TERT) in ctDNA extracted from the plasma of hepatocellular carcinoma (HCC) patients with LoD of 0.01%.[112] Wang et al. utilized ddPCR to detect aberrant DNA methylation in cfDNA extracted from the plasma of HCC patients with sensitivity and specificity of 78.57% and 89.38%, respectively.[113]

To further improve the sensitivity of ddPCR, Ou et al. built an integrated comprehensive droplet digital detection (IC3D) system combing droplet partitioning, fluorescent multiplex PCR system, and 3D droplet counting technology for ultra-rare ctDNA detection from large volumes of biological samples.[114] In their system, target-containing picoliter-sized droplets were first generated by flow-focusing principle and amplified off-chip by thermocycling, and then collected in bulk for high-throughput 3D particle analysis. Owing to the rapid 3D fluorescent scanning, the IC3D ddPCR system showed ultra-sensitive detection of oncogenic KRAS G12D mutant alleles with a sensitivity of 0.00125%–0.005% and a false-positive rate of 0%, which was 50 to 1000x higher than that of commercial liquid biopsy ddPCR (Bio-Rad) and qPCR platforms, respectively. Additionally, the IC3D System can analyze a 1 mL blood sample in just a few minutes, compared to 2 h for the Bio-Rad ddPCR System. When detecting the cancer cells spiked in whole blood, the IC3D platform also demonstrated direct cancer cell detection capability using their genetic markers without a pre-enrichment step. However, this ddPCR is still highly dependent on specialized instruments. To address this problem, Pan et al. used a microfluidic impact printer (MIP) to fabricate a disposable microfluidic chip combined with a ddPCR system for accurate DNA detection (Figure 3a).[115] By using a scanning fluorescence microscope, the genes dispersed in the planar droplet arrays can be sensitively and selectively

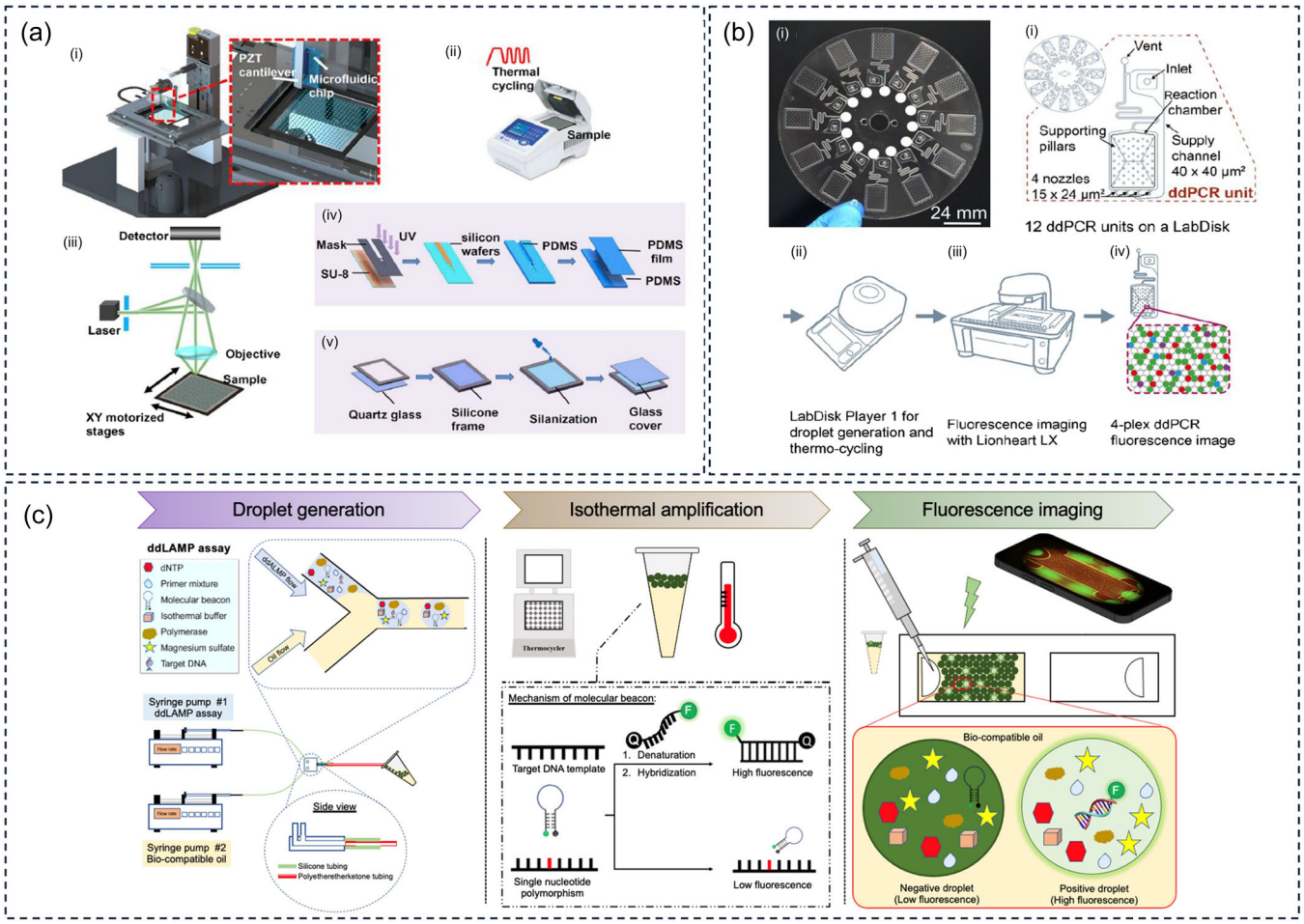

FIGURE 3 Droplet-based microfluidics for circulating tumor DNA (ctDNA)-based liquid biopsy. (a) Schematic diagrams of a microfluidic printing droplet digital polymerase chain reaction setup (MIP-ddPCR setup). Reproduced with permission.[115] Copyright 2019, Elsevier. (b) Workflow for an automated four-plex digital droplet PCR (ddPCR) in a LabDisk. Reproduced under terms of the CC-BY license.[116] Copyright 2021, Schlenker et al.[116] (c) Methods for performing ddLAMP using an assay featuring MB as a sequence-specific probe for single nucleotide polymorphisms (SNP) detection. Reproduced with permission.[117] Copyright 2022, American Chemical Society.

detected in the range of 0.464–464 copies/μL. To prove its practicability, this MIP-enabled PCR technology was applied to detect the expression of the p53 gene in colon cancer tissue, and the results were consistent with those of using conventional real-time PCR. Different from other complicated ddPCR systems, this low-cost microfluidic chip has no requirement for external pumps or valves, making it ideal for on-site assays. To achieve multiplex detection, Schlenker et al., developed a four-plex ddPCR in a LabDisk with 12 identical units for the detection of three cancer-associated KRAS point mutations (G12D, G12V, and G12A).[116] As shown in Figure 3b, each ddPCR unit consisted of an inlet, a venting structure, a supply channel, a reaction chamber with supporting pillars, and four nozzles for droplet generation by centrifugal step emulsification. The droplets can be generated and amplified in LabDisk Player 1 and analyzed by fluorescence imaging with an automated fluorescence microscope in all three-color channels. This four-plex ddPCR showed the lowest dilution points of 3.5 mutant DNA copies/μL for the KRAS G12V and G12A sequence, and 35 mutant DNA copies/μL for the G12D sequence.

Apart from ctDNA, CTCs and exosomes are considered alternative sources of nucleic acids in liquid biopsy. For CTCs/exosome-based liquid biopsy, ddPCR is often employed as a downstream analysis for nucleic acid characterization after CTCs/exosome enrichments.[118,119] Gao et al. detected EGFR mutations in lung cancer cells isolated with combined immunomagnetic beads (EpCAM, MUC1, and EGFR) through ddPCR.[118] Li et al. isolated exosomes from serum samples of HCC patients using a commercially available kit and detected tumor-specific TP53 mutations in exosomal DNA by ddPCR.[120] In some studies, ctDNA in liquid biopsy has been reported to be more sensitive than DNA extracted from CTCs, but not as sensitive as DNA derived from exosomes. According to Guibert et al., they used dd PCR to detect KRAS mutations in cfDNA extracted from plasma and in DNA extracted from CTCs during the treatment of KRAS-mutated lung adenocarcinoma.[121] The sensitivity was 78% for cfDNA and 34% for DNA extracted from CTCs. In contrast, Allenson et al. used ddPCR to detect KRAS mutations in cfDNA extracted from plasma and in DNA derived from exosomes at the

early stage of pancreatic ductal adenocarcinoma.[122] The sensitivities of cfDNA from locally, locally advanced, and metastatic PDAC patients were 45.5%, 30.8%, and 57.9%, respectively, while those of exoDNA were 66.7%, 80%, and 85%, respectively.

Although these ddPCR-based nucleic acid detections have extremely high accuracy and precision, most of the current ddPCR systems involve an off-chip PCR amplification step, which complicates the operation and brings inevitable contamination. Over the past decade, several commercially available ddPCR approaches have been developed, including Bio-Rad Qx200™, Biomark™ HD, QuantStudio™ 3D, Naica, RainDrop plus™, Droplet Digital™ PCR, and CONSTELLAATION®DPCR. However, the clinical application of ddPCR still faces several major challenges, including cost reduction, instrument platform integration, and experimental operation simplification.

Droplet isothermal amplification for ctDNA detection

As ddPCR may require complex instrumentation and thermal cycling procedures, it may not be available in field conditions or resource-poor settings. Therefore, thermal amplification techniques, such as loop-mediated isothermal amplification (LAMP), have been investigated to simplify the thermal cycling process and these techniques are expected to be alternatives to PCR. Typically, LAMP relies on the usage of multiple primers and a polymerase to amplify the target gene sequence at a constant temperature of 60–65°C for 30–60 min. The amplification efficiency of LAMP is 10–1000 times higher than that of PCR.[123] Tan et al. investigated a Hpall-edited absolute droplet LAMP method for accurate detection of the methylation level in ctDNA.[124] The DNA templates and sample solution were encapsulated into droplets by a Y-shape flow-focusing droplet-based microfluidic device. Then the generated droplets were collected for LAMP reaction and detection. As a result, hypermethylated DAPK1 can be detected at as low as 1% methylation level with an LoD of 5 aM (ca. 3 copies/μL). Twenty tissue samples from healthy individuals and cervical cancer patients were collected to evaluate the practical application of this droplet LAMP approach. The results showed that the methylation level of DAPK1 was significantly increased in cervical cancer patients and linearly correlated with cervical cancer progression ($R^2 = 0.9829$, $p < 0.0001$). Although this platform uses tissue samples, there is a great opportunity to apply it to liquid biopsy. Hsieh et al. developed a ddLAMP assay integrated with molecular beacon (MB) assay, 3D printed droplet generation, and smartphone imaging for sequence-specific DNA detection.[117] As shown in Figure 3c, the ddLAMP assay encapsulated droplets are generated by a 3D-printed millifluidic droplet generator with a Y-junction structure. LAMP assay was performed with the aid of MB as a sequence-specific fluorescent probe. The increased fluorescence intensity was measured by a fluorescence microscope with an EGFP filter and/or with a smartphone-based imaging system. This MB-ddLAMP approach can detect single nucleotide polymorphisms (SNP) with a sensitivity of 1% and an LoD of 4.39 copies/μL when using the OmpW DNA template. All the droplet-based microfluidics for ctDNA-based liquid biopsy are summarized in Table 3.

miRNA

miRNA is a type of endogenous noncoding RNA with regulatory functions, which can regulate gene expression at the levels of transcription, RNA processing, and translation. Typically, the mechanisms by which miRNAs regulate gene expression involve binding to mRNA, leading to miRNA destabilization or translational silencing. Numerous studies have shown that the abnormally expressed miRNAs can regulate gene expression in a targeted manner, thereby affecting the occurrence and development of tumors.[125] For example, some highly expressed miRNAs in tumor cells can make tumor cells proliferate indefinitely. According to He et al., miRNA from the mir-17 cluster was highly expressed in B-cell lymphoma samples, which can elevate c-Myc expression to accelerate cell proliferation and growth.[126] On the other hand, some abnormally expressed miRNAs are closely related to the invasion and metastasis of tumor cells. Ma et al. reported that the overexpress of miRNA-10b in nonmetastatic breast tumor cells can inhibit the encoding of homeobox D10, resulting in increased expression of the pro-metastatic gene, RHOC, thus leading to tumor invasion and metastasis.[127] Zhu et al. found that the suppression of miRNA-21 in metastatic breast cancer MDA-MB-231 cells could upregulate tumor suppressor genes, such as programmed cell death 4 and maspin, further reducing cancer invasion.[128] Therefore, the detection of miRNA expression levels in serum/plasma can be used to differentiate cancer patients from healthy individuals. The concentration of miRNAs varied by cancer clinical grades and subtypes. For instance, the expression of serum miRNA-21 in patients with early diffuse large B-cell lymphoma was higher than that in stages III and IV, and the differentiation of patients in different subgroups was obvious.[129] But it is worth mentioning that not all different miRNA expressions are associated with cancer, and chronic inflammation may be another reason,[130] so it is important to identify specific aberrantly expressed miRNAs in different cancer types. In addition, miRNAs are remarkably stable and abundant in serum, plasma, or exosomes. Based on these unique characteristics, miRNAs can not only serve as potential biomarkers in liquid biopsy for cancer diagnosis and prognosis but also are expected to be therapeutic targets for cancer treatment.

Conventional detection methods for miRNA-based liquid biopsy

Conventional methods for detecting miRNAs include northern blot,[131] in situ hybridization,[132] and microarrays,[133] which are less sensitive and cost-effective. Over the past decade, PCR-based methods such as RT-PCR, qPCR, and dPCR have been widely used for miRNA detection with high sensitivity, wider range, and greater

TABLE 3 Droplet-based microfluidics for circulating tumor DNA (ctDNA)-based liquid biopsy.

Targets	Detection method	Droplet generator	Biomarker	Disease	Sensitivity	References
ctDNA	Digital droplet PCR (ddPCR)	Bio-Rad QX 200 ddPCR system	KRAS and BRAF mutations	Colorectal cancer	0.23%	[107]
		Bio-Rad QX 200 ddPCR system	TP53 point mutations	Head and neck cancer	0.01%	[108]
		Bio-Rad QX 200 ddPCR system	HER2 copy number	Gastric cancer	0.733	[111]
		Bio-Rad QX 200 ddPCR system	TP53 and TERT point mutations	Hepatocellular carcinoma	0.01%	[112]
		Bio-Rad QX 100 ddPCR system	Methylation	Hepatocellular carcinoma	78.57%	[113]
	Integrated comprehensive droplet digital detection (IC3D)	Flow-focusing	KRAS G12D	Colorectal cancer	0.00125%–0.005%	[114]
	ddPCR	Microfluidic printer	P53 genes	Colorectal cancer	0.464 copies/μL	[115]
	ddPCR	Centrifugal step emulsification	KRAS point mutations G12D, G12V and G12	Colorectal cancer	3.5–35 mutant DNA copies in 15,000 wild-type DNA copies	[116]
	Hpall-edited droplet loop-mediated isothermal amplification (LAMP)	Y-shape flow-focusing	DAPK1 methylation level	Cervical cancer	Sensitivity 1% LoD 5 aM	[124]
	ddLAMP	Y-junction	Single nucleotide polymorphisms (SNP)	/	Sensitivity 1% LoD 4.39 copies/μL	[117]

Abbreviation: LoD, limit of detection.

precision,[134,135] but they are relatively time-consuming and labor-intensive. Other nucleic acid amplification-based methods, such as rolling circle amplification (RCA)[136] and LAMP,[137] have also been investigated for miRNA detection. Although isothermal amplification technology is relatively simple and efficient, the sensitivity of isothermal amplification technology is not enough to replace PCR in practical use. With the development of nanotechnology, various nanomaterials (e.g., gold nanoparticles,[138] quantum dots,[139] and 2D nanomaterials[140]) have also been employed as versatile platforms for miRNA detection. Due to their high surface-to-volume ratio and excellent optical and electrical properties, nanomaterials can be a powerful tool to boost the sensitivity of miRNA detection. However, miRNA-based liquid biopsy is still in its early stages, so it is important to develop more efficient and practical methods. In the next section, we will review recent studies on miRNA-based liquid biopsy by droplet-based microfluidics.

Droplet-based microfluidics for miRNA-based liquid biopsy

For circulating miRNA detection, total RNA was usually extracted from serum or plasma and reverse-transcribed to obtain the cDNA template of miRNA. ddPCR as a promising technique can enumerate miRNA copy numbers at limiting dilution without bias. Wang et al. developed a ddPCR system based on an oil-saturated PDMS microfluidic chip for the quantification of lung cancer-related miRNA from 10 to 10^5 copies/μL.[141] Cirillo et al. measured overexpressed circulating miR320a levels in serum samples of ovarian cancer patients using ddPCR, which was distinct from healthy controls with $p = 0.037$.[142] Jiao et al. successfully detected miRNA-21 levels in MCF-10A cells and MDA-MB-231 cells using a 3D-printed microfluidic chip-integrated droplet PCR method.[143] In their study, the 3D-printed chip consisted of a droplet generation part and a reaction part. T-junction geometry created droplets that went through dynamic thermal cycling through two heating blocks, dramatically simplifying the PCR process. However, droplet-based PCR always requires enzymes and thermal cycling for amplification, which is easily affected by the environment inside the droplets. Chen et al. developed an enzyme-free digital droplet auto-catalytic hairpin assemble (ddaCHA) system for miRNA quantification at single-molecule resolution.[144] The picoliter-sized droplets co-encapsulated with hairpin substrate and miRNAs were generated by a co-flow-focusing junction (Figure 4a). This ddaCHA system can realize amplification at room temperature without heating operations. By measuring the hybridization-induced fluorescence change, miRNAs can be sensitively detected with a linear range from 1 pM to 10 nM and an LoD of 0.34 pM. This LoD could be reduced to 10 fM when using high-throughput tracking computation of fluorescent droplets with a self-developed Python script. Similarly, Gines et al. optimized the amplification process of ddPCR by a DNA-based molecular program, which can specifically catalyze targeted miRNA with sensitivity down to femtomolar concentration.[145] Figure 4b shows a typical procedure for ddPCR mixed with molecular procedures. After amplification in droplets, the fluorescent positive droplets are calculated absolutely using Poisson statistics.

Analysis of intracellular miRNA at single-cell resolution is also crucial for revealing cell heterogeneity. Li et al. established a droplet microfluidic for multiple miRNA profiling in a single cell.[147] The integrated microfluidic chip allowed high-throughput cell isolation, cell lysis, the isothermal amplification of miRNA, and fluorescence detection in one chip. The single cells and reaction buffer can be encapsulated into droplets by the flow-focusing method with a capture efficiency of 80–150 s^{-1}. miRNA let-7a and miRNA 20 were quantified simultaneously by a laser-induced fluorescence method with a detection limit as low as 100 fM. When adapted in the clinic for liquid biopsy, the developed droplet microfluidic can successfully detect the expression of miRNA with a calculated amount of 0.66–7.66 zmol per CTCs. However, this method includes cell lysis inside the droplets, which could induce high background noise. The lysis buffer also harms amplification efficiency. To overcome these shortcomings, our group developed a digital droplet microfluidic flow cytometry based on biofunctionalized 2D MOF nanosensors (Nano-DMFC) for high-throughput detection of dual characteristic miRNAs of miRNA-21 (miR-21) and miRNA-10a (miR-10a) in single breast cancer cells (Figure 4c).[146] In our study, single cells were passively loaded into droplets through flow-focusing methods with an encapsulation efficiency of around 30%. Two-dimensional MOF nanomaterial-based FRET nanosensors were precisely injected into single-cell loaded droplets via an electro-based microinjector. After cellular endocytosis, the MOF-based FRET nanosensor will be turned on due to the recognition between dye-labeled nanoprobes on MOF nanosheets and the targeted miRNAs inside single cancer cells. By measuring the fluorescence recovery, dual miRNAs can be sensitively and specifically detected in a high-throughput manner at single-cell resolution in 10 mixed positive MCF-7 cells out of 10,000 normal cells in a bio-mimic serum sample. This Nano-DMFC platform demonstrated comparable accuracy (92.2%) to the current gold standard approach of EpCAM-based flow cytometry (92.5%) when analyzed with blood samples spiked with cancer cells. Notably, the precise implementation of nanomaterial-based FRET biosensors into droplets not only improved the sensitivity and specificity of detection but also avoided the lysis process of CTCs and the tedious isolation steps of miRNAs, preventing the interference signal from cell-free miRNA and the bulk solution. Another similar work was reported by Sun et al., who used gold nanoparticle-based SERS/FRET nanosensors encapsulated into microfluidic droplets for SERS-fluorescence dual-response detection of miR-21 within single intact CTCs.[148] Based on the sensing mechanism of SERS turn-on and fluorescence turn-off, the miR-21 can be sensitively detected with the lowest detectable concentration of 10 fM. Examples of droplet-based microfluidics for miRNA-based liquid biopsy are presented in Table 4.

Circulating tumor exosomes

Circulating tumor exosomes are small and stable extracellular vesicles (30–200 nm) secreted by tumor cells and found in

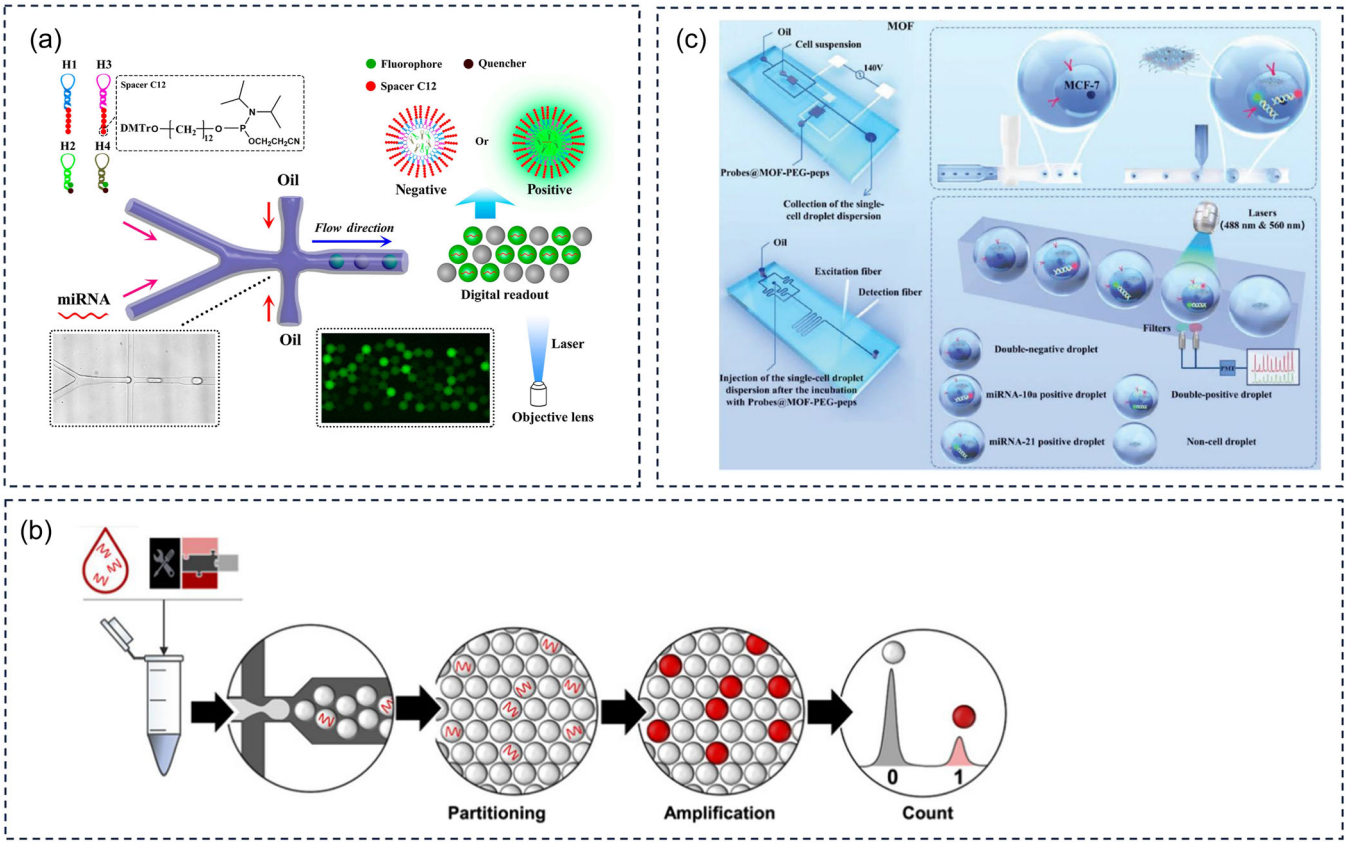

FIGURE 4 Droplet-based microfluidics for miRNA-based liquid biopsy. (a) Design and workflow of the droplet-generating co-flow focusing (CFF) junction and single-molecule microRNA (miRNA) imaging based on digital droplets. Reproduced with permission.[144] Copyright 2022, American Chemical Society. (b) Digital detection of microRNAs. Reproduced under terms of the CC-BY-NC license.[145] Copyright 2021, Schlenker et al.[145] (c) The scheme of Nano-DMFC with a sample processing unit and a miRNA detection unit to realize the encapsulation of single cell in the droplet, microinjection of nanosensors, and fluorescence detection of multi-miRNA in single circulating tumor cell (CTC) cell. Reproduced with permission.[146] Copyright 2022, Wiley.

TABLE 4 Droplet-based microfluidics for microRNA (miRNA)-based liquid biopsy.

Targets	Droplet generator	Detection method	Biomarker	Disease	Sensitivity	References
miRNA	Flow-focusing	Digital droplet PCR (ddPCR)	miR-126	Lung cancer	10 copies/μL	[141]
	Bio-Rad QX 200 ddPCR system	ddPCR	miR320a	Ovarian cancer	10.9 copies/μL $R = 0.95$	[142]
	T-junction	Droplet-based PCR	miR-21	Breast cancer	/	[143]
	Co-flow-focusing	ddaCHA	/	/	0.34 pM	[144]
	Flow-focusing	ddPCR; molecular programming	miRNA Let-7a	/	2.7 fM	[145]
	Flow-focusing	Isothermal amplification	miRNA let-7a; miRNA 20	Pancreatic cancer	100 fM	[147]
	Flow-focusing	MOF-based FRET nanosensors	miR-21; miRNA-10a	Breast cancer	miR-21: 0.54 nM; miR-10a: 0.94 nM 10 MCF-7 cells spiked in 10^5 normal cells	[146]
	Flow-focusing	AuNP-based SERS/FRET nanosensors	miR-21	Breast cancer	10 fM	[148]

circulation, carrying a variety of tumor-derived biological components, such as DNA, RNA, and proteins. As a carrier of intercellular communication, circulating tumor exosomes are usually produced and released in large quantities, and participate in multiple processes such as tumor growth, invasion, and metastasis.[149–151] It has been reported that tumor-derived exosomes can promote the formation of the tumor microenvironment through tumor-stromal cell interactions, which, in turn, leads to tumor progression.[152] Compared with CTCs and ctDNA, circulating exosomes with high abundance are widely distributed in various body fluids, such as serum, plasma, urine, saliva, and so on. The concentration of exosomes is usually over 10^9 vesicles/mL in blood, which is tremendously higher than that of CTCs and ctDNA.[153] Wang et al. compared the number of exosomes extracted from serum and plasma.[154] They found that serum not only had higher concentrations of exosomes but also higher chance of interference by wild-type DNA interference during detection. Plasma may contain lower amounts of exosomes but has higher sensitivity for mutation detection. The expression level of exosomes at the early stage of cancer patients is much higher than that of CTCs and tumor antigens,[155] indicating that exosomes can sensitively reflect the tumor status. Furthermore, exosomes can effectively protect nucleic acid substances in the form of secretory vesicles, which overcomes the problem of easy degradation of ctDNA and RNA in blood. Overall, circulating tumor exosomes are rich in information, large in quantity, easy to obtain from body fluid samples, expressed tumor biomarkers in early stages, and highly stable, which makes them ideal biomarkers for early diagnosis, prognosis, and monitoring of cancer.

Conventional detection methods for circulating tumor exosomes-based liquid biopsy

Current exosome isolation techniques mainly include ultracentrifugation-based methods,[156,157] size-based methods,[158] precipitation,[156,158] and immunoaffinity capture.[159] In short, ultracentrifugation-based methods such as differential centrifugation are the gold standard for isolating exosomes, which involve successive increases in centrifugal force to purify exosomes according to size and density.[157] However, it is time-consuming, has low yield, and may cause exosome damage due to high centrifugal force. Size-based methods, such as ultrafiltration, use nanomembranes to cut off large particles with a defined molecular weight or pore size.[158] This is a quick and straightforward approach but may cause exosome damage. The precipitation method is a method of separating exosomes through a hydrophilic polymer using a precipitation reagent, which is convenient to use but has low purity.[160] Commercially available isolation kits include Total Exosome Isolation Reagent (Invitrogen) and Exo Quick (Systems Biosciences). Immunoaffinity capture is a method of isolation using exosome-specific surface proteins and magnetic bead technology, which has high specificity but is limited by the high heterogeneity of exosomes.[159] Moreover, separation from magnetic beads will cause irreversible damage to exosomes. It can be seen that the main difficulties to be overcome in the traditional method of isolating exosomes are the long process time, low purity, and high risk of exosome damage. The analysis of exosomes is mainly based on gene and protein cargos.[161,162] Common detection methods include PCR-based methods, NGS-based methods, immunological assays, ELISA, and so on. Although exosome detection is still in its infancy, exosomes have great potential in the field of liquid biopsy due to their unsurpassed advantages. In the next section, we will review the latest research on droplet-based microfluidics for exosome-based liquid biopsy (Table 5).

Droplet-based microfluidics for circulating tumor exosomes-based liquid biopsy

Exosomes mainly contain DNA, mRNA, miRNA, and proteins. For exosomal nucleic acid detection, ddPCR is widely used as a powerful downstream analysis tool. For example, Li et al. isolated exosomes from serum by a commercially available ExoQuick™ kit, and used ddPCR to detect tumor-specific TP53 mutations in exosomal DNA of HCC patients with a sensitivity of 80%.[120] Bernard et al. isolated exosomes by ultracentrifugation and demonstrated that ddPCR using exosomal DNA was more sensitive than ddPCR using ctDNA for the detection of KRAS mutations in clinical samples of pancreatic cancer patients.[163] The detection rate of ddPCR using exosomal DNA and ctDNA was 61% and 53% in metastatic disease and 38% and 34% in localized disease, respectively. The results could be explained by the abundance of exosomes in the clinical sample. Wang et al. investigated cancer-related mutation in exosomal DNA from clinical samples of pancreatic cancer patients by ddPCR.[154] They found no significant difference in exosomal yield and KRAS mutation allele fraction between exosomes isolated from the membrane-based method or ultracentrifugation method. The sensitivity of ddPCR can be improved by inducing DNA denaturation before droplet formation. In pancreatic cancer patients, the total KRAS mutations in exosomal DNA detected by denaturation-enhanced ddPCR were 1.65-fold higher than standard ddPCR. In addition to traditional isolation methods, an acoustofluidic method combined with ddPCR was developed for salivary exosome isolation and exosomal DNA detection.[164] According to Wang et al., exosomes in saliva from HPV-OPC patients can be separated according to size based on the acoustic radiation force (F_r) induced by the SAW field and the drag force (F_d) induced by the fluid. The isolated exosomes were further analyzed by ddPCR, showing an LoD of 47.8 copies of HPV16 DNA/mL. The exosomes isolated by the developed acoustofluidic platform were of high purity and yield, which could benefit the downstream droplet-based genetic analysis.

In addition to DNA, exosomes also contain RNA, such as lncRNA, miRNA, and mRNA, which can be applied to the diagnosis of various tumors. For example, Wang et al. used ddPCR to detect urinary exosomal miR-29a with an LoD of less than 50 copies/μL, which was 120 times more sensitive than qPCR.[165] Shen et al. quantified the copy numbers of lncRNA in EpCAM-positive exosomes isolated with magnetic beads by ddPCR.[166] Cui et al. developed a flow-focusing and co-flowing combined microfluidic device to achieve one-step RT-PCR for the detection of miRNA in single exosomes.[167] In their

TABLE 5 Droplet-based microfluidics for circulating tumor exosomes-based liquid biopsy.

Targets	Droplet generator	Exosome source	Exosome isolation method	Detection method	Biomarker	Disease	Sensitivity	Reference
Exosome DNA	Bio-Rad QX 200 digital droplet PCR (ddPCR) system	Serum	ExoQuick™ kit	ddPCR	TP53 mutation	Hepatocellular carcinoma (HCC)	80%	[120]
	Bio-Rad QX 200 ddPCR system	Plasma	Ultracentrifugation	ddPCR	KRAS mutation	Pancreatic cancer	61%	[163]
	Bio-Rad QX 200 ddPCR system	Serum; plasma	Membrane-based method (ExoEasy Maxi Kit); ultracentrifugation	ddPCR; denaturation-enhanced ddPCR	KRAS mutation	Pancreatic cancer	42.9% (serum) 50% (plasma)	[154]
	QX100 Droplet Digital	Saliva	Size-based acoustofluidic device	ddPCR	HPV16	Oropharyngeal cancer	47.8 copies of HPV16 DNA/mL	[164]
Exosome RNA	Bio-Rad QX 200 ddPCR system	Urinary	/	RT-ddPCR	miR-29a	Castration-resistant prostate cancer	5–50 copies/μL	[165]
	Bio-Rad QX 200 ddPCR system	Serum	Total exosome isolation reagent EpCAM-specific magnetic beads	RT-ddPCR	lncRNA: RP11-77G23.5; PHEX-AS1	Lung cancer	/	[166]
	Flow-focusing; co-flowing	Plasma	Ultracentrifugation; magnetic beads	RT-PCR	has-miR-21-5p; miR-21	Lung cancer	has-miR-21-5p: 1 copy/droplet miR-21, 30 cycles	[167]
	Flow-focusing	Plasma	Immune magnetic beads based on chip isolation	RT-ddPCR	EGFR L858R and KRAS mutations	Lung cancer	Exosome: LoD 3.9×10^5 $R^2 = 0.9998$	[119]
	Flow-focusing	Cell	Ultracentrifugation; magnetic beads; mxene	Photothermal ddPCR	miRNA-21-5P; miRNA-375-3P; miRNA-574-3p	Prostate cancer	LoD: 190 copies	[168]
	Flow-focusing	Cell	Ultracentrifugation; biotin-avidin interaction; MoS_2	Photothermal ddPCR	miRNA-200b-3p; miRNA-21-5p; miRNA-22-3p	Lung cancer	0.02–3 copies per droplet	[169]
Exosome protein	Bio-Rad QX 200 ddPCR system	Plasma	Ultracentrifugation	ddPCR	EpCAM; PD-L1	Various cancers	$R^2 = 0.997$; LoD 0.0735 pg/mL	[170]
	Y-shape flow-focusing	Plasma	Ultracentrifugation; magnetic beads	exoELISA	GCP-1	Breast cancer	LoD 10^{-17} M 5 exosomes/μL	[171]

Abbreviation: LoD, limit of detection.

study, exosomes were first isolated by ultracentrifugation. Then, the isolated exosomes were further labeled with CD63-conjugated magnetic beads and co-encapsulated into droplets with RT-PCR mix and lysis buffer by co-flowing design. miRNA inside exosomes were released after lysis and amplified for PCR detection. By using synthetic has-miR-21-5p, the sensitivity of this platform can be as low as one copy per droplet. When using clinical samples from lung cancer patients, the exoRNA in the positive droplets could be detected after 30 cycles. However, most of the current work involves exosome isolation off-chip, which may require complex manual operations. To address this problem, Lu et al. developed an integrated microfluidic system for high-throughput on-chip exosome isolation and multiple lung cancer RNA analysis through ddPCR.[119] As shown in Figure 5a, the integrated chip has four sections, including the exosome isolation chamber, droplet generation structure, droplet reaction chamber, and the PMMA packing structure. Exosome isolation, lysis, and detection can all be completed in one chip without contaminations. Droplets containing the reaction mixture were generated by a flow-focusing method and analyzed by ddPCR. The high-throughput analysis was achieved by the large volume of the final reaction droplets at ~100,000, indicating its potential in liquid biopsy. On the other hand, another disadvantage of ddPCR is the complexity of the instrument. To overcome the dependence on equipment, Zhang et al. developed a photothermal ddPCR system driven by Mxene-integrated nanomaterials for duplex cell-derived exosomal miRNA expression profiling.[168] In Figure 5b, exosomes were first captured by magnetic nanoparticles and lysed for RNA extraction. Mxene-integrated nanomaterials were formed by adhering Mxene onto the surface of other magnetic beads. The hydrogel-based droplets as microcarriers containing cDNA templates, PCR mix, and photothermal Mxene-integrated nanomaterials were generated by a flow-focusing microfluidic chip. With a homemade automated NIR control module, the tunable thermal response of Mxene-integrated nanomaterials enables reverse transcription analysis and PCR thermal cycling to detect duplex miRNAs in a simple bench-top process. The results showed that the developed NIR-responsive ddPCR system can successfully distinguish prostate cancer cases from health controls with 7–210 folds higher exosomal miRNA (miRNA-21-5P, miRNA-375-3P, and miRNA-574-3p) expression levels in the former. To further extend this work, Parvin et al. combined MoS_2 with this photothermal hydrogel-based dPCR approach to detect exosomal miRNA expression in liver cancer.[169] The results showed that exosomal miRNA-200b-3p and miRNA21-5p expression was 19–31-fold higher and miRNA-22-3P expression was

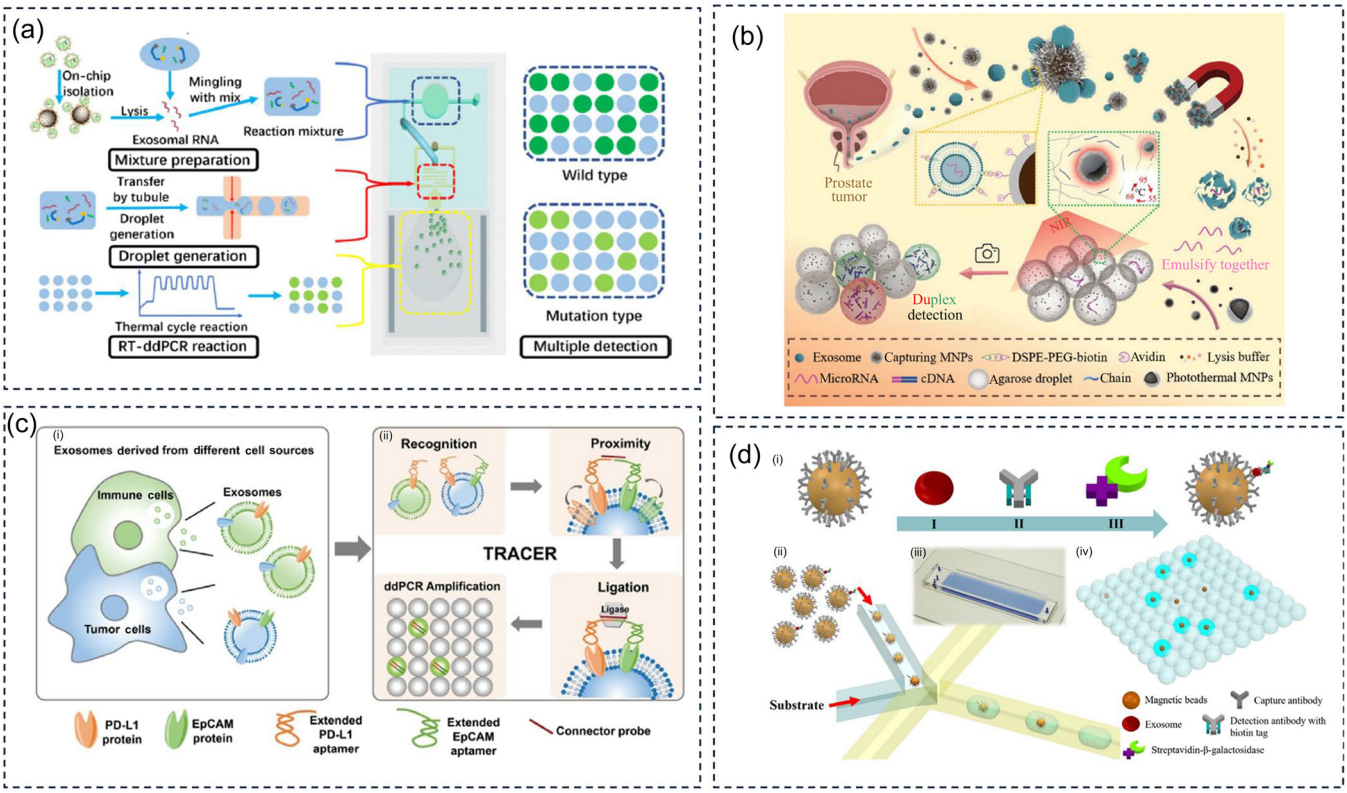

FIGURE 5 Droplet-based microfluidics for circulating tumor exosomes-based liquid biopsy. (a) Schematic diagram of the integrated exosome analysis system. Reproduced with permission.[119] Copyright 2021, Elsevier. (b) Schematics of exosome-derived microRNA (miRNA) detection approach. It consists of consecutive two-step of exosome capturing and photothermal dPCR processes. Reproduced with permission.[168] Copyright 2022, Wiley-VCH. (c) Working principle of the highly sensitive quantification of tumor-derived Exo-PD-L1 using aptamer-based PLA. Reproduced with permission.[170] Copyright 2020, Wiley-VCH. (d) Schematic showing the droplet digital ExoELISA for exosome quantification. Reproduced with permission.[171] Copyright 2018, American Chemical Society.

10-fold lower in liver cancer samples compared with healthy controls. It can be seen that this NIR-responsive hydrogel-based dPCR system simplifies the thermocycling process of PCR and prevents the evaporating and coalescence problems of the aqueous droplet, indicating a promising application for on-site detection.

For exosomal protein detection, Lin et al., developed a TRACER platform for the detection of tumor-derived exosomal PD-L1 by dual-aptamer activated proximity-induced ddPCR.[170] In Figure 5c, PD-L1 and EpCAM were simultaneously labeled with corresponding extended dual-aptamers to distinguish normal exosomes and tumor-derived exosomes. All the exosomes were isolated by standard differential ultracentrifugation from cell culture supernatants. In the presence of connector probe and ligase, the two extended aptamer can be closed ligated due to the close proximity. The ligation products can be further quantified by ddPCR, reflecting the number of tumor-derived Exo-$_{PD\text{-}L1}$. The ddPCR results showed a positive correlation of exosome concentrations with copy numbers of amplified PLA products ($R^2 = 0.997$) with a calculated LoD of 0.0735 pg/mL. By evaluating exosomes isolated from cancer patient plasma, the TRACER strategy could successfully identify exosomes from cancer patients and healthy donors (*t*-test, $p < 0.0001$). Besides, Liu et al. developed a droplet digital exoELISA approach for the quantification of exosomes at the single-cell level.[171] As shown in Figure 5d, the exosome suspension was first bound with a sufficient number of antibody-modified magnetic beads and detection antibodies with an enzymatic reporter, forming a single sandwich ELISA complex. After that, the prepared beads and enzymatic substrates were co-encapsulated into droplets by a Y-shape flow-focusing microfluidic ship. In the presence of an exosome immunocomplex, the substrate was catalyzed by enzymes and produced a fluorescent signal in the droplet. By measuring the signal change, exosomes can be sensitively detected with an LoD down to 5 exosomes/μL.

CHALLENGES AND FUTURE PERSPECTIVES

Today, liquid biopsy still faces many challenges. As biomarkers for liquid biopsy, CTCs, ctDNA, miRNA, and exosomes have their strengths and drawbacks. For example, although CTCs carry comprehensive tumor genetic information and can reflect the function and heterogeneity of tumor cells, they are difficult to enrich due to their rarity. ctDNA is a tumor-specific biomarker with comprehensive genetic information that can reflect tumor status in real time. Due to the advancement in DNA extraction technology and the development of PCR technology, ctDNA-based liquid biopsy has great potential in the applications of cancer screening, diagnosis, prognosis, and drug screening. However, in the early stage of cancer, the amount of ctDNA is very low, which puts forward high requirements for detection sensitivity. As a new type of biomarker, miRNA has the potential to integrate diagnosis and treatment that cannot be ignored. The rich tumor information and a huge quantity of exosomes make liquid biopsy highly sought-after. However, due to the late start of the research, miRNA- and exosome-based liquid biopsy currently lacks relevant research. In addition, the heterogeneity of tumor cells and molecules is a major obstacle in cancer diagnosis and treatment. It is difficult for a single detection method to fully reveal the progression and classification of tumors. Analysis of multiple biomarkers helps to understand the heterogeneity of cancer cells at different levels, such as genetics, transcriptomics, and proteomics. On the other hand, due to the different characteristics of biomarkers, the joint detection of multiple liquid biopsy methods can complement each other to achieve early screening, diagnosis, and prognosis of cancer. Nowadays, it is not enough to rely on CTC detection alone in clinical practice, and it needs to be combined with ctDNA detection for auxiliary diagnosis. It can be seen that the combined use of multiple liquid biopsy methods will be a major trend in the future. The precise diagnosis of cancer is like a big puzzle, which cannot be solved alone, and must be combined with multiple detections for comprehensive analysis.

The advent of droplet microfluidics has brought new insights into solving these challenges. First, droplet-based microfluidic technology can divide macroscopic samples into tens of thousands of microdroplets, further enhancing the advantages of microfluidic chips, such as low consumption, automation, and high sensitivity. Second, the formation of a large number of microdroplets provides a reliable platform for high-throughput biomarker detection at the single-cell/molecular level, which is very suitable for the study of tumor cell or molecular heterogeneity. Third, droplet-based microfluidics have great potential as integrated devices that can perform on-chip isolation and sample preparation steps. Currently, the separation of biomarkers is mainly performed off-chip, which may require complex manual operations and cause unavoidable contamination. Droplet-based microfluidics can quickly encapsulate isolated biomarkers into droplets at the single-cell level without contamination for various downstream detections, such as genome, transcriptome, and proteome, realizing a "sample-in-result-out" detection platform. Fourth, droplet-based microfluidics is capable of integrating multiple assays, which contributes to a deeper understanding of cancer from different levels, enabling comprehensive cancer diagnosis, prognosis, and monitoring within one chip. Based on these advantages, droplet-based microfluidics have broad prospects in the field of liquid biopsy.

However, droplet microfluidics based on "water in oil" technology is a "liquid–liquid phase" system. Compared with the traditional "solid–liquid phase" system microfluidics for cancer diagnosis, the stability and controllability of droplet-based microfluidics are relatively low. Especially when performing immunoassays, droplet-based microfluidics cannot implement washing steps to remove unbound antibodies or fluorescent labels, which will result in nonnegligible background noise within the droplet. Nevertheless, most droplet-based microfluidics rely on Poisson distribution to achieve single-cell/molecule/exosome isolation, which has a relatively low encapsulation efficiency and most of the droplets are empty. To improve the control of droplets, DMF, as a "solid–liquid phase" based droplet microfluidics, has received extensive attention. DMF can achieve precise manipulation of droplets, but low throughput is its main challenge. Furthermore, microchamber-based

microfluidics are also "solid–liquid" systems, in which single-cell/molecule/exosome can be trapped in nanoliter chambers. Compared with droplet-based microfluidics, the encapsulation of biomarkers by microchamber-based microfluidics is also limited by the Poisson distribution, but it has the advantage of simple washing steps and is more suitable for multiplex reactions. Compared with DMF, the detection throughput of microchamber-based microfluidics is significantly improved, but its control of droplets is not precise enough. Moreover, microchamber-based microfluidics are not suitable for quantitative analysis. In general, when the multiplexing capability and detection throughput increase, the liquid volume in the microchamber inevitably increases, thereby reducing the detection sensitivity.

Overall, droplet-based microfluidics has promising applications due to its high throughput, low contamination, high sensitivity, and single-cell/molecule/exosome analysis capabilities. Some droplet-based microfluidics have entered the stage of industrialization. Among them, the most representative product is ddPCR. Many manufacturers, such as Bio-Rad, Life Technologies, RainDance Technologies, and so on have launched commercial dPCR equipment. In addition, Illumina has introduced Drop-Seq technology to analyze mRNA transcripts in single-cell droplets. However, most of the current commercialized droplet-based microfluidic products contain multistep off-chip operations, making detection complex and prone to contamination. In future development, the easy-to-use and integrated droplet microfluidic platform will be the main direction. The emergence of artificial intelligence will also provide great potential for data analysis with high-throughput droplets.

ACKNOWLEDGMENTS

This work was supported by the Shenzhen Science and Technology Program-Basic Research Scheme (JCYJ20220531090808020), the Hong Kong Research Grants Council (RGC)-General Research Fund (PolyU 15216622 and PolyU 15217621), the Guangdong-Hong Kong Technology Cooperation Funding Scheme (GHP/032/20SZ and SGDX20201103095404018), the Hong Kong Polytechnic University Shenzhen Institute Bai Cheng Bai Yuan Fund (I2022A002), the Research Grants Council (RGC) Postdoctoral Fellowship Fund (PDFS2223-5S07) and the Hong Kong Polytechnic University Internal Fund (1-ZVVQ, 1-CD6J and 1-CD8M). This work was also supported by the University Research Facility in Life Sciences of the Hong Kong Polytechnic University.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

ORCID

Jingyu Shi http://orcid.org/0000-0001-7650-8212

REFERENCES

1. Ferlay J, Ervik M, Lam F, et al. *Global Cancer Observatory: Cancer Today*. International Agency for Research on Cancer; 2020.
2. Chi KR. The tumour trail left in blood. *Nature*. 2016;532:269-271.
3. Ashley EA. Towards precision medicine. *Nat Rev Genet*. 2016;17:507-522.
4. Haber DA, Velculescu VE. Blood-based analyses of cancer: circulating tumor cells and circulating tumor DNA. *Cancer Discovery*. 2014;4:650-661.
5. Jahr S, Hentze H, Englisch S, et al. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. *Cancer Res*. 2001;61:1659-1665.
6. Schwarzenbach H, Hoon DSB, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. *Nat Rev Cancer*. 2011;11:426-437.
7. Takizawa S, Matsuzaki J, Ochiya T. Circulating microRNAs: challenges with their use as liquid biopsy biomarkers. *Cancer Biomark*. 2022;35:1-9.
8. Dolgin E. Could tracking RNA in body fluids reveal disease? *Nature*. 2020;582:S2-S4.
9. Shin H, Choi BH, Shim O, et al. Single test-based diagnosis of multiple cancer types using exosome-SERS-AI for early stage cancers. *Nat Commun*. 2023;14:1644.
10. Simpson RJ, Lim JW, Moritz RL, Mathivanan S. Exosomes: proteomic insights and diagnostic potential. *Expert Rev Proteomics*. 2009;6:267-283.
11. Beebe DJ, Mensing GA, Walker GM. Physics and applications of microfluidics in biology. *Annu Rev Biomed Eng*. 2002;4:261-286.
12. Kovarik ML, Ornoff DM, Melvin AT, et al. Micro total analysis systems: fundamental advances and applications in the laboratory, clinic, and field. *Anal Chem*. 2013;85:451-472.
13. Shi J, Zhang Y, Yang M. Recent development of microfluidics-based platforms for respiratory virus detection. *Biomicrofluidics*. 2023;17:024104.
14. Seemann R, Brinkmann M, Pfohl T, Herminghaus S. Droplet based microfluidics. *Rep Prog Phys*. 2011;75:016601.
15. Guo MT, Rotem A, Heyman JA, Weitz DA. Droplet microfluidics for high-throughput biological assays. *Lab Chip*. 2012;12:2146-2155.
16. Joensson HN, Andersson Svahn H. Droplet microfluidics—a tool for single-cell analysis. *Angew Chem Int Ed*. 2012;51:12176-12192.
17. Meacham CE, Morrison SJ. Tumour heterogeneity and cancer cell plasticity. *Nature*. 2013;501:328-337.
18. Hajji I, Serra M, Geremie L, et al. Droplet microfluidic platform for fast and continuous-flow RT-qPCR analysis devoted to cancer diagnosis application. *Sens Actuat B*. 2020;303:127171.
19. Gao R, Cheng Z, deMello AJ, Choo J. Wash-free magnetic immunoassay of the PSA cancer marker using SERS and droplet microfluidics. *Lab Chip*. 2016;16:1022-1029.
20. Dimitriou P, Li J, Tornillo G, McCloy T, Barrow D. Droplet microfluidics for tumor drug-related studies and programmable artificial cells. *Glob Chall*. 2021;5:2000123.
21. Mazutis L, Gilbert J, Ung WL, Weitz DA, Griffiths AD, Heyman JA. Single-cell analysis and sorting using droplet-based microfluidics. *Nat Protoc*. 2013;8:870-891.
22. Coelho BJ, Veigas B, Bettencourt L, et al. Digital microfluidics-powered real-time monitoring of isothermal DNA amplification of cancer biomarker. *Biosensors*. 2022;12:201.
23. Choe G, Park J, Park H, Lee J. Hydrogel biomaterials for stem cell microencapsulation. *Polymers*. 2018;10:997.
24. Thorsen T, Roberts RW, Arnold FH, Quake SR. Dynamic pattern formation in a vesicle-generating microfluidic device. *Phys Rev Lett*. 2001;86:4163-4166.
25. Abate AR, Weitz DA. Air-bubble-triggered drop formation in microfluidics. *Lab Chip*. 2011;11:1713-1716.
26. De Menech M, Garstecki P, Jousse F, Stone HA. Transition from squeezing to dripping in a microfluidic T-shaped junction. *J Fluid Mech*. 2008;595:141-161.
27. Anna SL, Bontoux N, Stone HA. Formation of dispersions using "flow focusing" in microchannels. *Appl Phys Lett*. 2003;82:364-366.

28. Tan Y-C, Cristini V, Lee AP. Monodispersed microfluidic droplet generation by shear focusing microfluidic device. *Sens Actuat B*. 2006;114:350-356.
29. Anna SL. Droplets and bubbles in microfluidic devices. *Annu Rev Fluid Mech*. 2016;48:285-309.
30. Xu JH, Luo GS, Li SW, Chen GG. Shear force induced monodisperse droplet formation in a microfluidic device by controlling wetting properties. *Lab Chip*. 2006;6:131-136.
31. Utada AS, Fernandez-Nieves A, Stone HA, Weitz DA. Dripping to jetting transitions in coflowing liquid streams. *Phys Rev Lett*. 2007; 99:094502.
32. Oudeng G, Benz M, Popova AA, et al. Droplet microarray based on nanosensing probe patterns for simultaneous detection of multiple HIV retroviral nucleic acids. *ACS Appl Mater Interfaces*. 2020;12: 55614-55623.
33. Choi K, Ng AHC, Fobel R, Wheeler AR. Digital microfluidics. *Annu Rev Anal Chem*. 2012;5:413-440.
34. Cho SK, Moon H, Kim C-J. Creating, transporting, cutting, and merging liquid droplets by electrowetting-based actuation for digital microfluidic circuits. *J Microelectromech Syst*. 2003;12:70-80.
35. Bolognesi G, Saito Y, Tyler AII, Ward AD, Bain CD, Ces O. Mechanical characterization of ultralow interfacial tension oil-in-water droplets by thermal capillary wave analysis in a microfluidic device. *Langmuir*. 2016;32:3580-3586.
36. Jiang D, Park SY. Light-driven 3D droplet manipulation on flexible optoelectrowetting devices fabricated by a simple spin-coating method. *Lab Chip*. 2016;16:1831-1839.
37. Ding X, Li P, Lin SCS, et al. Surface acoustic wave microfluidics. *Lab Chip*. 2013;13:3626-3649.
38. Zhang Y, Nguyen NT. Magnetic digital microfluidics—a review. *Lab Chip*. 2017;17:994-1008.
39. Tan Y-C, Hettiarachchi K, Siu M, Pan Y-R, Lee AP. Controlled microfluidic encapsulation of cells, proteins, and microbeads in lipid vesicles. *J Am Chem Soc*. 2006;128:5656-5658.
40. Edd JF, Di Carlo D, Humphry KJ, et al. Controlled encapsulation of single-cells into monodisperse picolitre drops. *Lab Chip*. 2008;8: 1262-1264.
41. Kamalakshakurup G, Lee AP. High-efficiency single cell encapsulation and size selective capture of cells in picoliter droplets based on hydrodynamic micro-vortices. *Lab Chip*. 2017;17:4324-4333.
42. Tang SY, Joshipura ID, Lin Y, et al. Liquid-metal microdroplets formed dynamically with electrical control of size and rate. *Adv Mater*. 2016;28:604-609.
43. Yan Q, Xuan S, Ruan X, Wu J, Gong X. Magnetically controllable generation of ferrofluid droplets. *Microfluid Nanofluid*. 2015;19: 1377-1384.
44. Park S-Y, Wu T-H, Chen Y, Teitell MA, Chiou P-Y. High-speed droplet generation on demand driven by pulse laser-induced cavitation. *Lab Chip*. 2011;11:1010-1012.
45. Ling SD, Geng Y, Chen A, Du Y, Xu J. Enhanced single-cell encapsulation in microfluidic devices: from droplet generation to single-cell analysis. *Biomicrofluidics*. 2020;14:061508.
46. Huang Q, Mao S, Khan M, Lin J-M. Single-cell assay on microfluidic devices. *Analyst (Lond)*. 2019;144:808-823.
47. Lagus TP, Edd JF. A review of the theory, methods and recent applications of high-throughput single-cell droplet microfluidics. *J Phys D Appl Phys*. 2013;46:114005.
48. Pit AM, de Ruiter R, Kumar A, Wijnperlé D, Duits MHG, Mugele F. High-throughput sorting of drops in microfluidic chips using electric capacitance. *Biomicrofluidics*. 2015;9:044116.
49. Park S-Y, Chiou P-Y. Light-driven droplet manipulation technologies for lab-on-a-chip applications. *Adv OptoElectron*. 2011; 2011:909174.
50. Tan S-H, Sohel Murshed SM, Nguyen N-T, Wong TN, Yobas L. Thermally controlled droplet formation in flow focusing geometry: formation regimes and effect of nanoparticle suspension. *J Phys D Appl Phys*. 2008;41:165501.
51. Shin DJ, Wang TH. Magnetic droplet manipulation platforms for nucleic acid detection at the point of care. *Ann Biomed Eng*. 2014;42:2289-2302.
52. Wang Z, Zhe J. Recent advances in particle and droplet manipulation for lab-on-a-chip devices based on surface acoustic waves. *Lab Chip*. 2011;11:1280-1285.
53. Fidler IJ. The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. *Nat Rev Cancer*. 2003;3:453-458.
54. Budd GT, Cristofanilli M, Ellis MJ, et al. Circulating tumor cells versus imaging—predicting overall survival in metastatic breast cancer. *Clin Cancer Res*. 2006;12:6403-6409.
55. Smerage JB, Barlow WE, Hortobagyi GN, et al. Circulating tumor cells and response to chemotherapy in metastatic breast cancer: SWOG S0500. *J Clin Oncol*. 2014;32:3483-3489.
56. Rupp B, Ball H, Wuchu F, Nagrath D, Nagrath S. Circulating tumor cells in precision medicine: challenges and opportunities. *Trends Pharmacol Sci*. 2022;43:378-391.
57. Lu C-Y, Tsai H-L, Uen Y-H, et al. Circulating tumor cells as a surrogate marker for determining clinical outcome to mFOLFOX chemotherapy in patients with stage III colon cancer. *Br J Cancer*. 2013;108:791-797.
58. Harouaka RA, Nisic M, Zheng S-Y. Circulating tumor cell enrichment based on physical properties. *SLAS Technol*. 2013;18:455-468.
59. Song Y, Tian T, Shi Y, et al. Enrichment and single-cell analysis of circulating tumor cells. *Chem Sci*. 2017;8:1736-1751.
60. Nasiri R, Shamloo A, Akbari J. Design of a hybrid inertial and magnetophoretic microfluidic device for CTCs separation from blood. *Micromachines*. 2021;12:877.
61. Müller V, Riethdorf S, Rack B, et al. Prognostic impact of circulating tumor cells assessed with the CellSearch System™ and AdnaTest Breast™ in metastatic breast cancer patients: the DETECT study. *Breast Cancer Res*. 2012;14:R118.
62. Hyun KA, Koo GB, Han H, et al. Epithelial-to-mesenchymal transition leads to loss of EpCAM and different physical properties in circulating tumor cells from metastatic breast cancer. *Oncotarget*. 2016;7:24677-24687.
63. Fröhlich J, König, H. New techniques for isolation of single prokaryotic cells. *FEMS Microbiol Rev*. 2000;24:567-572.
64. Herzenberg LA, Parks D, Sahaf B, Perez O, Roederer M, Herzenberg LA. The history and future of the fluorescence activated cell sorter and flow cytometry: a view from Stanford. *Clin Chem*. 2002;48:1819-1827.
65. Emmert-Buck MR, Bonner RF, Smith PD, et al. Laser capture microdissection. *Science*. 1996;274:998-1001.
66. Kulemann B, Rösch S, Seifert S, et al. Pancreatic cancer: circulating tumor cells and primary tumors show heterogeneous KRAS mutations. *Sci Rep*. 2017;7:4510.
67. Ribeiro-Samy S, Oliveira MI, Pereira-Veiga T, et al. Fast and efficient microfluidic cell filter for isolation of circulating tumor cells from unprocessed whole blood of colorectal cancer patients. *Sci Rep*. 2019;9:8032.
68. Qiu S, Shen C, Jian X, et al. Single-cell level point mutation analysis of circulating tumor cells through droplet microfluidics. *Chin Chem Lett*. 2022;33:2701-2704.
69. Wei X, Chen K, Cai B, et al. An acoustic droplet-induced enzyme responsive platform for the capture and on-demand release of single circulating tumor cells. *ACS Appl Mater Interfaces*. 2019;11: 41118-41126.
70. Ruan Q, Ruan W, Lin X, et al. Digital-WGS: automated, highly efficient whole-genome sequencing of single cells by digital microfluidics. *Sci Adv*. 2020;6:eabd6454.
71. Ruan Q, Yang J, Zou F, et al. Single-cell digital microfluidic mass spectrometry platform for efficient and multiplex genotyping of circulating tumor cells. *Anal Chem*. 2021;94:1108-1117.

72. Strati A, Zavridou M, Economopoulou P, Gkolfinopoulos S, Psyrri A, Lianidou E. Development and analytical validation of a reverse transcription droplet digital PCR (RT-ddPCR) assay for PD-L1 transcripts in circulating tumor cells. *Clin Chem*. 2021;67:642-652.
73. Aya-Bonilla CA, Morici M, Hong X, et al. Detection and prognostic role of heterogeneous populations of melanoma circulating tumour cells. *Br J Cancer*. 2020;122:1059-1067.
74. Wang J, Sun N, Lee YT, et al. A circulating tumor cell-based digital assay for the detection of EGFR T790M mutation in advanced non-small cell lung cancer. *J Mater Chem B*. 2020;8:5636-5644.
75. Winter M, Cai Z, Winkler K, et al. Circulating tumour cell RNA characterisation from colorectal cancer patient blood after inertial microfluidic enrichment. *MethodsX*. 2019;6:1512-1520.
76. Ma Y, Luk A, Young FP, et al. Droplet digital PCR based androgen receptor variant 7 (AR-V7) detection from prostate cancer patient blood biopsies. *Int J Mol Sci*. 2016;17:1264.
77. Xu X, Zhang Q, Song J, et al. A highly sensitive, accurate, and automated single-cell RNA sequencing platform with digital microfluidics. *Anal Chem*. 2020;92:8599-8606.
78. Park J, Park S, Hyun KA, Jung HI. Microfluidic recapitulation of circulating tumor cell–neutrophil clusters via double spiral channel-induced deterministic encapsulation. *Lab Chip*. 2021;21:3483-3497.
79. Khajvand T, Huang P, Li L, et al. Interfacing droplet microfluidics with antibody barcodes for multiplexed single-cell protein secretion profiling. *Lab Chip*. 2021;21:4823-4830.
80. Dhar M, Lam JN, Walser T, Dubinett SM, Rettig MB, Di Carlo D. Functional profiling of circulating tumor cells with an integrated vortex capture and single-cell protease activity assay. *Proc Natl Acad Sci USA*. 2018;115:9986-9991.
81. Li Y, Zhang P, Li T, et al. A virtual-droplet system for sensing MMP9 activity of single suspended and adhered cancer cells. *Sens Actuat B*. 2020;308:127749.
82. Armbrecht L, Rutschmann O, Szczerba BM, Nikoloff J, Aceto N, Dittrich PS. Quantification of protein secretion from circulating tumor cells in microfluidic chambers. *Adv Sci*. 2020;7:1903237.
83. Cai B, Guo F, Zhao L, et al. Disk-like hydrogel bead-based immunofluorescence staining toward identification and observation of circulating tumor cells. *Microfluid Nanofluid*. 2014;16:29-37.
84. Yang X, Liu W, Chan DCH, et al. Fluorescent droplet cytometry for on-cell phenotype tracking. *J Am Chem Soc*. 2020;142:14805-14809.
85. Liu W, Zhang R, Huang S, et al. Quantification of intracellular proteins in single cells based on engineered picoliter droplets. *Langmuir*. 2022;38:7929-7937.
86. Del Ben F, Turetta M, Celetti G, et al. A method for detecting circulating tumor cells based on the measurement of single-cell metabolism in droplet-based microfluidics. *Angew Chem*. 2016;128:8723-8726.
87. Rivello F, Matuła K, Piruska A, Smits M, Mehra N, Huck WTS. Probing single-cell metabolism reveals prognostic value of highly metabolically active circulating stromal cells in prostate cancer. *Sci Adv*. 2020;6:eaaz3849.
88. Zhang W, Li N, Lin L, Li H, Lin JM. Metabolism-based capture and analysis of circulating tumor cells in an open space. *Anal Chem*. 2021;93:6955-6960.
89. Strati A, Nikolaou M, Georgoulias V, Lianidou ES. RNA-based CTC analysis provides prognostic information in metastatic breast cancer. *Diagnostics*. 2021;11:513.
90. Hassan S, Blick T, Williams E, Thompson R. Applications of RNA characterisation in circulating tumour cells. *Front Biosci Landmark*. 2020;25:4838874-4838892.
91. Rezaei M, Radfar P, Winter M, McClements L, Thierry B, Warkiani ME. Simple-to-operate approach for single cell analysis using a hydrophobic surface and nanosized droplets. *Anal Chem*. 2021;93:4584-4592.
92. Qu F, Zhao L, Li L, et al. Thermo-induced coalescence of dual cores in double emulsions for single-cell RT-PCR. *Anal Chem*. 2022;94:11670-11678.
93. Wu M, Singh AK. Single-cell protein analysis. *Curr Opin Biotechnol*. 2012;23:83-88.
94. Magbanua MJM, Park JW. Isolation of circulating tumor cells by immunomagnetic enrichment and fluorescence-activated cell sorting (IE/FACS) for molecular profiling. *Methods*. 2013;64:114-118.
95. Makridakis M, Vlahou A. Secretome proteomics for discovery of cancer biomarkers. *J Proteomics*. 2010;73:2291-2305.
96. Schiarea S, Solinas G, Allavena P, et al. Secretome analysis of multiple pancreatic cancer cell lines reveals perturbations of key functional networks. *J Proteome Res*. 2010;9:4376-4392.
97. Sorber L, Zwaenepoel K, Deschoolmeester V, et al. Circulating cell-free nucleic acids and platelets as a liquid biopsy in the provision of personalized therapy for lung cancer patients. *Lung Cancer*. 2017;107:100-107.
98. de Kock R, van den Borne B, Youssef-El Soud M, et al. Therapy monitoring of EGFR-positive non–small-cell lung cancer patients using ddPCR multiplex assays. *J Mol Diagn*. 2021;23:495-505.
99. Diehl F, Li M, He Y, Kinzler KW, Vogelstein B, Dressman D. BEAMing: single-molecule PCR on microparticles in water-in-oil emulsions. *Nat Methods*. 2006;3:551-559.
100. Zhou J, Zhao C, Zhao J, et al. Re-biopsy and liquid biopsy for patients with non-small cell lung cancer after EGFR-tyrosine kinase inhibitor failure. *Thorac Cancer*. 2019;10:957-965.
101. Forshew T, Murtaza M, Parkinson C, et al. Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA. *Sci Transl Med*. 2012;4:136ra68.
102. Lin C, Liu X, Zheng B, Ke R, Tzeng CM. Liquid biopsy, ctDNA diagnosis through NGS. *Life*. 2021;11:890.
103. Tewhey R, Warner JB, Nakano M, et al. Microdroplet-based PCR enrichment for large-scale targeted sequencing. *Nat Biotechnol*. 2009;27:1025-1031.
104. Cutarelli A, De Falco F, Uleri V, Buonavoglia C, Roperto S. The diagnostic value of the droplet digital PCR for the detection of bovine delta papillomavirus in goats by liquid biopsy. *Transbound Emerg Dis*. 2021;68:3624-3630.
105. Demuth C, Spindler KLG, Johansen JS, et al. Measuring KRAS mutations in circulating tumor DNA by droplet digital PCR and next-generation sequencing. *Transl Oncol*. 2018;11:1220-1224.
106. Palacín-Aliana I, García-Romero N, Asensi-Puig A, Carrión-Navarro J, González-Rumayor V, Ayuso-Sacido Á. Clinical utility of liquid biopsy-based actionable mutations detected via ddPCR. *Biomedicines*. 2021;9:906.
107. Zmrzljak UP, Košir R, Krivokapić Z, Radojković D, Nikolić A. Detection of somatic mutations with ddPCR from liquid biopsy of colorectal cancer patients. *Genes*. 2021;12:289.
108. van Ginkel JH, Huibers MMH, van Es RJJ, de Bree R, Willems SM. Droplet digital PCR for detection and quantification of circulating tumor DNA in plasma of head and neck cancer patients. *BMC Cancer*. 2017;17:428.
109. Tanaka J, Nakagawa T, Harada K, et al. Efficient and accurate KRAS genotyping using digital PCR combined with melting curve analysis for ctDNA from pancreatic cancer patients. *Sci Rep*. 2023;13:3039.
110. Gassa A, Fassunke J, Schueten S, et al. Detection of circulating tumor DNA by digital droplet PCR in resectable lung cancer as a predictive tool for recurrence. *Lung Cancer*. 2021;151:91-96.
111. Shoda K, Ichikawa D, Fujita Y, et al. Monitoring the HER2 copy number status in circulating tumor DNA by droplet digital PCR in patients with gastric cancer. *Gastric Cancer*. 2017;20:126-135.
112. Huang A, Zhang X, Zhou S-L, et al. Detecting circulating tumor DNA in hepatocellular carcinoma patients using droplet digital PCR is feasible and reflects intratumoral heterogeneity. *J Cancer*. 2016;7:1907-1914.

113. Wang J, Yang L, Diao Y, et al. Circulating tumour DNA methylation in hepatocellular carcinoma diagnosis using digital droplet PCR. *J Int Med Res*. 2021;49:300060521992962.
114. Ou CY, Vu T, Grunwald JT, et al. An ultrasensitive test for profiling circulating tumor DNA using integrated comprehensive droplet digital detection. *Lab Chip*. 2019;19:993-1005.
115. Pan Y, Ma T, Meng Q, et al. Droplet digital PCR enabled by microfluidic impact printing for absolute gene quantification. *Talanta*. 2020;211:120680.
116. Schlenker F, Kipf E, Borst N, et al. Centrifugal microfluidic integration of 4-plex ddPCR demonstrated by the quantification of cancer-associated point mutations. *Processes*. 2021;9:97.
117. Hsieh SA, Shamsaei D, Eitzmann DR, Anderson JL. Digital droplet loop-mediated isothermal amplification featuring a molecular beacon assay, 3D printed droplet generation, and smartphone imaging for sequence-specific DNA detection. *Anal Chem*. 2022;94:11949-11956.
118. Gao W, Huang T, Yuan H, et al. Highly sensitive detection and mutational analysis of lung cancer circulating tumor cells using integrated combined immunomagnetic beads with a droplet digital PCR chip. *Talanta*. 2018;185:229-236.
119. Lu Y, Tong Z, Wu Z, et al. Multiple exosome RNA analysis methods for lung cancer diagnosis through integrated on-chip microfluidic system. *Chin Chem Lett*. 2022;33:3188-3192.
120. Li Y, Wu J, Li E, et al. TP53 mutation detected in circulating exosomal DNA is associated with prognosis of patients with hepatocellular carcinoma. *Cancer Biol Ther*. 2022;23:439-445.
121. Guibert N, Pradines A, Farella M, et al. Monitoring KRAS mutations in circulating DNA and tumor cells using digital droplet PCR during treatment of KRAS-mutated lung adenocarcinoma. *Lung Cancer*. 2016;100:1-4.
122. Allenson K, Castillo J, San Lucas FA, et al. High prevalence of mutantKRAS in circulating exosome-derived DNA from early-stage pancreatic cancer patients. *Ann Oncol*. 2017;28:741-747.
123. Reyes JCB, Solon JAA, Rivera WL. Development of a loop-mediated isothermal amplification assay for detection of *Trichomonas vaginalis*. *Diagn Microbiol Infect Dis*. 2014;79:337-341.
124. Tan Y-L, Chen H, Wu Z-K, He J, Jiang J-H. Digital loop-mediated isothermal amplification-based absolute methylation quantification revealed hypermethylated DAPK1 in cervical cancer patients. *Anal Chem*. 2021;93:8077-8083.
125. Hwang HW, Mendell JT. MicroRNAs in cell proliferation, cell death, and tumorigenesis. *Br J Cancer*. 2006;94:776-780.
126. He L, Thomson JM, Hemann MT, et al. A microRNA polycistron as a potential human oncogene. *Nature*. 2005;435:828-833.
127. Ma L, Teruya-Feldstein J, Weinberg RA. Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. *Nature*. 2007;449:682-688.
128. Zhu S, Wu H, Wu F, Nie D, Sheng S, Mo YY. MicroRNA-21 targets tumor suppressor genes in invasion and metastasis. *Cell Res*. 2008;18:350-359.
129. Chen W, Wang H, Chen H, et al. Clinical significance and detection of micro RNA-21 in serum of patients with diffuse large B-cell lymphoma in Chinese population. *Eur J Haematol*. 2014;92:407-412.
130. O'Connell RM, Rao DS, Baltimore D. microRNA regulation of inflammatory responses. *Annu Rev Immunol*. 2012;30:295-312.
131. Bagga S, Bracht J, Hunter S, et al. Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. *Cell*. 2005;122:553-563.
132. Babapoor S, Horwich M, Wu R, et al. microRNA in situ hybridization for miR-211 detection as an ancillary test in melanoma diagnosis. *Mod Pathol*. 2016;29:461-475.
133. Alhasan AH, Kim DY, Daniel WL, et al. Scanometric microRNA array profiling of prostate cancer markers using spherical nucleic acid–gold nanoparticle conjugates. *Anal Chem*. 2012;84:4153-4160.
134. Raymond CK, Roberts BS, Garrett-Engele P, Lim LP, Johnson JM. Simple, quantitative primer-extension PCR assay for direct monitoring of microRNAs and short-interfering RNAs. *RNA*. 2005;11:1737-1744.
135. Tian H, Sun Y, Liu C, Duan X, Tang W, Li Z. Precise quantitation of microRNA in a single cell with droplet digital PCR based on ligation reaction. *Anal Chem*. 2016;88:11384-11389.
136. Tian W, Li P, He W, Liu C, Li Z. Rolling circle extension-actuated loop-mediated isothermal amplification (RCA-LAMP) for ultrasensitive detection of microRNAs. *Biosens Bioelectron*. 2019;128:17-22.
137. Sun Y, Tian H, Liu C, Sun Y, Li Z. One-step detection of microRNA with high sensitivity and specificity via target-triggered loop-mediated isothermal amplification (TT-LAMP). *Chem Commun*. 2017;53:11040-11043.
138. Chen T, Xu Y, Wei S, Li A, Huang L, Liu J. A signal amplification system constructed by bi-enzymes and bi-nanospheres for sensitive detection of norepinephrine and miRNA. *Biosens Bioelectron*. 2019;124-125:224-232.
139. Foda MF, Huang L, Shao F, Han H-Y. Biocompatible and highly luminescent near-infrared $CuInS_2$/ZnS quantum dots embedded silica beads for cancer cell imaging. *ACS Appl Mater Interfaces*. 2014;6:2011-2017.
140. Wang J, Lu J, Dong S, et al. An ultrasensitive electrochemical biosensor for detection of microRNA-21 based on redox reaction of ascorbic acid/iodine and duplex-specific nuclease assisted target recycling. *Biosens Bioelectron*. 2019;130:81-87.
141. Wang P, Jing F, Li G, et al. Absolute quantification of lung cancer related microRNA by droplet digital PCR. *Biosens Bioelectron*. 2015;74:836-842.
142. Cirillo PDR, Margiotti K, Mesoraca A, Giorlandino C. Quantification of circulating microRNAs by droplet digital PCR for cancer detection. *BMC Res Notes*. 2020;13:351.
143. Jiao Z, Zhao L, Tang C, Shi H, Wang F, Hu B. Droplet-based PCR in a 3D-printed microfluidic chip for miRNA-21 detection. *Anal Methods*. 2019;11:3286-3293.
144. Chen Z-P, Yang P, Yang Z-Z, et al. One-step digital droplet autocatalytic nucleic acid amplification with high-throughput fluorescence imaging and droplet tracking computation. *Anal Chem*. 2022;94:9166-9175.
145. Gines G, Menezes R, Nara K, Kirstetter AS, Taly V, Rondelez Y. Isothermal digital detection of microRNAs using background-free molecular circuit. *Sci Adv*. 2020;6:eaay5952.
146. Chen J, Oudeng G, Feng H, et al. 2D MOF nanosensor-integrated digital droplet microfluidic flow cytometry for in situ detection of multiple miRNAs in single CTC cells. *Small*. 2022;18:2201779.
147. Li L, Lu M, Fan Y, et al. High-throughput and ultra-sensitive single-cell profiling of multiple microRNAs and identification of human cancer. *Chem Commun*. 2019;55:10404-10407.
148. Sun D, Cao F, Yi X, et al. MicroRNA-21 expression in single living cells revealed by fluorescence and SERS dual-response microfluidic droplet platform. *Lab Chip*. 2022;22:2165-2172.
149. Dobra G, Bukva M, Szabo Z, et al. Small extracellular vesicles isolated from serum may serve as signal-enhancers for the monitoring of CNS tumors. *Int J Mol Sci*. 2020;21:5359.
150. Al-Nedawi K, Meehan B, Micallef J, et al. Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. *Nature Cell Biol*. 2008;10:619-624.
151. Balaj L, Lessard R, Dai L, et al. Tumour microvesicles contain retrotransposon elements and amplified oncogene sequences. *Nat Commun*. 2011;2:180.
152. Meehan K, Vella LJ. The contribution of tumour-derived exosomes to the hallmarks of cancer. *Crit Rev Clin Lab Sci*. 2016;53:121-131.
153. Kowal J, Tkach M, Théry C. Biogenesis and secretion of exosomes. *Curr Opin Cell Biol*. 2014;29:116-125.

154. Wang Z-Y, Wang R-X, Ding X-Q, Zhang X, Pan X-R, Tong J-H. A protocol for cancer-related mutation detection on exosomal DNA in clinical application. *Front Oncol*. 2020;10:558106.
155. He M, Zeng Y. Microfluidic exosome analysis toward liquid biopsy for cancer. *SLAS Technol*. 2016;21:599-608.
156. Rekker K, Saare M, Roost AM, et al. Comparison of serum exosome isolation methods for microRNA profiling. *Clin Biochem*. 2014;47:135-138.
157. Li P, Kaslan M, Lee SH, Yao J, Gao Z. Progress in exosome isolation techniques. *Theranostics*. 2017;7:789-804.
158. Patel GK, Khan MA, Zubair H, et al. Comparative analysis of exosome isolation methods using culture supernatant for optimum yield, purity and downstream applications. *Sci Rep*. 2019;9:5335.
159. Contreras-Naranjo JC, Wu HJ, Ugaz VM. Microfluidics for exosome isolation and analysis: enabling liquid biopsy for personalized medicine. *Lab Chip*. 2017;17:3558-3577.
160. Coumans FAW, Brisson AR, Buzas EI, et al. Methodological guidelines to study extracellular vesicles. *Circ Res*. 2017;120:1632-1648.
161. Melo SA, Luecke LB, Kahlert C, et al. Glypican-1 identifies cancer exosomes and detects early pancreatic cancer. *Nature*. 2015;523:177-182.
162. Madhavan B, Yue S, Galli U, et al. Combined evaluation of a panel of protein and miRNA serum-exosome biomarkers for pancreatic cancer diagnosis increases sensitivity and specificity. *Int J Cancer*. 2015;136:2616-2627.
163. Bernard V, Kim DU, San Lucas FA, et al. Circulating nucleic acids are associated with outcomes of patients with pancreatic cancer. *Gastroenterology*. 2019;156:108-118.
164. Wang Z, Li F, Rufo J, et al. Acoustofluidic salivary exosome isolation: a liquid biopsy compatible approach for human papillomavirus–associated oropharyngeal cancer detection. *J Mol Diagn*. 2020;22:50-59.
165. Wang C, Ding Q, Plant P, et al. Droplet digital PCR improves urinary exosomal miRNA detection compared to real-time PCR. *Clin Biochem*. 2019;67:54-59.
166. Shen X, Yang Y, Chen Y, et al. Evaluation of EpCAM-specific exosomal lncRNAs as potential diagnostic biomarkers for lung cancer using droplet digital PCR. *J Mol Med*. 2022;100:87-100.
167. Cui B, Liu C, Yao S. One-step RT-PCR for detection of microRNAs in exosomes using droplet microfluidics. *IEEE 33rd International Conference on Micro Electro Mechanical Systems (MEMS)*. IEEE; 2020:142-146.
168. Zhang L, Li W, Parvin R, et al. Screening prostate cancer cell-derived exosomal microRNA expression with photothermal-driven digital PCR. *Adv Funct Mater*. 2022;32:2207879.
169. Parvin R, Zhang L, Zu Y, Ye F. Photothermal responsive digital polymerase chain reaction resolving exosomal microRNAs expression in liver cancer. *Small*. 2023;19:2207672.
170. Lin B, Tian T, Lu Y, et al. Tracing tumor-derived exosomal PD-L1 by dual-aptamer activated proximity-induced droplet digital PCR. *Angew Chem Int Ed*. 2021;60:7582-7586.
171. Liu C, Xu X, Li B, et al. Single-exosome-counting immunoassays for cancer diagnostics. *Nano Lett*. 2018;18:4226-4232.

How to cite this article: Shi J, Zhang Y, Fan Y, Liu Y, Yang M. Recent advances in droplet-based microfluidics in liquid biopsy for cancer diagnosis. *Droplet*. 2024;3:e92. doi:10.1002/dro2.92