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Review

Analytical detection techniques for droplet microfluidics—A review

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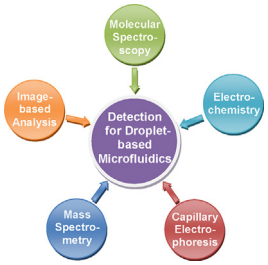
HIGHLIGHTS

- This is the first review paper focused on the analytical techniques for droplet-based microfluidics.
- We summarized the analytical methods used in droplet-based microfluidic systems.
- We discussed the advantage and disadvantage of each method through its application.
- We also discuss the future development direction of analytical methods for droplet-based microfluidic systems.

GRAPHICAL ABSTRACT



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ABSTRACT

In the last decade, droplet-based microfluidics has undergone rapid progress in the fields of single-cell analysis, digital PCR, protein crystallization and high throughput screening. It has been proved to be a promising platform for performing chemical and biological experiments with ultra-small volumes (picoliter to nanoliter) and ultra-high throughput. The ability to analyze the content in droplet qualitatively and quantitatively is playing an increasing role in the development and application of droplet-based microfluidic systems. In this review, we summarized the analytical detection techniques used in droplet systems and discussed the advantage and disadvantage of each technique through its application. The analytical techniques mentioned in this paper include bright-field microscopy, fluorescence microscopy, laser induced fluorescence, Raman spectroscopy, electrochemistry, capillary electrophoresis, mass spectrometry, nuclear magnetic resonance spectroscopy, absorption detection, chemiluminescence, and sample pretreatment techniques. The importance of analytical detection techniques in enabling new applications is highlighted. We also discuss the future development direction of analytical detection techniques for droplet-based microfluidic systems.

Contents

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1. Introduction

The past decade has witnessed the rapid progress of droplet-based microfluidics both in fundamental and applied researches, since the landmark work published by Stephen Quake and his coworkers [1]. Droplet-based microfluidics (also called plug-based microfluidics, segmented-flow microfluidics, and multiphase microfluidics) is the science and technology for manipulating and processing small (10^{-6} to 10^{-15} L) amounts of droplets or plugs carried by their immiscible phase [2–4]. Since the droplets are isolated and protected by the immiscible phase, each droplet could be regarded as a micro test tube or a microreactor to perform reaction and assay independently. Droplets can be generated [5,6], merged with each other [7,8], split [9], trapped [10,11], and sorted [12] with hydrodynamic, electrical, and optical-based droplet manipulation techniques. Droplet-based microfluidic systems not only inherit most of the advantages from conventional microfluidic systems (i.e. single-phase microfluidic systems), but also provide many attractive characteristics over them (see Fig. 1), such as: (1) the ability to process ultra-small reactors (down to femtoliter scale) [13]; (2) ultra-high throughput generation and manipulation of microreactors (up to 100 kHz) [12,14]; (3) elimination of Taylor diffusion and dilution of sample solutions [9]; (4) minimization of absorption of sample on channel surface [15]; and (5) enhanced mixing and mass transfer inside droplets [16].

Benefiting from these special advantages, droplet-based microfluidics has proven to be a promising technology platform for performing chemical and biological experiments with ultra-small volumes (nL to fL), and has been enabling new discoveries in these fields [2,4,17,18]. The main applications of droplet-based microfluidics are summarized as following. Single DNA and enzyme molecules can be trapped in ultra-small droplets, amplified via polymerase chain reaction (PCR) [19,20] and enzyme catalyzed reaction [21], and finally quantified with a digital counting manner. Such a method can be used in absolute quantification of samples with extremely low concentrations. Single cells or subcellular organelles can also be encapsulated into a droplet to distinguish the difference of enzyme activity [22,23], genes [22,24] and protein expression [25,26] at the single cell level. Using droplet-based systems, over thousands of reaction conditions, gene mutations, and chemicals can be screened with high throughput and low sample/reagent consumptions. These examples include protein crystallization screening [27–29], drug screening [30], catalyst screening [31], and direction evolution screening [32]. In droplet-based systems, since Taylor diffusion and dilution of sample and reagents are eliminated during their transferring and storage stages, the temporal concentration information of analytes can be preserved in droplets with high resolution and high fidelity. Such a feature is particularly suitable for in vivo sampling [33], measurement of dynamic release of organs [34,35], and collection of separated components in an electrophoresis system [36]. Since the mixing and mass transfer is enhanced within a droplet, droplet-based systems can be used in studies of rapid reaction dynamics [16] and on-line synthesis of microparticles with uniform sizes [37]. Despite these advancements, in most of the above-mentioned applications, model samples and ideal conditions were commonly used to demonstrate their feasibilities. Many challenges still need to be addressed before droplet-based microfluidics becomes a true and powerful tool to solve the real-world problems.

One of the challenges lies in the ability to analyze droplet content qualitatively and quantitatively. The analytical detection techniques for droplets play critical roles in the development and application of droplet-based microfluidic systems. In this review, the statuses and aspects of various analytical techniques used in droplet-based microfluidic systems are summarized, and their merits and demerits in the applications as well as the future development direction are also discussed. These analytical detection techniques include bright-field microscopy, fluorescence microscopy, laser induced fluorescence, Raman spectroscopy, electrochemistry, capillary electrophoresis, mass spectrometry, nuclear magnetic resonance spectroscopy, absorption detection, chemiluminescence, and sample pretreatment techniques. Since

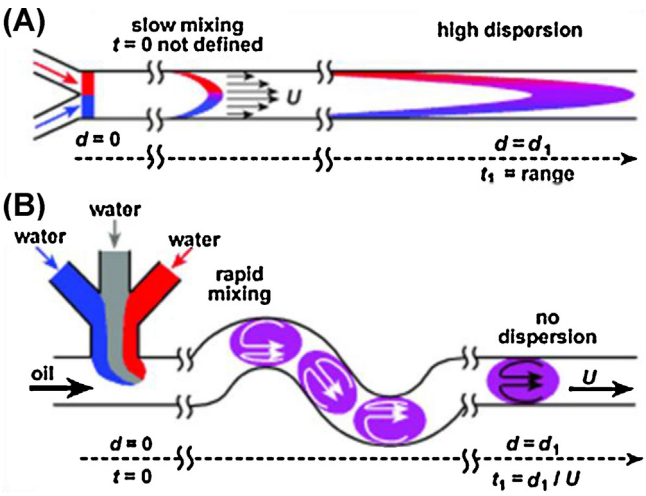

Fig. 1. Schematic comparison of a typical A + B reaction in a conventional single-phase microfluidic system (A) and in a droplet-based microfluidic system (B). Reprinted with permission from reference [9].

recently there have been several review articles published focusing on droplet manipulation techniques [3,15,17,38], droplet-based reactions [4], and chemical and biological applications of droplet-based systems [2–4,17,39], these contents are not introduced in detail in this paper. The digital microfluidic method, where the droplets are manipulated with electro-wetting on planar substrates rather than microchannels, is not covered as well [40].

2. Imaging-based droplet analysis

Imaging-based droplet analysis method is the most frequently-used technique for droplet analysis since microscopes are well-equipped in routine microfluidic and biological laboratories, and the imaging technique with bright-field or fluorescence detection is the most straightforward and convenient way for droplet analysis. Chemical distribution inside droplet can also be identified by imaging technique with multi-dimensionally spatial resolution. Imaging technique is particularly suitable for investigating physical and biological behaviors or processes of droplets as well as the species inside the droplets, such as generation or merging of droplets, generation of particle inside droplets, and growth of cells. In this section, the progress of imaging-based droplet analysis techniques and their applications are discussed.

2.1. Bright-field microscopy

With bright-field microscopy, the shape, size, color, trajectory and other status of droplets can be visualized conveniently. The generation and manipulation of droplets on microchips are complicated and fast processes [3,4]. The use of bright-field microscopes equipped with high-speed cameras could slow down these processes to facilitate the dynamics studies of these processes, with which time-lapse images showing physical status of droplets with sub-millisecond resolution could be obtained. Anna et al. [6] and Yobas et al. [41] investigated the droplet generation process in flow-focusing channels with high-speed cameras. Droplet size and generation frequency as the functions of flow rate were illustrated, and three different droplet generation regimes including squeezing regime, dripping regime, and jetting regime were observed (Fig. 2A). Besides the observation of droplet generation, high-speed imaging technique was also used to monitor and study the processes of rapid droplet merging [7], splitting [42], trapping [43,44], and sorting [12,45]. By adding dyes or colored tracers into droplets, rapid mixing [46,47] or reaction [48] process inside droplets can also be monitored with high-speed imaging technique. Ismagilov and co-workers [46,47] systematically studied the mixing phenomena in droplets in straight and winding microchannels using dyes as tracers. Chaotic mixing was observed in the winding channel and its kinetic model was established [47]. Huebner et al. [48] developed a droplet system to study the ultrafast kinetics with submillisecond resolution using a chromogenic reaction of Fe^{3+} with SCN^- (Fig. 2B). For a droplet with colored analyte, the bright-field microscopic imaging technique can be used to achieve quantitative analysis. Kreutz et al. [31] proposed a droplet-based method to screen new homogeneous catalyst using the oxidation of methane to methanol by molecular oxygen. In order to detect the formed methanol, they developed a colorimetric method using indicator droplets whose color changed with the concentration of methanol. The combination of bright-field microscopy with colored droplets can also be applied in reaction indexing [29] and particle encoding [49]. Kim et al. [49] used multi-color droplets and a double-emulsion system to encode microparticles for multiplex

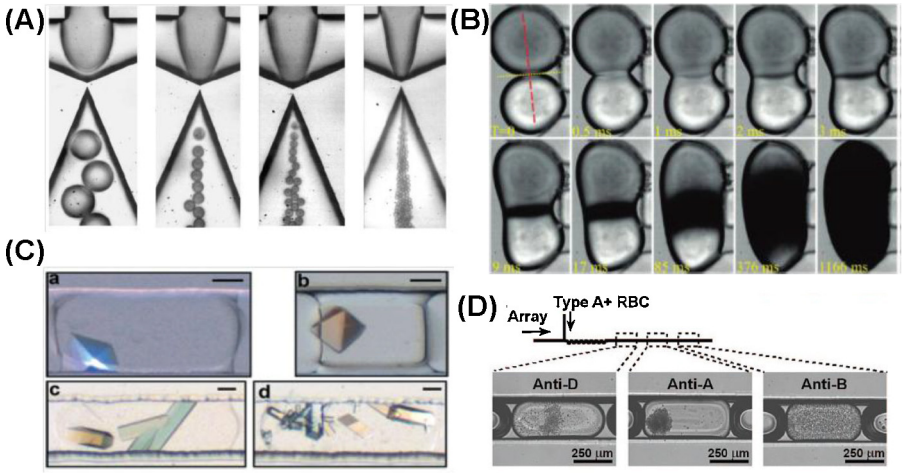

Fig. 2. Images of droplet analysis obtained with bright-field microscopy. (A) High-speed imaging of droplet generation process with flow-focusing geometry. Reprinted with permission from reference [41]. (B) A droplet system for studying ultrafast kinetics with submillisecond resolution using a chromogenic reaction of Fe^{3+} with SCN^-. Reprinted with permission from reference [48]. (C) Screening of protein crystallization conditions in droplets with the illumination of polarized light. Reprinted with permission from reference [50]. (D) Blood typing and subtyping using cell agglutination assay within droplets. Reprinted with permission from reference [53].

immunoassay. The possible number of codes could be 1771 if 20 core droplets with 3 different colors were used in one shell droplet.

Bright-field microscopy is the preferred analytical detection technique if heterogeneous reaction or assay is performed in droplet. A typical example for the former case is the droplet-based protein crystallization screening system, in which protein crystals in droplets could be easily identified and observed using a bright-field microscope with polarized light source. In the screening experiments, over hundreds of droplets need to be checked to seek suitable conditions for protein crystallization. With the illumination of polarized light, the protein crystals in droplets show different colors, making them be distinguished from the background and the precipitate [28,50] (Fig. 2C). Bright-field microscopy also provides a convenient method to study the nucleation and growth processes of proteins in droplets to understand the kinetics of protein crystallization [51]. Bright-field microscopy can also be used to detect the formation of blood clotting [52] and agglutination in droplets [53]. Performing blood test with droplet system is attractive when the available blood samples are limited, such as the blood typing of newborns. Kline et al. [53] developed a droplet-based microfluidic approach to perform ABO, D blood typing and subtyping using only microliter scale blood (Fig. 2D). Blood type was determined by seeking the droplets with cell agglutination and identifying the contained antibodies. In addition, the bright-field microscopic technique is also useful in the studies of growth and mobility behaviors of cells [11] and organisms [43] in droplets. Shi et al. [43] reported a droplet-based microfluidic system for single *Caenorhabditis elegans* assay. Single *C. elegans* were encapsulated into droplets and their mobility behaviors response to neurotoxin were characterized in parallel.

2.2. Fluorescence microscopy

Although the colorimetry with bright-field microscopy has been demonstrated to be a quantitative detection approach for droplet systems [31,48], its sensitivity is rather poor due to the short optical path length of droplets. In droplet-based biological applications, it is important to quantitatively read the information of a specific compound with low concentration and distinguish its expression level between different droplets. Fluorescence microscopy provides a simple and efficient way to fulfill this requirement. The detection sensitivity of fluorescence microscopy could be very high provided that high-sensitive cameras and appropriate filters are equipped. He et al. [23] reported a droplet-based method to study the enzyme activity of single cell. Single cell was trapped into a picoliter-volume droplet and lysed with laser pulse to release enzyme. The enzyme activity of single cell was assayed by measuring the fluorescence intensity of the enzyme-catalyzed product in the droplet with a fluorescence microscope. Schmitz et al. [11] developed a microfluidic device named as Dropspots for large-scale measurement of enzyme activities of single cells (Fig. 3A). The fluorescent intensities of hundreds of droplets were recorded simultaneously by fluorescence imaging and statistic information reflecting the heterogeneity in gene expression between cells can be obtained [11,22,54]. Recently, Noji and co-workers [21] successfully achieved single-enzyme assay of β-gal with a femtoliter-scale droplet microarray. In order to decrease the detection limit of fluorescence imaging, the stochastic noise of background was reduced by averaging 11 fluorescence images. Droplet-based system coupling with fluorescence microscopy is also a powerful platform for enzyme kinetics studies. With the advantage of chaotic mixing inside droplet, enzyme kinetic measurements with millisecond resolution were achieved in a droplet-based microchip [16]. The kinetic data at different reaction time was accessed by reading the fluorescence intensity at different position along the microchannel (Fig. 3B). The substrate concentrations inside droplets were controlled by varying the flow rate ratio of its stock solution and diluent solution before droplet formation. In another experimental design, substrate solutions with different concentrations were prepared using on-chip gradient generation systems based on multiphase laminar flow diffusion [55,56]. With a motivation of recording over thousands of droplets in one image rather than multiple images

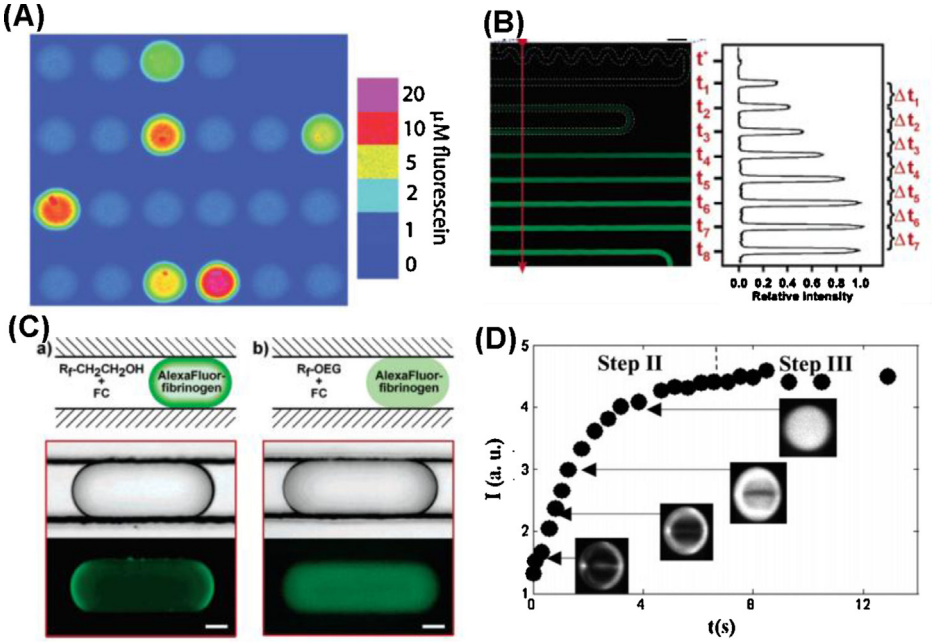

Fig. 3. Droplet analysis systems with fluorescence microscopy. (A) Single-cell enzyme assay within droplet microreactor. Reprinted with permission from reference [11]. (B) Study of enzyme exponential kinetics in droplet system. Reprinted with permission from reference [16]. (C) Protein absorption at the aqueous–fluorous interfaces with different of surfactants added into the fluorous phase. Reprinted with permission from reference [59]. (D) Investigation of droplet-based liquid–liquid extraction process with fluorescence imaging.

Reprinted with permission from reference [61].

[57], Hatch et al. [58] developed a wide-field fluorescence imaging system capable of detecting 1-million droplets in one image with a low-cost Canon digital camera and a macro-lens. The system was used to quantify the DNA molecules under droplet-based digital-PCR mode with high dynamic range.

Fluorescence imaging has outstanding advantages in studying the spatial distribution of interested molecules in droplets and surrounding phase. The control of protein adsorption on the interface between droplet and carrier phase is crucial in enzyme kinetics study and biochemical assay. Roach et al. [59] studied the protein adsorption on droplet–oil interface with fluorescently labeled proteins and confocal fluorescence microscopy (Fig. 3C). Their study indicated the nonspecific protein adsorption could be prevented by using oligoethylene-glycol capped surfactants in carrier phase. In the subsequent study, they designed a new surfactant to induce specific adsorption of proteins at the interface and used it to improve the success rate of protein crystallization [60]. The diffusion coefficient of protein molecule on the interface was quantitatively measured using fluorescence recovery after photobleaching (FRAP) experiments. Liquid–liquid extraction is a widely used technique in chemical engineering and analytical chemistry. Mary et al. [61] systemically investigated the liquid–liquid extraction process between moving droplets and carrier phase with fluorescence imaging technique (Fig. 3D). Three regimes of extraction including droplet-formation regime I, droplet steadily-moving regime II, and late time regime III were observed in the extraction process by analyzing the fluorescent molecular distribution inside the droplets.

2.3. Other imaging techniques

Fourier transform infrared (FT-IR) imaging is a label-free and nondestructive chemical imaging technique. Chan et al. [62] applied FT-IR imaging to visualize the moving process of water droplets in the microchannel with a temporal resolution of 120 ms. However, the sensitivity of FT-IR imaging for droplet analysis is rather low, which may limit its application in droplet-based biological assays. Electron microscopes frequently used in material research were also applied to characterize the polymer particles [63] and nano-particles [37] produced in droplet-based microfluidic systems.

3. Laser-based molecular spectroscopy

3.1. Laser-induced fluorescence (LIF) detection

One limitation of fluorescence imaging for quantitative analysis is that the frame rate of CCD camera is typically lower than the frequency of droplet generation. Thus, fluorescence microscopies are limited in the applications where individual droplet need to be accessed fast and continuously, such as high throughput screening and sorting [12]. LIF detection provides an effective way to solve this problem. In addition, LIF is one of the most sensitive detection techniques, with which the detection of single molecule has been successfully demonstrated [64,65]. Interestingly, the first use of LIF system to detect single molecule in levitated droplets was reported in 1993 [66], about 8 years before the proposal of the concept of droplet-based microfluidics. The main advantage of droplet system for single molecule detection is the ability to confine a single molecule in illumination volume and thus to maximize the detection efficiency [66,67]. In these works, droplets were generated by piezoelectric ejection and then levitated in a three-electrode system for fluorescence detection. Recently, Srisa-Art et al. [68] and Rane et al. [69] individually reported the counting of fluorescence-labeled single DNA molecule in microchip-based droplet systems. In their systems, restriction channels were designed at detection windows to squeeze droplets and thus increase the detection efficiency, because droplet volume was commonly larger than detection volume in confocal LIF systems. Jeffries et al. [70] compared the detection limit of confocal LIF system with that of orthogonal LIF system (Fig. 4A). The results revealed that the orthogonal design could evidently reduce the background and decrease the detection limit when large illumination volume was used for droplet detection (Fig. 4B). Besides high sensitivity, high throughput detection is another attractive feature of LIF systems. LIF systems are very suitable for applications where large number of droplet reactors need to be accessed, such as digital PCR [19,20,71,72], single cell analysis [25,30], low-abundance biomarker detection [26], and the screening of protein evolution [12,32]. An elegant example of these applications has been demonstrated by Weitz and co-workers [32], in which they used droplet-based system to achieve ultrahigh-throughput screening of protein evolution. About 10^8 individual enzyme reactions were screened in only 10 h using less than 150 μL reagent, which were 1000-fold increase in speed and 1-million-fold reduction in cost compared with conventional robotic screening systems. LIF system enabled the high-sensitive detection of enzyme reactions and high-throughput selection of the active enzymes [12]. Additionally, the dynamic range of LIF system is rather large, which could facilitate to quantify fluorescent species in droplets with large-scale concentration gradient. More recently, Cai et al. developed a flow injection-based droplet system to generate an array of droplets with large-scale concentration gradient and used it to perform enzyme inhibition assay [73]. With a single injection of 16-nL volume of analyte solution, an array of droplets with concentration gradient spanning 3–4 orders of magnitude could be generated. A LIF detector was used to achieve rapid detection of fluorescent product with large-scale concentration range.

Fluorescence resonance energy transfer (FRET) is a useful technique for biological molecular dynamics research such as protein-DNA interactions, protein-protein interactions, and protein conformational changes. Srisa-Art et al. [74,75] described the study of streptavidin-biotin binding kinetics with millisecond time resolution using droplet-based system coupled with FRET. Streptavidin and biotin were labeled with a FRET donor and acceptor, respectively, and the binding between the two molecules lead to detectable signal. The binding kinetics was studied by measuring the FRET signals at different points in the chip channel corresponding to different hybridization time. In the subsequent work, they applied the FRET technique to study the protein-protein interaction using an antigen-antibody model system [76]. Molecule fluorescence lifetime is an intrinsic characteristic of an individual fluorescent molecule that is affected only by its chemical environment. The same group developed a fluorescence lifetime-based method to image the fluidic mixing of two fluorescent dyes inside droplets with a temporal resolution of 1 μs [77,78]. The image was generated by reading the fluorescence lifetime values of different points in droplets and then assembling these values together according to their spatial positions. Fluorescence correlation spectroscopy (FCS) is another type of high sensitive fluorescence detection technique relying on the measurement of spontaneous intensity fluctuations of fluorescent species rather than the intensity itself. The FCS technique was applied to measure the size and velocity of DNA molecules [79] and nano-particles [80] confined in droplets.

3.2. Raman spectroscopy

One intrinsic drawback of LIF detection is the need of specific fluorescent labeling for non-fluorescent analytes before analysis. In contrast, Raman spectroscopy is a label-free detection technique,

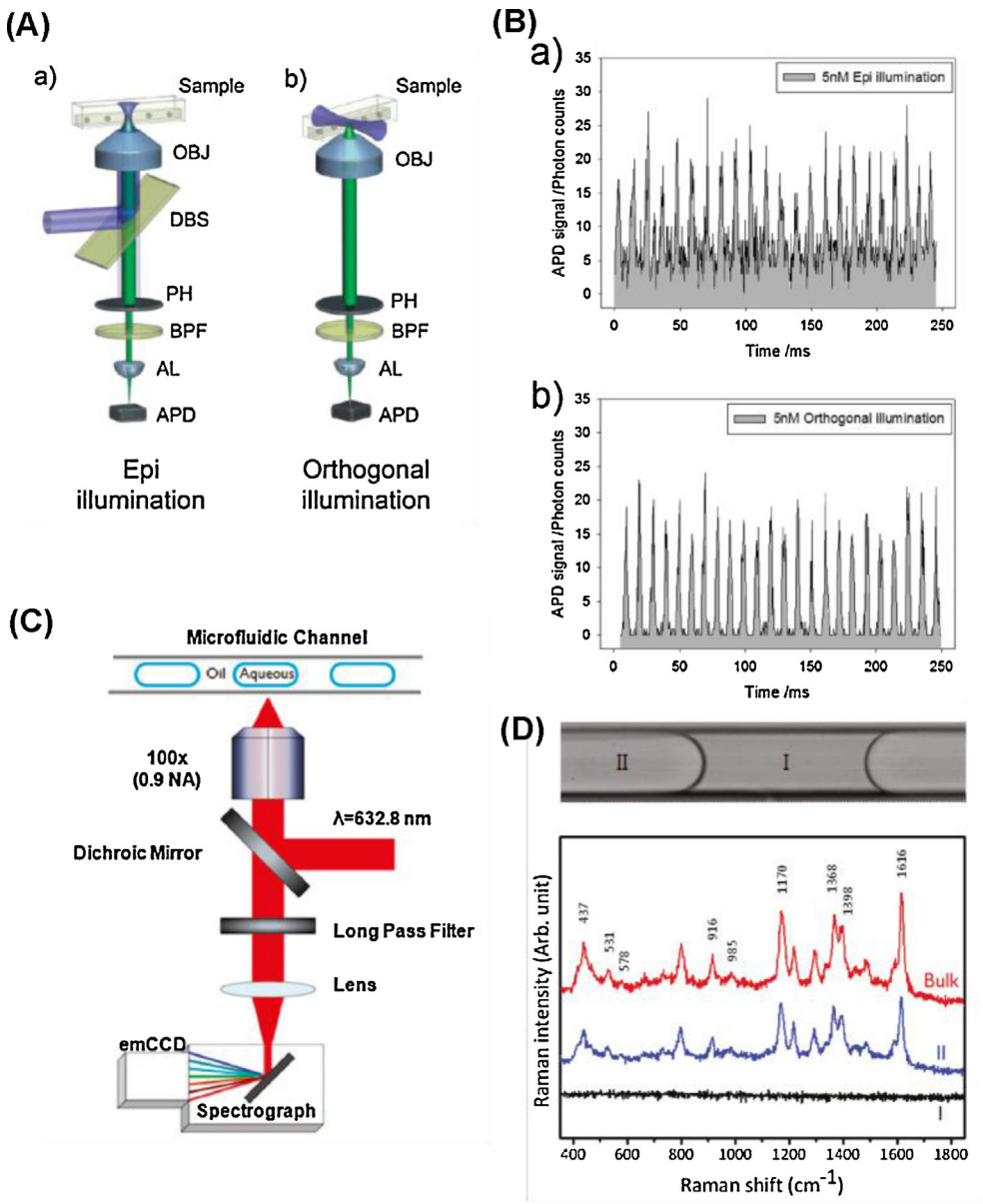

Fig. 4. (A) Schematic illustration of laser-induced fluorescence detection systems with confocal optical arrangement (a) and orthogonal optical arrangement (b). (B) Fluorescence signals of a series of 27-pL droplets with 5 nM fluorescein using confocal illumination (a) and orthogonal illumination (b). Reprinted with permission from reference [70]. (C) Schematic illustration of Raman spectroscopy system for droplet detection. (D) Typical Raman spectra of the oil phase (I), 75 ppb Malachite Green in droplet with silver nanoparticles (II), 75 ppb MG in bulk solution with silver nanoparticles (Bulk).
Reprinted with permission from reference [86].

with which the analyte can be recognized by the fingerprint of Raman shift and quantified using the corresponding peak intensity. Additionally, Raman spectroscopy holds other advantages including simultaneous detection of multiple analytes, high throughput detection, high spatial resolution, and high sensitivity when coupled with surface-enhanced technique [81]. The first application of Raman spectroscopy in droplet system was reported by Cristobal et al. [82]. Taking the advantage of high spatial resolution of Raman spectroscopy, the mixing of two non-fluorescent compounds inside droplets at different points along the microchannel was profiled. In other works, Raman spectroscopy was used to study the process of chemical reaction [83] and screen the reaction condition of UV-induced polymerization [84] performed in droplet systems.

The surface-enhanced Raman scattering (SERS) detection technique provides a promising alternative for high sensitive detection in droplet system. Meanwhile, droplet-based microfluidic technique also could significantly improve the repeatability and analysis throughput of SERS systems by eliminating the "memory effect", accelerating the mixing of sample with colloid suspension and activation agent (e.g. NaCl), and performing online calibration of wavenumber [85]. Strehle et al. [85] described a high-performance SERS detection system for measuring crystal violet inside droplets with limit of detection (LOD) of 1 μM and repeatability of 4.8 (RSD, $n = 80$). They observed that the "memory effect", induced by the adsorption and deposition of colloid/analyte aggregates at the optical windows in conventional SERS systems, was eliminated because the droplets and the microchannel surface were completely separated by carrier phase. Cecchini et al. [86] developed an ultra-high throughput SERS system for droplet detection with a maximum analysis speed of 32 droplets per second (Fig. 4C and D). Such a system may facilitate the study of millisecond enzyme dynamics and high throughput screening of protein

evolution in a label-free way. In the subsequent studies, SERS was applied to high sensitive detection of mercury (II) ions [87], paraquat [88], and several drugs [89]. Recently, Walter et al. [90] described a SERS-based droplet system for bacteria identification using the fingerprint of Raman spectroscopy. A database of 11200 spectra for a model system *Escherichia coli* including nine different strains was established with a record time of 1 s per spectrum. The validation accuracy could be up to 92.6% with a chemometric analysis, showing the great potential of this method for bacteria classification with high reliability and high throughput.

4. Electrochemical detection

Electrochemical detection is a well-established analytical detection technique in analytical chemistry and has been widely used in microfluidic systems because of its low cost, small size, high sensitivity, high speed, and ease of integrating with microchips. The physical characteristics of droplets such as size, frequency, velocity, and conductivity can be measured with electrochemical-based techniques [91–93]. Luo et al. [91] reported the first use of electrochemical technique to measure droplet size and ion concentration based on the impedance difference of droplets and carrier. The concentration of NaCl ranging from 0.02 mM to 1 M in picoliter-scale droplets could be quantified with this method. In addition to impedance-based electrochemical technique, capacitance analysis is another useful technique to determine the physical parameters of droplets [92]. By measuring the capacitance change when the droplets passed a specific designed sensing area, the size, the velocity, and the frequency of droplets could be obtained simultaneously. Liu et al. [93] developed a new electrochemical method to measure the droplet characteristics on the basis of the chronoamperometric analysis of an electro-active compound in carrier phase. Droplet size and frequency were obtained by measuring the periodic variation of mass-transport limited current produced when the droplets passed over the working electrode.

Electrochemical technique can also be used to obtain chemical information inside droplets, which is more important than physical information when the droplet systems are used to perform chemical or biological reactions and assays. Han et al. [94] described an amperometric-based electrochemical method to measure rapid enzyme kinetics in droplet system with pneumatic microvalves. The pneumatic microvalves were used to control the trajectory of droplets in microchannels and thus change the time of enzyme reaction. The Michaelis–Menten kinetics of catalase was successfully obtained with a single-run experiment and a sample consumption of less than 50 μL. However, their results indicated the amperometric analysis in droplet-based system was more complicated than that in single-phase microfluidic system. This could be attributed to the interference of oil phase to the electrochemical reaction happened on the surface of working electrode. Thus, in the further work, it may be necessary to separate the droplets from the oil phase or extract the droplets from the segmented flow before electrochemical detection of droplets. Several approaches have been developed to extract droplets from a segmented flow into an aqueous flow and then analyze them with capillary electrophoresis and mass spectrometry, which will be introduced in detail in the following sections of this paper.

5. Capillary electrophoresis (CE)

In the above-mentioned droplet systems with fluorescence and electrochemical detection methods, the droplet compositions were commonly simple and only single fluorescent or electro-active components were detected in one experiment. These methods are limited in the applications where multiple fluorescent or

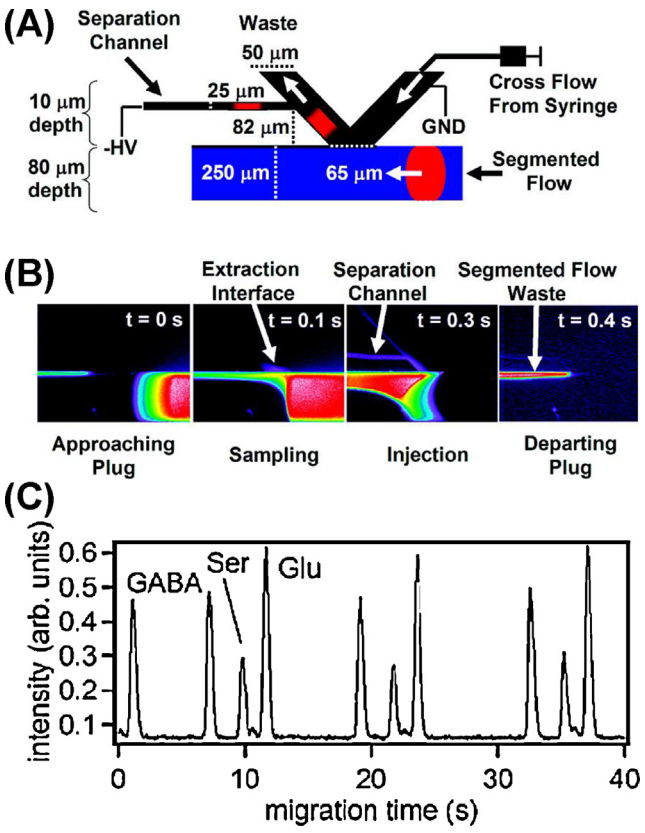

Fig. 5. (A) Schematic illustration of the microchip design for sampling and separation of the droplet content with capillary electrophoresis. (B) Fluorescence images showing the droplet extraction process. (C) Electropherograms of NDA derivatized amines in 17-nL droplets injected into the CE channel with 12 s intervals and separated with column length of 7.5 cm.

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electro-active components are existed in droplets, thus a separation process is required before these components enter the detector. Capillary electrophoresis (CE) is a powerful separation technique with high separation efficiency, high separation speed, and simple structure. CE has been widely used in many chemical and biological fields, such as gene sequencing, drug discovery, chiral recognition, and proteomic research. Although in the strict sense, capillary electrophoresis is a separation technique rather than a detection technique, recent studies demonstrated the substantial importance of CE technique in the analysis of biological samples with complex compositions in droplet-based analysis systems. Edgar et al. [95] demonstrated the feasibility of using a CE system with laser-induced fluorescence detection to analyze multiple components in droplets. Femtoliter-volume droplets were generated in a T-junction channel and then directly injected into an aqueous-phase CE channel. Three fluorescein isothiocyanate (FITC)-labeled amino acids including glycine, glutamate, and aspartate in droplets were successfully separated with an effective separation length of 3.2 cm. However, the separation efficiency was rather low due to the use of PDMS microchip, and the analysis throughput was difficult to increase because complicated and precise operations were required to prevent the leakage of oil into the CE separation channel.

In order to improve the separation efficiency and analysis throughput of CE-based droplet analysis systems, Kennedy and coworkers [96,97] developed an integrated glass chip-based system for performing automated droplet extraction and CE separation for droplet contents. A novel droplet extraction interface with K-shaped channel configuration was used to extract droplet from segmented flow to continuous aqueous flow channel. The surface

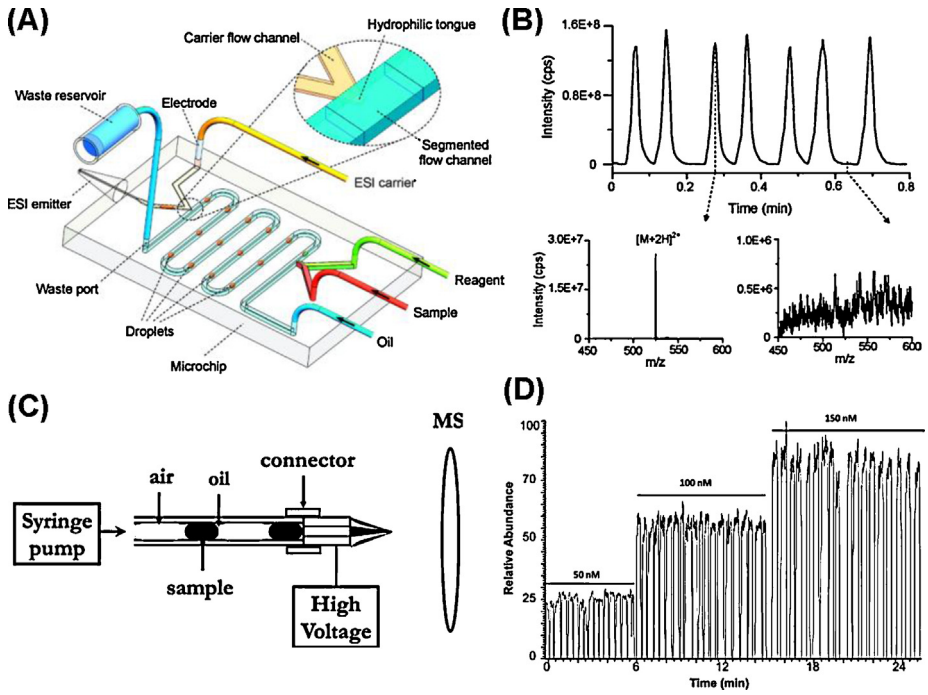

Fig. 6. (A) Schematic diagram of the integrated microchip with an electrospray emitter and a droplet extraction interface for droplet ESI-MS detection. The inset shows the hydrophilic tongue-based droplet extraction interface. (B) Extractive ion traces (m/z 524–526) for a series of 5-nL droplets with angiotensin (1 μM). The insets show the mass spectrum obtained from the peak apex and the baseline of the extractive ion traces. Reprinted with permission from reference [105]. (C) Schematic diagram showing the ESI-MS-based droplet analysis system by directly coupling the droplet cartridge to a commercial nanospray emitter. (D) Extracted ion traces for a series of 50-nL droplets with increasing concentrations of leucine-enkephalin. Ion signal is for MS^3 of leucine-enkephalin (MS^1 at m/z 556, MS^2 at m/z 397, and MS^3 at m/z 278, 323, 380). Reprinted with permission from reference [106].

of the chip microchannels for droplet generating and transferring were selectively modified to be hydrophobic, while the microchannels for droplet extraction and CE separation were kept to be hydrophilic. Such a design effectively prevented the oil from leaking into the CE separation channel during the continuous droplet extraction and CE separation processes (Fig. 5A). Using gated injection in the chip-based CE separation, six amino acids were separated with a separation efficiency of over 200,000 theoretical plates and a peak height repeatability of 4.4% (RSD, $n = 50$) [97]. This system was applied to in vivo monitoring of rapid change of neurotransmitter level in rat brain by combining with microdialysis-based sampling technique [97], which demonstrates the outstanding potential of the droplet-CE systems in biological research. In the subsequent study, the same group [98] developed a parallel CE separation system to further increase the throughput of droplet-based CE analysis. In this system, three droplet extraction interfaces and three CE separation channels were integrated in a single microchip, which allowed three droplets to be extracted to the CE channels and be separated simultaneously. An analysis throughput of 120 samples/10 min for an enzyme assay sample was achieved.

In the previously-reported droplet-based CE analysis systems, fluorescence detection was commonly used to detect the separated analytes. In the future studies, it is necessary to couple these systems with other detection techniques to broaden their application scope, such as electrochemical detection, Raman spectroscopy, and mass spectrometry.

6. Mass spectrometry

Mass spectrometry (MS) is an attractive analytical detection technique which can detect the samples qualitatively and quantitatively by measuring the mass-to-charge ratio of charged analytes. Compared with other detection techniques, MS provides outstanding advantages including label-free detection, capability of elucidating chemical structures with fragmentation, high sensitivity, and simultaneous detection of multiple analytes. Therefore, the use of MS in droplet analysis can greatly broaden the application of droplet-based microfluidics. Hatakeyama et al. [99] described the first utilization of matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS) for screening and optimizing reaction conditions in nanoliter-scale droplets. With the assistant of fluorinated carrier, 30-nL droplet reactors were deposited on the MALDI plate. A group of 44 chemicals were screened against a specific substrate with a total substrate consumption of 20 μg in 2-μL solution. The MALDI-MS technique was also applied to measure glucose level in an array of droplets collected from the Chemistrode [34]. However, as an offline analytical technique, the sample pretreatment process for MALDI-MS such as depositing droplets and matrix on MALDI plates, is time-consuming and labor-intensive, which significantly limits the throughput of droplet analysis.

Electrospray ionization mass spectrometry (ESI-MS) is an online and high-throughput MS technique, which has been widely used in microfluidic and capillary electrophoresis systems [100,101]. However, similar to capillary electrophoresis, the main challenge in coupling ESI-MS with droplet systems lies in the interference of oil phase to the electrospray process. Fidalgo et al. [102] used electrical pulse to extract droplets from segmented phase into aqueous phase by embedding a pair of microelectrodes between segmented flow and continuous aqueous flow. The electrical pulse was triggered by the fluorescence signals of the droplets, enabling the fluorescence-activated droplet extraction for selective droplet analysis. The droplet components were extracted into the continuous aqueous flow, delivered to a conventional electrospray emitter by the continuous aqueous flow, and directly detected by ESI-MS [103]. However, the sensitivity of this system was rather poor due to the high dilution of the sample droplet components in the long transferring line and the high flow rates of aqueous flow for

stabilizing the multiphase laminar flow profile. Kelly et al. [104] described a dilution-free droplet analysis system with ESI-MS by using an on-chip nano-ESI emitter. The droplet extraction was achieved by using an array of cylindrical posts between the segmented flow and aqueous flow to enable the droplets to pass through the apertures among the posts into the aqueous flow. The detection limit of this system was *ca.* 1 μM for a model peptide in 700-pL droplets, which was 100-fold lower than that of the previous work [103]. Recently, Zhu and Fang [105] developed another integrated chip-based droplet analysis system with ESI-MS detection, in which a monolithic ESI emitter and a hydrophilic tongue-based droplet extraction interface were fabricated on a glass chip (Fig. 6A). Using the hydrophilic tongue interface, droplets can be extracted under three modes including zero extraction, partial extraction, and entire extraction, which were controlled by adjusting the back pressure in the segmented flow channel with a back pressure regulator. Stable and reliable droplet extraction was achieved in a broad droplet frequency range of 0.1–10 Hz and droplet size range of 5–50 nL. The detection limit of the droplet analysis system was comparable to that reported by Kelly et al. [104] (Fig. 6B). In addition, we further applied this system to online monitoring of alkylation of a model peptide to demonstrate its potential in the study of reaction dynamics.

Kennedy and co-workers [106] reported a very different and simple method for achieving ESI-MS detection of droplets by directly coupling a droplet cartridge to a commercial nanospray emitter without the need of droplet extraction interface (Fig. 6C). In the droplet cartridge, droplets with different analytes were preloaded in a Teflon tubing and segmented with oil phase and gas bubbles. The carry-over between adjacent droplets in MS detection was minimized due to the isolation and protection of oil and gas. Since the droplets were analyzed without any dilution, low LOD of 1 nM for leucine-enkephalin was achieved with this method (Fig. 6D). In their subsequent work, the method was applied in high throughput and label-free screening of enzyme inhibitors to demonstrate its potentials in drug discovery [107]. A droplet analysis speed of 0.65 Hz was achieved with a volume of 10 nL for each droplet. In order to increase the quantification accuracy of ESI-MS for droplet reaction, an internal standard was added to the droplet to calibrate the concentration of reaction product. The ESI-MS detection for droplets could also be performed in offline mode by first segmenting the eluent from an online separation system into a series of droplets and storing them in a capillary, and then analyze these droplets with ESI-MS [108]. The offline ESI-MS droplet analysis system would provide a useful platform in applications where the separation speed and the MS analysis speed are not compatible, such as liquid chromatographic separation for digest protein sample and multi-stage MS identification for peptides in the elution fractions.

In droplet-based microfluidic systems, surfactants were frequently used to facilitate droplet generation, stabilize the droplets against fusion with each other, and provide a biocompatible environment for in-droplet reactions. However, in droplet analysis systems with MS detection, usually surfactants cannot be used because they will significantly inhibit the ionization efficiency in electrospray process and contaminate the mass spectrometer. Thus, there is a great demand to design and synthesize novel surfactants which are compatible with both droplet systems and MS systems [109].

7. Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is regarded as one of the most powerful analytical detection techniques for elucidating and identifying the chemical structures of analytes,

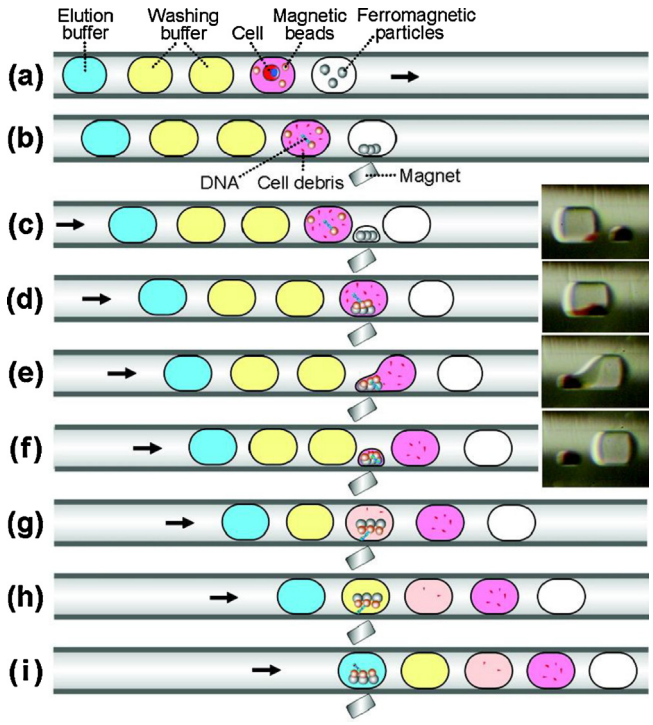

Fig. 7. Schematic illustration showing the process of genomic DNA purification from single cell on the basis of solid-phase extraction among picoliter-scale droplets. (a) Generation of an array of droplets containing ferromagnetic particles, magnetic beads and cell suspension, washing buffer, and elution buffer. (b) Binding of released genomic DNA to magnetic beads after cell lysis and protein digestion. (b–c) Extraction of ferromagnetic particles from droplet. (d) Attraction of DNA-bead complex to ferromagnetic particles. (e–f) Extraction of ferromagnetic particle/DNA-magnetic bead cluster from droplet. (g–h) Removing of interfering cell debris, proteins, and lipids with two washing droplets. (i) Recovery of genomic DNA in elution buffer droplet for subsequent PCR amplification.
Reprinted with permission from reference [22].

which is widely used in the fields of chemical synthesis, drug discovery, petroleum industry, and process control. Conventional NMR spectroscopy is suffered from low sensitivity, slow analysis throughput, and large sample consumption. Microcoil NMR is a recently innovative technique with improved sensitivity and analysis throughput by using a nanoliter to microliter microcoil probe and flow injection analysis (FIA)-based sampling technique [110]. Kautz et al. [111] described the use of microcoil NMR for analysis of compound libraries in droplet array. A sample analysis speed of 1.5 min/sample and a consumption of 2 μL for each sample were achieved. They also demonstrated that the sample dispersion and dilution effects existed in FIA-based sampling systems could be significantly minimized in the droplet-based NMR system due to the protection of the fluorinated oil carrier. The same group further combined the droplet-based NMR system with LC-ESI-MS system to identify unknown compounds in sample with complicated matrix [112]. With the integrated system, several metabolites in a cyanobacterial extract showing antibacterial activities were identified in a single 30-μg injection of the sample. However, several problems still need to be solved before NMR becomes a practical detection technique for droplet analysis systems. The sample pretreatment required for NMR, such as extracting the analytes from samples and re-dissolving them with deuterated solvents, are complicated and time-consuming. In addition, the sample volume required in NMR detection (commonly in the range of 100 nL–10 μL) is much larger than that in typical droplet-based microfluidic systems (pL to nL). Therefore, further improvement of the droplet-based NMR system should include the development

Table 1

Analytical method	Sensitivity	Analysis speed	Labeling required	Advantages	Disadvantages	Main applications
Bright-field microscopy	Poor	Good with high-speed camera	No	Convenient; Imaging the shape, size, color, and trajectory of droplets, and the interaction between droplets; Analysis of heterogeneous reactions inside droplets	Low sensitivity	Droplet generation and manipulation [6,41,44,45]; Mixing inside droplet [47]; Droplet encoding [49]; Protein crystallization [28,50]; Clotting and agglutination reaction [52,53]; Cell and organs [11,43]
Fluorescence microscopy	Good	Fair	Yes	Convenient; Good sensitivity; Quantification	Low analysis speed; Need derivatization	Single cell and single molecule analysis [11,21–23,54]; Enzyme kinetics [16,55,56]; Digital PCR [57,58]; Protein absorption on water–oil interface [59,60]
Laser-induced fluorescence	Excellent	Excellent	Yes	High sensitivity; High analysis speed; Quantification; Large dynamic range	Need derivatization	Single molecule detection [68,69]; Digital PCR [19,20,71,72]; Single cell analysis [25,30]; High throughput sorting and screening [12,32]; Enzyme inhibition assay [73]
Laser Raman spectroscopy	Poor/Good with SERS	Good	No	Label free; Good sensitivity with SERS; Good analysis speed	Matrix effect	Studying mixing and reaction process [82–84]; High throughput detection [86]; Bacteria identification [90]
Electrochemical analysis	Good	Good	Yes	Low cost; Simple structure; Small size; Good sensitivity	Need derivatization; Poor reproducibility	Measuring size, frequency, velocity of droplets [91–93]; Enzyme kinetics [94]
Capillary electrophoresis	–	Fair	Yes	Separation of multiple analytes	Need derivatization with fluorescence detection; Low analysis speed	Separation of amino acids; [95,96] In vivo monitoring of neurotransmitter [97]; Enzyme assay [98]
Mass spectrometry	Good	Good	No	Label free; Simultaneous detection of multiple analytes; Good sensitivity Capability of elucidating chemical structures	Matrix effect; Requiring sample pretreatment	Screening and optimizing reaction conditions [99]; Peptides [103–106]; Monitoring of alkylation reaction [105]; Enzyme inhibitor screening [107]; Off-line LC-ESI-MS [108]
Nuclear magnetic resonance spectroscopy	Poor	Poor	No	Label free; Capability of elucidating chemical structures	Requiring sample pretreatment; Low analysis speed	Rapid identification of compound libraries [111,112]
Absorption spectroscopy	Poor	Good	No	Label free; Good analysis speed; Quantification	Low sensitivity	Detection of aqueous K_2IrCl_6 [114]
Chemiluminescence detection	Good	Good	Yes	Good sensitivity; Simple setup	Limited applications	Detection of aluminum ion [116]

of automated sample pretreatment system for droplets, the minimization of microcoil probe and the increase of sensitivity of NMR detector.

8. Absorption and chemiluminescence detection

Absorption detection is a well-established and widely used analytical technique in conventional laboratories. Trivedi et al. [113] described the combination of absorption detection with droplet system for high-throughput detection of a chemical library in an array of droplets. Optical fiber-coupled LEDs were used as excitation sources and fiber-coupled photodetectors were used to record the signal. Using the LEDs with different emission wavelength, multi-wavelength absorption detection could be achieved simultaneously. Neil et al. [114] applied cavity-enhanced absorption spectroscopy in droplet analysis to increase the detection sensitivity and obtain more chemical information in droplets. A detection limit of 1.4 μM for 6-μL droplets of aqueous K_2IrCl_6 was achieved, due to the enhancement of light absorption between multiple-reflection mirrors.

Chemiluminescence (CL) is a high sensitive detection technique with simple optical structure and system setup. Shen et al. [115] described the use of chemiluminescence detection for measuring the content in an array of droplets trapped in micro-recesses on both sidewall of chip channel. Unlike commonly droplet systems where the droplets were used as individual microreactors, they used the organic droplet array as an extraction module to preconcentrate the analytes in a continuous aqueous sample stream. A detection limit of 1 nM for a model sample of butyl rhodamine B was achieved. This system was further applied to the determination of aluminum ion in water with a LOD of 1.6 μM [116]. However, to the best of our knowledge, the use of chemiluminescence detection for analyzing individual aqueous droplet has not been reported in droplet-based microfluidics. Interestingly, in a recent study, chemiluminescence detection was employed to study the chemical kinetics in levitated droplets [117]. The Michaelis–Menten constant of the reaction of pyruvate with nicotinamide adenine dinucleotide,

catalyzed by lactate dehydrogenase, was obtained with acoustically levitated droplets with a droplet volume of 5 μL.

9. Sample pretreatment

The sample pretreatment operations, such as liquid–liquid extraction, solid-phase extraction, filtering, desalting, purification, and chemical derivation, are important and essential steps in analytical systems, especially for samples with complicated matrixes. In droplet-based microfluidic systems, sample pretreatment procedures are also necessary for the samples with complicated matrixes before they are detected by ESI-MS, CE, and NMR spectroscopic systems. However, the sample pretreatment with complicated and multi-step operations is still a challenge for most droplet-based systems. Gu et al. [22] used DropLab technique [28] to achieve purification of DNA from single cells in picoliter-volume droplets based on magnetic-assisted solid-phase extraction (Fig. 7). The DropLab system can automatically perform complicated droplet manipulation operations required for sample pretreatment, including droplet generation, encapsulation of single cell in droplets, reagent addition, droplet fusion and fission. Another droplet system capable of performing multiplex droplet manipulation and sample pretreatment is SlipChip, which was proposed by Ismagilov and coworkers [118,119]. The SlipChip system can carry out multi-step operations for multiple samples in the nanoliter range in parallel by simply slipping one substrate of a SlipChip. The SlipChip system has been used in bead-based heterogeneous immunoassays in droplet array with multi-steps including immunoadsorption, bead washing, and enzyme amplification [119].

10. Conclusion

In this review, we introduced the development of analytical detection techniques in droplet-based microfluidic systems. The analytical performance, advantage, and disadvantage of each analytical technique are summarized in Table 1. We also discussed the new potentials of these techniques in the fields of high throughput screening, protein engineering, and proteomics. However, so far, the limited ability to read the chemical information in the small-volume droplets is still one of the main bottlenecks in applying droplet-based microfluidics to solve real-world problems. Mass spectrometry and other high-information-content detection techniques hold good potentials to address these problems, while their applications in droplet analysis are limited by the lack of reliable sample pretreatment techniques. Although solid-phase extraction operation has been realized in picoliter-scale droplets [22], the processing throughput is rather lower than those of droplet generation and detection. Therefore, the development of high-throughput sample pretreatment techniques for droplet systems will evidently expand the application range of droplet-based analysis. On the other hand, so far many powerful analytical techniques, such as liquid chromatography (LC), liquid chromatography–mass spectrometry (LC–MS), and capillary electrophoresis-mass spectrometry (CE–MS), are still not applied in droplet systems. The application of these techniques in droplet analysis is in great demand.

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