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Droplet microfluidics in (bio) chemical analysis

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Abstract

Droplet microfluidics may soon change the paradigm of performing chemical analyses and related instrumentation. It is not only the analysis scale and possibility for sensitivity improvement, reduced consumption of chemical and biological reagents, but also the vast increase of the speed in performing a variety of unit operations. At present, microfluidic platforms can reproducibly generate monodisperse droplet populations at kHz or higher rate with droplet sizes suitable for high-throughput experiments, single-cell detection or even single molecule analysis. In addition to being used as microreactors with volume in the micro- to femtoliter range; droplet based systems have also been used to directly synthesize particles and encapsulate biological entities for biomedicine and biotechnology applications. This minireview summarizes the various droplet microfluidics operations and applications for (bio) chemical assays described in the literature during the past few years.

Introduction

The past decade has seen increased interest in chemical reactions and development of tools for fluid flow control at the microscale. This multidisciplinary field involves fundamental concepts from different areas including physics and electrical engineering to biology. Emulsions are also heavily used in personal care products, foods, and topical drug delivery; however, these applications do not require uniformity of the droplet sizes. More demanding applications are now exponentially increasing and include, e.g., microreactors for catalysis and chemical synthesis, point-of-care diagnostics, drug delivery, cell/molecule compartmentalization and diagnostic testing (1-4). Most of the common laboratory unit operations can be performed quickly using minimum volumes of reagents and samples (volume reduction from milliliters to femtoliters). In addition short diffusion distances and higher surface to volume ratio can often reduce reaction times from hours to seconds or less.

While the origins of the droplet based microfluidics can be formally traced back to the segmented flow analysis and flow injection analysis concepts developed over 40 years ago, the current technology utilizing droplets in micrometer to nanometer diameter range relates more to the development of microfabrication techniques, micro-total-analysis and the lab-on-chip concepts (5-7). Droplet – based microfluidics is one subcategory of microfluidics, where drops serve as the transport and reaction vessels. The principle of the droplet formation is based on using of two immiscible liquids where the dispersed (droplet forming) phase is injected into the stream of a continuous phase, surrounding the droplets. Fast reaction times in such small compartments are induced by the high surface area to volume ratios, efficient heat and mass transfer and short diffusion distances. Most of the references deal with free floating droplets, which, unlike liquid plugs confined by the channel geometry require using stabilizing surfactants.

In this review, we are summarizing the recent advances in droplet formation and manipulation technology and chemical analysis applications with the stress on the new developments in the past 4 years. For more comprehensive review of the field prior to 2010 we recommend the excellent article by Huck et al. (8).

Principles of droplet formation

Emulsions (suspensions of droplets created using two immiscible fluids (9)) are widely used in a number of industrial areas including machining, civil engineering and mining. In turn, the formation of stable droplets are the key component of the droplet microfluidics. Regardless weather their final use is in microreactors or in particle/cell-based applications (10-13) the droplets must meet the desired properties, including the size, shape and monodispersity of droplets (14) and resistance to coalescence during the use and storage (15-17). Experiments with living cells or higher organisms droplets should also provide a biocompatible environment (18). The use of surfactants are essential for droplet and stability of the emulsion - resistance to coalescence (19). Their choice depends on the nature of the continuous phase influencing biocompatibility, viscosity defining the droplet formation through shear stress and exchange of gases (20). The most common continuous phases include hydrocarbon and fluorocarbon oils. In

order to stabilize hydrocarbon oils (mineral oil, decanol, hexadecane, etc.), commercially available surfactants such as Abil EM (cetyl dimethicone copolyol) and Span 80 (sorbitane mono-oleate) are used (21-24). Perfluorinated hydrocarbons (perfluorodecalin, perfluorotributylamine FC-43, FC-70 etc.) are often preferred over mineral oils, due to their immiscibility with organic and aqueous solvents, their high oxygen transport capability, physical and chemical stability. Fluorocarbon oils are typically denser than the dispersed phase (25). Droplet emulsions in the fluorinated continuous phase can be stabilized by perfluoropolyether (PEPEs) based surfactants such as Krytox, (a commercial lubricant from DuPont), consisting of PEPE tail and a carboxylic acid head group (26). This compound has been characterized in detail with regard to biocompatibility and long-term storage (26). In recent publications, several surfactants featuring polyethylene glycol (PEG), carboxy-PEPE, poly-L-lysine (pLL) or dimorpholino phosphate (DMP) head groups have also been used (26). Detailed information regarding the components of some of the described emulsions is listed in Table 1.

Table 1 Reported components of droplet emulsions in microfluidics

Type of emulsion	Continuous phase	Surfactants	Geometry
		Fluorinated oils	
W/O	1H,1H,2H,2H-perfluorodecyltrichlorosilane, Fluorinert FC 3283; FC 40	PEPE-PEG; PFPE−PEG−PFPE triblock copolymers; PFPE−PEG−Gold diblock surfactants (home-made); EA surfactant;	T-junction; Flow-focusing (27-30)
	Fluorinated HFE-7500	EA surfactant; Krytox–Jeffamine–Krytox A–B–A triblock copolymer	Flow focusing (31,32)
	Fluorinert FC 40	PFPE-PEG block copolymer	Flow-focusing (33)
	Fluorinert FC 70, FC 40	PEPE-PEG	T-junction; Flow-focusing (25)
		Hydrocarbon oils	
	Mineral	Span 80; Tween 80	main channel to the flow rate

			in the external access channel (34)
	Hexadecane	Span 80	Single T, K-junction (35,36)
O/W	Soybean; Hexadecane, Silicon	PEG, sodium dodecyl sulphate (SDS)	Flow-focusing (37,38)
	Mineral; Silicon	Pluronic F-68	T- junction (39)
	n-Hexadecane	SDS	T- junction (40)
G/L	Nitrogen	dispersed phase – deionized water (90% (v/v) glycerol; 2 wt % SDS)	Flow-focusing (41)
L/G	Propane, Butane, Air	2 wt % Tween-20	T-junction (42)

Droplet formation - Microfluidic dynamics

Parameters describing microfluidic systems can be described by the balance of inertial force, viscous drag forces, buoyant forces and interfacial tension forces. There are a few of them useful for description of the droplet formation in a microfluidic device - the Reynolds number (*Re*), Weber number (*We*), Bond numbers (*Bo*), Capillary number (*Ca*) and flow rate ratio. Each one describes a particular aspect of fluid flow within a device. They can be used together to predict if a particular microfluidic system will allow droplet formation to occur and if the device can be used for a specific application (43).

Reynolds Number

Re is a dimensionless number that characterizes the flow of fluid through a channel. It is the ratio between inertial forces and viscous forces which quantifies the relative importance of these two under given flow conditions. For flow in a pipe or around a spherical particle $Re=\rho VL/\mu$, where ρ is the fluid density, V is the mean velocity, L is a characteristic length and μ is the dynamic viscosity. *Re* can describe different flow regimes within similar fluids such as laminar and turbulent flows. Generally, in microfluidic systems operating with liquids, a value of the *Re* <<

2300, indicates a laminar flow under practically all experimental conditions. A turbulent flow at $Re > 2300$ may be observed in microfluidic systems operating with gases. (11)

Weber number

The Weber number describes the ratio of inertial forces to surface tension $We=\rho V^2 L/\gamma$, where ρ is the fluid density, V is the characteristic velocity of the flow, R is the characteristic size (e.g., the droplet diameter) and γ is the surface tension. If We is larger than the critical value, $We_{crit}=1.1$, the droplets are strongly distorted by the inertia forces and breakup may occur (44). In case of a very low We, inertia is negligible; for a moderate We, inertia deforms the droplet without disrupting it.

Bond number

The Bond number determines the relation between gravitational surface forces and surface tension forces $Bo=\Delta\rho g L^2/\gamma$, where $\Delta\rho$ is the difference in fluid densities (kg/m^3), g is the gravitational acceleration (m/s^2), L a characteristic length scale, and γ is the surface tension between two fluids (N/m). For Bo < 1, the gravity effects can be ignored (45).

Capillary Number

One of the most important parameters for droplet formation is the capillary number used to compare the viscous stress with respect to interfacial tension phenomena at the liquid-liquid interfaces (46). Capillary number is defined as $Ca=\mu V/\gamma$, where μ is the fluid viscosity, V is the velocity, and γ is the surface tension. At low values of Ca (<1) it appears that the surface tension forces are dominant over the viscous force and droplets are spherical. At high values of Ca (>>1) the viscous force plays an important role, leading to deformation of the droplets by the flow, characterized by asymmetric shapes (47).

In summary, Re describes the relative importance of inertial forces to viscous forces; Ca represents the effect of viscous forces to interfacial tension forces and We number compares the inertial effects to surface tension. Due to the fact that microfluidic systems are scaled in micrometer length, Re is less than 1. Thus the Ca and We values play a more important role and interfacial effects become dominant. It should be noted that the geometry of microfluidic

channels often play a key role in the droplet formation. A number of microfluidic configurations will be introduced in the next section.

Droplet generation

There are many methods for making emulsions; however, most have been developed for large batches in industrial use, leading to polydispersity. A variety of methods for droplets formation have been investigated and can be categorized into two groups, i.e., passive and active approaches. In both cases, the key parameters are the geometry of the microchannel, the flow rate ratio, and the capillary number to achieve droplet formation (48). Chen et.al. (49), define several methods for droplet formation including:

- hydrodynamics (T, Y – junctions, flow focusing, co-flowing)
- pneumatic pressure (the gas pressure is used as the shear force and diving force to form droplets)
- optically-driven (the principle using optical force to manipulate particles in microfluidic chips was successfully applied for the droplet generation)
- electrically driven techniques: dielectrophoresis (DEP) and (EWOD) electrowetting on dielectric (49, 50).

In this section, we will focus on the most important microfluidic configuration for droplet generation including T – junctions, flow focusing and co-flowing (hydrodynamic method).

T-Junction

The most common microfluidic geometry used for the generation of droplets is the T-junction. In this configuration, the inlet channel, containing the dispersed phase, intersects the main channel, containing the continuous phase. The channels are typically arranged orthogonally and droplets form at the channel intersection. This geometry can also be used for the formation of bubbles and/or double emulsions. Depending on the capillary number three main regimes in the T-junction microfluidics can be distinguished – dripping, jetting, and squeezing. At high *Ca*, the small droplets are formed, provided that the large viscous drag of the continuous phase shears the dispersed phase from the inlet of the channel. At low *Ca*, droplets larger than the microchannel could be obtained by blocking the microchannel downstream, as shown in Fig.1 (A).

Co-flowing Devices

There are two basic types of co-flow systems. Dripping and jetting are highly dependent on the flow rates of the liquids. At low flow rates, dripping is the predominant regime and is the product of absolute instability as the forces acting on the stream of water occur at a specific frequency and location in the stream. At higher flow rates, jetting is predominant and is the result of convective instabilities. If a monodisperse droplet formation is required, a system affected by absolute instabilities would be desired. In a microfluidic set up, this is achieved through careful control of the dynamics affecting the droplet formation. Droplet formation in co-flow systems highly depend on fluid velocities, viscosities, surface tension and the densities of the liquids. The geometry of the droplet formation region in the microfluidic device is important as well. Co-flow in microfluidics occurs as the dispersed phase is forced through a small capillary centered inside a larger diameter channel with the continuous phase flowing parallel to the dispersed phase as shown in Fig.1 (B). As the continuous phase surrounds the dispersed phase, the viscous shear force becomes stronger as the diameter of the dispersed phase increases. Eventually, the drag and interfacial tension forces become equal at the tip of the capillary and a droplet is formed and pinched off from the dispersed phase. By establishing an absolute instability in the system and optimizing all the variables, droplets can be formed at uniform frequencies and sizes.

Flow Focusing Devices

In this droplet formation arrangement, symmetric channels, supplying the continuous phase, surround the channel delivering the dispersed phase. As the dispersed phase flows into the open channel, the continuous phase forces the dispersed phase through a small orifice leading into a larger channel filled with the continuous phase. Droplets are formed as both phases are forced through the orifice. The continuous phase provides pressure to drive droplets through as well as the shear forces to separate the phases into different droplets. In flow focusing techniques, droplet size, velocity, and frequency can be tuned through the flow rates, controlling the inlet pressures, varying the phase viscosities, and the orifice size Fig.1 (C).
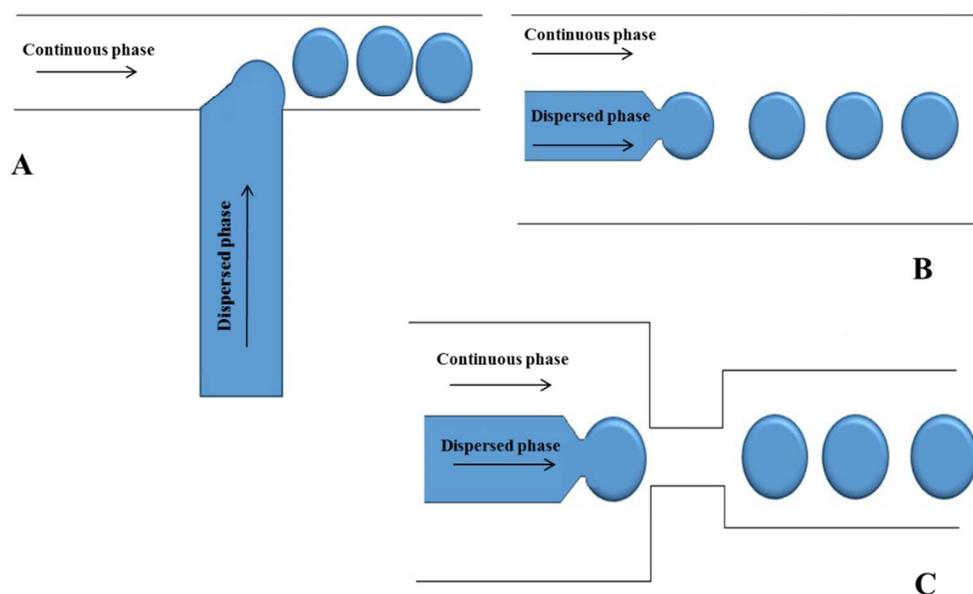
Fig.1. Schematics of microfluidic devices for making emulsions using A) standard T-junction flow focusing junction; B) co-flowing junction; C) flow focusing junction

Droplet manipulation

Division of droplets

Droplet splitting or division into two or more "daughter droplets" is an important operation required for droplet microfluidic manipulations. The operation principle depends on the chip geometry and fluids. It can be achieved using a T-shaped channel when a droplet flows to the bifurcation section where it is stretched by the extension flow (51). If the droplet is large, it will be thinned and split into two or more droplets as it passes through the channels. It was demonstrated that an asymmetric splitting can be achieved by changing the length of the branch channels. Fabricating the microfluidic tree network consisting of four Y-junction Zhao et.al. described the flow focusing junction approach for the droplet splitting (47). The droplets were divided along their length in the flow and number/size of the daughter droplets could be regulated by changing the flow rate of the splitting liquid. Other studies have shown that microfluidic devices allow nanoscale volume sampling from oil-segmented fluid plugs by passive splitting. The system allowed thousands of plugs to be asymmetrically split with no performance deterioration over time. The resulting plugs might be collected into separate channels for storage or other manipulation. In another approach fluid plugs splitting is based on

fluidic resistance (52). Kennedy et.al. have developed a microfluidic device in which nanoliter oil segmented fluid plugs were pumped into a loop and asymmetrically split according to the flow resistance. The device with microfabricated posts inside the recombination region downstream of the split kept the stream of drops from contacting and coalescing. It allowed to sample from plugs by asymmetrical splitting and collecting the split aliquots for further use (53).

Sorting

Droplet sorting is important for selection of specific droplets from a larger population for the next step of analysis. There are many ways of influencing the movement of the droplets in either serial or parallel fashion. The forces necessary for influencing the droplet movement are most often based on fluid flow, acoustic waves or coulombic attraction/repulsion. Dielectrophoresis is an example of an electrically driven technique, which can be operated in both serial and parallel modes. The process is based on a force exerted on a particle with different polarizability relative to the surrounding medium in an electric field gradient. Dielectrophoresis is a rapidly growing field as recently documented by special issues of scientific journals (54, 55). An example of a microfluidic device for high-speed sorting of water droplets using DEP has been described using microelectrodes prepared underneath polydimethylsiloxane (PDMS) channels of a microfluidic device. A sorting rate of over 1.6 kHz and a force of more than 10 nN has been reported (56). Similar approach has also been described for manipulation of water droplets using a microelectrode array and AC voltages across the electrodes with 25 V magnitude at frequency ranging from 50Hz to 1MHz (57). In most cases the electrodes are prepared in the form of thin metal layers with a predefined geometry. In an alternative approach "virtual" electrodes can be formed by illumination of a uniform photoducting layer, which becomes electrically conductive only upon illumination. In this way the electrode geometry can be easily changed by projecting different light patterns on the semiconducting layer (58). Recently, the optically induced dielectrophoresis (ODEP) platform was investigated by Hung et.al using a new voltage transformation ratio simplifying the control of the photoconductive effect and the resulting ODEP force (59). The results for the droplet manipulation showed that the numerical simulation conforms to the experimental data with a coefficient of variation of less than 6.2% (n = 3).

An important application of the rapid serial sorting is the cell characterization/separation in flow cytometry (fluorescent-activated cell sorting, FACS). Ultrasensitive fluorescence monitoring is used for each cell and the speed of analysis is limited by mainly by the serial detection process. While the speed of analysis can be increased by increasing the flow rate, at excessive flow velocities, the shear forces can damage or kill cells (60). Related to this limitation, Bai et.al. proposed using FACS microfluidic platform for droplet generation and protective cell encapsulation (61). Wu et.al. presented an integrated microfluidic droplet generation with fluorescence-activated droplet sorting (FADS) for single cell encapsulation, using hydrodynamically gated injection with stopped flow technique (62-64). This method allowed encapsulation of cyan fluorescence protein transfected HeLa cells, with the encapsulation and recovery rates of 94.1 and 85 %, respectively.

The application of acoustic forces (Surface acoustic waves - SAW) is mostly related to the continuous flow particle sorting (65, 66). Recently Li et.al. demonstrated sorting of water in oil droplets into multiple outputs using standing surface acoustic waves (SSAW). The device integrated microfluidic multichannel system with interdigital transducers (IDTs) to achieve droplet generation and sorting. The position of the pressure nodes could be changed by regulating the frequency of the SSAW excitation power with droplets sorted to multiple outlets at rates up to 222 droplets s^{-1} (67).

Fusion

Chemical and biological analysis using reactions in droplets can be used for a number of applications, including chemical synthesis, microfractionation in droplets, monitoring of reaction kinetics, formation of particles, etc. (68, 69). Controlling coalescence (fusion) of droplets containing samples or reagents is a critical step for such applications. In practice, it is necessary to bring the droplets into a physical contact and overcome the stabilizing effects of the surfactants (11). It should be noted that fusion of droplet depends on the viscosity ratio of the internal and external fluids as well as the presence of surfactants at the interface. There are several passive and active methods for controlling droplet fusion. Passive methods provide transfer of droplets into close proximity by the microchannel geometry to slow down the downstream drop until the upstream drop reaches it. Lower viscosities of the droplets enable a quick draining of the film between the immiscible fluids that facilitate coalescence of the

droplets. Conversely, higher viscosity leads to reduction of the mobility of the interface and reduced chance for coalescence of droplets (70).

Detection methods for droplet microfluidics

Fluorescence

Fluorescence measurements using fluorescence microscopy with a camera to image the analyzed droplets belongs to the most common detection modes. Marcoux et.al. developed a microfluidic device for measurement on enzyme activities of single-cells (20). The technique is based on stochastic confinement of cells into picolitre sized microreactor droplets. The enzyme activities were monitored by the fluorogenic reporter molecule 4-methylumbelliferyl-β-D-glucuronide bound to fluorescence imaging of the droplets. Moreover, this approach leads to fast accumulation of fluorescent tracer and reduction of the analysis time. Enzyme activity detection was also studied by the Juul's et.al (71). They fabricated and integrated a rolling-circle-enhanced-enzyme-activity for single-molecule-detection with a custom-designed microfluidic device allowing multiplexed detection of enzymatic events at the aberrant cells in a population. Gu et.al developed a multifunctional droplet-based microfluidic platform named DropLab (72). The present device provides complex picolitre-scale droplet manipulation and single-cell enzyme activity detection using fluorescent microscope. One of the simple microfluidic systems demonstrated a laminar, diffusive mixing flow combined with parallel droplet formation in a T-shaped channel (73, 74). Enzyme reactions have been also studied by Suiti et.al. with a nanoporous container (19 pL) allowing to carry out reactions under continuous flow (75). It has been shown that a 10 nm pore size allows capturing of proteins and diffusive exchange of small molecules. Ismagilov et al., described a SlipChip design based on multiplexed reactions for screening of different reagents in nanoliter volumes (76). Quantitative measurements of fluorescent intensities and high-resolution imaging were performed in these glass SlipChips. Hatch and co-authors successfully demonstrated a single microfluidic device for rapid droplet generation, thermocycling, and wide-field fluorescence imaging of 1-million droplets with a micro-lens digital camera (21). This system was applied for DNA detection under digital PCR. The PCR amplification resulted in approximately 30 000 copies of the compartmentalized gene in each droplet. In the next step the droplets fused with droplets delivering a cell-free coupled transcription–translation (IVTT) system and the reagents for a fluorogenic assay. Genes were

selectively recovered from droplets using Fluorescence-activated electrocoalescence. This system can sort at 2000 droplets s^{-1} by selecting mixtures of *lac*Z and *lac*Zmut genes (77).

Fluorescence imagining can also provide spatial distribution of the molecules in the droplets in a quantitative manner. This approach was used to characterize the development of the shape, size and the fluorescence intensity change inside the cells (78). Fluorescence imagining was also used in conjunction with dielectrophoresis and optical tweezers (79) for detection of the viability of cells in individual droplets (80). The presented platform included single cells encapsulation in picolitre droplets, incubation for several hours and imaging using automated microscopy to determine cell viability (Fig.2.) (81, 82).

The fluorescence lifetime imaging microscopy can also provide information about the internal environment of the observed droplets (83, 84). Bennet et.al has shown the capability of optical tweezers for manipulation of objects with temperature-sensitive fluorescence lifetime imaging microscopy. Droplets with encapsulated Rhodamine B were used in the experiment. In addition to fluorescence the encapsulated fluorophore dye provided the refractive index differential required for optical trapping (79).
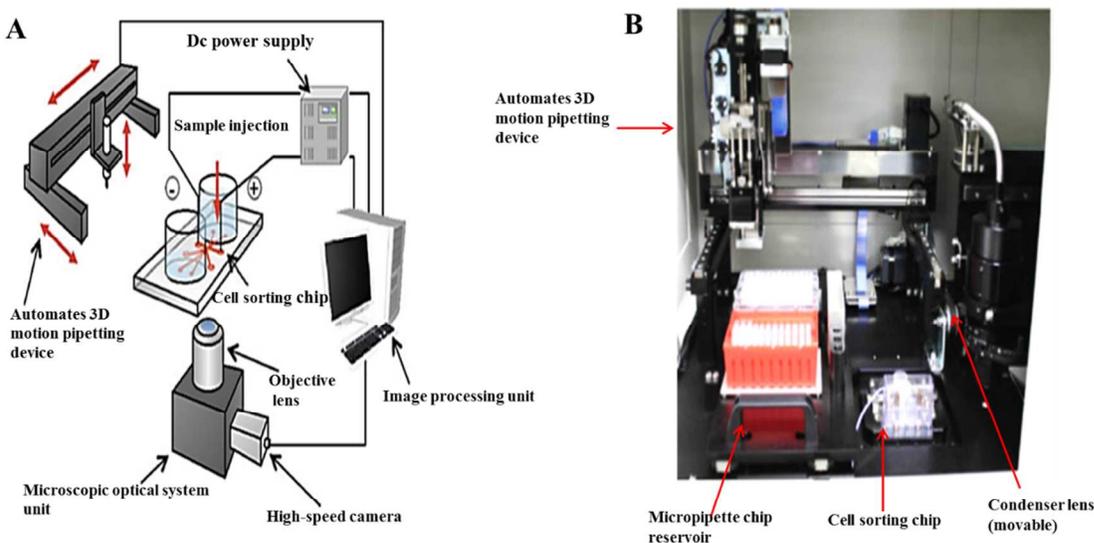

Fig. 2. Automated on-chip imaging flow cytometry system with disposable contamination-free plastic cultivation chip: A) schematic diagram, B) photograph. From reference (82); with permission.

Mass spectrometry

Mass spectrometry (MS) brings another dimension to droplet assays (85). In the simplest case (single quadrupole or linear time-of-flight based instruments) it can provide information about the mass to charge ratio of the analytes (86, 87). More complex instruments, combining two or more mass analyzers, can often provide substantial structural information via the analysis of molecular fragments. Current instruments featuring electrospray ionization (ESI) or matrix assisted laser desorption/ionization (MALDI) offer very good sensitivity in the low attomole range (88). The mass spectrometer is usually the most complex part of the analytical system the connected microfluidics act as the sample preparation tool (89-92). Since the sample volume consumed during the electrospray/mass spectrometry experiment is in micro- to nanoliter range, the droplet/MS analysis represents a viable approach for limited sample quantities (93, 94). Kennedy et.al successfully demonstrated the ESI-MC coupling to nanoliter scale flow reaction for label-free screening of reactions. The system was used for analysis of the cathepsin B inhibitor with ultralow reagent consumption and high-throughput (95). Yang et.al. developed a segmented flow protocol to combine nano- liquid chromatography separation with MC in proteomics research. The protocol is based on segmenting of the liquid chromatography (LC) effluent and analysing the segments off-line by mass spectrometry. A tryptic digests of a T. tengcongensis sample were analyzed using the segmented flow interface between nano-LC and MS (96). Fang et.al. developed a multifunctional microfluidic droplet-array system capable of performing multi-step sample pretreatment, introduction and ESI-MS detection using a capillary as a sampling probe and electrospray emitter to interface an oil-covered two dimensional (2D) droplet array to ESI-MS (97). Girault et.al. demonstrated a new approach for coupling droplet based microfluidics with MS using capacitive charge transfer across the boundary of immiscible liquids to monitor single-phase and biphasic reactions. An advantage of the presented system is using of dilution-free droplet ionization without the additional oil removal step, surfactant or sheath-flow (98). Kuester et.al. described a novel interface between droplet microfluidics and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry for label-free analysis of up to 26 000 individual aqueous droplets on a microarray plate (92). The system enabled the synchronization between of stage movement and the output of droplets from the capillary as well as the prevention of cross-contamination. The authors studied the efficiency of the system by monitoring the formation of an octapeptide (angiotensin II) from a decapeptide (angiotensin I)

during an enzymatic reaction within several hours (92). Baker et.al., successfully built a system consisting of a transfer capillary, a standard ESI needle, and a tapered gas nozzle addressing some of the challenges of integrated droplet microfluidics with MS detection. A transfer capillary was used to couple a microfluidic device to an ESI needle. The operation principle was based on applying a pulse of N_2 to the nozzle; a pressure differential induced at the outlet of the ESI needle pushed the droplets from the device, passing the ESI needle and entering the flow of N_2 to the MS inlet. Ionization potential and N_2 pressure were 3206 V and 80 psi, respectively. Open and closed devices were demonstrated from 630 nL to 2.5 μL aqueous droplets mixed with droplets of caffeine and theophylline internal standard transferred from the device to the ESI/MS. The ratio of the caffeine/theophylline peak areas has been detected in the range of 5-250 μg mL^{-1} (12.5-625 ng per droplet) with limit of detection (for caffeine) of 1 μg/mL (2.5 ng per droplet). The ability to rapidly mix droplets makes devices a promising platform for relative quantitation by MS (89). Smith et.al. developed ESI-MS to measure femtomole amounts of proteins with molecular weights of 1 to >100 kDa in an individual droplet (99). The droplets were generated at a frequency of 171 Hz in a flow-focusing geometry microfluidic design using fluorocarbon oil which was found suitable as the continuous phase. The produced droplets (107 μm or 650 pL) containing α-chymotrypsinogen A (50 μM) were collected off-chip to be stored overnight at 4 °C and re-injected into emitter devices before ESI/MS analysis (Fig. 3). The mixture of droplets occurring at >150 per minute provided high quality mass spectra of surfactant stabilized droplets.
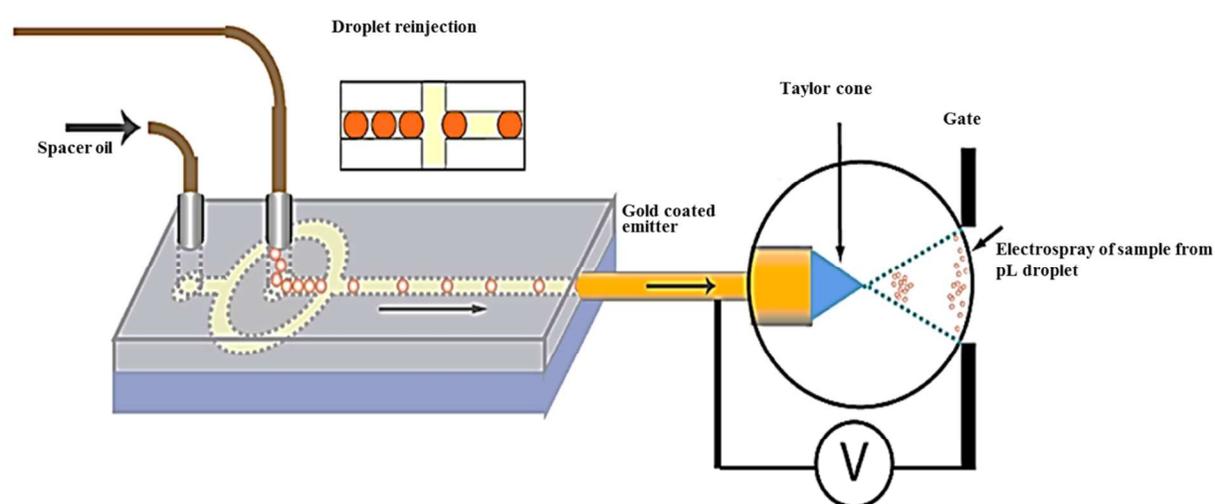
Fig. 3. Picoliter droplets containing α-chymotrypsinogen A (50 μM) were generated at 171 Hz in a flow-focusing droplet generator, after storage the droplets at 4 °C, they were re-injected into the emitter device for mass spectrometric analysis. From reference (99); with permission.

Kirby et.al., have broadened the application of the integrated system with in-line MS analysis and developed "microfluidic origami" microchemical reactions (91, 100, 101). This device included a microfluidic platform and nanoelectrospray ionization (nanoESI) emitter formed on a single flexible polyimide film substrate. This device was used to qualitatively monitor the reaction progress of the Morita–Baylis–Hillman reaction in real-time. It was possible to carry out the microchemical synthesis through applying an in-line interface connecting the microfluidics and MS.

Raman spectrometry

The Raman spectra can provide an information about the analyte structure, in the form of a spectral fingerprint as well as can be combined with other detection methods. While the tradiyional Raman spectrometry is inherently insensitive, recent developments in the surface enhanced Raman spectrometry (SERS) make this technique also suitable for microfluidic systems (102). Combining droplet techniques with SERS can significantly broaden the detection information in droplet systems (103). Cecchini and co-authors developed an ultra-high throughput SERS system for droplet reaction detection with submillisecond time resolution as a powerful tool. Such technique may allow the study of millisecond enzyme dynamics and high throughput screening of protein evolution in a label-free fashion (104). In the subsequent studies, SERS was applied to high sensitive detection of Rhodamine 6G (R6G) by enhanced Au nanoparticles-coated chitosan microbeads, and paraquat in water with limit of detection $2*10^{-9}$ M (105, 106). Qu et.al. demonstrated SERS detection approach by fabricating a novel Au/Ag bimetallic bell shaped droplet microfluidic SERS sensors on cellulose paper that could be integrated to portable analysis of substituted aromatic substances. In another paper, Walter et.al. developed a SERS based microfluidic lab-on a-chip for bacteria identification using the Raman spectroscopy fingerprinting. An advantage of this system is reducing the spectral recording time to 1 s with generating a database of 11200 spectra is established for a model system Escherichia *coli* including nine different strains. The validation accuracy of support vector machine was up to

92.6% according to chemometric analysis. This new method has also demonstrated the potential for bacteria classification with high reliability (107). Currently, Li et.al. reported a technique called "stamping surface-enhanced Raman spectroscopy" (S-SERS) for label-free, molecular sensing and imaging. This approach involves technique takes an advantige of a polydimethylsiloxane (PDMS) thin film used as a carrier for target molecules, which were deposited on the PDMS form dried and stamped onto an nanoporous gold disks with high-density of plasmonic hot spots as the Raman signal enhancer. This design has enabled SERS measurement of "sandwiched" target molecules (R6D and urea) for concentration ranging from 10 nM to 100 μM (108).

Electrical measurements

One of the main challenges in the development of droplet microfluidic devices relates to the measurement of the droplet size, frequency, conductivity, velocity and composition (109, 110). While the electrochemical detection is less universal than mass spectrometry, it provides a scalable, low power, cost-effective alternative to microscope/fast-camera systems commonly used for monitoring droplets, measuring droplet size and velocity. Moiseeva et.al. proposed a system to generate droplet trains that could be detected by measuring electrical impedance at thin-film electrodes (111). The developed system allowed simple low-cost measurments to be. Cahill et.al. developed the method for impedance measurement of the conductivity of aqueous droplets in a segmented-flow system (112). A thin-walled glass capillary with electrodes contacting the outer surface was used to provide the contactless measurement of conductivity of the liquid within the capillary. The developed system provided a low–cost and robust method well suited for in-line measurement of impedance spectroscopy in a segmented flow.

Electrophoresis

Recent years have seen the development of new methods for sampling and elecrophoretic analysis of aqueous plugs segmentetd in an oil stream. The applications include sample manipulation   for high-throughput screening, analysis of products of in-plug reactions, or microdialysis sampling (113-115). Chiu et.al. explored coupled microfluidic devices with capillary electrophoresis separation by following laser-induced fluorescence of single femtoliter-volume droplets. The droplets were generated using a T-channel connected to microinjectors and

moved towards the region with immiscible boundary isolating the CE separation channel. The device also incorporated patterned surface with differential wetting for selective deposition of a hydrophilic polymer in required regions of the chip (116). De Mello et.al. described a simple platform incorporating droplet-based microfluidic module for the precise injection of pL- to nL volume of sample to the capillary zone electrophoresis and capillary gel electrophoresis (117).

Biomolecules detection

Compartmentalization

The compartmentalization of reactions in picoliter-sized drop is effectively used for a wide range of experiments where individual droplets contain key reagents for further experiments. The droplet technology avoids eliminating the dispersion of reactants and decreases consumption of reagents (8). With lower concentration of samples, as well as the enclosed space of a droplet, compartmentalization allows control of the microenvironment for individual cell reactions. Massive parallel handling of millions of independent reactions enables the analysis of vast populations of single-cells to detect rare events. These technologies were employed by Tawfik and Griffith's et.al., where the fulfilment co-compartmentalization of single genes saved the genotype-phenotype linkage using aqueous droplets in oil emulsion (118). This system also allowed the study of in vitro transcription/translation solution for protein expression and enzymatic reactions. Fluorescence-based sorting of the droplets allowed timing of the initiation of an enzymatic reaction. Similar approach was demonstrated by Agresti et.al., who carried out co-compartmentalization of yeast cells expressing horseradish peroxidase (HRP) on the cell surface parallel with non-fluorescent substrate. This system has demonstrated a 10-fold increase of the catalytic efficiency of HRP and has also shown the possibility of using yeast cells surface display in conjunction with microfluids for enzyme engineering (119).

Microfluidic devices for PCR have been investigated by several groups. Geng et.al. described the compartmentalization of genomic DNA from single-cells along with primer-functionalized microbeads in agarose-in-oil droplets (120). Ellefson et.al. presented the phenotype compartmentalization partnered replication into water in oil droplets, where synthetic circuits were linked to the production of Taq DNA polymerase that evolved circuits. More efficiently drive Taq DNA polymerase production are enriched by exponential amplification during a

subsequent emulsion PCR step (121, 122). Another system was demonstrated by Zhao et.al. who described a microfluidic Slipchip device to compartmentalize target analyte bands *in situ* into separate microdroplets collected and moved for further analysis (123). The Slipchip generated droplets in parallel by slipping layers of a planar chip to different lateral positions. The system incorporated a microfluidic isoelectric focusing (IEF) separation followed by in situ compartmentalization as shown schematically in Fig.4 A-C.
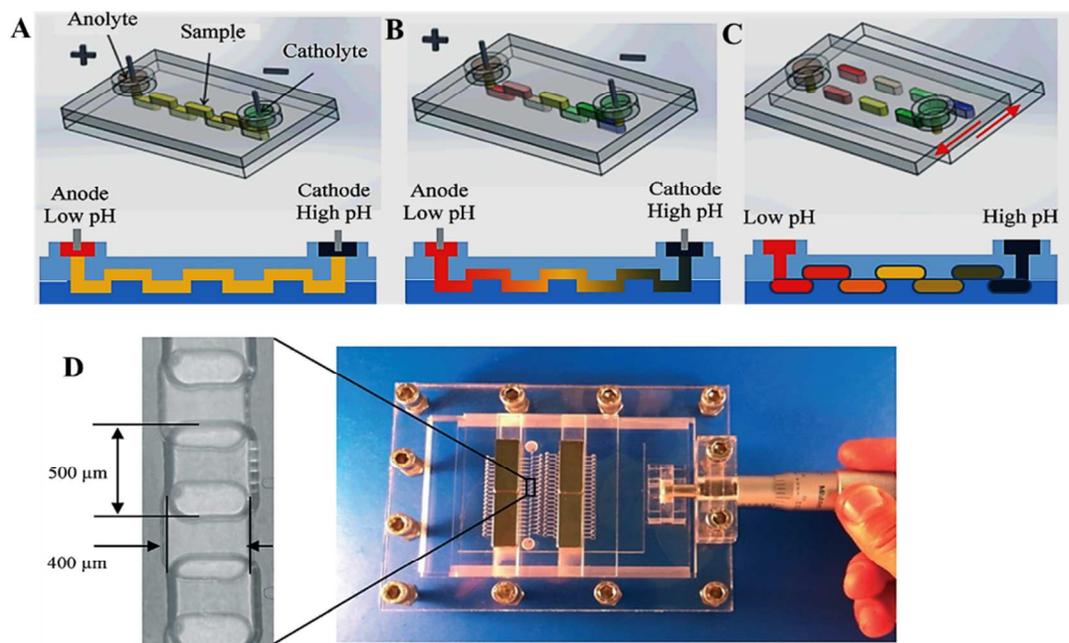
Fig. 4. Scheme of the IEF separation and in situ compartmentalization in a Slipchip. (A) Sample loading to a continuous "zig-zag" channel. (B) pH gradient is established and IEF is performed after application of an electric field. (C) In situ compartmentalisation after IEF separation. (D) The microdevice and platform made of PMMA, contained a "zig-zag" channel with dimensions of 400 μm × 250 μm × 5 cm. From ref. 123; with permission.

The device operation included the following steps: at first, an IEF separation was performed in a microfluidic zig-zag channel composed of a sequence of wells formed in the two halves of the Slipchip (Fig. 4. A) and the analyte focused along the channel by application of an electric field (Fig. 4 B). Slipping the chip disconnected the wells, leaving the analyte in isolated compartments or single droplets in each of the wells (Fig. C). The device was tested using standard pI markers as well with chip-based gel electrophoresis of a mixture containing 5 standard proteins (trypsin inhibitor, β-lactoglobulin A, carbonic anhydrase isozyme II, myoglobin, and lectin). It should be

noted that this method is capable to avoid remixing, is scalable and can be hyphenated with other analytical methods.

Single-cell detection in droplets

With the advancements in the field of cell biology and medicine, there is a considerable need for a device allowing multiparameter measurement and manipulation of single-cells (124). Microfabrication technologies are in many aspects well suited to bring such new tools for single-cell analyses avoiding problems related to common sample handling variability and contamination when the raw material from single-cells is diluted to microlitre volumes. With the development of microfluidic devices some of the problems can be minimized as far as devices comprise an interlinked series of channels, reaction microvalves, sample read-out devices, all of which are physically integrated on a microfabricated solid substrate (125, 126).

In this section we focus on recent reports of single-cell analysis using fluorescently tagged probes including antibodies (127), nucleic acid (128), gene expression at the single-cell level, enzyme kinetic reaction and bioimagining (129). Detection of an individual cells is performed using fluorescence microscopy imaging cells inside microfluidic devices. Rane et.al. (130) have developed a microfluidic chip for the pathigenic cell detection and quantification by screening the fluorescent droplets using confocal fluorescence spectroscopy (CFS). The experimental setup included a custom metal plate to act as an interface between the microfluidic chip and the CFS setup. A Peltier heater under the 'incubation zone' on the microfluidic chip was included on the metal plate to maintain a fixed incubation temperature throughout the experiment. The setup allowed to carry out a sample digitization, cell lysis, probe–target hybridization and subsequent fluorescent detection with dual laser excitation (488 nm and 633 nm) as well as simultaneous dual color detection (520 nm and 670 nm). A peptide nucleic acid fluorescence resonance energy transfer probe (PNA beacon) was used to detect 16S rRNA in E.*coli* cells. The same device could perform the "sample-to-answer" pathogen detection of single-cells (130). A number of analytical approaches have utilized the flow cytometry for both quantitative and qualitative multi-parametric analyses of single cell in genetics, diagnosis, and cancer research (131, 132). Some of the limitations common to conventional flow cytometry have been addressed by

microfluidics-based miniature flow cytometry devices (133). In particular, fluorogenic assays by measuring enzymes markers can now be used for single cell studies (134, 135).

Recent years have witnessed the development of microfluidic approaches for the monodisperse droplet formation and manipulation in association with fluorescence detection (136, 8). Arayanarakool et.al. developed a micro- and nanochannel network for the femtoliter aqueous droplet generation. The tiny droplets provide an ideal environment for single-enzyme activity assay where enzyme and substrate solutions were confined into femtoliter volumes helping in achieving the high product concentration without increasing background noise. Researchers have demonstrated single enzyme molecule encapsulation on the chip and measurement of the kinetic activity of single β-glucosidase molecules (137). Most recently, Guan et.al.have proposed a microfluidic method enabling a single molecule counting and digital protein detection in picoliter droplets via enzyme catalyzed signal amplification. The developed chip allowed to carry out an injection of two separate flows such as single β-galatosidase molecules and the fluorogenic substrate fluorescein di-β-D-galactopyranoside from different inlets. At a frequency of 6.6 kHz droplets were formed and approximately 200 000 droplets were collected as a monolayer on the chip for imaging (138).

Methods combining droplet-based microfluidics and enzyme-linked immunosorbent assy (ELISA) are becoming quite popular due to low limit of detection (139). An integrated microfluidic chip with bead-based immunoassay enables measuring the activity of single copy of enzyme and quantify an amount of biomarkers. The recently described prototype (139) allows determination of the prostate-specific antigen (PSA) in buffer at a concentration of 1.2 pg/mL; an improvement of nearly 2 orders of magnitude over the standard ELISA - Fig. 5.
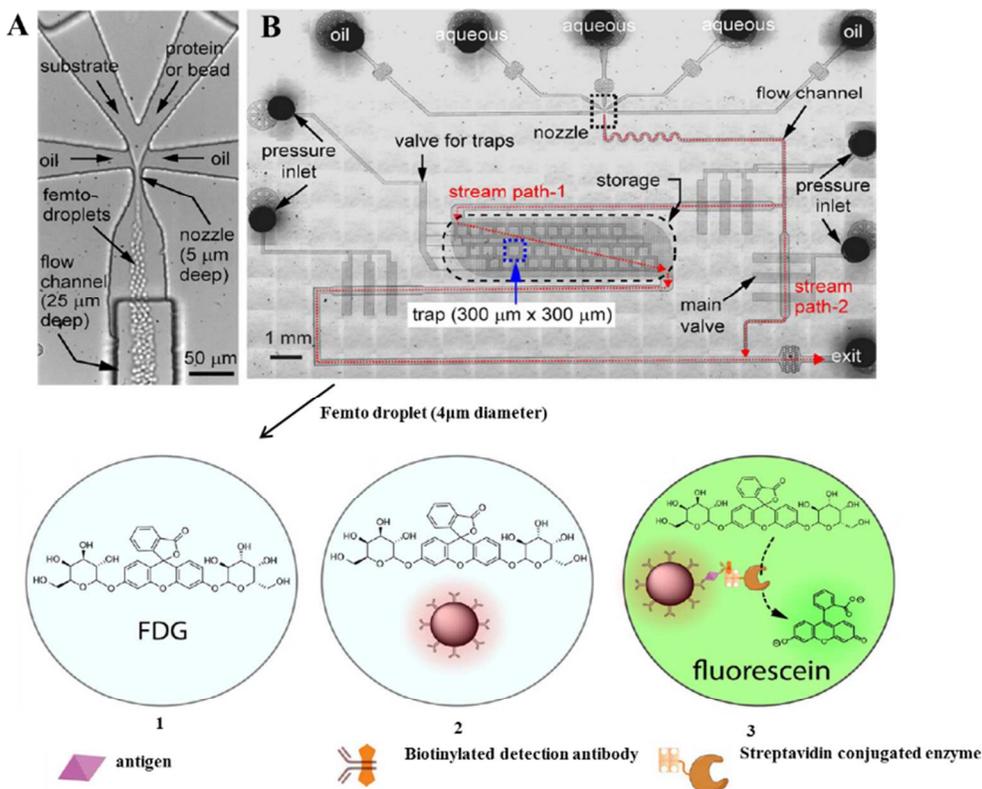
Fig. 5. The microfluidic device used for femto droplet generation and manipulation. (A) Femto droplet formation with a typical volume of 32 fL at a frequency of around $3.5*10^5$ Hz. (B) the multilayered PDMS device, where the upper layer consists of the nozzle (10 μm wide, 5 μm deep), flow channels (100 μm wide, 25 μm deep), and storage compartments (2mm wide, 7mm long 5 μm deep), with a capacity for $\sim 2 \ 10^{-5}$ femto droplets. The bottom layer houses the monolithic valves used to control droplet flow and isolate the traps. If this valve is opened, droplets flow out of the device by stream path 2 due to the lower flow resistance encountered. After that the on-chip incubation, three populations of femtodroplets are observed: (1) droplets containing no bead, (2) those containing a bead without immunocomplexes, and (3) those containing a bead with an immunocomplex exhibiting a positive fluorescence signal due to the enzymatic activity of a single β-galactosidase reporter. From ref. 139; with permission.

Microfluidic droplet PCR

Polymerase chain reaction is undoubtedly widely used process in different areas of DNA analysis ranging from evolutionary biology, environmental science, criminology, to microbial detection, clinical diagnostics and scientific research. While PCR is most commonly performed in homogeneous phase, the use of droplets as reaction vessels has been previously developed for pathogen detection (140). One of the most promising approach is the digital PCR, allowing the

combination of the limiting dilution and partitioning of the diluted sample into hundreds or thousands of reaction chambers with position statistics. Thus, the measurement of absolute number of DNA molecules can be easily achieved (141). Since its development, digital PCR has been utilized in a wide range of biological research including detection of bacteria, copy number variation and single cell genomics (135, 141-146). An integrated microfluidic device with real-time PCR (RT-PCR) analysis has been demonstrated for RNA transcripts detection (147,148), reverse transcription PCR combined with fluorescence microscopy (149,150), monoclonal template amplification for bead-based gene sequencing (120, 151), single RNA fragment detection (152, 153) and widely applied multiplex PCR (27, 120, 154, 155). The recent work of Geng et.al. (120) used microfluidic droplets for single-molecule sequencing. The somatically acquired carcinogenic translocations were detected at low concentrations ($<10^{-6}$) in biologic samples by using a bead-based hemi-nested digital PCR (dPCR). Authors have isolated and quantified various clonal forms with 10^{-7} limit of detection. In addition, the transfer replicas of short tandem repeat (STR) targets from a single cell genomic DNA into the co-encapsulated microbeads has been investigated. STR products, bound on the primer-functionalized microbeads co-encapsulated in the droplets were amplified by a secondary PCR reaction and detected by capillary electrophoresis fragment size analysis of target molecules. The microbead-based 9-plex PCR was optimized in bulk solution and in droplets using standard DNA solutions. A single cell STR typing method based on droplet microfluidics is shown in Fig.6.
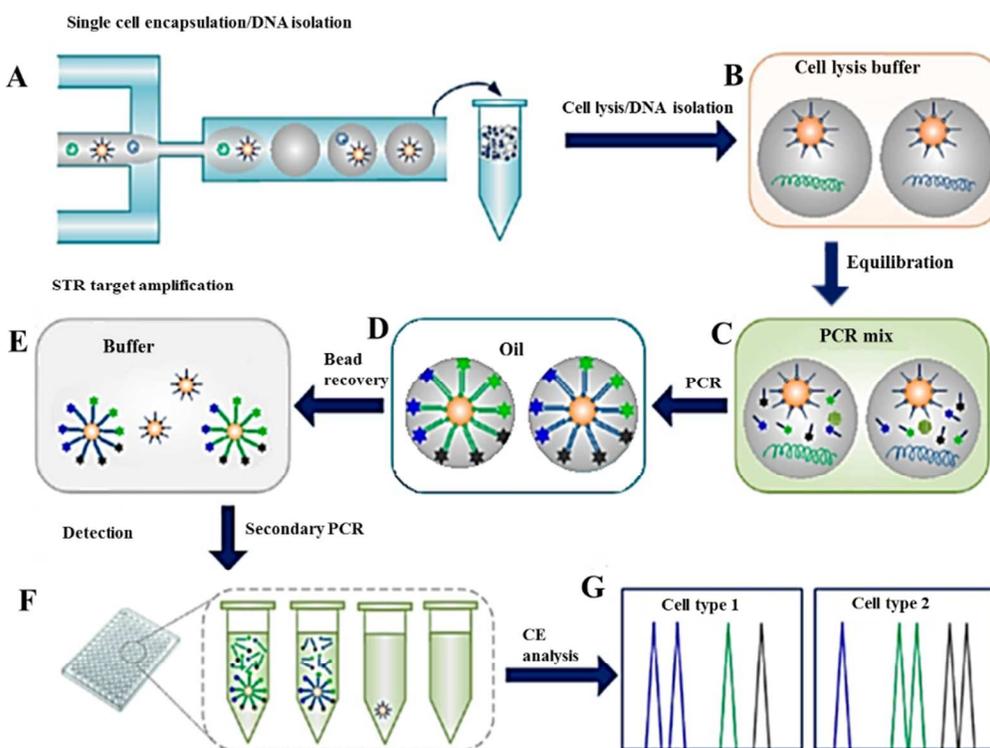
Fig. 6. Analytical procedure for single cell forensic STR typing. (A) Microfluidic droplet generator for encapsulation of cells together with primer-functionalized microbeads within agarose microdroplets. (B) Incubation of the gelled droplets in the cell lysis buffer to release genomic DNA into the gel matrix. (C) Diffusion of the PCR components into the gel droplets by equilibrating in the PCR mix. (D) After re-dispersion of the droplets in oil, emulsion PCR is performed with a thermal cycler. (E) Recovery of the beads by melting the agarose, after the first STR amplification. (F) Dilution of the secondary PCR as a starting point from single beads in standard PCR plates. (G) Capillary electrophoresis system for fragment size analysis. From ref. 120; with permission.

Compartmentalization of the sample prior to PCR amplification provides the basis for absolute quantification of target molecules (156). By dividing the diluted sample into a large number of small-volume reaction compartments, single copies of nucleic acid template can be confined in isolated compartments and PCR amplified followed by a "yes or no" readout. The number of the target molecules is then evaluated by performing a statistical analysis of the "positive" and "negative" signals. Digital PCR can be performed in a variety of formats, including well plates, microdroplets (157), pneumatically-controlled microchips (158,159), spinning discs (160,161), the SlipChip (76, 162-164) and LabDisk platform (165). A recent example of the single DNA molecule detection using an integrated self-priming compartmentalization on-chip digital PCR

device was described by Zhu et al. (166). In this design, the self-compartmentalization of the sample was based on surface tension differences in the chip fabricated in PDMS. The chip contained 5120 microchambers (5nl) detecting an average of 480 to 4804 template molecules with the optimal number of positive chambers between 400 and 1250 as evaluated by the Poisson distribution (166). In another study, Hindson et.al. investigated a high-throughput droplet digital PCR (ddPCR) system allowing processing of ~2 million PCR reactions in a 96-well plate workflow (146). This approach allowed accurate measurement of germline copy number variations, sensitive detection of mutant DNA in a 100,000-fold excess of a wild type DNA and quantitation of circulating fetal and maternal DNA in cell-free plasma. The linearity of the response to DNA concentration and accuracy over the dynamic range of the 20,000 droplet assay corresponded to more than 4 orders of magnitude of target DNA copy number per ddPCR. Copy number concentration and ratio with expanded uncertainties were evaluated using high density ddPCR and lower density microfluidic chamber based digital PCR platform (167). A relative expanded uncertainty under 5 % was obtained using ddPCR; a value much lower than currently typical for quantification of specific DNA target sequences.

Conclusions

This short review captures a snapshot of the field at this critical stage covering the recent advances in droplet microfluidics for (bio) chemical analysis with the focus on papers published in the past four years. A brief overview of the principle of droplet formation and manipulation, and detection methods in droplets including spectroscopy, fluorescence microscopy and electrical measurements is also provided. It is the belief of the authors that the rapid development of this field in the past decade will continue in the foreseeable future bringing solutions to many of the current bioanalytical challenges.

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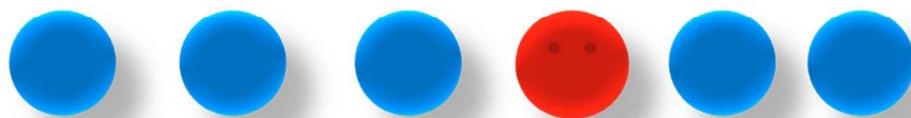
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