Building droplet-based microfluidic systems for biological analysis

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Abstract

In the present paper, we review and discuss current developments and challenges in the field of droplet-based microfluidics. This discussion includes an assessment of the basic fluid dynamics of segmented flows, material requirements, fundamental unit operations and how integration of functional components can be applied to specific biological problems.

Introduction

In the context of the current discussion, droplets are defined as water droplets dispersed in a continuous and immiscible oil phase or oil droplets dispersed in a continuous and immiscible water phase. These droplets are contained and motivated through microfluidic structures having dimensions most easily measured in microns. Interestingly, droplets can be considered one of the earliest tools used in biological analysis, and can be traced back to the time of van Leeuwenhoek when droplets of pond water were examined under a microscope. However, only recently have droplets become a reliable tool for performing biological operations such as analyte encapsulation, sampling, metering, dilution, reaction and detection [1–3].

A generic droplet-based microfluidic assay system is illustrated in Figure 1(A) [4]. Droplets are made in a reproducible and rapid manner, and are used to encapsulate reagents of interest (such as small molecules, nucleic acids, proteins, cells and organelles). Encapsulation can be used to initiate controllable mixing of reagents and their subsequent reaction. Droplets are then transported downstream to allow the reaction to progress and can be stored in sequence to allow incubation for extended periods of time [5]. Importantly, analysis of droplet contents can be performed in a non-invasive and sensitive fashion using a variety of spectroscopic methods [6]. Additionally, droplet contents can be collected and isolated by 'droplet-breaking' into the continuous phase and analysed using conventional analytical methods [7].

There are many advantages associated with performing biological analysis in 'segmented' rather than 'continuous' flows. First, since all reagents are contained within the dispersed phase, surface–molecule interactions are prevented (since the continuous phase wets the channel walls). This provides a high degree of control over the analytical sample, removes residence time distributions, and prevents cross-contamination between multiple samples. Secondly, rapid mixing of the contained fluid can be achieved via chaotic advection, allowing reaction kinetics to be probed with high precision [8]. Thirdly, since droplet volumes can be varied between a few femtolitres and hundreds of nanolitres, they can be made to 'match' the size of the biological entities such as cells or organelles. This facility has proved especially powerful in single cell, protein–protein interaction and cell heterogeneity studies [9–11]. Finally, since thousands of droplets (of various biological composition) may be generated per second, large experimental datasets can be assimilated, allowing sophisticated analyses of complex multicomponent systems [5,12–16].

In the present paper, we describe some of the scientific and technological challenges surrounding the construction of droplet-based microfluidic tools for biological analysis. It is important to realize that for basic applications, droplet-based systems are simple to construct and implement using precision machining (e.g. CNC milling) or soft lithography [17]. Indeed, in some cases, the advantages of segmented flows may be adequately leveraged using basic laboratory tubing [18–20]. Although such simple systems can be multiplexed and/or operated in parallel, when an assay involves multiple steps, the design, operation and control of fluidic architectures becomes increasingly complex [21–23]. Indeed, droplet-based fluidic systems are intrinsically non-linear [24,25]. Although non-linearity is challenging in the design process, it is accompanied by many unique and advantageous properties related to mixing [26,27], sorting [28,29], storing [30,31] and merging [32–36].

Materials and fluid dynamics

Oils, surfactants and chips

The application of droplet-based microfluidics to biological systems necessarily involves handling various analytical samples. These range from 'laboratory-quality' solutions to untreated bodily fluids, such as blood, urine, saliva or dialysate [37–40]. Additionally, the biomolecules contained

Key words: droplet-based microfluidics, fluid dynamics, segmented flow.
Abbreviations used: CE, capillary electrophoresis; LC, liquid chromatography.
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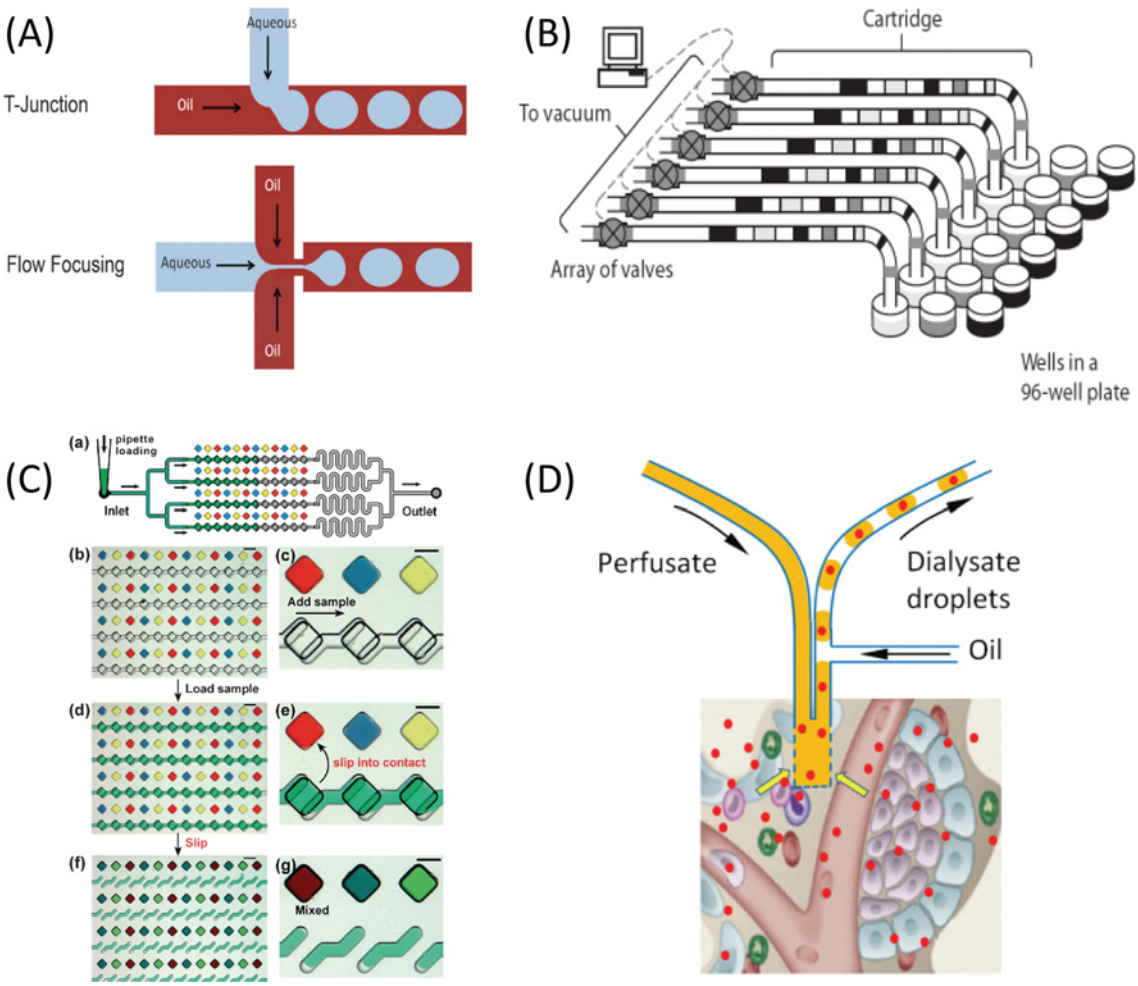
Figure 1 | **Droplet generation**

(**A**) Droplet generation at T-junction and flow focusing microfluidic geometries. Reproduced from Solvas, X.C.I. and deMello, A. (2011) Droplet microfluidics: recent developments and future applications. Chem. Commun. **47**, 1936–1942 with permission of the Royal Society of Chemistry. (**B**) Sampling of droplets into cartridges from a 96-well plate. Reprinted with permission from Linder, V., Sia, S.K. and Whitesides, G.M. (2005) Reagent-loaded cartridges for valveless and automated fluid delivery in microfluidic devices. Analytical Chemistry **77**, 64–71. © 2005 American Chemical Society. (**C**) Parallel droplet generation with using a microfluidic 'SlipChip'. Reproduced from Du, W.B., Li, L., Nichols, K.P. and Ismagilov, R.F. (2009) SlipChip. Lab Chip **9**, 2286–2292 with permission of the Royal Society of Chemistry. (**D**) *In situ* droplet sampling from bodily organ or tissue.



within such samples will be characterized by various degrees of hydrophilicity and lipophilicity, and may be dissolved in solvents such as water, ethanol, DMSO or acetonitrile. Accordingly, the careful selection of the continuous phase, surfactant, substrate material and channel surface coating are crucial in facilitating the effective and complete encapsulation of the analytical sample.

Commonly used continuous phases include hydrocarbon, silicon oil and fluorocarbon oils [2,7,41–45]. Of these, fluorocarbon oils are most popular because of their physical and chemical stability, their immiscibility with organic compounds and excellent oxygen permeability. Indeed, perfluorinated oils such as FC-40 ($C_{21}F_{48}N_2$), FC-70 (perfluorotripentylamine) and FC-3283 ($C_9F_{21}N$) are of high utility [8,46,47]. It should also be noted that perfluorinated oils are typically denser than the aqueous phases. This does not affect the manipulation and integrity of droplets within the microchannels, but does dictate special consideration for droplet preparation and collection [19,48].

Without doubt, the most important issue related to the continuous phase is the choice of an appropriate surfactant. In simple terms, surfactants play the key role in both stabilizing droplet interfaces and in controlling biocompatibility [49–51]. Span 80 (sorbitane mono-oleate) and Abil® EM surfactants are commonly used in conjunction with mineral oils [7,44], and Krytox (low-molecular-mass fluorine-end-capped homopolymers of hexafluoropropylene epoxide) with fluorous oils. Recently, a Krytox-based

surfactant containing a perfluoropolyether tail and carboxylic acid headgroup has been shown to provide excellent control over droplet emulsification, biocompatibility and stability [11,52]. The efficacy of the above surfactants can be seen by the success of droplet-based microfluidics for performing high-throughput and digital PCR, cell-culture, *in vitro* transcription and translation, and the ability to store droplets in close proximity for several days without coalescence. Despite these successes, it is evident that a wider range of surfactants is needed to improve accessibility and compatibility in various situations.

Traditional microfluidic substrate materials include silicon, glass, polymers, plastics, ceramics and metals [53]. The choice of substrate material is dictated by numerous factors, including cost, available fabrication infrastructure, chemical compatibility, thermal stability and ease of processing. For droplet-based microfluidics, a key requirement is that the continuous (carrier) phase should exhibit a high affinity for the channel surface. For water-in-oil droplets (typical of the biological systems discussed in the present paper), this necessitates that the surface exhibit a high degree of hydrophobicity. Accordingly, polymers such as PDMS (polydimethylsiloxane) have proved extremely popular in generating reproducible segmented flows. Silicon and glass are hydrophilic in nature and thus surface coatings must be employed to avoid interaction between aqueous droplets and channel walls. To this end, a number of studies have assessed the use of silane [54], aquapel [55] and Teflon [56] coatings. The latter is especially interesting since the coated surface is compatible with fluorous oils. Moreover, a new family of fluorinated polymers has recently been used to make microfluidic devices, alleviating the need for surface treatment in droplet-based applications [57].

Finally, it should be noted that other parameters will affect interfacial tension or even rheology both systematically and locally. Such parameters include temperature, surface roughness of the channel surface and the purity of the contained fluids. For example, by controlling temperature, thermoresponsive sol–gel droplets can be switched between liquid droplets and gel beads. These beads can be centrifuged, washed and redistributed, thus providing new operational abilities over pure liquid droplets [58,59].

A brief discussion of fluid dynamics

At the flow rates normally encountered in droplet-based microfluidics, laminar flows are dominant. The shear force between a stationary channel wall and a moving droplet (having a diameter bigger than the channel width) induces vortex circulations both inside the discrete phase and in the continuous phase. Moreover, when winding channel geometries are used, rapid mixing via chaotic advection is achieved spontaneously and allows efficient mixing on a millisecond timescale [26].

When a droplet encounters an expansion or contraction in channel geometry, deformation of the liquid interface curvature will induce a change of pressure differential between fluid interfaces, which is termed the Laplace pressure, ΔP. ΔP is equal to $\gamma(1/R_1 + 1/R_2)$, where R_1 and R_2 are orthogonal radii of curvature and γ is the surface tension. ΔP varies inversely with the radius of curvature, generating 'elasticity' in the liquid interface. This in turn will affect both droplet movement and the surrounding continuous fluid flow. In recent years, a variety of droplet manipulation units have been designed to utilize this elasticity to trap, merge and dilute droplets [15,33].

Finally, a moving droplet will also generate a differential pressure inside the containing microchannel [60–62]. This means that the total pressure decrease within a channel branch relies temporally and spatially on the presence of the droplets themselves. As such, droplet trafficking in one channel will be affected by droplets in connecting branches, and vice versa. This phenomenon has been studied in detail by Whitesides and co-workers who show that initial regular droplet spacings can be transformed into complex patterns [24]. It is important to note that such effects are significant when integrating multiple droplet operations.

Making droplets

Droplet generation is one of the most important steps in droplet-based microfluidics, especially when sampling low-volume or low-concentration samples. Not surprisingly, numerous studies have assessed a variety of approaches for droplet generation. These can be broadly categorized as on-chip generation, sequential generation, parallel generation and generation of droplets on demand.

On-chip methods can be categorized further as passive or active in nature. The passive approach, based on the fluid dynamics introduced above, typically involves the use of T-junction or flow focusing geometries [43,63] (Figure 1A). In the former, depending on the delicate balance between the continuous phase and discrete phase flow rates, two streams meeting at a junction will segment, with the phase having lower affinity for the channel surface forming the discrete phase. In the latter, three input flows are brought together and are compelled to flow through a narrow orifice. Confinement then causes the central stream to destabilize and segment. In both cases, the droplet size and frequency are determined by the combined effects of interfacial tension, input volumetric flow rates and the channel geometry at confluence. Adoption of passive methods allows droplets to be generated at rates between 10^{-2} and 10^4 Hz [64,65]. Importantly, the concentrations of reagents can be easily controlled by varying the flow rates of the pure fluid inputs. Unsurprisingly, the passive approach for droplet generation is the most widely used. Active droplet-generation methods involve the use of time-dependent perturbations of the fluid flow (using for example electric fields [66], pneumatic pressure [67], optical [68] and thermal [69] control). They generally exhibit lower droplet-generation rates, but have the advantage of being able to control droplet size and generation frequency without varying channel geometries or flow rates.

With respect to sample resources, on-chip droplet generation is inefficient, generally requiring pre-loading of

large sample volumes into external syringes. To address this limitation, the use of reagent-loaded cartridges has proved particularly useful [18]. In this case, nanolitres of analyte are dispensed as an array of plugs and stored in an external capillary or cartridge (Figure 1B). Plugs can then be delivered (when required) and merged with other reagent plugs (or streams) to initiate the assay process. Key benefits of this approach include significant reductions in sample wastage, the ability to screen large numbers of reaction conditions against target samples and the ability to incubate and store reagents over extended periods of time [19].

Approaches for the parallel generation of droplets are less common. Nevertheless, recent studies by the Ismagilov group at the University of Chicago have demonstrated efficient formation of arrays of hundreds or thousands of droplets in a single step, using a microfluidic device made of two layers (pre-patterned with wells and ducts) separated by an immiscible carrier fluid [70] (Figure 1C). The two layers are initially aligned to form 'channels' by the continuous overlapping of wells and ducts. Aqueous solution can then be loaded into the channels. When the layers are 'slipped' relative to one another, the wells and ducts part, and break the aqueous stream into an array of thousands of picolitre-volume droplets [71].

Finally, it should be noted that samples can be encapsulated *in situ*, with direct integration of the microfluidic device with the sample source. Recent innovations in this respect include integration of droplet generators with liquid chromatography [72] and electrophoresis columns/channels [73] and direct sampling from rat striata [74] (Figure 1D).

Droplet operations and system integration

In addition to droplet generation, other unit operations are required when performing complex biological assays. Such operations can be initiated and controlled using internal effects or external actuation.

Passive operational units

Dosing a droplet, to initiate a reaction for example, can be achieved either by merging the droplet with a continuous fluidic stream (followed by break-up to form a larger droplet) [75] or by directly merging the droplet with another droplet(s) [33] (Figure 2A). The former approach requires precise control of the continuous stream and a uniform spacing between droplets, whereas the latter requires synchronization and pairing of the droplets in time and space. Importantly, if the droplets to be merged are stabilized with surfactant, electrical or optical fields can be used to break the interface and initiate merging [66,76].

Droplet splitting at constricted T-junctions and at isolated obstacles has also been studied in depth and is relatively simple to implement [77] (Figure 2B). An elegant example of how splitting can be applied to high-throughput experimentation was recently shown by Hatch et al. [78] who used eight bifurcation junctions to split moving input droplets into 256 smaller droplets. By forming droplets at kHz rates, the authors were able to generate in excess of one million monodisperse droplets in less than 7 min. These droplets self-assemble into high-density packed configurations and are used to perform digital PCR.

When screening or assaying rare biological samples within microfluidic systems, it is challenging to vary the concentration of droplet contents in a rapid and controllable fashion. To this end, Niu et al. [79] have recently reported the fabrication and testing of a dilution module for high-throughput screening (Figure 2C). Via a process of droplet merging, mixing and re-splitting, droplet trains defining digital concentration gradients can be created at high speed. Significantly, the approach enables dilutions spanning over four orders of magnitude to be accessed on short timescales and thus has enormous utility when conducting high-throughput biological screens.

Finally, droplet storage and incubation can be achieved using a variety of approaches. For example, droplets may be 'parked' in sequence within long channels and external tubing or be paired with the confinement of microstructures [80,81] (Figure 2E). They can also be collected in large numbers into microfluidic reservoirs or off-chip vials, but at the risk of losing sequence information [48].

All of the above operations are passive in nature and therefore ideally suited for operation at high speed. Accordingly, they represent first-choice options when building up droplet-based microfluidic systems.

Active operational units

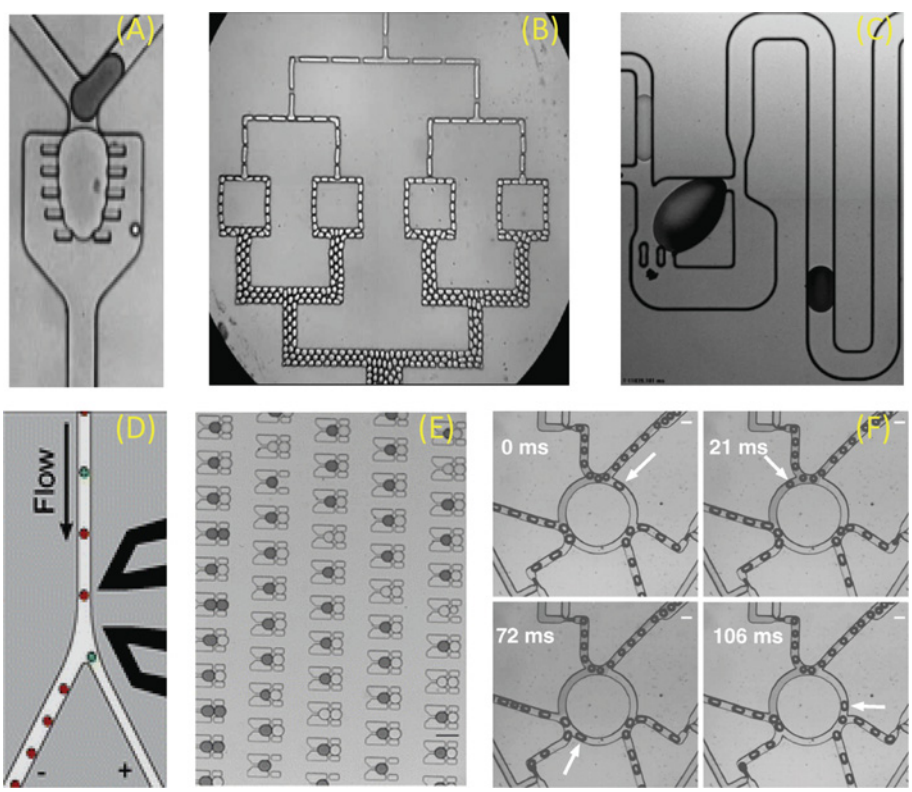
External actuation is essential for accomplishing processes that cannot be achieved using passive elements. An example of such a process is the sorting of droplets that contain analytes of interest. Droplet sorters come in a variety of flavours, including those based on electrostatic actuation [45,66,82] (Figure 2D), mechanical valves [83], surface acoustic waves [84] and optical operations [85]. Furthermore, it should be noted that, in most of the cases, actuation is integrated with detection feedback control based on simple 'on–off' strategies.

Integration of unit operations

In its simplest embodiment, system integration involves connection of multiple functional elements in either a sequential or parallel fashion, as shown in Figure 3. To date, sequential integration of droplet elements has been most studied (Figure 3B), with the serial combination of operations such as droplet generation, droplet merging, reagent mixing and reaction, incubation and analyte detection. By using such a strategy, complex chemical and biological processes can be performed with high efficiency. For example, a number of researchers have reported droplet-based microfluidic systems for performing PCR [86–88]. Hollfelder and co-workers described a radial microfluidic device for continuous-flow PCR, where reagents are encapsulated within 160 pl droplets surrounded by a continuous oil phase [7]. In this way, single-copy PCR was achieved in 15 min with amplification factors in excess of 5×10^6. Sequentially integrated systems have also been used to perform high-throughput cell-based assays [89],

Figure 2 | **Exemplar droplet operations**

(**A**) Droplet merging. Reproduced from Niu, X., Gulati, S., Edel, J.B. and deMello, A.J. (2008) Pillar-induced droplet merging in microfluidic circuits. Lab Chip **8**, 1837–1841 with permission of the Royal Society of Chemistry. (**B**) Sequential droplet splitting. Reprinted with kind permission from the authors of Link, D.R., Anna, S.L., Weitz, D.A. and Stone, H.A. (2004) Geometrically mediated breakup of drops in microfluidic devices. Phys. Rev. Lett. **92**, 054503. © 2004 The American Physical Society. (**C**) Controlled droplet dilution. Reprinted by permission from Macmillan Publishers Ltd: Nature Chemistry [Niu, X.Z., Gielen, F., Edel, J.B. and deMello, A.J. (2011) A microdroplet dilutor for high through put screening. Nature Chemistry **3**, 437–442], © 2011. (**D**) Active droplet sorting. Reproduced from Baret, J.C., Miller, O.J., Taly, V., Ryckelynck, M., El-Harrak, A., Frenz, L., Rick, C., Samuels, M.L., Hutchison, J.B., Agresti, J.J. et al. (2009) Fluorescence-activated droplet sorting (FADS): efficient microfluidic cell sorting based on enzymatic activity. Lab Chip **9**, 1850–1858 with permission of the Royal Society of Chemistry. (**E**) Droplet paring. Reproduced from Bai, Y.P., He, X.M., Liu, D.S., Patil, S.N., Bratton, D., Huebner, A., Hollfelder, F., Abell, C. and Huck, W.T.S. (2010) A double droplet trap system for studying mass transport across a droplet–droplet interface. Lab Chip **10**, 1281–1285 with permission of the Royal Society of Chemistry. (**F**) Bubble logic operations. Reproduced from Prakash, M. and Gershenfeld, N. (2007) Microfluidic bubble logic. Science **315**, 832–835 with permission from AAAS.



directed evolution to identify horseradish peroxidase mutants [90], synthesis of compound semiconductor nanoparticles [20], and dose–response screening of enzyme inhibitors [9]. Nevertheless, sequential processing is not ideal when dealing with extremely large numbers of droplets, owing to excessive system back pressures. Accordingly, collection of droplets in external containers (with subsequent reinjection for analysis after extended periods of time) has proved critical, allowing massively parallel experiments to be performed [48].

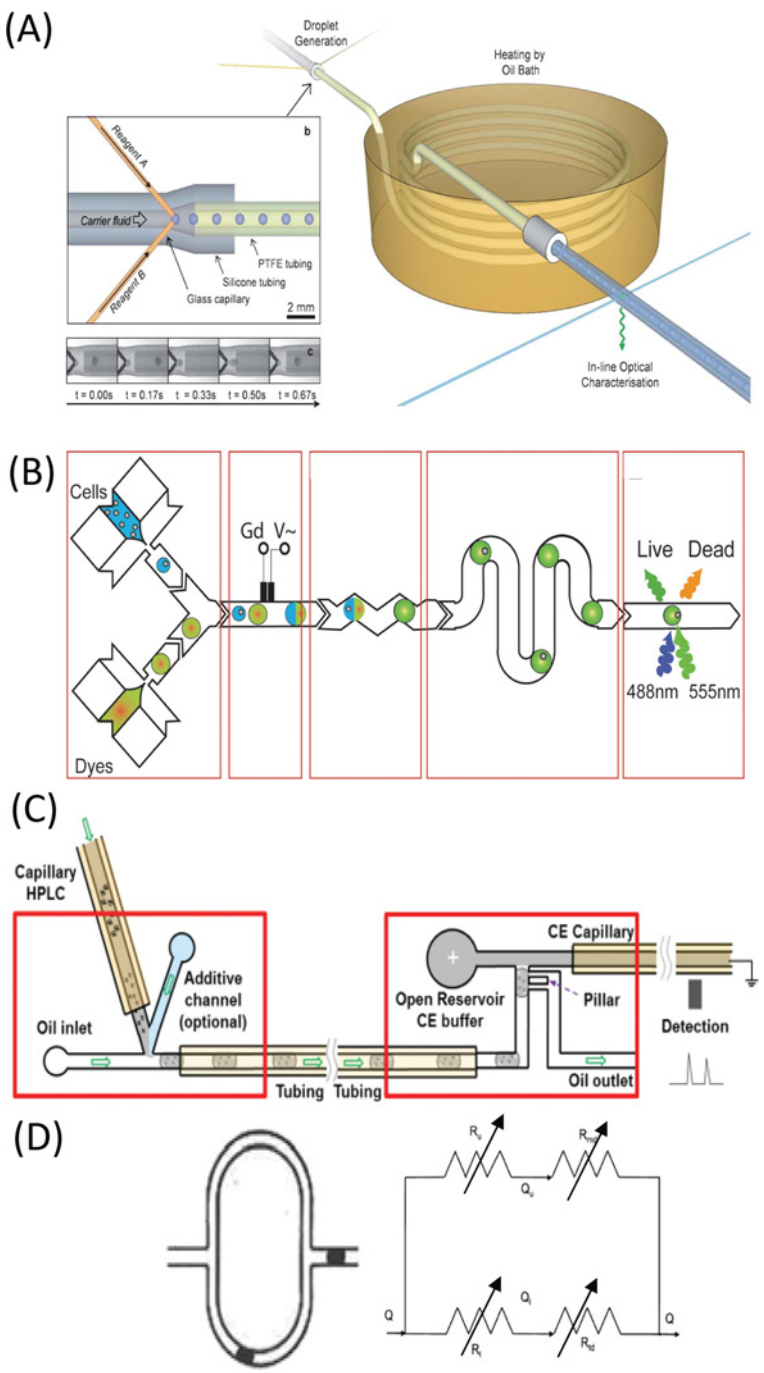
Droplet characterization

Characterization of large numbers of rapidly moving picolitre-volume droplets introduces new and significant experimental challenges for analyte detection. The most common approach involves the use of optical spectroscopy to probe the small volumes in a non-invasive and sensitive manner. A variety of optical techniques have been used to good effect in probing segmented flows. These include laser-induced fluorescence [10], UV–visible absorption [91], refractive index variation [92], IR spectroscopy [93], surface-enhanced Raman spectroscopy [94] and NMR [95]. In addition, high-speed cameras operating in brightfield mode are widely applied for system calibration and viewing droplet manipulation.

The other important method of characterizing droplet contents is to interface the microfluidic system with the other analytical method such as CE (capillary electrophoresis), LC

Figure 3 | **Integrated droplet systems**

(**A**) Tubing-based droplet systems for nanomaterial synthesis. Reproduced from Nightingale, A.M., Krishnadasan, S.H., Berhanu, D., Niu, X., Drury, C., McIntyre, R., Valsami-Jones, E. and deMello, J.C. (2011) A stable droplet reactor for high temperature nanocrystal synthesis. Lab Chip **11**, 1221–1227 with permission of the Royal Society of Chemistry. (**B**) Sequential droplet operations for cell-based assays. Reproduced from Brouzes, E., Medkova, M., Savenelli, N., Marran, D., Twardowski, M., Hutchison, J.B., Rothberg, J.M., Link, D.R., Perrimon, N. and Samuels, M.L. (2009) Droplet microfluidic technology for single-cell high-throughput screening. Proc. Natl. Acad. Sci. U.S.A. **106**, 14195–14200 with permission. (**C**) Droplet interfacing for chemical separations. Reproduced from Niu, X.Z., Zhang, B., Marszalek, R.T., Ces, O., Edel, J.B., Klug, D.R. and deMello, A.J. (2009) Droplet-based compartmentalization of chemically separated components in two-dimensional separations. Chem. Commun., 6159–6161 with permission of the Royal Society of Chemistry. (**D**) Droplet manipulation in parallel channels and equivalent circuit diagram. Reproduced from Fuerstman, M.J., Garstecki, P. and Whitesides, G.M. (2007) Coding/decoding and reversibility of droplet trains in microfluidic networks. Science **315**, 828–832 with permission from AAAS.



(liquid chromatography) and MS. In theory, this will yield a large amount of chemical and biological information, but such methods traditionally require sampling of continuous flows and volumes in excess of microlitres. A potential solution is to break up the segmented flow and its component into continuous phase; however, this removes the possibility of probing individual droplets. Fortunately, a number of studies have addressed this issue through the development of efficient interfaces that preserve droplet integrity. These include droplet-interfaced CE and LC protein, peptides and DNA separations [72–74] (Figure 3C) and droplet-interfaced MS [65,96,97]. These developments are exciting not only because of the fact that complex mixtures within individual droplets can be resolved, but also because of the potential for dramatically improving the performance of conventional analytical methods in the analysis of complex but rare samples.

Outlook and challenges

It is fair to say that droplet-based microfluidic systems have realized much of their early promise in the area of biological analysis. The compartmentalization of bulk samples into thousands of sub-nanolitre droplets using microfluidic structures affords a plethora of benefits that have already begun to have an impact on high-throughput biological experimentation. However, many challenges remain. Of note is the need to develop better detection methods able to probe small volume environments on short timescales. Indeed, whereas droplet-based microfluidic systems generate vast amounts of biological information, the majority of this is lost because of limitations in the detection protocol. Notable recent developments in this respect include the demonstration of single-molecule detection [98] and fluorescence lifetime imaging [99] of droplet contents. Moreover, the recent application of label-free detection techniques such as surface-enhanced Raman spectroscopy, surface-enhanced resonance Raman spectroscopy and FTIR (Fourier-transform IR) spectroscopic imaging to the analysis of droplets in microchannels is of significance [19,93,100]. Although vibrational spectroscopies are inherently less sensitive than fluorescence-based methods, their molecular specificity and quantitative nature make them invaluable in many chemical and biological applications. Related to the efficient analysis of droplet contents is the ability to sort, manipulate and probe droplets of special interest. Although examples of droplet sorting have been described above, the ability to integrate sorting with downstream droplet processing remains a significant challenge and an area of high priority for high-throughput screening applications.

As discussed above, the development of a wider range of continuous phases, surfactants, surface coatings and substrate materials is an ongoing process. This is of particular importance in biological analysis where assay timescales may range from seconds to days, and thus the need for efficient and spatially organized droplet storage (in chambers or capillaries) is paramount. Moreover, parallel systems such as that shown in Figure 3(D) are under increasingly intensive study. It is expected that future developments in this area will generate advanced functionalities for droplet manipulations.

A final and increasingly important aspect in the development of droplet-based microfluidic systems is the fabrication of small-footprint systems that can be efficiently integrated with both upstream and downstream analytical tools. Of special interest is the application of droplet-based systems to microdialysis. Microdialysis is a minimally invasive sampling technique that offers continuous *in vivo* measurement of drug or biomolecule concentrations in tissues and organs [101]. Current microdialysis techniques suffer from low temporal and spatial resolution and inefficient liquid handling, which limits wider application in pre-clinical and real-time diagnosis. Previous reports have demonstrated significant improvement in both temporal and spatial resolution through the use of droplet-based microfluidic sampling [74,102,103], and its use as a robust diagnostic tool is likely in the short term. To this end, we expect that droplet-based microfluidic systems will soon become fundamental components in a new generation of biomedical and analytical instrumentation.

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