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Abstract

Droplet-based microfluidic systems generate, manipulate and control sub-microliter droplets enclosed within an immiscible carrier fluid. Due to a number of remarkable features, such as the ability to precisely control the chemical and biological payload of each droplet and produce thousands of droplets per second, this technology is transforming how chemists and biologist perform high-throughput or massively-parallel experiments. In this Primer, we initially introduce and describe the basic features of droplet-based microfluidic systems and key issues that should be considered when developing new chemical and biological workflows. We provide a critical evaluation of how droplet-based microfluidic systems should be manufactured and the importance of integrating appropriate detection technologies to probe the small analytical volumes that are representative of the technology set. We then discuss issues related to data collection and management, providing guidelines on how large datasets should be processed and manipulated. Furthermore, we showcase some of the most successful and important applications of droplet-based systems in the biological and chemical sciences and consider issues that currently hinder progress in both technology development and application. Finally, we provide some opinion on future directions for the technology set and where its greatest impact will be felt in the coming years.

Introduction

Droplet-based microfluidic systems produce, load, manipulate and process sub-microliter droplets in a rapid and efficient manner. The interplay between hydrodynamic forces and interfacial tension **[G]** within microfluidic environments allows a continuous fluid flow to be transformed into a stream of droplets dispersed within an immiscible carrier fluid. Such systems have transformed the paradigm of experimentation within many areas of the chemical and biological sciences and are rapidly becoming an indispensable and embedded tool within contemporary laboratories[1].

Droplet-based microfluidic systems can be considered a subset of microfluidic technologies[2]. In basic terms, microfluidic systems are engineered fluidic devices in which flow is ordered and non-turbulent[3]. Although a number of divergent effects arise as fluidic systems are downsized, the most important are driven by the scale dependence of mass and heat transfer. First, the large surface-area-to-volume ratios that typify microfluidic systems ensure rapid heat transfer to and from contained fluids. Second, small instantaneous fluid volumes mean that mass transfer is almost always regulated by diffusion and that laminar flow (or low Reynolds number **[G]**) regimes are the norm. Practically, this ensures that fluid flows are predictable and that assay/reactions conditions can be controlled with precision. Early examples of microfluidic systems were designed to process a single fluid phase (for example, an aqueous solution), typically in a continuous hydrodynamic flow. Whilst continuous-flow systems have been shown to be valuable in many situations (such as when performing perfusion- or separation-based experiments), their analytical advantage is severely compromised by Taylor dispersion **[G]** , solute-channel wall interactions, the consumption of substantial volumes of sample/reagent and the need for extended channel lengths[4]. Unlike their continuous-flow counterparts, droplet-based systems possess a number of features that make them desirable platforms for performing quantitative experiments. As well as leveraging the scale dependencies of mass and heat transport, droplets may be formed in a robust fashion at kHz frequencies, with exquisite control over the size, location and molecular payload of each droplet. This means that large numbers of compartmentalized assay/reaction volumes may be created and processed in a rapid and reproducible manner, engendering the performance of complex biological and chemical workflows[5].

Since the first report of droplet production using a microfluidic system in 1997[6], droplet-based microfluidic systems have rapidly evolved as a technology set. Early activities in the field centred on the establishment of functional components for generating and manipulating droplets in a robust and high-throughput manner[7,8]. Subsequently, a diverse array of functional components for operations such as droplet generation, splitting, fusion, dilution, incubation, spacing, trapping, mixing, payload control and sorting were developed and

integrated within chip-based systems[9]. More recent endeavours have focused on the application of droplet-based microfluidic systems in the fields of chemistry, biology and materials science, where the ability to form and process enormous numbers of assay volumes allows the end-user to generate previously inaccessible or hard-to-get biological or chemical information. Examples of fields that have benefited from droplet-based microfluidic tools include single cell analysis[10], nanomaterials synthesis[11], directed evolution[12] and 3D cell culture[13].

The field has evolved considerably over the past two decades in terms of both the technology set and the areas of application. A plethora of systems and functional components have been developed to perform a wide variety of operations desired by the end-user. Computer-Aided Design (CAD) software and automation tools have provided an opportunity for further microfluidic applications. Indeed, the focus of activities within the field has transitioned from the development of basic methods and techniques to their use in a select number of timely and important applications in the chemical and biological sciences. This means that droplet-based microfluidic technologies are increasingly being viewed as basic experimental tools that engender new science, rather than being remarkable in their own right. Accordingly, whilst end-users might appreciate the role that droplet-based microfluidic systems could play in a given scenario, the challenges associated with building microfluidic devices able to perform bespoke experiments are significant and often overwhelming for those new to the field. This Primer attempts to introduce fundamental aspects of droplet-based microfluidics that should be considered when developing new chemical and biological workflows.

Specifically, we provide an overview of the fabrication methods, microfluidic technologies, detection methods and technical considerations associated with droplet-based microfluidic experimentation. We discuss issues related to data collection and management and provide guidelines on how large datasets should be dealt with. Subsequently, we highlight some of the most successful and important applications of droplet-based systems in the biological and chemical sciences. We then discuss issues that currently hinder progress in both technology development and application. Many of these are obvious to those working in the field but are often overlooked or ignored. Finally, we provide some opinion on future directions for the technology set, highlighting things that we should do better, new areas of application and also situations where droplet-based systems may have less advantage or utility.

Experimentation

We now provide an overview of some of the most important considerations when designing, fabricating and using droplet-based microfluidics systems. These considerations include choosing the most appropriate microfluidic device for generating droplets, the method of droplet generation, the discrete/continuous phase fluids, the functional operations required to perform a specific experimental workflow and the detection techniques used to probe droplets in rapid, sensitive and efficient manner.

Material Selection and Device Fabrication

When making a droplet-based microfluidic system the choice of the substrate material and the method of device fabrication depend on numerous factors, including considerations related to the required functionality of the final device, available microfabrication methods, desired chemical compatibilities and bio-compatibilities, thermal and electrical properties and the detection strategy to be employed during experimentation.

The majority of droplet-based microfluidic systems are fabricated as planar, chip-based devices incorporating a single, interconnected fluidic network. That said, capillary or tube-based systems can also be used to create segmented flows, without the need to involve complex microfabrication methods. Indeed, some of the earliest examples of droplet-based microfluidic systems involved the co-flow of immiscible fluids within tapered capillaries to generate monodisperse droplets[8], and polytetrafluoroethylene (PTFE) tubing (connected using PEEK cross-junction) remains the preferred format when performing high-temperature synthesis of nanomaterials — such as compound semiconductor nanoparticles[14] and organic/inorganic lead halide perovskites[11] — in droplets.

To date, chip-based systems for generating and processing droplets have almost exclusively been fabricated in polydimethylsiloxane (PDMS). The reasons for this are two-fold. First, since its introduction by Whitesides in the mid 1990s, soft lithography — the moulding of PDMS using master templates — has proved to the most popular method for making microfluidic structures[15]. PDMS-based devices can be fabricated in a rapid, flexible and low-cost manner. PDMS is optically transparent (in the visible and UV regions of the electromagnetic spectrum), durable, cheap, biocompatible and gas-permeable, and can therefore be used to perform a variety of chemical and biological experiments. Second, to ensure clean transport of encapsulated droplets through the system, the carrier fluid (and not the dispersed fluid) should preferentially wet the walls of the microfluidic channel. Since, the majority of droplet-based microfluidic experiments involve the use of aqueous discrete phases, the surface tension at the aqueous/channel surface interface should be higher than the interfacial tension at the aqueous/carrier fluid interface. When using PDMS, this

condition can be satisfied through surface treatment[16], and droplets may be manipulated without interaction with internal surfaces. In this regard, it should be noted that in theory, any combination of immiscible phases can be used to generate droplets within a microfluidic system. When droplets contain an aqueous payload, fluorinated oils, mineral oils or fatty acids are commonly used as the carrier fluid. When performing biological experiments, especially those involving cells, fluorinated oils are desirable because of their inert nature and gas permeability[17]. That said, and as will be discussed later, droplets are by nature metastable emulsions, and need to be stabilized if they are to be kept for extended amounts of time in incubation chambers or reservoirs. To avoid droplet coalescence, surfactants are used to reduce the interfacial tension between the two phases[18]. When using fluorinated oils as the carrier fluid, highly customizable surfactants are used, which often consist of multiple-block copolymers with long fluorinated tails. A popular family consists of fluoro-surfactants with a polyethylene glycol (PEG) head and two perfluoropolyether (PFPE) tails[19].

Whilst PDMS continues to be the material of choice when making droplet-based microfluidic devices, it is not without its drawbacks, most notably, its poor solvent resistance[20] and its propensity to absorb hydrophobic small molecules[21].These features limit the utility of PDMS-based devices in a range of droplet-based experiments, and thus other material solution are required. In this regard, other elastomeric materials (such as thermoset polyesters[22]), thermoplastics (such as polymethylmethacrylate [23], polycarbonate[24] and polystyrene[25]), amorphous polymers (such as cyclic olefin copolymer[26]), fluoropolymers (most notably, PTFE[27]) and glasses[28] can all be used as substrate materials for droplet-based microfluidic systems, with each material possessing specific advantages, such as outstanding solvent resistance, negligible biofouling or the ability to mass produce devices using techniques such as injection moulding or hot-embossing.

Droplet generation

Droplets with volumes ranging from a few femtoliters to hundreds of nanoliters can be generated using a number of different passive and active tools. In simple terms, droplets form through the transfer of energy to the liquid-liquid interface. This energy can come directly from the hydrodynamic flow itself (passive control) or via an external input (active control)[29]. Passive strategies that leverage geometric adaptations in microchannel features have proved to be especially powerful in enabling the robust formation of sub-nL volume droplets at high speeds. The most common passive methods for droplet production involve the use of T-junctions[7], flow focusing geometries[30] and co-flow structures[8] (**Figure 1**). Although different in their modes of action, each method involves the establishment of an interface between two immiscible fluids and the ensuing segregation of one of these fluids into droplets (the discrete or dispersed phase) that are surrounded by the other fluid (the continuous phase or carrier fluid). Control of the interfacial tension of the component fluids

with respect to the channel walls defines the identities of the discrete and continuous phases, ensuring that one fluid (the carrier fluid) will preferentially wet the surface of the microfluidic channels. Under such conditions, the discrete phase does not contact the channel surface due to a thin layer of carrier fluid between the droplet and surface. As has been noted and will be seen, this is a particularly advantageous feature of microscale droplet flows.

The T-junction (or cross-flow geometry) unites two immiscible fluid streams normally at 90° to each other (**Figure 1a**), with one fluid being sheared by the other to generate droplets[7]. Such a scheme is simple to implement, with droplet size being controlled by the relative volumetric flow rates of the input flows[31]. The flow-focusing geometry (**Figure 1b**) is an even more popular tool for droplet formation, since both droplet volume and production rates may be controlled over exceptionally wide ranges[30]. Here, concentric immiscible flows are accelerated prior to entering a narrow nozzle. Pressure and viscous stress acts to elongate the inner fluid, which eventually breaks either inside or downstream of the nozzle. Additionally, droplets may be generated passively using conventional capillaries or tubes (**Figure 1c**). In such formats, monodisperse droplet populations are typically produced by co-flowing immiscible fluids through a tapered capillary in which streamwise forces exceed interfacial tension, with droplet size being a function of the capillary tip diameter, carrier fluid velocity, extrusion rate and the viscosity of the component fluids. When using each of these methods, droplets may be produced at rates up to tens of kilohertz with size coefficients of variation (CV) between 2% and 5%. That said, whilst the cross-flow, flow-focusing and co-flow methods are the most widely adopted droplet generation methods, it should be noted that the size and size-distribution of the formed droplets can be sensitive to the flow rate of the incoming fluids, and thus passive droplet-generation methods based on variations in channel confinement are often desirable. In such step-emulsification methods[6,32], the size of the formed droplet is primarily controlled by the channel geometry and is essentially independent of flow rate.

Finally, higher order or multiple emulsion may also be generated using modified co-flow and flow-focusing droplet generators[33,34]. Multiple emulsions are multiphase structures in which primary droplets are loaded with smaller droplets in complex arrangements. Due to their complex and controllable internal structure, such materials have a myriad of applications in the pharmaceutical and consumer goods industries. For example, double emulsions can be easily created using cylindrical glass capillaries nested within a square glass tube. Here, an inner fluid is delivered through a tapered capillary, with a middle fluid being pumped through the outer coaxial region to form a coaxial flow at the exit. An outer fluid is supplied through the outer coaxial region from the opposite direction, with all fluids then being forced through

the exit to yield monodisperse double emulsions of controllable structure at kHz frequencies[33].

Operation

Droplet-based microfluidic systems are able to generate large numbers (thousands to hundreds of thousands) of isolated assay volumes in short times. However, it is equally important to be able to control and vary what goes into each droplet (its payload) in a rapid and robust fashion. Although this can be achieved in a number of ways, the most direct and simple way is to vary the relative volumetric flow rates of the various fluid inputs that will eventually form the discrete phase. Typically, payload control takes the form of regulating the amount or concentration of multiple species (for example, when synthesising small molecules or nanomaterials) in each droplet or loading a user-defined number of (larger) entities into droplets, such as cells[35], DNA strands[36], micro-organisms[37] or particles[38]. In both cases, variations in the flow rates and concentrations of the incoming co-flows allow control of the droplet payload[39]. Additionally, payload control can occur after the droplet formation process. This can be achieved passively by merging decompressing droplets within a channel expansion[40] or by taking advantage of the difference in hydrodynamic resistance of the continuous phase and the interfacial tension of the discrete phase within a pillar array[41]. That said, and for obvious reasons, droplets stabilized by surfactants can often be difficult to merge, and thus active strategies for payload control, such as the use of acoustic radiation[42] and electrical fields[43] are often more useful. In this regard, the process of picoinjection has proven especially useful in a wide range of applications[44]. Picoinjection operates by flowing droplets past a channel containing a pressurized reagent. If a droplet is protected by a surfactant layer, the fluid will normally not enter the droplet. However, application of an electric field can be used to destabilize and rupture the surfactant layer, enabling reagent entry for a short period of time. The process is highly robust and allows controlled addition of fL-pL volumes at kilohertz rates.

To perform complex experimental workflows, droplets must be manipulated and processed in a variety of ways after formation. Unsurprisingly, a range of functional components have been developed for this purpose. A selection of some of the most useful are shown in **Figure 2**. Numerous embodiments of each component are available to the experimentalist, with each operating in a passive and automated manner and being readily integrable with other components required within a given workflow. For example, droplets can be split at microchannel bifurcations[45], with the daughter droplet size being controlled by the relative flow rates (or outlet pressures) in the downstream channels. The distance between droplets within a flow may be made larger or smaller by adding or removing the carrier fluid through a side channel[46,47]. Such control is especially useful when performed upstream and prior to

droplet sorting. The capacity to sort and isolate droplets of interest is exceptionally important in a range of experimental workflows. Droplet sorting involves three primary operations. First, a detector is used to rapidly assess droplet phenotype (e.g. droplet content, size or deformability). Next, and depending on this result, the droplet is either ignored or control electronics are used to trigger some kind of flow perturbation that directs the droplet of interest away from the primary flow. A number of external perturbations can be used to sort, including dielectrophoretic forces[48], acoustic forces[49] or even mechanical valves[50]. Of these, dielectrophoretic sorters have proved to be the most popular, primarily due to the fact that droplets can be sorted at kHz rates, with the use of a gapped divider (between waste and collection flows) allowing sorting at frequencies as high as 30 kHz. Importantly, operation at such rates ensures that droplet sorting is no longer the rate determining step within a droplet-based workflow. Finally, it is noted that a wide range of other functional components, such as droplet mixers[45], diluters[51], synchronizers[52,53], traps and incubators[54,55] can be routinely used to create complex and integrated experimental workflows.

Droplet detection

Whilst droplet-based microfluidic systems are proficient at performing complex work flows in a robust manner, information relating to the identity and amount of contained species at the end of (or during) an experiment must be extracted and collected from individual droplets within the system. This is an immense challenge, since droplets have small volumes (almost always sub-nanoliter and potentially as small as few tens of attoliters) and move through the system at appreciable velocities. As we have seen, enormous numbers of droplets may be formed and processed within microfluidic platforms, and thus the primary goal is to ensure that droplets can be robustly assayed at speeds that match their generation rates.

Much effort has focused on integrating sensitive, rapid and robust detection methods with droplet-based microfluidic systems. Although a wide variety of detection techniques are available to the experimentalist, fluorescence-based methods are by far the most popular, due to their exquisite sensitivity, low limits of detection, fast response times and simple integration. Since, fluorescence measurements can be performed on microsecond timescales, kinetic or dynamic information can be extracted from rapidly moving droplets in an efficient manner[47,57], with simultaneous detection of multiple species being achieved via multicolour point detectors[58] and time-integrated[59] or time-resolved[60] imaging. That said, most molecules are not fluorescent and thus a range of other optical detection strategies have been reported. These include absorbance spectroscopy[61], X-ray absorption spectroscopy[62], Raman spectroscopy[63], surface enhanced Raman spectroscopy (SERS)[64], Fourier transform infrared spectroscopy (FT-IR)[65] and photothermal spectroscopy[66]. Despite these techniques normally exhibiting inferior limits of detection and sensitivities than fluorescence-based

methods, they are label-free in nature, and in many instances provide far richer information regarding molecular composition and structure. This particularly applies to vibrational spectroscopies, due to their molecular specificity and quantitative nature. In this regard, recent developments in SERS have allowed for dramatic reductions in acquisition times (and improvements in sensitivity), and thus enabled the extraction of detailed vibrational signatures from single droplets with sub-millisecond time resolution[67].

Finally, it should be noted that droplet analysis can also be performed off-chip. In this regard, the use of mass spectrometry (MS) to perform label-free detection is of particular importance. Unlike optical methods, MS analysis necessitates the transfer of droplets into a mass spectrometer. The primary challenge when doing this is to remove the carrier fluid (and surfactant) before droplet transfer[68]. This is important since the separative phase can cause Taylor cone **[G]** instability and contaminate the mass spectrometer. Notwithstanding, the use of MS is highly desirable due to its ability to measure the identity and abundance of molecular components within complex mixtures. Importantly, trains of pL-volume droplets can be continuously introduced into nanoelectrospray ionization (nESI) emitters over extended periods of time to allow for the analysis of over tens of thousands of droplets[69,70]. Additionally, droplet contents may be assayed using inductively coupled plasma (ICP)[71] and matrix-assisted laser desorption/ionization[72] (MALDI) based methods.

Results

In a general sense, droplet-based microfluidic systems are normally used to either manufacture materials or characterize a chemical/biological system. When used for manufacturing, the direct output of the device is the objective, and when used for analytical purposes, the goal is to extract information about the system of interest. In some situations, droplets are processed and sorted for downstream analysis, such as in nucleic acid cytometry[73], enzyme evolution[74] or single cell genomics[10]. In all cases, the advantages of speed, control and precision fluid handling are the primary advantages of the approach.

Material synthesis use cases

When synthesizing materials or particles, the microfluidic approach aims to produce materials with properties that could not be otherwise achieved, by exploiting phenomena unique to the microscale. Droplets exiting the device may be (or may contain) the final product, or may need to be further processed to complete the synthesis, for example via gelation to solidify liquid precursors or via dewetting to transform microfluidically-assembled double emulsions into unilamellar vesicles[75–77]. In this way, a droplet acts as a structural

template from which the final particle is obtained, and thus the method is called droplet templating[75,77].

Single emulsion templating

When using single emulsions to manufacture particles, the primary benefit is the ability to combine and compartmentalize distinct reagents within monodisperse droplets of a desired size. For example, hydrogel particles are valuable in single cell analysis[78], but must have a controllable chemical composition and size. Additionally, and depending on the chemistry, gelation may occur immediately upon reagent mixing, such that if performed in bulk, a solid gel block in the shape of the container would result. By contrast, with single emulsion templating, millions of identical gel particles can be synthesized on short timescales[79]. Reagents can be combined as co-flows, so that they do not mix prior to emulsification[76]. Once inside a droplet, reactants mix via diffusion and chaotic advection to initiate gelation, solidifying into particles of equal size and shape[76,77]. Elaborate microfluidic networks comprising co-flow[80], picoinjection[44] and droplet fusion[81], can be used to combine reagents in defined sequences, in a way not possible with bulk mixing. In addition, microfluidically generated droplets or particles can be post-processed to further enhance their properties. For example, particles may be functionalized with enzymes[82], antibodies[83] and oligonucleotides[84] and geometrically distorted via centrifugation[85].

Single emulsion templating is a surprisingly general process, able to fabricate particles composed of hydrophilic, hydrophobic or fluorophilic building blocks[77,86]; the only constraints being that the chemicals used must not foul or degrade microchannel surfaces, must be encapsulated in a carrier phase with sufficient immiscibility such that droplets readily form and must generate an emulsion with enough stability such that particles have sufficient time to solidify. Normally, this is accomplished by using miscible fluids for droplet interiors and an immiscible fluid for the carrier phase, although even miscible inner and outer phases can be used, as in aqueous two-phase systems [87,88]. The use of harsh solvents, such as low molecular weight hydrocarbon oils or organic solvents, precludes the use of plastic or PDMS devices[20]. In such situations, coated channels, fluorinated elastomers and glass capillary devices can be effective alternatives[89–91].

Multiple emulsion templating

When a single emulsion droplet (such as water-in-oil W/O) is encapsulated in another droplet of an immiscible phase (like O/W), the result is a core shell structure called a double emulsion (in this case, W/O/W)[75,92] (**Figure 3**). If this double emulsion is encapsulated in another droplet, a triple emulsion will be formed[75,92]. Although this process could in principle be continued indefinitely, to date, the highest order multiple emulsions generated microfluidically are W/O/W/O/W/O quintuple emulsions[92]. A key feature of multiple emulsions, is that they consist of at least two immiscible phases physically segregated in

accordance with how the fluids were combined microfluidically. This affords unique opportunities for particle templating since the chemistries of the phases can be independently selected to optimize for different objectives. For example, an important application of double emulsion templating is the formation of microcapsule delivery vehicles for active compounds[93,94]. Here, a core phase can be selected to solubilize and stabilize the compound, whilst the shell is composed of an immiscible phase that acts as a barrier to the external environment[76,93,94]. Moreover, shell composition can be tuned to allow rupture upon application of a temperature, pH or chemical cue[75,93]. Shell chemistries can be selected to undergo physical or chemical transformations, to increase the types of structures that can be generated. For example, solvent evaporation or dewetting can transform double emulsions with lipid shells into unilamellar liposomes, polymer surfactants into polymersomes **[G]** , and colloidal surfactants (Pickering emulsions) into armoured droplet colloidosomes **[G]**[75,95]. Each type of core-shell structure can have unique properties tuned to the use case, whether in therapeutics (drug delivery), agriculture (pesticides) or cosmetics (enzymes)[93]. The number and content of the cores can be independently controlled, to generate multiple emulsions with one or many cores,[96,97] to enable triggered reactions by merging cores in the multiple emulsions, or to fabricate particles with non-spherical shapes, such as biphasic Janus particles[98].

On the microscale, interfacial tension and wettability **[G]** control fluid flow and, when immiscible fluids are used, which fluid is dispersed and which is the carrier phase. A hydrophobic channel will naturally favour the generation of water-in-oil droplets, while a hydrophilic channel will generate oil-in-water droplets[99]. With multiple emulsions, droplets of both polarities must be formed, often repeatedly and in different regions of a device[92]. In such instances, different regions should have different surface wettabilities. Creating wettability patterns can be laborious[100–103], and the reliance on wettability for droplet encapsulation can be unreliable due to surface fouling[104]. A different strategy involves hydrodynamic confinement techniques, such as flow focusing[105]. Here, a bespoke arrangement of nozzles and sheath fluids is used to hydrodynamically keep the dispersed phase away from the channel walls. Such devices produce single and double emulsion droplets with polarity being dictated by channel geometry and not wettability[105]. However, they can be difficult to fabricate and scale, and often require flow conditions that limit the size and uniformity of the double emulsions[105,106].

Scaling up production

When using droplets for emulsion or particle synthesis, a common issue is the small space-time yield. Typical devices will produce up to 1 mL of droplets per hour[107–109], which limits the use of these techniques in high volume applications. In such instances, the best approach is to scale out production via massive parallelization[110]. A strength of the photolithographic

process used to fabricate planar devices is that hundreds (or even thousands) of droplet generators can be fabricated within an area of only a few square centimetres[79,111]. By supplying these components with fluids via distribution networks, each generator is subject to identical flow conditions and will produce droplets of equivalent size and structure, thereby scaling production rates by orders of magnitude[79]. Moreover, stacking arrays enables the integration and parallel operation of tens of thousands of droplet generators[79].

Analytical use cases

The ability to generate and manipulate millions of picoliter droplets with control opens up a myriad of analytical applications. In general, these applications fall into two broad categories: screening, in which the devices are used to interrogate and isolate a sub-population, or whole population analysis, in which all population members are characterized in detail. In both cases, the ability to efficiently and cost effectively execute millions of distinct reactions is the key feature that makes droplet-based microfluidics enabling for these applications.

Screens

The efficacy of a screen is normally limited by the number of entities that can be tested: the more tested, the more likely an uncommonly valuable entity will be discovered[112]. On one end of the spectrum are selections involving flow cytometry[113], which can screen through millions of entities with ease, but are limited in the kinds of assays they can utilize. On the other end of the spectrum are reactions performed in well plates, in which a variety of information rich and sensitive assays can be used, but in which only hundreds of entities can be screened[114]. Droplet-based microfluidic screening attempts to combine the best attributes of each approach, such as the throughput advantages of flow cytometry with the analytical flexibility and control of well plates[115]. The concept finds its origins in in vitro compartmentalization[116], in which droplets serve as a minimal reaction volume for testing population members. Droplets are loaded with genes encoding the library members and the reagents needed for expression and testing. In this way, the droplets link the genotype of a population member (the gene encoding it) with the phenotype (the assay result from a droplet). Using microfluidic tools, droplets can be probed and sorted at kilohertz rates[117]. In this way, throughputs rivalling flow cytometry are achieved, but without relying on cells that may interfere with the assay. The approach has found particular utility in enzyme screening and evolution since, prior to its invention, such screens were usually limited to well plates and, thus, had limited power in identifying uncommon/rare efficient variants. The result of such a screen is usually a cell or gene sequence representing the best variant, which can then be analysed and subjected to additional rounds of mutagenesis and screening; a process known as directed evolution[118]. In addition to enzymes, droplet-based microfluidic screens are useful for cell and pathway engineering[119–121], metagenomic bioprospection **[G]** [122,123] and drug discovery and combination testing[124–126], as will be discussed later.

While droplet-based microfluidic screening is a fundamental advance over prior methods, there are nevertheless constraints that limit its utility and generality. The overall process can be complex, requiring multiple devices and steps to express, test and sort the library[10,127]. Moreover, because these steps must usually be tailored to the screen, it is difficult to build robust platforms that can be applied generally. Moreover, whilst the fluorinated oils and surfactants used to compartmentalize droplets are intended to maximize cell viability, biomolecule function and analyte retention, they are not perfect. For example, without suitable surfactants, proteins denature at the water-oil interface, rendering them non-functional. Additionally, the droplet environment can often be hostile to mammalian cells, which may become stressed and dye within hours or days[128], and many molecules, especially small hydrophobic molecules, leak out of droplets[129]. Accordingly, the droplet approach remains experimental and requires significant planning and development. Another constraint is that to date droplet-based screens have been primarily limited to fluorescence-based assays. Since droplets are small (with diameters no bigger than a few tens of microns) and must be analysed rapidly to realize the throughput advantage, they yield tiny optical signals, with fluorescence-based techniques most normally providing the requisite sensitivity[127]. This precludes many assays commonly used in well plates, since they rely on readouts that are incompatible with kilohertz droplet analysis and sorting. Consequently, there has thus been a push to expand the types of readouts that can be performed with droplet microfluidics using enzyme-coupled assays[130], aptamers[131] and cell-based reporters[132].

As noted earlier, an exciting new direction has been the use of unbiased mass spectrometry with droplet-based microfluidic screening[29]. The challenge here lies in integration, since the approach is destructive and usually takes multiple seconds to measure one sample. To overcome this issue, droplets can be split and the two halves maintained in registry in a delay line; one going to the MS for analysis and the other to the sorter[133]. Using electrospray ionization, a target molecule can be quantified in the analysis droplet and used to make a sorting decision for the sister droplet. Even when selecting just a single target molecule for quantitation the approach can only sort a few droplets per second, limiting the number of entities that can be tested to a few thousand[133]. Alternatively, droplets prepared microfluidically can be printed to an MS-compatible substrate and analysed[134,135]. High speed sorting can be used to ensure that every printed droplet contains a cell, overcoming the issue of Poisson loading **[G]**. Using fast MALDI imaging, sensitive quantitation of thousands of molecules in parallel can be obtained for every printed droplet within a few minutes. Because the spots exist on a fixed grid, the signal can be analysed off-line and hits recovered by manual or automated sampling, allowing hundreds of hits to be identified from tens of thousands of variants. Because this approach does not require the target to be defined ahead of time, it affords unique advantages, including the ability to quantify

substrate, intermediate and final product concentrations. In addition, the ability to quantify hundreds of other analytes allows the discovery of unexpected side products or novel activities through indirect sensing[134]. Accordingly, this approach offers the potential of providing a truly universal readout for enzyme screening with droplet-based microfluidics.

Whole population analysis

In screening experiments, a population of cells is characterized according to a minimal feature, such as the presence of a particular nucleic acid or surface protein, and sorted based on that feature for further study. This is an efficient process that can scale to millions of cells, but is biased by the feature chosen for the sorting decision. Often, cells of interest may comprise a system that is poorly understood, having no known feature on which to define the sorting decision. In these instances, unbiased, whole population single cell analysis provides an exciting way forward. The principle here is to skip sorting and instead perform detailed analysis on every cell in the population. This has only recently become practical with the advent of modern ultra-high throughput and information rich measurement techniques, such as mass spectrometry and DNA sequencing, and modern computing capabilities to process the petabytes of generated data.

When applying this idea to single cell multi-omics **[G]**, the challenge lies in recovering information about all individual cells in a cost-effective manner. For single cell genomics, the sequencing step is expensive and time consuming, and thus cannot be performed on each cell individually; rather, all cells must be batched into one sequencing run. Initial methods relied on well-plate indexing, conventionally applied to separate nucleic acid samples, but in which the samples contained, instead, single cells[136]. Unique DNA barcodes were attached to all nucleic acids obtained from each well, thereby allowing them to be traced back to a single cell. Performed in well plates, this approach proved expensive and limited to just a few hundred cells. Valve-based microfluidics was used to automate the process[137], providing some cost and data quality advantages, but failed to significantly increase throughput[138,139]. Here, again, the ability of droplet-based microfluidics to encapsulate single cells, perform efficient molecular biology, and scale to millions of droplets has afforded potent advantages over prior methods[138,140,141]. The community has embraced this platform, building on top of it a slew of molecular techniques for measuring myriad properties at the single cell level, including genotype, epigenotype, chromatin structure, transcriptome and internal and surface proteins[10]. Some of these methods can be multiplexed, such as genome and surface protein, and transcriptome and surface protein. In all cases, the result of microfluidic processing is to barcode the nucleic acids of the cells representing the different forms of information desired, so that it can be analysed in one sequencing run and deconvoluted back to single cells via the barcode. The general approach is applicable to most cell types, including mammalian, archaeal, bacterial and fungal cells. For multicellular organisms and solid tissues, cells can

be enzymatically disaggregated or nuclei can be extracted and analysed[142,143]. The impact of the technique is far reaching, in virology, microbiology, drug discovery, cell engineering and diagnostics[10,144,145], with companies in this space already worth billions of dollars and constituting the greatest commercial successes of droplet-based microfluidics to date. Additionally, the speed and efficiency with which the approach allows single cells to be analysed has stimulated a true revolution in cell biology, facilitating detailed cell atlases for all organs of organisms, such as such as humans[146], mice[147], fruit flies[148], *c.* elegans[149] and zebrafish[150]. These atlases provide an invaluable resource on which to base new hypotheses and interpret results that is reminiscent of the scientific impact of the first sequenced human genome.

Applications

As already shown, a range of functional droplet-based microfluidic technologies have been developed over the past two decades, and the technology set is now employed to excellent effect in a diversity of fields within the chemical and biological sciences. We now discuss some of the most important areas of application in more detail. At a basic level, the fact that pL-volume droplets can be made and manipulated at kHz frequencies, makes them ideally suited to compartmentalize and analyse large numbers of small entities (such as nucleic acids and cells) on an individual basis. Unsurprisingly, a number of interesting biological applications have been developed based on this concept.

Droplet digital PCR

Real-time quantitative PCR (qPCR) is used to examine the progression of amplification after each cycle using fluorescent reporter molecules, and is the benchmark for determining variations in gene expression levels. Unfortunately, variations in amplification efficiency with different primer pairs and targets necessitates external calibrators or normalization to endogenous controls. Additionally, qPCR is sensitive to inhibitors in the sample, which limits the accuracy and sensitivity of the technique for absolute quantitation; typically, sensitivity for the detection of mutant genomic DNA (gDNA) diluted in wild-type gDNA is no better than 1%[151]. Droplet digital PCR (ddPCR)[36,152] circumvents these limitations by using a large number of microfluidically-produced droplets. Here, target DNA is compartmentalized into tens of thousands to millions of pL-nL volume droplets (at a concentration of less than one target gene per droplet) together with one or more fluorogenic probes and amplified by PCR. The end-point fluorescence from each droplet is then measured, which is a binary positive-negative signal, and the absolute concentration of target genes determined by fitting the fraction of fluorescent droplets to a Poisson distribution. Such an approach is orders of magnitude more precise and sensitive than real-time qPCR and significantly more robust to

PCR inhibitors. Importantly, instruments for ddPCR are commercially available from companies such as Bio-Rad and Stilla Technologies, and allow for multiplexed detection. Droplets may be produced on one device, thermocycled off-chip and then analysed on a second device[152], or produced (using a gradient of confinement[32], for example), packed into 2D droplet arrays, thermocycled and analysed on a single-chip[153]. ddPCR has been used to quantify cell-free circulating tumor DNA for early-stage cancer diagnosis[154,155], detect of pathogenic bacteria[156] and viruses[157] and non-invasive prenatal testing[158], among many other applications.

Single-cell analysis

Phenotyping and sorting

Single cells can be compartmentalized in droplets following Poisson statistics[35,159] and a desired phenotype detected, typically using a fluorescence-based assay. Single cells within droplets can be incubated either in on-chip delay lines (for incubation times less than 1 hour) and analysed on the same microfluidic chip or incubated off-chip (for much longer incubation times) and then reinjected into a second microfluidic device for analysis. Assay reagents and, if required, cell lysis reagents[160] are typically co-flowed with cells prior to droplet formation. In some cases, it is necessary to add detection reagents into droplets after incubation (for example to allow time for cells to secrete proteins to be detected) via droplet fusion or picoinjection. Assays are typically based on measuring the fluorescence of whole droplets, or localized fluorescence on beads or cells within droplets. Enzymatic activity is typically measured using fluorogenic analogues of substrates for the studied reaction[161] or by coupling the studied reaction to a fluorogenic reaction[162]. Assays for binding activity are normally based on measuring localized fluorescence within droplets on single cells[163,164], single beads[165] or multiple beads[164].

Importantly, droplets containing cells with a desired phenotype can be recovered using fluorescence-activated droplet sorting (FADS)[48] or absorbance-activated droplet sorting (AADS) within the microfluidic system[166], or through the use of commercial fluorescence-activated cell sorters (FACS)[132]. Such approaches have been used for ultrahigh-throughput screening of microorganisms for directed evolution, bioprospection and metagenomic screening, but also for other applications, notably the screening of antibody secreting cells.

Dynamic phenotyping

It is possible to immobilize tens of thousands of droplets containing single cells in two-dimensional (2D) arrays of tightly packed droplets[167–169]. In this way, droplets may be imaged

over extended periods of time to allow for quantitative and dynamic single-cell phenotyping[170]. The use of static droplet arrays allows measurement of the frequency of cells as well as extraction of additional functional characteristics, such as secretion rates and affinity **[G]** of antibodies[168,170,171] and cytokines[172] at the single-cell level, using for example, immunoassays based on fluorescence relocation[168,169]. Such systems are well-equipped to provide dynamic snap-shots of complex immune responses, such as cell-mediated killing[173], antibody secretion and affinity/specificity after immunization[168,174,174], infection[175] or auto-immunity[176]. The acquired data can also be used to advance understanding through modelling and simulation[177], providing deeper insights into the biological system under study[167,175]. In addition, osmotically induced changes in droplet volume can be used to probe the metabolism of single cells, while simultaneously imaging the cells to measure both growth and division[167]. Indeed, such concepts have been used to study the metabolic cost of rapid adaptation of single yeast cells[178]. Lower density 2D droplet arrays, where droplets are immobilized at specific positions using flow traps[54] or surface energy anchors[179], can also be used for temporal monitoring of single cells and colonies derived from single cells[180].

Sequencing

Droplet-based microfluidics has undoubtedly revolutionized single-cell transcriptomic analysis, allowing the analysis of tens-of-thousands of single-cells in a single experiment. The principal instruments used for high-throughput single-cell RNA sequencing (scRNA-seq), the inDrop[84,181], Drop-seq[182] and 10× Genomics Chromium (10×)[183] systems, are all based on a similar operating principle; namely, single cells are co-encapsulated with single beads carrying barcoded cDNA primers in droplets (**Figure 4**). Once compartmentalized, cells are lysed and the barcoded primers hybridized to the released mRNA and used to prime reverse transcription, resulting in distinctively barcoded cDNA. Since all primers on a single bead contain the same barcode, cDNAs from the same cell will carry the same barcode. After next-generation sequencing (NGS), reads from the same cell can be directly identified via this barcode. Additionally, the barcoded primers also have a unique molecular identifier (UMI) to correct for amplification bias[184,185]. Finally, advanced bioinformatic tools are used to cluster cells according to gene expression profiles, revealing rare cell types that are almost always overlooked when using bulk or low-throughput methods. Nevertheless, scRNA-seq techniques differ in several respects. Drop-seq uses rigid methacrylic polymer beads, with the barcoded primers being synthesized using on-bead, split-and-pool reverse-direction phosphoramidite synthesis, whereas the inDrop and 10× systems use elastomeric hydrogel beads (themselves synthesized by polymerization in microfluidically-generated droplets), with barcoded primers being produced by split-and-pool ligation (10×) or split-and-pool primer extension (inDrop) (although split-and-pool ligation can also be used with inDrop[186]). Injection of closely packed hydrogel beads advantageously avoids Poisson

distribution limitations of beads in droplets[187]. In drop-seq the primers on the beads are used to capture mRNA in droplets, and cDNA synthesis is performed in bulk after breaking the emulsion, whereas when using inDrop and 10× primers are released from beads by UV photocleavage or by dissolution, respectively, with cDNA synthesis being performed in the droplet. These systems can be used for sequencing total mRNA (priming on the poly(A) tail) and/or targeted RNA-sequencing, for example, for paired VH-VL chain sequencing of antibody genes[164,188] or paired ➔-➔ chain sequencing of T cell receptors (TCRs)[189–192].

While single cell transcriptome sequencing methods reveal unique cell states, underlying differences are determined by regulation of gene expression in the nucleus. Droplet-based barcoded single-cell sequencing has been adapted to study chromatin accessibility using ATAC-seq[193] including in combination with RNA-seq and modulation of chromatin structure via histone modification using ChIP-seq[186,196]. Droplet-based barcoded single-cell RNA-seq can also be combined with the analysis of cellular phenotype using CITE-seq[197]. In CITE-seq, cell-surface proteins are labelled with oligonucleotide-tagged antibodies prior to encapsulation and in the droplets the antibody tags are captured by barcoded primers provided by beads and associated with the same barcode as the cellular mRNAs.

Antibody discovery

Single antibody secreting cells in droplets can be screened using FADS[48] based on different assays —for example, binding to a purified soluble antigen[164], binding to multiple soluble antigens (to determine cross-reactivity or binding specificity), binding to cell-surface antigens (on bacterial or eukaryotic cells)[163,164] target antigen inhibition[198], cellular internalization, opsonization **[G]** and modulation of cellular signalling pathways[164]. Significantly, millions of non-immortalized plasma B cells or activated memory B cells from immunized mice or human donors can be screened per experiment. Sorted B cells can then be recovered and paired V_H-V_L sequencing of antibody genes from recovered single cells performed in microtiter plates or re-compartmentalized in droplets for single-cell RNA-seq in a droplet-based microfluidic system (see below)[164]. A similar system has also been used for high-throughput functional screening of single cells transfected with lentiviral libraries **[G]** of antibody fragments pre-selected by phage display **[G]** from a large naïve library (10^{10} clones): here activated reporter cells were co-compartmentalized in droplets containing single lentiviral-transduced antibody secreting cells and screened using FADS to identify rare agonist antibodies of the costimulatory receptor CD40 and functional anti-Her2 × anti-CD3 bispecific antibodies[199].

Directed evolution and metagenomic screening

Directed evolution **[G]** exploits Darwinian evolution in the laboratory to generate proteins (and nucleic acids) for industrial or biomedical applications. It involves iterative cycles of mutation and/or recombination of genes, followed by selection or screening for genes that encode proteins where a desired activity has been improved[200]. Droplet-based microfluidics is an immensely powerful tool for directed evolution as it allows for ultrahigh-throughput screening[201–204]. Large libraries of mutated/recombined genes can be created, transformed into microorganisms for expression and compartmentalized in droplets for single-cell screening (**Figure 5**). Screening of a range of microorganisms, including bacteria[48,160,161], yeasts[140,205] and filamentous fungi[206], has been demonstrated. Alternatively, single genes can be expressed in droplets using cell-free expression systems (in vitro transcription translation **[G]** or IVTT systems)[206]. In vitro transcription **[G]** (IVT) systems can be used similarly for screening and directed evolution of RNAs[207–212]. Single genes must be PCR-amplified in droplets prior to IVT or IVTT, which requires the addition of new reagents after amplification via droplet fusion or picoinjection. Whether expression is in cells or using cell-free systems, the binding or catalytic activity of the expressed protein (or RNA) is assayed in the droplet, typically using a fluorescence assay, with the droplets exhibiting the highest activity being selected.

Droplet-based microfluidic systems can increase screening throughput by over three orders of magnitude, and reduce costs by six orders of magnitude when compared to conventional microplate-based screening systems[140]. They have been used for the directed evolution of a range of enzymes, including peroxidases[140], hydrolases[160], phosphotriesterases[213], ➔-L-threofuranosyl nucleic acid (TNA) polymerases[214], esterases[215], dehydrogenases[166], oxidases[163,216], sulfatases[217] and aldolases[12,161], as well as catalytic RNA (X-motif ribozyme)[207] and multiple fluorogenic RNA aptamer biosensors[208–212]. Significantly, directed evolution in droplet-based microfluidic systems is faster than in microplates and evolution can continue when microplate-based systems fail due to reaching an apparent local fitness plateau, from which escape is only possible via screening of a larger number of variants. For instance, directed evolution of an artificial aldolase using droplet-based microfluidics improved catalytic activity 10-times faster than using microplate assays[12] and the best enzyme from a stalled microplate screen was improved to give a greater than 10^9 rate enhancement, similar to that of natural class I aldolases[161]. Ultra-high-throughput screening in droplet-based microfluidic systems can be used in a similar way to screen natural microorganisms for a desired enzymatic activity [123] and to screen large metagenomic libraries[122].

3D microtissues

Going beyond single cells, the analysis of individual spheroids (three-dimensional cellular aggregates that mimic tissues) compartmentalized in droplets can also be parallelized using surface energy anchors[179] to immobilize drops within 2D

arrays[218]. Different conditions can be tested in a single device by the merging of new droplets with spheroid-containing droplets, allowing, for example, screening of the effect of a drug over a large concentration range in a single experiment.

Material synthesis

The benefits of using droplet-based microfluidic systems for chemical production are also now well recognized. In simple terms, the ability to create and homogenise solute and temperature gradients on short timescales, whilst preventing surface-molecule interactions ensures that the chemist is able to control reaction conditions in a way that is simply not possible on the macroscale. Unsurprisingly, droplet-based reactors have been used to excellent effect in the synthesis of small molecules[219], semiconducting polymers[220], catalysts[221,222] and biomimetic materials[223]. However, they have proved especially enabling in the synthesis of nanoscale materials, where the ability to control particle nucleation and growth is essential to the production of bespoke materials, with user-defined optical and electronic properties[224] (**Figure 6**). Since the first report of nanoparticle synthesis in a droplet-based reactor[225], the generic platform has been used to create a wide range of complex, nanoscale materials that are either difficult or impossible to make using conventional wet-chemistry methods. Notable examples of such materials include, inorganic semiconductor nanoparticles[225], metal halide perovskite nanocrystals[11,226], conjugated polymer nanoparticles[227], carbon dots, noble metal nanomaterials[228] and rare earth upconversion nanoparticles[229]. Whilst the ability to directly produce high quality materials was the initial driver for adopting droplet-based platforms, their real strength lies in their ability to explore complex reaction parameter spaces on timescales many orders of magnitude shorter than those associated with conventional (bench-top) methods. Here the ability to integrate sensitive analytics, reaction control architectures and efficient machine learning algorithms is key[228].

Artificial cells and the origin of life

Droplet-based microfluidic systems have also proved to be a useful tool in investigating various questions related to the origin of life. For example, they have revealed that compartmentalization of an unfavourable synthetic reaction in pL-volume aqueous droplets can improve reaction thermodynamics and mesoscale compartmentation[230] and could have helped to overcome the thermodynamic unfavorability of certain synthetic reactions, which has led to criticism of the prebiotic broth theory for the origin of life. The Belousov–Zhabotinsky (BZ) reaction, a well-known chemical oscillator, compartmentalised in microfluidically-produced droplets has also been used as a model to study complex nonlinear phenomena: the diffusion of chemical intermediates between compartments triggers specific reactions leading to collective dynamics more typical of biological systems

(reviewed in[231]). Darwinian properties and their trade-offs have also been studied in thousands of autocatalytic RNA reaction networks by adapting droplet-based barcoded single-cell RNA-seq (see above) for single-droplet RNA sequencing[232]. Finally, permeation measurements using continuously generated microfluidic droplet interface bilayers have unveiled the enantioselectivity of lipid bilayers[233], largely overlooked in computational modelling although paramount for drug design, notably.

Reproducibility and data deposition

As discussed, droplet-based microfluidic experimentation involves the multidisciplinary integration of numerous concepts, physical features, peripherals and design elements. Designing and fabricating microfluidic devices includes principles in mechanical engineering (in terms of fluid dynamics and structural design), electrical engineering (in physical design and electronic integration), chemistry (surface chemistry), and computer science (notably, control theory, automation and machine learning), as well as far-reaching applications. Due to the highly interdisciplinary nature of microfluidics and limited economic incentives, there is a recognised lack of standards with regard to design principles, formats, operations and fabrication methods across the field. Furthermore, whilst many researchers have reported individual devices that generate consistent results under specific conditions[234], reproducibility across labs and platforms is not ubiquitous. To achieve cross-lab reproducibility, an understanding of the sources of variability, standardization and reporting standards need to be addressed.

Sources of Variability

Each step of the microfluidic process introduces some degree of variability, due to the design element heterogeneity, diverse manufacturing methods and varied operating conditions. Moreover, as the system increases in complexity, errors in each primitive are compounded. These sources of variability are summarized in **Box 1**.

Design Element Heterogeneity

When incorporating a primitive into a design, researchers typically search through examples of functional components published in the literature and account for manufacturing techniques, specific applications and operation methods. These considerations often leave the researcher with limited options for characterized microfluidic devices. Moreover, due to the large number of parameters in the design process, finding the optimal solution requires fine-tuning of multiple parameters. One way to standardize this process is to limit the degrees of freedom when designing microfluidic systems. Additionally, an expansive

microfluidic database containing designs, experimental conditions and performance metrics is necessary to build on established research and increase accessibility of the microfluidics field. These databases could be used to train a machine learning tool that maps designs and operation to device performance for automated design of microfluidics. Currently, a limited number of repositories do exist, such as *Metafluidics*[235], however these are not widely used or standardized, possibly due to a limited amount of information required when uploading a design.

Diverse Manufacturing Methods

As discussed, wide variety of methods are used to manufacture microfluidic devices, including photolithography[236], micromachining[237], replica molding[238], laser ablation[239], 3D printing[240], chemical etching[241], hot-embossing[242] and injection molding[242]. Each of these methods has different workflows, and within a method, depending on the equipment and standards adopted by each group, workflows may differ. Adding a further layer of complexity and potential failure points, different bonding and surface treatment methods can also be used. To minimize the variability both within and across manufacturing methods, reporting unambiguous manufacturing techniques is necessary. Additionally, it is also advantageous to automate established techniques to decrease human interaction and increase quality control[244].

Execution and Operation

The operation of microfluidic systems is almost always application-driven. Whilst different applications may often use similar primitives, their operation methods will differ in the fluids and fluid manipulations used. For example, surfactants are often used to stabilize droplets, but the use of different surfactants or varying concentrations will almost always alter the behavior of a device[245]. In this regard, and as discussed previously, although droplets are widely considered to act as isolated compartments, mass transfer to and from droplets will occur to some extent, causing temporal variations in composition[18]. Furthermore, many studies have shown droplet volume change during cell incubation[246]. Accordingly, standard operating conditions should be established to reduce variability between methods, by forming operating classes that describe key experimental conditions. As these processes become automated, there will be reduced human interaction which should decrease batch-to-batch variability[247]. Additionally, with automated processes, there is an opportunity to develop standardized and automated testing to ensure quality control and calibration of microfluidic devices.

Reporting Standards

As in many science and engineering disciplines, there are no clear and consistent reporting standards across the field of droplet microfluidics. However, to aid the wide adoption of microfluidics devices, full reporting of experimental procedures is conventional. In this regard, three key technical elements need to be described: standards, workflows and metrics. Standardization across the droplet microfluidics field is essential. These standards need to describe the manufacturing, functional and operational aspects of the devices, including a complete description of device performance[248], physical device features and composition[56] and design and fabrication constraints[243]. We propose that journals require researchers to report which standards were implemented in their experiments or classify work by standard type. Clear workflows that show designer[249], manufacturer[250] and user[251] perspectives also need to be developed and reported. These workflows should motivate the need for complex devices and demonstrate how a core set of microfluidic operations can enable a wide swathe of applications. This is analogous to instruction set architectures (ISA) in modern microprocessor engineering[252]. Finally, reporting defined metrics to online databases is necessary for easy comparison between different devices[253]. These metrics should encompass both the complexity of manufacturing and device performance, thereby providing benchmarks for microfluidic operations to be quantitatively compared and help inform where design effort should be placed to maximize future performance. Minimally, the performance metrics include droplet size, polydispersity, throughput and dynamic range[254].

Limitations and optimisations

The utility and potential of microfluidically produced droplets in biological and biochemical screening are now well-recognised. However, it must be remembered that the implementation of reliable and robust protocols for both chemical and biological assays in droplet-based microfluidic systems is based on the assumption that droplets generated and dispersed in a continuous carrier fluid are able to act as stable and biocompatible reaction volumes over the timescale of a particular experiment. The problem with such an assumption is that dispersions of one liquid phase in another are intrinsically unstable. The interfacial tension associated with a liquid-liquid interface contributes to the free energy of the system. For a given dispersed phase volume, minimization of the free energy of the system is equivalent to minimizing the interfacial area between both phases[18]. It is straightforward to show that the equilibrium condition is simply defined by two bulk phases separated by a minimal surface; a spherical cap in the absence of gravity. Accordingly, a dispersion will inevitably age towards this state. As noted previously, to stop this happening, surfactants can be used to stabilize the dispersion by providing kinetic barriers to the decay towards the state of minimal energy and prevent the coalescence of colliding droplets. In this regard, it should not be forgotten that stabilisation is not an equilibrium process and that the dispersion will

ultimately and inevitably and spontaneously move towards a state of minimal energy (**Figure 7**). The goal is therefore to provide formulations that guarantee the meta-stability of the system over timescales larger than the duration of the desired experiment.

The optimisation of a formulation involves consideration of four fundamental aspects, namely the stability of the microcompartments (mechanical stabilisation against coalescence), the stability of the payload of the compartment (chemical stability of encapsulation against ripening), the biocompatibility of the system (the ability to perform biochemical reactions without affecting equilibrium or reaction kinetics[18]) and the compatibility of the formulation with the operational conditions of the microfluidic system itself (in terms of the device material but also in terms of the rheological properties of the complex fluid that must be reliably actuated). These aspects may appear independent at first glance, but they are in fact intimately intertwined. For example, the mechanical stabilisation of droplets must be achieved immediately after their production. To ensure compartmentalization at kHz generation frequencies, the droplet interface should be covered with surfactants on a millisecond time scale[255,256]. However, the downstream manipulation of droplets to enable complex multistep protocols involves operations such as droplet fusion[41], pico-injection[44] or emulsion breakup for the recovery of the encapsulated compounds[257] and therefore a reversible or at least controllable interfacial stabilisation is required. At another level, the required biocompatibility for cellular assays and manipulations necessitates that respiratory gases be exchanged and transported through the fluid phases, whilst nutrients and essential metabolites should be retained within the droplets compartment. Accordingly, molecular exchange must not be simply removed but rather controlled. Without reconstructing the history of formulation optimisation, surfactant stabilised water-in-fluorocarbon oils emulsions appear as the most appropriate system for most droplet-based microfluidic applications. Respiratory gases are highly soluble in fluorocarbon oils, while organic molecules have a much lower solubility in fluorocarbon oils than in organic oils. Many of these have dynamic viscosities comparable to that of water (~ mPas), which aids flowability in micron-sized channels. Fluorocarbon oils provide an excellent basis for technological solutions since they are compatible with polydimethylsiloxane substrates, as measured through swelling[20]. Additionally, the low relative permittivity of fluorocarbon oils (~ 5–10)[258] ensures dielectric contrast with aqueous solutions, which is important for electroactuation of droplets by dielectrophoresis[48,259], and their high compressibility provides for a contrast in sound velocity, which is important for actuation by surface acoustic waves[260]. For these reasons, formulations based on aqueous fluids in fluorocarbon oils have emerged as the most appropriate and convenient system for droplet-based microfluidics. Emulsion stabilisation is most normally achieved using block copolymer surfactants containing fluorophilic and hydrophilic moieties[18]. The equilibrium interfacial tensions of the oil/water interface are 1–20 mN/m[261] and directly affect the manipulation of droplets. For example, droplet splitting within

a microchannel constriction is enhanced when capillary numbers **[G]** are increased[262], with increased velocities or lower interfacial tensions reducing the reliability of droplet manipulation, and formulations based on block copolymers have been shown to be relevant for ultra-high throughput manipulations, at rates of several kHz[117].

The absence of charges on the hydrophilic side of the droplet interface has been shown to be important in reducing protein adsorption[263], cell death[159] and molecular transport[129]. One of the key features of water-in-fluorocarbon oil emulsion is their ability to enhance molecular retention of hydrophilic molecules. Organic oils were quickly proven unreliable candidates for droplet-based microfluidic applications because of significant molecular exchange between droplets[264]. The reason for such exchange directly relates to the thermodynamic equilibration of chemical potential amongst droplets[265]. Further, minimal emulsions have been used unravel the fundamentals of molecular transport in water-in-fluorinated-oil emulsions. Using such an approach, it has been quantitatively shown that transport results from permeation of the solute across the oil phase[129]. The oil acts as a permeable membrane and a slight solubility of the solute in the oil phase is sufficient to provide a driver for chemical equilibration, with the time scale of the process depending on the partition coefficient of the solute between the dispersed and continuous phase. This simple model explains why fluorocarbon oils are preferred over organic oils[264] and why hydrophilic molecules are better sequestered than hydrophobic molecules, thereby providing guidelines on how to optimize probes to be used in droplet-based experiments[266]. It also explains why additives such as Bovine Serum Albumin[264], sugars[264] or salts[129], as well as buffers and pH[267] affect the kinetics of payload exchange. Put simply, adding a molecule to a mixture modifies the chemical potential of all species and therefore changes the equilibrium constants and partition coefficient. The surfactants themselves are a major factor controlling partitioning, with the timescale of the kinetics of transport being shown to be inversely proportional to the surfactant concentration[129]. This has important consequences on formulations. For example, high surfactant concentrations that allow rapid stabilization of interfaces[254,255] will enhance payload exchange, and thus a balance must be found for formulation optimization. In this regard, stabilizing methods based on nanoparticles have been envisioned to improve cell adhesion, biocompatibility and even payload exchange[268,269]. However, currently they fail to produce compartments that are easily manipulated, since the rheological properties of the Pickering emulsions generated are incompatible with flows within micron-sized channels[270]. To conclude, it is clear that the feasibility of any assay within a droplet format must account for the payload exchange timescale, which will impact the implementation of the technology for drug screening, especially for those applications where hydrophobic molecules are targeted.

As discussed, the vast majority of detection methods used in droplet-based microfluidic experiments are fluorescence-based. Although absorbance detection has been used to

probe segmented flows, and indeed has even been used for droplet sorting at moderate throughputs, the reduced optical pathlengths associated with microfluidic systems severely compromise both sensitivity and limits of detection[61]. Other label-free methods, such as photothermal spectroscopy, have also been used to probe droplets, but whilst sensitive and fast, they require further development before they can be used in a routine manner. We previously highlighted the potential utility of mass spectroscopy for probing complex biological systems. In this regard, it is important to note that single-droplet electrospray ionization mass spectrometry (ESI-MS) has been demonstrated[271] and even used to trigger mass-activated droplet sorting (MADS)[133], but at extremely low throughput (0.7 s^{-1}). Accordingly, further improvements in this and other label-free techniques would greatly enlarge the range of applications of droplet-based microfluidics. In this regard it should be noted that piezo-acoustic dispensing has recently been used to isolate individual cells in sub-nanoliter volumes on fluorinated surfaces for highly parallel single-cell proteomic sample preparation. Although, such workflows have yet to be transferred to microfluidic formats, the basic method enables the processing thousands of single cells in parallel for high-throughput, high-information content analysis[272].

While 2D droplet arrays allow for time-resolved imaging of droplets over extended periods of time, it is currently not possible to couple such dynamic analysis to droplet sorting. In the future, it may be possible to couple dynamic analysis of droplets in such arrays to FADS, for example by photoactivation of a fluorophore in droplets with desired properties prior to FADS. Similarly, it is not possible to map phenotypic data from individual droplets onto single-droplet sequencing data, for example to map single-cell phenotypic data (from imaging) onto single-cell sequencing data. Methods to enable this, for example based on combining 2D droplet arrays with DNA microarrays carrying barcoded primers/adapters/transposition sequences, would be extremely valuable.

As considered in the previous section, transcriptomics (scRNA-seq) is currently the best developed and most widely used single-cell omicsapplication of droplet microfluidics. Other omics applications are less well developed, in particular proteomics, which is currently largely limited to analysing proteins on the surface of cells using DNA-tagged antibodies (CITE-seq)[197] and metabolomics, which is effectively inaccessible. Proteomic analysis is in general limited by the absence of high-throughput MS-based analysis in droplet-based microfluidic systems and the absence of next-generation protein sequencing systems. That said, progress has recently been made in the development of single-molecule protein sequencing technologies[273,274] and could rapidly open the way to droplet-based single-cell protein sequencing. Furthermore, it would be highly advantageous to access different sorts of omic data from the same cell[275]. However, the ability to perform such multi-omic analysis is currently relatively limited within droplet-based microfluidic systems. Accordingly, there is a clear need to expand the range and performance of omics techniques and multi-omic

analyses that can be performed in such systems. Finally, single-cell sequencing, where beads are used to deliver barcoded primers, is expensive and limited to the analysis of approximately 10,000 cells per experiment. Bead-free methods of single-droplet barcoding may therefore prove to be a rather attractive alternative for some applications. Indeed, PCR amplification of single barcodes in drops, followed by fusion of the droplets with droplets containing target cells, has previously been demonstrated for single-cell genomic DNA sequencing[141,276].

Outlook

We hope that this primer has to some extent highlighted the impact of droplet-based microfluidics in the chemical and biological sciences. It is evident that within a period of less than two decades, the technology set has matured to a level where droplet-based technologies can now be viewed as basic tools that are accessible to many, and when used properly engender new chemical or biological insight. Although droplet-based microfluidic platforms can provide many advantages for the experimentalist, they are not a panacea. Their adoption must be driven by clear and compelling benefits with respect to factors such as analytical performance, accessibility, cost and information yield. In this spirit, it is critical to acknowledge that droplet-based microfluidic tools are not all empowering (or even useful) in many scenarios, and thus it is critical to correctly identify the applications and experiments where most benefit can be garnered.

When discussing any disruptive technology, a focus is inevitably placed on features that advance the state-of-the-art and how these features might transform what the user can do or achieve. Unsurprisingly, much less attention is paid to limitations, challenges or weaknesses. These issues are often quite apparent to the those skilled in the art, but almost always less obvious to those new to the field. Although some of the most important limitations of the platform have already been highlighted, it is worth emphasizing these again, since they will undoubtedly play a significant role in the future development and application of the technology. First, and as discussed previously, droplets are imperfect vessels in which to perform chemical and biological experiments. Payload exchange will always occur to some extent, and whilst this can be advantageous (for example, when exchanging respiratory gases in cellular experiments), it is most normally problematic. In this regard, it is encouraging that some progress has been made in the design and application of bespoke surfactants and continuous phases, with the development of dendronized fluorosurfactants[277] (able to form robust droplets that are stable and resistant to inter-droplet material transfer) and fluorinated Pickering emulsions[278] (mitigating both inter-droplet transport of small molecules and adsorption of macromolecules at the droplet interface) being notable recent advances. Further developments in this area will likely provide enhanced control over both

the degree and timescale of payload exchange, opening up a plethora of new opportunities in high-throughput small-molecule screening. Moreover, the ability to regulate both intra-droplet and inter-droplet molecular transport is likely have significant utility in the design, fabrication and functionality of complex droplet-derived synthetic cells able to more closely mimic the features and biological function of natural cells[279–281].

It is clear that the adoption of droplet-based microfluidic technologies has most impact when performing complex biochemical experiments, with the gains associated with throughput, control, precision and sample usage being undeniable. Whilst the literature is replete with examples of such systems, the realisation of robust droplet-based platforms is normally a result of extensive empirical investigations and trial-and-error optimisations. One may argue that such an approach has been enormously successful, however it is also clear that developed platforms are likely to be sub-optimal with regard to performance. Accordingly, automation of aspects of the microfluidic design process would potentially transform both performance metrics and accessible workflows. In this regard, it is important to note that the in silico design of microfluidic circuits is far from trivial and significantly more challenging that the methods used to design integrated circuits. Electronic design automation software tools (from providers such as Ansys and Cadence Design Systems) allow engineers to design, test and optimise circuits prior to chip fabrication. This is currently far from routine for microfluidic circuit design, since fluid physics is challenging to abstract. That said, activities in this space are advancing rapidly. For example, *DAFD* (*Design Automation of Fluid Dynamics*) is an open source simulation tool that leverages machine learning to design and predict the performance of droplet generators[253]. Such an approach allows the rapid design of single components, but can also be extended to support additional fluidic operations in a simple and direct manner. Not only does this ensure predictable device performance, but also in principle allows non-expert users or automated systems to design and fabricate devices for specific applications. Indeed, it is inevitable that in the short-medium term machine learning will transform both the design of microfluidic systems and the way in which complex chemical and biological workflows are performed and analysed[282]. The intuition of the expert microfluidicist will be encapsulated through machine learning, ensuring that many of the current barriers to the adoption of droplet-based microfluidic platforms will removed in both R&D and commercial scenarios.

Despite their utility, it is a simple fact that droplet-based microfluidic devices are rarely inexpensive and simple to produce or access. As discussed, a diversity of techniques can be used to produce microfluidic devices, but end-use scenarios are highly variable. This means that it is unlikely that device costs will approach those associated with conventional lab consumables, such as the 96-well plate, in the short term. Nevertheless, we have already seen significant commercial success in regard to the utility of droplet-based microfluidics; the most obvious example being in the field of single cell analysis. The ability to efficiently

encapsulate and process hundreds of thousands of single cells on short timescales has revolutionised the field, allowing quantitative analyses on a previously unimaginable scale and providing new insights into cellular processes. Such commercial applications of the technology set will almost certainly expand dramatically in the coming years, driving the standardization and modularization of microfluidic components, and ensuring that cost of microfluidic of microfluidic consumables will be progressively reduced. Finally, even though droplet-based microfluidic systems have already been shown to be adept at performing a range of functional operations and complex experimental workflows, technical innovations will continue to provide for enhancements in analytical throughput, sensitivity and operational sophistication. Whilst these will be welcome, the effective integration of sensitive, information rich detectors and machine learning algorithms will likely be far more important in transforming the ability of droplet-based platforms to rapidly process complex chemical and biological workflows at unimaginable rates and with unrivalled precision.

To conclude, we believe that droplet-based microfluidics has already proved itself to be a disruptive technology, allowing chemists and biologists to rethink and reimagine the structure and complexity of experimental workflows. Further, we expect that the continued adoption of droplet-based microfluidic tools by end-users, will encourage and accelerate the development of microfluidic embedded instruments, that will be used by experimentalists who may not necessarily be interested or care about the underlying technology, but are rather driven by the desire to generate high quality chemical/biological information as fast as possible.

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Author contributions

Introduction (T.M., A.dM.); Experimentation (T.M., A.dM. and J-C.B.); Results (C.M. and A.A.); Applications (K.S., A.G., T.B., J-C.B., T.M. and A.dM.); Reproducibility and data deposition (D.A. and D.D); Limitations and optimizations (T.B., J-C.B., A.dM., A.A., D.D. and A.G.); Outlook (A.dM., A.A., J-C.B., D.D. and A.G.); Overview of the Primer (T.M, A.dM., A.A., J-C.B., D.D. and A.G.).

Competing interests

A.A. founder of Mission Bio and Fluent Bio. J-C.B. is founder and shareholder of Emulseo. D.D. is a founder and shareholder of Lattice Automation, Inc., Asimov Inc. and BioSens8 Inc. A.G. A.D.G. is a founder and shareholder of HiFiBiO Therapeutics, Biomillenia (now Design Pharmaceuticals), Cyprio and Minos Biosciences

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Box 1. Summary of sources of variability in droplet-based microfluidic experimentation.

Sources of variability can be categorised into the design, manufacturing, and operation of microfluidic devices. Those listed are common examples within each category that account for the majority of variability cases.

Design

- Various design parameters
  - Channel depth
  - Generator width
  - Input channel widths

Manufacturing

- Different manufacturing parameters
- Microfluidic materials
- Bonding techniques
- Surface treatment methods

Execution and operation

- Fluid viscosities
- Surfactant concentrations in oil
- Droplet leakage
- Flow control and rates

Terms

Interfacial tension

The force of attraction between molecules at the interface of two fluids.

Reynolds number

A dimensionless parameter quantifying the ratio of inertial forces to viscous forces in a system; useful in predicting whether a flow will be laminar or turbulent.

Taylor dispersion

An effect in which a shear flow can increase the effective diffusivity of a species. In simple terms, shear acts to smear out the concentration distribution in the direction of the flow, enhancing the rate at which it spreads in that direction. An effect in which shear acts to smear out the concentration distribution in the direction of the flow, enhancing the rate at which it spreads in that direction.

Taylor cone

The shape of a fluid jet generated during electrospraying (such as during the sample ionization for mass spectrometry).

Polymersome

An artificial vesicle in which the vesicle membrane is composed of amphiphilic block or amphiphilic block or triblock copolymers, with high stability and tunable size.

Colloidosome

A solid microcapsule formed by the self-assembly of colloidal particles at the interface of emulsion droplets.

Wettability

Describes the ability of a liquid to spread over a surface. It is normally quantified through measurement of the contact angle between the liquid and surface.

Bioprospection

A systematic and organized search for useful products derived from bioresources including plants, microorganisms and animals that can be developed further for commercialization or overall benefit to society.

Poisson loading

An encapsulation strategy in which droplet occupancy follows a Poisson distribution.

Multi-omics

An analysis approach that combines data from multiple omic sources, such as genomics, proteomics, transcriptomics, epigenomics and metabolomics, to study living systems in a concerted manner.

Affinity

The strength of the binding interaction between two molecules. Affinity can be described by the dissociation constant (K_D) or by the standard free energy change ($\Delta G°$): $\Delta G° = -RT\ln K_D$ where R is the gas constant and T the absolute temperature.

Opsonization

Opsonization is an immune process that uses opsonins (extracellular proteins) to mark foreign pathogens for elimination by phagocytes.

Lentiviral libraries

Libraries of genes cloned into vectors derived from lentiviruses, which infect by inserting DNA into the host cell genome, and which can infect non-dividing cells.

Phage display

A method to select large libraries of genes encoding proteins, where genes are inserted into a phage coat protein gene, resulting in phage particles with the protein displayed on the surface and the gene that encodes it inside the phage particle, generating a connection between genotype and phenotype.

In vitro transcription translation (IVTT)

Coupled in vitro transcription and in vitro translation allowing protein synthesis outside of the cellular environment, thus enabling rapid expression of small amounts of functional proteins.

In vitro transcription (IVT)

Allows template-directed synthesis of bespoke RNA molecules in µg to mg quantities outside of the cellular environment.

Capillary number

A dimensionless number used to quantify the ratio of viscous forces to capillary forces between two immiscible liquids.

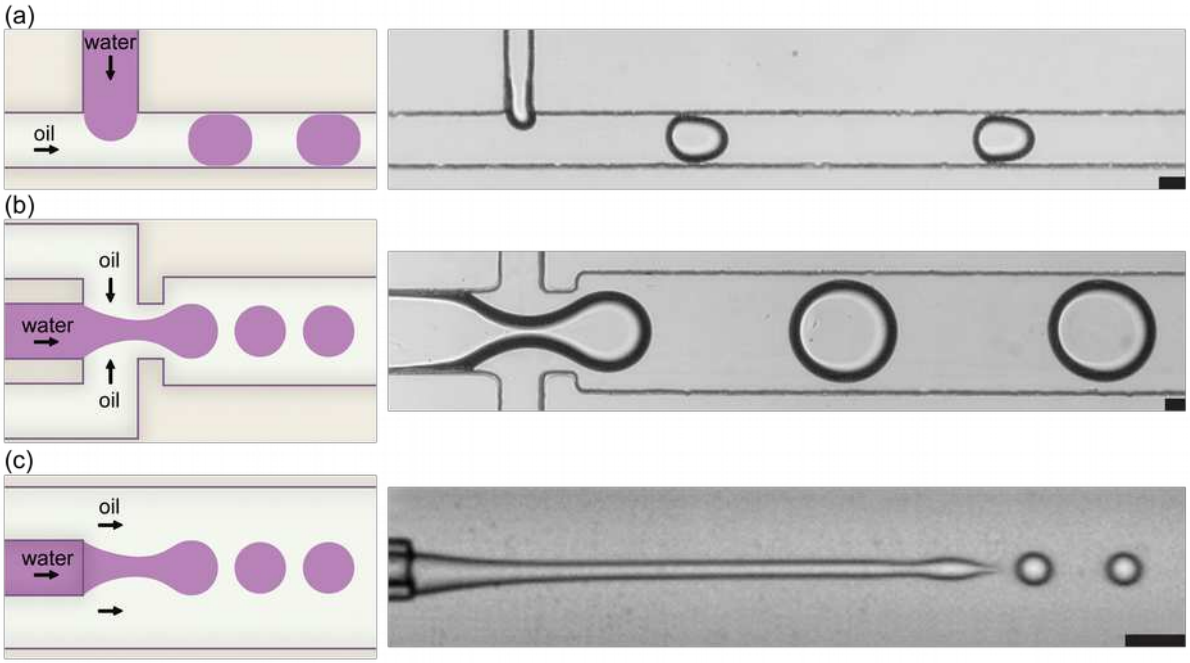

Figure 1 Examples of three common passive droplet generation modes.
(a) *Cross-flow droplet generator*: the dispersed phase is delivered into the continuous phase in an orthogonal fashion. As the dispersed phase enters the continuous phase flow, shear forces elongate the "head" of the dispersed phase entering the main channel, until a segment eventually separates and relaxes into a sphere or plug due to interfacial tension. (b) *Flow focusing geometry*: axisymmetric (3D) or planar (2D) immiscible flows are accelerated prior to entering a narrow nozzle or constriction. Pressure and viscous stress acts to elongate the inner fluid, which eventually breaks either inside or downstream of the nozzle. (c) *Co-flow geometry*: dispersed and continuous phase fluid streams are united in a parallel fashion, most usually by co-flowing immiscible fluids through a tapered capillary in which streamwise forces exceed interfacial tension. Each method is adept as producing monodisperse droplet populations, with CV values (the ratio of standard deviation to the mean of the droplet radius)) normally between 2 and 5%. Schematics are presented on the left-hand side and images of droplet generation shown on the right-hand side. Scale bars are 50 microns. Image presented in Figure 1(c) is adapted with permission from A.S. Utada, Phys. Rev. Lett. 99, 094502.

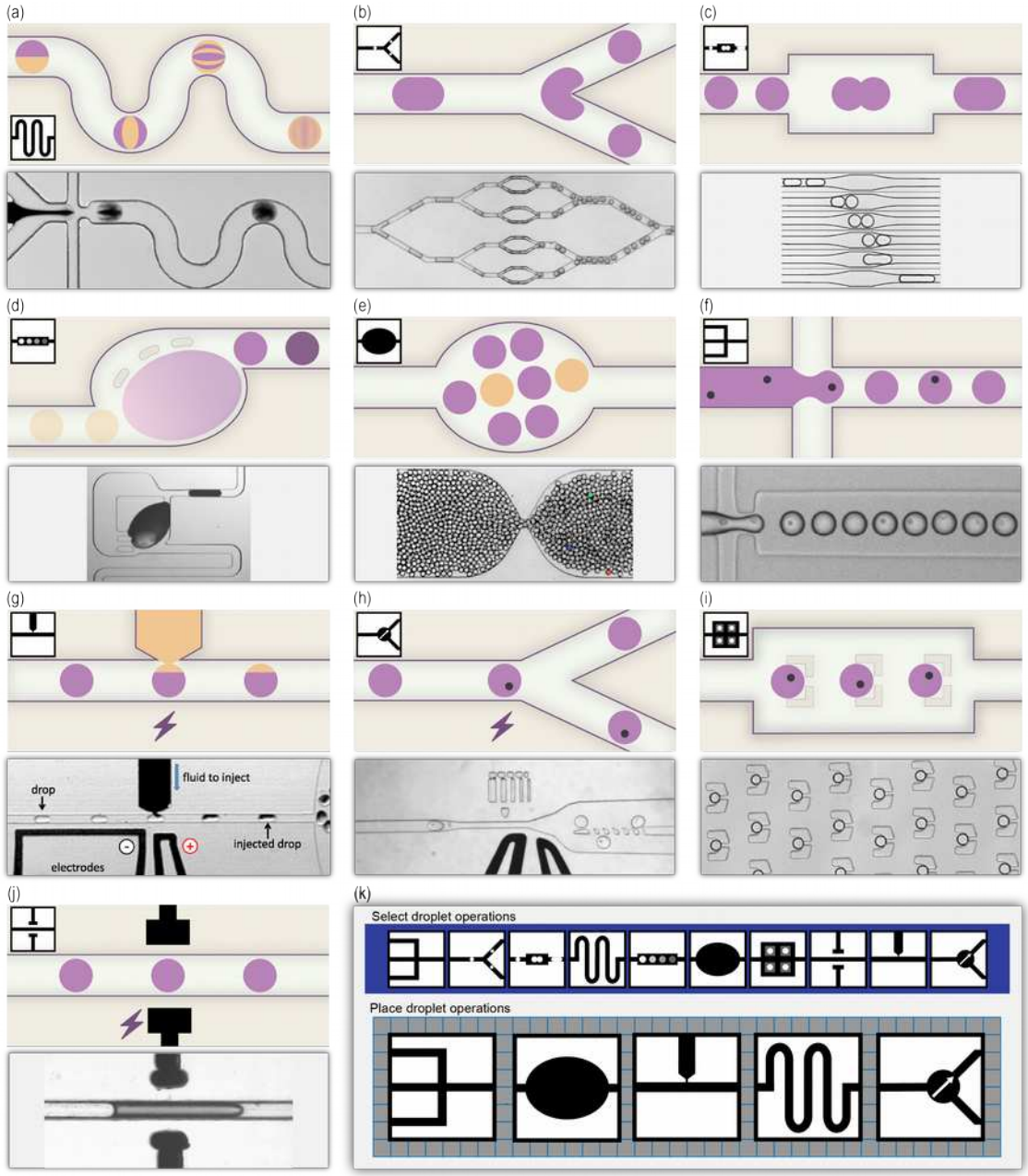

Figure 2 Droplet manipulations and unit operations.

(a) *Mixing:* Rapid payload mixing can be realized via chaotic advection by motivating droplets along a winding microfluidic channel. (b) *Splitting:* droplets can be split at microchannel bifurcations, with the daughter droplet size being controlled by the relative flow rates or outlet pressures in the downstream channels. (c) *Merging*: Droplets may be merged within a microchannel expansion with coalescence occurring during the separation phase after initial impact. Image adapted from ref. 40, American Physical Society. (d) *Dilution*: Through a process of droplet merging, mixing and re-splitting, a trapped droplet is combined with a series of smaller droplets to generate a sequence of output droplets that define a digital concentration gradient. Image adapted from ref. 51, Nature Springer. (e) *Incubation*: Microchannel constrictions and expansions redistribute droplets repeatedly along a delay-line allowing on-chip incubation of droplets. Droplet shuffling minimizes the distribution of incubation times. Image adapted from ref. 55, Royal Society of Chemistry. (f) *Encapsulation:* Cells or particles can be encapsulated into droplets (in a semi-controllable manner) by diluting them into the dispersed phase prior to droplet formation. (g) *Injection*: Controlled fluid volumes may be added to pre-formed droplets by using an electric field to trigger injection from a pressurized side-channel. Image adapted from ref. 44, National Academy of Sciences. (h) *Sorting:* deflection of droplets using dielectrophoretic forces can be used to sort droplets at kHz rates. Image adapted from ref. 166, Nature Springer. (i) *Trapping*: hydrodynamic trapping structures can be arrayed to trap (and subsequently release) droplets for extended periods of time. Image adapted from ref. 54, Royal Society of Chemistry. (j) *Droplet Sensing*: droplets passing specific regions can be detected using phenomenon like capacitance difference between droplets and oil using integrated conductive ink electrodes. Image adapted from ref. 281, Royal Society of Chemistry (k) *Interactive CAD Environment*: Using design software, droplet unit operations can be selected, composed, and physically placed in a workflow to create a specific microfluidic protocol[56].

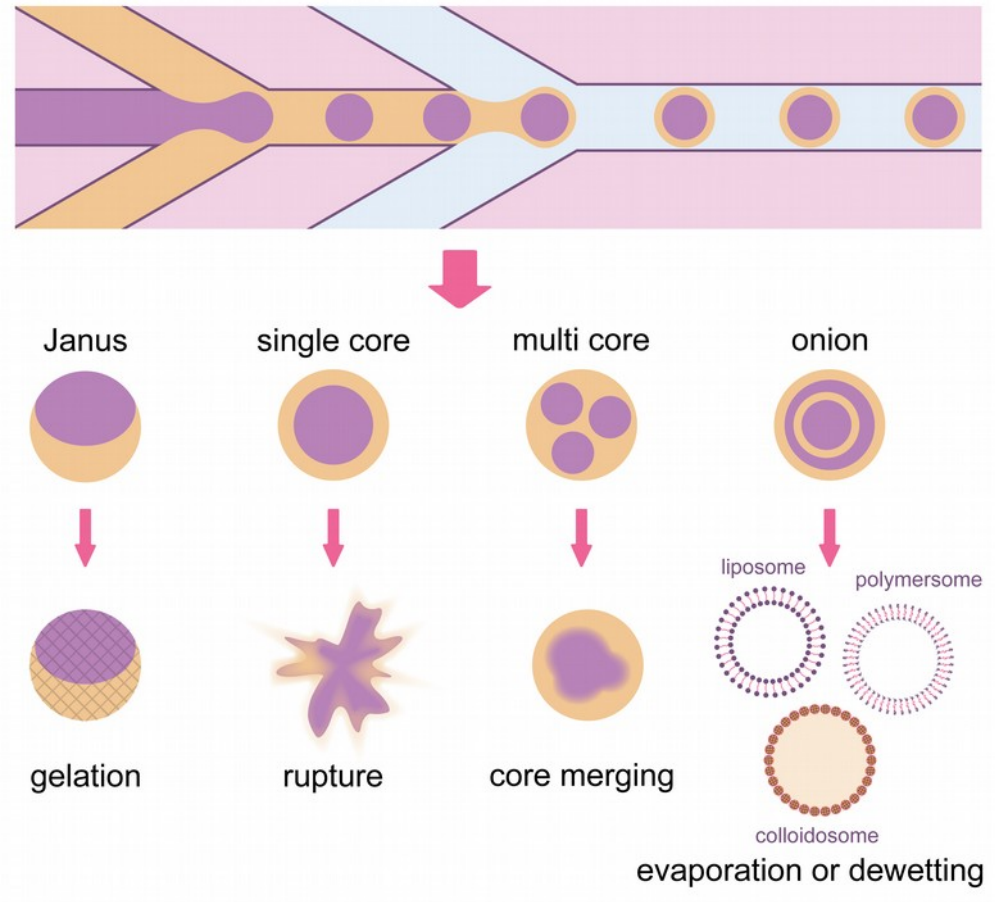

Figure 3 Multiple emulsion templating.
An example of multiple emulsion generation, in which a single emulsion droplet (e.g. W/O) is encapsulated in another droplet of an immiscible phase (e.g. O/W); the result being a core shell structure called a double emulsion (e.g. W/O/W)[75,92]. Other templates comprise biphasic Janus particles, multi-core double emulsions and onion-shaped multiple emulsions. An important application in this regard is the controlled delivery of active compounds[93,94] through shell composition tuning to allow rupture upon application of a temperature, pH, or chemical cue[75,93]. Such external signals can also trigger gelation to form monodisperse gel particles or induce core merging in a multi-core double emulsion, starting a chemical reaction[96,97]. Solvent evaporation or dewetting can transform multiple emulsions with lipid shells into unilamellar liposomes, polymer surfactants into polymersomes, and colloidal surfactants (Pickering emulsions) into armoured droplet colloidosomes[75,95]. Inspired by ref. 75.

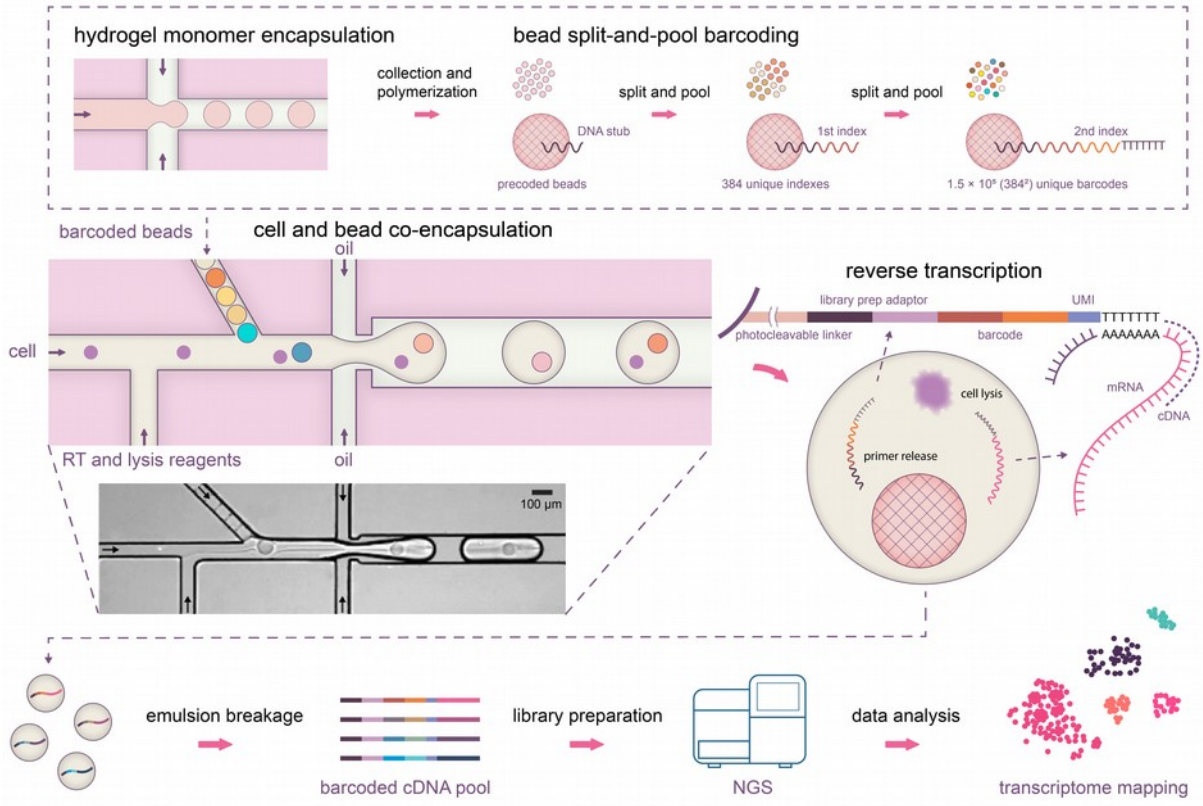

Figure 4 Droplet-based single-cell RNA sequencing (scRNA-seq).
The schematic outlines the basic experimental workflow associated with the inDrop method[84,181]. An aqueous acrylamide/bis-acrylamide solution carrying an acrydite-modified DNA primer is emulsified by hydrodynamic flow focusing using a microfluidic device to yield highly monodisperse droplets, which are collected off-chip and polymerized into hydrogel beads (HgBs) with the DNA primer covalently attached. The barcodes are constructed on the beads by two rounds of split-and-pool synthesis by primer extension, 384 barcodes at each round, generating $1.5\ 10^5$ (384^2) barcodes. Single barcoded HgBs are then co-encapsulated with single cells together with lysis buffer and RT reagents. In the droplets, primers are released from the beads by photocleavage (or restriction enzyme cleavage) prior to reverse transcription of the mRNA released from the cells. After in-drop reverse transcription, the emulsion is broken and subsequent steps in sequencing library preparation are performed in bulk, followed by NGS and data analysis. From top to bottom: hydrogel beads are prepared and barcoded with cDNA primers following a split-and-pool strategy. Each bead is then co-encapsulated with single cells, in a medium containing reverse transcription and lysis reagents. Once compartmentalized, cells are lysed and the barcoded primers are hybridized to the released mRNA and used to prime reverse transcription, resulting in distinctively barcoded cDNA. Since all primers on a single bead contain the same barcode cDNAs from the same cell will carry the same barcode. After NGS, reads from the same cell can be directly identified via this barcode, and the corresponding transcriptome mapped.

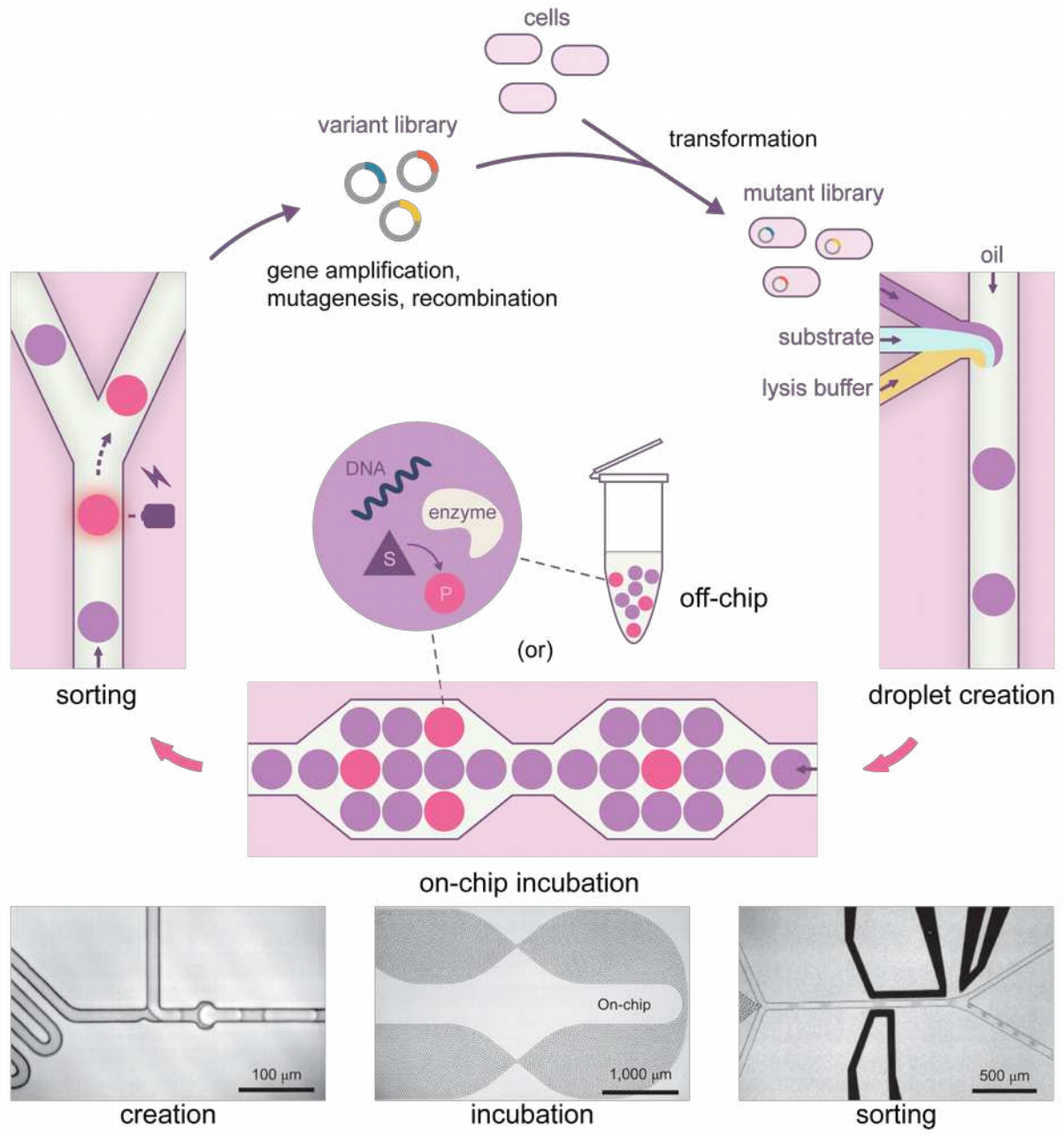

Figure 5 Droplet-based microfluidic platform for the directed evolution of enzymes.

Schematic of a microfluidic system for the directed evolution of aldolases[12,161]. A library of variant enzyme genes is cloned into an expression vector and transformed into bacterial cells. The cells are then screened using a droplet-based microfluidic protocol comprising three steps: (1) droplet formation to compartmentalize single cells with the fluorogenic substrate and lysis reagents; (2) incubation off-chip (for long incubations) or on-chip (for shorter incubations and enhanced temporal resolution); and (3) fluorescence-activated droplet sorting (FADS). With on-chip incubation all three steps are integrated on a single device. The aqueous droplets provide a physical link between DNA and proteins from lysed cells and product (*P*) generated from a fluorogenic substrate (*S*), and so allow genotype–phenotype coupling. DNA is recovered from the sorted droplets and amplified by PCR, with optional mutation and/or recombination prior to re-cloning into an expression vector for the next round of directed evolution.

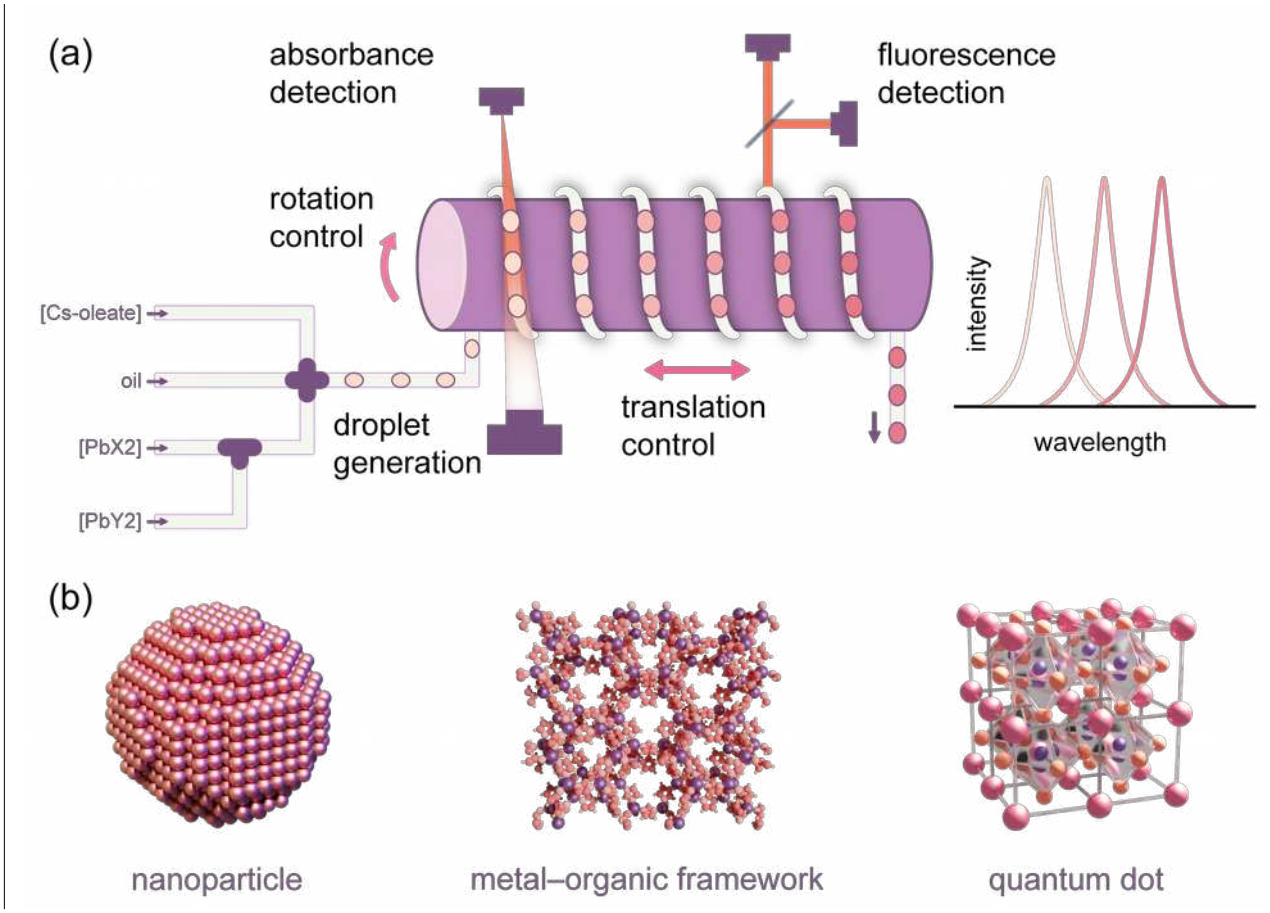

Figure 6 Droplet-based microfluidic synthesis of materials with bespoke properties.
Schematic of a representative microfluidic system for the synthesis of cesium lead halide perovskite nanocrystals. Reagents are fed by syringe pumps and pre-mixed in a user-controlled manner at a cross junction, to form a segmented flow. Rapid heating to a desired temperature is achieved by coiling the tubing around a grooved metal rod. Control of the residence time of the droplet in the heated zone permits control of the reaction time. The system is integrated with in-line absorbance and fluorescence detection for real-time characterization of products properties. Such systems are applicable to a broad range of materials such as metal nanoparticles,[229] metal-organic frameworks[222] or luminescent quantum dots.[11,227] Images inspired by ref. 11.

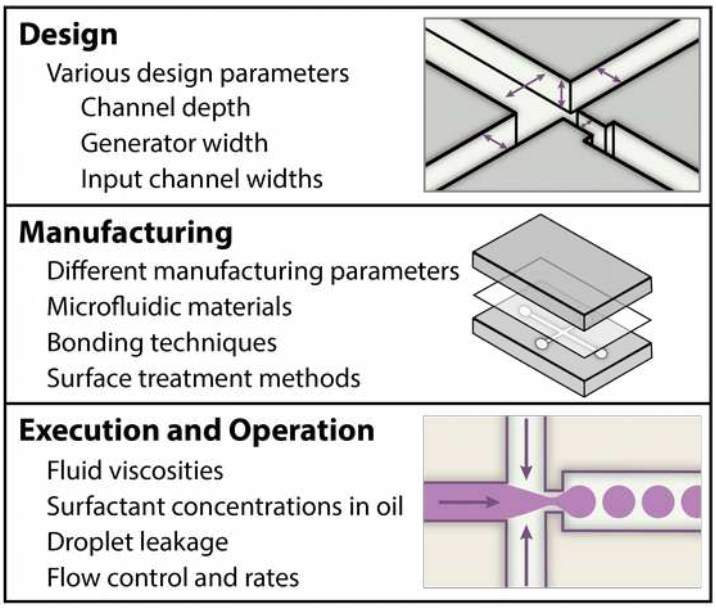

Figure 7 Summary of sources of variability in droplet-based microfluidic experimentation.
Sources of variability can be categorised into the design, manufacturing, and operation of microfluidic devices. Those listed are common examples within each category that account for the majority of variability cases.

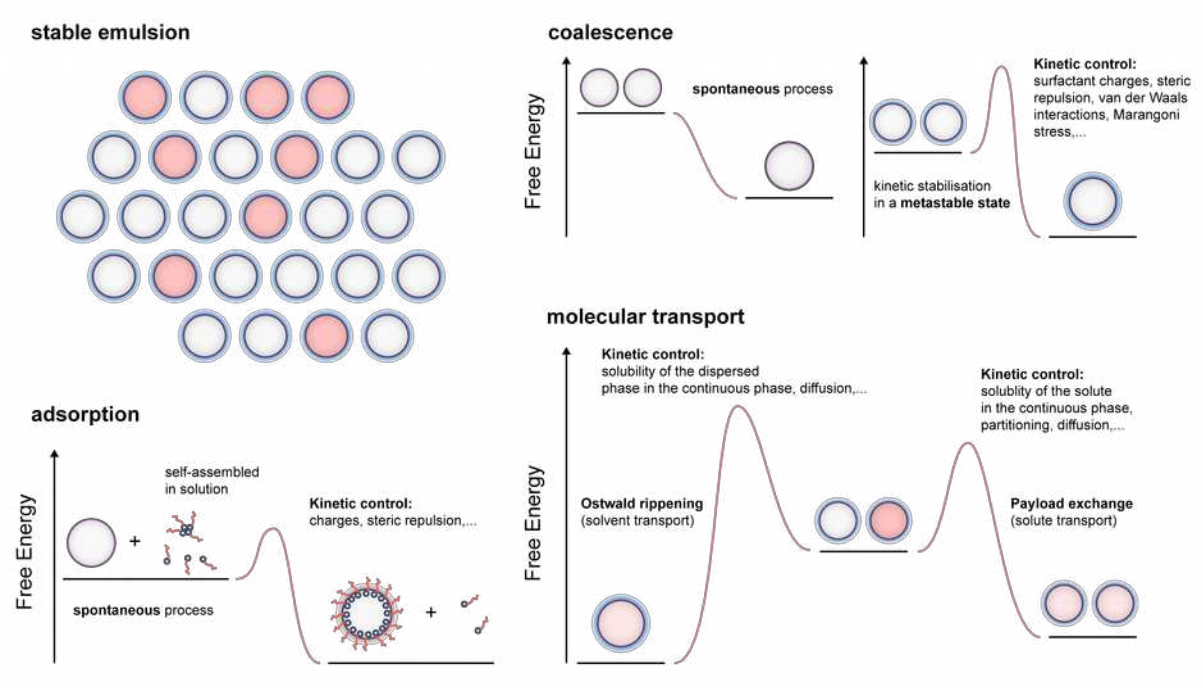

Figure 8 Physical phenomena affecting the stability of microfluidic emulsions.
Molecular adsorption at the droplet interface, droplet coalescence, and molecular transport between droplets or between the droplet and the continuous phase are phenomena impacting the stability of the emulsion and payload control. Formulation optimization involves consideration of mechanical stabilization against coalescence, the stability of the payload of the compartment against ripening, the biocompatibility of the and the compatibility of the formulation with the operational conditions of the microfluidic system itself (in terms of the device material and the rheological properties of the complex fluid). The experimentalist may prevent or promote each phenomenon through variations in surfactant type and concentration, the choice of discrete, continuous phase and substrate material, and other experimental parameters.