Lab on a Chip

CRITICAL REVIEW

Droplet microfluidics for high-throughput biological assays†

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Droplet microfluidics offers significant advantages for performing high-throughput screens and sensitive assays. Droplets allow sample volumes to be significantly reduced, leading to concomitant reductions in cost. Manipulation and measurement at kilohertz speeds enable up to 10^8 samples to be screened in one day. Compartmentalization in droplets increases assay sensitivity by increasing the effective concentration of rare species and decreasing the time required to reach detection thresholds. Droplet microfluidics combines these powerful features to enable currently inaccessible high-throughput screening applications, including single-cell and single-molecule assays.

Introduction

Many technologies and resources developed over the past several decades have greatly impacted medical research, therapeutics, and diagnostics. Some applications of these technologies and tools would greatly benefit from increased throughput and sensitivity. For example, small compound libraries are powerful resources that can be screened to discover new drugs, but drug discovery can require screening of as many as one million variants.[1] This large number makes compound screens extremely expensive and time-consuming, and thus less accessible. As another example, diagnostic assays that detect pathogens in the bloodstream can easily identify species that typically infect at high concentrations, but are severely hampered or completely obstructed by the long turnaround times required to detect species that occur at concentrations as low as 5 cells per mL.[2] Such low signal-to-noise ratios restrict the possible targets of medical diagnostics, and thus limit their medical utility. These applications, and many others, require screening of small volumes at high rates and with high fidelity.

Droplet microfluidics addresses the need for lower costs, shorter times, and higher sensitivities by using water-in-oil emulsion droplets to compartmentalize reactants into picolitre volumes,[3–7] instead of the microlitre volumes commonly used with standard methods. These droplets increase throughput by reducing the volume and increasing the rate at which assays can be performed. Other microfluidic approaches that handle similar volumes use chambers for compartmentalization.[8–11] While these approaches are very powerful, using chambers can introduce the risks of fouling and cross-contamination between samples. Applying fluidic control directly to the reactants may also complicate sample manipulation and retrieval. In contrast, the oil that carries droplets prevents undesirable interactions between reactants and solid surfaces, and facilitates rapid manipulation by separating the reactants from the fluidics.

We will describe those aspects of droplet-microfluidic technology that enable high-throughput screens and sensitive assays. We will discuss the unique advantages that the droplet-microfluidic approach provides, as well as its limitations. To highlight these advantages, we will describe representative examples of applications that are challenging to perform with conventional high-throughput screening methods, and are facilitated by droplet-microfluidic techniques.

Droplet microfluidics

Assays generally require multiple steps, such as compartmentalization, manipulation, and measurement. Executing these steps using droplet microfluidics can maximize throughput.

Microfluidic devices can be used to compartmentalize reactants by using an inert carrier fluid, usually oil, to encapsulate small volumes of aqueous reagents in droplets and separate the fluidics from the droplet contents. Such devices can produce monodisperse droplets,[5,12] ranging in volume from 0.05 pL to 1 nL, or from 5 μm to 120 μm in diameter. Droplets can encapsulate cells, DNA, and other particles or molecules that are in the inner aqueous phase (Fig. 1a).[13] If desired, molecules and particles can be singly encapsulated in individual droplets.[14]

Following an initial encapsulation step, water-in-oil droplets can be manipulated in several ways. Existing droplets can be collected off-chip in a microtube (Fig. 1b), then reinjected into another microfluidic device (Fig. 1c).[15] Picolitre volumes of additional reagents can be injected into existing droplets at rates of ~1 kHz.[16] As aqueous droplets flow in carrier oil past a channel containing the aqueous reagents to be added, the interface between each passing droplet and the picoinjection channel can be controllably destabilized, so that some volume is added to the droplet during the brief transit time (Fig. 1d).

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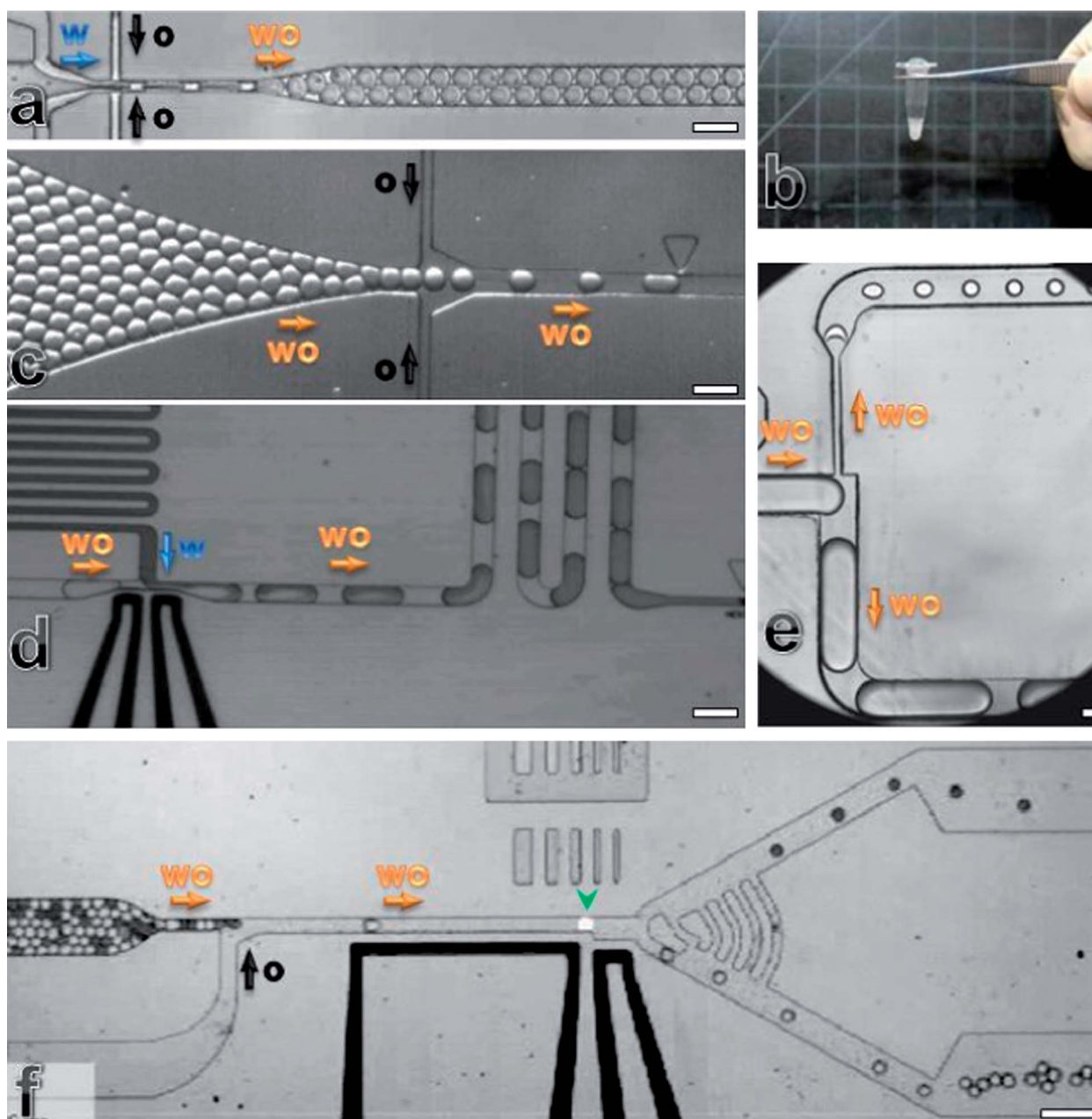
Fig. 1 Manipulations with droplet microfluidics. (a) A droplet-producing device. An aqueous solution is coflowed with carrier oil to produce water-in-oil emulsion droplets that encapsulate any cells, particles, or molecules present in the aqueous solution. (b) One million droplets, each 25 μm in diameter, are visible as ~20 μL of emulsion stored in a 200 μL microtube. (c) A reinjection device. Previously formed droplets are taken from off-chip storage and reinjected into a microfluidic device. Oil is added as a spacer between the closely packed droplets. (d) A picoinjection device. Droplets (wo) flow through a T-junction, past a side channel containing a second, blue-dyed aqueous solution (w) to be injected into the droplets. Electrodes (thick black lines) are charged to produce an electric field that locally destabilizes the interface of the droplet passing by the side channel, so that the aqueous solution briefly fuses with the droplet, injecting additional fluid. The dye quickly diffuses throughout the droplet. (e) A splitting device. Droplets flowing through a T-junction split into two droplets, with the size of each daughter droplet determined by the geometry of the device. (f) A sorting device. Dark droplets containing blue dye and light droplets containing a fluorescent dye are pooled together and reinjected into a device. A laser spot (bright spot below the arrowhead) interrogates the droplets. Electrodes (thick black lines) are charged according to the emitted fluorescence, to deflect only the light droplets into the lower channel. Scale bars denote 100 μm. Arrows indicate the direction of flow for different phases: aqueous fluid (w), carrier oil (o), and water-in-oil emulsion droplets (wo).

Reagents can also be combined by coalescing droplets and allowing their contents to mix.[17] Conversely, other geometries can subtract volume by splitting droplets, with volume ratios controlled by differential channel resistance (Fig. 1e).[18] This allows controllable quantities of a single droplet to be split off, enabling multiple assays of the same droplet contents, even if the assays are mutually incompatible.[19] Finally, drops can be detected on-chip and efficiently sorted, allowing selection of subpopulations based on a variety of readouts (Fig. 1f).[20–22]

These microfluidic techniques can be combined to perform nearly any biological reaction or assay that can be performed in a conventional microtiter well plate. Indeed, many of the most commonly used assays in biological research have already been demonstrated in droplet microfluidics,[6,13,23–27] and even more are in development. There are several key reasons to use droplets rather than well plates: small volumes, high speeds, low noise, and isolation of droplet contents from solid surfaces and fluidic control. Small volumes conserve expensive reagents, and high speeds significantly reduce the time required to assay extremely large libraries. Droplets achieve low noise by reducing reaction volumes; any background noise present in a solution will be decreased proportionally to the volume. Various materials can be used to form picolitre-sized compartments, but the inert oil–water interface of a droplet has the advantage that it shields droplet contents from the solid walls of a microfluidic device. Droplets thus minimize fouling and cross-contamination. These advantages are exemplified by many applications.

High-throughput screens can especially benefit from small volumes. A typical screen using conventional robotics involves pipetting volumes as small as ∼10 μL of reagents into each well of a 384-well microtiter plate, and adding ∼10 μL of each variant from a library into different wells. This results in a total reagent volume on the order of millilitres for one microtiter plate, and significantly higher as the number of plates increases. In contrast, the same screen using droplet microfluidics would involve emulsifying volumes as small as ∼1 pL of each variant from a library, and adding ∼1 pL of reagents to each variant droplet.[27] This represents a reduction in volume by up to 7 orders of magnitude. Since reagent volume is often the limiting factor for how many reactions can be screened, this volume reduction is a critical factor that helps enable droplet microfluidics to achieve extremely high throughput.

Droplet microfluidics can often also achieve higher speeds by enabling reaction detection after shorter time courses. If a single molecule or cell is present in a 1 μL volume, its concentration will be extremely low. If that 1 μL volume is emulsified into one million droplets, each 1 pL in volume, one of those droplets will contain the single molecule or cell, and its concentration in that droplet will be effectively increased one million-fold. Since reaction rates increase with effective concentration, reaction times that are normally on the order of hours in bulk may decrease to seconds or minutes in droplets.[28,29] In addition, the minimum concentration of reaction products required for detection will be reached more quickly in smaller volumes. The detection step itself can also be performed rapidly, at rates of ∼1000 reactions per second. This rate ultimately limits the number of samples that can be screened, as well as the time resolution of any measurements. If there are ∼10^5 samples to be assayed, then time series measurements may be taken every few minutes. For a single endpoint measurement, it is realistic to screen ∼10^8 samples per day.

An additional benefit for cell-based assays is the automatic association of genotype with functional output through compartmentalization rather than through a physical bond. This is in contrast to screens that involve fluorescence-activated cell sorting, and could replace membrane-binding systems such as yeast surface display or phage display.[13,21,30] Droplets thus offer a particular advantage for secretion assays that may be difficult to adapt to such membrane-binding systems.

Droplet microfluidics does however introduce a significant disadvantage: heterogeneous assays are difficult to perform. For example, many secretion assays normally incorporate washing steps; adapting these to droplets is very difficult. Encapsulating homogeneous liquid reactants and taking a single measurement is straightforward, but removing a buffer entirely from a cell and exchanging it for a different buffer requires more complicated techniques currently under development.

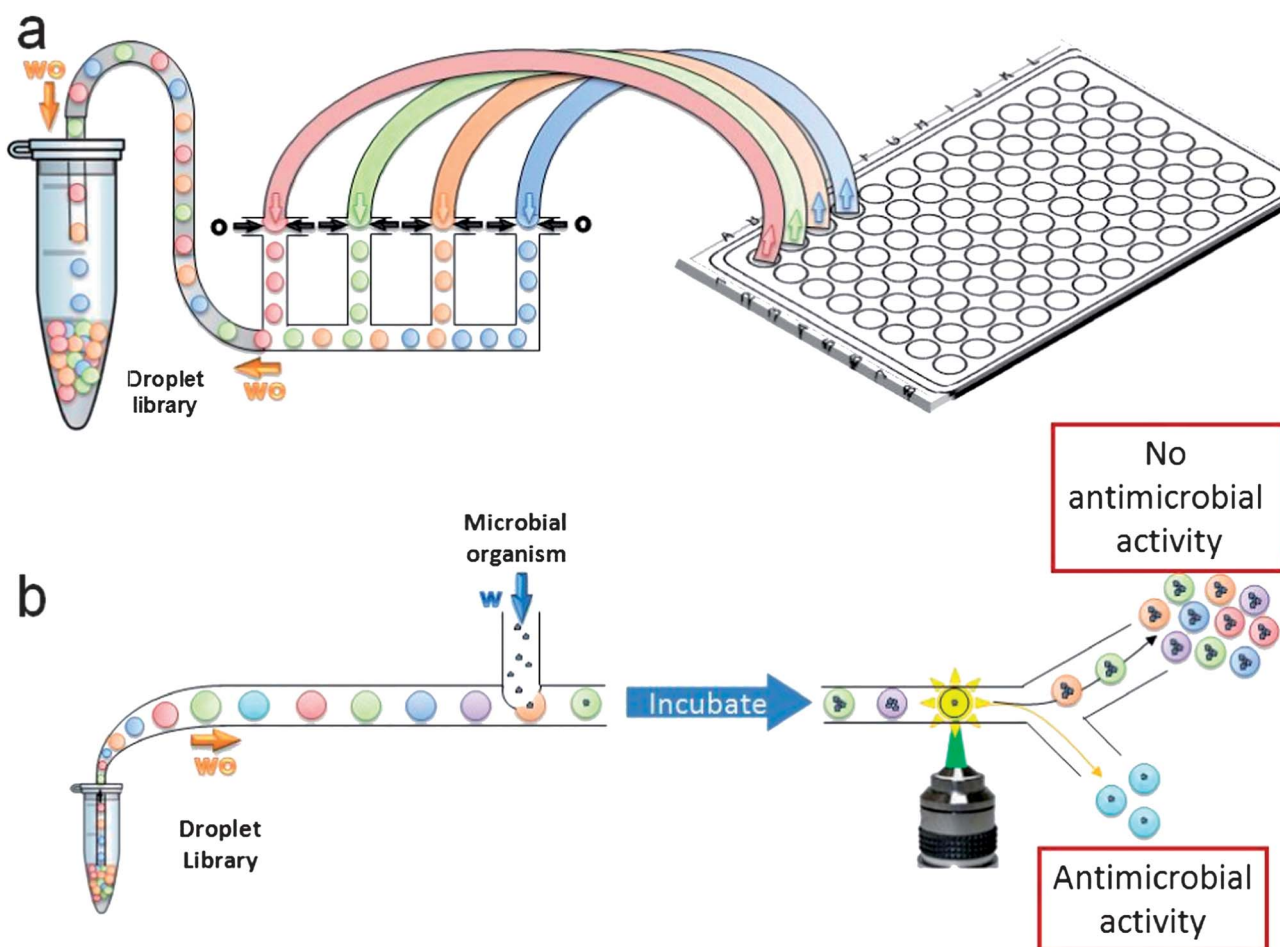## Droplet libraries and compound screens

Conventional high-throughput screens use microtiter plate wells to store a large number of unique elements; each element is then tested for some chemical or biological property. However, high-throughput screens in droplet microfluidics require that the unique elements be stored, manipulated, and tested in droplets. Thus, droplet-based screens require the encapsulation of each element in droplets prior to performing the screen itself. Although it is possible to emulsify and screen each element individually, this would take more time than performing a conventional screen; instead, after encapsulation, droplets of different elements can be pooled into a "droplet library," ready for subsequent use in a single screening assay that includes all library elements. Since each element is emulsified into many droplets, the same library can be used for multiple screens, where each assay uses an aliquot, or small portion, of the library, and each aliquot includes many droplets of each library element.[27]

As an example, small compounds dispensed in microtiter plates, totaling tens of microlitres of each element, may be encapsulated to form a droplet library of billions of droplets at a rate of several minutes per plate. This is achieved by parallelizing microfluidic devices and interfacing them with microtiter plates, as shown in Fig. 2a. An aliquot of one-thousandth of this library contains thousands of copies of each library element and this constitutes sufficient statistics for one assay. To perform a screen, the aliquot of droplets is reinjected into a microfluidic device, a target such as a microbial organism is added to each drop, and after some reaction occurs during an incubation step, the droplets are detected and sorted at a typical rate of 1000 Hz (Fig. 2b). A library of one million compounds can be screened in a day as opposed to months using conventional screens, while using one-thousandth of the amount of reagents that is conventionally used.

When the contents of a microtiter plate are emulsified and pooled to form the droplet library, sample labeling encoded by spatial positioning in the original plate is lost; thus, some other means must be developed to track the library contents. One solution is to associate a unique barcode with each element and include it with the element in the plate prior to encapsulation. Each droplet then contains a barcode that indicates which element is encapsulated in the droplet. Potential barcodes include nucleic acid sequences and fluorophore combinations.[31,32]

The advantage of using fluorophore combinations as barcodes for droplet libraries is that they can be read in real-time concurrently with the result of the assay. However, the number of distinguishable fluorophore combinations is limited by the dynamic range of the optical setup that detects the fluorophores. In contrast, nucleic acid sequences cannot be read in real-time but can accommodate arbitrarily large library sizes. Many

Fig. 2 Droplet library production and small compound screen. (a) The wells of a microtiter plate are interfaced with the aqueous inlets of multiplexed droplet-producing devices (colored arrows) to simultaneously emulsify the contents of each well. The droplets are pooled together to form a droplet library. Each well contains a unique barcode, represented in the figure as a different color, that is carried by the droplets from that well and is used to decode the contents of each droplet. (b) A droplet library of small compounds is screened for antimicrobial activity. An aliquot of the library is reinjected into a microfluidic device and a single microbial cell is added to each droplet. After incubation, the droplets are screened for growth arrest of the microbe. The small compounds encapsulated in the selected droplets are identified by their barcodes and noted as potential antimicrobial drugs. Arrows indicate the direction of flow for different phases: aqueous fluid (w), carrier oil (o), and water-in-oil emulsion droplets (wo).

screens do not require real-time decoding of the barcodes: since the goal of most screens is to isolate a rare element out of a large library, only a small pool of elements are selected and these can be decoded after the screen is completed. Thus, barcodes such as nucleic acid sequences are often a good fit for large screens.

The diverse chemistry of library elements may affect whether chemicals leak out of the water-in-oil emulsion droplets and this could lead to crosstalk between droplets. This occurs if the inert oil and surfactant are permeable to the compound. Chemicals may also affect the physical stability of the droplets encapsulating them. This can happen for example if the chemicals counteract the stabilizing effect of the surfactant, resulting in coalescence during fluidic manipulation, thermocycling, or long-term storage. Appropriately engineered devices and surfactants can mitigate these issues of instability, leakage, and crosstalk, but these issues must be evaluated and minimized in droplets whenever new reagents are used in a library.[15]

Many high-throughput assays screen genetic elements. These may be DNA in cells or viruses, or nucleic acids that encode

proteins. In the case of genetic elements, each cell, particle, or molecule constitutes a single element of the library; thus, if a suspension of all elements mixed together is sufficiently diluted before encapsulation, then each droplet will contain just one element, and the droplet library can be formed using a single microfluidic device. Moreover, the unique genetic sequence that is the object of the screen also serves as a barcode, so that no additional barcoding is required when encapsulating the library. Genetic screens have wide-ranging applications, including directed evolution,[33] SNP measurement and identification,[11] *in vitro* translation,[6] and metagenomic analyses.[10]

Cell growth assays and cell–cell interactions

Cell growth assays are widely used in biological and pharmacological screens because growth is often a valuable indication of the effect of a tested condition on a targeted cell. Cell growth is quantified by measuring cell density, and these measurements are usually limited by a density detection threshold, under

which the existence of cells cannot be detected. A major advantage of using droplets for cell growth assays is therefore the drastic reduction of the volume in which the cells are grown, which proportionally increases the density of a given population of cells. Thus, the minimum number of cells that can be detected in droplets is lower than in bulk. As a numeric example, the density of a single cell in a 10 μm drop is equivalent to that of a billion cells in a 1 μL well. For many assays, this means that in droplets, the presence and growth of single cells are easily detected.[13]

Assaying cell growth at the single-cell level is crucial for example in diagnostics, where a sample suspected of containing a pathogen is cultured until the population density of the pathogen is high enough to detect, at which point the source of the infection can be identified.[2] Because the density is so much larger, performing this test in droplets can substantially decrease the time required to detect an infection, in some cases even down to the doubling time.

Monitoring single-cell growth in droplets is also important for detecting unusual growth rates.[34–36] Currently, since growth is measured for populations of many cells, the fastest-growing cells dominate the population, and cells with lower growth rates cannot be easily monitored. However, cells with intermediate or fluctuating growth rates may prove to be extremely important once they are isolated and studied using droplet-based screens. For example, detecting rare growth events could improve our understanding of emerging antibiotic resistance, or emerging viral epidemics.

Culturing conditions inside droplets may differ from those in bulk, even with the same medium and external environment. For example, the amount of nutrients available to each cell is initially much smaller than in bulk, the permeability of the emulsion to gases is different from that of bulk aqueous media, and the boundaries of the droplet are generally less adherent than the plastic in a culture flask.[13,37] Correspondingly, culturing cells in such environments may require optimization per cell type: droplet size must be tuned to adjust nutrient depletion, carrier oil and storage conditions must be chosen to adjust oxygen availability, and beads inside droplets may be necessary as growth substrates for certain cell types.

Bacterial persistence screens

One example of an application where single cell growth is important is screening for bacterial persistence.[34] A population of bacteria may contain some bacteria with weak antibiotic resistance, and some with high antibiotic resistance. The latter will be more fit and thus highly persistent, while the former will have intermediate persistence. In large volumes, individuals with intermediate persistence will be outgrown by the more fit bacteria before their persistence can be detected and studied. To isolate intermediate persistent bacteria using conventional methods, cultures are diluted and spread on agar plates so that newly formed colonies are isolated from each other.[38] Several colonies can be harvested from each plate after a time course of up to several days. Colony harvesting is a laborious and poorly scalable process. Moreover, if the persistence is not stable over several generations, colonies may not be detected at all. In contrast, if single bacteria are encapsulated in droplets, even bacteria with the weakest and most transient persistence can be detected and selected at rates of 1000 Hz.

Plaque assays and virus–host interactions in drops

Another example of a cell growth assay that can be improved by using droplets is the virus plaque assay.[39] Plaques are macroscopically visible patches on a culture plate where cells have died due to a spreading viral infection. In well plates, these plaques expand for as long as 48 hours before the virus infects enough cells to be observed,[40] while in droplets, infections of single cells can be detected after much shorter incubation times. Moreover, single virus particles that are able to infect new host cells but are deficient in some other aspect of propagation would not be detected with conventional plaque assays, yet such viruses may be crucial for understanding the emergence of viral epidemics. To assess host–virus interactions in droplets, host cells can be encapsulated and incubated to establish a baseline before viruses are added to the droplets. After the droplets are incubated through one replication cycle of the virus, they are screened for changes in biological function, such as the host cell death or virus copy number (Fig. 3).
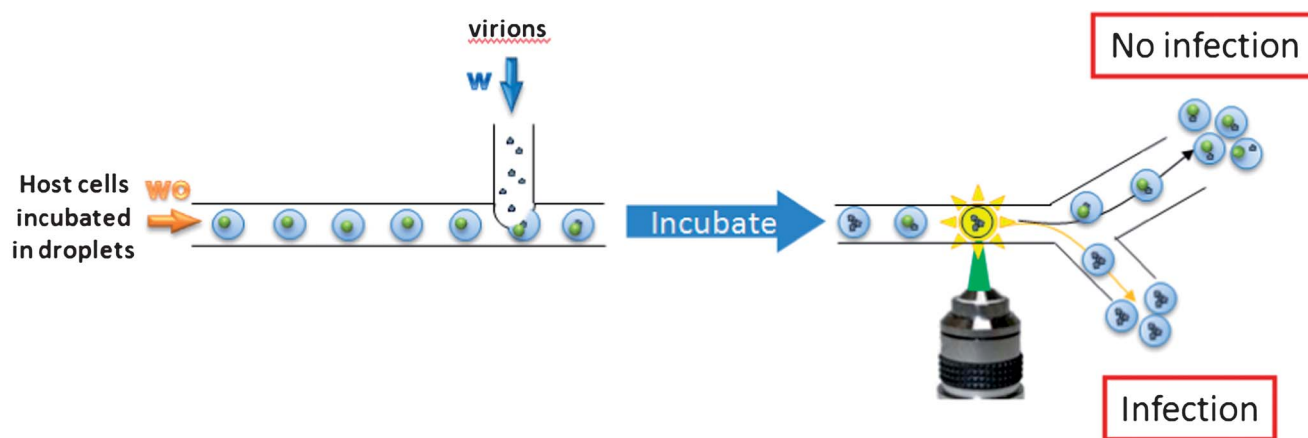
Fig. 3 Droplet-based plaque assay. Media containing virions are picoinjected into droplets containing host cells. After incubation, droplets are screened for successful virus replication and release, and infectious virions are selected. Arrows indicate the direction of flow for different phases: aqueous fluid (w), carrier oil (o), and water-in-oil emulsion droplets (wo).

Antibody screens

Antibodies are used in many fields ranging from biomedical and pharmaceutical research to medical diagnostics and therapeutics.[41] They are able to specifically target different molecular domains, which is essential for applications such as quantifying specific proteins and identifying pathogens. In the standard method to develop a monoclonal antibody,[42] antibody-secreting B cells are first isolated from the spleen of a mouse immunized against the desired target. Each B cell produces a single species of antibody, and only a relatively small fraction of species will bind to the desired target. Because a single cell would die before it could fill a standard plate well with a detectable concentration of antibodies, B cells must first be fused with immortal cells to form hybridomas, which can be cultured to generate cell lines. To generate pure antibodies with the desired target specificity, these cell lines must be derived from single cells. Such monoclonal cultures are obtained through several steps. Cultures are initially polyclonal, containing a mixture of several types of hybridomas. These cultures are screened for the desired antibodies, and if the antibodies are found in a given culture, the specific cells responsible for producing them must be singly isolated and cultured again to produce monoclonal cultures, with each clone derived from the divisions of a single hybridoma cell. All cells within each homogeneous clone will produce identical antibodies that have the same binding affinity to the desired target. Obtaining and validating monoclonal cultures typically takes 10–12 weeks. Droplet microfluidics can bypass the repeated culturing and screening steps by encapsulating and screening single antibody-producing cells directly. A single cell in a 0.05–1 nL droplet can produce a detectable quantity of antibodies in hours or even minutes, compared to days or longer in a 0.3–1 mL well.[13,29] In addition, droplet microfluidics can perform the screening step at a much higher rate. At typical sorting rates of ~150 droplets per second, ~10^6 individual cells can be analyzed and sorted in one day. In a typical plate-based screen, ~200 different cells can be analyzed in 10–12 weeks.[42] Thus, relative to standard screening methods, direct droplet screening of single cells enables much higher throughput.

An antibody screen must distinguish between secreted antibodies that bind to the target and those that do not. In plates, this is done by washing: a solid surface displaying the targets is provided, the desired antibodies bind to the surface, and the non-specific, undesired antibodies are washed away. The specifically bound antibodies that remain can then be detected optically.[42] This type of washing-based assay is a heterogeneous assay. Such assays are typically difficult to accomplish in droplet-based microfluidic systems, in which washing steps are difficult, and must therefore be modified for use in droplets. To overcome this limitation, a droplet-based solution replaces the washing-based assay with an assay based on signal concentration. This method encapsulates cells together with a bead loaded with target molecules. As the cells secrete antibodies, only those antibodies that are specific to the target bind to the co-encapsulated bead. Thus, specific antibodies produce a tightly localized signal inside a droplet, while non-specific antibodies remain distributed throughout a droplet and produce a diffuse signal (Fig. 4). A microfluidic sorting device can then select those droplets that contain the desired antibodies, along with the cells that produced those antibodies. Droplets that encapsulate cells with beads thus represent a heterogeneous system that can yet be manipulated fluidically. This system demonstrates that assays commonly performed in microtiter plates can be adapted for use in microfluidic droplets.

This droplet-microfluidic approach can potentially address a second inefficiency, that of hybridoma production. Fewer than 1 in 100 000 B cells that are subjected to fusion become viable hybridomas.[42] This dramatically limits the number of potential antibodies that can be isolated. Moreover, this may in fact not be a randomly distributed success rate. For example, some desired B cells, such as those that secrete at a very high rate, or those that secrete antibodies against a particular target, might be resistant to fusion. Hybridoma screening methods are inherently unable to access any B cells that do not stably undergo fusion. Since droplet microfluidics increases the screening rate so dramatically, it becomes feasible to screen single cells, and therefore becomes feasible to screen large numbers of B cells directly, without first immortalizing them through hybridoma generation. This would allow extremely deep mining of the antibody space, and increase the chances of isolating useful antibodies from a single screen. Droplet microfluidics can also screen cells for which there are no robust fusion partners, such as human B cells.[43] Cells from a patient recovering from an infection could be especially valuable.[44] Once the desired B cells are selected, their antibodies can be produced on an industrial scale by retrieving the antibody-encoding gene sequences from the B cells and cloning these sequences into an expression system.

Directed evolution of enzymes

Directed evolution is a powerful method for developing new variants of enzymes and proteins in general.[33,45] This method generates a DNA library of mutations of a known enzyme, clones the library into bacteria, and screens the enzymes produced by the bacteria for some desired property, such as increased catalytic efficiency, solubility, or specificity for particular substrates. If the library contains a very large number of mutations, a few variants may have the desired activity. PCR can then be used to retrieve the sequences of those variants from the selected bacteria. To facilitate detection of enzymatic activity, the mutation library is often cloned into a special strain of bacteria that has been engineered with a reporter, which produces detectable changes proportional to the level of enzymatic activity. These changes may be based on fluorescence or growth.[33]

For large libraries of putative novel enzymes, encapsulating the bacteria library in droplets can increase screening throughput by reducing the reaction volume.[13,21,30] This increases the effective concentration of reporter molecules, and thus the incubation time required before detection. Droplets also facilitate handling of large sample numbers. It is particularly significant that through compartmentalization, droplets can maintain the association between observed enzymatic activity and its genetic source. When screening enzymes, it is generally desirable to recover the DNA sequences that code for any selected enzymes. With droplet microfluidics, it is straightforward to simultaneously sort out both phenotype and genotype.
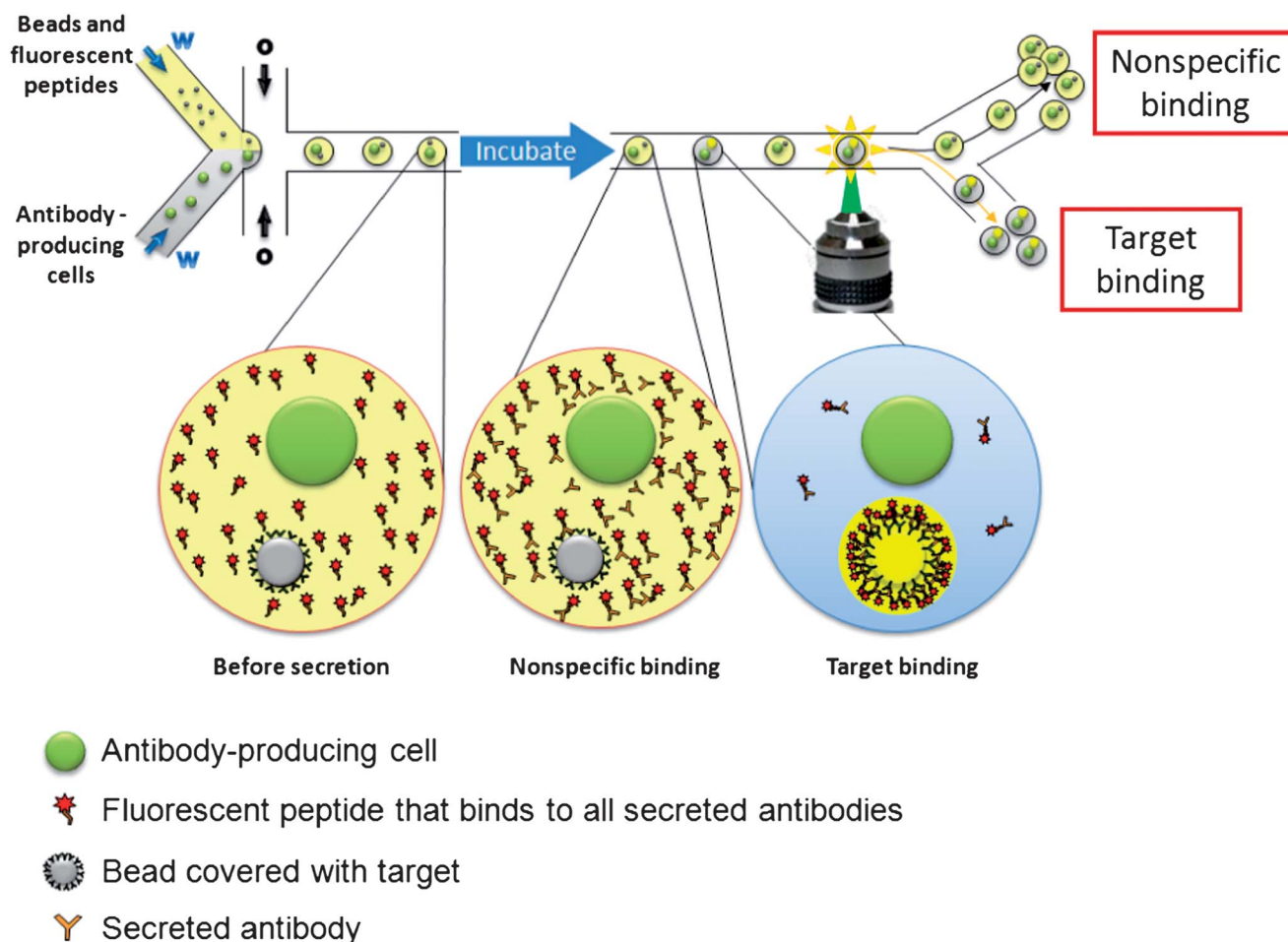
Fig. 4 Droplet-based antibody screen. Immediately before encapsulation, antibody-producing cells are coflowed with a mix of target-covered beads and fluorescent peptides that bind to all secreted antibodies. Before incubation, the peptides fill all droplets with homogeneous fluorescence. After incubation, in droplets containing antibodies that bind specifically to the target, the antibodies will primarily be attached to the bead, so the peptides bound to the antibodies will produce fluorescence concentrated in a small, bright spot within the droplet. In droplets containing non-specifically binding antibodies, the fluorescence will remain homogeneously distributed throughout the droplet. Droplets are then screened for antibody binding specificity. Arrows indicate the direction of flow for different phases: aqueous fluid (w), carrier oil (o), and water-in-oil emulsion droplets (wo).

In addition to libraries generated by mutagenesis, environmental samples such as soil, water, and insect guts are a promising source for novel enzymes.[46] Environmental sources harbor a diverse reservoir of uncharacterized bacteria and archaea that have been shown to carry potentially useful DNA polymerases,[47] restriction endonucleases,[48] and metabolic enzymes.[49] To systematically screen environmental samples, extracted DNA could be sheared into short fragments, then cloned into the same reporter-engineered bacteria used for mutation libraries. However, extremely large libraries would be required to fully probe the high diversity found in environmental samples. The sheared DNA fragments should be approximately the size of a typical gene, about 1 kilobase. Since bacteria genomes are usually on the order of 1 megabase, a few million randomly sheared 1 kb fragments would be necessary to cover most of a single genome. One gram of soil contains on the order of 10^3 different genomes,[50] so a library would have to contain $\sim10^9$ fragments to cover all the genetic diversity in 1 g of soil. Droplet microfluidics can access such large numbers.

PCR-based analysis of single, rare templates

The polymerase chain reaction (PCR) has wide-ranging applications, especially in medical diagnostics. PCR produces many copies of a DNA template, and is therefore crucial for analyzing small amounts of molecules or cells that must be amplified to higher levels before downstream detection or sequencing steps.[51] For standard volumes and preparation methods, target templates are amplified from an initial concentration of $\sim10^6$ molecules per mL. This fairly high template concentration is necessary because PCR may amplify non-specific DNA that does not match the desired sequence, especially under suboptimal conditions. The concentration of specific template, or signal, must be sufficiently high to overcome this noise.[51] However, clinical samples such as blood from sepsis patients may carry pathogenic targets at concentrations as low as 1–10 microbes per mL, against a background of 10^7 white blood cells per mL.[2] Even if the white blood cells are somehow removed, the reaction will easily be contaminated by aerosols, dust, and other incidental sources of noise. Careful and specialized sample preparation is

therefore necessary to eliminate extraneous DNA and minimize noise.[52,53]

Instead of increasing the signal-to-noise ratio by taking extra steps to reduce noise, an alternative approach is to increase the effective concentration of target molecules, which constitute the signal (Fig. 5a).[24,30] Droplet microfluidics enables single-template PCR by using this approach.[24,25,54,55] For a 1 mL blood sample containing 10 microbes and 10^7 white blood cells, compartmentalization into 1 pL droplets will produce a few droplets containing ~1 microbe at a concentration equivalent to 10^6 cells per mL, and ~10 white blood cells at 10^7 cells per mL. These droplets will have a signal-to-noise ratio of ~1 : 10, and thus will be easily distinguished from the vast majority of the droplets that contain no microbes and thus a much lower signal-to-noise ratio. The sample as a whole will be easily distinguished from a healthy blood sample that contains no microbes and produces only low signal-to-noise droplets. The same sample containing 10 microbes would have a signal-to-noise ratio in bulk of ~1 : 10^6, which would be very difficult to distinguish from a sample containing no microbes.

In addition to noise, another challenge facing single-template PCR is that the enzymatic activity of the polymerase can be inhibited by certain molecules, such as heme, EDTA, and humic acid.[56] Increasing the effective concentration of the signal will improve not only the signal-to-noise ratio, but also the signal-to-PCR inhibitor ratio. Droplets are thus less susceptible to

inhibitor concentrations that would obstruct PCR in bulk. This is especially relevant for blood and soil samples, which often have high concentrations of PCR inhibitors.

Following compartmentalization, emulsion PCR can be performed to amplify some identifying part of the microbial genome to a detectable level.[23,54] Droplets containing the amplified pathogen can then be detected to diagnose bacterial or fungal infection. This approach is generally applicable to diagnosing any disease where abnormal DNA species circulate at very low levels in the bloodstream. Another example is certain types of cancer, where circulating tumor cells may be present in the blood at 1 cell per mL.[57]

In the same way that compartmentalization enables detection of rare DNA sequences, it also enables higher resolution measurement of sequence distributions within a sample. PCR amplification is biased against sequences with high complexity, such as G/C-rich sequences, and amplifies these sequences with lower efficiency.[58] Thus, the relative concentrations of different sequences cannot be accurately determined from the final concentrations of their PCR products. With emulsion PCR, since each DNA molecule is singly confined within its own droplet, the amplification of each molecule can run to completion without competition from other sequences, regardless of how slowly each reaction runs.

Single-cell PCR would also be highly informative in metagenomic studies.[49,59–61] Metagenomics analyzes the genomic sequences in mixed populations of microbes to determine what genes are present and how they affect the function of the population as a whole.[46] Interesting populations include collections of diverse but poorly characterized bacteria or virus species from biomedically relevant environmental samples such as soil,[62] sewage wastewater,[63] or the human gut.[64–66] Typical metagenomic datasets determine all the genes present in such a population by fragmenting the microbial DNA in a sample and sequencing each fragment. This method yields insight into the distribution of species in a population, the metabolic functions that the population can perform, and other biologically relevant information.[46] However, because short DNA fragments from multiple cells are mixed together, all information about which fragment originated from which cell is lost, and there is no way to associate sequences coming from the same cell. Whole-genome assembly from single cells in an environmental sample is therefore impossible.[67] Even if several identical cells in a complex population allow for assembly from overlapping fragments, assembly is still an enormous computational challenge.[68,69]

Droplets can be used to associate distant portions of the genome from a single cell. Encapsulating single cells or viruses while their genomes are still intact ensures that all the DNA within a given droplet must have originated from the same cell, no matter how fragmented it becomes in later steps, as long as the droplet remains intact. The droplet effectively replaces the cell membrane or virion capsule as a container for the whole genome.

Once single cells or viruses from a population are encapsulated in droplets, single-cell PCR can target an interesting gene previously detected in the population and amplify that gene in every droplet where it is present. Droplet microfluidics can then sort out those droplets containing the PCR products (Fig. 5b). Each of these droplets must also contain the original genome that carried the gene that produced those PCR products. The selected
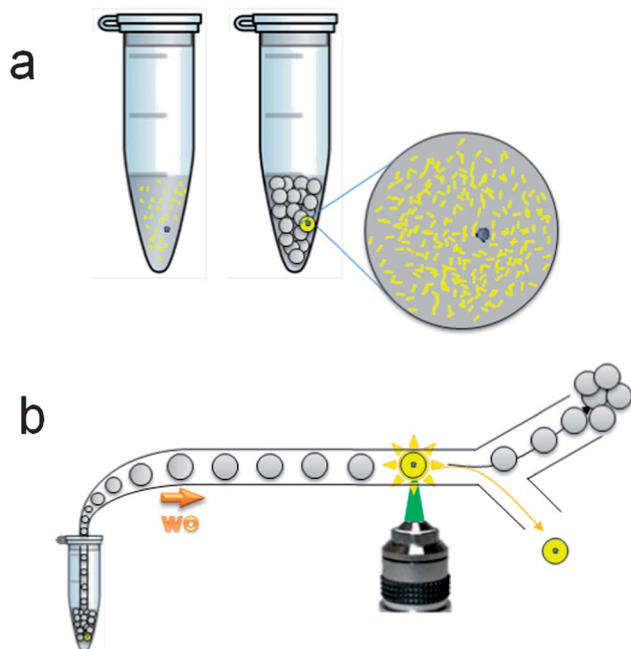
Fig. 5 PCR-based selection of rare templates. (a) A single, rare template (dark spot) is amplified, producing a fixed number of PCR products (bright fragments) that may be fluorescently stained. In bulk (left), the PCR product concentration is low, while in droplets (right), the effective concentration is much higher, since all of the products are confined to the single droplet that contains the single template. (b) After the PCR thermocycle is performed in a standard microtube, droplets are reinjected into a microfluidic device and screened for successful PCR product formation. The single droplet containing the rare template is retrieved for downstream assays such as sequencing. The arrow indicates the direction of flow for water-in-oil emulsion droplets (wo).

droplets can then be coalesced and subjected to whole genome sequencing using conventional methods in bulk. Because the selected genomes of interest will constitute a population of much lower complexity than the original environmental sample, whole genome assembly will be much more feasible. In fact, if the selected genomes were very rarely occurring variants within the original population, whole genome assembly would be impossible without screening in droplets. Droplet microfluidics thus enables whole genome sequencing of rare single cells of interest from complex environmental samples.

Limitations compared to bulk assays

While droplet-based microfluidics provides enormous opportunities for high-throughput biological assays, it also has some constraints that limit its applications. Droplet microfluidics is especially well-suited for ultra-high-throughput assays, which can process as many as $\sim 10^8$ samples per day. However, not all biological assays require such high throughput. It is significantly more efficient to use droplet microfluidics for assays involving at least 10^5 samples. For fewer samples, tools such as 1536-well microtiter plates are generally sufficient. Indeed, droplets suffer from some limitations compared to bulk assays in microtiter plates. For example, while droplets do produce very small volumes, they also produce very high surface area-to-volume ratios. Thus, the oil–water interface that forms each droplet must be both stable and inert. Finding the right surfactant that will maintain such an interface is often a challenging chemistry problem. Similarly, while a library of a billion droplets can easily be handled in a single microtube, droplets cannot take advantage of the spatial barcoding that is available in the two-dimensional arrays of microtiter plates. Thus, droplet libraries require a suitable barcoding system, which can be difficult to develop.

There are also some specific cases where other methods may present advantages over droplet microfluidics. An array of microfluidic chambers and valves arranged in an *n*-by-*n* matrix can efficiently map out the full combinatorial space of pairwise interactions within a library,[70] but performing the same exploration in droplets would likely require an impractical number of parallel dropmakers and picoinjectors. Heterogeneous assays involving washing steps are routinely performed in bulk, but are difficult to adapt to droplet microfluidics. Flow cytometry machines are often used to sort single cells into individual microtiter plate wells, but the technology has not yet been developed to steer single droplets exiting a microfluidic device into individual wells. More generally, interfaces between droplet microfluidic devices and the macroscopic world remain to be developed.

Despite these limitations, droplet microfluidics nevertheless has great potential for many biological assays. Indeed, these limitations do remain challenges, and are the subject of further development.

Conclusions

Droplet microfluidics offers several distinct advantages that can be leveraged in many ways to improve a wide range of important biological applications. All of the applications we have discussed here can benefit from the ultra-high throughput attainable by droplet microfluidics through the use of picolitre volumes and kilohertz rates for sample manipulation and detection. Ultra-high throughput has particularly significant implications for cost when screening large libraries of precious small compounds. Small volumes lead to an enormous increase in effective concentrations and signal-to-noise ratios, which is critical for performing single-cell assays, such as those used to study bacterial persistence, virus–host interactions, and cell growth in general. High sensitivity is also critical for detecting and analyzing small amounts of biological molecules, such as antibodies, enzymes, and single genomes. Compartmentalization in droplets can be useful as a method for linking phenotype to genotype, in place of genetic techniques such as surface display. Finally, droplet microfluidics not only magnifies the scale of existing high-throughput screens, but also enables new types of experiments. The single-cell population studies that we have discussed are only one example of many novel studies that should become feasible.

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