Lab on a Chip

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Journal:	*Lab on a Chip*
Manuscript ID	LC-PER-04-2020-000347.R1
Article Type:	Perspective
Date Submitted by the Author:	27-May-2020
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High-Throughput Screening by Droplet Microfluidics: Perspective into Key Challenges and Future Prospects

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Abstract:

In two decades of development, impressive strides have been made for automating basic laboratory operations in droplet-based microfluidics, allowing the emergence of a new form of high-throughput screening and experimentation in nanoliter to femtoliter volumes. Despite advancements in droplet storage, manipulation, and analysis, the field has not yet been widely adapted for many high-throughput screening (HTS) applications. Broad adoption and commercial development of these techniques require robust implementation of strategies for the stable storage, chemical containment, generation of libraries, sample tracking, and chemical analysis of these small samples. We discuss these challenges for implementing droplet HTS and highlight key strategies that have begun to address these concerns. Recent advances in the field leave us optimistic about the future prospects of this rapidly developing technology.

Main Text:

1.0.0. Introduction

Droplet microfluidics enables the translation of chemical and biological assays to scales and rates unachievable in conventional laboratory workflows. The impetus for using water-in-oil emulsions grew initially from a desire to develop low-cost alternatives to robotic liquid handlers for high-throughput experimentation. Microfluidic segmentation presents impressive $10^3 – 10^6$ fold volume reductions of bioassays compared to bulk workflows.[1] Additionally, use of droplets provides solutions to some drawbacks of single phase microfluidic systems such as sample diffusion, analytical throughput, surface fouling, and inefficient mixing.[2,3]

Droplets are relatively simple to generate in a wide range of volumes, compositions, and throughputs. They may be stored, transported, and analyzed, facilitating modular operations.[4] Droplet-based sample manipulations may exceed 500 Hz, which is orders of magnitude faster than robotic “high throughput” liquid handling (>5 Hz).[5,6]

High throughput screening (HTS) is traditionally defined as the rapid analysis of unique samples, exceeding 10^3 samples/day, for the identification and selection of hits. While anything on pace with robotic handling may be comfortably classified as high throughput, the screening achieved with 1536 well plates (>10^5 samples/day) has been used to define “ultrahigh” throughput.[7] Droplet microfluidics allow for these ultrahigh rates for both screening and sample manipulations, positioning droplet technology as a strong contender for the next generation of chemical and biochemical HTS and experimentation methods.

In this perspective we highlight the key challenges and applications for HTS in droplet microfluidics. We focus on continuous flow approaches for sample processing and analysis, and omit digital microfluidics which is reviewed elsewhere.[8,9] In particular, we aim to highlight the challenges that have hindered the broader adoption of droplet microfluidics for HTS, provide insight into the progress that has been made addressing key challenges, and discuss the potential for future improvements. The purpose of the highlighted publications here is not to provide a tutorial for performing droplet-based HTS, but rather to emphasize the body of developments made

in preliminarily addressing practical concerns. The challenges we have outlined here should serve as a guide to critical considerations and potential applications for droplet microfluidics for HTS, and to highlight how far the field has come in the past decade.

Harnessing the throughput capabilities of droplet microfluidics has been stymied by the challenges of multi-device integration, droplet leakage, in-droplet library generation, sample tracking, and analytical readout limitations. The field has seen the emergence of a few key strategies that have been effective at addressing these challenges and show promise for significantly broadening the applicability of droplet HTS. Developments in droplet analysis platforms, dosing strategies, and integrated workflows have opened the doors to novel applications and opportunities in commercialization. We argue that the field is well positioned for wider adoption and application in the coming decade, should these challenges continue to be addressed.

1.1.0. Droplet Sample Processing

In the well plate-screening paradigm, samples are retained in wells on a 96, 384, or 1536-well plate. These plates allow reactions to be performed in volumes as low as two microliters. The spatial separation of samples in discrete wells provides the ability for thousands of parallel reactions to take place in a compact, traceable, and addressable format. With chemistry and biology confined to these wells, researchers can culture and sequence cells, add reagents, aliquot samples, perform sample cleanup, and analyze with a wide range of instrumentation. The ability to manipulate and analyze each sample based on its position is critical to the utility of this format. For a novel screening platform to compete, it is essential that it reliably perform all of these functions; however, to be widely adopted, such a screening technology must also provide key advantages over these existing workflows.

Research in droplet microfluidics has largely addressed each of the core functionalities of well plate screening.[4] Spatial separation is achieved by breaking fluids into individual droplets using either a T-junction or flow focusing geometry. (**Figure 1A**). The resultant droplets ranging in volume from single femtoliters to tens of nanoliters,[10–13] an impressive reduction from the microliters needed in well plates. A variety of microfluidic geometries allow multiple substances to be co-encapsulated in these droplets as they are made, at controlled ratios and with minimal sample to sample variation.[14–16] Droplet generation in the kilohertz range dwarf the fluid handling

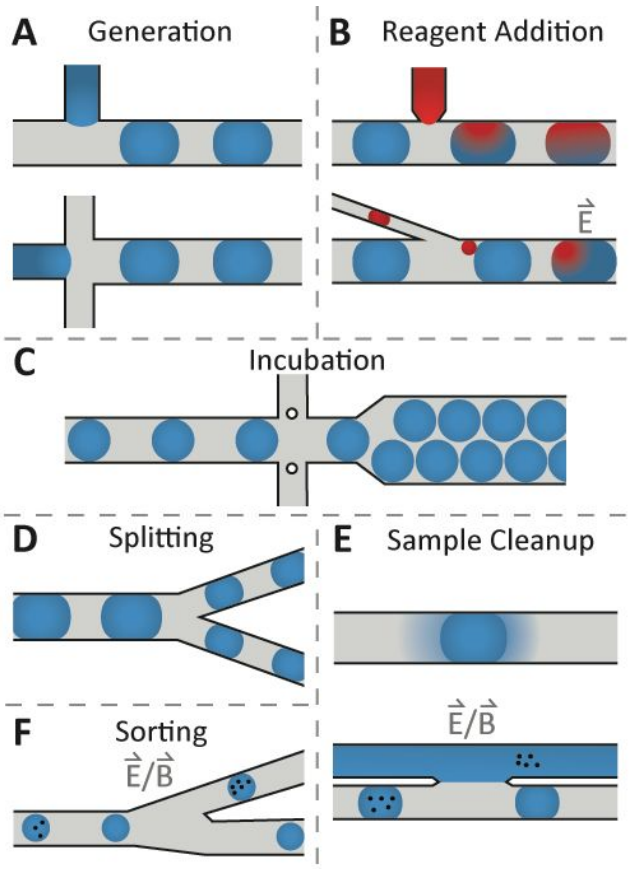

Figure 1. Common droplet unit operations with flow proceeding left to right. A) Droplet generation by T-junction or flow focusing. B) Reagent addition by direct injection and pairwise merging. C) On-chip incubation by oil drain and channel expansion. D) Splitting by channel bifurcation. E) Sample cleanup by continuous phase partitioning or solid phase extraction. F) Sorting by dielectro- or magneto-phoresis.

capabilities of robotics and proposed methods for parallelization hold the potential to push these speeds even higher.[17]

Once these droplets have been formed, it is critical to maintain their spatial and chemical separation in bulk format. Structure is maintained through the use of surfactants,[18,19] which mimic the function of phospholipid membranes in biological systems, i.e. they limit droplet merging and chemical transfer when in contact.[18] Use of surfactants also allows for droplet stability at a range of temperatures, enabling complex reactions such as droplet-based polymerase chain reaction (PCR),[20] or elevated temperature incubations for extended periods.[21]

In well plates, the spatial separation of samples has facilitated the use of robotics for the addition of reagent to individual reactions. The reproducibility and reliability of such systems is critical to modern screening technologies. In droplet microfluidics, overcoming the challenge to create a comparable form of reagent addition has been critical to the use of the technology for complex chemical reactions. Reagent addition has been achieved by direct injection into droplets, pairwise droplet merging, and double emulsion strategies.[22] Direct injectors (e.g. Pico-injectors) bring single-phase reagent into contact with a cross flow of droplets, allowing for brief merger between the aqueous components, then re-segmentation after the addition of the reagent (**Figure 1B**).[23–25] When surfactant stabilized, reagent addition by pico-injection may be actuated by use of an external electric field.[24] Direct injection into droplets has been demonstrated over a wide range of volumes and the throughput exceeds that of robotic handling by several orders of magnitude.[26,27] When placed in series, multiple reagents may be added to each droplet in rapid succession.[23]

While direct injection has allowed complex reaction workflows to be performed in droplets, it does not allow for combinatorial reaction screening to be performed in the same way that it may be in plate-based screening. With direct injection, the same reagent must be added to every single droplet, meaning different compounds or samples may not be added to each droplet. One method to address this is to combine two libraries of droplets to produce a combinatorial set of samples. To this end, droplet pairing and merging strategies have been developed for both continuous flow and stationary droplet handling. In continuous flow, two streams of droplets may be combined in pairs and merged to produce an output stream of combined droplets (**Figure 1B**).[22,28–30] In stationary droplet handling, microwell based strategies pair one or more droplets in a single well prior to electro-coalescence, producing deterministic combinations of the original droplets.[31–34]

In any chemical reaction, sample incubation is critical to allow the desired reaction to occur prior to screening. In the droplet format, this may be achieved by removing droplets from a device and storing them in bulk. When integrated into multistep microfluidic processes on a single device, it is necessary to reduce fluidic velocity on-chip to allow for increased on-device time. Largely, this is accomplished by draining oil and allowing droplets to close-pack (**Figure 1C**).[35,36] Devices with integrated delay lines are useful for simplifying workflows to single devices when incubation is necessary. On-chip incubation lines are often aided by channel expansion or multi-layer fabrication, where an increase in channel volume contributes to both decreased fluidic velocity and increased droplet containment.[14,36,37]

Sampling from well plates in traditional screening workflows is common; small volumes of sample may be pulled for analysis, further reactions, or long-term storage. In droplet format, sampling can be performed by splitting droplets in flow using microchannel bifurcations (**Figure 1D**),[10,25] electrostatic forces,[38] or acoustics waves.[39]

Solid and liquid phase extractions are regularly performed in plate format to prepare samples for analytical assessment, and similar sample cleanup has been demonstrated in droplets (**Figure 1E**).[26,40–44] Droplet splitting may be used to perform solid phase extractions, allowing selective retention of targeted analytes in droplets via bead based capture and wash workflows.[45,46]

Once a sample is analyzed it is often necessary to further manipulate the sample. On well plates, this function is trivial assuming that the analytical method does not consume all of the sample. For flowing droplets, however, collecting desired samples for further use requires a

collection or sorting method. The most common strategy for doing so is dielectrophoresis (DEP) where a droplet may be deflected by an electric field to exit a device via a selected channel (**Figure 1F**).[47,48] Other sorting strategies have been achieved using external magnetic fields,[49,50] acoustic waves,[51] or through other physical properties of droplets.[37,52] Coupling these sorting techniques with rapid analytical assessment can provide high throughput approaches to hit identification and selection, and enables downstream discovery and analysis.[53–55]

Well plate screening methodologies are a well-established, robust approach to HTS but microfluidic droplets are quickly becoming a viable alternative. Droplets represent an attractive opportunity to scale down expensive chemical use in HTS, reduce unsustainable consumable use in the screening process, and rapidly profile thousands of samples, all while using automated workflows that run at rates that are orders of magnitude higher than industry standard techniques.

2.0.0. Droplet HTS: Challenges and Ongoing Innovation

Arguably, the advances in droplet manipulation over the past decade have positioned droplet microfluidics to deeply alter the methods by which HTS is performed. Droplet microfluidics has the ability to emulate many of the key unit operations in traditional well plate screens, with impressive advantages in sample size and throughput. Despite these advantages, droplets have not yet gained traction as a common method for HTS. Efforts to commercialize these systems have been limited to only a few niche applications.[56] In this section, we will discuss the major challenges to droplet HTS, and how they have been addressed (Table 1).

Table 1. Critical challenges to droplet HTS and their common solutions

Challenge	Synopsis	Current Resolutions
Multi-device integration	Tethering multiple unit operations induces droplet shearing and merging	Gentle droplet reinjection,[29,57] Single device integration[14,58]
Molecular transport	Small molecules may transport between droplets	Dendritic,[59] nanoparticle[60] surfactants Substrate derivatization[61]
Library generation	Compound libraries are difficult to dose into droplet populations	Combinatorial droplet merging[33,34] Bead associated libraries[62]
Droplet tracking	Droplet identities are difficult to trace back to original sample information	Fluorescently barcoded beads[63] DNA encoded particles[64]
Droplet analysis	Label-free droplet interrogation techniques are lacking	Raman detection[65] Mass spectrometry[31,66]

2.1.0. Droplets as Robust Microreactors

2.1.1. Enabling Multi-unit Integration

Complex biological assays often require multiple additions, extractions, and temperature conditions, all within the same sample. With surfactant stabilization, droplets are stored, reinjected, incubated, and thermocycled in bulk within just a few milliliters of volume.[18] Sequential devices are used for tethering unit operations to translate complex workflows into droplet-based schemes.[67] Clever droplet storage solutions allow reliable storage and transfer with minimal loss to shearing and merging,[29,57] allowing complex multi-step reactions to be performed in droplets.[27] Unfortunately, each step of these processes introduces variability in droplet volume, contents, and stability that can disturb downstream functions. Unit operations often rely on the monodisperse input of bulk-generated emulsions, and size variation can disrupt the reliability of these devices. As a result, some work has gone into removing merged or damaged samples from complex droplet

workflows,[27] and such necessary steps will likely become more common as researchers attempt more and more complex biochemical reactions within droplets.

2.1.2. Molecular Transport

To effectively process and analyze samples discretely, it is critical that droplets reliably maintain their contents through the course of any incubation or manipulation without cross contamination or sample loss. Experiments have revealed conditions where droplets allow cross-talk. Early research with mineral oil as the continuous phase for droplet segmentation revealed that some analytes demonstrated transfer into this hydrophobic oil.[68] Fluorinated oil and surfactants offered an alternative carrier phase to contain analytes.[69] Chemical leakage from droplet samples has also been observed in these systems. Several groups have investigated molecular retention and leakage in droplet emulsions using fluorescent detection with fluorophores as model analytes (**Figure 2A**).[19,70–72] Buffer pH, fluorophore solubility and hydrophobicity, and micellar transport have all been proposed as contributors to analyte loss both into the surrounding oil phase and into neighboring droplet samples. No definitive mechanism of molecular transport has been agreed upon.[73]

Investigation into both the fundamental mechanism and prevention of cross-talk may expand droplet functionality to workflows inaccessible due to chemical leakage.[72] The addition of bovine serum albumin or sugar additives has been shown to increase fluorophore containment,[68,74] and the modification of fluorophores with permanently-charged sulfonate groups appears to discourage chemical loss between samples.[61,70,75] The use of multiple surfactant layers, sizes, and structures have been demonstrated as effective solutions in certain contexts.[71] Dendronized polymer surfactants have recently been demonstrated for both efficient droplet stabilization and small molecule containment.[59] The use of nanoparticle surfactants has been shown to eliminate micelle formation, leaving carrier phase solubility as the only driving force for chemical transport.[60] As such, commercial nanoparticle surfactant/oil mixtures have been introduced to meet broader interest in these systems.[76,77]

Small molecules appear to be the primary concern for partitioning between droplets.[70] Larger biomolecules such as nucleic acids and proteins have been shown to remain within original droplets.[72] Droplet stability and containment will likely continue to complicate the use of surfactant-stabilized droplets to HTS for small molecules, particularly in the context of probing diverse chemical libraries and reaction conditions. Relying solely on fluorescence analysis is likely to be limiting in the detailed molecular studies needed to understand cross-talk. Label-free analysis, such as droplet Raman and mass spectrometry, is beginning to provide wider insight into how the chemical contents of droplets change over time, and may facilitate increased characterization of droplet cross-talk.[66,78,79] Such studies could facilitate both the selection and development of compatible oils, additives, and surfactant combinations that reduce the cross-contamination of these analytes.

2.2.0. Library Distribution into Droplets

The generation of unique reactions within each droplet is necessary for droplet HTS. Granular control and variation of droplet contents is challenging in continuous flow, where the addition of varied contents must match the frequency of droplet formation or droplet introduction. Low frequency droplet generation techniques, such as well plate based sampling[80,81] or singular droplet generation[82,83] provide the capability to create libraries of chemically distinct droplets from larger sample pools, but lack the throughput of their continuous flow counterparts.

The most basic method for varying chemical composition in droplets for HTS is to modulate composition of the dispersed phase as droplets are created. Generating a library in this fashion is performed by combining multiple reagent streams prior to droplet emulsification.[69] Varying the flow rates of each combined stream allows a wide array of droplet compositions to be created rapidly, and this technique has been used to sample and optimize reaction conditions in

microfluidic droplets.[84] Modulating conditions by this approach may be used to sample a wide array of concentrations for a few reagents but it does not allow for the distribution of large chemically or biologically diverse libraries.

To date, library distribution in droplets has been largely addressed stochastically, with the addition of particles (e.g. cells, functionalized particles, genetic material) to the dispersed phase. Using Poisson loading of these particles, unique chemical or biological contents may be encapsulated randomly in droplets.[85] Effective droplet loading is modulated by the concentration of the particle, such that statistically driven encapsulation occurs (Figure 2B). Poisson-driven library distribution has found particular use in methods for emulsion PCR, where individual gene sequences may be captured and amplified in droplet samples and used to get precise reads on copy number for rare or isolated DNA.[86] Poisson loading of single cells has proven useful for studying low-frequency genotypes and phenotypes in droplets.[14,87] Additionally, bead bound DNA-encoded compound libraries have recently been adapted to droplet microfluidics to dose individual droplets with small molecule libraries.[58] With this approach, virtually any material that may be attached to

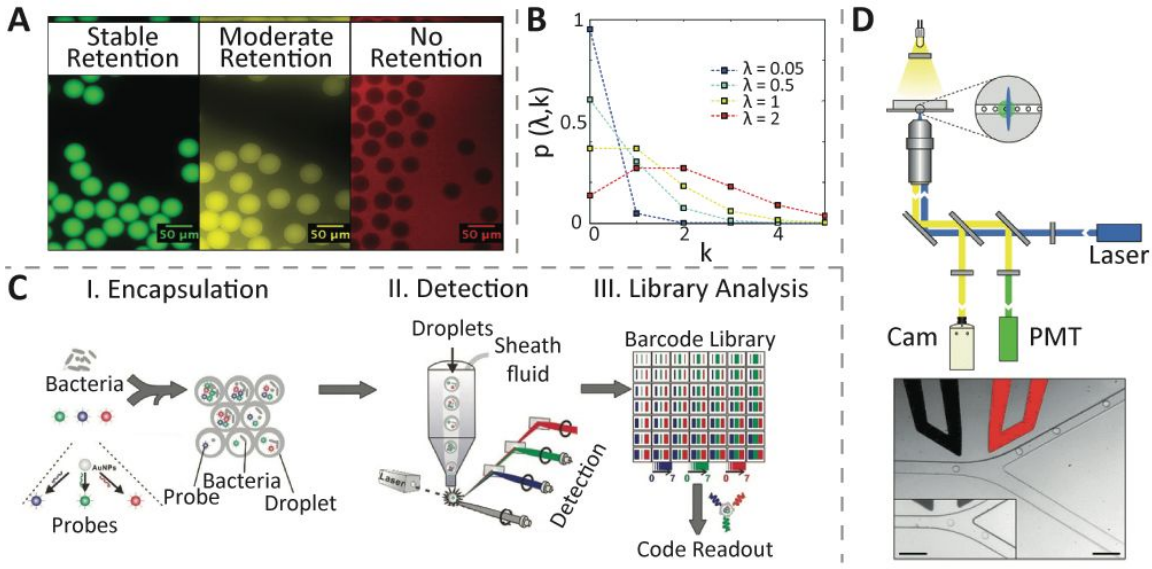

Figure 2. Challenges facing droplet HTS. A) Molecular transport between droplets is a critical challenge in droplet systems where droplets act as individual microreactors. Here, fluorescently labelled dyes display differential partitioning out of an aqueous droplet, complicating assay analysis. Reproduced from ref. 70 with permission from The American Chemical Society, copyright 2015. B) Library distribution in droplets is often achieved through Poisson distribution of particles and cells. Statistical loading of cells per droplet, *k,* may be easily modelled for given λ values to estimate the probability of multiple library members being encapsulated together in a single droplet. Reproduced from ref. 85 with permission from The Royal Society of Chemistry, copyright 2015. C) Droplet barcoding enables facile tracking of droplet contents. Specific bacterial species may be detected and identified by co-encapsulated fluorescent probes for analytical readout and screening of 2D barcodes. Reproduced from ref. 97 with permission from John Wiley & Sons, copyright 2019. D) Rapid fluorescent detection and dielectrophoretic sorting enables high throughput screening and sorting of droplets at the picoliter to nanoliter scale. Fluorescent activity measured by a photomultiplier tube triggers an on-chip electric pulse to deflect a droplet into a collection channel at 30,000 Hz. Reproduced from ref. 54 with permission from The Royal Society of Chemistry, copyright 2009.

a particle may be distributed into droplet samples, provided the endpoint analysis is sensitive enough to detect the loaded material.

Poisson-dependent sample encapsulation necessarily means that the majority of the generated samples will be empty.[85] However, in ultrahigh throughput screening applications with assessment rates as high as 10^4 samples/second, these empty droplets are acceptable by virtue of the rate at which droplet samples may be screened. The regular occurrence of empty droplets is an advantage in that it provides a repetitive negative control signal in the screen, which boosts the reliability of statistical sample analysis. Additionally, precise 1:1 loading is not always necessary for screening accuracy, depending on the size of the distributed library and the throughput of the analysis techniques.[88] At lower analytical throughputs where large numbers of empty droplets would be more time costly, or in instances where the co-occurrence of multiple encapsulation events is enabling for the assay at hand (such as single cell sequencing applications where a bead and cell must be co-encapsulated), hydrodynamic methods for deterministically loading particles into droplets may be used. Particles, such as beads or cells, are inertially focused in microfluidic channels prior to droplet formation to provide increased droplet loading efficiencies. Hydrodynamic strategies have allowed up to ~80% of droplets to be produced with the desired particle or cell.[89] Magnetophoretic and fluorescent sorting of droplets have also been used to further enrich for encapsulated materials.[50,90,91]

Generating libraries through combinatorial means has recently been demonstrated in droplets.[27,34] Droplet populations may be generated for each desired condition then paired and merged. A pairwise approach has been used to combine droplets containing cells and barcoded beads,[33] as well as combine small molecule libraries.[34] Such deterministic merging has been performed both in flow and in static droplet arrays; it may be used to pair droplets of varied size, shape, and contents to create more complex combinatorial libraries.[92] In flow, paired droplets have been used to perform *in vitro* expression of DNA in droplet samples,[27] as well as add pre-determined concentrations of reagent to existing droplet reactions.[93]

A recent example of droplet pairing comes in the form of droplet printing, which combines the spatial resolution of well plate formatting with the throughput and size advantages of droplet fluidics. Using automated positioners, droplets are deposited in pre-determined locations for subsequent assay and screening.[94–96] Additional droplets may be deposited and merged at pre-determined locations on an array of microwells.[31] By coupling printing to a continuous flow microfluidic fluorescent sorting device, researchers have been able to individually address and deposit pre-defined quantities of material into specific microwells.[32] With this advance, individual droplets may be addressed with tailored addition, and the distance between micro well formatting and high throughput droplet processing in flow has reduced further. These tactics demonstrate competitive technology compared to well plate strategies, even in the more difficult contexts of compound additions and screening.

2.3.0. Tracking Droplets

While droplet processing and manipulation is faster than traditional robotics-based techniques, this speed often comes at the cost of facile sample tracking. Droplets arranged in a channel may be indexed and tracked by their position, but fluidic resistance inherently prevents more than a few thousand samples to be stored at a time. “Sipper”-based droplet generation uses negative pressure to pull nanoliter sample arrays into a capillary, and has been used to reformat well plate samples into droplets prior to assay and analysis,[80,81] but it represents a comparatively low throughput approach to droplet monitoring because each individual droplet sample must be created and indexed in a linear array.[80,81] Similarly, printed droplet microarrays (**Section 2.2.0**) may be addressed and assayed on a site by site basis, but the association of droplets with a physical position lowers sample quantity to just a few thousand samples. Furthermore, precise optics and robotics are required for careful tracking and manipulation of the printed samples.[31,32]

To reliably assay droplets stored in surfactant stabilized emulsions, droplet contents must be directly associated with assay output. Droplet sorting and barcoding offer solutions that address this challenge. Droplet sorting allows the collection of samples that meet threshold criteria.[54] Fluorescent barcoding of droplets has been used to track combinatorial additions of small molecules in droplet arrays, as well as cell mixtures in bulk droplets (**Figure 2C**).[97] Recent work to address the band overlap of available fluorophores has expanded the number of fluorescent combinations to span more than 1000 distinct optical readouts, which may be associated with specific bead (and therefore droplet) content.[63] Particles outfitted with unique oligonucleotide barcode sequences have also been used to label droplets and track their contents to associate them with single droplet samples.[98,99] In widely utilized sequencing methods, beads containing short oligo barcodes appended to PCR primers are co-encapsulated with single cells. When cells are lysed and sequenced in the presence of a bead, this barcode is appended to the PCR product in each droplet, associating a unique code with each read that can be traced back to that single droplet. Barcoding strategies have allowed massively multiplexed sequencing of rare cell populations where members with low abundance would otherwise be lost in the noise of the dominant cells in the system.[62] A similar barcode handle has been recently utilized in HTS of bead-based small molecule libraries.[58] By coupling split-and-pool bead based chemical synthesis to short oligo synthesis, MacConnell *et. al* was able to associate DNA barcodes with unique synthetic products, capture the bead in droplets, and demonstrate screening of up to 30,000 members of a small molecule compound library in one hour.[58,64] Hits were collected via fluorescent sorting and hit compounds could be recovered and identified using the associated DNA barcode.

Though these barcoding strategies have proven successful, many workflows are not compatible with these bead-based approaches. Surface functionalization is limited by the available surface area of the bead itself, which can limit the concentration of the associated bead-bound library member in the droplet. Nevertheless, these strategies represent a creative and effective method by which to keep track of droplets in flow, even in the context of stochastic mixing and re-ordering during droplet storage. Future successful droplet applications will necessarily need to employ similar techniques to accurately track the contents and the processing of droplets in these systems.

2.4.0. Droplets for Rapid Analytical Processing

Rapid analysis is a critical bottleneck in HTS. Well plate screening allows individual samples to be tested photometrically in under a second, and separation techniques enable the assessment of multiple analytes within the same run. Commercial technology has emerged for the automation of analysis in 384 well plates for LC-MS workflows, but the throughput still lags behind droplets and liquid handling is limited to >1 μL.[100] Recent technologies such as the Agilent RapidFire demonstrate throughput up to ~0.1 Hz,[101] and represent significant progress towards faster HTS in well plates, albeit at high instrument costs. Multiple injections in a single experimental run (MISER) is a viable LC technique that increases throughput up to ~0.05 Hz.[102,103] Emerging technologies such as acoustic injection for MS (ECHO MS) provide up to 3 Hz throughput from a well plate.[104] While these emerging technologies improve analytical throughput considerably, they are still tied to the complex robots and relatively large volumes of well plates in standard plate-based screens. The challenge for analysis in droplet microfluidics lies in obtaining high information content in minimal reaction volumes at high rates.

2.4.1. Optical Analysis

The analysis of droplet content has relied heavily on a few techniques that have been applied widely. Most notably, droplet HTS has been performed extensively using fluorescent detection (**Figure 2D**). Laser induced fluorescence (LIF) is a common technique for droplet analysis as it is easily applied to droplet analysis; LIF approaches have rapid acquisition rates, which allow for multiple sample points across a single droplet even at high throughputs and boast

low limits of detection and high sensitivity. When coupled to DEP sorting, LIF based droplet selection has been demonstrated as fast as 30 kHz with >99% accuracy,[105] throughput on-par with fluorescence activated cell sorting >100 kHz.[106] However, LIF based workflows require fluorescent reporters, which are often challenging to incorporate into chemical and biological assays. In many chemical or biochemical screens, the native target is not fluorescently active and adding a fluorescent tag may change the activity of interest. To address this limit, secondary reporters have been utilized to give a signal output from non-fluorescently active assays.[55,107,108] Applications of these reporter systems are limited and must be designed on an *ad hoc* basis.

For droplet microfluidics to expand into a more versatile technology for HTS, analytical techniques other than direct LIF must be adapted to the droplet paradigm. Many alternative approaches have been investigated for the analysis of droplet samples. Absorbance based detection schemes have been explored as a complimentary strategy for droplet analysis.[55] However, the low specificity and high limits of detection limit this approach to reactions that produce a change significant enough to be detected above the background reaction matrix. Absorbance detection is more challenging in droplets due to the limited path length through which to detect across a droplet. To counter this, more sensitive methods such as differential detection photothermal interferometry have been reported for absorbance readouts from droplets.[109] High-speed charged coupled device (CCD) cameras have been employed for the screening of colorimetric outputs in droplets.[107,110] Though CCD cameras have been used primarily to interrogate droplet size, a system in which droplets change turbidity or refractive index may be accessible for this type of detection as well.[111]

Spectroscopic techniques such as droplet Raman and infrared detection, as well as droplet nuclear magnetic resonance (NMR) spectroscopy, have been developed but are under-utilized in droplet HTS. Raman has been applied to single cell detection in droplets but has not been widely employed for many HTS workflows due to high noise levels and low sensitivity.[65] Strategies for improvement of sensitivity and selectivity are a major topic of research,[112,113] which may lead to improved detection methods for DEP triggering.[65] Infrared detection has been applied to imaging droplets in flow, but HTS has not been demonstrated, likely due to the high interference from the aqueous background.[114,115] NMR analysis of microliter segmented plugs has been shown as a multiplexed detection from LC effluent,[116] but has similarly not been applied to HTS. Droplet NMR spectroscopy may be an exciting future development with the improvement of miniaturized high-field magnets.[117,118]

2.4.2. Mass Analysis

While optical detection in droplets has proven both rapid and versatile, it is challenging to make analytical determinations in systems where optical changes are not readily observed in droplets. Mass spectrometry (MS) analysis of droplets has been explored as an alternative approach, expanding the tool set for droplet analysis through label free detection. Droplet MS has been applied using both electrospray ionization (ESI)[81,119,120] and matrix-assisted laser desorption ionization (MALDI).[95] ESI of droplets is particularly attractive, as it may be integrated into the outflow of a microfluidic system. As mass spectrometers become faster and more sensitive, techniques such as tandem mass spectrometry have enabled selective analysis within droplet samples. Throughputs for droplet ESI are modest in comparison to optical detection; recently 300 pL droplet samples have been analyzed at up to 10 Hz using nano-ESI[66] and 800 pL have been shown to be detectable at up to 33 Hz with software modifications to commercially available mass spectrometers.[121] Droplet ESI is also currently limited by droplet size; most studies have thus far operated in the 10 – 50 nL range,[80,81,122–124] though recent work using nano-ESI has shown droplet detection in volumes as small as 65 pL.[66] A major challenge in coupling microfluidics with ESI is incompatibility between scan speeds of mass spectrometers and droplet size and flow rates; droplets are often too small to maintain sustained electrospray for thorough droplet analysis. While integrating droplet ESI into microfluidic devices and achieving analytical speeds on par with

droplet processing speeds remains a challenge, droplet ESI is an attractive analytical technique for the rapid, label free analysis of microfluidic volumes.

MALDI-MS is also developing as a technique for analyzing droplets. To date, several groups have deposited droplets onto a surface and analyzed by MS-imaging techniques.[95,96] Droplet MALDI has the advantage of flexibility in droplet size as the laser ablation area is often smaller than droplet diameters commonly used. Similarly, MALDI is not fully destructive like ESI, allowing researchers to return to samples of interest after analysis. Haidas and coworkers reported the ability to spot and analyze up to 2400 droplets on a standard glass slide,[31] and others have fabricated microwell plates for droplet deposition at higher densities.[32] However, unlike ESI, droplet MALDI cannot be performed as an online technique for analysis, which restricts throughput. Additionally, analysis can be challenging to perform on stabilized droplets as the use of surfactants may suppress MALDI.[125,126] Nevertheless, droplet MALDI offers an attractive alternative to ESI in instances where facile sample recovery and reconstitution are critical.

3.0.0. Applications of Droplet Microfluidics

While current limitations in analytical readout and droplet processing prove challenging to implementing the workflows necessary for HTS, several applications of droplet screening stand out as particularly reliable and widely adopted. Notably, droplets have shown significant utility in high-throughput screening for enzyme evolution and activity assays, DNA/RNA sequencing and detection, and most recently small molecule-protein interactions.

Enzyme evolution, directed evolution, and protein engineering are all related fields that have been driven by high-throughput screening.[27,55,108,127,128] Individual droplets doped with cell, enzyme, or protein variants may be processed using the unit operations described above and analyzed to determine catalytic activity. These screens have primarily relied on fluorescence-activated droplet sorting, which has enabled high sorting rates (**Figure 3A**),[27,54,108,129] but limits the scope to enzymes that may be probed by fluorescence. A few screens have used ESI- or MALDI-MS to demonstrate proof of principle but MS has not been fully integrated into comprehensive biocatalytic screens.[31,123] Droplet screening has also been implemented effectively as a tool for bioprospecting, the search for valuable natural-product chemistry in biological organisms (**Figure 3B**). Droplets are particularly advantageous for these applications since many bacterial strains may not be cultured in bulk but may proliferate individually in droplets.[130,131] Similar HTS campaigns have enabled the enrichment of natural catalytic activities on non-native substrates in impressive libraries of $>10^8$ cells.[132]

Perhaps the most adopted application for droplet HTS has been in the implementation of droplets for single cell isolation and sequencing.[133] By capturing single cells in droplets with oligonucleotide barcoded beads and PCR materials, researchers have been able to identify and sequence rare cell populations that would otherwise have been lost in the noise of the other species around them.[62,133,134] The co-encapsulated beads allow sequence reads to be associated with individual droplets, allowing rapid elucidation of the genetic makeup of complex cell populations (**Figure 3C**).[98] Droplet sequencing technologies show remarkable promise in the field of personalized medicine, cancer diagnostics, and micro biome research, and have been widely adopted.

Similarly, single DNA and RNA detection has been revolutionized by droplet microfluidics and remains one of the few examples of droplet technology that has been successfully commercialized.[135] Like cells, individual nucleic acid oligos may be captured within a droplet of PCR reagents. With the advent of droplet PCR techniques, simple thermocycling results in a fluorescent signal in any droplet containing the probed DNA sequence, allowing the detection of DNA and RNA at a single molecule level.[17,136,137] Droplet genomic detection shows significant promise as a technique for rapid, personalized diagnostics where nucleic acids may be used as a marker for the presence or absence of the probed condition.

Most recently, advances in bead and droplet-based small molecule libraries have set the stage for rapid drug discovery in droplet microfluidics.[99,138] Coupled with fluorescent sorting techniques, thousands of compounds may be rapidly assessed for biological activity such as enzyme inhibition. HTS of small molecule interactions further alleviates the need to perform expensive, well plate-based compound library screens (**Figure 3D**).[58,64,139]

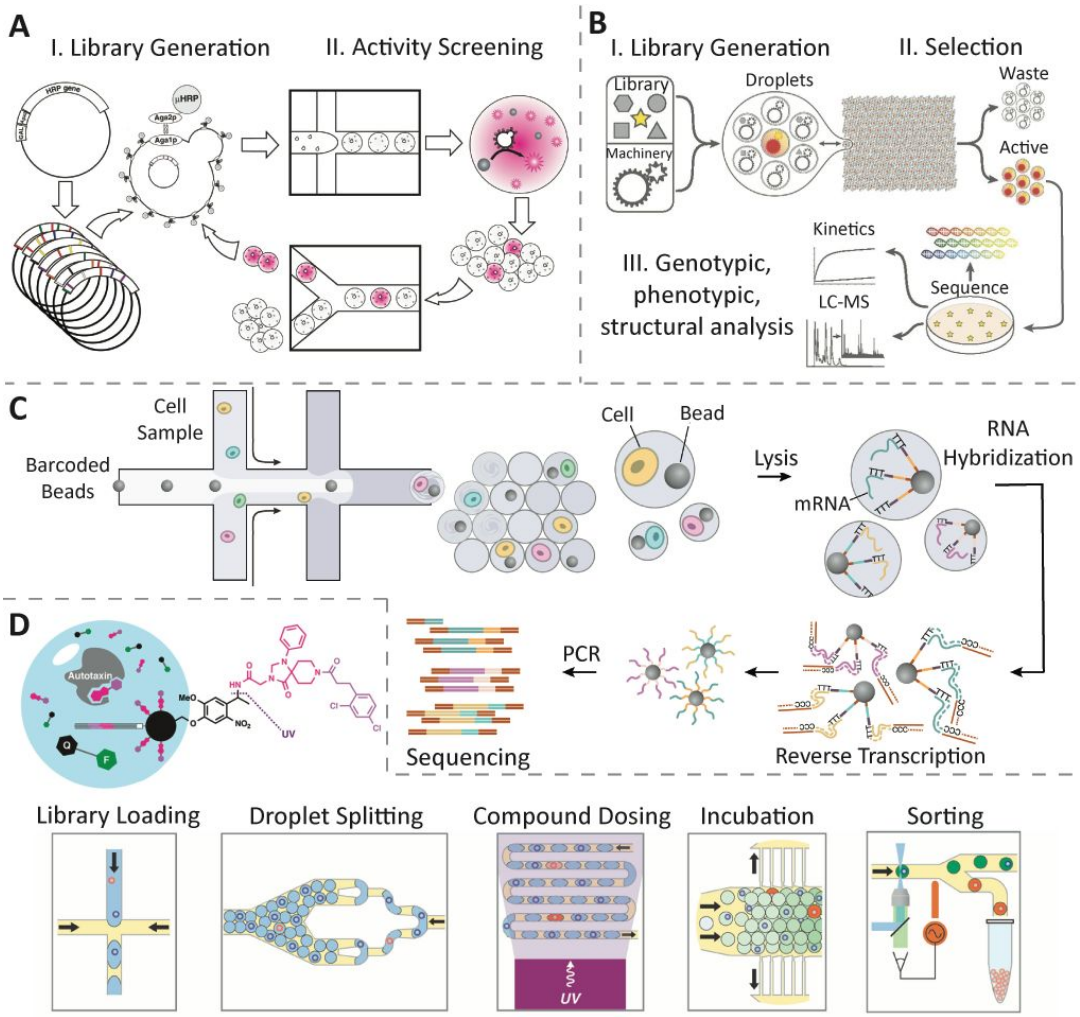

Figure 3. Applied droplet microfluidics. A) Droplet enzyme evolution. Reproduced from ref. 128 with permission from The National Academy of Sciences, copyright 2010. B) Droplet bioprospecting. Reproduced from ref. 132 with permission from The National Academy of Sciences, copyright 2017. C) Droplet genomic sequencing. Reproduced from ref. 98 with permission from Elsevier, copyright 2015. D) Droplet small molecule screening. Reproduced from ref. 64 with permission from The American Chemical Society, copyright 2019.

4.0.0. Future Prospects

While it is still an actively evolving area of research, droplet microfluidics has seen gradual acceptance as a technique for HTS. Commercialization of droplet techniques,[135] coupled with novel analytical methods, are driving the field towards the adoption of droplet screening as an attractive alternative to well plate workflows.

To continue the development of droplet microfluidics for complex applied and commercial workflows, a few critical considerations must be addressed. Firstly, maintaining droplet integrity continues to hinder workflows that require multiple unit operations and varied multistep processing. Tracking and labeling droplets continues to present a challenge, but barcoding strategies and new analytical methods appear to be paving the way for identification of droplets and their contents under a variety of conditions. Most significantly, the analytical methodologies that may be applied to droplet microfluidics have dramatically improved in the past five years, and novel detection methods are providing versatile analysis of these microfluidic samples.

Mass spectrometers are continuing to improve in terms of mass resolution, sensitivity, and scan speeds. Increasingly complex droplet samples may be analyzed with lower limits of detection and higher sensitivity than ever before. As analytical assessment options continue to improve, the possibilities for droplet analysis also expand. Recently, our group demonstrated an ESI-based system in where droplets may be dielectrophoretically sorted based on their mass identities,[124] which we believe will be a useful for label free analysis and HTS in complex droplet systems. MALDI-MS based analysis has seen faster and more reliable droplet deposition techniques in recent years, and shows promise for bridging the gap between the advantages of well plates and the throughput of droplets.[32]

Additionally, droplet analysis by Raman and NMR spectroscopy has the potential to be a versatile label free detection technique. However, it is necessary to resolve challenges to detection in complex samples and optical/magnetic interrogation. Electrochemical detection similarly shows promise for droplet analysis.[140] Electrochemical detectors have been widely applied in the analysis of HPLC effluent and have been shown to be sensitive and rapid. Broadly, electrochemical detection does not have the broad applicability as other analytical techniques but may fill a necessary gap in droplet analysis compared to optical detection.

Droplet microfluidics has been an emerging HTS technology for years but has been slow to take its place among the more conventional microwell plate assays. In order to do so, it must be capable of matching these systems in analytical utility and system reliability. While many of the necessary unit operations to perform such assays have been explored, the limited analytical readouts provided by LIF detection have stunted the broader adoption of these techniques. However, the success of several strategies for HTS in droplets, particularly with respect to enzyme evolution, bioprospecting, genomic detection and sequencing, and small molecule screening is driving interest in the field. With an expanded toolbox available for droplet manipulation, recently established techniques for droplet tracing and dosing, and an increasing set of analytical tools at our disposal, we see significant potential for droplet microfluidics as the “next generation” of automated screening workflows.

Author contributions: EMP and DHM wrote and edited the manuscript. SS and RTK conceptualized the perspective and contributed to editing.

Conflict of interest: RTK has an interest in a company that seeks to commercialize droplet ESI - MS.

Funding: This work was supported through NSF 1604087.

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