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Droplet microfluidics for synthetic biology

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Synthetic biology is an interdisciplinary field that aims to engineer biological systems for useful purposes. Organism engineering often requires the optimization of individual genes and/or entire biological pathways (consisting of multiple genes). Advances in DNA sequencing and synthesis have recently begun to enable the possibility of evaluating thousands of gene variants and hundreds of thousands of gene combinations. However, such large-scale optimization experiments remain cost-prohibitive to researchers following traditional molecular biology practices, which are frequently labor-intensive and suffer from poor reproducibility. Liquid handling robotics may reduce labor and improve reproducibility, but are themselves expensive and thus inaccessible to most researchers. Microfluidic platforms offer a lower entry price point alternative to robotics, and maintain high throughput and reproducibility while further reducing operating costs through diminished reagent volume requirements. Droplet microfluidics have shown exceptional promise for synthetic biology experiments, including DNA assembly, transformation/transfection, culturing, cell sorting, phenotypic assays, artificial cells and genetic circuits.

Introduction

Synthetic biology exploits engineering principles and innovations in molecular biology, cell biology, and bioinformatics to design and construct modified organisms with desired properties. Synthetic biology has demonstrated significant potential, for example, in the biomanufacturing of a wide variety of commercially-viable products including pharmaceuticals, biofuels, chemicals, and biomaterials.[1] Primary techniques to genetically modify organisms can be grouped into two distinct categories: directed evolution and rational design. Directed evolution uses randomized mutagenesis to generate diverse DNA libraries through a variety of approaches such as gene shuffling, error-prone PCR, and the use of chemical mutagens or irradiation. Conversely, rational design (potentially leveraging biological part characterization data and modeling/computer-aided design tools) implements a set of specific genetic variants through targeted DNA synthesis/assembly/editing approaches. With either technique, exogenous constructs (if any) are introduced into host organisms by transformation/transfection. The resulting derivative organisms are then analyzed for the detection and quantification of desired products (and/or other relevant proteins/RNAs/metabolites/*etc.*). Fig. 1 shows the key steps involved in the synthetic biology process for the selection of highest-performing pathway amongst many possible configurations, the successful implementation of which demands significant expertise and high-throughput instrumentation for biological design, manufacture, and performance screening. Most experiments are performed manually and are very labor-intensive, consume large amounts of expensive reagents such as enzymes and synthetic DNA, are limited in throughput, and have poor reproducibility. Robotic liquid-handling stations can overcome the throughput and reproducibility limitations, however they are very expensive, hard to maintain, and consume the same amount of reagents as manual experiments (Table 1).

Microfluidic systems overcome many of the drawbacks of both manual and robotic systems, as they are capable of high throughput, low reagent consumption, and automation. Droplet-based microfluidics, in which sub-microliters to picoliters of aqueous phase are encapsulated into monodisperse droplets, are especially useful for applications requiring parallel experiments at minimal reagent costs. In addition, droplet-based microfluidic technologies enable high-throughput and controlled processes for cell-free, artificial cells, and genetic circuits applications. Previous review articles have covered the fundamentals of droplet-based microfluidics, droplets-in-flow technologies for biological assays, and flow-based microfluidic methodologies for synthetic biology.[2–4] This review focuses on recent advances in droplet-

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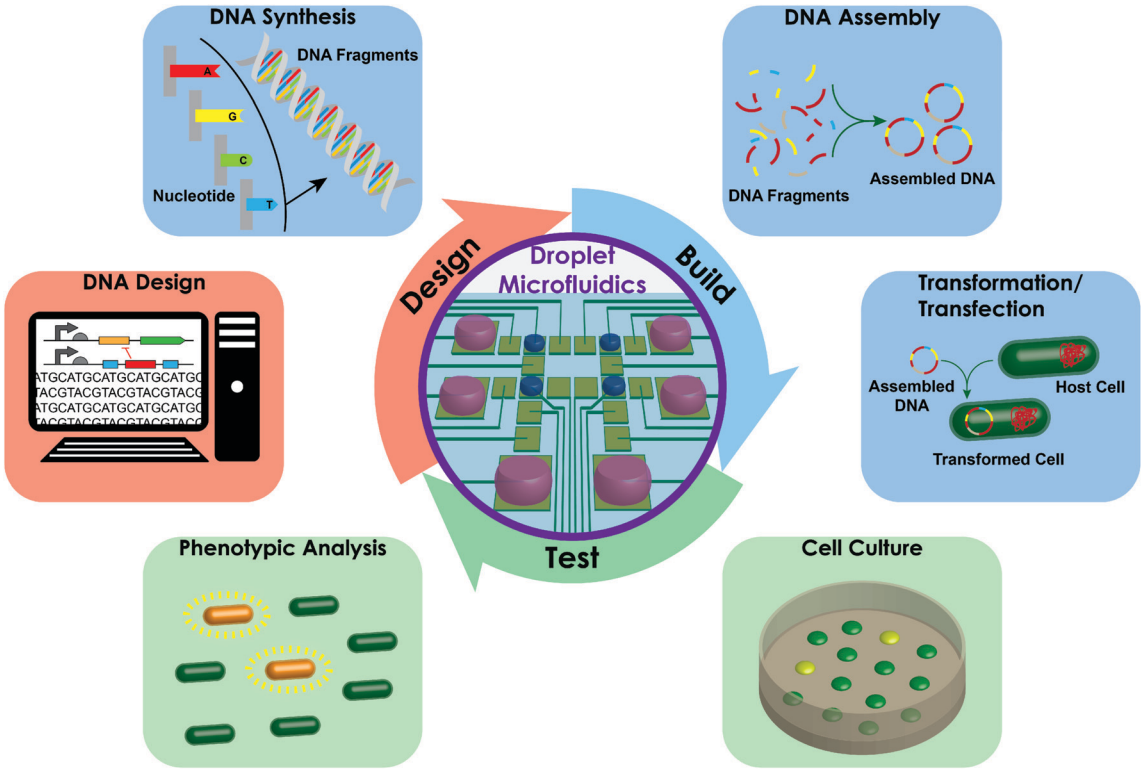

Fig. 1 Molecular biology and analytical steps involved in synthetic biology using droplet microfluidic systems. Biological design/build/test engineering cycles include key steps such as DNA synthesis, DNA assembly, DNA transformation, cell culture, and phenotypic analysis, which often require costly and labor-intensive manual processes. Microfluidic systems have the potential to overcome such drawbacks, through enabling high-throughput automated processing with low reagent consumption requirements.

Table 1 Comparison of conventional and microfluidic approaches to synthetic biology

Synthetic biology steps	Conventional high throughput method	Droplet microfluidics approach
Metabolic pathway design	- Biological computer-aided design and manufacture tools (bioCAD/CAM)[121] - Sequence and design repositories	- N/A
de novo oligonucleotide synthesis	- Controlled pore glass (CPG) column - Microarray with printing technology[122]	- Not available
Gene assembly	- Sequential assembly protocol in an automated robotic system[123] - Microarray with micro-wells[22,124,125]	- Digital microfluidics (MDF)-based multi-step droplet merger[10,11,55] - Droplet-in-flow merger
Transformation	- Microtiter plate heat shock and electroporation - Automated transformation plating	- DMF-based electroporation and heat shock[49] - Droplet-in-flow-based electroporation and heat shock
Outgrowth/culture	- Microtiter plate incubation in robotic systems	- Off-device surfactant-stabilized droplet incubation - On-device incubation - Streaking droplets on an agar plate[62]
Colony picking	-Colony picking robots	- Fluorescence-activated droplet sorter (FADS)[3,69] - DMF-based sorter - Passive droplet traps
Phenotypic assay	- Microtiter plate reader - Mass spectrometry (MALDI-MS, LC-MS, *etc.*) - Fluorescence-activated cell sorting (FACS)	- FADS[3,69] - Droplet-in-flow to mass spectrometry[18,80,81] - DMF to mass spectrometry[19]
Cell-free and artificial cell systems and genetic circuits	- Vesicle bioreactors[126] - Manual formation of bilayer - Observation of genetic circuits as a cell population in solution or on plates	- Flow focusing and microfluidic jetting for formation of vesicles[16,92] - Droplet interface bilayers[95] - Droplets on microarray surface[15] - Encapsulation of genetic circuits in droplets[115,119]

based microfluidic systems directed explicitly towards synthetic biology applications, which may become the fastest growing segment of the worldwide synthetic biology market.[5] Fundamental microfluidics advances, and applications to systems biology, sequencing, single-cell analysis, and drug screening, (that merely have the potential for future application to synthetic biology) have been purposefully excluded.

Droplet microfluidic formats

A variety of microfluidic systems, each having inherent advantages and disadvantages, support synthetic biology applications spanning DNA assembly to single-cell phenotyping. Droplet-based microfluidics predominantly fall into two broad droplet formation categories: continuous and digital. Continuous methods employ flow-focusing, in which an outer flow stream, often an oil phase with a droplet-stabilizing surfactant, encapsulates an inner aqueous phase to generate droplets.[6,7] Continuous methods can rapidly generate monodisperse droplets (up to 1–10 kHz), and are especially useful for high-throughput screening applications.[8,9] However, they allow limited control over addition or subtraction of reagents once droplets are formed. In contrast, digital droplet formation methods, while offering lower-throughput, provide on-demand droplet manipulation and control.[10,11] Digital microfluidics frequently use electrowetting-on-dielectric (EWOD) in which a voltage applied to an electrode pad (lowering the contact angle of the droplet on the hydrophobic surface and macroscopically converting the surface to hydrophilic) provides a droplet-driving force.[12,13] Droplets can also be manipulated using dielectrophoretic (DEP) forces using an array of electrodes.[14] EWOD or DEP systems, often referred to as digital microfluidics (DMF), can dispense, transport, merge, and split each discrete droplet in an on-demand and programmable manner. Droplets may also be formed on a patterned substrate surface, providing easy compartmentalization of biological molecules and reagents without complex instrumentation.[15] An additional technique, microfluidic jetting, provides the compartmentalization of biomolecules in a phospholipid membrane, and is used in artificial cell applications.[16]

Selection of surfactants

Surfactants are an essential component of droplet microfluidics, as they stabilize the droplet interface. The choice of surfactant also impacts many other aspects including molecular biology reactions, cell culture, and functional assays (*e.g.*, mass spectrometry).[17] A good surfactant should also minimize leakage or non-specific adsorption of analytes in a droplet including nucleotides, enzymes, metabolites, and cells. Baret provides an excellent review of this topic.[17] The choice of surfactant is guided mainly by the interfacial chemistry between the hydrophobic (oil, air) and hydrophilic (water) phases. For mineral oil, silicone oil, or air based droplet systems, non-ionic detergents such as polyethylene glycol ester (*e.g.*, Triton X-100) and sorbitol ester (*e.g.*, SPAN 80) are widely used. The drawback of the hydrocarbon-based oil systems is that small organic molecules are soluble in them, and hence, can be lost from the droplet interior. Most droplet microfluidics rely on fluorocarbon oils and fluorocarbon surfactants, as these are biocompatible and have low solubility for hydrophobic molecules. Fluorocarbon oils also have a high gas solubility, required to support cell culture. While fluorocarbon surfactants have proven effective for molecular biology steps and cell culture, they are not compatible with mass spectrometry (for example, the otherwise preferred surfactant PicoSurf1 is incompatible with mass spectrometry).[18,19] For mass spectrometry applications, nanoparticle-based surfactants could be a potential option.[20] For artificial cell applications, phospholipid is typically used instead, in an organic phase such as chloroform, hexane, and octanol.[21]

DNA construction in droplets

DNA synthesis (*de novo* as well as oligo assembly)

For complex pathway engineering efforts, multiple iterations of the biological design/build/test (DBT) engineering cycle are typically required (Fig. 1). The past decade has seen incredible advances in the synthesis of DNA. Companies have used microarray technologies to bring down the cost of DNA synthesis, through paralyzing reaction chambers and reducing reagent volumes.[22] Microfluidics offers an alternative to microarray DNA synthesis, and has advantages in that each reaction chamber can be individually controlled and potentially integrated with other capabilities.[23] Much of the effort towards DNA synthesis miniaturization have employed channel-based devices, because these technologies allow several rounds of reagent changes and rinses.[23–26] Droplet microfluidic systems have not been used for *de novo* synthesis of oligonucleotides to date.

The use of droplets could significantly lower the reagent use for DNA synthesis. The DMF platform used by Yehezkel *et al.* was successfully applied to the assembly of double-stranded DNA, starting with 160 bp single-stranded oligos that are taken through a series of additions in 300 nL droplets.[10] Beyond oligo assembly, such a system, if scaled using a solid-phase substrate, could potentially implement the consecutive rounds of reaction and washing steps required for standard phosphoramidite-based DNA synthesis.[27] Ongoing efforts are needed to further increase the number of sample preparation steps that can be performed on-chip to translate these technologies into fully integrated systems.

DNA assembly (double-stranded)

The first step in engineering a multi-gene pathway is assembling of genes and other genetic elements such as promoters. Limited sizes of DNA fragments practically achievable with current DNA synthesis techniques necessitates additional steps to further assemble these fragments into larger constructs. Modern DNA assembly methods (*e.g.*, Gibson,[28] Golden-Gate[29]), which do not require sequential reagent additions or washing steps, have greatly enabled on-chip DNA construction. The high cost of DNA sample preparation *via* benchtop methods, using large reagent volumes and several pipette tips, can make rational generation of large

combinatorial libraries cost prohibitive.[30] Microfluidics offers a method for systematically selecting the gene fragments and assembly reagents to mix for quickly producing these combinatorial libraries in discrete droplets.[31] A single-layer pincher valving system was developed by Ochs and coworkers to precisely regulate fluid flow in microchannels (Fig. 2a).[32] These valves were used to control the mixing of four different DNA fragments prior to droplet formation. The DNA fragments were encapsulated along with Golden Gate assembly reagents and collected into separate PCR tubes for assembly. Another system utilized on-demand droplet dispensing valves to generate 1 nL droplets containing one of eight different DNA fragments.[33] At this point, droplet pairs were dispensed into separate wells of a microtiter plate where droplets were merged by centrifugation and assembled *via* Gibson assembly. This system was able to successfully produce a 16-plex combinatorial library verified by PCR. The pico-injection technique, which uses electrical fields to temporarily de-stabilize droplets to allow injection of reagents, can be also used to generate combinatorial library, or multi-step reactions on a chip.[34,35] These systems using PDMS valves can quickly create combinatorial libraries in <1 nL droplets and offer great potential for scalability. However, these microfluidic systems were primarily used for reagent segmentation necessitating droplet collection into PCR tubes or microtiter wells for downstream sample processing, such as thermocycling to complete gene assembly. The closest current commercial products come to these results come from acoustic dispensing technologies.

Acoustic printers employ sound waves to eject droplets from a source microplate and dispense onto a collection substrate. A Labcyte Echo 550 acoustic dispenser was recently utilized to mix reagents for performing Gibson and Golden-Gate assembly reactions in 50 nL reaction volumes.[36] This technology has several benefits over traditional robotic liquid handlers including excellent throughputs, versatility, small reaction volumes and non-contact transfer. However, this open-air system can be plagued by evaporation at small volumes and does not offer integration afforded by microfluidic technologies. The use of acoustic dispensing for reagent addition or removal from these microfluidic devices could be an exciting advance for improving both throughput and versatility.

Several systems include additional functionality beyond DNA assembly, using DMF to integrate all of the fluidic steps necessary to generate, mix, and transport droplets in one device. A complete DMF system was capable of de-novo assembly of 160 bp parts in droplets to construct a YFP reporter library (Fig. 2b).[10] The oligonucleotides were assembled using a programmable order polymerization (POP) assembly process in which DNA fragments are iteratively added to elongate an initial DNA template. This group adapted the PCB-based DMF platform developed by Advanced Liquid Logic (later acquired by Illumina, Inc.) to perform these operations. This technology still necessitated DNA removal from the device for sequencing, plasmid construction, and analysis. The chip was also applied to the combinatorial assembly of a variant library of the Azurine gene.[10] This system successfully completed 24 assembly reactions requiring 50-fold less reagents and 10 times less time than conventional manual library preparation. The same device could also be used for performing single-molecule PCR to allow for cell-free DNA cloning. The Illumina device incorporated heater bars below the chip to provide the different thermal zones necessary for *de novo* DNA construction and PCR. Another system was described that combined continuous and digital microfluidics to implement DNA assembly and subsequent step of transformation. The hybrid DMF/droplet system allowed generation of 16 plasmid combinatorial libraries.[11] This platform employed DMF to dispense and mix discrete 200 nL droplets containing different DNA fragments and ligase reagents. Droplets were then transferred to a PDMS valve controlled region for assembly and electroporation. The device was compatible with a variety of commonly used assembly methods including Golden Gate, Gibson, and yeast assembly.

The successful construction and delivery of DNA is reliant on the quality of input DNA fragments, typically necessitating purification prior to assembly. DNA cleanup is a tedious operation currently performed off droplet microfluidic chips. Integration of these procedures on-chip would improve the utility of these bioprocessing devices. Along with in-line strategies for validating successful DNA sequences, microfluidics could become a valuable research tool for rationally designing and screening gene variants.

Transformation/transfection of cells

The transformation of exogenous DNA into cells is a critical step in synthetic biology experiments. After construction of DNA libraries, screening these libraries for finding constructs with the desired activities remains a major scale-limiting bottleneck, both in terms of cost and time. Several strategies are available for delivering genes into cells including electroporation,[37,38] heat-shock,[39–41] microinjection,[42] cellular constraining,[43] sonoporation,[44] nanoparticles,[45] and viral transduction.[46,47] All of these have been adapted to a microfluidic format,[48] and a smaller subset to droplet microfluidics.[37–39] The key parameters to determine success of this step in a microfluidic format are: yield, ease of implementation, ease of integration, and reproducibility. Heat shock is the easiest to implement in any microfluidic format, because the heating/cooling elements can be off-chip. Electroporation and heat-shock are the most common strategies for gene delivery into bacteria, and work by increasing the permeability of a cells membrane to allow DNA uptake. Implementing these transformation methods in microfluidic devices result in transformation efficiencies equal to or better than their bulk counterparts at much lower reagent volumes.[41,49,50] Kwon *et al.* used a microarray spotter for dispensing 60 nL droplets containing THLE-2 human liver cells encapsulated in a hydrogel array onto a 532 element micropillar array. The micropillars were then mated with a complementary microwell array where various metabolizing-enzyme genes where delivered *via* recombinant adenoviruses.[51] This

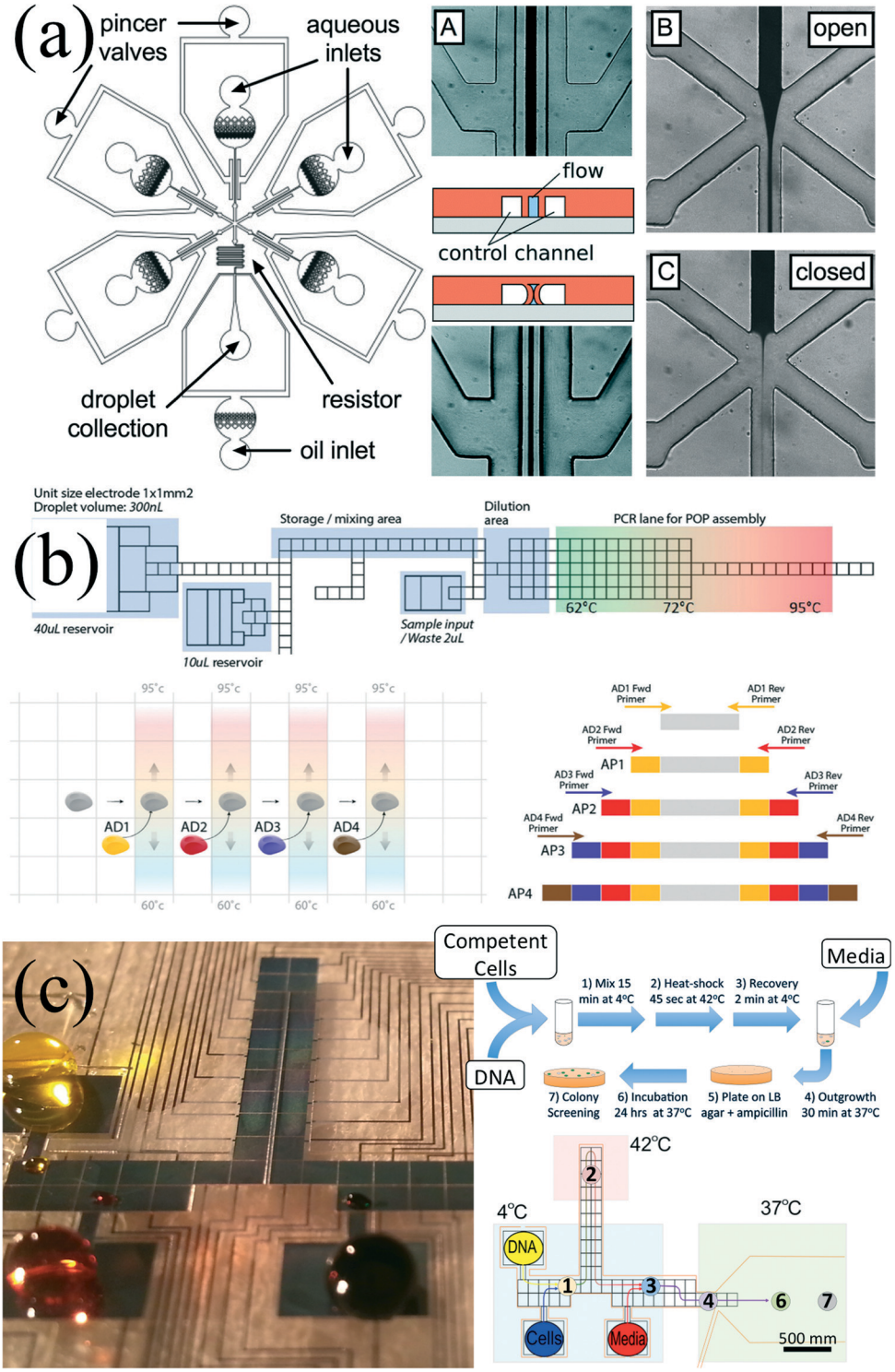

Fig. 2 Examples of microfluidic devices for synthetic biology applications. (a) Microfluidic valving system for combinatorial DNA mixing. A pincer valve allows modulation of the flow rates of 5 aqueous solutions (containing various DNA fragments). This allows different concentrations of DNA fragments to be encapsulated in discrete droplets. Adapted from ref. 32 with permission from the royal society of chemistry. (b) Schematic representation of a single lane (out of eight total) for a digital microfluidic chip that performs DNA assembly and single-molecule amplification (top). Process flow diagram for construction of a yfp reporter gene (bottom). The device iteratively adds two DNA pairs and assembly reagents included in assembly droplets 1 through 4 (ad1–ad4) to a template DNA (gray). Following addition of each assembly droplet the reaction droplet is thermo-cycled to produce assembly products 1 through 4 (ap1–ap4) adapted from ref. 10. (c) A digital microfluidic system that optimizes chemical-heat shock conditions for gene delivery (left). A schematic of the device (bottom-right) shows system mixing of droplets containing DNA (yellow), cells (blue) and media (red) and transportation across different temperature zones (colored squares). Device performs the steps of bench-top heat-shock (top-right) as represented by each circle labeled 1 through 7. Reprinted with permission from P. C. Gach, S. C. C. Shih, J. Sustarich, J. D. Keasling, N. J. Hillson, P. D. Adams and A. K. Singh, *ACS Synth. Biol.*, 2016, **5**, 426–433. Copyright 2016 American Chemical Society.

system was used to screen 84 combinations of these CYP450 enzymes for their effects on drug metabolism. Alternatively, a droplet-arraying chip was created by selectively functionalizing a glass slide with a super-hydrophobic polymer layer to create 500 μm microwells.[52] Depositing cell suspensions or buffer onto the chip following liquid aspiration results in discrete 24 nL droplets in the microwells which can then be covered by an electrode array to perform electroporation. This platform was used to successfully transfect HeLa cells with plasmids containing genes for green or red fluorescent proteins and 293T cells with sfRNAs by the CRISPR/Cas9 system. Along with allowing for the generation of 16 plasmids, the hybrid DMF/PDMS valve system developed by Shih *et al.* afforded in-line electroporation following DNA assembly.[11] This technology utilized the electrodes used for DMF for providing the DC pulses for electroporation in the channel-based region of the device. At this point, droplets were manually removed from the device and cultured off-chip. The DMF electrodes utilized for moving droplets cannot also be employed for electroporation, due to the presence of the dielectric layer. Madison *et al.* showed that while an electroporation electrode partially covering DMF electrodes slightly reduces transport velocities, droplet movement can still be repeatedly obtained.[53] Further studies with this device demonstrated the successful delivery of *pGERC* plasmid DNA into *E. coli* at efficiencies up to $8.6 \pm 1.0 \times 10^8$ cfu μg^{-1}.[54] Alternatively, Moore and colleagues modified the Illumina digital microfluidic system with similar electroporation electrodes to enable multiple multiplex automation genetic engineering (MAGE) cycles to deliver DNA into *E. coli* cells.[55] In addition to droplet transport and mixing by DMF, magnetic beads were bound to the *E. coli* cells to allow the several washing steps and media transfers required for MAGE. Another technique directly interfaced pin headers with a microfluidic chamber to improve the controllability of and ease of electroporation.[50] A standard micropipette was used to deliver 1 to 2 μL mixtures of DNA and cells. This system successfully delivered genes to microalgae[50] and Jurkat T cells[56] with efficiencies of ~6× and ~10×, respectively, higher than their conventional counterparts. The authors credited these improvements due to the decreased electric current afforded by the smaller reaction volume.

Other digital microfluidic systems have implemented heat-shock to enable DNA delivery.[39,49,57] Heat-shock was performed on a device that enabled the transformation of up to 100 droplets coupled with on-chip culture and fluorescence-based droplet screening (Fig. 2c).[49] On-chip heat-shock was implemented with this system by integrating micro-Peltier thermal modules with the device to provide different hot/cold temperature zones. This system highlights the usefulness of digital microfluidic format for accurately controlling the duration of each heat-shock step to produce optimal transformation efficiency. The combination of DNA assembly, transformation, and screening technologies could allow for the rapid production and characterization of engineered biological libraries of recombinant DNA, proteins, or whole cells. However, droplet microfluidic technology has yet to integrate single-cell encapsulation, culture, and sorting following transformation. The technologies described in the section "phenotypic analysis in droplets" currently perform transformation and library construction off-chip prior to droplet generation and screening. Conversely, a technology like the single-cell printer, which accurately dispenses single cells onto a microtiter or agar plate, would pair well with current microfluidic genetic engineering platforms.[58]

Droplet screening

High throughput colony screening in droplets

In traditional benchtop methods, after transforming exogenous DNA into cells, the cells are incubated and plated on an agar plate supplemented with antibiotics to provide the desired selection pressure. Colorimetric methods such as β-galatosidase-driven blue/white colony screening may be used to screen for desired colonies for further culturing and phenotypic analysis. This process can be highly laborious and time-consuming. Colony picking robots are available with a throughput typically of more than 1000 colonies per hour, but are not cost-accessible for many academic labs. Droplet microfluidics' ability to encapsulate a single cell (effectively a clonal unit) at high frequency (>kHz) and ability to screen and sort in high throughput manner (up to millions of droplets per day), can greatly expedite progress.[59,60]

Single cell encapsulation in passive droplet microfluidics (Fig. 3a (ii)) follows a Poisson distribution. To make it sufficiently unlikely to capture two or more cells in the same droplet, the concentration of the cell suspension going into the droplet generating inlet has to be so low that it typically results in two third of droplets being empty (without a cell). To improve these statistics, various active and more controlled encapsulation methods are being developed.[61] An interesting approach is to use droplet microfluidics and a simple motorized system to streak droplets with a single cell on agar plates (Fig. 3b).[58,62] After incubating the droplets on an agar plate for cell growth, active colonies were effectively screened and recovered by aligning the mask, which was printed based on fluorescence image of the agar plate. This method allowed for better coverage of rare species from soil microbial samples compared to conventional methods.

Fluorescent phenotypic analysis in droplets

An important step in any synthetic biology or genetic engineering cycle is to culture and screen cells for expression of proteins, peptides, or chemicals of interest. It is desirable to measure both the amount and activity of the expressed chemical or protein. Fluorescence is a widely used method, as it is readily integrated with microfluidic platforms. Examples include the measurement of protein amount by expressing a protein with a fluorescent tag (*e.g.*, GFP) and the measurement of enzyme activity by using fluorogenic substrates.[7,49,63] Fluorescent-activated droplet sorting (FADS, Fig. 3a), analogous to fluorescence-activated cell sorting (FACS), has

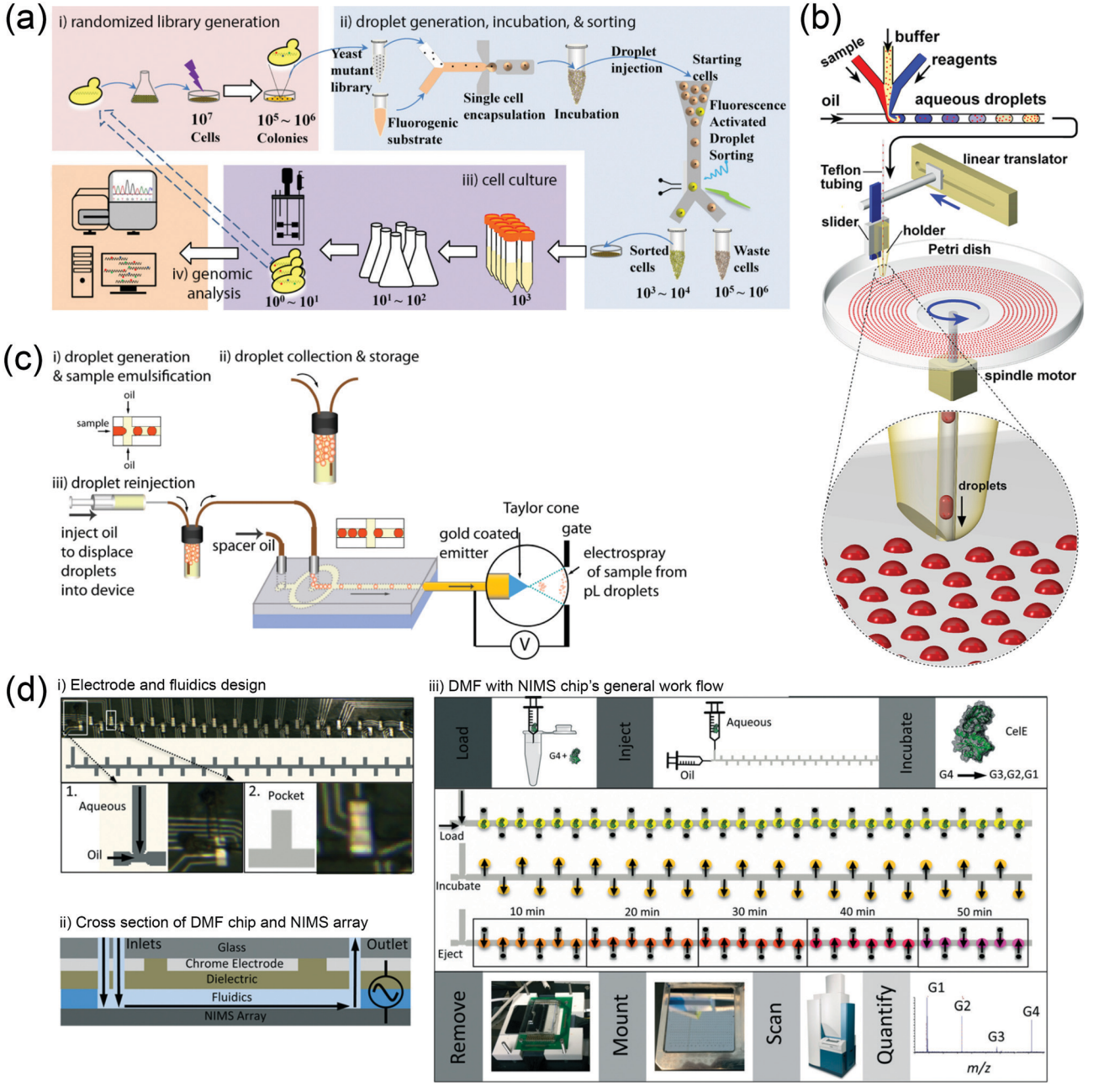

Fig. 3 (a) An example of a droplet fluorescence sorter and its application to directed evolution. A single yeast cell is emulsified following uv-mediated random mutagenesis. Its desired activity is interrogated *via* a fluorescence-based sorting system at very high frequency. Cells with desired activities are further cultured and cycled into another iteration of directed evolution. Reprinted with permission from ref. 6. Copyright 2015 national academy of sciences. (b) Schematic of an automated system for streaking droplets onto an agar plate. Droplets are deposited on a rotating agar plate while the droplet outlet slides on a linear translator to generate spiral droplet arrays. From ref. 62 copyright 2016 american society for microbiology, license number: 4156701161332. (c) An example integration of droplet microfluidics with electrospray ionization mass spectrometry (esi-ms) for high-throughput screening. Droplets are directed to a capillary gold-coated emitter for electrospray ionization for mass spectrometry. Reprinted with permission from C. A. Smith, X. Li, T. H. Mize, T. D. Sharpe, E. I. Graziani, C. Abell and W. T. S. Huck, *Anal. Chem.*, 2013, **85**, 3812–3816. Copyright 2013 American Chemical Society. (d) An example of DMF connecting with mass spectrometry (μnims). Reproduced from ref. 19 with permission from the royal society of chemistry.

recently been used for high-throughput screening.[3] Consistently sized surfactant-stabilized nano-liter droplets (that can trap a single cell) can be incubated for days, with reagents/chemicals added as desired to the droplets at selected time intervals.[64] This allows for accurate microbial performance assessments and subsequent droplet sorting to collect microbes with desirable phenotypes typically at ~1 kHz, and potentially up to 30 kHz.[65] These characteristics give FADS an access to various phenotypic screenings such as screening for cell culture[66] and single cell secretion level of proteins and

metabolites,[6,67,68] that are not accessible to FACS as FACS assay is inherently limited to fluorescence within the cell or on the cell membrane.[69] Huang *et al.* used droplet microfluidics to screen 10^5 to 10^6 of UV-irradiated *S. cerevisiae* variants with desired α-amylase secretion rates (Fig. 3a).[6] The *S. cerevisiae* cell culture was plated on an agar plate and irradiated with 254 nm UV light to increase random mutations. These cells were re-suspended and mixed with α-amylase fluorogenic substrate in a droplet generator, encapsulating a single yeast cell in a droplet. After off-chip incubation, the droplets were screened and sorted using FADS, resulting in 8 clones with high α-amylase secretion rates. These variants were subjected to whole-genome sequencing, and identified total 330 mutations of interest. FADS has been used in similar workflow to optimize enzyme activities,[7,63,70,71] protein secretion,[6,68] metabolite production,[67] as well as to profile single-cell gene expression.[63,72,73]

An alternative to water-in-oil (w/o) droplets, double emulsions of water-in-oil-in-water (w/o/w) droplets can be generated using droplet microfluidics by subjecting the surfactant stabilized w/o droplets into the second droplet generator with aqueous liquid (typically water with detergent such as TWEEN 20 or SDS) as continuous phase.[74] The w/o/w droplets allow high throughput sorting using commercially available FACS machines, and thus increasing its accessibility to non-experts in microfluidics as well as assessing higher throughput sorting capability of FACS machine typically at 10 kHz or above.[63,70] With continual throughput improvement[65] and the ability to barcode droplets,[72,73] FADS, and related fluorogenic strategies (such as oxidase activity assays and Watson-Crick base pairing systems[67,70]), have great potential for application to synthetic biology challenges.

Mass spectrometry phenotypic analysis in droplets

Beyond fluorescence-based methods, label-free (*e.g.*, mass spectrometry) screening is desirable for broader applicability. Integration using automated liquid-handling system or acoustic printing system has increased the throughput significantly.[75,76] However, the sample volume required for each well in a microtiter plate for an acoustic printer is about 10 μL or more. Droplet microfluidics can bypass the microtiter plate sample preparation workflow by encapsulating the analytes in droplets in sub-nanoliter sample volume. There have been many efforts to integrate microfluidics with mass spectrometry.[77–79] A specific example to highlight includes the integration of droplet-based microfluidics with electrospray ionization mass spectrometry (ESI-MS) (Fig. 3b)[18] for functional assessment of enzymatic cocktails (cytochrome C, α-chymotrypsinogen A, carbonic anhydrase, and lysozyme). The surfactant-stabilized droplets including the enzyme of interest and substrates are re-injected into a PDMS-based microfluidic device and spacer oil is used to create additional gap between droplets. Droplets are directed to a capillary gold-coated emitter for electrospray ionization mass spectrometry. Gasilova *et al.* integrated droplet microfluidics with ESI-MS by drilling a spyhole on the top of the device and applying high voltage pulses on the other side of the spyhole to generate eletrospray.[80] This method demonstrated screening of biochemical reactions such as tryptic digestions with high sensitivity at 10 Hz sampling rate without a dilution or oil removal step. Another approach is to integrate droplet microfluidics to matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) by interfacing droplet outlet to a microarray MALDI plate on a motorized *xy*-stage.[81] The optical sensor at the droplet outlet synchronized the *xy*-stage movement to pattern droplet samples onto 26 000 hydrophilic spots on the MALDI plate without cross contamination. This was applied to study enzymatic digests of angiotensin. Heinemann *et al.* recently demonstrated the integration of DMF with nanostructure-initiator mass spectrometry (NIMS) (Fig. 3c).[19] In a hybrid flow-in/DMF device, droplets containing glycoside hydrolase and a tetra-saccharide probe were screened for glycosidic bond hydration kinetics. EWOD was used to direct each droplet to an on-chip incubation position for specified amount of time (Fig. 3b, iii) before subsequent routing to a designated spot on the NIMS pad for analyte deposition. The throughput of the DMF portion of the platform could be increased by scaling up the device (*e.g.*, increased numbers of electrode pads).

Cell-free, artificial cell systems and synthetic genetic circuits

Additional examples using droplet microfluidics for synthetic biology applications include cell-free and artificial cell systems. Beyond bacterial, fungal, and mammalian cell systems, researchers have used *in vitro* cell-free systems in controlled microfluidic environments to investigate biochemical reactions, gene expression, and protein synthesis.[82–86] For example, Kapsner *et al.* recently demonstrated that transcriptional noise level is strongly dependent on the ratio of templates to polymerases, through the investigation of transcriptional circuits compartmentalized in oil-encapsulated microdroplets (Fig. 4A).[83] In addition, Ho *et al.* studied the effect of a poly(vinyl) alcohol surfactant for encapsulating mammalian cell-free expression (CFE) systems in double emulsion templated vesicles, using glass capillary droplet microfluidics.[87]

Fully synthesized artificial cells to mimic/model natural systems would be useful not only for engineering of biological organisms and products but also for reproducible reconstitution studies of biomolecular processes and functional organization in biology without the complicated cellular environment,[88] although the controlled formation of functional artificial cell membranes and encapsulation of biologically active components has proven challenging. While droplet-based microfluidic systems have demonstrated robust and repetitive generation and characterization of artificial lipid bilayer membranes (*e.g.*, vesicles),[89–91] other technical challenges remain, such as stability of the bilayer or the exchange of the materials.[21] Stachowiak *et al.* has developed a

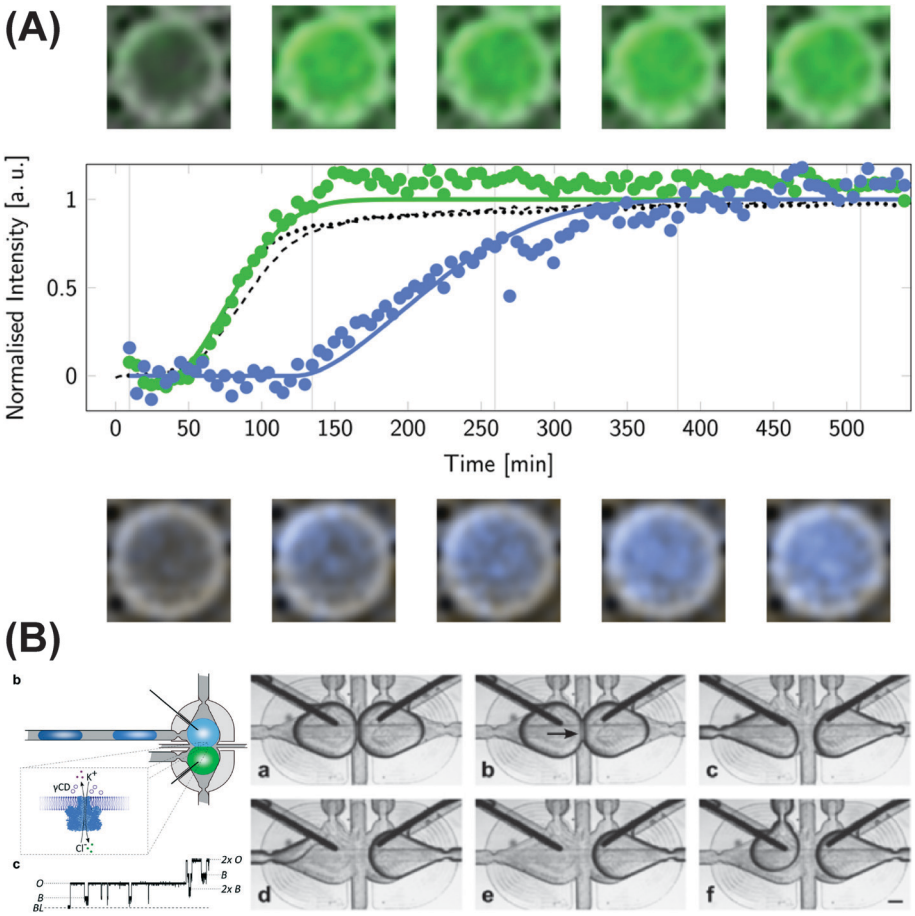

Fig. 4 Droplet-based microfluidics for synthetic biology in cell-free systems and artificial cells. (a) RNA transcriptional reaction circuits in microdroplets. Circuit reactions are measured as a fluorescence increase in droplets (indicated in the graph as green and blue points). Reprinted with permission from K. Kapsner and F. C. Simmel, *ACS Synth. Biol.*, 2015, **4**, 1136–1143. Copyright 2015 American Chemical Society. (b) Droplet-based measurements of artificial cell membrane functionality. Lipid bilayers are formed between two trapped water-in-oil droplets. Incorporation of a single pore of α-hemolysin into a lipid bilayer results in a step increase of 50 pa in current, and the presence of an inhibitor, γ-cyclodextrin, results in the decrease of the current by approximately 60%. Microdroplets are exchanged as shown in the series of micrographs (scale bar = 200 μm). Adapted from ref. 95 with permission from the Royal Society of Chemistry.

method to form giant unilamellar vesicles by microfluidic jetting.[16] Do Nascimento *et al.* have fabricated Pluronic vesicles with controlled permeability utilizing multi-layered microfluidic flow-focusing devices.[92] Ho *et al.* have used a deformable membrane to compress microfluidic double emulsion droplets to alter oil thickness towards mechanosensitive artificial cells.[93] For more robust control and study of membrane transport than multi-phase emulsions[94] can provide, microfluidic platforms have been developed to form lipid bilayers repeatedly between two lipid monolayer coated droplets,[95,96] membrane proteins have been characterized through the use of a droplet microarray on a static surface,[15] and fast and sensitive measurements (monitoring fluorescence[83] or electrical current[95]) (Fig. 4B) of membrane transport have been enabled by microscale compartmentalized lipid bilayers.[97] To fully utilize the artificial cell technology, it would also be important to develop robust and high-throughput methodologies for manipulate and assemble a population of artificial cells in a controlled manner.[98–100]

The ultimate goal of synthetic biology is to have the capacity and capability to easily design and build any desired biological system.[101–103] Microfluidic methodologies have been often adapted to test and characterize genetic circuits such as oscillators with controlled environments.[104–112] Encapsulation of biochemical circuitry for characterization of synthetic circuits is important for the development of sophisticated and programmable artificial biomimetic systems,[113,114] and droplet microfluidic technology enables uniform and high-throughput production of micro compartments.[115–117] Sugiura *et al.* have produced microfluidic open-reactor system towards dynamic control over artificial biomimetic systems far from equilibrium in chemical and biomedical studies.[118] Towards artificial multi-cellular hybrid systems, Schwarz-Schilling *et al.* have demonstrated gene expression of genetic circuits (AND gate and sender circuits) in linear chains of microdroplets containing either bacteria or cell-free gene expression systems, and studied communication between bacteria and artificial cellular compartments.[119] With

these recent development and adaptation of microfluidic techniques shown above, rapid growth of the fields of artificial cells and genetic circuits is expected for synthetic biology applications such as therapeutic detections and bacterial drug delivery.[110,120]

Future outlook

As the field of synthetic biology grows, so does the need for high throughput experiment and screening platforms. Metabolic pathway optimization is typically performed with a trial and error approach. Maximizing titer, rate, and yield can require the evaluation of thousands of gene combinations. Consequently, faster and more integrated microfluidic systems will be required to enable the targeted fabrication and screening of hundreds of thousands of specific DNA constructs, which at present remains inaccessible with current low-throughput on-demand fluidic manipulation technologies (required for combinatorial parts mixing) and the current maximum numbers of valves or electrodes per device (constraining the total number of steps that a single device can perform). As another example, while microfluidic devices can precisely control individual cells, affording real-time analysis of cell–cell interactions and cellular function and heterogeneity (difficult or impossible to obtain with bulk/population studies in culture tubes or microtiter plates), further work (*e.g.*, altered material biocompatibilities and gaseous exchange rates) towards accurately scaling-down cell culture to microfluidic devices will be required to better mimic/model/predict cellular behavior (*e.g.*, growth rates, nutrient consumption, target molecule production) at relevant bioreactor volume/scales.

Conflicts of interest

There are no conflicts to declare.

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