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Review

Digital microfluidics as an emerging tool for bacterial protocols

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ABSTRACT

Bacteria are widely studied in various research areas, including synthetic biology, sequencing and diagnostic testing. Protocols involving bacteria are often multistep, cumbersome and require access to a long list of instruments to perform experiments. In order to streamline these processes, the fluid handling technique digital microfluidics (DMF) has provided a miniaturized platform to perform various steps of bacterial protocols from sample preparation to analysis. DMF devices can be paired/interfaced with instrumentation such as microscopes, plate readers, and incubators, demonstrating their versatility with existing research tools. Alternatively, DMF instruments can be integrated into all-in-one packages with on-chip magnetic separation for sample preparation, heating/cooling modules to perform assay steps and cameras for absorbance and/or fluorescence measurements. This perspective outlines the beneficial features DMF offers to bacterial protocols, highlights limitations of current work and proposes future directions for this tool's expansion in the field.

Introduction

Digital microfluidics (DMF) is a fluid handling technique that utilizes electrostatic forces to manipulate nano- to microliter liquid droplets along electrodes insulated with a dielectric layer [1–3]. Unlike most conventional microchannel-based microfluidic systems, DMF does not require valves, pumps or mixers for fluid handling. DMF devices come in two primary formats; i) the one-plate format in which droplets are manipulated on an open surface (Fig. 1A) and ii) the two-plate format in which droplets are sandwiched between two plates (Fig. 1B). In both formats, the bottom plate comprises a substrate and a patterned conductive layer that is used to form an array of driving electrodes. Electrodes are generally insulated with a dielectric layer, and hence the term electrowetting on dielectric (EWOD) is often used to describe this kind of device. The dielectric layer enables field gradient or charge buildup for droplet actuation [1]. In the one-plate format, the droplet is placed and actuated on top of the electrode array; to complete the circuit a grounded wire or wires are used. Several geometries and setups have been employed, a general example is shown in Fig. 1A. Albeit simple, the one-plate format allows for direct access to the sample making sensor integration trivial. Meanwhile in the two-plate format (Fig. 1B), a second substrate is used (top plate) that restricts the droplets in the z-axis, squeezing them to a pancake shape. In addition, the top plate is often coated with a continuous conductive layer that serves as the ground plane to complete the circuit. Transparent materials are often selected for top plates in order to optically visualize droplets. While the two-plate format is more complex, it comes with several advantages: (i) the ability to split and dispense droplets, (ii) limiting evaporation of the loaded liquids, (iii) minimizing exposure of the user to the sample and sample contamination, (iv) allowing the use of several oil-based filler media without the risk of leaking. These characteristics make the two-plate format ideal for the applications discussed in this review. In most modern DMF devices, a hydrophobic coating is used as well, which allows for droplet movement with limited resistance. Liquids, surrounded by air or a water immiscible medium (oil), are electromechanically manipulated by applying electrical potential between the ground plane and at least one driving electrode [4]. Sequential activation of electrodes in a specific order can allow for dispensing, combining, mixing and/or splitting of droplets (Fig. 1C). This powerful tool can perform automated multistep liquid handling procedures to achieve various analytical techniques including dilutions [5], precipitation [6] and magnetic separations [7], with limited user input.

Over the last 15 years, there has been growing interest in the use of DMF to perform bacteria-related protocols from applications of synthetic biology to diagnostic testing. What makes DMF appealing for these types

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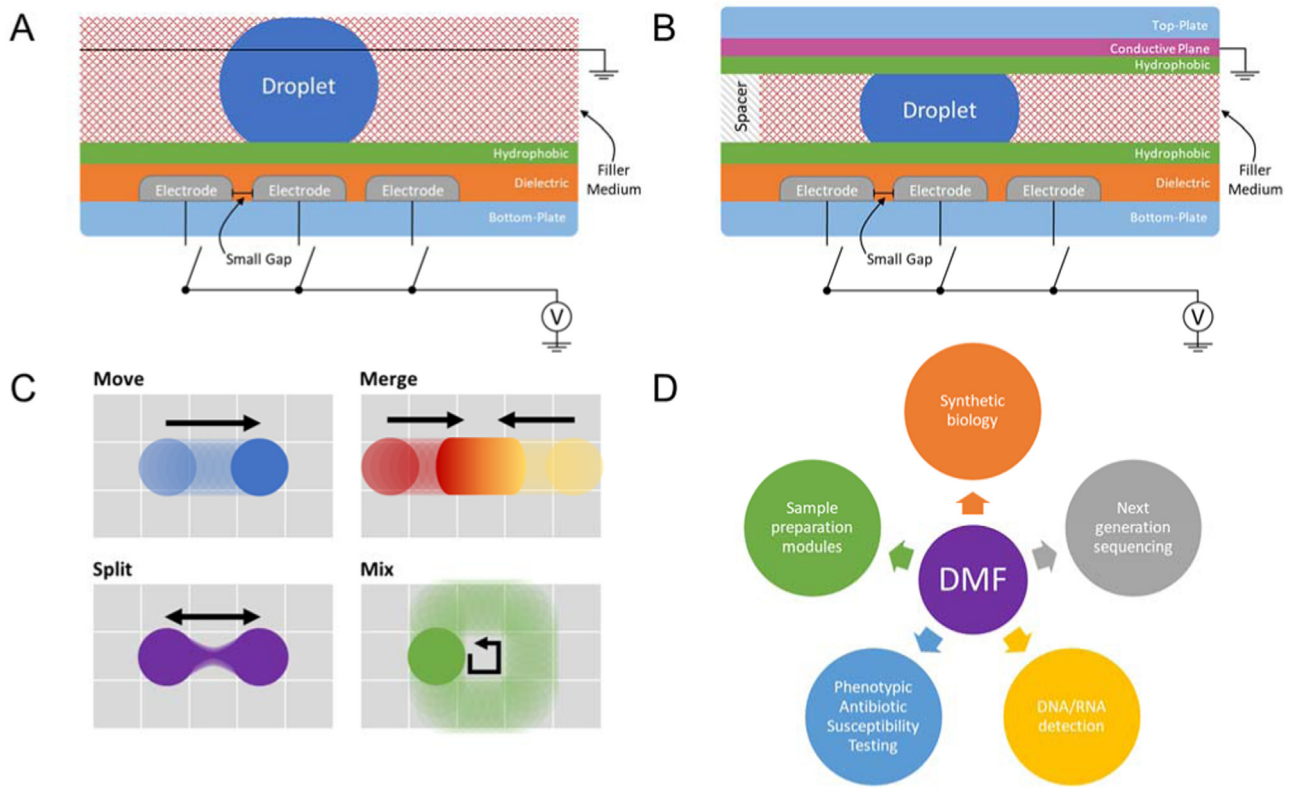

Fig. 1. (A) Side-view of a one-plate DMF device. (B) Side-view of a two-plate DMF device. (C) Top-view of DMF device demonstrating the different droplet manipulation that can be achieved on chip. Arrows represent the direction of droplet movement. (D) Research fields that have utilized DMF discussed in this perspective.

of protocols is the small volume requirements, which limits reagent consumption and costs. Automation of protocol steps is another advantage of DMF that helps minimize user input and streamline protocols that include multiple pipetting and mixing steps. By minimizing user input the inherit error that accompanies it is minimized as well. In addition, the most common device setup, the two-plate format, offers a layer of protection for the sample and the reagents, and by extent reduces the risk for contamination. DMF can also be interfaced or integrated with various analytical instruments for absorbance and fluorescence measurements [8,9], electrochemistry [10,11], mass spectrometry [12] and nuclear magnetic resonance [13] enabling applications including cell culture [8], quantitative assays [11,12], chemical analysis [13] and diagnostic testing [10]. Most importantly, unlike microchannel-based microfluidic systems, a variety of processes can be achieved on a single DMF device, without the need for redesign, demonstrating DMF's versatility compared to conventional microfluidic platforms.

Practically, DMF platforms have demonstrated limitations in performing protocol steps beyond dispensing, combining, mixing, and splitting of droplets. For example, many protocols require sample preprocessing before transferring solutions on chip, such as cell lysis and centrifugation. To help mitigate sample handling steps, some DMF protocols have been integrated with microchannel-based microfluidic systems, generating hybrid devices [14], as well as capillary interfaces [15,16], to utilize various sample preparation modules and expand the capabilities of the system to perform complex protocols.

An important finding in expanding DMF as a tool for bacterial protocols is the demonstrated device biocompatibility [17], allowing for bacterial culture protocols over extended periods of time on chip [8]. Early iterations of bacterial growth protocols required the periodic transfer of DMF devices into and out of incubators, which is impractical and inefficient due to the need of additional instrumentation and manual interventions. According to more recent reports, it appears that the field is shifting toward instruments with on-chip temperature control [18]. By incorporating heating and cooling modules directly on the DMF instrument, optimal growth conditions or temperature-sensitive protocol steps can be achieved without having to relocate the DMF device.

Finally, DMF has been paired with various sensors and detectors to monitor protocols over time and obtain results. The small size of DMF devices provides compatibility with instruments such as microscopes or plate readers, which often have rather limiting specifications, eliminating the need for custom holders or special alterations to the hardware. Furthermore, the flexibility provided by the digital nature of DMF allows for chip layouts to be repurposed without the need for redesign. This reduces the overall time spent on device modifications since adjustments can simply be implemented through simple software changes. Although this facile transfer of devices to various readout tools is a useful feature, there is the drawback of additional instrumentation requirements and manual intervention, similar to the drawback indicated above for incubators. The literature is moving toward integration of DMF devices with built-in modules to obtain measurements such as fluorescence, absorbance, etc. all in one package.

In this perspective, we review the recent DMF literature for applications to the modification, culture and analysis of bacteria (Fig. 1D), particularly in areas of synthetic biology (ligation, transformations and inductions), diagnostics (nucleic acid detection and antibiotic susceptibility testing) and sample preparation for downstream bacterial protocols (next generation sequencing and single bacterium capture/selective retrieval), highlighting the progress in the field and areas where there are needs for innovation in the future.

Synthetic biology

There has for many years been great enthusiasm for the translation of traditional synthetic biology (SynBio) protocols to DMF. There are two main approaches to SynBio, a bottom-up approach that uses a combination of chemical and biological building blocks to create biomimetic

systems, and a top-down approach that uses metabolic and genetic engineering tools to add new functions to living cells. We are aware of only a single report of a bottom-up SynBio protocol that used DMF to perform cell-free biology by, Liu et al. [19], while in contrast there are numerous reports that have described top-down SynBio approaches using DMF to manipulate solutions containing cells (*i.e.*, bacteria and yeast) in order to produce a protein of interest. The cloning technique is among the most used tools that allow scientists to study the functions of genes. Molecular cloning methods have been performed using DMF and today reports have included Golden Gate [20] and Gibson [21] assemblies. The DMF community seems to have focused on three SynBio protocols, which include i. ligations, ii. transformations and iii. inductions; in the following subsections we review DMF implementations of these procedures.

Ligation

To our knowledge, there are only two DMF synthetic biology publications to date that focus on ligation using bacterial systems. In 2008, Liu et al. [22] reported the first effort to perform DNA ligation using DMF. The DMF device was built with coplanar electrodes made of optically transparent indium tin oxide (ITO); in other words, the bottom plate contained both the activation and ground electrodes all on the same plane. The chip was used to combine sub-microliter unit droplet volumes containing either the target DNA or the vector DNA with a mixture containing the ligase and ligation buffer. The reaction mixture was incubated at room temperature on chip, before being removed from the DMF device. The solution was then heated to deactivate the ligase and was used to transform chemically competent cells that were cultured off chip.

A couple of years later the same group reported a similar setup and ligation protocol that was multiplexed (Fig. 2A) [23]. The DMF device was modified to allow for multiple ligations to occur using the same vector DNA and a variety of 4 insert DNA samples (Fig. 2B). In addition, the authors added 0.2 μL of silicone oil (octamethyltrisiloxane) for every 5 μL of aqueous solution in order to form a thin oil-shell around each droplet. The oil-shell reduced the evaporation of the aqueous droplet while it improved the movement of the different solutions used for the ligation on the DMF device. Lastly the efficiency of the DNA ligation was optimized by varying the mixing sequences, as well as the waiting time between the two mixing steps.

Transformation

Transformations are the processes that often follow the *in vitro* assembly of genetic material to alter the genes of a bacterium by inserting an exogenous sequence through its cell membrane. The primary method for insertion of new DNA is through heat shock, which weakens the cell membrane allowing the DNA to enter the cell. An alternative method is electroporation, which uses an electrical pulse to generate transient holes in the cell membrane allowing DNA to pass into the cell; after the pulse, the cell's membrane-repair mechanisms close the generated holes. Another requirement for transformation is having cells in a state of competence, a condition that makes the cell transiently permeable to DNA. Competence may occur naturally for short periods of time under certain conditions such as starvation or induced in a laboratory.

The first of multiple reports demonstrating the use of DMF (Table 1) to perform bacterial transformations was from Au et al. [8], using a custom DMF device dubbed as the BAY (Bacteria, Algae, Yeast) microbioreactor. The main purpose of the device was to allow for culture of a variety of microorganisms, including *E. coli,* in droplets of a few microliters. DMF was used to transport and mix droplets (every 2.5 min), while the temperature was set at 37 °C using a hotplate. As mentioned by the authors, the use of the hotplate resulted in less precise control of the temperature (compared to an incubator), but that did not seem to have a substantial impact on the reported growth profiles of the bacteria. The device also included a window that allowed the user to monitor the optical density (OD) of the culture and thus ensure bacteria were in the log phase. Hence, the BAY device was transferred to plate reader every hour to record the OD of the droplet. Once the culture reached the log phase, a *YFP* reporter gene was transformed using the heat shock method, whereby the BAY device was moved manually between a hot plate (42 °C) and an ice bath (0 °C) with approximately one-minute intervals.

Soon after the BAY paper was published, Madison et al. [24] reported a more integrated system that was based on a commercially available DMF instrument from Advanced Liquid Logic Inc. (acq. by Illumina Inc.), which was already equipped with heaters and magnets (used for magnetic separations). The DMF platform was used to perform Software Automated Genomic Engineering (SAGE) protocols that enabled genetic modification of cells using DMF by implementing the multiplex automated genomic engineering (MAGE, Fig. 3A) protocol, which was previously developed by the same group [25]. With the reported platform, *E. coli* were transformed with *GalK* recovery oligonucleotides using electroporation via dedicated custom on-board gold electrodes. The efficiency of the electrotransfer of the oligos was assessed by monitoring the OD of the sample using a fiber-optic-based mini spectrophotometer in reflectance mode. The reflectance spectroscopy strategy was straightforward to use and proved to be an important addition to the MAGE methodology. On the other hand, the DMF device had to be filled with silicone oil, which could limit the aerobic growth of the culture.

Several years later the same group reported an improvement on the electroporation method using a simple DMF device with only 22 electrodes [28], as well as an upgraded version of the DMF device and layout that included electroporation electrodes and heat shock regions for the complete automation of the MAGE protocol (Fig. 3B,C) [26]. Another key advancement found in the latter report was the addition of streptavidin-coated magnetic particles that were used to bind *E. coli* using biotinylated lectins. This process was crucial since it allowed for efficient isolation of the bacteria on the DMF device, which enabled the successful exchange of culture media on device as well.

A fourth report of using electroporation to transform bacteria was contributed by Shih et al. [29] Unlike the previous device architectures, these authors reported a unique microfluidic device that combined DMF with conventional microfluidic channels (often referred to as a hybrid device format). In addition, unlike all the reports discussed above, this was the first time DNA assembly (Golden Gate or Gibson) and transformation were reported using a single DMF setup. It should be noted that in this device, DMF was only responsible for dispensing and mixing the initial solutions, while queuing (up to 16 droplets) and incubation of the assemblies was performed inside a serpentine microchannel. At the end of the channel a cell inlet was included to allow the user to introduce and mix the assembled DNA with a bacterial culture while a set of electrodes located right after it was used to electrotransform the DNA into the cells. Last the cells could be retrieved and plated for colony growth.

Almost in parallel, Gach, et al. [27] reported a similar hybrid device that was used for DNA assembly (Golden Gate), transformation, cell culture and protein expression. Unlike the previous report, the authors chose heat shock to perform the transformation, which was also implemented on the DMF portion of the hybrid device (Fig. 3D). By partitioning the chip into three regions and using three different thermoelectric systems, the authors were able to maintain different temperature conditions in each region (Fig. 3E). Using DMF, droplets were driven back-and-forth between the cool (4 °C) and warm (42 °C) regions to achieve transformation of the assembled DNA, while the incubation of the transformed bacteria was done inside the microfluidic channel that was held at 37 °C. The embedded microchannel also allowed an improvement in throughput, as well as replenishing oxygen during long-term culture (1 to 5 days).

While the system described by Gach, et al. [27] allowed for fast temperature transitions (required in heat shock transformation), it also required three thermoelectric (TEC) units, which were not as efficient and required more real estate underneath the DMF device. To overcome this

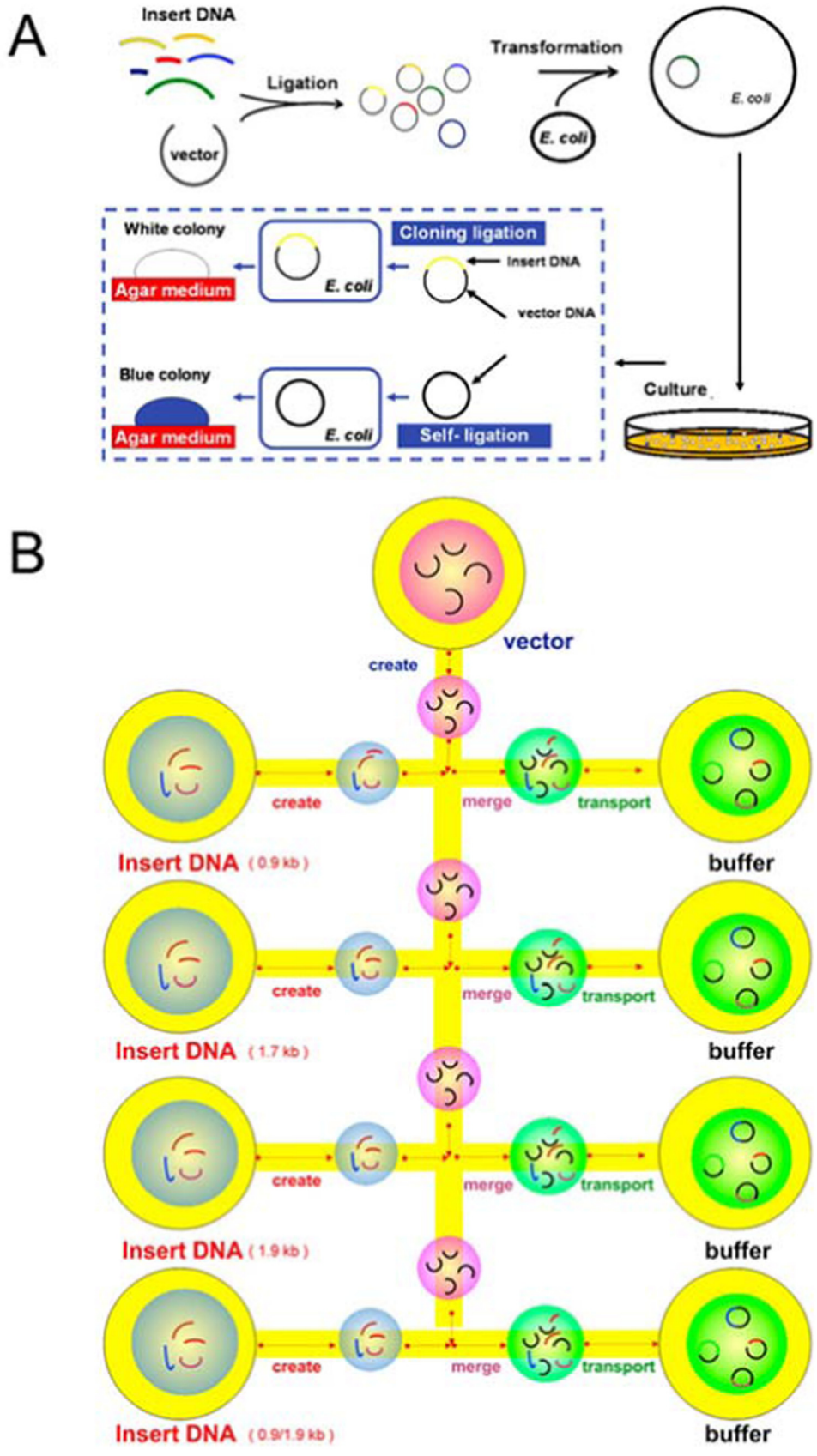

Fig. 2. (A) Schematic of DNA ligation and cloning. During the ligation step, insert DNA is combined with a vector DNA to form a cloning ligation. The circular DNA is then transformed into competent cells. (B) Cartoon image of a DMF device used to perform a ligation protocol. Reproduced from Lin et al. [23] with permission from SLAS.

Table 1
Summary of E. coli transformation systems using DMF. N.S. refers to information that is not specified.

Technology	Key Innovation	DNA construct	Transformation type (heat shock, electroporation)	Droplet volume	Transformation efficiency monitoring	Peak efficiency	Reference
40-channel relay control board	Bacterial, Algae, Yeast (BAY) microbioreactor	YFP reporter gene	Heat shock	3.15 μL	Plate reader (optical density measurement)	N.S.	[8]
Advanced Liquid Logic (ALL) platform	Software Automated Genomic Engineering (SAGE) protocol and Multiplex Automated Genomic Engineering (MAGE)	GalK recovery oligonucleotides	Electroporation (using gold electrodes)	0.7 μL	Fiber-optic-based mini spectrophotometer (optical density measurement)	9.7 ± 3.4 % (Average transformation efficiency)	[24]
32-channel relay control board	Electroporation/ Electrowetting-on-dielectric device	pGERC plasmid (kanamycin resistance)	Electroporation (using gold electrodes)	0.2 μL	Cells recovered and grown off-chip in presence of kanamycin and observed for colony growth	8.6×10^8 CFU/μg	[28]
Advanced Liquid Logic (ALL) platform	Multiplex Automated Genomic Engineering (MAGE)	bla (beta-lactamase) gene (carbenicillin resistance)	Electroporation (using gold electrodes)	0.35 μL	Cells recovered and grown off-chip in presence of carbenicillin and observed for colony growth	9 ± 9% (average transformation frequency)	[26]
Arduino-based control board	Hybrid device (DMF + serpentine microchannel)	Combinatorial library of 16 plasmids each	Electroporation	0.2 μL	Cells recovered and grown off-chip in presence of kanamycin and observed for colony growth	4.5×10^6 CFU/μg	[29]
Arduino-based control board	Hybrid device (DMF + serpentine microchannel)	GFP (Green Fluorescent Protein), BFP (Blue Fluorescent Protein) or RFP (Red Fluorescent Protein) plasmids	Heat shock	0.235 μL	Fluorescence microscopy	4.3×10^6 CFU/μg	[27]
Arduino-based control board	World-to-Chip Interface	GFP, RFP, and Endoglucanase (EGL) plasmids	Heat shock	1 μL	Cells recovered and grown off-chip in presence of kanamycin or kanamycin and chloramphenicol and observed for colony growth Plate reader used for fluorescence measurements	1.48×10^5 CFU/μg	[30]
Arduino-based control board	"One-pot" Golden Gate DNA Assembly	Combination of 6 DNA fragments into a 14 kb plasmid conferring the violacein biosynthesis pathway	Heat shock	250 nL	Cells recovered and grown off-chip in presence of kanamycin and observed for colony growth	~3.5×10^6 CFU/μg	[31]
Arduino-based custom control board	Automated Induction Microfluidics System (AIMS) with realtime OD monitoring	RFP and EGL plasmids	Heat shock	1.42 μL	Optical density using a 600 nm LED and a light sensor Plate reader used for fluorescence measurements	N.S.	[32]

issue, the Shih group replaced the multiple TEC units with a single unit that was controlled by a PID feedback loop [30,31]. The feedback loop in the system described in these more recent reports enables quick heating and cooling of the DMF device, which then allows the user to perform heat shock transformations without the need to move the droplet between the different temperature regions. Specifically, in the first report [30], a 3D-printed plunger was also included that allowed for replenishment of liquid lost throughout the protocol due to evaporation. In the second report [31], the plunger was replaced by a syringe pump, while the DMF device design was simplified and produced with low-cost tools (printed circuit boards) with the goal to provide a cost-effective alternative to the previously used chromium coated glass devices from the former study.

Induction

Induction is the process of turning genes "ON" by using molecules that inactivate repressor proteins and as a result activate the transcription of one or more genes. In the laboratory, induction protocols are quite labor-intensive, requiring a number of iterations to determine the appropriate conditions for the expression of the desired genes. This is the final step of molecular cloning, and as of writing, there is only a single report that describes the process of induction using a DMF platform. In 2018, Husser et al. [32] reported an autoinduction DMF platform (Fig. 4A) that was dubbed 'AIMS' (Automated Induction Microfluidics System, Fig. 4B). Previously transformed bacterial suspensions were loaded into the DMF device and cultured at 37 °C by placing the platform

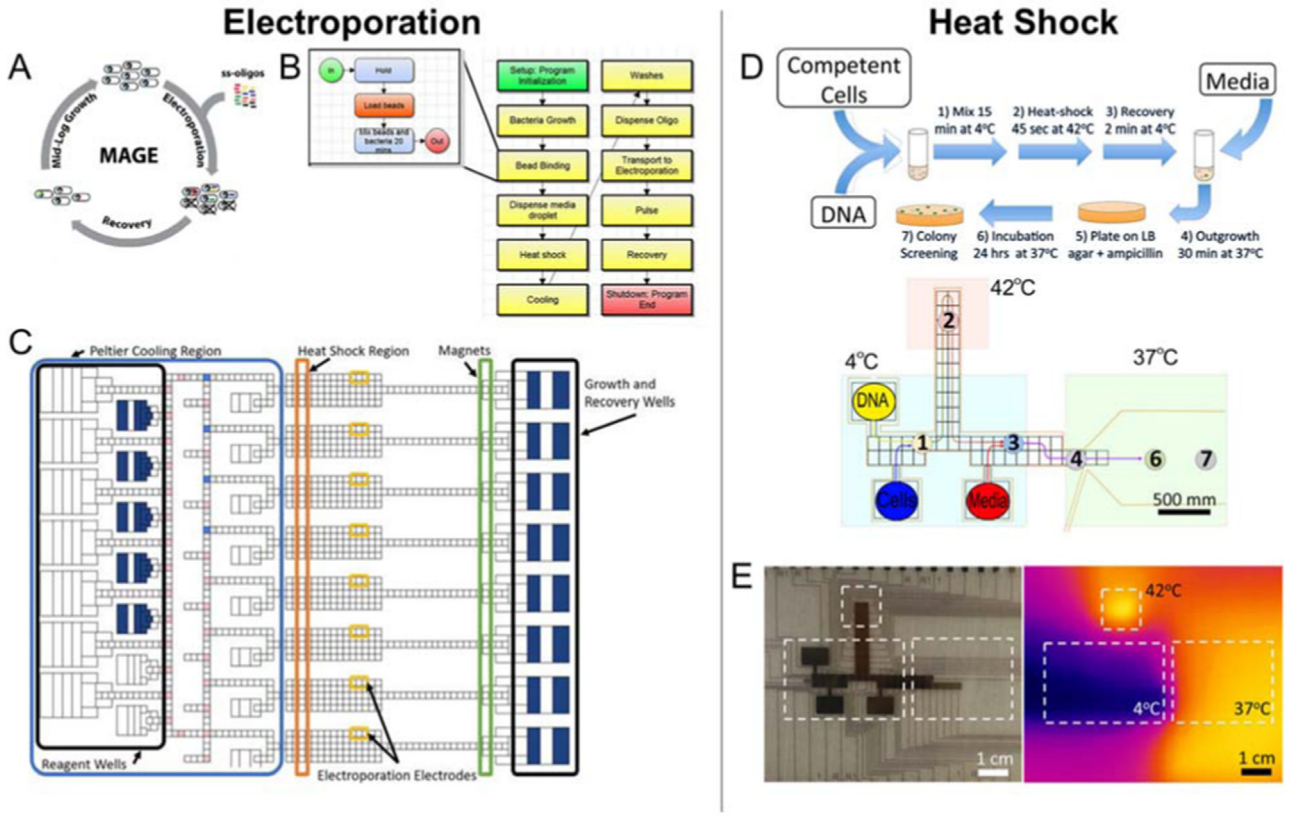

Fig. 3. (A) Summary of MAGE; oligonucleotide DNA is combined with electrocompetent E. coli cells that are electrotransformed, recovered, and cultured on-chip. (B) Software script showing MAGE cycle workflow and subroutine for bead binding. (C) Diagram of electrode layout of the electrowetting cartridge, outlining regions per functionality for MAGE applications. Reproduced from Madison et al. [24] and Moore et al. [26] with permission from AIP Publishing. (D) Schematic of the basic operations of a benchtop (top) and digital microfluidic transformation (bottom). The microfluidic chip performs droplet generation, merging and relocation to thermally controlled regions. Fluidic channels are represented by the orange outlines, electrodes are black and Peltier elements are colored boxes. Numbered circles on the device schematic correspond to heat-shock steps listed in the benchtop schematics procedure. (E) Brightfield (left) and infrared images (right) of the DMF device, indicating the different temperature regions used for heat shock transformations. Reproduced from Gach et al. [27] with permission from the American Chemical Society.

in an incubator. Droplets containing the bacterial culture were transported between the culture area and the measurement electrode where the bacterial growth was monitored by OD using an LED (600 nm) and a photodiode pair. This system allowed the user to trigger the induction process of *β-glucosidase (BGL)* or *red fluorescent protein (RFP)* genes (Fig. 4C) once the OD of the suspension surpassed the threshold of 0.4. Then induction was initiated by mixing the culture with isopropyl *β*-D-1-thiogalactopyranoside (IPTG), a molecule that triggers transcription of the *lac operon*. By splitting the 'mother droplet' containing the bacterial culture, the authors were able to mix sub-droplets of cultured bacteria with varying concentrations of IPTG and to optimize the IPTG concentration for their induction protocol (Fig. 4D). After 4 hours of incubation, protein expression was evaluated in a plate reader, either in intact bacteria via RFP expression, or in chemically lysed samples treated with 4-methylumbelliferyl *β*-d-glucopyranoside (MUG), allowing BGL to turn over a fluorescent reporter (Fig. 4E).

As of writing, AIMS is an outstanding demonstration of the capabilities of a DMF system; however, it remains a proof-of-principle system that is not fully automated/integrated. The primary advantages of AIMS are (1) on-device OD reading, (2) in-line bacterial culture and induction in unit droplet format, and (3) analysis of enzyme expression and activity. Meanwhile, the disadvantages of the current system include the limited incubation time (approx. 5 h), low throughput, and the absence of in-line absorbance or fluorescence measurements (requiring that devices be repeatedly inserted into a stand-alone plate reader).

Diagnostics

Nucleic acid testing is routinely performed for diagnosis of bacterial, viral, and fungal infections, as well as environmental monitoring (*e.g.*, water quality testing). Through the detection of genes that are strain-specific and genes that code for antibiotic-resistance markers, bacterial identification and antibiotic resistance profiling can be achieved, respectively. Nucleic acid detection approaches to guide antibiotic administration decisions can be limiting since the detection of an antibiotic resistance gene does not confirm its expression and the absence of a gene does not guarantee antibiotic susceptibility, since there are many different nucleic acid sequences conferring resistance, which evolve as novel mutations appear. To overcome the limitations of genotypic testing, phenotypic detection strategies, relying on bacterial metabolism, structure, appearance, protein expression and counts, have been explored for antibiotic susceptibility testing (AST) and bacterial classification/identification [33]. Both genotypic (Table 2) and phenotypic detection approaches have been developed for bacterial diagnostics on DMF, each demonstrating different areas of innovation and challenges for their translation on chip.

Genotypic—Nucleic acid amplification and detection

Nucleic acid detection techniques often rely on amplification to detect low gene concentrations. Such techniques have been adapted to

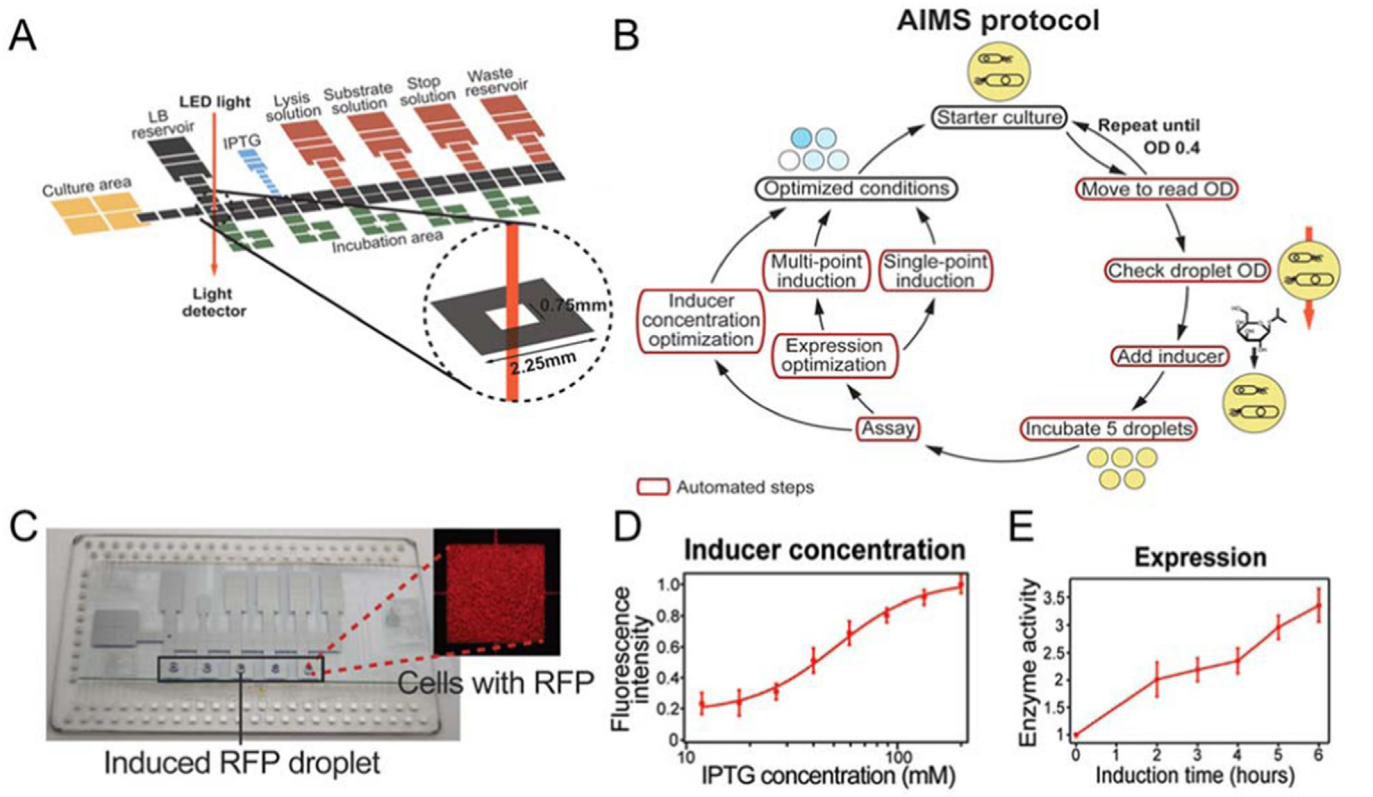

Fig. 4. (A) DMF device used for automated bacteria induction. The chip has multiple areas including ports for loading reagents and broth, a culture area, and an incubation area. The chip also has an electrode that allows for light to pass through and perform OD measurements. (B) Automated Induction Microfluidics System (AIMS) protocol indicating all the steps required for induction that can be performed with the DMF device. (C) Photograph of a DMFdevice with droplets induced with IPTG containing an expanded inset showing cells expressing RFP. (D) Dose-response curve of IPTG for AIMS protocol optimization. (E). Induction profile demonstrating BGL3 protein expression over time. Reproduced from Husser et al. [32] with permission from the American Chemical Society.

DMF protocols, including real-time (RT)-PCR [34–36], as well as isothermal amplification [37–39] of bacterial DNA. Other amplification-free techniques have been achieved for the detection of low nucleic acid concentrations on DMF for pathogen detection [40] and water monitoring [41]. There have been reports of detection of one [35–37,39], two [34,41], three [38] and four [40] bacterial genes on DMF, demonstrating the multiplexed detection capability of the platform.

In an early example of this type of work, Kalsi et al. detected the presence of a gene coding for extended spectrum β-lactamases in *E. coli* through isothermal recombinase polymerase amplification (RPA) [37]. DNA extraction from cultured bacteria was performed prior to dispensing on chip. Droplet temperature was regulated through a built-in sensing and heating system. Continuous droplet mixing on DMF during amplification allowed for DNA detection as low as a single copy in around 15 minutes over 4-orders of magnitude using a fluorescence microscope, an approximate 100-fold improvement compared to a benchtop assay. Building on this system, the same team developed multiplexed detection of three genes coding for extended spectrum β-lactamase and carbapenem resistance in Gram-negative bacteria [38]. Unlike the previous iteration, this platform had an integrated fluorecence detection system that used built-in LEDs and a camera, limiting the need for a microscope. Work later published by this group demonstrated the detection of the same extended spectrum β-lactamase gene in *K. pneumoniae* that was spiked in urine [39]. Heat lysis of bacteria was achieved using a thermomixer and 500-fold DNA pre-concentration via magnetic beads occurred on a separate unit that was interfaced directly with the DMF platform. A detection limit of 10^4 CFU/mL, suitable for urinary tract detection, was reported with a 30 min sample-to-answer time. While this work demonstrated promise for use of this system with clinical specimens, the pre-processing largely took place off-chip.

On a different note, RT-PCR protocols have been developped for pathogen detection on DMF [34–36]. These protocols can achieve automated thermal cycling of the whole device surface at each step (using embedded resistive elements and passive cooling) [36], or through the repeated transfer of the reaction mixture between two segregated areas of different temperatures on a DMF device (heated using resistive heaters, kept at different temperatures) [34,35]. The detection of nucleic acids from *S. aureus* was demonstrated in single- [35,36] and duplex systems (with *M. pneumoniae*) [34] with customized integrated fluorecence detection modules, allowing for one self-contained instrument to perform assay steps. Some reported platforms have the capacity for magnetic bead handling for sample concentration and solution exchange on chip, which was applied for the detection of yeast in a similuated clinical whole blood sample [34] and heat-inactivated *methicillin-resistant S. aureus* (MRSA) in nasal swabs that were lysed off-chip [35], showing promise for future clinical applications.

There have also been reports of a two-plex amplification-free system for 16s rRNA detection in two *Langionella* strains on DMF for water monitoring applications [41]. This approach utilizes magnetic bead probe capture (Fig. 5A) and fluorescent probes for detection (Fig. 5B) using a fluorescence microscope. This method can detect down to 122 pM synthetic rRNA in 30 min (Fig. 5C), showing high sensitivity and speed. Another DMF platform paired with a fluorescence microscope (Fig. 5D), was employed for the simultaneous detection of multiple 500 nM DNA sequences (Fig. 5E) [40]. A unique design feature of the DMF device employed in this work is the presence of on-chip 3D microblade struc-

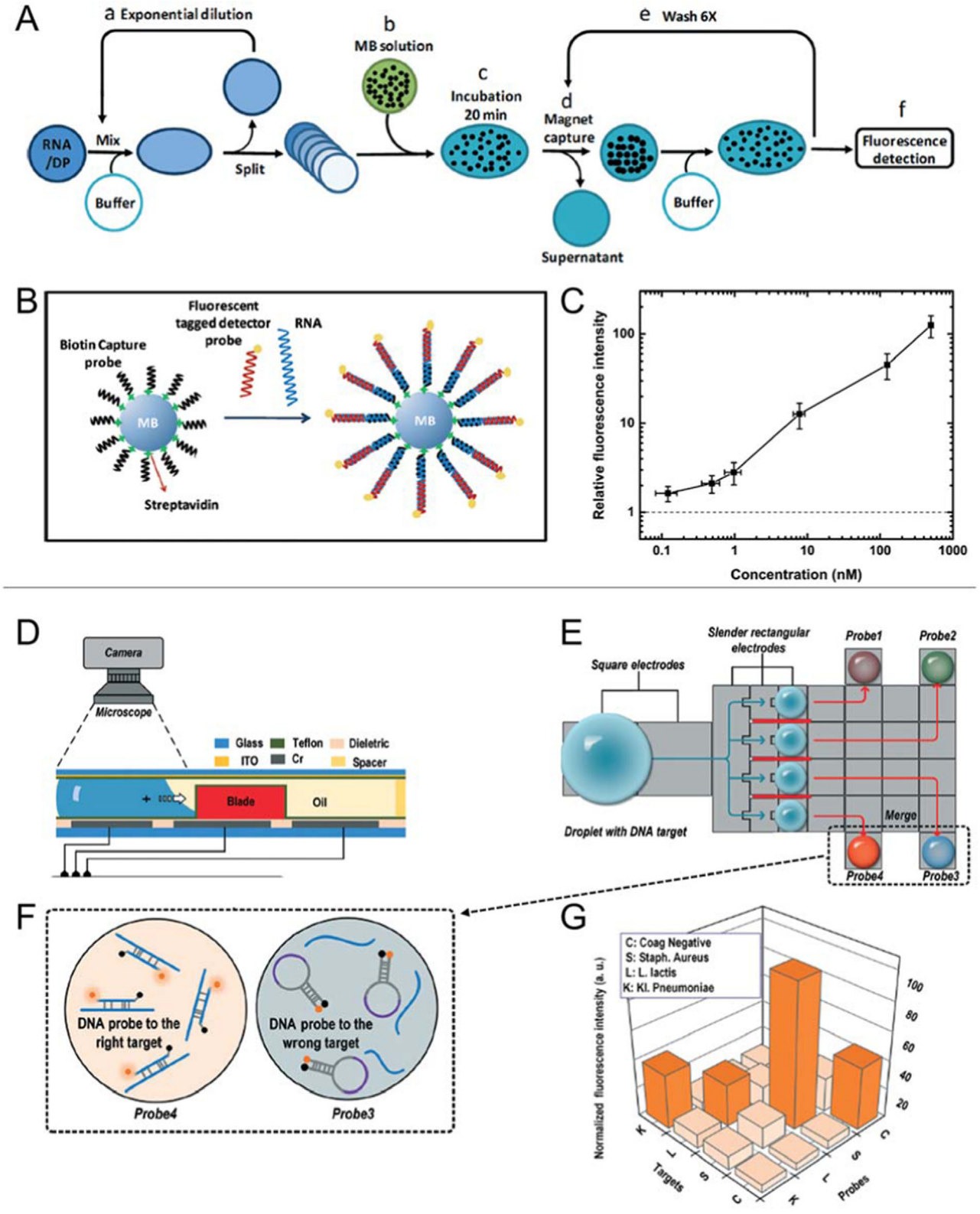

Fig. 5. (A) Schematic of DMF protocol for the dilution, hybridization and detection of 16s rRNA. DP (detector probe) and MB (magnetic bead) are displayed in the subsequent figure. (B) MB functionalized with capture probes and fluorescent tagged detector probes allow detection of target 16s rRNA via fluorescence microscopy. (C) Relative fluorescence intensity vs. L. pneumophila RNA concentration. Reproduced from Foudeh et al. [41] with permission from the Royal Society of Chemistry. (D) Side-view schematic of DMF device containing 3D microblades for droplet splitting. (E) Top-view schematic of the same device in previous figure demonstrating the incubation of 4 separate probes to perform molecular beacon assay with fluorescence DNA detection. (F) Molecular beacon assay demonstrating detection of right and wrong probes. (G) Fluorescence profiles of mixtures of probe–target combinations for 4 different pathogens. Reproduced from Dong et al. [40] with permission from the Royal Society of Chemistry.

Table 2
Summary of nucleic acid amplification and detection techniques on DMF. N/A refers to information that is not applicable, LOD is the limit of detection reported.

Technique	Application	Bacterial gene(s) detected	Time	Heating	Fluorescence detection	Detection range	Reference
RT PCR	Bacterial, mycoplasma and yeast	2 genes (*S. aureus* with *M. pneumoniae*, mycoplasma)	18 min (40-cycle PCR)	Resistive heaters, amplification by moving droplets between two areas of different temperatures on chip	Custom integrated module (a light emitting diode and a photodiode)	1 to 100 000 copies (307 pg to 3.07 fg DNA)	[34]
	Antibiotic-resistant bacteria in nasal swabs.	1 gene (Methicillin-resistant *S. aureus*)	12 min (40-cycle PCR)	Resistive heaters, amplification by moving droplets between two areas of different temperatures on chip	Custom miniature fluorimeter, (a light emitting diode and a photodiode	Optimized with 400 pg in 600 nL PCR mix	[35]
	Point-of-care detection of S. aureus DNA.	1 gene (*S. aureus*)	Not specified	Polysilicon heaters	CMOS-integrated single-photon avalanche diode (SPAD)	1 to 10 000 copies per droplet (1.2 nL)	[36]
Isothermal amplification: Recombinase polymerase amplification (RPA)	Antibiotic resistance in *E. coli*	1 gene (extended spectrum β-lactamase)	~15 min (time-to-positivity)	Resistive heating	Fluorescence microscope	Single copy LOD (4 orders of magnitude detection range)	[37]
	Gram-negative antibiotic resistance genes.	3 (extended spectrum β-lactamase, with carbapenemases)	~7 min (time-to-positivity)	Resistive heating	Custom camera setup	1000 copies	[38]
	Antibiotic resistance in *K. pneumoniae* for diagnosis of urinary tract infections	1 (extended spectrum β-lactamase)	~30 min (sample-to-answer time)	Resistive heating	Custom camera setup	10 copy LOD (purified DNA) 10^4 CFU/mL LOD (lysed bacteria)	[39]
Amplification free	Point-of-care detection of genes from pathogens causing sepsis.	4 genes (*S. aureus*, with *K. pneumoniae*, Coag. negative, *L. Lactis*)	N/A	N/A	Fluorescence microscope	500 nM tested	[40]
	Bacterial rRNA for environmental water monitoring.	2 genes (*L. pneumophila*, with *L. israelensis*)	~30 min (total analysis time)	Thermoelectric heating	Fluorescence microscope	0.5 μM to 122 pM	[41]

tures for simple, robust and accurate droplet splitting, which is necessary for the generation of multiple droplets to detect the different target sequences. The platform employs different molecular beacons (Fig. 5F) to detect four septic pathogens (*S. aureus, L. lactis, K. pneumoniae* and coagulase negative bacteria) on one device (Fig. 5G). Future work in amplification-free detection systems on DMF should focus on demonstrating the feasibility of these platforms with real-world samples, as matrix effects could impact detection limits, assay time and processing requirements.

While the speed, sensitivity, mixing and multiplexing capabilities of DMF have demonstrated its potential for use in bacterial nucleic acid detection protocols, there is still a way to go to demonstrate a fully integrated system that can perform detection directly from clinical and/or environmental specimen on a DMF device. The current literature has demonstrated detection of synthetic nucleic acid sequences, cultured samples of bacteria with off-chip DNA extraction and some preliminary data showing on-chip processing of spiked biological specimens. For such a system to become practical for widespread use, its application in clinical and/or environmental samples should be demonstrated next.

Phenotypic—Antibiotic susceptibility testing

Standard clinical AST often relies on the broth microdilution method, whereby samples of bacteria are incubated 16-20 h with 2-fold dilution series of antibiotics in a 96-well plate format [42]. Optical density measurements are then obtained using a plate reader to determine the minimum inhibitory concentration (MIC) of an antibiotic. This method relies on trained personnel to work aseptically while performing multiple pipetting steps, requires multiple instruments, only provides an endpoint measurement and consumes larger solution volumes than if it were performed in a microfluidic chip format. In order to perform MIC determination on DMF, a key feature is biocompatibility of devices with prolonged culture of bacteria and to sustain the volume of the liquid media the culture is suspended in, while heating at 37 °C. Culture of bacteria over numerous hours on a DMF device was first demonstrated by Au et al. with *E. coli*, using a petri dish as a humidified chamber to mitigate evaporation [8]. This work showed promise and opened up possibilities for protocols requiring bacterial culture on DMF.

Recently, two DMF publications presented AST protocols with comparable results to the standard method, while utilizing real-time monitoring of bacterial growth. Qiu and Nagl integrated an optical oxygen sensor and a heating module on their DMF instrument to culture bacteria and to measure extracellular dissolved oxygen produced by viable bacteria to determine MIC values (Fig. 6A) [43]. The dispensing, dilution and mixing of solutions was automated, simplifying user input. The DMF device was also filled with mineral oil, which limited evaporation and allowed sustained bacterial culture for 16 h. Mineral oil was selected as a filler medium since it has poor vapor and gas permeability, allowing each droplet on chip to act as a microincubator whose changes in oxygen levels could be attributed to bacterial viability and proliferation. This system was validated using an *E. coli* strain and three different antibiotics.

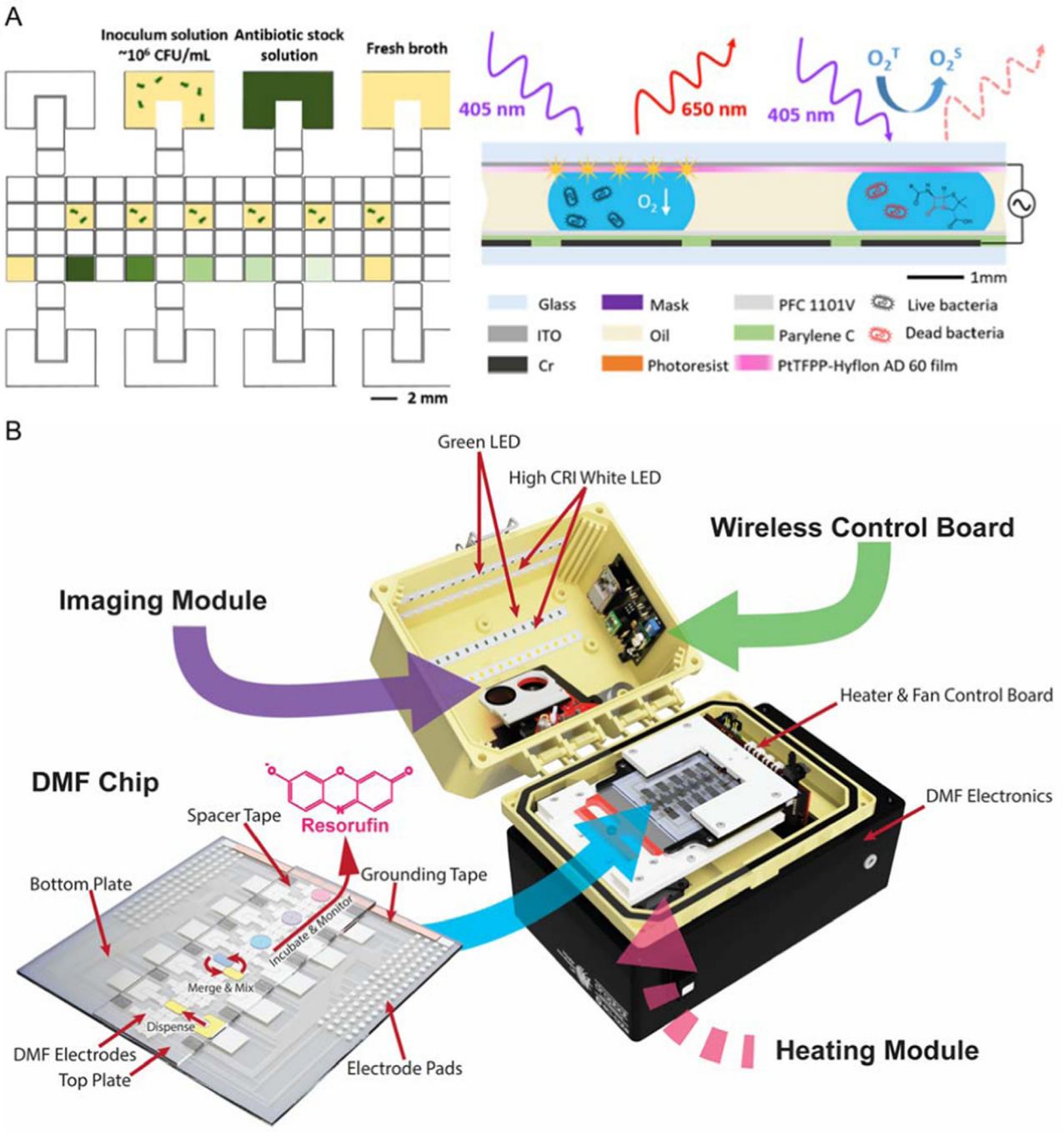

Fig. 6. (A) Top view schematic of antibiotic dilutions and incubation with bacteria to for perform AST on DMF (left) and side view of oxygen sensing system employed for detection of bacterial viability (right). Reproduced from Qiu and Nagl [43] with permission from the American Chemical Society. (B) Assays for automated AST and BC on DMF via the detection of fluorescent resorufin. Reproduced from Sklavounos, Nemr et al. [18] with permission from the Royal Society of Chemistry.

In parallel, Sklavounos, Nemr et al. developed an integrated DMF instrument capable of performing AST, as well as bacterial classification (BC), using a built-in fluorescence detector with simplified optics (Fig. 6B) [18]. Metabolic markers were incorporated in culture broth for detection of bacterial growth and MIC determination through fluorescence measurements using a low-cost color camera. Evaporation was mitigated for culture of bacteria up to 18 h by engulfing droplets in a thin layer of low-viscosity water-immiscible oil, a simpler approach than flooding the device with a filler oil. Various protocols including AST, BC of two bacterial strains simultaneously, a proof-of-concept multiplexed system for breakpoint testing (two antibiotics), as well as *E. coli* and coliform classification demonstrated the versatility DMF offers for completing various assays without requiring chip redesign. This work also demonstrated the ability to culture Gram-positive *S. aureus*, as well as Gram-negative *K. pneumoniae, P. mirabilis* and two *E. coli* strains.

The two integrated AST systems improved automation, solution consumption and minimized instrumentation requirements compared to the standard method. The well-plate format still provides higher throughput than the abovementioned systems, which is an area that could be expanded further to allow for multiple replicates and sample testing at the same time. Having devices with larger droplet capacities could also expand the use of these platforms from AST and BC to AST with com-

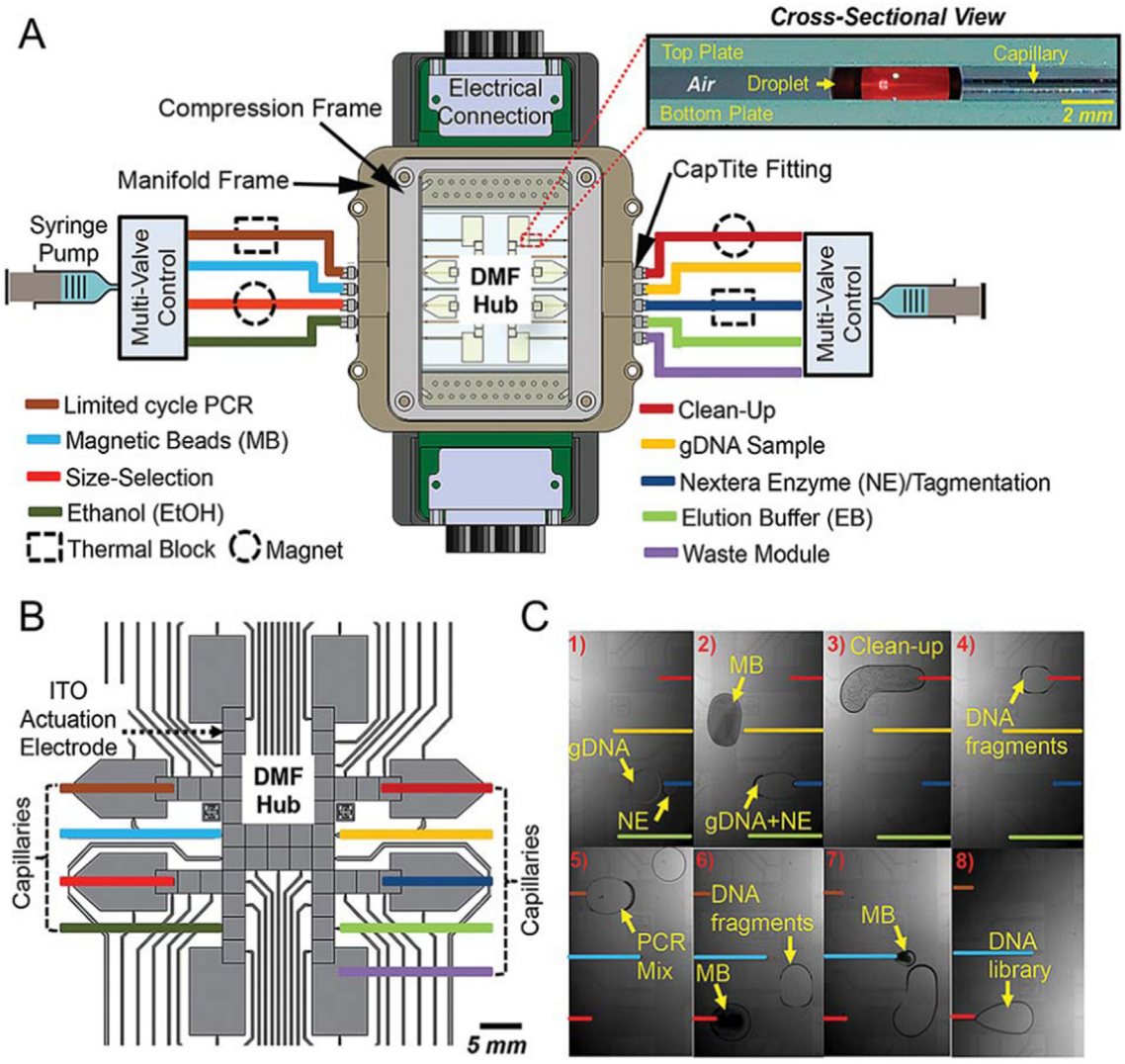

Fig. 7. (A) Top view of and cross-sectional view of a DMF-capillary interface for controlled dispensing and preparation of various solutions (shown in different colors) for next generation sequencing. Magnets and thermal blocks allow sample preparation in tubing and syringe pumps enable liquid handling. (B) Schematic of DMF device highlighting ITO actuation electrodes allowing movement of solutions to and from capillaries. (C) Series of frames from a movie showing the preparation of a sequencer-ready DNA library using the developed platform: 1) Mixing of droplets containing Nextera Enzyme (NE) and gDNA, 2) Merging of NE and gDNA reaction solution with magnetic beads (MB), 3) Actuation of clean-up module. 4) Droplet post-clean up containing DNA fragments, 5) DNA fragments combined with PCR Mix droplet for PCR, 6,7)post-PCR mixture added to varied volumes of MBs for size-selection, 8) DNA library droplet. Reproduced from Kim et al. [44] under Creative Commons Attribution License.

bined bacterial identification. Bacterial identification provides species-specific determination of bacteria, which is more specific than BC. Using a similar approach to BC with species-selective culture broths, as demonstrated for *E. coli* [18], could allow for a more expansive analysis of the samples being tested. Finally, demonstrating AST on clinical specimens and reducing assay times would also enable DMF to be used as a widespread tool for AST, BC and bacterial identification.

Sample preparation interfaced with downstream bacterial protocols

DMF's ability to automate fluid handling steps such as droplet dispensing, mixing and splitting are attractive features of the instrument for sample preparation applications. In addition, DMF design can be tailored for interfacing with other instruments/techniques for downstream sample handling/analysis. Kim et al. reported a fully-integrated DMF sample-in library-out platform (Fig. 7A) to prepare DNA libraries for next-generation sequencing (NGS) [44]. NGS is a powerful tool for microbial community profiling, as well as pathogen discovery and characterization, though it is an expensive and multistep technique. By interfacing DMF with a network of capillaries (Fig. 7B), liquid samples could be handled interchangeably as droplets or in a continuous flow format to achieve sample dispensing, fractionation and separation (Fig. 7C). Formatted sequencing libraries of human, as well as bacterial genomic DNA were prepared on DMF for downstream analysis by Illumina sequencing. Using 5 ng of total DNA, *E. coli* libraries with even genome coverage, good quality scores and over 99% alignment with references were generated. A *de novo* assembly of antibiotic resistant *K. pneumoniae* was also achieved, demonstrating the capabilities of the method.

Work by Liu et al. demonstrated whole genome amplification of bacterial DNA from *C. glutamicum* and *P. somerae* DNA on DMF for downstream sequencing using a MinION sequencer, a miniature platform for fast, real-time and long-read nucleic acid sequencing [45]. Whole genome amplification was performed to increase the total amount of

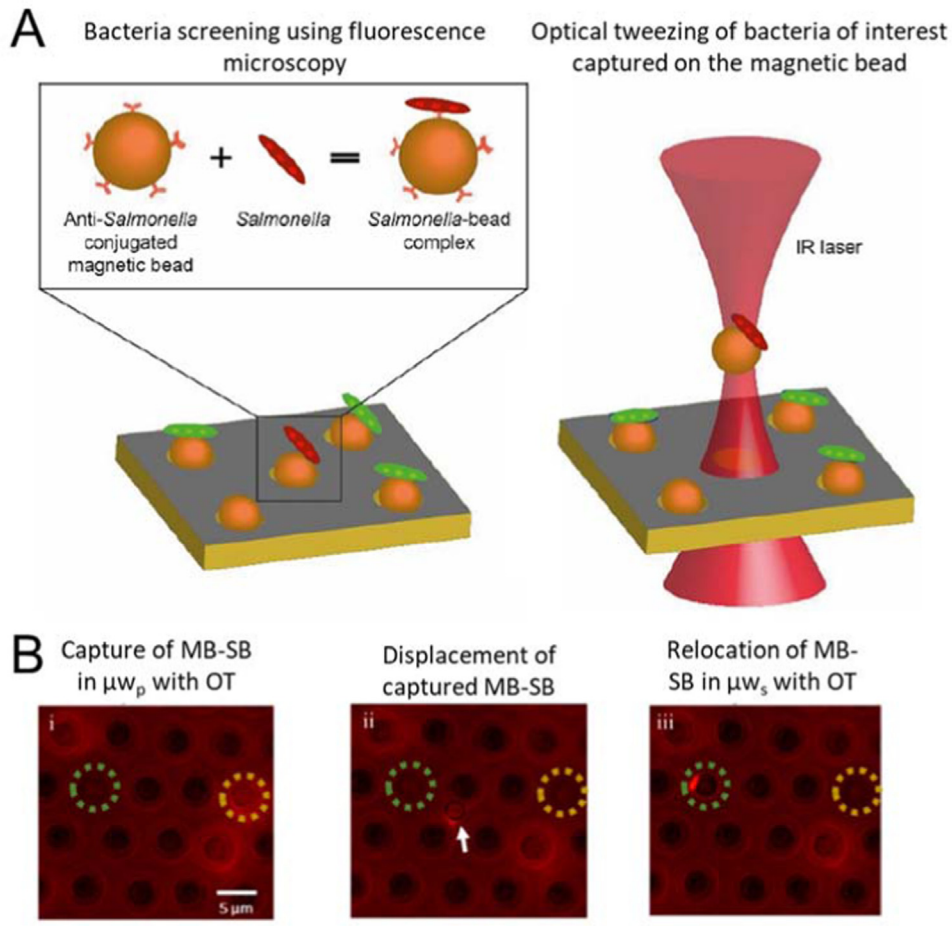

Fig. 8. (A) Schematic demonstrating the binding of Salmonella with a magnetic bead conjugated with anti-Salmonella antibodies, followed by optical tweezing of the captured bacterium using an infrared (IR) laser. (B) Fluorescence microscopy images demonstrating: i) the capture of a magnetic bead bound with a single bacterium (MB-SB) expressing mCherry in a primary microwell (μw_p, yellow circle) with optical tweezer (OT), ii) the displacement of captured MB-SB, shown with the white arrow, iii) the relocation of MB-SB in a secondary microwell (μw_s, green circle) with OT. Reproduced from Kumar et al. [46] under Creative Common CC BY license attribution.

bacterial DNA; required for successful sequencing of the extracted bacterial genome. Components of a commercially available whole genome amplification assay kit were used to perform the assay on DMF. Assay components were dispensed, combined in desired ratios and incubated on chip at room temperature with droplet mixing to achieve improved amplification inside an oil filler medium to prevent evaporation. The chip was then transferred to a hot plate and on ice to perform various heating and cooling steps post-amplification. Samples were subsequently transferred to a microfuge tube and sequenced using a MinION sequencer. A drawback for this system is that it is relatively unintegrated (unlike other recent examples of DMF systems used in other applications [18,30]), with a number of processes that required different temperatures, which were all performed manually. On the other hand, this was the first report of a WGA protocol performed on DMF demonstrating the remarkable capabilities of the technology to handle and process precious samples, such as ones with minute analyte amounts.

Another example of sample preparation on DMF is the capture of individual bacteria on chip with selective retrieval using optical tweezers (OT) [46]. Antibody-coated magnetic beads targeting *Salmonella typhimurium* allowed the single cell capture of fluorescent *S. typhimurium* (Fig. 8A). Individual beads bound to bacteria were initially captured inside of microwells using an external magnet and then each bead was relocated via OT guided by bacterial fluorescent expression. Behavior of individual bacteria could be monitored in real-time via imaging on DMF (Fig. 8B). Single bacteria could then undergo transfers and proliferation on an agar patch that was affixed atop the DMF device, allowing for downstream analysis of viable bacteria.

Overall, interfacing sample preparation on DMF with downstream bacterial protocols can improve streamlining and automation of procedures. This can ultimately minimize user-input and increase user-friendliness, helping reduce experimental error and sample contamination, which is of particular concern when working with viable bacteria. While functionality of these systems is a major advantage, manufacturing of multimodal systems can pose challenges. Fabrication complexity and customized hardware needs may limit the ability to mass produce materials and increase production costs.

Outlooks

The advances in DMF platforms for bacterial protocols have demonstrated the expansive capabilities of the technology. As these platforms become more intricate with built-in features and hybrid interfaces, their versatility and user-friendliness will grow vastly for bacterial protocols and beyond. This could allow for handling and processing of complex samples (such as clinical specimen), as well as generating all-in-one platforms to perform extensive protocols (*e.g.*, assembly, transformation, and induction on one integrated instrument). This also opens new avenues in the development of portable and miniaturized instrumentation, which is desirable at the point-of-care and in remote areas. At the same time, adding more protocols and procedures to a single system increases its complexity. This may introduce hardware and assay protocol design challenges moving forward. Other considerations for implementing bacterial protocols on DMF include preventing complete evaporation of liquids, ensuring that particular reagents (especially non-aqueous solutions) can move on devices and confirming that any surfactants used as supplements to the various liquids do not interfere downstream, with the sample preparation or analysis.

DMF detection approaches for bacterial protocols have to date mostly utilized imaging, fluorescence and absorbance measurements. Future instrument iterations could include other detectors such as electrochemical sensors [10,11], surface plasmon resonance [47], etc. These modalities have previously been paired with DMF in other applications,

so their repurposing for bacterial applications should be straightforward. With the growing DMF toolkit for bacterial protocols, further applications may include investigating polymicrobial populations, studying antibiotic synergy, as well as direct testing of clinical samples.

Last, looking at the commercial landscape, we can identify that there is a lot of momentum generated around the need for the level of automation that DMF offers. Solutions include the ePlex System from GenMark (used for DNA amplification and detection), the Miro Canvas from Miroculus (used for Next Generation Sequencing library preparation), and Alto from Nicoya (used for DMF enabled SPR measurements). As the industry moves in parallel with academic advances, we envision that DMF is going to be ubiquitous in the years to come and will help hundreds of scientists advance the technology and their research.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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