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A Droplet Microfluidic Platform for Automating Genetic Engineering

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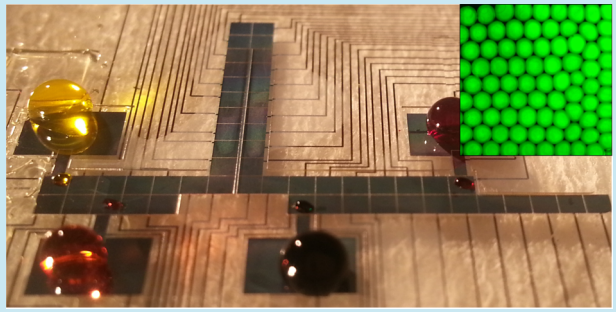
Supporting Information

ABSTRACT: We present a water-in-oil droplet microfluidic platform for transformation, culture and expression of recombinant proteins in multiple host organisms including bacteria, yeast and fungi. The platform consists of a hybrid digital microfluidic/channel-based droplet chip with integrated temperature control to allow complete automation and integration of plasmid addition, heat-shock transformation, addition of selection medium, culture, and protein expression. The microfluidic format permitted significant reduction in consumption (100-fold) of expensive reagents such as DNA and enzymes compared to the benchtop method. The chip contains a channel to continuously replenish oil to the culture chamber to provide a fresh supply of oxygen to the cells for long-term (~5 days) cell culture. The flow channel also replenished oil lost to evaporation and increased the number of droplets that could be processed and cultured. The platform was validated by transforming several plasmids into *Escherichia coli* including plasmids containing genes for fluorescent proteins GFP, BFP and RFP; plasmids with selectable markers for ampicillin or kanamycin resistance; and a Golden Gate DNA assembly reaction. We also demonstrate the applicability of this platform for transformation in widely used eukaryotic organisms such as *Saccharomyces cerevisiae* and *Aspergillus niger*. Duration and temperatures of the microfluidic heat-shock procedures were optimized to yield transformation efficiencies comparable to those obtained by benchtop methods with a throughput up to 6 droplets/min. The proposed platform offers potential for automation of molecular biology experiments significantly reducing cost, time and variability while improving throughput.



KEYWORDS: *digital microfluidics, molecular biology, transformation, cell culture*

Genetic engineering has become a workhorse tool in biology, and the assortment of technologies used to clone DNA has grown substantially in the last 20 years allowing a plethora of new applications.[1,2] The applications span basic research such as expression of proteins for structure/function studies as well as commercial efforts such as production of therapeutics (*e.g.*, antibodies) and industrial enzymes.[3] The basic steps of genetic engineering involve the steps of plasmid and competent cell addition, transformation by heat-shock, electroporation or a viral vector, outgrowth, addition of selection marker, culture and expression.[4] These steps are typically performed manually requiring a lot of pipetting and plating. They can be automated but require very expensive robotic liquid-handling workstations. The fairly large volume required in microtiter plates also makes the process fairly expensive with respect to the cost of DNA and enzymes.

Microfluidics is a powerful technology that has allowed adaptation of standard laboratory methods to improve automation, throughput and drastically reduce reagent consumption.[5] Various molecular biology steps have been performed using microfluidic systems including synthesis of DNA,[6,7] cell sorting,[8] single cell analysis,[9] and cellular assays.[10] Microfluidic devices have also been developed to provide gene delivery to cells by chemical heat-shock and electroporation. Chemical heat-shock has the advantage over electroporation in

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its simpler equipment, efficient operation with high-conductivity medium, application to electrosensitive cell types, and with ultracompetent cells can achieve comparable transformation efficiencies.[11] Droplet microfluidics is a type of microfluidics in which individual aqueous droplets, separated by an immiscible phase such as oil or air, can be generated and manipulated on-chip.[12,13] Droplet microfluidics allows numerous chemical and biological assays to be performed in individual picoliter to microliter chambers with great reproducibility and at much higher throughput than standard methods.[14] These droplets can be manipulated within microchannels by controlling the carrier oil phase (droplets-in-flow) or by application of electric potentials (digital). Electrowetting on dielectric, also termed digital microfluidics, is a type of droplet microfluidics in which an array of individually actuatable electrodes is employed to manipulate discrete droplets over a device. It provides simple fabrication, biocompatibility, operational customization, automation, and consistent droplet volume generation.[15−17] Au and co-workers demonstrated a digital microfluidic platform that could transform bacteria with a fluorescent gene.[18] However, this device did not possess integrated thermal elements, necessitating physical transfer of the device between a hot plate, ice bath, and incubator and then moving the droplets to agar plates for culture. In reports published to date, only the gene introduction step is consistently executed on the device, leaving other key procedures performed off-chip, including; incubation, post-transformation addition of selection antibiotics, culture, and analysis.

We propose a hybrid droplet microfluidic platform for automation of steps required for genetically modifying and screening organisms. We use the digital format-using electrodes for manipulation of droplets, to merge and move the droplets between different temperature zones and a flow channel to improve the throughput and replenish oxygen during culture. We demonstrate the versatility of the system by transformation a multitude of plasmids and a Golden Gate DNA assembly reaction into industrially relevant organisms: *Escherichia coli*, *Saccharomyces cerevisiae* and *Aspergillus niger*.

RESULTS AND DISCUSSION

Microfluidic Device Operation. We have developed a droplet microfluidic platform for automating the standard procedures of chemical heat-shock (Figure 1). The chip as depicted in Figure 1B has the following basic components: (1) inlets for introducing reagents, (2) electrodes for making and manipulating droplets, (3) temperature zones for heat-shock transformation, (4) culture zone, and (5) a fluid channel connected to a pump for continuous addition of oil.

The device was fabricated with chrome electrodes and SU-8 channels to yield a sturdy, biocompatible platform (Figure 2B). This material is also nonreactive with the biocompatible fluorinated oil (HFE 7500) used as the bulk phase. Channels were coated with Aquapel to provide a hydrophobic surface minimizing adhesion of aqueous droplets and for reducing cross-contamination between droplets. Two strategies were employed for reagent delivery to the reservoirs: pipetting prior to assembly or constant fluid addition during operation, as described previously.[19] Reagent storage on device consists of a reservoir, which supports up to 3 μL volume at a time. From these reservoirs, individual 235 ± 11 nL droplets were generated by actuation of the electrode at a rate up to 1 droplet/s. A custom C++ program was written to control an Arduino board which provides electrode actuation employed for all droplet

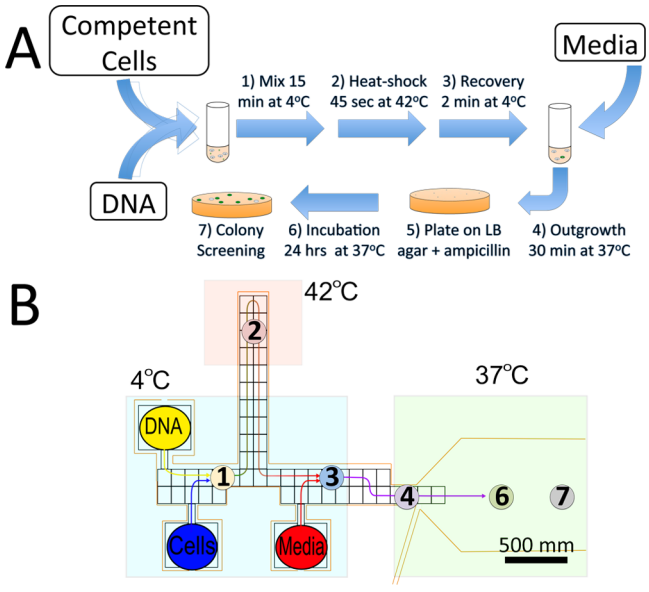

Figure 1. Schematic of the basic operations of (A) benchtop and (B) microfluidic transformation. The microfluidic chip performs droplet generation, merging and relocation to thermally controlled regions. Fluidic channels are represented by the orange outlines, electrodes are black and Peltier elements are colored boxes. Numbered circles on the device schematic correspond to heat-shock steps listed in the benchtop schematics procedure.

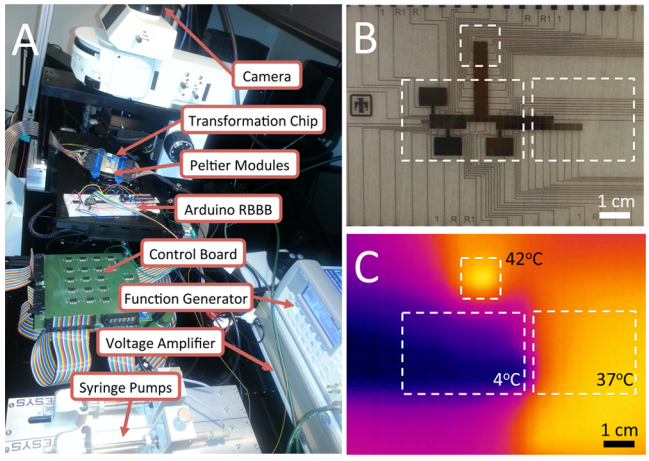

Figure 2. (A) The workstation setup for the transformation device. (B) Brightfield and (C) infrared images of the DMF device for cell transformation.

manipulations including droplet generation, merging, and movement.[19] Three Peltier modules attached to the bottom of the digital microfluidic chip provide three controllable thermal zones. The chip was designed such that the three temperature zones had minimal overlap and stable and discrete temperature regions could be maintained on the device for extended periods as confirmed by thermal imaging. (Figure 2C). At the end of the digital microfluidic reaction region there is a chamber for long-term culture of cells on the device. A syringe pump connected to the channel *via* a capillary provided a constant flow of oil (0.1 μL/s) at this interface to propel the droplet from the last electrode into the culture chamber. The constant addition of oil to the culture chamber has the additional benefits of replenishing oil lost to evaporation and providing cells fresh oxygen during prolonged experiments.

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Genetic Engineering of *E. coli*. Heat-shock on the digital microfluidic device consists of 6 steps: (1) generation of droplets containing DNA and cells, (2) mixing droplets of DNA and cells, (3) DNA/cell droplet actuation to the heat-shock region, (4) dispensing of a culture medium droplet, (5) mixing of DNA/cell droplet with a medium droplet, and (6) DNA/cell/medium droplet transfer to culture region (Figure 3). Three

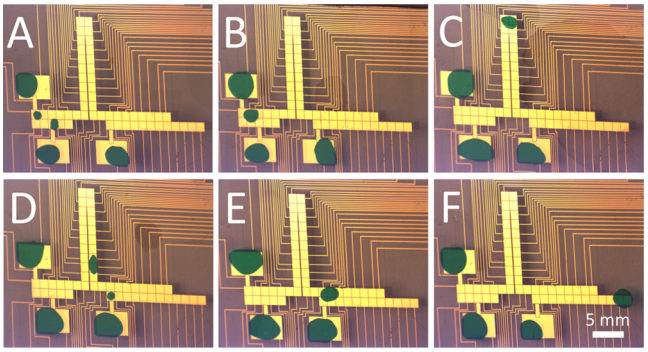

Figure 3. Image sequence of on-chip DMF transformation. (A) Images of filled reservoirs and generation of droplets containing DH5α cells or GFP plasmid, followed by (B) mixing of droplets at 4 °C. (C) Droplet transferred to 42 °C region of device and then (D) moved to 4 °C device region during generation of droplet consisting of LB and AMP. (E) Droplets are then merged and allowed to recover at 4 °C, then (F) transferred to a 37 °C culture chamber.

temperature zones on the chip were created to achieve (1) cold temperature (4 °C) for DNA/cell mixing and recovery, (2) hot temperature (37 or 42 °C) for heat-shock, and (3) warm temperature (30 or 37 °C) for cell culture (Figure 2A,B). The programmability of digital microfluidics and Peltier units to control droplet manipulation and thermal regulation, respectively, allows easy adjustments of the operating procedures to accommodate various transformation protocols required for different cell hosts and gene type. Automated operation paired with flow-based droplet collection allowed a large number of droplets to be continuously processed in a serial and controllable manner.

We tested our hybrid droplet microfluidic device by transforming chemically competent *E. coli* cells (DH5α) with a GFP-expression plasmid (pRSET-EmGFP). Unless otherwise stated, five individual 705-nL droplets were produced for each experiment. We started by performing chemical heat-shock following recommended protocols for the benchtop method,[20] with minimal modifications to accommodate the droplet-based system. Complete details about the transformation protocols, strains and plasmids, and resulting transformation efficiencies are provided in Tables 1 and 2. In this trial, 235-nL droplets containing 1×10^{10} cells/mL and 0.1 ng/μL DNA were merged and mixed at 4 °C for 15 min, moved to 42 °C for 45 s, and following a 2 min recovery at 4 °C which merged with a 235-nL droplet containing medium, 0.05% w/v F-127 Pluronic[21] and antibiotics (ampicillin) and mixed at 37 °C for 30 min (Protocol 1). This mixed droplet was relocated to a 37 °C culture chamber for 24 h. All droplets possessed GFP positive cells following on-chip culture with ~20-fold fluorescence increase compared to droplets containing cells cultured without the GFP gene (Figure 4A–D). However, when droplets were cultured on an agar plate following on-chip transformation, the transformation efficiency (Figures 4F, 5A.1) was significantly lower than the standard benchtop procedure under the same conditions (Figure 4FG, 5A.1) (two sided *t*-test, $t(4) = 8.106$, $p = 0.001$). Through simply changing the heat-shock temperature to 37 °C (Protocol 2) or heat-shock duration to 10 s (Protocol 3) on the microfluidic device, the transformation efficiencies were substantially increased (Figure 5A.2, 5A.3). These results are likely due to faster temperature change in a droplet compared to the 50 μL volume in a centrifuge tube. This is consistent with recommended heat-shock procedures for microtiter plates, where shorter heat-shock times are generally employed.[22,23] These results demonstrate that transformation efficiencies in our chip are comparable to those achieved in benchtop experiments (two sided *t*-test, $t(4) = 1.232$, $p = 0.3$) with the benefit of using ~100 times less reagents.

Improving Heat-Shock Throughput. While recapitulating standard heat-shock procedures with the microfluidic chip produced high transformation efficiencies, this approach afforded extremely low throughput (~1 droplet/h). Time intensive steps in traditional heat-shock include a 15 min DNA/cell mixing period, 2 min post heat-shock recovery and at least a 30 min outgrowth step. Several heat-shock protocols have demonstrated that the length of various steps can be reduced, though resulting in compromised transformation efficiencies. In protocols 3–6 we adjusted the duration at each transformation step to investigate the effect on the transformation efficiency (Figure 5A, Table 1). Employing ampicillin as a selection marker allows direct droplet transfer to the culture region, bypassing the outgrowth period, because ampicillin is a bacteriostatic thereby giving cells time to take up the DNA and replicate in its presence with no statistically significant effect on the transformation

Table 1. Heat-Shock Protocols

protocol name	description	selection	DNA–cell mixing duration/temperature	heat-shock duration/temperature	recovery duration/temperature	outgrowth duration/temperature	culture duration/temperature
1	*E. coli* long heat-shock at 42 °C	Ampicillin	15 min/4 °C	45 s/42 °C	2 min/4 °C	30 min/37 °C	1 day/37 °C
2	*E. coli* long heat-shock at 37 °C	Ampicillin	15 min/4 °C	45 s/37 °C	2 min/4 °C	30 min/37 °C	1 day/37 °C
3	*E. coli* quick heat-shock	Ampicillin	15 min/4 °C	10 s/42 °C	2 min/4 °C	30 min/37 °C	1 day/37 °C
4	*E. coli* quick mixing and outgrowth	Ampicillin	10 s/4 °C	10 s/42 °C	2 min/4 °C	0 min	1 day/37 °C
5	*E. coli* quick mixing, heat-shock and outgrowth	Ampicillin	10 s/4 °C	10 s/42 °C	2 min/4 °C	0 min	1 day/37 °C
6	*E. coli* quick mixing, heat-shock, recovery and outgrowth	Ampicillin	10 s/4 °C	10 s/42 °C	10 s/4 °C	0 min	1 day/37 °C
7	*E. coli* kanamycin resistance	Kanamycin	10 s/4 °C	10 s/42 °C	10 s/4 °C	30 min/37 °C	1 day/37 °C
8	*S.cerevisiae* heat-shock	Uracil	10 s/30 °C	10 s/30 °C	10 s/4 °C	0 min	4 days/30 °C
9	*A. niger* heat-shock	Hygromycin B	10 s/4 °C	10 s/30 °C	10 s/4 °C	30 min/30 °C	5 days/30 °C

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Table 2. Transformation Efficiencies

cell type	plasmid	DNA conc (ng DNA/μL)	selection	format	protocol #	transformation efficiency (CFU/μg DNA)	st dev
E. coli	pRSET-emGFP	0.1	Amp	DMF	1	1.3×10^6	1.5×10^5
E. coli	pRSET-emGFP	0.1	Amp	DMF	2	2.2×10^6	3.9×10^5
E. coli	pRSET-emGFP	0.1	Amp	DMF	3	4.3×10^6	2.2×10^5
E. coli	pRSET-emGFP	0.1	Amp	DMF	4	4.1×10^6	4.0×10^5
E. coli	pRSET-emGFP	0.1	Amp	DMF	5	1.2×10^6	1.5×10^5
E. coli	pRSET-emGFP	0.1	Amp	DMF	6	8.9×10^5	8.0×10^4
E. coli	none	0	Amp	DMF	6	0	0
E. coli	pBbB2k-GFP	0.1	Ken	DMF	7	4.3×10^5	9.9×10^4
E. coli	none	0	Ken	DMF	7	0	0
E. coli	pProm1_BCD1-GFP	0.1	Ken	DMF	7	successful	on-chip culture
E. coli	pRSET-emGFP	0.1	Amp	Bench	1	3.9×10^6	5.2×10^5
E. coli	pRSET-emGFP	0.1	Amp	Bench	2	3.2×10^6	3.8×10^5
E. coli	pRSET-emGFP	0.1	Amp	Bench	3	8.1×10^5	1.6×10^5
E. coli	pRSET-emGFP	0.1	Amp	Bench	4	7.4×10^5	1.0×10^5
E. coli	pRSET-emGFP	0.1	Amp	Bench	5	1.4×10^5	2.1×10^4
E. coli	pRSET-emGFP	0.1	Amp	Bench	6	7.3×10^4	1.7×10^4
E. coli	none	0	Amp	Bench	6	0	0
E. coli	pBbB2k-GFP	0.1	Ken	Bench	7	1.3×10^4	1.0×10^3
E. coli	none	0	Ken	Bench	7	0	0
E. coli	pRSET-emGFP	0.001	Amp	DMF	6	2.3×10^6	8.1×10^5
E. coli	pRSET-emGFP	0.01	Amp	DMF	6	1.3×10^6	2.0×10^5
E. coli	pRSET-emGFP	0.1	Amp	DMF	6	9.2×10^5	2.1×10^5
E. coli	pRSET-emGFP	1	Amp	DMF	6	1.9×10^5	8.7×10^4
E. coli	pRSET-emGFP	0.001	Amp	Bench	1	1.0×10^7	2.2×10^6
E. coli	pRSET-emGFP	0.01	Amp	Bench	1	5.8×10^6	1.3×10^6
E. coli	pRSET-emGFP	0.1	Amp	Bench	1	3.4×10^6	4.4×10^5
E. coli	pRSET-emGFP	1	Amp	Bench	1	9.4×10^5	1.5×10^5
E. coli	pBAD-mTAG BFP2	0.1	Amp	DMF	6	7.7×10^5	2.2×10^5
E. coli	pRSET-emGFP	0.1	Amp	DMF	6	8.9×10^5	1.0×10^5
E. coli	pDsRed_T3-S4T	0.1	Amp	DMF	6	6.7×10^5	1.4×10^5
E. coli	pBAD-mTAG BFP2	0.1	Amp	Bench	1	3.3×10^6	5.5×10^5
E. coli	pRSET-emGFP	0.1	Amp	Bench	1	3.9×10^6	5.2×10^5
E. coli	pDsRed_T3-S4T	0.1	Amp	Bench	1	3.0×10^6	5.2×10^5
S. cerevisiae	pL211-mCherry	20	Uracil	DMF	8	1.8×10^4	6.0×10^3
S. cerevisiae	none	0	Uracil	DMF	8	0	0
S. cerevisiae	pL211-mCherry	20	Uracil	Bench	8	3.5×10^4	5.0×10^3
S. cerevisiae	none	0	Uracil	Bench	8	0	0
A. niger	peGFP-glaA	20	Hygro-B	DMF	9	successful	on-chip culture

efficiency ($(4.1 \pm 0.4) \times 10^6$ CFU/μg DNA) (two sided *t*-test, $t(4) = 1.738$, $p = 0.2$). Reducing the DNA/cell mixing time and recovery period on the chip to 10 s each (Protocol 6) resulted in a 5-fold reduced transformation efficiency and yielded cultured droplets with ~19-fold higher fluorescence over control droplets (Figure 5A.5). This is significantly better than reducing benchtop incubation times by the same factor, which resulted in a 54-fold lower transformation efficiency (two sided *t*-test, $t(4) = 17.520$, $p < 0.0001$). This is likely a result of the enhanced mixing and improved thermal transfer in the droplets that quickens DNA delivery and cell recovery. Although a 5-fold reduction in the transformation efficiency is significant (two sided *t*-test, $t(4) = 13.498$, $p = 0.0002$), this approach allows heat-shock to be completed in just 30 s. Furthermore, by serial processing of 3 droplets at a time we further increased the droplet preparation speed to 1 droplet every 10 s which enables a throughput of 360 droplets/h. As a demonstration of large sample processing this protocol was used for preparing 100 individual droplets in just 17 min. Filled reagent reservoirs were maintained throughout operation by continuously supplying fluids with a syringe pump. Droplet collection with fluidic flow was a critical aspect to high volume sample collection, which would otherwise require a prohibitively large number of digital microfluidic electrodes. Following 24 h culture, all of the heat-shocked droplets remained intact and possessed GFP-expressing *E. coli* cells (Figure 4H,I). The only anomaly was the presence of an additional droplet following culture, likely the result of over compression of a droplet during incubation. Efforts are underway to develop a larger digital microfluidic chip with larger number of electrodes operating in a linear and/or parallel manner to improve the throughput by 2 orders of magnitude.

Optimization of DNA Concentration Used for Transformation. Changing the quantity of DNA added to cells has a direct correlation to the number of transformants generated and resulting transformation efficiency.[24] We varied the plasmid DNA concentration in each droplet to examine its effect on *E. coli* transformation in the chip. These experiments were performed on the microfluidic chip operating at the highest throughput (Protocol 6). As the DNA concentration in each DNA/cell droplet was changed from 0.001 ng/μL to 1 ng/μL the microfluidic device produced transformation efficiencies ranging from $(2.4 \pm 0.8) \times 10^6$ CFU/μg DNA to $(1.9 \pm 0.9) \times$

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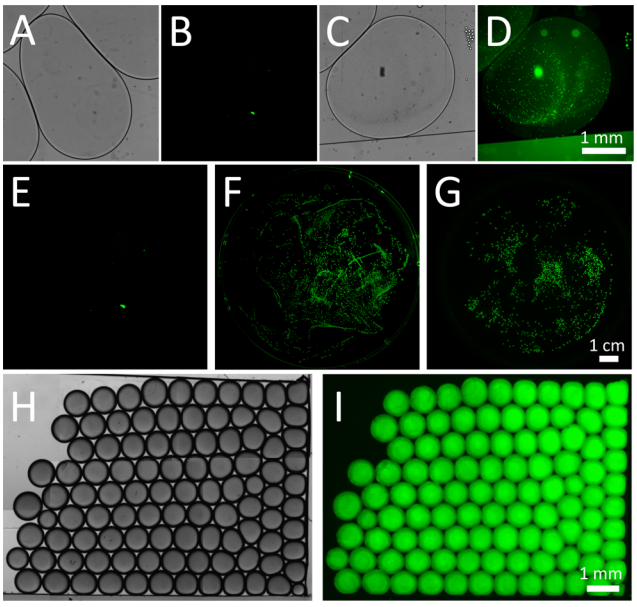

Figure 4. Brightfield and fluorescence images of a droplet containing DH5α cells and pRSET-EmGFP plasmid DNA (A, B) without heat shock and (C, D) following transformation within the DMF device. Fluorescence images of LB agar plates containing DH5α cells (E) without heat shock and (F) following transformation with GFP plasmid DNA by the DMF device, and (G) the conventional tube-based method. (H) Brightfield and (I) fluorescence images of culture chamber containing 100 droplets containing DH5α cells following heat shock with GFP plasmid and 24 h culture at 37 °C. Final image is a composite of six micrographs stitched together.

10^5 CFU/μg DNA, respectively (Figure 5B). These colony counts equate to each heat-shocked droplet with 0.001 ng/μL to 1 ng/μL DNA generating 1.7 ± 0.5 to 114 ± 50 transformed cells, respectively. Conversely, standard benchtop heat-shock using Protocol 1 gave transformation efficiencies 3.8 to 4.8 times greater than the microfluidic chip at DNA concentrations of 0.001 to 1 ng/μL, respectively (Figure 5B). The benchtop and microfluidic systems each generate similar logarithmic regression curves with increasing DNA concentrations with the benchtop approach yielding greater transformation efficiencies though at significantly prolonged heat-shock durations and reagent consumption, as discussed above. Importantly, all droplets cultured on the microfluidic chip for 24 h following heat-shock with 0.001 ng/μL to 1 ng/μL DNA possessed GFP-expressing *E. coli*.

Transformation Results for Other Plasmids. To demonstrate the versatility of platform, cells were transformed with plasmids containing genes for blue fluorescent protein (pBAD-mTag_BFP2) or red fluorescent protein (pDsRed_T3-S4T). DH5α *E. coli cells* transformed with 0.1 ng/μL pBAD-mTag_BFP2 or 0.1 ng/μL pDsRed_T3-S4T under identical conditions as the pRSET-EmGFP plasmid above (Protocol 6) produced slightly lower transformation efficiencies (Table 2). Successful transformation of pBAD-mTag_BFP2 and pDsRed_T3-S4T plasmids could also be confirmed after 24-h on-chip cultivation. Following imaging with a blue filter set or red filter set the pBAD-mTag_BFP2 and pDsRed_T3-S4T transformed cells generated droplets with heightened fluorescence with respect to control droplets, 15.0 ± 3.5 and 6.7 ± 0.8 fold increased RFU, respectively (Figure 6).

Kanamycin Selection. To this point, ampicillin was used as the selection antibiotic, which allowed immediate cultivation

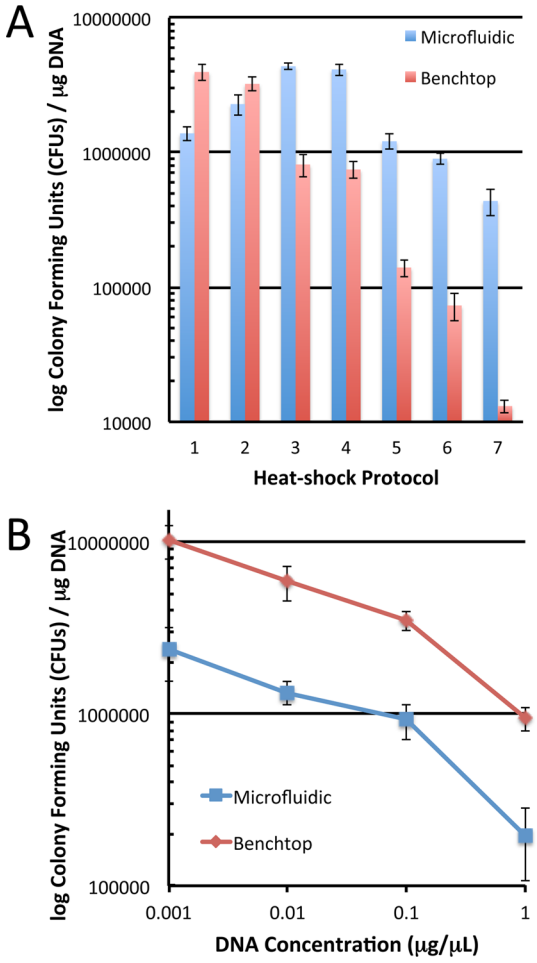

Figure 5. (A) Plot of transformation efficiencies obtained under various heat shock procedures and (B) with different concentrations of GFP plasmid DNA. Heat-shock protocols are as follows: (1) long heat-shock at 42 °C, (2) long heat-shock at 37 °C, (3) quick heat-shock, (4) quick heat-shock and outgrowth, (5) quick mixing, heat-shock and outgrowth, (6) quick mixing, heat-shock, recovery and outgrowth, and (7) kanamycin resistance.

following heat-shock. However, during prolonged culture *E. coli* can secrete beta-lactamase that inactivates the ampicillin resulting in potential growth of nonselective cells. To avoid this scenario, other antibiotics such as carbenicillin or kanamycin are used. Kanamycin is a bacteriocidal antibiotic and immediately kills cells.[20] To ensure cell survival, an outgrowth step in antibiotic-free medium is required following heat-shock and prior to the addition of kanamycin (Protocol 7). The outgrowth period in the microfluidic device was implemented by holding the droplet on the last electrode for 30 min following heat-shock.. After outgrowth, 150 μg/mL kanamycin was added and the droplet was moved to the culture region for 24 h. GFP fluorescence increased by ~18-fold over control droplets demonstrating successful transformation and culture of GFP cells.

Golden Gate DNA Assembly Reaction. To demonstrate the compatibility of the microfluidic heat-shock device with DNA assembly reactions, we examined the on-chip transformation of a Golden Gate assembly reaction for pProm1_BCD1-GFP expressing an ampicillin resistance gene into *E. coli*. DNA assembly was initially performed off-chip using the Golden Gate assembly method[7] and then 0.1 ng/μL pProm1_BCD1-GFP

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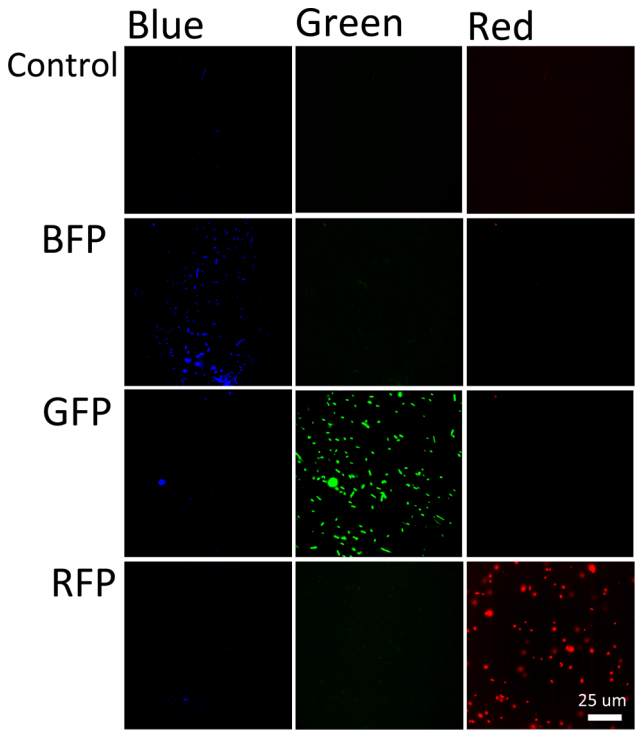

Figure 6. Fluorescence images of DH5α cells transformed with blue fluorescence protein (BFP), green fluorescence protein (GFP) or red fluorescence protein (RFP) following 1-day culture at 37 °C on the DMF device.

was added to the microfluidic device and kanamycin selection heat-shock performed on five droplets (Protocol 7). Following 24 h on-chip culture, all droplets possessed GFP positive cells with ~15-fold fluorescence increase *versus* control droplets.

***Fungal* Transformation.** *Yeast Transformation.* Eukaryotic systems are frequently used for expressing proteins and products that are difficult to synthesize in bacterial systems with desired yields. Introduction of exogenous DNA into *S. cerevisiae* is routinely performed by heat-shock or electroporation. A common procedure for delivering DNA into *S. cerevisiae* is to heat-shock competent cells with DNA in a PEG solution at 30 °C followed by cultivation on an agar plate.[25] Flexibility of the microfluidic heat-shock device was demonstrated by adapting its operation to transform 2×10^7 cells/mL *S. cerevisiae* JBEI-4714 with 20 ng/μL mCherry generating plasmid (pL211-mCherry). The same heat-shock steps as the 30-s serial droplet transformation protocol was used except for changes in reagent use and operating temperatures, Protocol 8. For on-chip culture, a 4-day incubation period was used to account for the longer replication time of yeast cells. Following 4-day incubation, all five of the cultured droplets possessed mCherry expressing yeast cells and produced ~10-fold fluorescence increase over control droplets (Figure 7).

Filamentous Fungi Transformation. Filamentous fungi, such as *A. niger*, is another important system for molecular biology and chemical synthesis.[26] Although laborious sample preparation was required to generate protoplasts,[27] once prepared these cells can be transformed using similar protocols as with yeast heat-shock above. Transformation of *A. niger* (ATCC11414) protoplasts at 9×10^6 cells/mL on the digital microfluidic device was examined by testing the incorporation of 20 ng/μL plasmid DNA expressing GFP and Hygromycin B resistance (peGFP-glaA). A 30 min outgrowth step was included

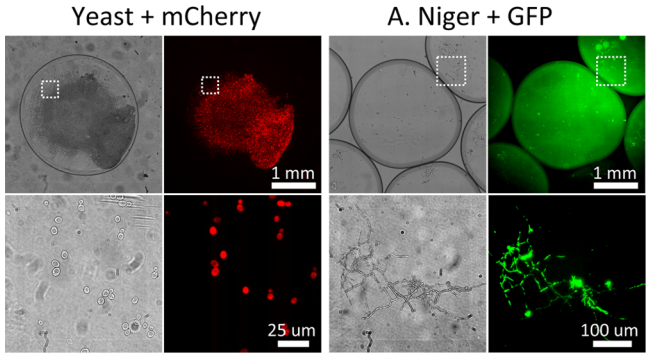

Figure 7. Brightfield and fluorescence images of droplets containing CEN.PK cells transformed with mCherry (left) or *Aspergillus niger* cells transformed with GFP (right) following 5-day culture at 30 °C on chip. Bottom images show individual cells in magnified areas of the droplets.

prior to the addition of Hygromycin B to afford sufficiently high gene expression required to retain cell viability in its presence (Protocol 9). Five droplets were processed on the device and each droplet contained GFP positive cells following 5-day culture in the microfluidic chip (Figure 7). Various cell morphologies indicative of fungal growth were observed in the droplet cultures including individual mycelium (hyphal mats), mycelium clumps and individual *A. niger* spores all expressing GFP. Similar morphologies were observed along with larger conidiophores when *A. niger* cells were transformed using the traditional benchtop method with bilayer agar culture.[27] Agarose hydrogels have previously been generated by digital microfluidics, which could potentially be adapted for recapitulating the bilayer agar culture of transformed *A. niger* cells.[28]

CONCLUSIONS

A temperature-controlled hybrid microfluidic platform was developed to implement heat-shock transformation, antibiotic selection, culture and assay in discrete droplets. Micro Peltier modules were attached to the bottom of the chip to provide the different temperatures required during heat-shock and culture. Automated operation was used for droplet generation, mixing and movement across the various thermal regions of the device. This high level of droplet control allowed the entire transformation process in a single device including cell/DNA mixing, heat-shock, media addition, culture, and outgrowth. A flow-based droplet collection system was included with the digital microfluidic sample preparation to increase the quantity of droplets that could be processed and cultured. The introduction of a continuous stream of oil had the additional benefit of allowing continued replenishment of oil to the culture chamber, which prevented droplet evaporation and provided a fresh supply of oxygen to the cells allowing long-term cell culture. The device was used to optimize the heat-shock parameters of *E. coli* to afford high transformation efficiencies, high throughput sample processing and introduction of various DNAs. The same device was used to transform *S. cerevisiae* and *A. niger* cells with exogenous DNA. This system along with existing technologies that allow DNA synthesis, DNA assembly and various analytics provide a powerful suite of technologies for screening gene variants and systematic investigation of DNA delivery strategies. We expect that the microfluidic platform described here will find great utility in cellular engineering applications benefiting from the automated transformation, culture, and assessment of numerous samples.

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METHODS

Device Assembly and Operation. A digital microfluidic device was fabricated as described in the Supporting Information (Device fabrication). Prior to operation the device was connected to tubing, Peltier units and droplet actuation electronics (Figure 2A). Real-time segmented control of device temperature was achieved by three Peltier heaters/coolers attached to the bottom of the device by thermal adhesive tape and controlled by an Arduino microcontroller (Modern Device, Providence, RI). A total of three Peltier elements were attached to the device: (1) a cooler module for DNA/cell suspension mixing and cell recovery covered by an aluminum block, (2) 2 mm Peltier for heat-shock and (3) 24.5 mm Peltier for cell culture (TE Technology, Inc., Traverse City, MI). Two techniques were employed for liquid addition of DNA, cell suspension, media and oil to the device: pipetting before assembly, and continuous flow during operation. For the majority of experiments, 3.0 μL liquid was pipetted directly onto the device reservoirs and the channels were flooded with HFE 7500 then topped with an ITO glass slide and secured with Nd magnets #B228 (K&J Magnetics, Pipersville, PA). For experiments requiring more reagents, the ITO glass slide was first bound to the device then ends of capillary tubes connected to a neMESYS syringe pump (Cetoni GmbH, Korbussen, Germany) were glued into channels associated with the device reservoirs using 5 min Epoxy Gel (Devcon, Danvers, MA). This configuration uses a neMESYS syringe pump to continuously inject reagents directly into the reservoirs through a capillary imbedded in a channel on the device (similar to what is described in Shih *et al.*[19]). Following assembly of either technique, a capillary was inserted into a fifth channel at the entrance of the culture chamber from which HFE 7500 with 0.5% v/v Picosurf (Dolomite Microfluidics, Charlestown, MA) was flowed continuously to remove droplets from the reaction chamber and replenish evaporated oil. During on-chip culture, the oil flow rate was set to 0.01 μL/s, which used only 860 μL oil for 24-h cell culture. Finally, the device was connected to a custom-built control board activated by a RBBB Arduino microcontroller (Modern Device, Providence, RI). A customized C++ program controlled the driving potentials of the Arduino board to allow automation of droplet movement in the device.[19] Droplets were manipulated on the digital microfluidic device by applying a 100 V rms driving potential in the form of an 8 kHz sine wave to the designated actuation electrodes as described previously.[19]

Microfluidic Transformation. Information regarding the preparation of cells and plasmids are available in the Supporting Information. All strains and plasmids used in this report are listed in Supplementary Table S1 and available on the JBEI public registry (https://public-registry.jbei.org/folders/205).[29] A detailed list of the durations and temperatures of each heat-shock step is available in Table 1.

E. coli. The transformation chip reservoirs were first loaded with all reagents using either a pipet or the syringe pump: (1) 3 μL thawed chemically competent DH5α cells at 1×10^{10} cells/mL, (2) 3 μL DNA solution (0.001−1.0 ng DNA /μL), and (3) 3 μL Luria−Bertani (LB) broth with selection antibiotics (see below). A 235 nL droplet containing cells was first mixed with a 235 nL DNA droplet at 4 °C. This droplet was then moved to a 37 or 42 °C chip region for 10 or 45 s. The cell/DNA droplet was then merged with a 235 nL media droplet containing 0.05% w/v F-127 Pluronic. For ampicillin selection, 300 μg/mL ampicillin was included with the media and droplet was immediately moved to the devices culture region. Conversely, kanamycin selection required 30 min outgrowth following media delivery prior to addition of a 235 nL droplet containing 200 μg/mL kanamycin. Once delivered to the culture region the droplets were incubated for at least 30 min at 37 °C.

S. cerevisiae. A modified procedure from the Frozen-EZ Yeast Transformation II Kit was employed for transforming *S. cerevisiae* JBEI-4714 cells with mCherry-expressing DNA (Zymo Research Corp., Irvine, CA). The transformation chip reservoirs were initially filled with reagents: (1) 3 μL thawed chemically competent JBEI-4714 cells in EZ 2 solution at a density of 2×10^7 cells/mL, (2) 3 μL mCherry DNA (20 ng/μL) in EZ 3 solution (40−50% PEG), and (3) 3 μL medium without uracil. As per Zymo protocols for yeast transformation the entire chip was heated to 30 °C. A 235 nL droplet containing competent cells in EZ 2 solution was first mixed with a 235 nL DNA droplet mixed with EZ 3 solution 30 s. The heat-shocked droplet was then merged with a 235 nL droplet containing media minus uracil and 0.05% w/v F-127 Pluronic and moved to the devices culture region where it was incubated for 4 days at 30 °C.

A. niger. Necessary reagents for *A. niger* transformation were first loaded: (1) 3 μL thawed protoplast at 9×10^6 cells/mL, (2) 3 μL peGFP-glaA DNA (20 ng/μL) in 40% PEG, (3) 3 μL minimal medium with 1 M sorbitol, and (4) 3 μL minimal medium with 1 M sorbitol and 400 μg/mL Hygromycin B. A 235 nL droplet containing protoplast was first mixed with a 235 nL DNA droplet at 4 °C for 1 min. This droplet was then moved to a 30 °C chip region for 30 min. The cell/DNA droplet was then merged with a 235 nL media droplet with 0.05% w/v F-127 Pluronic and given 30 min outgrowth at 30 °C. At this point the droplet was merged with the 235 nL Hygromycin B droplet and moved to the devices culture region. Here the droplet was incubated for 5 days at 30 °C.

Transformation Efficiency. *Off-Chip Culture.* To assess the transformation efficiency of *E. coli* by the digital microfluidic chip, a 705 nL droplet was pipetted from the culture region following 30 min outgrowth. This droplet was immediately diluted with 50 μL LB broth and spread on an LB agar plate containing antibiotics and incubated for 24 h at 37 °C. Fluorescence images of the transformed colonies were taken using a FluorChemQ MultiImage III system equipped with a CCD camera (Alpha Innotech, Santa Clara, CA). Transformation efficiencies were calculated from the average of 5 droplets and represent the number of colony forming units (CFUs) per μg plasmid DNA. These transformation efficiencies were directly compared to the conventional benchtop method performed following previous procedures.[20] Transformation efficiencies were directly compared by unpaired Students *t*-test to determine statistically significant changes. All transformation efficiencies are available in Table 2.

On-Chip Culture. The efficiency of microfluidic transformation and on-chip culture was initially evaluated by imaging droplets with a 4× objective following 1 to 5 days culture. A total droplet fluorescence value obtained by image analysis using ImageJ software was utilized to assess the quality of transformation with fluorescent protein generating DNA. The mean droplet signal was then background subtracted to generate the droplet fluorescence value. Droplet fluorescence is reported as the fold change with respect to the signal produced by control droplets containing cells not transformed with fluorescent genes. High magnification images of cells were obtained by transferring droplets from the device to a coverslip by a pipet.

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The droplet was covered by a second coverslip and then transferred to a microscope for imaging by a 100× objective. All images were taken with an inverted IX73 microscope (Olympus, Center Valley, PA) and iXON+ EMCCD camera (Andor Technology LTD, Belfast, UK).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.6b00011.

Details for reagents, device fabrication, cell preparation, plasmid preparation and transformation protocols. (PDF)

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Notes

The authors declare no competing financial interest.

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