SOFTWARE AUTOMATED GENOMIC ENGINEERING (SAGE) ENABLED BY ELECTROWETTING-ON-DIELECTRIC DIGITAL MICROFLUIDICS

Melissa Sandahl[1], Srikoundinya Punnamaraju[1], Andrew Madison[2], John Harrington[1], Matthew Royal[2], Richard Fair[2], Allen Eckhardt[1], Arjun Sudarsan[1], and Michael Pollack[1]

[1]Advanced Liquid Logic, Inc., Research Triangle Park, NC, USA

[2]Department of Electrical and Computer Engineering, Duke University, Durham, NC, USA

ABSTRA CT

Software automated genomic engineering (SAGE) enables arbitrary genetic modification of bacteria on a fluidic platform that implements the multiplex automated genomic engineering (MAGE) process [1]. Electrowetting-on-dielectric (EWD) digital microfluidics is well suited for SAGE because of its inherent reconfigurability, small reagent volumes, and parallel processing capability [2]. We report on the first demonstration of bulk cell transformation of *E. coli* by an electroporation device integrated with an EWD microfluidics system, which achieved up to 9.8% transformation efficiency (evaluated as the ratio of transformed cells to survived cells) while maintaining fluid transport capability. Toward the goal of enabling efficient MAGE cycling with real time feedback control, monitoring of cell recovery and growth was implemented via reflectance spectroscopy with a limit of detection of about 10^8 cells/ml. Furthermore, simulated MAGE cycles showed that bacteria remained viable for at least 90 cycles (27 days) on-chip.

KEYWORDS

Digital microfluidics, Genome engineering, Electroporation, Lab-on-a-Chip

INTRODUCTION

Recent demand from the synthetic biology community for scalable and parallelizable systems capable of automating efficient genome engineering experimentation is currently unmet. Although electrowetting-on-dielectric (EWD) digital microfluidic (DMF) platforms have demonstrated excellent performance in terms of scalability, automation, reconfigurability, and parallelizability, compared to continuous flow platforms, efficient gene transfer methods remain to be demonstrated in this class of lab-on-chip (LOC) environment [2]. Thus, our current research thrust involves adapting proven gene transfer methods to DMF systems. In particular, our focus has narrowed to on-chip electroporation as utilized by multiplex automated genome engineering (MAGE), a process that allows for directed evolution of cell lines through the repeated introduction of synthetic DNA (Fig 1: a) [1]. Although on-chip genome transformation devices have been reported for chemical transformation [3, 4], simple, hydrostatic electroporation [5], as well as, continuous-flow electro-sonoporation [6], to our knowledge, electroporation has not yet been integrated with an EWD microfluidics platform.

ON-CHIP ELECTROPORATION

Compared to viral vector methods, chemical-based methods, and physical methods such as direct bombardment and injection, electroporation is perhaps one of the simplest and most efficient methods of gene transfer known to date [6]. Moreover, on-chip electroporation device architectures are amenable to fabrication procedures routinely practiced in the development of EWD lab-on-chip systems, *e.g.* printed circuit board (PCB), flex circuits, thin metal film deposition, and electroforming. Thus, the choice of utilizing electroporation as a means of gene transfer in a PCB-based EWD platform is natural.

The digital microfluidic system for SAGE consisted of 4 main modules: (1) Assay Development Environment (ADE) software for developing custom droplet operations such as droplet generation, transport, merging, automated switching between electrowetting and electroporation, and post pulse recovery; (2) instrumentation containing key components such as electrowetting hardware, heaters, cooler, and magnets; (3) fluidics cartridges with patterned electroporation electrodes integrated on a PCB bottom plate with electroporation electrode contact pads for connection to an external

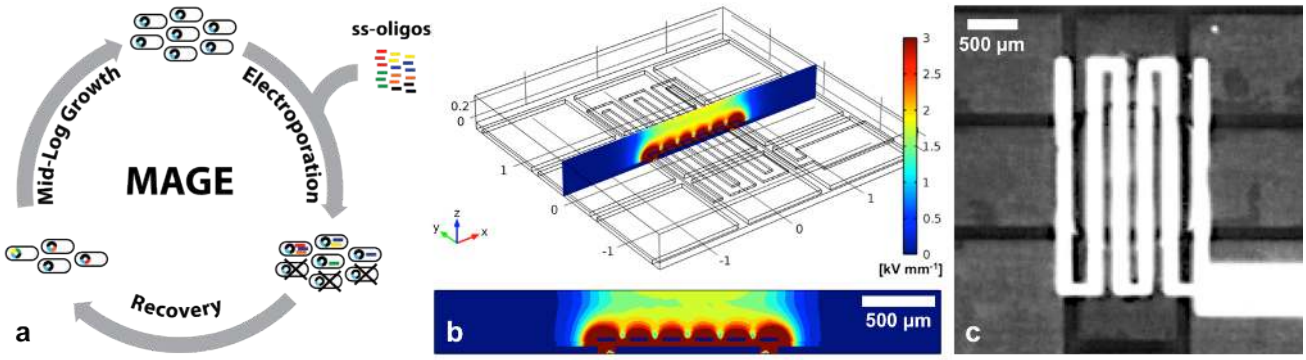

Figure 1: (a) Summary of MAGE; oligonucleotide DNA is combined with electrocompetent E. coli cells that are electrotransformed, recovered, and cultured on-chip [1]. (b) Finite element model (FEM) simulations of the electric field generated by the on-chip electroporation electrodes (c) implemented in Advanced Liquid Logic's SAGE DMF system.

high voltage pulser; (4) Bio-RAD micropulser with high voltage cables clipped to contact pads on the cartridge PCB and top plate. Of the various patterns of electroporation electrodes tested with various modes of pulsing (Fig 2: c-h), significant post pulse bacteria transformation was achieved with a 6-stripe serpentine electrode pattern (Fig 2: c). This design included 6 ~ 125 micron wide stripes, separated by ~ 125 microns on top of electrowetting electrode and dielectrics) and with reverse polarity double pulses polarity on a ~ 700 nL droplet with bacteria and oligonucleotide. Droplet area coverage by these 6 stripe electrodes was up to 70%.

Proper design of integrated EWD and electroporation devices requires consideration of the spatial electric field profile needed for electroporation and the physical footprint of the wires that are mounted over the EWD actuator. When mounted directly on the EWD dielectric, the copper electroporation wires electrostatically shield the droplet contact line from the applied EWD bias; this reduces the electrowetting force and slows down or prevents droplet actuation. Thus, we postulate that the optimum EWD/electroporation device geometry is one in which the volume of the droplet exposed to a target electric field is *maximized* and the total area of the electroporation wires is *minimized*. Finite element model (FEM) electrostatic simulation of the prototype geometry shown in Fig 1: c suggests that around 50% of a 700 nL droplet will be exposed to an electric field of 2.25 ± 0.5 kV mm^{-1} when a 1 kV bias is applied between the serpentine wires and the top plate electrodes.

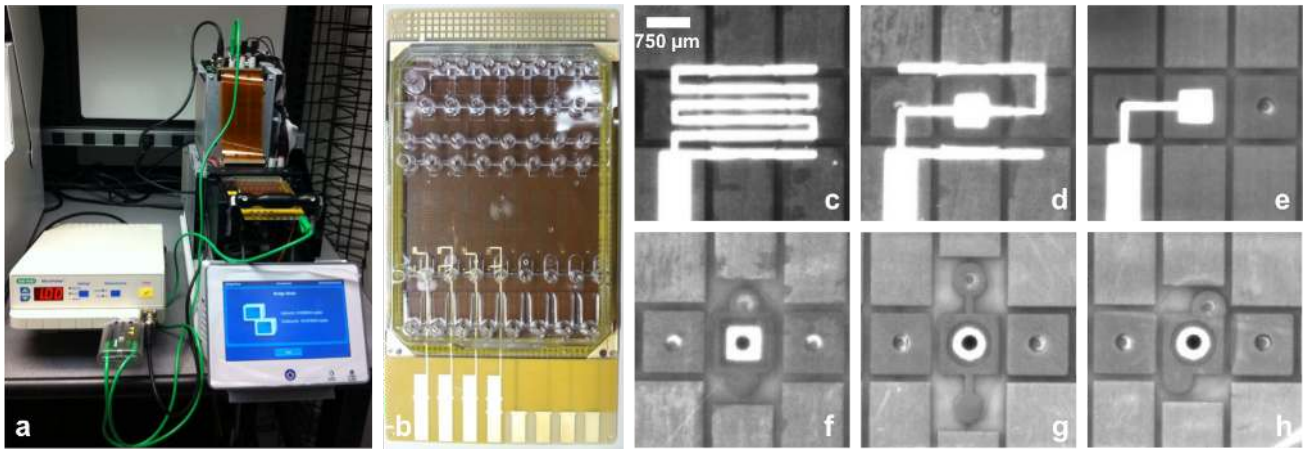

Figure 2: (a) Image of Advanced Liquid Logic's SAGE digital microfluidic system. (c) Image of SAGE prototype cartridge. (c-h) Images of electroporation electrodes integrated on second prototype PCB.

EXPERIMENTAL

Electroporation: To test the efficiency of electroporation on a cartridge with embedded electroporation electrodes, *E. coli* EcNR2-Bla (-) bacteria and the Bla restore oligonucleotide were used (obtained from Harvard University). In this strain of EcNR2, the Bla gene contains a point mutation to knock out the bacteria's resistance to ampicillin. Using MAGE, ampicillin resistance can be restored using Bla restore, a 90-base oligonucleotide to remove the mutation. Transformants were analyzed by growing cells on agar plates that contained ampicillin.

E. coli EcNR2-Bla(-) bacteria were grown off-cartridge in LB growth media to mid-log growth, bound to 1 µm paramagnetic beads coated with mannose binding lectin (obtained from Harvard University), and heat shocked to release MAGE proteins. Samples were then cooled and bacteria bound to beads were separated from the growth media and resuspended in deionized (DI) water with 2 µM Bla restore oligonucleotides. On-bench electroporation was performed in a standard 1x1x3 mm cuvette through application of an exponentially decaying pulse (τ = 6 ms, 1.8 kV peak-voltage) with a Bio-Rad Micropulser. Bench prepared samples were tested for electroporation on cartridge by actuating 700 nL (2x) droplets of bead-bound bacteria and oligonucleotides to electroporation electrodes (Fig 1: c). These droplets were pulsed twice (τ = 6 ms, 0.8 kV peak-voltage) with the devices shown in Fig 2: c-h; the polarity of the second pulse was reversed to counter the non-uniformity inherent in the electroporation field. After electroporation, on-cartridge and on-bench samples were recovered in LB growth media for 3 hours on bench. Samples were then diluted and plated on agar plates to determine survival rates and transformation efficiency. Survival was determined by comparing the number of bacteria with non-pulsed samples after 3 hours of recovery. Transformation efficiency is calculated as the percentage of surviving bacteria that were transformed.

Reflectance Spectroscopy: A simple reflectance spectroscopy strategy was adapted to ALL's DMF platform for on-chip observation of MAGE progress. A white light source (Fiber Lite PL-900) was coupled to the EWD cartridge by a 6:1 (ex:em) bifurcated optical fiber that is terminated, at the sample end, with a flat reflectance probe. The probe was aligned to the LOC cartridge by 3D printed mounts that snap onto ports of the recovery and growth reservoirs. The returning arm of the optical fiber was coupled with Ocean Optics' USB 4000 Mini-spectrometer.

Bacteria concentration was calibrated against the logarithm of the ratio of reflected light measured through a droplet to that measured through the cartridge filler fluid using bacteria suspensions of known concentrations. The optical

density of known samples was verified at 630 nm with a plate reader. Once the reflectance system was calibrated, a solution of bacteria was diluted to $<10^8$ cells/mL and monitored for growth dynamics at 600 nm in LB media for 3 hours.

MAGE Cycle Simulation: On cartridge bacteria culture was demonstrated through 90 growth-dilution cycles spanning 27 days. A droplet of *E. coli* EcNR2 bacteria in LB growth media at 20 million cells/mL was incubated on cartridge at 32°C for 7 hours, then diluted 128-fold by serially diluting the droplet 1:1 with fresh media droplets seven times to simulate the loss of bacteria during pulsing. These cycles were repeated, with droplets collected at varying intervals to determine bacteria concentration within the droplet at the end of a growth cycle. On day 27, the cartridge was no longer electrowetting properly but the droplet was collected and bacterial viability was confirmed by continuing growth on bench. (Fig 3: d).

RESULTS AND DISCUSSION

Electroporation was performed on cartridge and was found to be comparable to bench-top electroporation for the EcNR2-Bla (-) /Bla restore system (Fig 3: a). Average bench electroporation experiments revealed transformation efficiency of 9.7 ± 2.2% while on-chip transformation efficiency of 6 ± 3.4% was achieved on cartridge on the 6 stripe serpentine electrode pattern. Under optimal conditions, a single sample reached 9.8% transformation efficiency, performing as well as on bench electroporation. Optimization of on-chip electroporation will be needed to improve consistency across sample replicates.

Alternative pulsing strategies were utilized to compensate for field non-uniformity present in the on-chip electroporation devices. The electrostatic shielding effect that the electroporation electrodes impart to the EWD actuation electrodes requires that wires of minimal width be used. In turn, as electroporation wires narrow, field non-uniformity tends to increase, as the overall electroporation structure deviates from the ideal, parallel plate electrode case that has been demonstrated in continuous microfluidic formats [5, 6]. Thus, multiple pulses of alternating polarity have emerged as a means of rectifying an intrinsically non-uniform electroporation field.

It is predicted that optical monitoring will be extremely helpful in guiding MAGE experiments. To this end, it appears that reflectance spectroscopy is a viable approach to tracking such experiments in a digital microfluidics platform. For optimal transformation efficiency, bacteria must begin a MAGE cycle at mid-log phase of growth, as the oligonucleotides introduced during electroporation are incorporated into the genome of bacteria that are undergoing

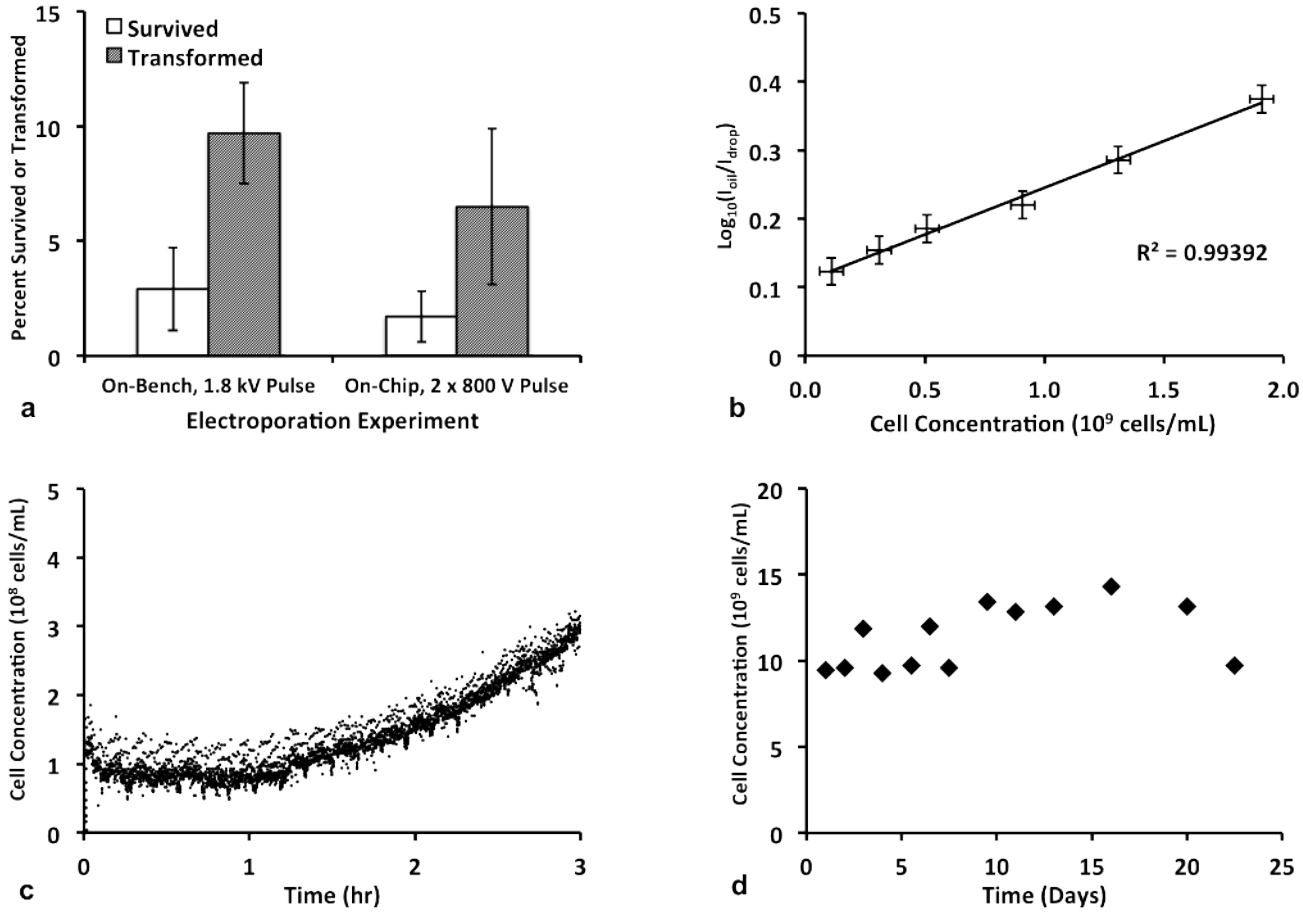

Figure 3: Electroporation and in-process reflectance data; (a) Comparison of bench-top and on-cartridge electroporation of EcNR2-Bla(-) bacteria; (b) Calibration curve of cell concentration and optical density as measured in the reflectance system described above; (c) Time versus cell concentration indicating cell growth in an on-chip recovery reservoir; (d) Cell concentration at the end of the growth period for 16 simulated MAGE cycles over a period of 1 week.

DNA replication [1]. Optically monitoring recovery and growth phases of MAGE provides a direct way of measuring bacteria readiness at the start of each cycle. The calibration of bacteria concentration and optical density revealed a highly linear response (R^2=0.99392) between $2x10^9$ and 10^8 cells/mLwith a limit of detection of 10^8 cells/mL for measurements made of cells in recovery reservoirs using reflectance spectroscopy (Fig: 3 b-c).

Sustained, directed evolution of cell populations through many cycles of MAGE relies heavily on the ability of the fluidic environment to support bacteria growth for a long period of time. Media deoxygenation or bio-fouling are just two pitfalls that could potentially halt an otherwise well planned MAGE run. Thus, it is important to validate the ability of the LOC environment to handle such a task. As Fig 3: d indicates, simulated MAGE cycling was found to be capable of growing EcNR2 bacteria 27 continuous days with as many as (N = 90) growth/dilution iterations on cartridge (Fig: 3 d). The digital microfluidics platform is suitable for extended periods of cell culture.

CONCLUSION

We report the first demonstration of bulk electroporation in an EWD microfluidics platform, optical measurement capabilities for monitoring on-chip cell growth, and on-cartridge cell viability for 90 simulated MAGE cycles. The demonstration of these functions in an EWD microfluidic system is integral to realizing SAGE in a lab-on-chip platform. In moving forward, focus will shift from improving the efficiency of on-chip electroporation to incorporating more steps of MAGE including, magnetic bead-based washing of electroporated cells and software development for handling in-process feedback of optical measurements.

ACKNOWLDEGEMENTS

The authors gratefully acknowledge financial support from the Living Foundries Program supported by the Defense Advanced Research Projects Agency of the United States of America. Additionally, we would like to express gratitude to our collaborators from the Church Lab at the Wyss Institute at Harvard University as well as the Horowitz/Griffin Lab at Stanford University for invaluable discussions that have helped to guide our progress.

REFERENCES:

[1] H. H. Wang, *et al.*, *Nature,* vol. 460, pp. 894 – 898, Aug. 2009.
[2] R. B. Fair, *Microfluidics and Nanofluidics*, vol. 3, no. 3, pp. 245-281, Jun. 2007.
[3] S. H. Au, *et al*., *Biomed Microdevices*, vol. 13, pp. 41-50, 2011.
[4] I. Barbulovic-Nad, *et al.*, *Lab Chip*, vol. 10, pp. 1536-1542, April 2010.
[5] M. Wu, *et al.*, *Anal. Chem.,* vol. 85, no. 9, pp. 4483-4491, April 2013.
[6] W. Longsine-Parker, *et al.*, *Lab Chip*, vol. 13, pp. 2144-2152, April 2013.

CONTACT

R. B. Fair, rfair@ee.duke.edu