Software Automated Genomic Engineering (SAGE) Enabled by Electrowetting-on-Dielectric Digital Microfluidics

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

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

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

ABSTRACT

Software automated genomic engineering (SAGE) enables arbitrary genetic modification of cells 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 13% 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 cells remained viable for at least 16 cycles (7 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 required 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 microfluidics platform included in this study was built using a standard copper PCB process with

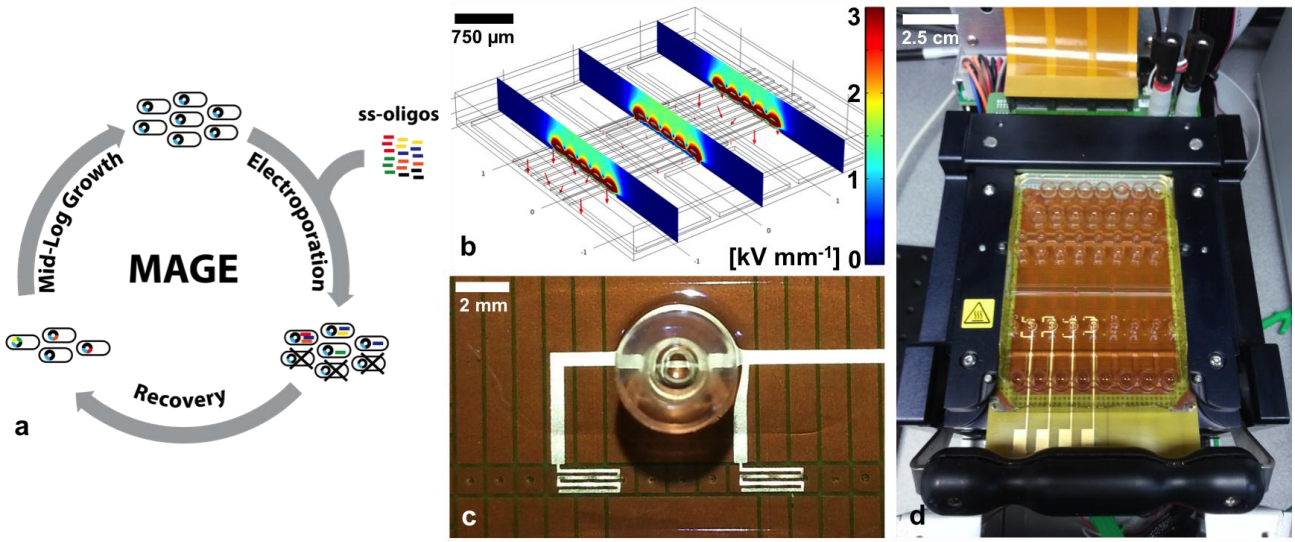

Figure 1: (a) Summary of MAGE; oligonucleotide DNA is combined with electrocompetent E. coli cells that are electrotransformed, recovered, and cultured on-chip [1]. (b) Finite element model (FEM) simulations of the electric field generated by the on-chip electroporation device implemented in a custom PCB/flex circuit cartridge (c) built for Advanced Liquid Logic's (ALLs) digital microfluidics platform (d).

hydrophpbic surface-coated polyimide as the EWD dielectric and a hydrophobic/conductive polymer surface-coated cyclic olefin polymer (COP) top plate. The integrated electroporation devices (Fig 1: b-d) were constructed by transferring serpentine shaped copper wires from a flex substrate directly to the polyimide layer of the EWD PCB. Once the electroporation wires were seated in place, a hydrophobic surface coating was applied to the integrated device stack. The top and bottom plates were then bonded together to complete the EWD/electroporation platform. During operation, the electroporation bias is applied between the serpentine wires and the ground plane located at top plate. In this configuration, the electroporation and EWD circuits share a common ground, the top plate, which means that upon application of the electroporation bias, the electric field within the fluid layer of the device is oriented in the vertical direction, normal to the plane of the PCB (Fig 1: b).

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.

IN-PROCESS MONITORING VIA REFLECTANCE SPECTROSCOPY

A simple reflectance spectroscopy strategy was adapted to ALL's DMF platform for on-chip observation of MAGE progress. For SAGE, cell concentration during the recovery phase of MAGE provides a great process indicator. Once electrotransfer of the ss-oligos has taken place, droplets are transported directly to recovery reservoirs where the transformed cells are grown back up to substantial populations. Throughout the recovery and growth phases of MAGE, the turbidity of the cell suspension increases. Increases in turbidity are measureable via reflectance spectroscopy as a reduction in the intensity of light returning to the spectrometer. Particularly for cells, observation of light attenuation at 600 nm during recovery and growth phases may indicate a successful cycle of MAGE and provides information that can be used in a decision to exit the growth phase and begin the next MAGE cycle.

The reflectance spectroscopy strategy applied herein is quite simple (see Fig 2: a-d). A white light source (Fiber Lite PL-900) is coupled to the EWD cartridge by a 6:1 (ex:em) bifurcated optical fiber (Fig 2: a) that is terminated at the sample end with a flat reflectance probe (Fig 2: c). The probe is aligned to the cartridge by 3D printed mounts that snap onto ports of the recovery and growth reservoirs. The returning arm of the optical fiber couples with Ocean Optics' USB 4000 Minispectrometer (Fig 2: b). Hence, the optical path originates at the white light source and extends through six 600 µm excitation fibers to the top plate of the recovery reservoir and into the cell broth; it then reflects against the PCB surface and is captured by a single emission fiber that directs the attenuated signal to the spectrometer. Any absorption or scattering that occurs from interaction with the cells or the PCB interface attenuates the returning excitation signal. Initial readings, through the silicone oil filler fluid, provide a baseline of PCB reflectance that is subtracted from each measurement of turbidity.

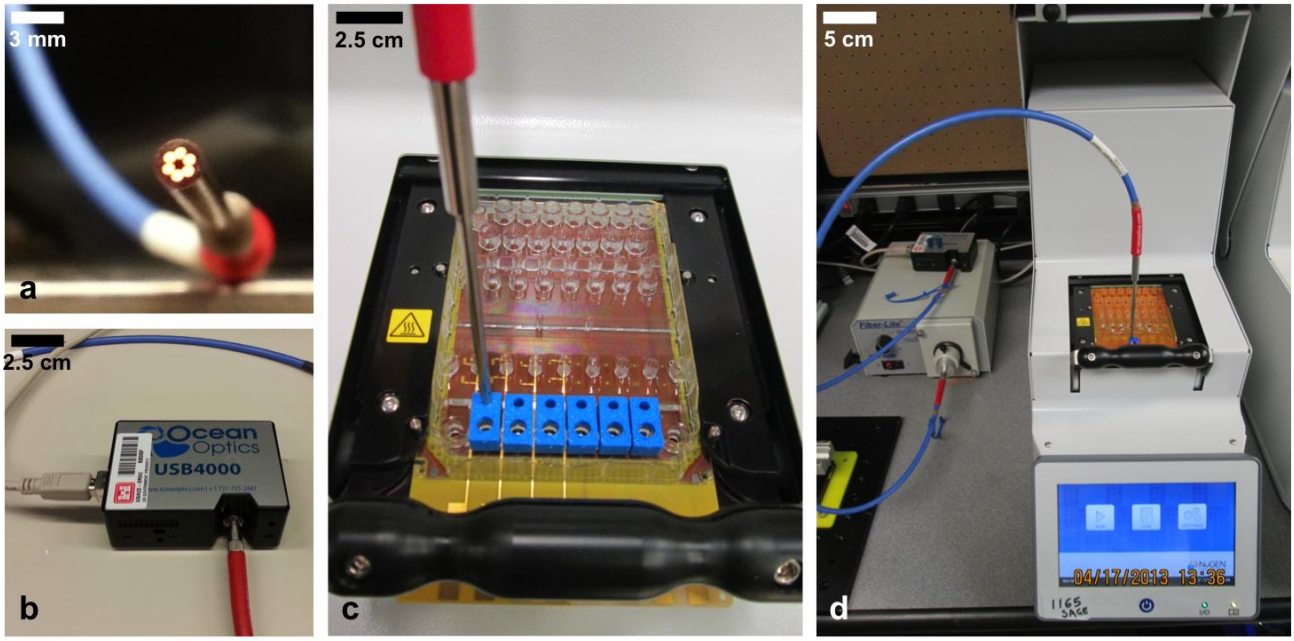

Figure 2: Optical measurement system for in-process monitoring; (a) 6:1 (ex:em) reflectance probe; (b) Ocean Optics USB 4000 Mini Spectrometer; (c) Cartridge and 3D printed probe mounts; (d) ALL digital microfluidics platform shown with Fiber Lite PL-900 white light source, Ocean Optics Mini Spectrometer, and reflectance probe.

EXPERIMENTAL

Electroporation: *EcNR2-(GalK-)* cells were grown off-cartridge in LB growth media to mid-log growth phase, then separated from the growth medium and re-suspended in de-ionized (DI) water with 10 ng/µL *GalK* recovery 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. For comparison, on-chip electroporation was tested by actuating 700 nL (2x) droplets of oligonucleotides and cells to electroporation electrodes (Fig 1: c). These droplets were pulsed twice (τ = 6 ms, 0.75 kV peak-voltage) with the devices shown in Fig 1: b-d; the polarity of the second pulse was reversed to counter the non-uniformity inherent in the electroporation field. After electroporation, droplets were actuated to recovery reservoirs containing galactose-rich LB growth media for 3 hours, while on-bench samples were transferred to a similar recovery medium. Samples were then plated and resulting colonies tallied and compared with plates of initial cell populations for calculation of mortality and transformation efficiency.

Reflectance Spectroscopy: The reflectance measurement system described above was calibrated for observation of cell growth in the recovery reservoirs of ALLs digital microfluidics platform. Cell concentration was calibrated against the logarithm of the ratio of reflected light measured through a droplet to that measured through the silicone oil using cell suspensions of known concentrations. The optical density of the known samples was verified at 600 nm with a plate reader. Once the reflectance system was calibrated, a solution of cells was diluted to $<10^8$ cells/mL and monitored for growth dynamics at 600 nm in LB media for 3 hours.

MAGE Cycle Simulation: Cell morbidity associated with electroporation was simulated by repeated 128-fold dilutions on-chip. For each cycle, cells were grown for 8 hours in a reservoir on the cartridge and concentration was measured off-chip by a plate reader from an aliquot. 16 cycles were achieved over a period of 7 days (Fig 3: d).

RESULTS AND DISCUSSION

An average on-chip transformation efficiency of 9.7 ± 3.4 % was demonstrated for two 750 V, τ = 6 ms reversed polarity pulses. By comparison, average bench-top electroporation experiments revealed a comparable average transformation efficiency of 9.0 ± 1.8 % for a single 1.8 kV, τ = 6 ms pulse. The calibration of cell concentration and optical density revealed a highly linear response (R^2=0.99392) and a limit of detection of 10^8 cells/mL for measurements made of cells in recovery reservoirs. Simulated MAGE cycling was found to be capable of many (N > 16) growth/dilution iterations.

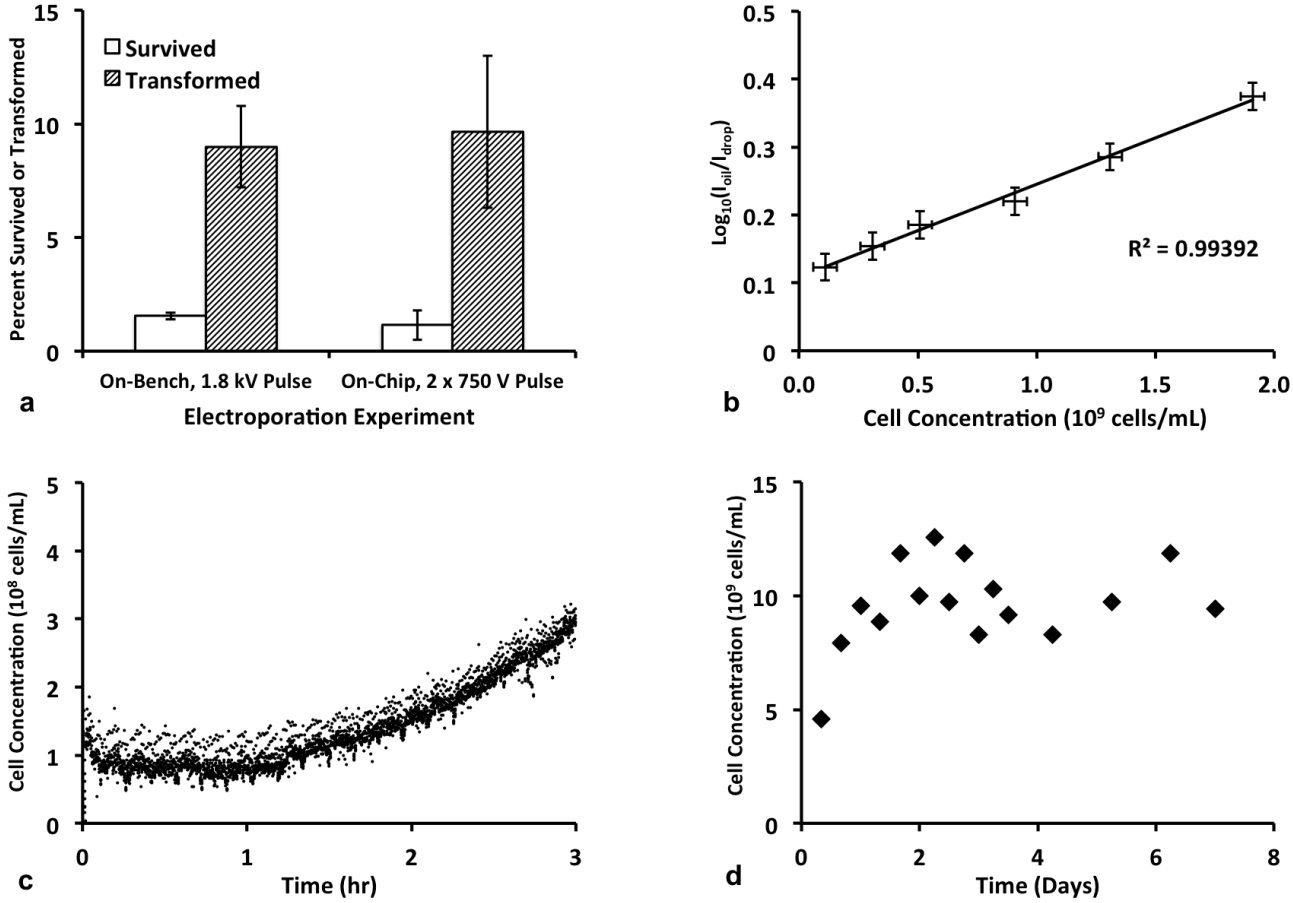

Figure 3. Electroporation and in-process reflectance data; (a) Comparison of bench-top and on-cartridge electroporation of EcNR2-(GalK-) cells; (b) Calibration curve of cell concentration and optical density as measured in the reflectance system described above; (c) Time versus cell concentration indicating 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.

As Fig 3: a suggests, on-chip electroporation was found to be comparable to bench-top electroporation for similar cell/oligonucleotide combinations (*EcNR2-(GalK-)* / *GalK* recovery). However, 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. As outlined in [1], selection pressure is often applied in the recovery and growth phases of MAGE. In the case presented here, *EcNR2-(GalK-)* cells are incapable of converting ß-galactose, as they are deficient of the gene encoding for ß-galactosidase. Electroporation in the presence of *GalK* recovery oligonucleotides remedies this deficiency. However, any original cells that go untransformed can be selected against by recovering electroporated droplets in galactose-rich LB media, as these cells will lack any carbon source to convert. Thus, optically monitoring recovery and growth phases of MAGE in conjunction with the application of selection pressure provides a direct way of measuring the transformation process *in-situ.*

Sustained, directed evolution of cell populations through many cycles of MAGE relies heavily on the ability of the fluidic environment to support cell 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 during initial stages of process development. As Fig 3: d indicates, the digital microfluidics platform under test in this set of experiments 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 at least 16 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.

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CONTACT

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