Individually addressable multi-chamber electroporation platform with dielectrophoresis and alternating-current-electro-osmosis assisted cell positioning

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A multi-functional microfluidic platform was fabricated to demonstrate the feasibility of on-chip electroporation integrated with dielectrophoresis (DEP) and alternating-current-electro-osmosis (ACEO) assisted cell/particle manipulation. A spatial gradient of electroporation parameters was generated within a microchamber array and validated using normal human dermal fibroblast (NHDF) cells and red fluorescent protein-expressing human umbilical vein endothelial cells (RFP-HUVECs) with various fluorescent indicators. The edge of the bottom electrode, coinciding with the microchamber entrance, may act as an on-demand gate, functioning under either positive or negative DEP. In addition, at sufficiently low activation frequencies, ACEO vortices can complement the DEP to contribute to a rapid trapping/alignment of particles. As such, results clearly indicate that the microfluidic platform has the potential to achieve high-throughput screening for electroporation with spatial control and uniformity, assisted by DEP and ACEO manipulation/trapping of particles/cells into individual microchambers.

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I. INTRODUCTION

Electroporation has received much attention in the biological field as a powerful tool which can physically introduce foreign molecules into the intra-cellular space with enhanced transfection efficiency.^{1,2} Application of a high electric field impulse induces a transmembrane potential exceeding a critical threshold, creating nanopores in the cell membrane, which enable the introduction of membrane-impermeable exogenous molecules into the cells or conversely the release of intracellular components from the cells.^{3,4} The transmembrane potential, $\Delta\psi_E$, induced by external electric field can be approximated as $\psi_E = 1.5aE\cos\theta$. Herein, a is the cell radius, E the external electric field, and θ the angle between the direction of electric field and the radial direction with the origin located at the cell center.^{3,5} The specific parameters of the applied electric field (i.e., pulse amplitude, pulse duration, and number of pulses) determine whether the electroporated cell membrane can be resealed rapidly, ensuring cell viability (i.e., reversible electroporation) or if it will be disrupted permanently resulting in cell death (i.e., irreversible electroporation/cell lysis). Both states of electroporation are useful in different applications. For example, reversible electroporation may be used to deliver genes,^{6,7} proteins,^{8,9} or drugs^{10,11} while an irreversible electroporation is necessary for extracting cellular materials from the cells¹² or tumor ablation.¹³ Determination of the optimal applied field conditions is challenging due to the large number of parameters and strong dependence of the outcome on the specific cell-type, device and environmental conditions.

Microfluidic based electroporation units can overcome several of the problems associated with conventional systems, such as high applied voltages (kV), complicated handling procedure

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and high cost, owing to their unique characteristics of miniaturization. A number of specific microfluidic based electroporation platforms have recently been developed for gene transfer,^{8,14,15} cell lysis,^{16,17} single cell level analysis,^{18–26} as well as more general array or flow-through systems capable of high throughput screening.^{27–32} However, development of electroporation microsystems is still in its early stages, necessitating further research to guarantee maximum harnessing of the unique potential of microscale physics to produce highly sophisticated microfluidic platforms which can maintain precise control of cell manipulation, high uniformity and efficiency, and real-time monitoring the cell transfection behaviour.³³

In order to improve the efficiency of the electroporation assay, it is highly advantageous to exploit the microfluidic platform capability of integration of multi-functionality,³⁴ e.g., combining dielectrophoresis (DEP)-assisted cell manipulation with electroporation^{14,25,35} or the coupling of electroporation with capillary electrophoresis (CE) and electrochemical detection.³⁶ Optically induced DEP has previously been used to manipulate cells to precise locations where electric field is enhanced, resulting in high transfection efficiency.^{25,37} The transport rate of the molecules crossing the electroporated cell membrane is further enhanced by electrophoresis³⁸ or hydrodynamic vortex flow³⁹ compared to the case of molecular diffusion alone.

In the current study, we use DEP for both cell/particle manipulation and pre-concentration. Its ease of integration with other electrical functionalities, simplicity in design, biocompatibility in terms of cell viability, and the ability to shift from positive DEP (p-DEP) to negative DEP (n-DEP), i.e., attractive to repulsive response, are a few of the many advantages of this powerful technique.⁴⁰ In addition, at sufficiently low frequencies, alternating-current-electro-osmosis (ACEO), a non-linear electrokinetic effect which results from the action of the applied electric field on its own induced electric double layer (EDL),^{41,42} becomes apparent. Since ACEO is a long range convective force, it may complement the short range DEP forces, resulting in an enhanced trapping/alignment of the particles.⁴³

Here, we develop a novel, multifunctional platform with potential for high throughput electroporation which integrates dielectrophoretic trapping/positioning and electroporation mechanisms in a multi-chamber array where each chamber may be individually activated. The key advantage of our platform lies in its ability to produce a spatial gradient of electroporation parameters (i.e., pulse amplitude, duration, and number) and to increase electroporation efficiency due to DEP-assisted cell trapping or positioning. Additionally, the use of transparent indium tin oxide (ITO) electrodes, rather than the common opaque metal electrodes, enables visualization of cell transfection in real time. As a proof of concept, platforms with an array of 40×2 microchambers were fabricated and tested to demonstrate the feasibility of integrating the two functionalities: (1) the manipulation of the particles/cells using DEP with/without ACEO and (2) gradient electroporation using both delivery membrane-impermeable foreign molecules and release of intracellular molecules. For proof of concept purposes, it was suffice to separately test these two functionalities, although, it may be sometime desirable to perform these in a consequent order (e.g., manipulation of cells to desired locations followed by electroporation). In the following, we describe the experimental methods in Sec. II, the results and discussion in Sec. III, and concluding comments in Sec. IV.

II. MATERIALS AND METHODS

A. Design of electroporation platform

The developed microfluidic platform consists of two ITO-patterned glass substrates which sandwich a patterned SU8 structure and a film of polydimethylsiloxane (PDMS; Sylgard 184 Silicone elastomer kit, Dow Corning) (Fig. 1). The patterned SU8 structure defines the geometry of the channels and micro-chamber array and serves as a spacer between the top and bottom substrates. The corresponding patterned ITO electrode array potentially enables the individual activation of each microchamber to produce an electroporation gradient. However, for simplicity reasons, every four microchambers were shared with one bottom electrode connection. Its parallel plate design may be exploited to produce a uniform intensity within each of the microchambers. Additionally, by simply not applying a pulse, a “control” chamber may be formed

FIG. 1. (a) Schematics of the combined DEP-electroporation microfluidic platform; (b) Cross-sectional layered view; and (c) top-view micrograph of the platform.

with *identical* conditions to those existing within the active cell chambers. Each microchamber and electrode is $400\ \mu\text{m}$ in length, $200\ \mu\text{m}$ in width and is separated from adjacent chamber by $150\ \mu\text{m}$ via the SU8 wall. In order to prevent leakage of solution between the two plates, a patterned PDMS film was coated onto the top ITO electrode. The gap ($65\ \mu\text{m}$) between top and bottom ITO electrodes is determined by the thickness of PDMS film ($50\ \mu\text{m}$) and the SU8 structure ($15\ \mu\text{m}$).

Due to the parallel plate electrode structure, the electric field is uniformly distributed within the microchamber array, except at the edges of the chambers, as demonstrated using a finite-element based three-dimensional (3D) simulation (ComsolTM 4.3) shown in Fig. 2. The field magnitude at the edge of the electrode is approximately three times higher than those at the center of the chamber. Hence, in addition to uniform electroporation, this electrode design enables the manipulation of cells/and or particles using DEP forcing. In particular, Fig. 2(c) indicates the existence of high electric field gradients along the microchamber edges. In particular, the edge of the ITO electrode, which coincides with the entrance of each microchamber, may act effectively as an on-demand gate to the microchamber. However, this necessitates further improvement of the microchamber isolation (e.g., eliminating or using a much thinner PDMS film) as with the current $50\ \mu\text{m}$ thick top PDMS layer, particles/cells can flow over the microchambers.

B. Fabrication process of electroporation platform

The layered view of the fabricated platform is illustrated in Fig. 1(b). To fabricate the top and bottom electrode layer, ITO coated glass slides were pre-cleaned with Acetone/Methanol/deionized water. Prior to patterning the bottom ITO electrode layer, metal of Au/Ti (200 nm/30 nm) was deposited onto the electrode pads by a lift-off process which enables easy alignment during the multi-step photolithography process. Afterwards, the ITO was patterned using standard photolithography and wet etching processes.⁴⁴ To create the walls of the microfluidic channels including the micro-chamber array, $15\ \mu\text{m}$ thick SU-8 negative photoresist (SU-8 2015, MicroChem) was then patterned on the ITO electrode array. For the top cover plate, a bare ITO coated glass was spin-coated (5000 rpm) with a $50\ \mu\text{m}$ thick PDMS film as an intermediate layer. In order to leave the top area of the microfluidic channel and chamber array exposed to ITO, a 1 mm-width Scotch tape was simply attached to the top cover plate before PDMS coating. After peeling off the tape, the PDMS film was cured on hotplate at 90°C for

FIG. 2. Numerical simulation of the electrostatic problem; (a) 3D geometry of the periodic cell; (b) the norm of the electric field; and (c) gradient of electric field squared. A uniform electric field is obtained within the microchamber except at the edges where strong field gradients exist.

10 min, and 1.0 mm-diameter inlet holes were mechanically drilled. The fabricated top and bottom plates were then assembled and packaged using a mechanically screwed holder.

C. Preparation of cell suspension, fluorescent probes

Normal human dermal fibroblasts (NHDF-Ne, Lonza Walkersville, Inc.), Passage 8, were maintained in cell culture medium (L-glutamine containing Dulbecco's Modified Eagle medium, Gibco) supplemented with 10% fetal bovine serum (FBS, HyClone), 1% Pen-Strep (Biological Industries), 1% minimum essential medium non-essential amino acids (Gibco) and 0.1 mM 2-mercaptoethanol (Gibco) at 37 °C, and 5% CO₂ environment. The cells were consistently harvested at 80% confluency by using Trypsin (Invitrogen). The separated cells were re-suspended in cell medium with a nominal cell concentration of 1.5×10^6 cells/ml for electroporation. For the assay of intracellular release and short term cell cultures, red fluorescent protein expressing human umbilical vein endothelial cells (RFP-HUVECs, Angio-Proteomie), Passage 8, were cultured in the cell medium supplemented with 5% FBS, 1% Pen-Strep, 1% minimum essential medium non-essential amino acids, and 0.1 mM 2-mercaptoethanol at 37 °C and 5% CO₂ environment.

D. DEP-activated cell/particle manipulation

In order to examine the DEP-assisted cell/particle manipulation, 1 μ m fluorescent polystyrene beads (Fluoro-Max, Thermo Scientific) were diluted to volumetric concentrations of $2 \times 10^{-4}\%$ in Deionized (DI) water ($\sigma = 0.6 \mu\text{S/cm}$) before being loaded into the platform. The crossover frequency (COF) of the particles in DI is approximately 2.2 MHz as measured using an interdigitated electrode array.⁴⁵ Different AC field frequencies with a sinusoidal waveform demonstrating p-DEP (20 kHz and 20V_{pp}) and n-DEP (5 MHz and 20V_{pp}) responses, respectively, were applied to one row of the chamber array using function generator (33250A, Agilent), while the other row was left as a control (no field). Due to the multiple stages of loading/exchange of medium in electroporation, it is necessary for the cells/particles to remain

fixed within the microchambers under pressure-driven flow conditions. This was demonstrated for a pressure driven flow of $2 \mu\text{l}/\text{min}$ using $1 \mu\text{m}$ and $2 \mu\text{m}$ fluorescent polystyrene beads with various AC field frequencies ranging from 1 kHz to 5 MHz with $20V_{\text{pp}}$ amplitude.

Cells suspended within physiological cell medium ($\sigma = 16 \text{ mS}/\text{cm}$) were manipulated only at high frequencies (5 MHz and 50 MHz) and $20V_{\text{pp}}$ to observe repulsion due to n-DEP. This is due to electrolysis along with bubble generation occurring at low frequencies⁴⁶ while in the intermediate frequency range the DEP may induce electroporations on cells.^{17,25,47} The motion of particles and/or cells through the microchannel/chamber interface was recorded using an Andor Neo sCMOS camera attached to a Nikon TI inverted epi-fluorescent microscope with $10\times$ objective lens.

E. ACEO-activated cell/particle manipulation

At sufficiently low frequencies (on the order of a few kHz), ACEO becomes a significant contributor, resulting in steady fluid flow velocity that depends on the applied frequency and scales quadratically with the voltage. The frequency dependence of the fluid velocity shows a maximum, tending to zero as the frequency goes to zero or infinity. The maximum velocity is obtained at an angular frequency $\omega_0 \approx (\lambda_D L/D)^{-1}$ (where, λ_D is the Debye length, L the characteristic length scale, and D the diffusion coefficient of the background ionic species) which typically range from $0.1\text{--}10 \text{ kHz}$ depending on the conductivity of the medium. It is noted that this characteristic frequency lies significantly below the charge relaxation time of the electrolyte, $\omega\epsilon/\sigma \ll 1$ ($\epsilon/\sigma = \lambda_D^2/D$, wherein, ϵ and σ are the permittivity and conductivity of the medium, respectively) so that the EDL may be presumed to be in quasi-equilibrium. For simplicity reasons we follow the analyses of Ramos *et al.*⁴¹ and Green *et al.*,⁴² where it is assumed that electrolysis does not occur at the electrode surfaces and the electrodes may be considered as ideally polarizable. Additionally, in accordance with the weak field assumption wherein the applied voltages are small relative to the thermal potential, we neglect any surface conduction or convection within the EDL so that the double layer may be modelled as a linear capacitor and the hydrodynamic and electrostatic problems are decoupled ($Pe = 0$), thus, significantly simplifying the analysis. Since the EDLs are thin relative to the other channel dimensions ($\lambda_D/L \sim O(10^{-3})$), we can solve the Laplace equation for the electric potential

$$\nabla^2 \phi = 0, \quad (1)$$

in conjunction with the following boundary condition at the ITO electrode surfaces

$$\sigma_m \frac{\partial \phi}{\partial y} = i\omega C_{DL}(\phi - V_j), \quad (2)$$

which describes the oscillatory Ohmic charging of the induced EDL as a response to an AC potential applied at the electrodes, of magnitude V_j (j is the electrode's index), and frequency ω . The parameter C_{DL} represents the capacitance per unit area of the total EDL (diffuse plus Stern layers), expressed as $C_{DL} = \Lambda C_d$, where $\Lambda = (1 + C_d/C_s)^{-1}$, C_s and C_d are the Stern and diffuse layer capacitances, respectively. Based on the Debye-Huckel theory, it can be approximated as $C_{DL} = \epsilon/\lambda_D$ (assuming a Stern capacitance is much larger than the diffuse layer capacitance, i.e., $\Lambda \approx 1$). On the bare glass substrate, SU8 and PDMS walls, as well as between the boundaries of the periodic cell, an insulating/symmetry condition is used,

$$\frac{\partial \phi}{\partial y} = 0. \quad (3)$$

Solution of the boundary value problem (Eqs. (1)–(3)) then yields the electric potential distribution within the channel domain. Based on the thin EDL approximation, the velocity field may be obtained using the unforced Stokes equation, accounting for the electric forcing by simply

prescribing an effective slip velocity at the electrodes. The time-averaged slip velocity boundary condition on top of the electrodes is^{41,42}

$$\langle u \rangle = -\frac{\varepsilon}{4\eta} \Lambda \frac{\partial}{\partial x} \left[(\phi - V_j)(\phi - V_j)^* \right], \quad (4)$$

wherein the symbol* indicates the complex conjugate and the brackets $\langle \rangle$ stands for time averaging over a single period. Finally, at the other physical boundaries, i.e., glass substrate, SU8, and PDMS walls, the no slip boundary conditions were used since the linear electroosmotic flow (EOF) contribution has a zero time-average effect under AC forcing.

The 3D numerical simulation was performed in COMSOL 4.3. A general partial differential equation (PDE) module was used to solve the electrostatic equations (Eqs. (1)–(3)). The creeping flow module, in conjunction with a slip boundary condition was used to solve the hydrodynamic flow. User-refined triangular and tetrahedral mesh elements were used in both main channel and microchamber. The entire geometry was divided into three sub-sections (microchamber, main channel and the gap formed between the SU8, and the top ITO surface) in which maximum and minimum mesh element size varied. The maximum and minimum lengths were limited to 25 μm and 140 nm, respectively.

F. Electroporation of cells and viability

The electroporation effect was quantified by analyzing the percentage of cells stained by propidium iodide (PI) and/or fluorescein diacetate (FDA) (Invitrogen). Since the membrane is impermeable to PI, its uptake by cells is an indication that exogenous molecules are diffused across the membrane due to electroporation.^{14,23,48} Since PI staining is indifferent to cell viability, we used FDA staining of cells in order to distinguish between reversible and irreversible electroporation which results in cell death. Hence, intersection of these two tests, i.e., cells that are simultaneously stained by both PI and FDA, is an indication of “successful reversible (reversible) electroporation” and thus their percentage indicates the electroporation efficiency. PI with concentrations of 20 $\mu\text{g/ml}$ was added to the cell suspension loaded into the platform. Electroporation parameters were varied by altering the electric pulse parameters applied to the microchamber array using an electrical voltage source (Keithley 2636). For simplicity, identical pulses were applied to every four chambers in each experiment to increase the number of cells (~ 60) to be quantified. A time window of 30 min was used to observe the cell’s uptake of PI, during which the cells could attach onto the ITO surface. This was followed by staining of cells by FDA prepared with a concentration of 100 $\mu\text{g/ml}$ within the cell medium. To do so, 5 μl of FDA solution was introduced into the microfluidic device at a relatively low flow rate (approximately, 5 $\mu\text{l/min}$), so as to keep most cells in their original position, after which viable cells were stained by FDA for a few minutes. Thereafter, the electroporated cells in the chamber array were optically monitored for an additional 5 min. During the entire test, a Nikon TI inverted epi-fluorescent microscope with a 10 $\times$ objective lens and an Andor Neo sCMOS camera were used for visualization of cell’s response by PI and FDA molecules, with sets of red fluorescence filter (EX525/540, DM 565, BA 605/55) and green fluorescence filter (EX465/495, DM:505, BA:515–555) for PI and FDA, respectively. The captured images were processed using image processing software (ImageJ, NIH). To prevent analyzing migrating ghost cells, we kept track of the captured cells in each chamber during the entire image processing. Also the cells located at the edges of chambers were neglected in the analysis because of the non-uniform and intensified electric field. The change of intensity of the cell nucleus, as a result of PI penetration, can be normalized as follows:

$$I^* = \frac{I - I_0}{I_f - I_0}, \quad (5)$$

where I^* is the normalized intensity, and I , I_0 , and I_f are the intensities of a given PI-stained cell, non-stained cell, and fully PI-stained cell (i.e., dead cells which were stained with PI before electroporation), respectively. For each experimental conditions (i.e., electroporation

parameters) over 100 cells, distributed through several chambers, were simultaneously examined. Each test was repeated at least three times.

G. Release of intracellular molecules by electroporation and cell culture

Due to the toxicity of PI, cells stop growing when the nucleus is stained by PI. In order to examine the cell growth after electroporation and as an additional indication of cell viability, as well as to better determine the boundary between reversible electroporation and cell-lysis, RFP-expressing cell suspension within the medium was used. The release of RFP molecules from the cell indicates a direct disruption of the cell membrane by irreversible electroporation without additional steps of loading molecules (e.g., Calcein AM) inside the cell.^{17,28} The loaded cells were then exposed to the same electroporation parameters as in the PI uptake experiments. After electroporation, the decay of the fluorescent intensity of the cell indicating the release of RFP molecules, was monitored by time-lapse image capturing, in which the interval between frames is set at 2 s, for 40 min using inverted epi-fluorescent microscope with a 10 $\times$ objective lens and a set of red fluorescence filter. The intensity of each cell was extracted from the recorded time-lapse images and further analysed using MATLAB program. The platform was then placed in the incubator for 24 h and was optically inspected for cell growth. The recorded intensity of each using Matlab program.

III. RESULTS AND DISCUSSION

A. DEP manipulation of particles/cells

Figure 3 shows typical motions of 1 μm -diameter polystyrene particles and cells activated by p-DEP/n-DEP forces without background flow. The DEP forces are most pronounced at the

FIG. 3. (a) Schematics of the p-DEP and n-DEP response of microparticles/cells within the microchamber. 1 μm particles are either (b) trapped or (c) repelled from the microchamber edges via p-DEP or n-DEP, respectively; Similar n-DEP behavior is experienced by cells before (d) and after (e) applying DEP. (Multimedia view) [URL: <http://dx.doi.org/10.1063/1.4873439.1>] [URL: <http://dx.doi.org/10.1063/1.4873439.2>]

chamber entrance and edges, wherein large field gradients exist (Fig. 2(c)). Thus, DEP is shown to enable both particle trapping at the chamber entrance and edges by p-DEP at 20 KHz (Fig. 3(b)) or their repulsion towards the center of the chamber and away from the entrance by n-DEP at 5 MHz (Fig. 3(c) (Multimedia view)). In the case of cells, the used physiological buffer solution with high conductivity results in the cells being restricted only to n-DEP.^{40,45,49} Lowering buffer conductivity could potentially yield a p-DEP response but at the same time may result in loss of cell viability because of the toxicity of DEP buffer solution.⁴⁵ In Fig. 3(e) (Multimedia view), we show that application of n-DEP conditions (5 MHz with 20Vpp) causes the cells located at the chamber edges to move towards its center and away from the entrance, resulting in more uniform electric field conditions. It is advantageous to operate the chip under relatively high frequencies when cells within physiological medium are involved, since Faradaic reactions at the electrodes⁴⁶ and cell electroporation induced by the AC field itself⁴⁷ are both diminished with increasing frequencies. The geometry of the current chip ensures localization of the DEP forces at the microchamber edges and entrance and facilitates particle/cell manipulation under either p-DEP or n-DEP modes, although the mode of DEP (n-DEP or p-DEP) is not controlled by the geometry.

B. ACEO assisted particle trapping

The onset of ACEO occurs at frequencies on the order of $\sim 10^2\text{Hz}$ – 10^3Hz , corresponding to the characteristic RC time, defined as $\lambda_D L/D$ wherein for DI water, $\lambda_D \sim 100\text{ nm}$, $L \sim 100\text{ }\mu\text{m}$, and $D \sim 10^{-9}\text{ m}^2/\text{s}$ so that $\omega \sim 100\text{ Hz}$. The ACEO streamline pattern obtained from numerical solution of Eqs. (1)–(3) at a frequency of 100 Hz, within a realistic 3D geometry (Fig. 2(a)) representing the current microchamber setup is depicted in Figure 4 and shows three distinct types of vortices: a single vortex with its axis aligned along the microchamber entrance (i.e., “microchamber entrance vortex”) which coincides with the edge of the bottom ITO electrode, an in plane vortex pair that drives colloids from the main channel into the microchambers and an additional two vortices, each with its axis aligned along the microchamber side edges (“microchamber side vortex”). From symmetry considerations, two stagnation lines are formed along the center of the microchamber as evident in Fig. 4(b) which illustrates the cross-sectional streamline pattern of the microchamber side vortices and scalar plot of the velocity norm.

The combined role of ACEO and DEP is demonstrated (Figs. 5(a)–5(d) (Multimedia view)) for the case of $1\text{ }\mu\text{m}$ (diameter) particles at 20 kHz and 20Vpp (with some small unintentionally pressure-driven background flow due to unbalanced reservoirs levels), depicting the time evolution of the entrance vortex and the in-plane vortex pair along with their trapping at the microchamber edges wherein the largest p-DEP forces exist (in accordance with Fig. 2(c)). Such a combined effect is also demonstrated in Figures 5(e) and 5(f) (Multimedia view) for $2\text{ }\mu\text{m}$ -diameter polystyrene particles suspended in DI water with pressure-driven background flow (volumetric flux of $2\text{ }\mu\text{l/min}$). There, the long range ACEO flow, which results in a vortex

FIG. 4. 3D Numerical simulation of the hydrodynamic problem of ACEO. (a) top-view showing the streamline pattern with 3 distinct vortex type; cross-sectional streamline pattern of the (b) microchamber side vortices and scalar plot of the velocity norm indicating the location of the stagnation lines and (c) microchamber entrance vortex.

FIG. 5. (a)–(d) Dynamics of 1 μm (diameter) particles at 20 kHz and 20V_{pp}, without pressure driven background flow, depicting the time evolution of the entrance vortex and the in-plane vortex pair along with their trapping at the microchamber edges wherein the largest p-DEP forces exist (in accordance with Fig. 2(c)); (e)–(f) Dynamics of 2 μm (diameter) particles at 1 kHz and 20V_{pp}, with pressure driven background flow, depicting their trapping at the microchamber entrance due to p-DEP and their accumulation at the stagnation line formed at the center of each microchamber and driven by its side vortices (see Fig. 4(b)). (Multimedia view) [URL: <http://dx.doi.org/10.1063/1.4873439.3>] [URL: <http://dx.doi.org/10.1063/1.4873439.4>]

along the entrance, feeds particles from the main channel towards the chamber entrance region, where they are trapped due to the short range attractive p-DEP forces. In addition, due to the ACEO vortex pair created within the chamber, the particles are accumulated along its center line which coincides with the vortex stagnation line. Since the cells suspended within physiological cell medium were manipulated only at high frequencies (5 MHz and 50 MHz), in order to avoid electrolysis and cell electroporation, ACEO was hindered.

C. PI uptake by electroporation

Electroporation optimization and cell viability were performed by applying a gradient of electroporation pulse parameters (i.e., a single square pulse with 10 different amplitudes from

2 V to 20 V and durations of 0.5, 1, and 3 ms). Neglecting the voltage drop across the electrodes EDLs, this voltage range corresponds to an electric field range of 0.3 to 3 kV/cm. Figure 6(a) shows the micrographs of the PI/FDA stained cells in the chamber with various electric pulse parameters 40 min after electroporation. For ease of visualization, the bright field image (gray scale) was merged with two different fluorescent images taken at the same location, wherein the red and green colors represent PI and FDA stained cells, respectively. The results clearly illustrate that as both pulse amplitude and duration are increased, the number of PI-stained cells increases, while cell viability decreases (Figs. 6(a) and 6(b)). At pulse durations over 1 ms, pulse amplitudes exceeding 14 V (~ 2.15 kV/cm) led to considerable PI-uptake of the cells with visibly wrinkled cell membrane morphology, indicating considerable irreversible electroporation and cell death ($\sim 95\%$). At lower amplitudes, less than 4 V (~ 0.61 kV/cm), the electroporated cells under all three pulse durations had negligible PI-uptake which indicates the absence of electroporation. It is also noted that in the control group, i.e., no applied voltage, more than 95% of cells remained unstained by PI.

FIG. 6. Electroporation of PI into the NHDFs at various pulse amplitudes and durations with single pulse of 0.5, 1, 3 ms pulse duration. (a) Micrographs of cells in microchamber stained with PI (red) and FDA (green) 40 min after electroporation. For visualization, the image in bright field (gray-scale) and two different fluorescent images at the same location were merged together. (b) Transfection efficiency of PI and cell viability, (c) Electroporation efficiency, and (d) Normalized intensity of PI stained cells are shown versus pulse amplitude at different pulse durations. The grey and blue transparent rectangular backgrounds represent appropriate regimes of reversible electroporation at pulse durations of 1 and 0.5 ms, respectively.

The electroporation efficiency (Fig. 6(c)) can be quantified as the percentage of cells that are both PI and FDA stained, i.e., indicating both successful electroporation and cell viability. Each of the graph in Fig. 6(c) corresponds to a fixed pulse duration, and exhibit a maximum at a voltage roughly corresponding to the intersection point of the PI and FDA curves in Fig. 6(b). At pulse durations of 0.5, 1, and 3 ms, maximum average values of 50.1% (~ 18 V), 52.2% (~ 10 V), and 9.8% (~ 6 V) are obtained, respectively. Hence, it can be conjectured that a shorter pulse duration results in higher electroporation efficiency with maximum value obtained at higher voltages. The grey and blue transparent rectangular backgrounds represent appropriate reversible electroporation regimes (the criteria chosen was above 20% of both PI and FDA stained cells) at pulse durations of 1 and 0.5 ms, respectively. The left and right sides exceeding these rectangular areas represent non-activated and irreversible electroporation, respectively.

FIG. 7. Electroporation of RFP-expressing HUVECs. (a) Micrographs of the RFP-expressing HUVECs showing bright field with a red fluorescent filter before/after 40 min/after 24 h from the application of electroporation at increasing pulse amplitudes (4 V, 10 V, 16 V) with single pulse of 0.5, 1, 3 ms pulse duration. (b) The effect of pulse amplitude and duration on the fluorescent intensity of RFP-expressing HUVECs indicating the release of RFP molecules due to the electroporation/cell lysis. The intensity is normalized by the initial intensity of the cell. The grey and blue transparent rectangular backgrounds represent appropriate regimes of reversible electroporation at pulse durations of 1 and 0.5 ms, respectively.

This assessment is further quantified by the associated fluorescence intensity which reflects its free diffusion across the electroporated membrane (Fig. 6(d)). As expected, higher pulse amplitudes and duration result in higher intensities. At the regime of reversible electroporation, the PI-uptaken cells has intensities with the range of $0.45 < I^* < 0.65$, approximately.

D. Release of intracellular molecules by electroporation

To assess the release of intracellular molecules by electroporation, RFP-expressing HUVECs were exposed to similar conditions of electroporation. Figure 7(a) shows cell morphology of RFP-expressing HUVECs in bright field with fluorescent filter (maximum excitation/emission at 535 nm/620 nm) after different durations from application of electroporation at increasing pulse amplitudes (4 V, 10 V, and 16 V) with single pulse of 0.5, 1, and 3 ms duration. Before electroporation, most cells have a significant fluorescent signal due to the RFP molecule content within the cell. The decay of the fluorescent signal after electroporation suggests that electroporation induces the release of the RFP molecules across the membrane. At the 4 V pulse

FIG. 8. Time evolution of RFP-expressing HUVECs intensity after electroporation at increasing pulse amplitudes with a single pulse of 3 ms duration. (a) Time-lapse fluorescence image after electroporation at increasing pulse amplitudes (4 V, 8 V, 16 V). (b) The time evolution of the averaged normalized fluorescence intensity.

amplitude, which according to the PI-uptake experiments corresponds to non-activated electroporation in all pulse durations, the cells maintained both their intact morphologies and their fluorescent intensities 40 min after electroporation resulting in viable cells after 24 h. For pulse amplitudes of 16 V, the treated cells exhibited a wrinkled morphology and lost their intensity when compared to 4 V pulse amplitude. Of course no cells were grown after 24 h, demonstrating the irreversible damages of cells. At the intermediate 10 V pulse amplitude, for durations of 0.5 ms and 1 ms, which are supposed to be in the range of reversible electroporation region, the fluorescent intensity decayed slightly compared to the 4 V pulse amplitude and control group (0 V) with cells sustained viable also after 24 h. Figure 7(b) illustrates the decay of normalized measured intensities of RFP-expressing HUVECs at various electroporation conditions. The normalized intensity decay of the control group is less than 2% (i.e., the normalized intensity after electroporation is 0.98 ± 0.02) which confirms the nonappearance of decay in the absence of electroporation. Overall, there is a good agreement between the results of RFP molecule release and subsequent cell culturing with those of the PI-uptake experiments.

Figure 8 shows the time evolution of RFP-expressing HUVECs fluorescent intensity after electroporation. When a strong electric field (16 V and 3 ms) was applied, a rapid decay of cell intensity (decay of $\sim 50\%$ in 5 min), indicating cell lysis, was observed. At these conditions, bubble generation, due to the electrolysis of water, was also obtained. At moderate electric fields (8 V and 3 ms) slow decay of cell intensity was observed (decay of $\sim 30\%$ in 40 min) without any bubble generation indicating that this parameter lies in the intersectional regime between reversible and irreversible electroporation. At low electric fields, corresponding to the regime of non-activated electroporation, the average intensity decay was around 3% in 40 min which is almost same value of the fluorescent intensity in the control group. Based on the above results, we confirm that real-time monitoring of the release of RFP molecules across the cell membrane is worthy in terms of assessing electroporation efficiency.

IV. CONCLUDING REMARKS

The present study focused on examining the capability of the unique microfluidic platform to perform as a high throughput electroporation assay. The unique design results in uniform electric fields, regardless of the microchamber lateral dimensions (provided they are large enough relative to the cell dimension) and ensures the increase of electroporation uniformity. Another key advantage of this platform is the microchamber array design which allows the potential isolation of multi-microchamber environments, parallelism, and individually addressable electrodes which can be used to generate on-chip electroporation gradients. Furthermore, the entrance of each microchamber may act as an on-demand gate which can be opened/closed under the application of DEP by improving microchamber isolation (e.g., eliminating or using a thinner PDMS sealing film). Specifically, since the microchamber entrance and edges constitute regions of high field gradients, external control of the frequency of the applied field can enable either trapping (p-DEP) at the microchamber edges and entrance or repulsion (n-DEP) of colloids/cells towards the center of the microchamber. The unique geometry means that particles/cells can be retained within the microchambers under external pressure-driven flows (e.g., exchange of buffer solution) in either n-DEP or p-DEP modes. For cells, where physiological buffer solutions restrict the response to n-DEP, the repulsive force concentrates cells at the center of the chamber, increasing electroporation uniformity and minimizing cell loss during flushing. We show that particle/cell concentration could potentially be further enhanced by exploiting the convective contribution of ACEO, although the ACEO effect on cells in physiological buffer were hindered due to electrolysis, if the chip is operated at low enough frequencies corresponding to the RC time of the effect. Since the ACEO is a long range convective force, it serves to rapidly feed particles either to the p-DEP traps or away from the microchamber edges and into its center in the case of n-DEP.

Reversible assembly of the chip is another advantage and is suitable to long-term cell culturing. Because the two plates are sandwiched by a screwed holder, the top plate can be easily

separated and cells can be further cultured in the incubator or additional foreign molecules introduced. The platform can also be sterilized and the same chip re-used more than 10 times. An improvement to the current design would be to eliminate the gap ($\sim 50\ \mu\text{m}$) formed between the SU-8 pattern and the top PDMS film, hence, potentially increasing the microchamber isolation and suppressing any cross-contamination between adjacent microchambers.⁵⁰

A spatial gradient of electroporation parameters was successfully produced into the microchamber array to extract optimal reversible electroporation conditions for NHDF cells using PI and FDA staining and the release of intracellular RFP molecules using RFP-expressing HUVECs. As such, the preliminary results clearly indicate that the developed generic microfluidic platform has the potential to achieve high-throughput screening for electroporation with spatial control and uniformity, assisted by DEP and ACEO manipulation/trapping of particles/cells into individual microchambers.

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