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Real-time Imaging and Tuning Subcellular Structures and Membrane Transport Kinetics of Single Live Cells at Nanosecond Regime

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Abstract

We developed an electric-field exposure microchannel system with 230-nanometer thin-layer gold electrodes, and interfaced it with a single living cell imaging station and a 10-nanosecond-electric-pulse (10nsEP) generator. This design allows us to image intracellular molecules and structures, membrane transport and viability of single leukemic cells (HL60) while the cells are exposed to 10nsEPs of 0–179 kV/cm, permitting the study of subcellular responses at nanosecond regime. The electrodes confine a thin-layer section of the cells exposed to 10nsEPs, offering unprecedented high spatial resolution (230-nm at z-direction of E and imaging plane) for imaging intracellular molecules of single cells affected by 10nsEPs. We found that nucleic acids, membrane transport rates and viability of single cells depend on the number and electric-field-strength (E) of 10nsEPs, showing the cumulative effect of 10nsEPs on intracellular molecules and structures and suggesting the possibility of tuning them one-pulse-at-a-time. Using lower E (51 kV/cm) of 10nsEPs, we could manipulate nucleic acids of single living cells without disrupting their cellular membrane and viability. As E increases to 80, 124 and 179 kV/cm, membrane integrity and viability of cells exhibit higher dependence on the number of 10nsEPs in a non-linear fashion, showing that critical E and pulse number are needed to surmount cellular transport barriers and membrane integrity.

Keywords

Fluorescence imaging of single living cell; nanosecond electric pulses; subcellular structures; membrane transport kinetics; ultrasensitive analysis

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On-line Supporting Information Available:

A. Experimental Section: Designs and constructions of single living cell imaging station, E-exposure microchannel system and 10 nsEP generator; Real-time Fluorescence Spectroscopic Measurements.

B. Four figures (Figs. 1S–4S) are included.

Figure 1S: Schematic illustrations of E-exposure microchannel system for imaging subcellular structures and membrane transport kinetics of single living cells as the cells were exposed to 10nsEPs;

Figure 2S: EtBr accumulation kinetics in living human leukemic cells;

Figure 3S: Study of subcellular structures and membrane transport rates over time in the absence of electric field (control experiments);

Figure 4S: Design and characterization of E-exposure conventional cuvette system for bulk cell study.

C. Four real-time videos (Movies 1–4) show 330 sequential EtBr fluorescence images of single cells in Figures 3B–6B(a–h), respectively. These sequential EtBr fluorescence images of single cells were recorded simultaneously as the 330 of 10nsEPs of 51, 80, 124, and 179 kV/cm were applied, illustrating direct observation of subcellular structures and membrane transport of cells induced by given number and E of 10nsEPs. The videos were recorded in real time by CCD camera with a time interval between each image at 1.333 s, including 1 s time interval, 0.100 s acquisition time of CCD image, and 0.233 s readout time.

1. Introduction

Tuning membrane transport and subcellular structures of single living cells in real time holds great promise for selectively regulating cellular and subcellular pathways, determining the fate of cells, and engaging smart drug delivery. Generally, a mammalian cell includes a conductive cytoplasm and a surrounding dielectric layer of plasma membrane.^{1,2} As cells are exposed to pulsed electric fields (PEFs), accumulated charges on the surface of the cell membrane can alter the voltage across the cell membrane (transmembrane potential), which is normally 60–110 mV in healthy cells. Once the transmembrane potential surpasses a critical value (~ 1 V), it can cause irreversible formation of membrane pores and permanent damage of membrane structures, leading to cell death.^{1,2} Such phenomena are typically named as classical plasma membrane electroporation, referred as conventional electroporation. In contrast, if the transmembrane potential is below the critical value and pulse duration is sufficiently short, the formation of membrane pores is temporary and reversible. Thus, the cells can survive. Such effects are called as electroporabilization, which has been widely used for delivery of macromolecules (e.g., genes and drugs) into the cells. Nonetheless, terminology of electroporation and electroporabilization is not clearly defined and strictly used in the literature because of historic reasons.³

Currently, the primary methods for the study of cellular responses (e.g., membrane porosity, viability, apoptosis) to PEFs include imaging accumulation of submicrometer latex beads inside cells using transmission electron microscopy (TEM), and detecting various intracellular fluorescent dyes using flow cytometry and fluorescence microscopy.^{4–8} These measurements were not conducted in real time as cells were exposed to PEFs.^{5,9–11} Thus, it prohibits one from probing immediate and temporary effects of PEFs on cellular structures (e.g., temporary formation of membrane pores), membrane transport and their underlying mechanisms.

Conventional pulsed electric-field (E) exposure systems typically consist of a cuvette with two electrodes inside that are a centimeter, millimeter or sub-millimeter apart; cell suspensions in a cuvette are exposed to the PEFs.^{5,10–14} Typically, long duration electric pulses (e.g., millisecond, microsecond, sub-microsecond) were used,^{1,11} which led to disruption of cell membranes, eliminating the possibility of probing their effects on intracellular molecules and subcellular structures. The long durations of electric pulses also generate significant thermal effects on cells, which prohibits their unique effects on the cells from being studied and prevents sequential electric pulses from being used to study their cumulative effects on cellular functions.

Recent development of ultrashort electric pulse generators offers new opportunities to effectively reduce thermal effects, deliver trace doses of electric energy into subcellular structures, and study fast kinetics of cellular responses at nanosecond (ns) regime.^{2,5,10,11,15,16} As described by Cole,¹⁷ for cells (spherical like objects) with an ideal dielectric membrane and homogeneous cytoplasm that are well suspended and dispersed at a low concentration in a medium (single-shell model), the charging time (τ_c) of its membrane can be described by Eq. [1]

$$\tau_c = \left[\frac{\rho_m}{2} + \rho_c \right] C_m \frac{D}{2} \quad [1]$$

Where ρ_m and ρ_c are the resistivity of the medium and the cytoplasm of cells, respectively; C_m is capacitance of the membrane per unit area; D is the cell diameter. Thus, charging time of the outer plasma membrane of the cell is about 75 ns, assuming a spherical cell with a diameter of 10 μm , its cytoplasm and medium resistivity of 100 $\Omega\text{-cm}$, and an ideal dielectric

membrane layer (no current leakage) with capacitance of $1 \mu\text{F}/\text{cm}^2$.^{1,2,10} When the pulse duration is shorter than charging time of cell membrane, the pulses cannot generate charges on the surface of cell membrane, instead creating charges on membranes of intracellular organelles (e.g., nuclear envelope, mitochondrial membrane) because they are smaller and have shorter charging time than the cells, as described in Eq. [1].

Therefore, ultrashort electric pulses, such as 10 ns electric pulses (10nsEPs), allow electric field to penetrate into cell membrane and affect intracellular organelles and structures, offering the possibility of probing their effects on intracellular molecules and structures of living cells without disrupting cellular plasma membrane.¹⁰ The shorter duration (τ) of electric pulse also provides lower energy as described in Eq. [2]

$$W = E^2 \tau / \rho \quad [2]$$

Where W , E , τ , and ρ represent the electric energy, electric field strength, pulse duration, and electric resistivity of the cell suspension, respectively.^{10,15} The small pulse duration (τ) can significantly reduce energy and thermal effects, which allows a small dose of energy and multiple sequential pulses to be delivered into the cells one-pulse-at-a-time.

Recent studies have demonstrated that ultrashort electric pulses (60, 30 and 10 ns) can induce intracellular responses, such as eosinophil sparklers,^{2,18} apoptosis,^{12,13,19} calcium release,^{8,16} membrane perturbation,^{4,10} phosphatidylserine translocation,²⁰ and cytotoxic effects.^{10,19,21} However, several crucial questions about the possibility of quantitatively timing and tuning subcellular structures and functions and membrane transport rates of single living cells using cumulative effects of 10nsEPs remain to be addressed.^{3,10,15} For example, (i) Can one manipulate intracellular biomolecules and structures of single living cells using 10nsEPs while maintaining cells alive and membranes intact? (ii) Can one use sequential 10nsEPs to generate cumulative effects of PEFs on cellular function and to tune membrane transport rates? (iii) Can one selectively alter specific cellular structures and disrupt specific membrane barriers by carefully controlling the number and E of sequential 10nsEPs? These are vital questions to be addressed in order to develop powerful tools using ultrashort electric pulses for study and manipulation of intracellular molecules and structures for biomedical applications (e.g., smart drug delivery, effective electrochemotherapy).¹⁰

To address these questions, in this study, we develop a new E -exposure microchannel system, interface and synchronize it with a single living cell imaging station and a 10nsEP generator. We study the dependence of membrane transport rates, conformations of intracellular nucleic acids and viability of individual human leukemic cells (HL60) on the electric field strength (E) and sequential number of 10nsEPs, aiming to explore the possibility of tuning intracellular molecules and membrane transport kinetics of individual live cells by controlling the number and E of 10nsEPs. We investigate the dependence of these cellular responses to sequential 10nsEPs using our microchannel system and conventional cuvette system, and compare both measurements to validate the suitability and unique advantages of our microchannel exposure system. Unlike conventional cuvette systems,^{5,10–14} our synchronized microchannel system allows us to image intracellular molecules and membrane transport rates of single living cells induced by sequential 10nsEPs in real time with high spatial and temporal resolution, offering new opportunities to investigate temporary and reversible effects of 10nsEPs on intracellular molecules and structures and their underlying mechanisms.

We select human leukemic cells (HL60) for this study because they represent a type of cancer cells grown in suspension.²² HL60 cells exhibit nearly uniform size and spherical shape;

thereby they can serve as a model system to investigate the effects of 10nsEPs on intracellular molecules and structures. Note that irregular shape and size of cells may contribute to distinctive E-effects on cells.²³ We use ethidium bromide (EtBr) to identify and visualize intracellular nucleic acids by specifically intercalating them with EtBr, allowing directly probe the effects of 10nsEPs on intracellular nucleic acids using fluorescence microscopy and spectroscopy. Intracellular nucleic acids play vital roles in cellular functions. Therefore, study of effects of 10nsEPs on intracellular nucleic acids of single living cells in real time will pave the way for probing their effects on cell functions and exploring the possibility of utilizing 10nsEPs to control and direct cellular functions.

2. Experimental Section

2.1. Cell Culture and Preparation

A human promyelocytic leukemic cell line (HL60) (ATCC) was cultured using RPMI-1640 medium containing 1% glutamine, 20% fetal bovine serum, and 1% penicillin and streptomycin in a CO₂ incubator (37°C, 5% CO₂). The cell viability (> 98%) was monitored daily using the Trypan blue assay (0.2%) (Sigma),²⁴ and cells (< 2×10⁶ cells/mL) were passaged weekly. The cultured cells were collected by centrifugation (1000 rpm, 5 min), washed twice with 3 mL Hank's balanced salt solution without Ca²⁺ and Mg²⁺ (HBSS, ATCC), and re-suspended in HBSS buffer.⁸ The cell suspension (2×10⁶ cells/mL) was then incubated with 10 μM EtBr in the CO₂ incubator for 25 min and further incubated for 10 min in a microchannel between two Au electrodes prior to imaging and exposing them to 10nsEPs at room temperature. EtBr (Sigma) solution was prepared in ultra-pure deionized water (Nanopore, 18 MΩ) and triple filtered using 0.2 μm sterilized membrane filters.

2.2. Synchronization of a Single Living Cell Imaging Station with a 10nsEP Generator

A homemade microscope slide holder with two electric contacts directly connects two Au electrodes of a microchannel on a quartz microslide with a 10nsEP generator, and secures the slide on the microscope stage (Figure 1A). The thickness of each thin-layer Au electrode is 230 nm and the gap between the two Au electrodes in each pair is 19.1 μm. The depth of each channel is (5 ± 0.5) μm below the slide surface, and a cooling reservoir of 10 μm above the pair of electrodes is constructed to directly connect with the cell suspension in the E-exposure microchannel (Figure 1S in Supporting Information). Designs and constructions of single living cell imaging station,^{25–35} E-exposure microchannel system and 10 nsEP generator^{15,36} are fully described in Supporting Information.

A wave-generator (WaveTek) is utilized to synchronize: an electronic shutter used for controlling the fluorescence excitation beam; a shutter of the CCD camera that controls exposure time (TTL signal) and the MOSFET switch of the 10nsEP generator (Figure 2). As the CCD camera acquires sequential fluorescence images of single cells, the electronic shutter opens and allows single living cells to be exposed to the excitation beam and 10nsEPs, simultaneously. This synchronization allows sequential fluorescence images of single cells between the two electrodes to be acquired as the cells are exposed to the consecutive 10nsEPs, permitting direct observation of cellular responses to 10nsEPs in real time. Such real-time imaging capability is particularly critical to capture temporary, reversible and cumulative effects of sequential pulses on cells in real time. The synchronization of the electronic shutter in front of the excitation beam with the shutter of CCD camera prevents EtBr and living cells from being exposed to the fluorescence excitation beam while the fluorescence images are not acquired, reducing the photodecomposition of EtBr and other possible effects of excitation beams on living cells. An oscilloscope (Tektronix, TDS-3052) is used to acquire the 10nsEPs in real time (Figure 1B).

2.3. E-Exposure Cuvette System for Study of Bulk Cells

Design and characterization of a 10nsEP generator (ARC Technology) for exposing of cell suspensions in a conventional E-exposure cuvette (Biosmith) (Figure 4S in Supporting Information) were described previously.¹⁵ The pulse generator provides voltages up to 40 kV and E up to 200 kV/cm for a cuvette that contains two Al-electrodes with a 0.2-cm gap between them, and produces 10nsEPs with sub-ns rise and fall time, similar to the one in Figure 1C. The sequential number, interval time and voltage of pulses are controlled by a controller and monitored by an oscilloscope. The 0.3 mL cell suspension (2×10^6 cells/mL) in the cuvette was exposed to the 330 sequential 10nsEPs of 51, 80, 124, and 179 kV/cm. The viable and apoptotic cells were investigated at given pulse number and E using Trypan blue assay and annexin V-FITC (fluorescein isothiocyanate) assay,^{37,38} respectively.

2.4. Study of Cell Viability Using Trypan Blue and Annexin V-FITC Assays

After single cells in the microchannel were exposed to the 330 sequential 10nsEPs of 0, 51, 80, 124, and 179 kV/cm, the membrane integrity and viability of single cells were studied by incubating the cells with Trypan blue (0.2 %) for 5 min. Single cells were imaged using phase contrast optical microscopy. The cells that were penetrated and stained by Trypan blue were classified as dead cells, owing to disintegration of cell membranes. The cells that remained unstained by Trypan blue showed intact cell membrane and were determined as live cells. The viability of cells exposed in the cuvette system was also studied using Trypan blue assay. The cell suspensions exposed to given pulse number of 51, 80, 124, and 179 kV/cm in the cuvette exposure system were sampled, and the number of live (unstained) and dead (blue) cells were counted using a hemacytometer to determine the viability and mortality of bulk cells at single cell resolution at given E and pulse number, respectively.

The cells, exposed to 330 consecutive 10nsEPs of 0 and 51 kV/cm in the cuvette, were cultured in RPMI-1640 medium in the incubator for 5 days. The cells were sampled at given times, incubated with annexinV-FITC (5%) for 20 min at room temperature, well rinsed with HBSS, and then imaged using fluorescence microscopy. The number of apoptotic cells (FITC stained, green fluorescence cells) and non-apoptotic cells (non fluorescence cells) were counted to determine the percentages of non-apoptotic cells over time.

2.5. Statistical Analysis

Each experiment is repeated at least three times and representative data is presented. For each experiment conducted in the microchannel system, at least 15 cells were studied at single cell resolution in real time. Kinetic measurements of single cells in Figure 3S-B and Figures 3B–6B are presented in Figure 7. The results from multiple measurements for each experiment described in Figures 3S and 3–6 are summarized in Table I. For each experiment carried out in the cuvette system for the study of cell viability (Figures 8B and 9), more than 300 cells at each given pulse number and E were investigated at the single-cell resolution to gain sufficient statistics. The results are compared with those measured using the microchannel system (Figure 8A), ensuring that the viability study of single cells in the microchannel represents bulk cells at single cell resolution. We selected representative healthy cells with the same phase (S-phase) of cell cycles for each experiment.

Recent studies show that 10–14 measurements of individual events (e.g., single molecules) are sufficient sample sizes to extrapolate ensemble properties with accuracy of analytical measurements at single-entity level.³⁹ Thus, the sample sizes presented in this study are adequate for the study of bulk cells at the single-cell resolution. Unlike ensemble measurements, single-cell imaging allows us to unmask heterogeneous nature of individual cells in bulk solution and probe intracellular molecules and structures of individual cells with both spatial and temporal resolution.

3. Results and Discussion

3.1. Real-Time Fluorescence Spectroscopic Measurement of Accumulation of EtBr in Bulk Cells

A time-course transient of fluorescence intensity of EtBr incubated with the cell suspension (2×10^6 cells/mL) in the absence of electric field ($E = 0$) (Figure 2S in Supporting Information) shows that fluorescence intensity of EtBr increases with time and reaches a plateau at 35 min. Viability of the cells that had been incubated with EtBr over a hour were investigated using Trypan blue and annexin V-FITC assays, showing that over 95% and 80% of cells are alive and non-apoptotic cells, respectively. These results illustrate that EtBr can transport into the living HL60 cells and accumulation of EtBr in living cells reaches equilibrium at 35 min in the absence of electric field. Thus, we determine the minimum incubation time prior to exposing the cells to 10nsEPs, and use change of fluorescence intensity of intracellular EtBr induced by 10nsEPs to determine their effects on the cells.

EtBr emits weak fluorescence intensity in aqueous solution. As EtBr molecules enter the nuclei and intercalate with nucleic acids, the structure of EtBr becomes more rigid and its quantum efficiency and lifetime increases, leading up to a 25-fold increase in fluorescence intensity and longer fluorescence lifetime.⁴⁰ Therefore, change of fluorescence intensity of intracellular EtBr has been widely used to monitor membrane transport of living bacterial cells.^{27,30,33,41} In contrast, EtBr is considered to be impermeable to living eukaryotic cells and has been used to stain dead and dying eukaryotic cells.^{42–44} However, such a live/dead cell assay has been found somewhat unreliable because EtBr is not as large and highly charged as other dyes (e.g., ethidium homodimer and propidium iodide).⁴⁴ Thus, it is possible that EtBr could passively diffuse (or actively transport) into some types of living eukaryotic cells.

3.2. Non-invasive Manipulation of Intra-nuclear Nucleic Acids of Single Living Cells

The cells were incubated with EtBr over 35 min, allowing accumulation of intracellular EtBr reach the equilibrium, prior to their exposure to the pulses. Phase-contrast optical image in Figure 3A shows such a cell aligned between two electrodes in the E-exposure microchannel, right before the pulse was applied. As the cell was exposed to the 330 sequential 10nsEPs of 51 kV/cm, their sequential EtBr fluorescence images were acquired in real-time (Figure 3B and Movie 1 in Supporting Information), which illustrates that intra-nuclear nucleic acids are lit up by intercalated EtBr. Interestingly, the fluorescence intensity of intracellular EtBr decreases as pulse number increases, which is similar to those observed in the absence of electric field ($E = 0$) (a control experiment in Figure 3S in Supporting Information). The result suggests that decreased fluorescence intensity is attributed to photodecomposition of EtBr and membrane transport rates of EtBr into the nuclei induced by the pulses remain essentially unchanged.

Notably, an intra-nuclear bright fluorescence cluster was observed after 100 pulses (Figure 3B: c–h). Since the fluorescence intensity of single cells exposed to the pulses of 51 kV/cm is the same as that of the cells in the absence of the electric field (Figure 3S in Supporting Information), the observed intra-nuclear localized bright fluorescence clusters are most likely attributed to the condensation of intra-nuclear nuclei acids induced by the 10nsEPs, leading to more compact structure and higher fluorescence intensity. As described previously, 10nsEPs can allow PEF to penetrate into the cells and affect subcellular structures (e.g., nuclei) and the charge of intra-nuclear molecules (e.g., nucleic acids). Such effects may cause charge redistribution of intra-nuclear nucleic acids and alter their dipoles and electrostatics and hydrophobic interactions, leading to their conformational and structural changes. Note that 10nsEPs is too short (10 ns) to move any molecules (EtBr or DNA) around. Thus, the bright

intracellular fluorescence clusters are not attributed to the redistribution of intracellular EtBr by the pulses.

A phase-contrast optical image of the cell (Figure 3C), acquired right after its exposure to the 330 sequential pulses of 51 kV/cm, was used to compare with the one prior to its exposure to the pulses (Figure 3A), showing that cellular morphologies remain essentially unchanged during the 330 pulses. Study of fluorescence intensity (Figure 7b) and viability (Figures 8A–b and 9) of the cell upon pulse number shows that the cell membranes remain intact and the cells are alive and non-apoptotic after its exposure to the pulses of 51 kV/cm, which are discussed in the later sections.

3.3. Dependence of Subcellular Structures on E and Pulse Number

Using the same approaches as described in Figure 3, we acquired sequential EtBr fluorescence images of single cells as they were exposed to the consecutive 330 pulses of 80, 124 and 179 kV/cm, aiming to investigate the dependence of subcellular structures and intra-nuclear nucleic acids on E and pulse number. The results are shown in Figures 4–6 and described below. The dependence of their membrane transport kinetics (influx rates of EtBr) and viability of cells on E and pulse number are presented separately in the later sections (Figures 7 and 8).

At E of 80 kV/cm, the sequential EtBr fluorescence images of individual cells (Figure 4B:a–h and Movie 2 in Supporting Information) show the formation of much brighter intra-nuclear EtBr fluorescence clusters (nucleic acids intercalated with EtBr), as the number of pulses increases. The phase-contrast optical images acquired before and after the 330 consecutive 10nsEPs (Figures 4A and C) show more notable changes of cellular morphology than those observed in Figure 3. The locations of intra-nuclear EtBr fluorescence clusters of the single cell at the 330th pulse (Figure 4D) match with the dark dense spots observed in optical images of the cell (Figure 4C).

As E increases to 124 kV/cm, the sequential EtBr fluorescence images of single cells (Figure 5B:a–h and Movie 3 in Supporting Information) show much brighter and more compact intra-nuclear EtBr fluorescence clusters than those observed in Figure 4B:a–h, as only 30 sequential pulses are applied. As pulse number increases up to 120 and beyond, the cellular and nuclear membranes begin being disrupted by sequential pulses in a pulse-number-dependent manner (Figures 5B: c–h), suggesting that the electroporation of membranes occurs. The phase-contrast optical images acquired before and after the 330 pulses (Figures 5A and C) illustrate remarkable changes of cellular morphology and dense spots inside nuclei, which match with observed bright and compact intra-nuclear EtBr fluorescence clusters (Figure 5D).

As E increases further to 179 kV/cm, the sequential EtBr fluorescence images of single cells (Figure 6B and Movie 4 in Supporting Information) show multiple bright and compact intra-nuclear EtBr fluorescence clusters as 20 sequential pulses are applied. As pulse number increases up to 100 and beyond, we observe that: (i) The cellular and nuclear membranes begin to be disrupted by the pulses; (ii) Fluorescence intensity of intra-nuclear EtBr of individual cells increases dramatically; and (iii) Entire nuclei are illuminated by fluorescence emission of EtBr (Figures 6B: c–h). The phase-contrast optical images acquired before and after the 330 sequential 10nsEPs show astonishing changes of cellular morphology (Figures 6A and C), illustrating the rough cellular surface, a clear outline of the nucleus and three highly dense compact spots (Figure 6C). Since the entire nucleus is fully illuminated by fluorescence emission of over-flown EtBr, the fluorescence clusters observed in Figure 6D look hazy and are not as well-defined as those in Figures 4–5D. Nonetheless, three highly dense compact spots (Figure 6C) overlap with bright intra-nuclear EtBr fluorescence in Figure 6D.

Taken together, we observed high dependence of intra-nuclear structures on the E and pulse number of 10nsEPs. At lower E (51 kV/cm), we observed less bright intra-nuclear EtBr fluorescence clusters (intra-nuclear nucleic acids) of single cells at the 300 pulses. As E increased to 80, 124 and 179 kV/cm, we observed brighter intra-nuclear EtBr fluorescence clusters of single living cells at fewer pulse number of 150, 30 and 20 pulses, respectively. These results show the cumulative effects of sequential pulses on intracellular structures and illustrate that such effects increase as the E and sequential pulse number increase. Thus, the fewer pulses of higher E are needed to alter intracellular structures in living cells. Notably, the locations of intra-nuclear EtBr fluorescence clusters (nucleic acids intercalated with EtBr) in Figures 4–6: D, match with the dark dense spots observed in optical images of the cells (Figure 4–6: C). Such observations further suggest that intra-nuclear nucleic acids of single cells might become more compact as being exposed to 10nsEPs, because the compact nucleic acids could lead to increased fluorescence intensity of intracellular EtBr in fluorescence images (Figures 4–6: D) and dense spots in optical images (Figures 4–6: C).

The ultrathin Au electrodes (230 nm) directly fabricated on the quartz slide with the depth of microchannel of (5 ± 0.5) μm beneath the slide and a cooling reservoir 10 μm above the pair of electrodes (Figure 1S), allow a thin-layer section of cells to be exposed to 10nsEPs. Since only a section of the cells, rather than the entire spherical volume of the cells, were exposed to 10nsEPs, only intracellular molecules and structures on the exposure plane of 230 nm affected by the pulses led to higher EtBr fluorescence intensity. Thus, the fluorescence intensity of intracellular EtBr in the parts of the cells that are above and below the exposure plane (section) remains nearly unchanged. This approach offers unprecedented high spatial resolution in the z-direction of imaging plane and pulsed electric-field to investigate the intracellular molecules and structures affected by 10nsEP, allowing us to observe these interesting phenomena in real time. The small electrodes also reduce the charging current and decrease the amounts of unwanted chemicals that might be generated by possible electrochemical reactions, which avoids changes in ion concentrations and pH in the buffer that might otherwise trigger the cellular responses. Unlike previous studies in which aluminum (Al) and stainless steel electrodes were used,^{4,5,10,18} Au electrodes are used in this study because they are less likely to generate metal ions or oxides upon the application of PEFs, which eliminates possible toxic chemical effects on cells. These experimental approaches allow us to observe solo effects of 10nsEPs on cellular and subcellular structures and functions.

3.4. Dependence of Membrane Transport Kinetics on E and Pulse Number

To quantitatively analyze the dependence of membrane transport kinetics (rates) upon E and pulse number observed in Figures 3S and 3–6, we plot fluorescence intensity of intracellular EtBr of the single cells versus pulse number for E of 0, 51, 80, 124, and 179 kV/cm (Figure 7). We use the change of fluorescence intensity of intracellular EtBr of single cells verse time (slopes in Figure 7) at given E and pulse number of 10nsEPs to measure the transport (influx) rates of EtBr, and compare them with the rates in the absence of electric fields ($E = 0$) to determine the effect of E and pulse number of 10nsEPs on cell membranes. Fluorescence intensity of intracellular EtBr in single cells was calculated by subtracting the integrated fluorescence intensity of the pixel area (e.g., dashed-line square area in Figure 3B) where no cell was present (EtBr in solution) from the same size of pixel area (e.g., solid-line square area in Figure 3B) in the same image, where the cell was present. This subtraction allows the deduction of dark-noise of CCD camera and photobleaching of EtBr. The results from multiple measurements, similar to those described in Figures 3S and 3–6, are summarized in Table I, which shows high dependence of membrane transport rates on E and pulse number. The results also illustrate significant variability of individual measurements of single cells (high standard deviation of measurements), suggesting variation of individual cells and underscoring the importance of probing of bulk cells at the single-cell resolution.

In the absence of electric field ($E = 0$; Figures 7: a), the fluorescence intensity of intracellular EtBr of single cells decreases with time at the rates of 1.3×10^4 , 7.9×10^3 and $2.6 \times 10^3 \text{ s}^{-1}$ during 0–70, 70–110 and 110–330 images, respectively, suggesting that intracellular EtBr was photobleached by the fluorescence excitation beam. This experiment serves as a control for measuring the effect of photodecomposition on fluorescence intensity of intracellular EtBr of single cells over time, as well as for probing the dependence of membrane transport rates of EtBr on E and pulse number of 10nsEPs. At E of 51 kV/cm (Figures 7: b), fluorescence intensity of intracellular EtBr of single cells decreases with time (pulse number) at the rates of 7.8×10^3 , 2.8×10^3 , and $7.9 \times 10^2 \text{ s}^{-1}$ during 0–50, 50–110, and 110–330 pulses, which is slower than the rates observed at given time in the absence of electric field (Figures 7: a). The membrane transport rates induced by pulses were calculated by subtracting the fluorescence intensity in the absence of E from that in E (51 kV/cm) at the given times, to deduct the contribution of photobleaching. We found that the membrane transport rates increase with time at 5.0×10^3 , 6.7×10^3 , and $1.6 \times 10^2 \text{ s}^{-1}$ during 0–50, 50–110 and 110–330 pulses, respectively. This result further confirms what we observed and discussed previously in Figure 3, suggesting that the 330 sequential pulses of 51 kV/cm cannot provide sufficient energy to either disrupt well-organized membranes or substantially increase the influx of EtBr into cells to overcome photodecomposition of EtBr. Thus, fluorescence intensity of the cell decreased over time.

At E of 80 kV/cm (Figures 7: c), fluorescence intensity of intracellular EtBr of single cells decreases with time during 0–120 pulses and then increases with pulse number. The subtracted membrane transport rates increase with time at 7×10^3 , 2.2×10^4 and $6 \times 10^4 \text{ s}^{-1}$ during 0–120, 120–210, and 210–330 pulses, respectively, showing cumulative effect of pulses on membrane transport rates. The membrane transport rates during 0–120 pulse of 80 kV/cm are similar to those observed at 51 kV/cm. However, as pulse number increases from 120 to 330, the membrane transport rates induced by the pulses of 80 kV/cm are 100-fold higher than those induced by 51 kV/cm, suggesting that pulses number at given E (120 pulses of 80 kV/cm) has reached critical values to disrupt well-organized membranes, leading to influx of EtBr into cells and nuclei.

At E of 124 kV/cm (Figures 7: d), fluorescence intensity of intracellular EtBr of single cells decreases with time during 0–20 pulses and then increases with pulse number. The subtracted membrane transport rates increase with time at 7.4×10^3 , 4×10^4 and $3.3 \times 10^4 \text{ s}^{-1}$ during 0–20, 20–180 and 180–330 pulses, respectively, showing cumulative effects of pulses on membrane transport rates. The membrane transport rates during 0–20 pulses of 124 kV/cm are similar to those observed at 80 kV/cm. However, the rates induced by the pulses of 124 kV/cm are nearly 6-fold and 2-fold higher than those by 80 kV/cm during 20–120 and 120–330 pulses, respectively, suggesting that influx rates of EtBr into the cells are less dependent on E once the transport barriers and membranes are broken down at critical E of given number, which is 20 pulses of 124 kV/cm.

At E of 179 kV/cm (Figures 7: e), fluorescence intensity of intracellular EtBr of single cells decreases with time during 0–10 pulses and then increases with pulse number. The subtracted membrane transport rates increase with time at 1.2×10^4 , 9.3×10^4 , 4.3×10^4 , and $5.6 \times 10^4 \text{ s}^{-1}$ during 0–10, 10–110, 110–200, and 200–330 pulses, respectively, showing cumulative effect of pulses on cellular membrane transport rates. Interestingly, the membrane transport rates during 0–10 pulses of 179 kV/cm are nearly the same as those observed at 124 and 80 kV/cm. In contrast, the membrane transport rates induced by 179 kV/cm pulses are about 3-fold higher than those by 124 kV/cm during 10–110 pulses. Surprisingly, the rates at 110–200 and 200–330 pulses are similar to those observed at 80 and 124 kV/cm, suggesting that influx rates of EtBr into the cells are far less dependent on pulse number once the transport barriers and membranes are totally broken down at critical pulse number of given E , which is 10 pulses of 179 kV/cm.

In summary, the plots in Figure 7 show the high dependence of fluorescence intensity of intracellular EtBr of single cells on pulse number and E of 10nsEPs. Notably, the fluorescence intensity increases with pulse number at given E, as well as increases with E at given pulse number, in a non-linear fashion, suggesting that a critical number of pulses at given E and the critical E at given number of pulses are needed to offer sufficient energy to disrupt transport barriers of cells. By comparing the transport (influx) rates of EtBr into the cells in the absence and presence of PEFs, we can determine the effect of 10nsEPs on cell membranes and membrane transport kinetics. For instance, as the cells were exposed to the pulses of 51 kV/cm, transport rates of EtBr into the cells remained nearly the same as those in the absence of PEFs, showing that the plasma membranes were not significantly affected by 10nsEPs. On the other hand, the plasma membranes were damaged and disrupted by 10nsEPs at given pulse number of 80, 124, 179 kV/cm, showing that the influx rates of EtBr into the cells were higher than those measured in the absence of PEF. Thus, by measuring the influx rates of EtBr into single cells, we quantitatively determine transport kinetics of intact membranes of single cells, as well as assess the degree of damaged membranes of individual cells.

3.5. Dependence of Membrane Integrity and Cell Viability on E and Pulse Number

After the cells had been exposed to the 330 sequential pulses of 0, 51, 80, 124, and 179 kV/cm in the microchannel (Figure 3S and Figures 3–6), we determined their plasma membrane integrity and viability using Trypan blue assay, aiming to investigate their dependence on E and pulse number. The optical images of single cells (Figures 8A: a–b) illustrate that cells exposed to the 330 sequential pulses of 0 and 51 kV/cm are not permeable to Trypan blue, showing that cellular plasma membrane remain intact and single cells are alive. In contrast, Trypan blue is observed in the cells exposed to the 330 sequential pulses of 80, 124 and 179 kV/cm (Figures 8A: c–e), indicating that the cellular plasma membrane is disrupted by the pulses and the cells are dead. Since representative cells from each independent experiment are shown in Figure 8A, they exhibit normal size variation among single cells. Nonetheless, the cells exposed to the higher E appear slightly larger (Figure 8A), which might be attributed to a higher degree of disintegration of cellular plasma membranes at the higher E.

We also investigated the dependence of membrane integrity and viability of individual cells on E and pulse number of 10nsEPs using a conventional E-exposure cuvette system (Figure 4S in Supporting Information), aiming to validate that the real-time study of effects of 10nsEPs on single cells using our newly designed E-exposure microchannel system indeed represents the behavior of bulk cells at single cell resolution. Plots of percentages of viable cells (= number of viable cells divided by total number of cells) versus pulse number at 0, 51, 80, 124, and 179 kV/cm in Figure 8B show a high dependence of percentages of viable cells upon E and pulse number of 10nsEPs. In the absence of electric field ($E = 0$) (Figure 8B: a), the percentages of viable cells remain nearly constant at 95% over time. At E of 51 kV/cm (Figure 8B: b), the percentages of viable cells decrease slightly from 95% to 93% during 0–230 pulses and to 88% as the pulse number increases to 330, indicating a majority of cells are alive after being exposed to the 330 sequential pulses of 51 kV/cm. These results further confirm our observation in Figure 3, showing that the cells exposed to the 330 sequential pulses of 51 kV/cm are alive and cell plasma membranes are intact.

As E increases to 80, 124 and 179 kV/cm (Figure 8B: c–e), percentages of viable cells show the higher dependence on pulse number. At E of 80 kV/cm, the percentages of viable cells decrease from 95% to 84%, 55%, 29%, and 17% as pulse number increases from 0 to 120, 160, 230, and 330, respectively, showing 83% of cells are dead after exposure to the 330 pulses of 80 kV/cm. As E increases to 124 kV/cm, the percentages of viable cells decrease with pulse number even more rapidly than those observed at 80 kV/cm. It decreases to 57%, 18%, 4%, and 0%, as pulse number increases to 100, 170, 230, and 330 pulses, respectively. The result

shows that none of cells survive after exposure to the 330 pulses of 124 kV/cm. As E increases further to 179 kV/cm, the percentages of viable cells decrease even more rapidly with pulse number than those observed at 124 kV/cm. It decreases to 56%, 5%, 0.3%, and 0% as pulse number increases to 40, 180, 230, and 330, respectively, showing that all cells are dead after exposure to the 230 sequential pulses of 179 kV/cm. These results agree well with what we observed in Figures 4–6, showing that the disruption of cellular plasma and nuclear membranes occurs at given pulse number and E of 10nsEPs as discussed above.

Notably, Trypan blue staining assay is primarily suited for detecting the integrity of plasma membrane and identifying dead cells. To further determine the viability of cells (non-apoptotic cells) that were not permeable to Trypan blue (Figure 8A: a and b), we cultured the cells that had been exposed to the 330 pulses of 51 kV/cm in the cuvette exposure system and in the absence of E , followed the cell growth over 5 days and characterized apoptotic cells using annexin-V-FITC assay (Figure 9). Annexin-V, a high affinity phospholipid binding protein, was conjugated with FITC. No fluorescence was observed in viable (not-apoptotic) cells (Figure 9A–b), because phosphatidylserine molecules were located at the inner face of cytoplasmic membrane of viable cells and could not bind with extracellular annexin-V-FITC. In contrast, fluorescence of annexin-V-FITC was observed in the apoptotic cells (positive control experiment; Figure 9A–c) because phosphatidylserine molecules redistributed from the inner face of the membrane to the outer leaflet during apoptosis of cells, allowing them to bind with extracellular annexin-V-FITC.

We found that the cells exposed to the 330 pulses of 51 kV/cm grew over time and percentages of non-apoptotic (viable) cells are above 97%, which is slightly lower than the cells that had not been exposed to the electric field (Figure 9), showing that the cells exposed to the 330 pulses of 51 kV/cm are indeed viable and alive. The result in Figure 9B agrees well with what we observed in our single-cell microchannel exposure system (Figures 8A and B: b), showing that the majority of cells are indeed viable after exposure to the 330 sequential pulses of 51 kV/cm and EtBr molecules can indeed transport into viable cells. These results further demonstrate that we can manipulate intra-nuclear nucleic acids of single living cells using the 51 kV/cm pulses without altering cell viability and disrupting cellular plasma membrane (Figure 3).

Taken together, these results show high dependence of cell viability on E and pulse number of 10nsEPs in a non-linear manner (Figure 8), which agrees well with high-dependence of membrane transport rates of single cells on E and pulse number (Figures 3–7). These results also show the cumulative effects of sequential 10nsEPs on cellular membrane integrity and viability. At the lower E (0–51 kV/cm), the cumulative energy of the 330 consecutive 10nsEPs is not sufficient to disrupt the interaction of well-organized membrane molecules and the cells survive. This result further validates that we can manipulate intra-nuclear nucleic acids of living cells without harming its viability and plasma membranes. As E increases to 80, 124 and 179 kV/cm, the fewer number of sequential 10nsEPs is needed to break down the plasma membrane transport barriers and disrupt membranes, which results in the cell death. This result indicates that the critical energy is required to disrupt the interaction of membrane molecules and temporarily or permanently damage well-organized cellular membrane structures. These interesting observations suggest that sequential number of 10nsEPs at given E are needed to offer sufficient energy to increase plasma membrane transport and porosity. The plausible explanation of such interesting observations is that cumulative effects of sequential pulses might induce charge redistribution of plasma membrane molecules (e.g., phospholipids, membrane proteins), which might lead to the change of their dipoles and disruption of their interactions and well-organized structures and thereby result in increased membrane porosity.

The 10nsEPs would be too short to charge the cellular plasma membrane if the plasma membranes were an ideal dielectric membrane as assumed in single-cell model (Eq. 1). Notably, ions transport through cellular plasma membrane; thereby the plasma membrane is not an ideal dielectric membrane, which may explain the dependence of porosity and transport rates of plasma membrane on higher E and pulse numbers of 10nsEPs. Nonetheless, effects of the pulses on subcellular structures may trigger cell death (apoptosis), leading to the higher porosity and disintegration of plasma membrane, so-called indirect effect of 10nsEPs on plasma membrane. However, indirect effects would not occur instantaneously as the pulses were applied, as what we observed at the higher E (> 51 kV/cm) in these real-time measurements, suggesting possible direct effects of 10nsEPs on the plasma membrane, and further demonstrating the importance of real-time studies of effects of 10nsEPs on cellular function and the need of further development of adequate model systems.

4. Summary

In summary, we have developed a high-resolution and real-time single-cell E-exposure microchannel system that is devoid of thermal and chemical effects on the cells. The system is equipped with 230-nm thin-layer Au electrodes and synchronized with a single living cell imaging station and 10nsEP generator, which allows us to directly image intracellular molecules and structures, membrane transport kinetics, and viability of individual living cells, induced by sequential 10nsEPs in real-time, and study their dependence on the number and E of 10nsEPs. We investigate the cellular responses to sequential 10nsEPs using our microchannel system and conventional cuvette system, and compared both measurements to validate the suitability and unique advantages of our microchannel exposure system. We found that: (i) Intra-nuclear nucleic acids of single living cells can be manipulated without disrupting the cellular plasma membrane and altering cell viability using low E (≤ 51 kV/cm) of sequential 10nsEPs. (ii) Subcellular structures, membrane transport rates and viability of single cells highly depend on the number and E of the pulses, showing cumulative effects of 10nsEPs on cellular functions and subcellular structures and demonstrating the possibility of selectively depriving individual living cells using 10nsEPs. (iii) Critical energy levels are required to surmount energy barriers of membrane transport and membrane integrity, which can be achieved by controlling the number and E of sequential 10nsEPs. These new findings show the cumulative effects of 10nsEPs on intracellular molecules and structures of single cells and demonstrates the possibility of tuning them with high spatial (230-nm at z-direction of E and imaging plane) and temporal resolution (10 ns) using 10nsEPs. Studies are underway to further identify specific components of intracellular structures affected by 10nsEPs and their related cellular functions and pathways, aiming to better understand the effect of 10nsEPs on cellular functions and their underlying mechanisms, and explore the possibility of selectively altering cellular functions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Design and characterization of microchannel E-exposure system and 10nsEP generator:

(A) Photo of the microscope quartz slide fabricated with a microchannel between two Au electrodes (gap = 19.1 μm), and a microchamber cooling reservoir above the microchannel. The slide is placed on the microscope stage and directly connected two Au electrodes with the 10nsEP generator. Scale bar = 22 mm. The schematics are illustrated in Figure 1S in Supporting Information.

(B) Schematic diagram of design of the 10nsEP generator.

(C) Representative 10nsEP acquired by an oscilloscope shows a nearly rectangular pulse with a voltage of 500 V, a duration of 10 ns, and voltage rise and fall time of nearly 2 ns.

A.

B.

Figure 2.

Schematics of the interface and synchronization of a single living cell imaging system with a 10nsEP generator:

(A) A homemade microscope plate with two electric contacts is used to directly connect two Au electrodes of microchannel on the slide with the 10nsEP generator and to secure the slide on the microscope stage (Figure 1A). The wave-generator is used to synchronize: an electronic shutter that controls the fluorescence excitation beam; the shuttle of CCD camera that controls exposure time of camera (TTL signal); and the MOSFET switch of the 10nsEP generator, allowing single cells to be imaged using fluorescence microscopy, as the sequential 10nsEPs are applied between two electrodes. The pulses are measured in real time using an oscilloscope. (B) Schematic waveforms of TTL signal of CCD camera, the electronic shutter, and the 10nsEP show that TTL signal opens the electronic shutter in front of excitation beam and activates

MOSFET switch, allowing single cells to be exposed to the fluorescence excitation beam and 10nsEP simultaneously, as their fluorescence images being acquired by CCD camera.

Figure 3.

Manipulating intracellular molecules of single living cells without disrupting their plasma membrane using sequential 10nsEPs of 51 kV/cm. Images of a single living cell (HL60) incubated with 10 μ M EtBr: (A) and (C) are phase-contrast optical images of the cell before the first pulse and after the 330th pulse, respectively; (B) is the fluorescence image of the cell at the first pulse. (B: a–h) are the snapshots of the sequential EtBr fluorescence images of the cell at the (a) 1st, (b) 50th, (c) 100th, (d) 150th, (e) 200th, (f) 250th, (g) 300th, and (h) 330th pulses. The intensity scale of the fluorescence images is 160–320 analog-to-digital (ADC). The arrow shows the direction of PEF. The scale bar is 10 μ m for all images. Real-time video of EtBr fluorescence images of the cell as their exposure to the pulses of 51 kV/cm is shown in Movie 1 in Supporting Information.

Figure 4.

Dependence of subcellular structures, intra-nuclear nucleic acids and membrane transport of single living cells on the number of 10nsEPs of 80 kV/cm. Images of a single cell incubated with 10 μ M EtBr: (A) and (C) are phase-contrast optical images of the cell before the first pulse and after the 330th pulse, respectively; (B) and (D) are the fluorescence images of the cell at the first pulse and 330th pulse, respectively.

(B: a–h) are the snapshots of the sequential EtBr fluorescence images of the cell at the (a) 1st, (b) 150th, (c) 160th, (d) 180th, (e) 200th, (f) 250th, (g) 300th, and (h) 330th pulses. The intensity scale of fluorescence images in (a–d) and (e–h) is from 110 to 270 and 470 ADC, respectively. The arrow directs the PEF. The scale bar is 10 μ m for all images. Real-time video of EtBr fluorescence images of the cell as their exposure to the pulses of 80 kV/cm is shown in Movie 2 in Supporting Information.

Figure 5.

Dependence of subcellular structures, intra-nuclear nucleic acids and membrane transport of single cells on the number of 10nsEPs of 124 kV/cm. Images of a single cell incubated with 10 μ M EtBr: (A) and (C) phase-contrast optical images of the cell before the first pulse and after the 330th pulse, respectively; (B) and (D) are the fluorescence images of the cell at the first pulse and 330th pulse, respectively

(B: a–h) are the snapshots of the sequential EtBr fluorescence images of the cell at the (a) 1st, (b) 30th, (c) 120th, (d) 150th, (e) 200th, (f) 250th, (g) 300th, and (h) 330th pulses. The intensity scale of fluorescence images in (a–d), (e–f), and (g–h) is from 130 to 400, 450 and 550 ADC, respectively. The arrow represents the direction of PEF. The scale bar is 10 μ m for all images. Real-time video of EtBr fluorescence images of the cell as their exposure to the 330 sequential pulses of 124 kV/cm is shown in Movie 3 in Supporting Information.

Figure 6.

Dependence of subcellular structures, intra-nuclear nucleic acids and membrane transport of single living cells on the number of sequential 10nsEPs of 179 kV/cm. Representative images of a single cell incubated with 10 μ M EtBr: (A) and (C) are phase-contrast optical images of the cell before the first pulse and after the 330th pulse, respectively; (B) and (D) are the fluorescence images of the cell at the first pulse and 330th pulse, respectively.

(B: a–h) are snapshots of the sequential fluorescence images of the cell at the (a) 1st, (b) 20th, (c) 100th, (d) 150th, (e) 200th, (f) 250th, (g) 300th, and (h) 330th pulses. The intensity scale of fluorescence images in (a–d), (e), (f), (g), and (h) is from 170 to 500, 600, 700, 900, and 1200 ADC, respectively. The arrow shows the direction of PEF. The scale bar is 10 μ m for all images. Real-time video of EtBr fluorescence images of the cell as their exposure to the pulses of 179 kV/cm is shown in Movie 4 in Supporting Information.

Figure 7. Dependence of membrane transport kinetics of single living cells (HL60) on E and pulse number of 10nsEPs. Plots of integrated fluorescence intensity of single cells in Figure 3S in Supporting Information and Figures 3–6B versus pulse number for E at: (a) 0, (b) 51, (c) 80, (d) 124, and (e) 179 kV/cm. The time interval between each pulse is 1.333 s.

Figure 8.

Dependence of cell viability on E and pulse number of 10nsEPs:

(A) Images of single cells incubated with 0.2% Trypan blue for 5 min after single cells in the microchannel system (Figure 3S-C and Figures 3–6C) were exposed to the 330 pulses of E at (a) 0, (b) 51, (c) 80, (d) 124, and (e) 179 kV/cm, respectively. The scale bar is 10 μ m for all images.

(B) Plots of percentages of viable cells versus pulse number of E at (a) 0, (b) 51, (c) 80, (d) 124, and (e) 179 kV/cm. Cells were exposed to the given number and E of 10nsEPs using the conventional cuvette exposure system (Figure 4S in Supporting Information).

Figure 9.

Characterization of apoptotic and non-apoptotic (viable) cells (HL-60) that were exposed to the 330 sequential 10nsEPs of 51 kV/cm.

(A) Determination of apoptotic and non-apoptotic cells using Annexin V-FITC assay: (a) optical and (b) fluorescence images of a non-apoptotic cell; and (c) fluorescence image of an apoptotic cell. The scale bar = 5 μm .

(B) Plots of percentages of non-apoptotic cells versus cell-cultured time: (a) cells without exposure to the pulses (blank control); (b) cells after exposure to the 330 sequential pulses of 51 kV/cm. Cells exposed to the 330 pulses of 51 kV/cm were cultured in the medium for 5 days. Apoptotic and non-apoptotic cells in the medium were then characterized over time using Annexin V-FITC assay.

Table 1

Summary of Dependence of Membrane Transport Kinetics on E and Pulse Number

E (kV/cm)	Pulse Number	Fluorescence Intensity Rate (s^{-1})	Membrane Transport Rate (s^{-1})
0	0–70	$-(1.3 \pm 0.6) \times 10^4$	0
	70–110	$-(7.9 \pm 5.2) \times 10^3$	0
	110–330	$-(2.6 \pm 1.0) \times 10^3$	0
51	0–50	$-(7.8 \pm 2.0) \times 10^3$	$(5.0 \pm 2.0) \times 10^3$
	50–110	$-(2.8 \pm 0.6) \times 10^3$	$(6.7 \pm 0.6) \times 10^3$
	110–330	$-(7.9 \pm 5.6) \times 10^2$	$(1.6 \pm 5.6) \times 10^2$
80	0–70	$-(6.2 \pm 5.7) \times 10^3$	$(7.3 \pm 4.3) \times 10^3$
	70–120	$-(6.6 \pm 6.3) \times 10^3$	$(0.7 \pm 1.2) \times 10^4$
	120–210	$(1.5 \pm 2.4) \times 10^4$	$(2.2 \pm 1.9) \times 10^4$
	210–330	$(6.7 \pm 7.7) \times 10^4$	$(5.7 \pm 5.9) \times 10^4$
124	0–20	$-(5.4 \pm 3.3) \times 10^3$	$(7.4 \pm 3.3) \times 10^3$
	20–110	$(3.0 \pm 2.7) \times 10^4$	$(4.1 \pm 2.7) \times 10^4$
	110–180	$(3.7 \pm 2.1) \times 10^4$	$(4.0 \pm 2.1) \times 10^4$
	180–330	$(3.1 \pm 1.5) \times 10^4$	$(3.3 \pm 1.5) \times 10^4$
179	0–10	$-(0.3 \pm 6.5) \times 10^3$	$(1.2 \pm 0.7) \times 10^4$
	10–110	$(8.2 \pm 9.6) \times 10^4$	$(9.3 \pm 9.6) \times 10^4$
	110–200	$(4.1 \pm 0.3) \times 10^4$	$(4.3 \pm 0.3) \times 10^4$
	200–330	$(5.3 \pm 5.7) \times 10^4$	$(5.6 \pm 5.7) \times 10^4$