# Nanosecond Pulsed Electric Fields Trigger Intracellular Signals in Human Lymphocytes

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## ABSTRACT

We present evidence for a new technological approach to signaling and triggering of *intracellular* events in biological cells. Remotely applied nanosecond, megavolt-per-meter, pulsed electric fields perturb the intracellular environment, causing calcium release, phosphatidylserine externalization, and apoptosis. This technology does not require direct electrical contacts to biological material. Ultra-short pulses with durations less than the charging time constant of the plasma membrane generate voltages across structures in the cell interior, enabling intracellular manipulations with stimuli that are remotely generated and controlled. The work reported here suggests possibilities within the reach of state-of-the-art bioelectrical engineering and nanotechnology for utilizing nanopulse-triggered intracellular electroperturbations in remote bio-systems deployed as reporters and effectors, and for other applications such as wound healing.

***Keywords***: nanosecond, megavolt-per-meter, calcium burst, electroperturbation, phosphatidylserine translocation

## 1 INTRODUCTION

This paper summarizes ongoing studies of a new approach for inducing electric field effects within biological cells, without contacts or probes directly attached to the cells. We describe basic observations demonstrating that properly tailored external electrical pulses of short (ns) duration produce clearly demarcated internal effects, such as programmed cell death (apoptosis).

Regulated changes in electrical potential, particularly the transmembrane voltages at the interfaces between intracellular compartments, between the cytoplasm and the external environment, and between neighboring cells, comprise an integral part of the network of mechanisms through which living systems respond to their internal and external environments. The development of practical methods for controlling these cell signaling pathways requires minimally invasive means for accessing them *inside* cells. DC and low frequency electric fields affect cell activities through non-specific effects mediated through receptors in the external membrane.

We demonstrate here that pulsed power physics and electronics offer a convenient and versatile tool for reaching *into* the cell — ultra-short (ns), high-field (MV/m) electrical pulses. Fast-rising pulses of very brief duration bypass the plasma membrane dielectric and at the same time eliminate the complications of heating even with very large pulse amplitudes (high power but *low* total energy).

Critical to this approach is addressing the biology demonstrating the *intracellular* effects of ultra-short external pulses, which bypass the cytoplasmic membrane. The charging time constant for the plasma membrane for many eukaryotic cells is on the order of 100 ns [1]. For these cells a nanosecond pulsed electric field is an *intracellular* stimulus [2], in contrast to the situation with the longer, slower-rising (>μs) pulses used for conventional electroporation [3].

Intracellular effects of nanoelectropulse exposure reported previously include permeation of eosinophil granules [4], calcium bursts in T cells [5], and various indicators of apoptosis in several cell lines [2], [6], [7]. Engineering biologically effective devices and systems in this new ultra-short, high-field regime will require deepening and extending this knowledge.

In this report we concentrate on two immediate effects of remote nanoelectropulse perturbation of the intracellular environment in human lymphocytes (Jurkat T cells): increases in cytoplasmic calcium concentration [5] and translocation of phosphatidylserine (PS) to the external face of the cell membrane [2]. Because calcium ions serve as regulatory messengers in a wide variety of processes across the physiological landscape of the cell, understanding how to manipulate calcium ion release with remotely delivered electrical signals is of great interest. We monitor intracellular calcium concentration changes in live cells during pulse exposure with the calcium-sensitive fluorochromes Calcium Green-1 and rhod-2.

PS externalization modifies the thermodynamic and mechanical equilibria associated with the membrane lipid bilayer and marks the cell physiologically for removal by phagocytic agents. We track this event in real time with FM1-43, a cationic styryl fluorescent dye that localizes in the outer leaflet of the plasma membrane lipid bilayer, and which exhibits increased binding when the PS fraction increases on the external face of the membrane [8].

Intracellular calcium modulation and maintenance of membrane phospholipid asymmetry represent important intra- and extracellular signaling pathways. The ability to control these and other physiological signals remotely, through externally generated electric pulses, opens new avenues for bioengineering in the nanosecond, nanometer regime.

## 2 MATERIALS AND METHODS

*Cell lines and culture conditions.* Human Jurkat T lymphocytes (ATCC TIB-152) were maintained as described previously [2].

*Pulse generator and pulse exposures.* A MOSFET-based, inductive-adding pulse generator with a balanced, coaxial-cable pulse-forming network and spark-gap switch designed and assembled at the University of Southern California, provided trapezoidal electrical pulses to cell suspensions (2 x 107 cells /mL) at room temperature in growth medium in rectangular electroporation cuvettes with a 1-millimeter electrode separation (Bio-Rad).

For microscopic observations, cells were placed in a rectangular channel 100 μm wide, 30 μm deep, and 12 mm long, with gold-plated electrode walls, microfabricated with photolithographic methods on a glass microscope slide. A fast MOSFET MicroPulser [9] was mounted on the microscope stage for delivery of pulses directly to the microchamber electrodes in ambient atmosphere at room temperature.

*Fluorescence microscopy.* Observations of live cells stained with Calcium Green (Molecular Probes) were made with an epifluorescence microscopy system as described previously [5]. To reduce nonspecific cytoplasmic staining with the calcium-sensitive cationic fluorochrome rhod-2, cells were loaded at 2 μM in growth medium for 5 minutes at 25 °C, 25 minutes at 4 °C, 30 minutes at 37 °C, resuspended in growth medium without rhod-2, and incubated for an additional 60 minutes at 37 °C.

For $Mn^{2+}$ quenching experiments, cells were incubated in 250 μM $MnCl_2$ in growth medium for 15 minutes at 37 °C. To stain mitochondria, cells were loaded with JC-1 (Molecular Probes; 750 nM) or MitoTracker Green (Molecular Probes; 50 nM). FM1-43 (Molecular Probes; $\lambda_{ex}$ = 480 nm, $\lambda_{em}$ = 580 nm) was added to the medium at 5 μM 20 minutes before observations of PS externalization.

EGTA and ionomycin were obtained from Calbiochem. $LaCl_3$, $GdCl_3$, cyclosporin A, verapamil, and ruthenium red were from Sigma-Aldrich, and BAPTA-AM and thapsigargin were from Molecular Probes.

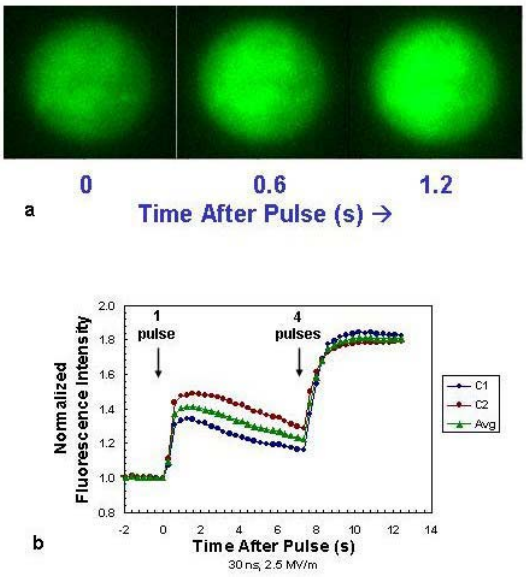


Figure 1: (a) Calcium Green fluorescence images of calcium burst in Jurkat T cells after single pulse, 30 ns, 2.5 MV/m. (b) Normalized calcium burst intensity (Calcium Green fluorescence) after 1 and 4 pulses, 30 ns, 2.5 MV/m.

## 3 RESULTS

*Calcium bursts.* Observations with Calcium Green show a uniform release of calcium throughout the cell within milliseconds after the leading edge of a nanosecond electric pulse (Fig. 1). Pulse-induced calcium bursts are not affected by EGTA in the medium or by the calcium channel blockers $La^{3+}$, $Gd^{3+}$, or verapamil, or by the mitochondrial permeability transition inhibitor cyclosporin A, or by the mitochondrial calcium transport blocker ruthenium red, but they are inhibited by thapsigargin and cytochalasin D [5].

Cells loaded with rhod-2, a calcium-sensitive fluorochrome with a larger calcium dissociation constant than Calcium Green-1 (570 nM versus 190 nM), present a somewhat different picture (Fig. 2). The nanoelectropulse-induced rhod-2 fluorescence increase is more gradual, spanning several seconds, and in many cells the fluorescence intensification occurs at multiple distinct locations in the cytoplasm. These localized rhod-2 bright spots are not stained by JC-1 or MitoTracker Green, indicating that they are not mitochondria.

Cells loaded with the calcium chelate BAPTA or the calcium competitor and fluorescence quencher $Mn^{2+}$ do not show pulse-induced Calcium Green or rhod-2 fluorescence intensification.

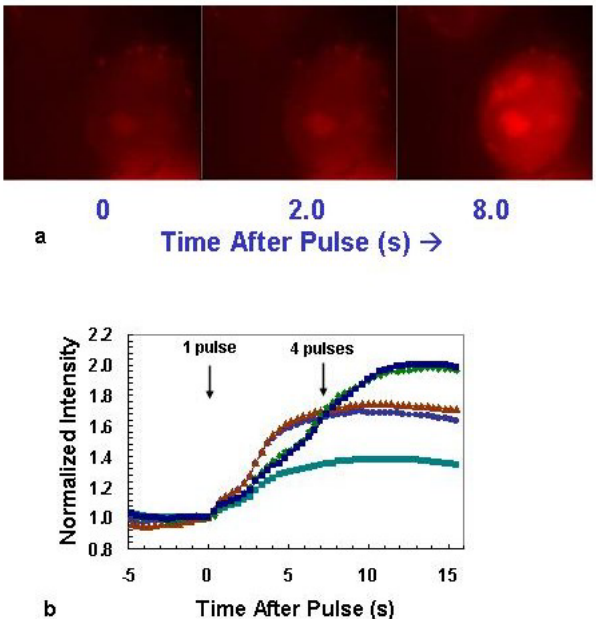


Figure 2: (a) Rhod-2 fluorescence images of calcium burst in Jurkat T cells after single pulse, 30 ns, 2.5 MV/m. (b) Normalized calcium burst intensity (rhod-2 fluorescence) after 1 and 4 pulses, 30 ns, 2.5 MV/m.

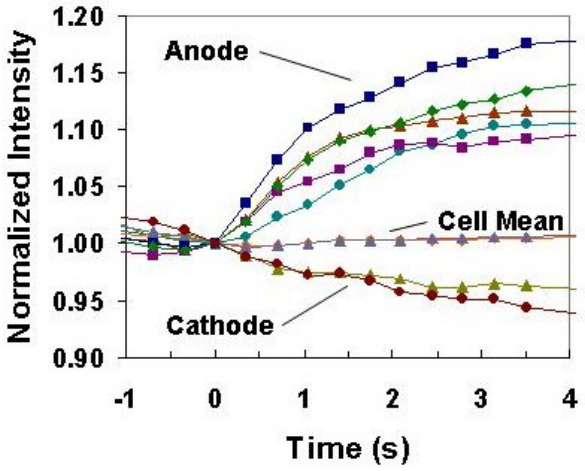


Figure 3: FM-1-43 fluorescence intensity at selected points in a cell after pulsed electric field exposure, 4 pulses, 30 ns, 2.5 MV/m, normalized to the integrated intensity of the whole cell. Points at the anode pole of the cell brighten relative to the integrated intensity of the whole cell, while the response of points at the cathode pole is less than the cell mean.

Longer, microsecond pulses porate the external membrane and permit entry of propidium iodide, $Ca^{2+}$, $Na^{+}$, and $Mn^{2+}$ into the cell. No influx of these species is detected after nanosecond pulses [2], [5].

*Membrane phospholipid rearrangement.* Nanoelectropulse-induced changes in the intensity and distribution of the membrane-staining dye FM1-43 begin less than one second after pulse exposure. An initial brightening at the anodic pole of the cell appears to dissipate within seconds, but over the next several minutes the fluorescence intensity of FM1-43 in the membrane increases all around the cell (Fig. 3).

These observations would be expected if the interaction of the pulsed field with the cell causes an immediate rearrangement of membrane phospholipids at the pole of the cell nearest the anode, including a translocation of some PS molecules from the cytoplasmic side of the membrane, where they normally are exclusively found, to the outer surface of the cell. This PS externalization would lead to the binding of additional FM1-43, but the initial local concentration disturbance would be quickly dissipated by lateral diffusion in the bilayer. Over the next few minutes, levels of FM1-43 in the membrane would rise, adjusting to the new equilibrium concentration of PS in the membrane

## 4 DISCUSSION

A conservative interpretation of the evidence reported and summarized here leads to the conclusion that nanoelectropulses cause the release of calcium from intracellular compartments and the translocation of PS in the plasma membrane through directly physical and immediate mechanisms — without any measurable poration of the cell membrane.

Nanoelectropulses could trigger intracellular calcium release in a number of ways. Our data suggests the involvement of the calcium compartments of the endoplasmic reticulum (which cannot be re-supplied in the presence of thapsigargin) and some components of the cytoskeleton (which cannot be dynamically maintained in the presence of cytochalasin D).

Two mechanisms for pulse-induced PS externalization may be considered: nanopore-facilitated "lateral" diffusion and direct electrostatic transport across the dielectric barrier of the lipid bilayer (Fig. 4).

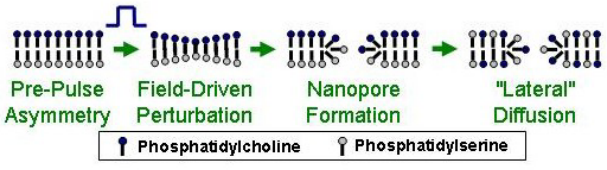


Figure 4: Formation of an aqueous pore with nanometer dimensions permits "lateral" diffusion of phosphatidylserine from the inner leaflet to the external face of the cell. Transmembrane voltages greater than 1 V may provide sufficient energy (50 kT) to drive the charged phospholipid head group directly across the hydrophobic membrane dielectric; no pore formation would be required.

A simple dielectric shell model for nanoporation (nanometer-diameter, nanosecond-duration electropores) predicts PS externalization at both the anode and cathode poles of a nanoelectropulsed cell [5]. FM1-43 fluorescence intensification after pulse exposure always occurs at the anode pole and has never been observed at the cathode pole, consistent with direct, electrostatically driven migration of the negatively charged PS head group across the membrane lipid bilayer.

Ultra-short, high-field electric pulses are not immediately physically damaging to cells and not necessarily lethal in the medium or long term (although they induce apoptosis under the right conditions). At low pulse counts they produce no gross morphological changes. Nanoelectropulsed cells remain in place, actively metabolizing, potentially serving as dynamic environmental sensors and transducers. Appropriately prepared cells, or bio-inspired nanomachines differentially sensitive to a defined set of pulse regimens (Fig. 5), could execute on remote command a variety of biochemical tasks, as standalone "biobots" or as components of integrated biomicroelectromechanical systems or other "lab on a chip" implementations.

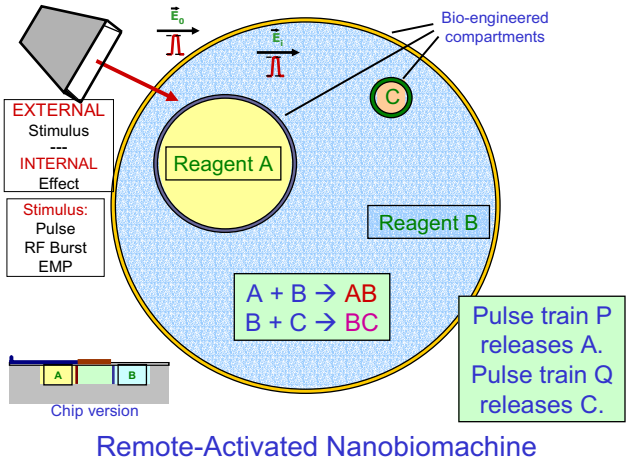


Figure 5: Nested dielectric shells with biochemically active components — a bionanoelectromechanical system.

## 5 ACKNOWLEDGEMENTS

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