REPORT DOCUMENTATION PAGE

AFRL-SR-AR-TR-04-

0458

valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE (DD-MM-YYYY)	2. REPORT TYPE	3. DATES COVERED (From - To)
30/08/2004	~~Annual~~ Technical (Final)	01/08/2003 – 31/07/2004

4. TITLE AND SUBTITLE

Ultra Short Pulse Electroporative Physics and Technology (Bio-Inspired Theme)

5a. CONTRACT NUMBER

5b. GRANT NUMBER
F49620-01-1-0495

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Gundersen, Martin A.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Southern California
Electrical Engineering-Electrophysics
3737 Watt Way, PHE 512
Los Angeles, CA 90089-0271 NE

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

USAF, AFRL
Air Force Office of Scientific Research
4015 Wilson Blvd, Room 713
Arlington, VA 22203-1954

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release: distribution is unlimited.

20040910 057

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The project has demonstrated that ultra-short, high-field pulses are a useful tool for study of cellular physiology, and a potential therapeutic instrument for malignant cells. The research has established that intracellular effects including apoptosis can be induced by the application of short, intense (but low total energy) electric pulses. Experiments on human cells have produced convincing evidence that these applied fields nondestructively alter subcellular processes and can be investigated using biophotonic studies for imaging of morphological and functional changes at subcellular levels. The results suggest a promising pathway toward achieving the ultimate goal of selectively disabling tumor or other undesirable cells. In addition to demonstrating that ultrashort pulses have potentially important applications for cell and cancer biology investigations, and for understanding the application of pulsed fields to living matter, these results demonstrate that an effective and productive multidisciplinary collaboration between science, engineering, medicine and biology has been created.

15. SUBJECT TERMS

nanosecond high-field electric pulse, electroperturbation, electroporation, pulse-Induced phospholipid translocation, nanoelectropulse

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Dr. Robert Barker
Unclassified	Unclassified	Unclassified	UL	8	19b. TELEPHONE NUMBER (include area code) 703-696-8574

"Ultra Short Pulse Electroporative Physics and Technology

(Bio-Inspired Theme)"

AFOSR Grant No. F49620-01-1-0495

M. Gundersen, P.I. mag@usc.edu

OBJECTIVES

The objective proposed was to conduct electroporation and electroperturbation experiments using a range of electrical pulse parameters to determine the response of cells to the fields, and determine sensitivity to fields including RF and tailored electromagnetic fields. Questions to be addressed included: Will the application of ultra-short pulses result in intranuclear effects on genes, cell wall membranes, and/or other organelles? Can simulations be used to exploit the data and guide further experiments? Will it be possible to visualize through biophotonic experiments and simulations the effect of fields on cell membranes? Can data be applied to theoretical models of cell wall structure, leading to a quantitative understanding of cell membrane transport processes? Can the short pulses be effective in controlling processes at the cell nuclear and mitochondrial membranes? Will there be device applications incorporating the cell response to the fields, that can form the basis for bio-inspired devices? Will there be new therapies for cells, such as cancer treatment, that will be enabled through a combination of the applied fields, biophotonic diagnostics, and simulations?

STATUS OF EFFORT

Initial investigations of the responses of mammalian cells to ultra-short, high-field electric pulses established an experimental framework in which reproducible, physiologically significant effects can be observed, including the induction of programmed cell death (apoptosis). Subsequently we demonstrated that the responses of different cell types to nanosecond, megavolt-per-meter pulse exposure are different, and we identified two pulse-induced events that occur within milliseconds of pulse delivery and which represent important biological signals --- phosphatidylserine (PS) externalization (rearrangement of plasma membrane phospholipids) and calcium bursts (intracellular calcium release). In order to identify the mechanisms through which nanoelectropulses perturb cellular homeostasis, we are currently engaged in characterizing pulse-induced PS translocation and calcium bursts using real-time, live-cell fluorescence microscopic methods of analysis. In addition we are proposing to expand the work systematically to other cell lines and, in collaboration with a leading hematologist and oncologist, to develop pulse delivery systems for treatment of diseased cells and tissues in whole organisms, and to investigate potential therapeutic applications for this technology. Details are briefly summarized below on **nanoelectropulse-induced apoptosis** and two immediate intracellular consequences: **increases in cytoplasmic calcium concentration** and **translocation of phosphatidylserine (PS) to the external face of the cell membrane**.

ACCOMPLISHMENTS/NEW FINDINGS

Described in this report are results of nanoelectropulse investigations into several cell lines. A companion report for the grant "Pulsed Electric Fields For Biological Weapons Defense", AFOSR Grant No. F49620-02-1-0073, describes research into pulsed power supporting these studies (that is, detail about pulse generators incorporated into a microscope for real-time investigations).

Nanoelectropulse-induced apoptosis

Our experiments earlier in the project [Vernier et al., IEEE TDEI, 2003] demonstrated nanoelectropulse-induced apoptosis in actively metabolizing Jurkat T lymphoblasts in culture medium, and we also showed that another mammalian cell type, rat glioma C6, is relatively resistant to the effects of ultra-short, high-field electric pulses. Pulse-induced calcium bursts have been observed in human lymphocytes, including Jurkat and normal CD4+ T cells, human multiple myeloma cells, and resting and activated normal human peripheral blood mononuclear cells (PBMCs). Pulse-driven PS externalization studies have concentrated on Jurkat T lymphoblasts.

Increases in cytoplasmic calcium concentration (*Calcium bursts).* 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 monitored intracellular calcium concentration changes in live cells during pulse exposure with the calcium-sensitive fluorochromes Calcium Green-1 and rhod-2, using an epifluorescence microscopy system as described previously [Vernier et al., BBRC, 2003]. To reduce nonspecific cytoplasmic staining with the calcium-sensitive cationic fluorochrome rhod-2, cells were loaded with the acetoxymethyl ester at 4 °C to allow dye molecules to pass through the membranes of intracellular structures, then incubated at 37 °C to permit hydrolysis of the ester linkages, resuspended in growth medium without rhod-2, and incubated for an additional 60 minutes at 37 °C.

Observations with Calcium Green show a uniform, dose-dependent release of calcium throughout the cell within milliseconds after the leading edge of a nanosecond electric pulse. 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 [Vernier et al., BBRC, 2003].

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. 1). 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. The simplest

interpretation of these observations is that the calcium released in the initial burst is quickly taken up by intracellular buffers, most likely 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.

We have also observed nanoelectropulse-induced calcium bursts in normal human CD4+ T cells and in the human multiple myeloma cell lines RPMI 8226 and NCI-H929 (Fig. 15). In some cases a pulse-induced oscillation of intracellular $[Ca^{2+}]$ with a period of several seconds is seen in multiple myeloma cells, but never in Jurkat cells.

Comparisons of the calcium burst sensitivity of resting and phytohemagglutinin-activated peripheral blood mononuclear cells (PBMCs) with Jurkat and multiple myeloma cells (Fig. 16) reveal substantial differences in the responses of these different cell types. Dose-response studies and systematic comparisons of these and other cell types are needed to identify the origins of these differences and to evaluate their potential scientific and therapeutic significance.

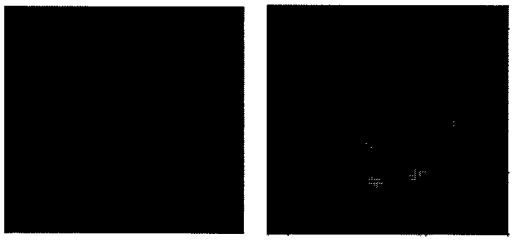

Figure 1. Rhod-2-stained Jurkat T cell before and 15 s after exposure to 4 pulses, 30 ns, 2.5 MV/m, revealing localized increases in $[Ca^{2+}]_i$ after the initial burst.

Lower-field but 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 [Vernier et al., BBRC, 2003], [Vernier et al., Biophys. J., 2004], but recently we have observed entry of the dye YO-PRO-1, to which early apoptotic cells become permeable while still excluding propidium iodide, after nanoelectropulse exposures, providing direct evidence that even very short pulses affect the plasma membrane, although the disturbances are perturbative rather than porative in the conventional sense.

Translocation of phosphatidylserine (PS) to the external face of the cell membrane. *Membrane phospholipid rearrangement.* 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 tracked pulse-induced PS translocation 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. FM1-43 (Molecular Probes; λ_{ex} = 480 nm, λ_{em} = 580 nm) was added to the medium at 5 μM 20 minutes before observations of PS externalization to permit equilibration of the partition of FM1-43 between the aqueous medium, where it is essentially non-fluorescent, and the membrane.

Nanoelectropulse-induced changes in the intensity and distribution of FM1-43 fluorescence, indicating PS translocation, 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. 2). The relative amount of fluorescence increase is a function of the pulse amplitude (applied electric field) and the number of pulses.

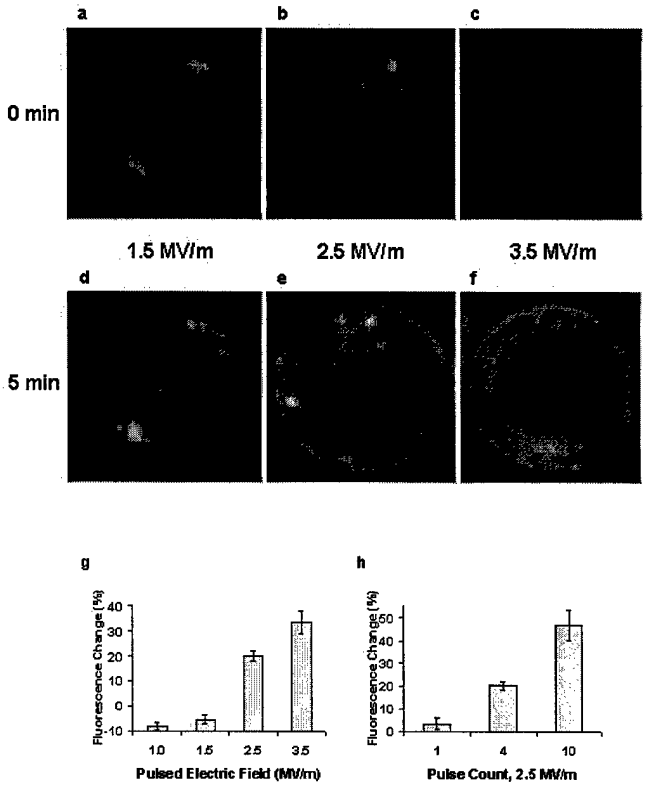

Fig. 2. PS externalization response with increasing pulsed field strength and pulse count and after disruption of Ca^{2+} distribution and actin polymerization. Representative Jurkat cells stained with FM1-43 are shown before (**A, B, C**) and 5 min after (**D, E, F**) exposure to 4, 30 ns pulses at 1.5, 2.5, and 3.5 MV/m. Increased FM1-43 fluorescence indicates translocation of PS to the external face of the cytoplasmic membrane. (**G**) Integrated, whole-cell, FM1-43 fluorescence changes 5 min after exposure to 4, 30 ns pulses of 1.0, 1.5, 2.5, and 3.5 MV/m. (**H**) Integrated, whole-cell, FM1-43 fluorescence changes 5 min after exposure to 1, 4, and 10, 30 ns pulses of 2.5 MV/m delivered to separate groups of cells with a pulse repetition rate of 4 Hz. Data is from at least 20 representative cells from three independent experiments. Error bars

We interpret these observations to mean that 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, consistent with electrophysical models, and a translocation at the point of this disturbance of 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 leads to binding of additional FM1-43 at the anode pole, but the initial local concentration disturbance is dissipated within seconds by lateral diffusion in the bilayer. Over the next few minutes, levels of FM1-43 in the membrane rise, in response to the overall increased concentration of PS in the external leaflet of the lipid bilayer. Rat glioma C6 cells, both attached to a substrate and in suspension, are much more resistant to pulse-induced PS externalization than are Jurkat cells. Ongoing work with higher applied fields (6 MV/m) and shorter pulses (3 ns) is consistent with a membrane dielectric charging model and with the dose-response relationships observed with lower fields and "longer" (but still less than the charging time constant of the plasma membrane) pulses.

PERSONNEL SUPPORTED

Professor Martin Gundersen	Professor/Department Chair
Dr. Aimin Li	Research Associate
Mr, Matthew Behrend	Undergraduate Research Assistant
Mr. Peter Gabrielsson	Technician
Ms. Katherine Chiu	Undergraduate Research Assistant
Ms. Sarah Salemi	Graduate Research Assistant
Ms. Mya Thu	Graduate Research Assistant
Mr. Yushun Zhang	Research Lab Technician

PUBLICATIONS

These publications and presentations were supported in part by other grants including, principally, the AFOSR support for biodefense studies (AFOSR Grant No. F49620-02-1-0073, described in a separate report).

Journal Papers

Vernier, P. T., A. Li, L. Marcu, C. M. Craft, and M. A, Gundersen, "Ultrashort pulsed electric fields induce membrane phospholipid translocation and caspase activation: differential sensitivities of Jurkat T lymphoblasts and rat glioma C6 cells, IEEE Trans. Dielect. Elect. Ins. 10:795—809, 2003.

Vernier, P. T., Y. Sun, L. Marcu, S. Salemi, C. M. Craft, and M. A. Gundersen, Calcium bursts induced by nanosecond electric pulses, *Biochem. Biophys. Res. Commun.* 310:286-295, 2003.

Vernier, P. T., M. Thu, L. Marcu, C. M. Craft, and M. A. Gundersen, Nanosecond electroperturbation -mammalian cell sensitivity and bacterial spore resistance, in press, *IEEE Trans. Plasma Sci.*, August 2004.

Vernier, P. T., Y. Sun, L. Marcu, C. M. Craft, and M. A. Gundersen, Nanoelectropulse-induced phosphatidylserine translocation, in press, *Biophys. J.* 86, 2004.

Vernier, P. T., Y. Sun, L. Marcu, C. M. Craft, and M.A. Gundersen, "Nanosecond Pulsed Electric Fields Perturb membrane Phospholipids in T Lymphoblasts," in press FEBS Letters.

Vernier, P. T., Y. Sun, L. Marcu, C. M. Craft, and M. A. Gundersen, "Nanosecond Pulsed Electric Fields Perturb Membrane Phospholipids in T Lymphoblasts," FEBS lett. 572:103-108, 2004.

Papers in Conference Proceedings

X. Gu, A. Kuthi, M. Behrend, P.T. Vernier, and M.A. Gundersen, "Compact Pulse Generator for Nanosecond Electroperturbation of Biological Cells," 26th IEEE International Power Modulator Conference, San Francisco, CA, May 23-26, 2004.

Kuthi, P. Gabrielson, M. Behrend and M. Gundersen, "Nanosecond Pulse Generator Using a Fast Recovery Diode," 26th IEEE Power Modulator Conference, San Francisco, CA, May 23-26, 2004.

Kuthi, M. Behrend, T. Vernier and M. Gundersen, "Bipolar Nanosecond Pulse Generation Using Transmission Lines for Cell Electromanipulation," 26th IEEE Power Modulator Conference, San Francisco, CA, May 23-26, 2004.

P.T. Vernier, Y. Sun, L. Marcu, C.M. Craft, and M.A. Gundersen, "Nanosecond Pulsed Electric Fields Trigger Intracellular Signals in Human Lymphocytes," Nanotech 2004, Boston, MA, March 7-11, 2004, Technical Proceedings of the 2004 NSTI Nanotechnology Conference and Trade Show, Vol. 1, Ch. 1, pp. 7-10, 2004.

Y. Sun, P.T. Vernier, M. Behrend, L. Marcu, and M.A. Gundersen, "Microscope Slide Electrode Chamber for Nanosecond, Megavolt-Per-Meter Biological Investigations", Nanotech 2004, Boston, MA, March 7-11, 2004, Technical Proceedings of the 2004 NSTI Nanotechnology Conference and Trade Show, Vol. 1, Ch. 11, pp. 485-488.

P. T. Vernier, L. Marcu, Y. Sun, S. Salemi, C. M. Craft, and M. A. Gundersen, "Real-time imaging of mammalian cells in nanosecond, megawatt, millijoule pulsed electric fields", BiOS 2004 (SPIE), San Jose, Jan. 2004.

INTERACTIONS/TRANSITIONS

A. PARTICIPATION/PRESENTATIONS AT MEETINGS, CONFERENCES, SEMINARS, ETC.

Oral Presentations, Invited

"Physics and Applications of Pulsed Power", M. Gundersen, presented to the Physics Department of the Naval Postgraduate School, August 2003.

"Pulsed Power: Physics, and Two Diverse Applications", M. Gundersen, Lawrence Berkeley Laboratory, February 17, 2004.

Contributed Oral Presentations, Conference

"Non-invasive intracellular electroperturbation of human lymphocytes," Vernier, P.T., Y. Sun, L. Marcu, S. Salemi, C. M. Craft, and M. A. Gundersen, "Workshop on High-Field Effects and Fast Pulse Responses in Bio-Systems, IEEE Conference on Electrical Insulation and Dielectric Phenomena, Albuquerque, 2003.

"Field-dependent nanosecond electroperturbation of Jurkat T lymphoblasts," Vernier, P. T., Y. Sun, L. Marcu, C. M. Craft, and M. A. Gundersen, Scientific Conference, Society for Physical Regulation in Biology and Medicine, San Antonio, 2004.

"Nanosecond, megawatt, millijoule pulses selectively perturb but do not porate mammalian cells," Vernier, P. T. and M. A. Gundersen, Air Force Office of Scientific Research, Chemistry and Life Sciences Directorate, Bio-Inspired Concepts Review, Annapolis, MD, 2003.

"Fluorescence microscopy studies of a peripheral benzodiazepine receptor-targeted molecular probe for brain tumor imaging," Marcu, L., P. T. Vernier, C. H. Manning, S. Salemi, A. Li, C. M. Craft, M. A. Gundersen, and D. J. Bornhop, Diagnostic Optical Spectroscopy, European Conference on Biomedical Optics, Munich, Germany, 2003.

"Germination of *Bacillus atrophaeus* spores after exposure to ultra-short, high-field electric pulses," Thu, M., P. T. Vernier, M. Behrend, S. Salemi, C. M. Craft, and M. A. Gundersen, ElectroMed 2003, San Antonio, 2003.

"Compact pulse generator for nanosecond electroperturbation of biological cells," Gu, X., A. Kuthi, M. Behrend, P. T. Vernier, Q. Zhou, and M. A. Gundersen, IEEE 26th International Power Modulator Conference, San Francisco, 2004.

"A catheter electrode for ultra-short, high-field pulses," Thu, M., M. R. Behrend, P. T. Vernier, Y. Sun, A. Kuthi, L. Marcu, C. M. Craft, and M. A. Gundersen, IEEE 26th International Power Modulator Conference, San Francisco, 2004.

"Real-time imaging of mammalian cells in nanosecond, megawatt, millijoule pulsed electric fields," Vernier, P. T., L. Marcu, Y. Sun, S. Salemi, C. M. Craft, and M. A. Gundersen, BiOS 2004 (SPIE), San Jose, 2004.

"Nanosecond pulsed electric fields trigger intracellular signals in human lymphocytes," Vernier, P. T., Y. Sun, L. Marcu, C. M. Craft, and M. A. Gundersen, Nanotech 2004, Boston, 2004.

"Nanoelectropulse perturbations of calcium and phospholipid distribution in human lymphocytes," Vernier, P. T., Y. Sun, L. Marcu, C. M. Craft, and M. A. Gundersen, Bioelectromagnetics Society 26th Annual Meeting, Washington, 2004.

"CMOS-compatible MEMS on multi-project wafers: fabrication and characterization, National Institute of Standards and Technology," Vernier, P. T., Semiconductor Electronics Division, Electronics and Electrical Engineering Laboratory, Gaithersburg, MD, 2003.

"Nanosecond, megawatt,millijoule pulses selectively perturb but do not porate mammalian cells," Vernier, P. T. and M. A. Gundersen, Air Force Office of Scientific Research, Chemistry and Life Sciences Directorate, Bio-Inspired Concepts Review, Annapolis, MD, 2003.

"Non-invasive approaches to nano-biology through advanced pulsed power," Y. Sun, P. T. Vernier, M. Behrend, L. Marcu, and M. A. Gundersen, Workshop on High-Field Effects and Fast Pulse Responses in Bio-Systems, IEEE Conference on Electrical Insulation and Dielectric Phenomena, October 19-22, Albuquerque, 2003.

B. CONSULTATIVE AND ADVISORY FUNCTIONS TO OTHER LABORATORIES AND AGENCIES

C. TRANSITIONS. DESCRIBE CASES WHERE KNOWLEDGE RESULTING FROM YOUR EFFORT IS USED, IN A TECHNOLOGY APPLICATION.

NEW DISCOVERIES, INVENTIONS, OR PATENT DISCLOSURES

"Method For Intracellular Modifications Within Living Cells Using Pulsed Electric Fields

USC File No. 3237A

HONORS/AWARDS

Matthew Behrend will begin his graduate studies at USC in the Fall and will be supported by two of the most prestigious national engineering fellowships, namely a Hertz Foundation Fellowship and a National Defense Science and Engineering Graduate Fellowship. This October he will be honored with the Outstanding Electrical Engineering Student Award, given annually by Eta Kappa Nu, the national Electrical Engineering honor society. In May he was awarded the Undergraduate Research Award at the International IEEE Power Modulator Conference. He also received this award in 2002.