
# AFRL-RH-FS-TR-2015-0027

## General Dynamics Information Technology Directed Energy Bioeffects Research II Task Order 1 Final Report

## Contract No. FA8650-13-D-6368

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Chief, Bioeffects Division
Human Effectiveness Directorate
711th Human Performance Wing
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**14. ABSTRACT**

Understanding the biological effects of directed energy is a primary goal of the Air Force Research Laboratory, 711th Human Performance Wing, Human Effectiveness Directorate, Bioeffects Division, Radio Frequency Bioeffects Branch (711 HPW/RHDR) at JBSA Fort Sam Houston, Texas. General Dynamics Information Technology (GDIT) scientists, with specialized expertise in biological and biophysical research, conducted studies to identify critical biochemical or molecular changes following exposure to directed energy (DE) prior to or during mission operations that assisted in the prediction of health degradation. We completed research efforts directly aimed at identifying any biological impact from RF exposures ranging from direct current (DC) – terahertz (THz) frequencies, and to nanosecond duration pulses. Interestingly, each different type of RF exposure caused sets of unique biological responses. The investigation and classification of each response allowed GDIT scientists to develop models to predict those responses. This body of research focused upon directed energy bioeffects. This report describes in detail the different research efforts and associated deliverables generated for each project assigned to GDIT under the Directed Energy Bioeffects Research II program for Task Order 1 (Directed Energy Bio-Mechanisms).

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## LIST OF ACRONYMS

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|---|---|
| 711 HPW/RHDR | Air Force Research Laboratory, 711th Human Performance Wing, Human Effectiveness Directorate, Bioeffects Division, Radio Frequency Bioeffects Branch |
| AFI | Air Force Instruction |
| AFOSR | Air Force Office of Scientific Research |
| AFRL | Air Force Research Laboratory |
| AP | Action Potential |
| BP | Bipolar |
| CHO | Chinese Hamster Ovary |
| $CO_2$ | Carbon Dioxide |
| DAG | Diacylglycerol |
| DC | Direct Current |
| DE | Differential Expression |
| D1 | One-day-old |
| D5 | Five days after Neuronal Dissociation |
| EF | Electric Field |
| EH | Environmental Heating |
| EMG | Electromyography |
| EMI | Electromagnetic Interference |
| EMP | Electromagnetic Pulse |
| EP | Electric Pulse |
| GDIT | General Dynamics Information Technology |
| GHz | Gigahertz |
| HAP | High Average Power |
| HPM | High Power Microwave |
| $IP_3$ | $Inositol_{1,4,5}$-trisphosphate |
| IR | Infrared |
| JNLWD | Joint Non-Lethal Weapons Directorate |
| MMW | Millimeter Wave |
| MP | Monopolar |
| mRNA | Messenger Ribonucleic Acid |
| miRNAs | MicroRNAs |
| MTT | 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium |
| MW | Microwave |
| nsEP | Nanosecond Electrical Pulse |
| nsPEF | Nanosecond Pulsed Electrical Field |
| OCR | Oxygen Consumption Rate |
| PHN | Primary Hippocampal Neurons |
| PGC-1α | Peroxisome Proliferator-activated Receptor $\gamma$ Coactivator 1 |
| PI | Propidium Iodide |
| $PIP_2$ | Phosphatidylinositol (4,5)-bisphosphate |
| PLC | Phospholipase C |

| | |
|---|---|
| PM | Plasma Membrane |
| RF | Radio Frequency |
| RFR | Radio Frequency Radiation |
| RH | Relative Humidity |
| RNA | Ribonucleic acid |
| siRNA | Small Interfering Ribonucleic Acid |
| THz | Terahertz |
| THz-TDS | Terahertz Time-Domain Spectroscopy |

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## EXECUTIVE SUMMARY

Understanding the biological effects of directed energy is a primary goal of the Air Force Research Laboratory, 711th Human Performance Wing, Human Effectiveness Directorate, Bioeffects Division, Radio Frequency Bioeffects Branch (711 HPW/RHDR) at JBSA Fort Sam Houston, Texas. General Dynamics Information Technology (GDIT) scientists, with specialized expertise in biological and biophysical research, conducted proteomic, genomic, and metabolomics studies that identified critical biochemical or molecular changes following exposure to directed energy (DE) prior to or during mission operations that assisted in the prediction of health degradation. We completed research efforts directly aimed at identifying any biological impact from RF exposures ranging from direct current (DC) – terahertz (THz) frequencies, and to nanosecond duration pulses. Interestingly, each different type of RF exposure caused sets of unique biological responses. The investigation and classification of each response allowed GDIT scientists to develop models to predict those responses. This body of research addressed the following specific aims:

- Generation of basic scientific data detailing the fundamental biological effects of RF radiation from DC-THz
- Observation of the short and long term biological responses to radio frequency radiation (RFR) to understand the entire lifecycle of exposure
- Development of fundamental theories on the interactions of directed energy technologies with biology at the cellular and organism levels
- Help evolve current military exposure standards to provide maximal exploitation of RF technologies while protecting civilian and soldier safety
- Incorporating the research data into effects databases to support development of decision support tools

This report describes in detail the different research efforts and associated deliverables generated for each project assigned to GDIT under the Directed Energy Bioeffects Research II program for Task Order 1 (Directed Energy Bio-Mechanisms).

# 1.0 INTRODUCTION

The objective of this contract was to conduct exploratory and developmental research and provide support relating to health and safety standards for human exposure to directed energies and human vulnerabilities to such exposures. These studies assessed the physiological, behavioral, and short term effects of radio frequency radiation (RFR) technologies.

All research involving the use of human subjects have been approved by the Air Force Research Laboratory's Institutional Review Board in accordance with Air Force Instruction (AFI) 40-402[1] and Air Force Research Laboratory (AFRL) Instruction 40-402.

All experiments involving animal procedures were approved by the Institutional Animal Care and Use Committee of the 711th Human Performance Wing, JBSA Fort Sam Houston, Texas, USA, and were conducted in accordance with the National Research Council's "Guide for the Care and Use of Laboratory Animals," prepared by the Committee for the Update of the Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research, Division on Earth and Life Studies, National Research Council of the National Academies.[2]

[1] Department of the Air Force. (2005). *Protection of Human Subjects in Biomedical and Behavioral Research* (AFI 40-402). Retrieved January 21, 2014, from http://www.fas.org/irp/doddir/usaf/afi40-402.pdf

[2] National Research Council (NRC). (2011). *Guide for the Care and Use of Laboratory Animals*. Washington, DC: National Academies Press.

## 2.0 TASK ORDER 1: DIRECTED ENERGY BIO-MECHANISMS

The development of directed energy devices for a variety of military uses continues to grow at an accelerated rate. These technologies are being developed for battlefield military applications including potential use in electronic warfare, imaging, and decontamination. Essentially, the increased application and active use translates into an increasing possibility of the warfighter and non-combatants being exposed to multiple types of directed energy. To assess the potential impact of a subset of directed energy on humans, General Dynamics Information Technology (GDIT) scientists in conjunction with scientists at the Air Force Research Laboratory, 711th Human Performance Wing, Human Effectiveness Directorate, Bioeffects Division, Radio Frequency Bioeffects Branch (711 HPW/RHDR) engaged in determining the molecular bioeffects of three distinct types of directed energy including: millimeter wave (MMW) radiation, nanosecond electrical pulses (nsEP), and terahertz (THz) radiation. The following technical report sections will specifically detail the research performed with these types of directed energy. In sum, the GDIT bioeffects team was responsible for investigating processes and reporting results for the cellular and molecular responses of directed energy applications as they apply to providing knowledge on human health and understanding biomarkers of exposure. Applying this knowledge will enable us to develop and test strategies to prevent adverse effects of overexposure to the warfighter and protect their operational performance to ensure safety and protection of bystanders.

### 2.1 Air Force Office of Scientific Research/Nanosecond Electrical Pulse Membrane Damage

Recent studies have demonstrated that exposure of cells to nanosecond pulse electric fields (nsPEF) form small, but recoverable pores within the plasma membrane, termed nanopores, which form at electrical discharge that allow passage of small ions for minutes after exposure. Despite much research, direct empirical evidence of such pores and the mechanism(s) behind their formation remains unclear. To address this gap in the knowledge, GDIT researchers studied the process of nanoporation, including determining the biological and biophysical effects of nsPEF on cell membranes (both plasma membrane and organelle membranes), cell death processes (i.e., autophagy and apoptosis), and intracellular structure changes (cytoskeleton) after such nsPEF events. The projects aimed at accomplishing these tasks are described below.

#### 2.1.1 Importance of Membrane Composition in Cellular Response to Nanosecond Pulsed Electrical Field

In order to more completely understand the properties of cell membranes that contribute to nanopore formation following nanosecond pulsed electrical field (nsPEF) exposure, GDIT investigators examined the effect of the cholesterol and protein content of the plasma membrane (PM) on cell responses. Specifically, we tested the effects of altering PM lipid microdomains, including planar lipid rafts and caveolae. First, we altered the cholesterol composition of the PM by treating with varying concentrations of free methyl-β-cyclodextrin, a cholesterol acceptor that

specifically extracts cholesterol from cellular membranes. Second, we altered the caveolin content of the PM using small interfering ribonucleic acid (siRNA) to specifically reduce the expression of caveolin isoforms 1 and 2.

Decreasing the cholesterol and/or caveolin content of the PM disrupts lipid raft domains and results in a more fluid bilayer; therefore, we hypothesized that if membrane fluidity (and/or the lipid microdomain) affects nanopore formation, decreasing cellular cholesterol and/or caveolin expression will result in an increase in the formation of nanopores following nanosecond electrical pulse exposure. In order to test this hypothesis, we altered the cholesterol and/or caveolin content of the PM of cells, exposed them to nsPEF and assessed changes in nanopore formation and membrane permeability by examining the cellular toxicity (MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) toxicity evaluation) and determining membrane permeability changes by investigating the influx of small molecules (monitoring propidium iodide and calcium movement) into the cell body. Results showed that depletion of membrane cholesterol was correlated to sensitization of cells to nsPEF. Specifically, the PM becomes disrupted at a less intense electric field when membrane cholesterol levels are reduced. In contrast, disruption of caveolae, by depleting caveolin proteins, produced cells that were less sensitive to nsPEF. Future studies will be conducted to determine the differences in the various lipid microdomains with respect to nsPEF-induced cellular effects. However, the results of the current study suggest that PM lipid microdomains are important determinants in the cellular response to nsPEF. The results of this study were published in one abstract and one oral presentation submitted to 711 HPW/RHDR.

(b) (6) (2014, December). *Sensitivity of Cells to Nanosecond Pulsed Electric Fields is Dependent on Membrane Lipid Microdomains* (AFRL-RH-FS-AB-2014-0054, P.A. Case No. TSRL-PA-2014-0148, 2 Dec 14). Abstract for 1st World Congress on Electroporation and Pulsed Electric Fields in Biology, Medicine and Food & Environmental Technologies scheduled to take place in Portorož, Slovenia on 6-10 Sep 2015. Submitted to 711 HPW/RHDR.

(b) (6) (2015, August). *Sensitivity of Cells to Nanosecond Pulsed Electric Fields is Dependent on Membrane Lipid Microdomains* (AFRL-RH-FS-OP-2015-0042, P.A. Case No. TSRL-PA-2015-0121, 27 Aug 15). Oral Presentation for 1st World Congress on Electroporation and Pulsed Electric Fields in Biology, Medicine and Food & Environmental Technologies scheduled to take place in Portorož, Slovenia on 6-10 Sep 2015. Submitted to 711 HPW/RHDR.

### 2.1.2 Intracellular Effects of Nanosecond Electrical Pulse – Mitochondrial Activity and Biogenesis

The unique cellular response to nanosecond pulsed electric field (nsPEF) exposure, as compared to longer pulse exposure, has been theorized to be due to permeabilization of intracellular organelles including the mitochondria. In order to specifically address the effects of nsPEF on mitochondira, GDIT investigators utilized a high-throughput oxygen and pH sensing system (Seahorse® XF24 Extracellular Flux Analyzer) to assess mitochondrial activity within

Jurkat and U937 cells after nsPEF. The XF Analyzer uses a transient micro-chamber of only a few µL in specialized cell culture micro-plates to enable oxygen consumption rate (OCR) and extracellular acidification rate to be monitored in real-time. Results show that nsPEF exposures of ten pulses of 10-ns pulse width and at 50 kV/cm e-field induce an increase in OCR in both U937 and Jurkat cells. Results also show that high pulse numbers (>100) caused a significant decrease in OCR. Higher amplitude 150 kV/cm exposures had no effect on U937 cells and yet they had a deleterious effect on Jurkat cells, matching previously published 24 hour survival data. These results suggest that the exposures were modulating metabolic activity in cells possibly due to direct effects on the mitochondria themselves. To validate this hypothesis, mitochondria were isolated from U937 cells and exposed similarly; results showed no significant change in metabolic activity for any pulse number. In a final experiment, cells were exposed in calcium-free buffer and no significant enhancement in metabolic activity was observed. These results suggest that direct permeabilization of the mitochondria is unlikely a primary effect of nsPEF exposure and instead, likely mediated through calcium-mediated intracellular pathway activation..

In order to more specifically analyze mitochondrial responses to nsPEF, we examined mitochondrial biogenesis following nsPEF exposure. We hypothesized that nsPEFs activate the induction of the peroxisome proliferator-activated receptor $\gamma$ coactivator 1 (PGC-1α) gene expression, thereby increasing mitochondrial biogenesis of the exposed cells and controlling reactive oxygen species levels. To show the correlation between the increased mitochondrial biogenesis and the PGC-1α gene expression, RNA interference (RNAi) target selection was used to knock-down the expression of the messenger ribonucleic acid (mRNA) for PGC-1α. Results showed reduced levels of OCR in PGC-1α knock-down pulsed cells. This suggests that nsPEFs stimulate mitochondria biogenesis through the activation of PGC-1α. The results of this work were published in two abstracts and two oral presentations submitted to 711 HPW/RHDR.

(b) (6) . (2014, January). *Investigation of a Direct Effect of Nanosecond Pulsed Electric Fields on Mitochondria* (AFRL-RH-FS-OP-2014-0010, P.A. Case No. TSRL-PA-14-0015, 30 Jan 14). Oral Presentation for SPIE Photonics West 2014 Conference, San Francisco, CA (1-6 Feb 14). Submitted to 711 HPW/RHDR.

(b) (6) (2014, February). Investigation of a Direct Effect of Nanosecond Pulse Electric Fields on Mitochondria (AFRL-RH-FS-PC-2014-0006, P.A. Case No. TSRL-PA-14-0033, 28 Feb 14). *Proc. SPIE 8941, Optical Interactions with Tissue and Cells XXV; and Terahertz for Biomedical Applications, 89411S,* SPIE Photonics West 2014, San Francisco, CA (1-6 Feb 14). Submitted to 711 HPW/RHDR.

(b) (6) (2014, March). *Nanosecond Pulsed Electric Fields Stimulate Mitochondrial Biogenesis Through the Activation of PGC-1α* (AFRL-RH-FS-AB-2014-0004; P.A. Case No. TSRL-PA-14-0043, 6 Mar 14). Abstract for Annual Joint Meeting of the Bioelectromagnetics Society (BEMS) and the European BioElectromagnetics Association (EBEA) held 8-

13 June 14 in Cape Town, South Africa (BioEM 2014 Annual Meeting). Submitted to 711 HPW/RHDR.

(b) (6) (2014, May). *Nanosecond Pulsed Electric Fields Stimulate Mitochondrial Biogenesis Through the Activation of PGC-1α* (AFRL-RH-FS-OP-2014-0031, P.A. Case No. TSRL-PA-2014-0071, 30 May 14). Oral Presentation for Annual Joint Meeting of the Bioelectromagnetics Society (BEMS) and the European BioElectromagnetics Association (EBEA) to be held 8-13 June 14 in Cape Town, South Africa (BioEM 2014 Annual Meeting). Submitted to 711 HPW/RHDR.

### 2.1.3 Intracellular Effects of Nanosecond Electrical Pulse – Autophagic Repair of Nanosecond Pulsed Electrical Field-Induced Damage

Previous work from GDIT investigators and others demonstrated significant changes in cellular membranes following exposure of cells to nanosecond pulsed electrical field (nsPEF), including nanoporation and increased intracellular calcium concentration. While it is known that nsPEF exposure can cause cell death, how cells repair and survive nsPEF-induced cellular damage is not well understood. To address this gap in knowledge, GDIT researchers investigated whether autophagy is stimulated following nsPEF exposure to repair damaged membranes, proteins, and/or organelles in a pro-survival response. We hypothesized that autophagy is activated to repair nsPEF-induced plasma membrane damage and overwhelming this compensatory mechanism results in cell death. Activation of autophagy and subsequent cell death pathways were assessed measuring toxicity, gene and protein expression of autophagy markers, and by monitoring autophagosome formation and maturation using fluorescent microscopy. Results show that autophagy is activated at subtoxic nsPEF doses, as a compensatory mechanism to repair membrane damage. However, prolonged exposure results in increased cell death and a concomitant decrease in autophagic markers. These results suggest that cells take an active role in membrane repair, through autophagy, following exposure to nsPEF. The results of this work were published in one abstract, one poster presentation, and one manuscript submitted to 711 HPW/RHDR.

(b) (6) (2014, March). *Activation of Autophagy in Response to Nanosecond Pulsed Electric Field Exposure* (AFRL-RH-FS-AB-2014-0003; P.A. Case No. TSRL-PA-14-0035, 3 Mar 14). Abstract for Annual Joint Meeting of the Bioelectromagnetics Society (BEMS) and the European BioElectromagnetics Association (EBEA) held 8-13 June 14 in Cape Town, South Africa (BioEM 2014 Annual Meeting). Submitted to 711 HPW/RHDR.

(b) (6) (2014, May). *Activation of Autophagy in Response to Nanosecond Pulsed Electric Field Exposure* (AFRL-RH-FS-PO-2014-0018, P.A. Case No. TSRL-PA-2014-0070, 30 May 14). Poster for Annual Joint Meeting of the Bioelectromagnetics Society (BEMS) and the European BioElectromagnetics Association (EBEA) to be held 8-13 June 14 in Cape Town, South Africa (BioEM 2014 Annual Meeting). Submitted to 711 HPW/RHDR.

[redacted] (2015, March). Activation of Autophagy in Response to Nanosecond Pulsed Electric Field Exposure (AFRL-RH-FS-JA-2015-0001, P.A. Case No. TSRL-PA-2015-0010, 8 Jan 15). *Biochemical and Biophysical Research Communications Journal, 458*(2), 411-7. Doi: 10.1016/j.bbrc.2015.01.131. [Epub 2015 Feb 7]. Submitted to 711 HPW/RHDR.

### 2.1.4 Intracellular Effects of Nanosecond Electrical Pulse – Cytoskeleton

Nanosecond pulsed electric fields (nsPEFs) perturb membranes of cultured mammalian cells in a dose-dependent manner with different types of cells exhibiting characteristic survivability. Adherent cells appear more robust than non-adherent cells during whole-cell exposure. To address this difference in cell survivability, we hypothesized that cellular elasticity is based upon the actin cytoskeleton and is a contributing parameter to the observed differences in adherent and non-adherent cells, and the alteration of a cell's actin cortex will significantly affect viability upon nsPEF exposure. GDIT investigators compared Chinese hamster ovary (CHO) cells that were (a) untreated, (b) treated with latrunculin A to inhibit actin polymerization, or (c) exposed to nsPEFs which have been probed using atomic force microscopy force-indentations. Results showed that exposure to 50 or 100 pulses of 10 ns duration and 150 kV/cm in a single dosage approximately lowered the average CHO cell elastic modulus by half, whereas latrunculin lowered it by more than 75%. Latrunculin pre-treatment disrupted the actin cortex enough that it negated cumulative damage by equally fractionated (i.e., two rounds of 50 pulses each, separated by 10 min) dosages of nsPEFs as seen in untreated and dimethyl sulfoxide-treated cells with propidium uptake, phosphatidylserine externalization, and 24 h viability according to 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) and CellTiter Glo assays. The results of this study suggest a correlation among cell stiffness, cytoskeletal integrity, and susceptibility to recurrent exposures to nsPEFs, which emphasizes a mechanobiological underpinning of nsPEF bioeffects. The results of this study were published in one manuscript submitted to 711 HPW/RHDR.

[redacted] (2014, February). Disruption of the Actin Cortex Contributes to Susceptibility of Mammalian Cells to Nanosecond Pulsed Electric Fields. *Bioelectromagnetics*, *35*(4), 262-272. Submitted to 711 HPW/RHDR.

### 2.1.5 Biophysical Measurements of Membrane Nanoporation

The mechanism behind the induced breakdown of plasma membranes by nanosecond electrical pulses, termed nanoporation, remains unknown. Current theories treat the interaction between the electrical field and the membrane as an entirely electrical event pointing to multiple plausible mechanisms. However, by investigating the biophysical interaction between plasma membranes and nanosecond electrical pulse (nsEP), we have identified a non-electric field driven mechanism that could be responsible for nanoporation of plasma membranes. In this investigation, we used a non-contact optical technique, termed probe beam deflection technique (PBDT), to characterize acoustic shockwaves generated by nsEP traveling through tungsten wire electrodes. The results of this study showed that these acoustic shockwaves are the consequence

of the nsEP exposure imparting electrohydraulic forces on the buffer solution. When these acoustic shockwaves occur in close proximity to lipid bilayer membranes, it is possible that they impart a sufficient amount of mechanical stress to cause poration of the membrane. This research establishes for the first time that nsEP discharged in an aqueous medium generate measureable pressure waves of a magnitude capable of mechanical deformation and possibly damage to plasma membranes. These findings provide a new insight into the long-unanswered question of how electric fields cause the breakdown of plasma membranes. The results of this study were published in one oral presentation, one proceeding, and one abstract submitted to 711 HPW/RHDR.

(b) (6) (2014, January). *Nanosecond Electrical Pulses Can Generate Pressure Transients Capable of Sonoporation* (AFRL-RH-FS-OP-2014-0011, P.A. Case No. TSRL-PA-14-0016, 30 Jan 14). Oral Presentation for SPIE Photonics West 2014 Conference, San Francisco, CA (1-6 Feb 14). Submitted to 711 HPW/RHDR.

(b) (6) (2014, February). Characterization of Acoustic Shockwaves Generated by Exposure to Nanosecond Electrical Pulses (AFRL-RH-FS-PC-2014-0005; P.A. Case No. TSRL-PA-14-0024, 21 Feb 14). *Proc. SPIE 8941, Optical Interactions with Tissue and Cells XXV; and Terahertz for Biomedical Applications*, 89411O, San Francisco, CA (1-6 Feb 14). Submitted to 711 HPW/RHDR.

(b) (6) (2014, November). *Exploration of the Biophysical Interaction Mechanisms(s) and Downstream Physiological Consequences of Cellular Exposure to Intense Electric Pulses* (AFRL-RH-FS-AB-2013-0051, P.A. Case No. TSRL-PA-2013-0149, Nov 14). Abstract for AFOSR Workshop. Submitted to 711 HPW/RHDR.

## 2.2 Biological Effects Associated with Terahertz Radiation

### 2.2.1 Development of State-of-the-art Terahertz Exposure Chamber

Terahertz (THz) imaging and sensing technologies are increasingly being used at international airports for security screening purposes and at major medical centers for cancer and burn diagnosis. The emergence of new THz applications has directly resulted in an increased interest regarding the biological effects associated with this frequency range. Knowledge of THz biological effects is also desired for the safe use of THz systems, identification of health hazards, and development of empirically-based safety standards. In order to expand our knowledge of the biological effects of THz radiation on biological systems, GDIT investigators developed a state-of-the-art exposure chamber that allowed for highly controlled and reproducible studies of THz biological effects. This innovative system incorporated an industry grade cell incubator system that permitted a highly controlled exposure environment, where temperatures could be maintained at 37 °C ± 0.1 °C, carbon dioxide ($CO_2$) levels at 5% ± 0.1%, and relative humidity levels at 95% ± 1%. To maximize the THz power transmitted to the cell culture region inside the

humid incubator, a secondary custom micro-chamber was fabricated and incorporated into the system. This micro-chamber shields the THz beam from the incubator environment and could be nitrogen-purged to eliminate water absorption effects. Additionally, a microscope that allowed for real-time visualization of the live cells before, during, and after THz exposure was integrated into the exposure system. This system was utilized for important biological experiments, as described in section 2.2.2 (below). Additionally, this work resulted in one oral presentation and one conference proceeding submitted to 711 HPW/RHDR.

(b) (6) (2014, January). *State-of-the-art Exposure Chamber for Highly Controlled and Reproducible Terahertz (THz) Biological Effects Studies* (AFRL-RH-FS-OP-2014-0013, P.A. Case No. TSRL-PA-14-0017, 30 Jan 14). Oral Presentation for SPIE Photonics West 2014 Conference, San Francisco, CA (1-6 Feb 14). Submitted to 711 HPW/RHDR.

(b) (6) (2014, February). State-of-the-art Exposure Chamber for Highly Controlled and Reproducible THz Biological Effects Studies (AFRL-RH-FS-PC-2014-0010; P.A. Case No. TSRL-PA-14-0031, 28 Feb 14). *Proc. SPIE 8941, Optical Interactions with Tissue and Cells XXV; and Terahertz for Biomedical Applications, 89411H,* SPIE Photonics West 2014, San Francisco, CA (1-6 Feb 14). Submitted to 711 HPW/RHDR.

### 2.2.2 Cellular Effects of Terahertz Radiation

In recent years, a surge in the development of many terahertz (THz) sensing and imaging technologies occurred leading to its increased use in military and civil operations. Therefore, understanding the biological effects associated with exposures to this radiation has become increasingly important. Water and many biological macromolecules have characteristic lifetimes in the picosecond range. As a result, these biomolecules strongly absorb THz radiation. Due to this strong absorption, THz radiation can exert a diverse range of effects on biological structures. For example, THz radiation has been shown to impact the structure, functional activity, and dynamics of macromolecules such as deoxyribonucleic acid and proteins. THz-molecular interactions can affect several gene expression pathways and, consequently, can alter the cell's biochemical and physiological characteristics. Indeed, THz radiation has been shown to influence the expression of several genes in different cell types. However, a complete view of the global transcriptional responses and the intracellular canonical pathways specifically triggered by THz radiation has not been elucidated.

To address this question, GDIT investigators examined the cellular response of human cells exposed to low- and high-power 2.52 THz radiation. During exposures, we maintained the cells under controlled standard tissue culture conditions using our recently developed THz exposure system, which integrated a modified, industry grade, cell culture incubator to an optically pumped molecular gas THz source (described in section 2.2.1). We used a custom spectrum analyzer to evaluate the frequency-power spectrum, and a pyroelectric array and calorimeter to characterize the absolute power, width, and intensity profile of the THz beam. We monitored

THz power as direct current (DC) voltage-logged values (LabVIEW™ IV log) during all THz exposures. To determine the heating profiles during THz exposure, we measured temperature changes in the unexposed (control sham) and THz-exposed cells using thermocouples. We then used these values to set the thermally-matched bulk-heating controls. To measure the effects of THz radiation on cells, we assessed changes in cellular viability, gene expression profiles using mRNA microarrays, and we identified the THz-induced signaling pathways for each frequency using bioinformatics. Importantly, we show that THz radiation alters the expression of specific mRNAs, microRNAs (miRNAs) and intracellular signaling pathways, which were not observed in a thermally-matched bulk-heating. Our data provide valuable new insights that give a global, comparative picture of the genes and intracellular signaling pathways triggered in cells exposed to THz radiation at different frequencies. Additionally, the results of these studies imply that THz radiation may be a useful, non-contact tool for the selective control of specific genes and cellular processes. This work was published in three abstracts, two proceedings, one poster presentation, one oral presentation, and one manuscript submitted to 711 HPW/RHDR.

(b) (6)

(2014, April). *Terahertz Stimulate Specific Signaling Pathways in Human Cells* (AFRL-RH-FS-AB-2014-0006; P.A. Case No. TSRL-PA-14-0045, 4 Apr 14). Abstract for IRMMW-THz Conference, 39th International Conference on Infrared, Millimeter and Terahertz Waves on September 14-19, 2014 at The University of Arizona, Tucson, AZ. Submitted to 711 HPW/RHDR.

(b) (6)

(2014, June). Terahertz Stimulate Specific Signaling Pathways in Human Cells (AFRL-RH-FS-PC-2014-0014, P.A. Case No. TSRL-PA-2014-0083, 24 Jun 14). *Proceeding for 39th International Conference on Infrared, Millimeter, and Terahertz Waves (IRMMW-THz 2014)*, September 14-19, 2014 at The University of Arizona, Tucson, AZ. Submitted to 711 HPW/RHDR.

(b) (6) (2014, August). *Investigation of the Effects of Low- and High-power 2.52 THz Radiation on Human Keratinocytes* (AFRL-RH-FS-AB-2014-0027, P.A. Case No. TSRL-PA-2014-0073, 4 Aug 14). This abstract is for BiOS Photonics West 2015 held in San Francisco, CA (7-12 Feb 15). Submitted to 711 HPW/RHDR.

(b) (6) (2014, August). *Effects of Different Terahertz Frequencies on Gene Expression in Human Keratinocytes* (AFRL-RH-FS-AB-2014-0026; P.A. Case No. TSRL-PA-2014-0072, 4 Aug 14). This abstract is for BiOS Photonics West 2015 held in San Francisco, CA (7-12 Feb 15). Submitted to 711 HPW/RHDR.

(b) (6)

(2014, September). *Terahertz Stimulate Specific Signaling Pathways in Human Cells* (AFRL-RH-FS-PO-2014-0026, P.A. Case No. TSRL-PA-2014-0101, 9 Sep 14). This poster is for the 39th International Conference on Infrared, Millimeter, and Terahertz

Waves (IRMMW-THz-2014), September 14-19, 2014, at the University of Arizona, Tucson, AZ. Submitted to 711 HPW/RHDR.

(b) (6) (2015, February). *Investigation of the Effects of Low- and High-Power 2.52 THz Radiation on Human Keratinocytes* (AFRL-RH-FS-OP-2015-0006, P.A. Case No. TSRL-PA-2015-0022, 2 Feb 15). This document was submitted to BiOS Photonics West 2015 held in San Francisco, CA (7-12 Feb 15). Submitted to 711 HPW/RHDR.

(b) (6) (2015, February). Effects of Different Terahertz Frequencies on Gene Expression in Human Keratinocytes (AFRL-RH-FS-PC-2015-0016; P.A. Case No. TSRL-PA-2015-0036, 25 Feb 15). *SPIE Proc. 9321, Optical Interactions with Tissue and Cells XXVI, 93210Q*, 2015 SPIE Photonics West to be held in San Francisco, CA on 7-12 Feb 15. Submitted to 711 HPW/RHDR.

(b) (6) J. (2015, May). *Terahertz Radiation: A Non-contact Tool for the Selective Stimulation of Biological Responses in Human Cells* (AFRL-RH-FS-JA-2015-0015, P.A. Case No. TSRL-PA-2015-0096, 23 Jul 15). Manuscript submitted for publication to *IEEE Transactions of Terahertz Science and Technology*. Submitted to 711 HPW/RHDR.

### 2.2.3 Terahertz Effects on Skin

Terahertz time-domain spectroscopy (THz-TDS) methods have been used to characterize the optical properties of skin and its primary constituents, including water, collagen, and keratin. However, similar experiments have not been performed to investigate whether the type of melanocytes and the melanin pigment they synthesize influence the skin's optical properties. To address this gap in knowledge, GDIT investigators used transmission-based THz-TDS techniques to measure the optical properties of *in vitro* pigmented human skin tissue models. Skin tissue models were cultured for three weeks to promote gradual differentiation and melanogenesis, and spectra were collected at various time intervals. Frequency-domain analysis techniques were performed to determine the index of refraction ('n') and absorption coefficient ($\mu_a$) for each skin sample over the frequency range of 0.1-2.0 THz. For all samples, we found that as frequency increased, 'n' decreased exponentially and $\mu_a$ increased linearly. Additionally, we observed that skin samples with higher levels of melanin exhibited greater 'n' and $\mu_a$ values than the non-pigmented samples. Our results provide evidence that melanocytes and the degree of melanin pigmentation contribute to the skin's optical properties. The results of this work were published in one journal article submitted to 711 HPW/RHDR.

(b) (6) (2014, September). *The Optical Properties of Melanin-pigmented Human Skin Tissue Models at Terahertz Frequencies* (AFRL-RH-FS-JA-2014-0012; P.A. Case No. TSRL-PA-2014-0108, 18 Sep 14). Manuscript submitted for publication. Submitted to 711 HPW/RHDR.

## 2.3 Overexposure Biomarkers

Previous studies in our laboratory of the effects of microwaves on the central nervous system demonstrated that exposure to certain frequencies can produce non-homogeneous deposition of energy and thermal profiles in animal tissues. Thus, GDIT investigators initiated a study to define the mechanisms of response to microwaves (MW) and determine the presence of biomarkers in a rat model of MW overexposure. The objective of this effort was to analyze the rat brain and plasma for changes in expression of biological molecules as a result of a single, high-power 2.07 gigahertz (GHz) exposure. To accomplish this goal, GDIT investigators conducted a study to identify genomic and proteomic biomarkers produced in rats after overexposure to high power microwaves (HPM) using an S-band transmitter tuned to 2.07 GHz. Localized specific absorption rate and dosage experiments were performed to establish the power settings and exposure time used to elicit an effect. Heat control rats were exposed to a high temperature exposure in an environmental chamber to produce a core temperature increase as seen in microwave-exposed rats. High throughput genomic and proteomic analysis methods were utilized on both brain and plasma to define coding and non-coding nucleic molecules and proteins at endpoints of 6 and 24 hr post-exposure. Examination of the transcriptomic and proteomic data (comparing sham (SM) and environmental heating (EH) controls to the MW conditions) identified significant, differential expression (DE) at endpoints of 6 and 24 hr post-exposure. Specifically, ribonucleic acid (RNA) microarray analysis of brain tissue identified 7 MW DE genes and 6 EH DE genes for the two endpoints. MicroRNA microarray analysis of 6 hr post-exposure plasma identified 4 MW DE microRNAs and 20 EH DE microRNAs, while two-dimensional differential gel electrophoresis of the plasma identified 18 DE MW proteins and 13 DE EH proteins for the two endpoints. These HPM effects to the brain tissue are of concern because damage to brain tissue following other types of insults (i.e., mild traumatic brain injury) can be progressive over time. Therefore, having a plasma-based biomarker assay would complement other clinical diagnostic tests and help determine the existence and extent of any underlying and progressive damage to the brain and other internal organs following a HPM event. Future studies using successive HPM exposures with this rat model are suggested to validate the persistence of the observed biomarkers and probable new arising biomarkers at additional time points (e.g., up to two weeks post-exposure), combined with histological and behavioral studies to evaluate neurodegenerative effects. The results of this study were published in two technical reports submitted to 711 HPW/RHDR.

(b) (6) (2014, January). *Literature Search for Biomarkers Phase II Protocol* (19 Nov 13). Submitted to 711 HPW/RHDR.

(b) (6) (2015, July). *Molecular Bioeffects of 2.06 GHz Microwave Exposure in the Laboratory Rat (Rattus norvegicus)* (AFRL-RH-FS-TR-2015-XXXX). Unpublished technical report. Submitted to 711 HPW/RHDR.

## 2.4 Cellular Effects of Electromagnetic Pulse Exposure

Electromagnetic pulses (EMPs) are short duration bursts of high intensity radio frequency that are frequently observed following nuclear explosions and/or similar large scale blasts. In the context of the warfighter, EMP-based technologies are useful to disable electronics, including computer systems and automobiles. However, the risk of EMPs to humans is not well characterized, and significant biological endpoints must be identified in order to expand the use of EMP-based systems in the field. Given the biological effects observed following nanosecond pulsed electrical field (nsPEF) exposure, we hypothesized that exposure to EMPs could have similar cellular effects. Specifically, we sought to determine the effects of EMPs on biological membrane disruption, cell death, and permeability to ions.

In contrast to previous studies with nsPEF which were performed with monopolar (MP) pulses, EMPs are bipolar (BP) oscillations. Multiple studies have shown that BP electric pulses in the microsecond range are more effective at permeabilizing cells while maintaining similar cell survival rates as compared to MP pulse equivalents. To study permeabilization effectiveness at the nanosecond range, MP or BP pulses were delivered to single Chinese hamster ovary (CHO) cells and the response of three dyes, Calcium Green-1, propidium iodide (PI), and FM1-43, was measured by confocal microscopy. Results show that BP pulses were less effective at increasing intracellular calcium concentration or PI uptake and cause less membrane reorganization (FM1-43) than MP pulses. Twenty-four hour survival was measured in three cell lines (Jurkat, U937, CHO) and over ten times more BP pulses were required to induce death as compared to MP pulses of similar magnitude and duration. Flow cytometry analysis of CHO cells after exposure (at 15 min) revealed that to achieve positive FITC-Annexin V and PI expression, ten times more BP pulses were required than MP pulses. Overall, unlike longer pulse exposures, BP nsPEF exposures proved far less effective at both membrane permeabilization and cell killing than MP nsPEF.

Nanoelectroporation of biomembranes is an effect of high-voltage, nanosecond-duration electric pulses (nsEP). It occurs both in the plasma membrane and inside the cell, and nanoporated membranes are distinguished by ion-selective and potential-sensitive permeability. It is unclear if BP pulses induce nanoporation similarly to MP pulses. GDIT investigators aided in the discovery of a novel phenomenon of bioeffects cancellation that puts nsEP cardinally apart from the conventional electroporation and electrostimulation by milli- and microsecond pulses. We compared the effects of 60- and 300-ns monopolar, nearly rectangular nsEP on intracellular $Ca^{2+}$ mobilization and cell survival with those of bipolar 60 + 60 and 300 + 300 ns pulses. For diverse endpoints, exposure conditions, pulse numbers (1–60), and amplitudes (15–60 kV/ cm), the addition of the second phase cancelled the effects of the first phase. The overall effect of BP pulses was profoundly reduced, despite delivering twofold more energy. Cancellation also took place when two phases were separated into two independent nsEP of opposite polarities; it gradually tapered out as the interval between two nsEP increased, but was still present even at a 10-μs interval. The phenomenon of cancellation is unique for nsEP and has not been predicted by the equivalent circuit, transport lattice, and molecular dynamics models of electroporation. The existing paradigms of membrane permeabilization by nsEP will need to be modified. Importantly, cancellation impacts nsEP applications in cancer therapy, electrostimulation, and biotechnology, and provides new insights into effects of more complex waveforms, including

pulsed electromagnetic emissions. The results of our studies were published in two manuscripts, two technical reports, and one white paper submitted to 711 HPW/RHDR.

(b) (6) (2014, January). Cancellation of Cellular Responses to Nanoelectroporation by Reversing the Stimulus Polarity. *Cellular and Molecular Life Sciences*. [Epub ahead of print]. Doi: 10.1007/s00018-014-1626-z. Retrieved April 28, 2014, from http://www.ncbi.nlm.nih.gov/pubmed/24748074. Submitted to 711 HPW/RHDR.

(b) (6) (2014, January). Bipolar Nanosecond Electric Pulses are less Efficient at Electropermeabilization and Killing Cells than Monopolar Pulses. *Biochemical and Biophysical Research Communications*, *443*(2), 568-573. Submitted to 711 HPW/RHDR.

(b) (6) (2014, December). *Ultrawideband Electromagnetic Pulse Systems for Biological Studies.* Final Report (16 Dec 2013). Norfolk, VA: Old Dominion University. Submitted to 711 HPW/RHDR.

(b) (6) (2014, September). *Cellular Effects of Bipolar Electric Pulses.* Unpublished technical report. Submitted to 711 HPW/RHDR.

(b) (6) (2014, August). *White Paper on Development of a Three-Dimensional Tissue System of nsEP Exposure Using Skin Culture Models*. Submitted to 711 HPW/RHDR.

### 2.5 Joint Non-Lethal Weapons Directorate/Nanosecond Electrical Pulse

The Joint Non-Lethal Weapons Directorate (JNLWD) is actively pursuing the use of very short electrical pulses to inhibit neural conduction for prolonged incapacitation. This phenomenon has recently been demonstrated in both rat and porcine subjects, suggesting it may scale from small to large targets. It has been hypothesized that the loss of motor movement following stimulation was caused by a conductance block of motor signals to the lower extremities via the spinal cord. Studies by GDIT investigators developed a model where simulations can be used to calculate the electric fields at the spinal cord of a rat and pig model during nsEP stimulation. Modeling efforts were aimed at speeding up the code (increasing accuracy) and developing "real" simulations relative to the envisioned device geometry in order to analyze possible human exposure scenarios.

In addition to modeling the effects of short electrical pulse exposure on animal models, physiological studies were performed by GDIT investigators to collect *in vivo* data. This study, entitled "Effects of Voltage Pulses Applied to Body Surface on Limb Movements in the Rat (*Rattus norvegicus*)," is intended to fill knowledge gaps of high voltage pulse effects on motor

inhibition by validating experiments completed by Old Dominion University and addressing ranges of effectiveness for delivered stimuli. Force and electromyography (EMG) data were collected during an experiment in which 1-kV 10-µs pulses were applied to seven different locations on a rat to investigate the modification of muscle activity resulting from brain stimulation. Initial results showed that the high-voltage pulses did not change force or EMG as measured in the experiment. GDIT investigators are actively involved in improving and troubleshooting experimental apparatuses, measurement systems, and techniques to secure, anesthetize, and instrument the animals in order to improve data collection on future animals. This work resulted in three technical reports submitted to 711 HPW/RHDR.

(b) (6) (2014, June). *Nanosecond Electrical Pulse Bioeffects: Simulation of the Electric Field at the Spinal Cord* (AFRL-RH-FS-TR-2014-0038). JBSA Fort Sam Houston, TX: Air Force Research Laboratory, 711th Human Performance Wing, Human Effectiveness Directorate, Bioeffects Division, Radio Frequency Bioeffects Branch.

(b) (6) (2015, January). *Summary of Exploratory Data Analysis of Force and Electromyography Results from an Experiment on High-Voltage Pulses Applied to the Rat.* White Paper. Submitted to 711 HPW/RHDR.

(b) (6) (2015, April). *Interim Status Report for JNLWD/NSEP*. Submitted to 711 HPW/RHDR.

### 2.6 Measurement of Neurological Impact of Radio Frequency Exposures

The somatic neuronal plasma membrane is the gatekeeper for all biological processes in neurons. Its extensions form the axons and dendrites responsible for all cell–to-cell communication within the central and peripheral nervous systems. External influences such as a directed energy fields, including high average power (HAP) microwave, nanosecond pulsed electric field (nsPEF) or infrared (IR) irradiation have a profound impact on plasma membrane structure and therefore its function. Directed energy exposures have recently demonstrated a potential to alter neuronal membrane function by dose-specific stimulation or inhibition of action potentials (APs). nsPEF are known to cause a rapid increase in intracellular calcium, which is believed to enter via the formation of stable nanopores in the plasma membrane. IR stimulation with pulse durations on the order of micro- to milliseconds, as well as HAP microwave impact, has been shown to directly stimulate nerves without any chemical pre-treatment or genetic alteration. This effect has very recently been theorized to result from a rapid increase in the local temperature that depolarizes the plasma membrane through capacitive charging. While similar cellular responses have been observed for both very-low electrical and thermal stimulation, it is unknown whether the mechanisms are the same on the fundamental mechanistic level. By understanding the biophysical similarities and differences between these distinct sources of cellular perturbation, a greater understanding and optimization of the cellular responses may be obtained. However, most of the reported results of directed energy exposure experiments were presented as whole body physiological effects or as changes in plasma membrane properties of a single non-excitable cell. In order to study directed energy effects on neuronal tissues, GDIT

researchers created a unique neurophysiological laboratory equipped with a state-of-the-art tissue slicer and electrophysiological microscope system. This system is capable of capturing changes in intracellular calcium dynamics and electrophysiological conductance deep within live brain slices or dense neuronal cultures. The preliminary studies leading to the development of this system are described below.

### 2.6.1 Preliminary Cellular Studies of Phosphatidylinositol$_{4,5}$-Bisphosphate Hydrolysis and its Relationship to Calcium Influx

Studies by AFRL, GDIT, and others have demonstrated that small nanometer-sized pores (nanopores) are preferentially formed after exposure to nanosecond pulsed electric fields. Further, we have reported that nanoporation of the plasma membrane directly affects the phospholipids of the cell membrane, ultimately culminating in phosphatidylinositol$_{4,5}$-bisphosphate ($PIP_2$) intracellular signaling. $PIP_2$, located within the internal layer of the plasma membrane, plays a critical role as a regulator of ion transport proteins, a source of second messenger compounds, and an anchor for cytoskeletal elements. To expand upon our earlier studies, GDIT investigators generated data that demonstrates that nanosecond pulsed electric fields (nsPEFs) initiate electric field dose-dependent $PIP_2$ hydrolysis and/or depletion from the plasma membrane through the observation of the accumulation of inositol$_{1,4,5}$-trisphosphate ($IP_3$) in the cytoplasm and the increase of diacylglycerol (DAG) on the inner surface of the plasma membrane. The phosphoinositide signaling cascade involves activation of phospholipase C (PLC) and protein kinase C, which are responsible for a multitude of biological effects after nsPEF exposure. These results expand our current knowledge of nsPEF induced physiological effects, and serve as a basis for development of novel tools for drug independent stimulation or modulation of different cellular functions.

Importantly, the interaction between nsPEF-induced $Ca^{2+}$ release and nsPEF-induced $PIP_2$ hydrolysis is not well understood. To better understand this interrelation we monitored intracellular calcium changes and generation of the $PIP_2$ hydrolysis byproducts $IP_3$ and DAG. Following exposure to nsPEF, we found that, in the absence of extracellular $Ca^{2+}$, the population of $IP_3$ liberated during nsPEF exposure is diminished compared to the response in the presence of calcium. However, the production of DAG in the absence of extracellular $Ca^{2+}$ was not statistically different from cells exposed in the presence of extracellular calcium suggesting that the change in intracellular calcium concentration is not solely driving the observed response. Interestingly, the DAG produced in the absence of $Ca^{2+}$ is the strongest near the membrane regions facing the electrodes, whereas the presence of extracellular $Ca^{2+}$ leads to a whole cell response. The reported observations of $Ca^{2+}$ dynamics combined with $IP_3$ and DAG production suggest that nsPEF may cause a direct effect on the phospholipids within the plasma membrane.

Despite the promising results of this preliminary study, the exact mechanism(s) causing the observed depletion of $PIP_2$ remained unknown. Complicating this problem, the formation of nanopores can result in the observed increase in intracellular calcium. While elevated intracellular calcium can cause activation of PLC (a known catalyst of $PIP_2$ hydrolysis), $PIP_2$ depletion has been shown to occur in the absence of both extracellular calcium and in calcium depleted intracellular environment. These observations have led to the hypothesis that the high

electric field itself may be playing a direct role in the hydrolysis of $PIP_2$ from the plasma membrane. To test this hypothesis, we used immunohistochemistry to quantify the total amount of free $IP_3$ in Chinese hamster ovary cells, giant unilaminar vesicles containing $PIP_2$, and free $PIP_2$ after applying nsPEF. In addition, we established a relationship between the applied "dose" and $IP_3$ production. Furthermore, we used molecular dynamics simulations in both the bare lipids and bilayers containing ion channels. We found that, at electric fields above electroporation thresholds and membrane poration, $PIP_2$ can be depleted from the plasma membrane by externalization. Taken together, the results of these studies suggest that nsPEF induced calcium influx through nanopores likely facilitates $PIP_2$ hydrolysis. This work was critical in refining our understanding of the role of $PIP_2$ in the cellular response to nsPEF and also in determining the fundamental biological effects of high electric field exposures. The results of these studies were published in two abstracts, one poster, two proceedings, one journal article, one unpublished manuscript, and one unpublished technical report submitted to 711 HPW/RHDR.

(b) (6) (2014, January). *Dose Dependent Translocation of Fluorescent Probes of $PIP_2$ Hydrolysis in Cells Exposed to Nanosecond Pulsed Electric Fields* (AFRL-RH-FS-PO-2014-0003, P.A. Case No. TSRL-PA-14-0014, 30 Jan 14). Poster for SPIE Photonics West 2014 Conference, San Francisco, CA (1-6 Feb 14). Submitted to 711 HPW/RHDR.

(b) (6) (2014, February). Dose Dependent Translocations of Fluorescent Probes of $PIP_2$ Hydrolysis in Cells Exposed to Nanosecond Pulsed Electric Fields (AFRL-RH-FS-PC-2014-0007; P.A. Case No. TSRL-PA-14-0032, 27 Feb 14). *Proc. SPIE 8941, Optical Interactions with Tissue and Cells XXV; and Terahertz for Biomedical Applications, Photonics West 2014,* San Francisco, CA (1-6 Feb 14). Submitted to 711 HPW/RHDR.

(b) (6) (2014, January). 600 ns Pulse Electric Field-induced Phosphatidylinositol$_{4,5}$-bisphosphate Depletion. *Bioelectrochemistry, 100,* 80-87. Submitted to 711 HPW/RHDR.

(b) (6) (2014, December). *Investigating the Potential of Pulsed Electric Fields to Initiate the Intracellular Phosphatidylinositol$_{4,5}$-biphosphate Depletion* (AFRL-RH-FS-AB-2014-0059; P.A. Case No. TSRL-PA-2014-0157, 15 Dec 14). Abstract for the 59$^{th}$ Biophysical Meeting on 7-11 Feb 15 in Baltimore, MD. Submitted to 711 HPW/RHDR.

(b) (6) (2014, August). *Investigating the Potential of Nanosecond Pulsed Electric Fields (nsPEF) to Directly Cleave the $PIP_2$ Molecule in Biological and Artificial Environments* (AFRL-RH-FS-AB-2014-0016; P.A. Case No. TSRL-PA-14-0087). Abstract for SPIE Photonics West, San Francisco, CA, 7-12 Feb 15. Submitted to 711 HPW/RHDR.

(b) (6) (2015, February). The Role of $PIP_2$ and the $IP_3$/DAG Pathway in Intracellular Calcium Release and Cell Survival During Nanosecond Electric Pulse Exposures (AFRL-RH-FS-PC-2015-0015; P.A. Case No. TSRL-PA-2015-0039, 25 Feb 15). *Proc. SPIE 9326, Energy-based Treatment of Tissue and Assessment VIII, 932611*, SPIE Photonics West held in San Francisco, CA on 7-12 Feb 15. Submitted to 711 HPW/RHDR.

(b) (6) (2015). *Dose Dependent Translocations of Fluorescent Probes of $PIP_2$ Hydrolysis in Cells Exposed to Nanosecond Pulsed Electric Fields*. Unpublished Manuscript. Submitted to 711 HPW/RHDR.

(b) (6) (2015). *Measure of Neurological Impact of Directed Energy Exposure* (AFRL-RH-FS-TR-20xx-xxxx). Unpublished technical report. Submitted to 711 HPW/RHDR.

### 2.6.2 Cell Swelling and Blebbing

Cell swelling and blebbing has been commonly observed following nanosecond pulsed electric field (nsPEF) exposure. The hypothesized origin of these effects is nanoporation of the plasma membrane followed by transmembrane diffusion of extracellular fluid and disassembly of actin structures. However, recent data suggests that simple passive movement of ions into the cell through pores is not solely responsible for the observed swelling and blebbing. Specifically, as described in the previous section, recent studies have shown that phosphatidylinositol (4,5)-bisphosphate ($PIP_2$) is depleted following nsPEF exposure. This observation is critical as $PIP_2$ is heavily involved in osmoregulation by modulation of ion channels and also serves as an intracellular membrane anchor to cortical actin and phospholipase C (PLC). However, the role of this lipid in cellular swelling/blebbing after nsPEF exposure has not been explored. Importantly, GDIT investigators found the "classical" $G_{q11}$-dependent $PIP_2$ hydrolysis (receptor mediated) and nsPEF-induced $PIP_2$ depletion caused cells to swell and bleb. Edelfosine, a blocker of endogenous PLC, prevented $PIP_2$ hydrolysis during receptor-mediated experiments. Importantly, in nsPEF-exposed cells, a significant reduction in swelling was observed. Edelfosine treatment completely prevented blebbing in all experiments except 20 nsPEFs where only a single bleb on the side facing the anodic electrode formed. Based on these observations, we hypothesize that $PIP_2$ depletion and PLC activity is primarily responsible for the observed cellular swelling/blebbing after nsPEF exposure. Given the rather critical role $PIP_2$ depletion appears to play in the observed response of cells to nsPEF exposure, it remains unclear how its well-studied downstream effects and, specifically, ion channel regulation may contribute to unknown mechanisms of the lasting "permeabilization" of the plasma membrane. The results of this study were published in an abstract, an oral presentation, and a poster submitted to 711 HPW/RHDR.

(b) (6) (2014, May). *nsPEF-induced $PIP_2$ Depletion, Consequential PLC Activity, and Actin Cytoskeletal Cortex Remodeling*

*Responsible for Cellular Swelling and Blebbing* (AFRL-RH-FS-AB-2014-0007, P.A. Case No. TSRL-PA-2014-0078, 30 May 14). Abstract for upcoming Gordon Research Conference Bioelectrochemistry, Cellular and Organismal Responses to Endogenous and Exogenous Fields, 6-11 July, University of New England in Biddeford, ME. Submitted to 711 HPW/RHDR.

(b) (6) (2014, October). *nsPEF-induced $PIP_2$ Depletion, PLC Activity, and Actin Cytoskeletal Cortex Remodeling Responsible for Cellular Swelling and Blebbing* (AFRL-RH-FS-PO-2014-0028; P.A. Case No. TSRL-PA-2014-0117, 7 Oct 14). Oral Presentation for upcoming Bioelectrics 2014 (11th International Bioelectrics Symposium) in Columbia, Missouri on13-16 Oct 14. Submitted to 711 HPW/RHDR.

(b) (6) (2014, June). *nsPEF-induced $PIP_2$ Depletion, Consequential PLC Activity, and Actin Cytoskeletal Cortex Remodeling Responsible for Cellular Swelling and Blebbing* (AFRL-RH-FS-PO-2014-0020, P.A. Case No. TSRL-PA-2014-0082, 24 Jun 14). Poster for Gordon Research Conference Bioelectrochemistry, Cellular and Organismal Responses to Endogenous and Exogenous Fields, 6-11 July, University of New England in Biddeford, ME. Submitted to 711 HPW/RHDR.

### 2.6.3 The Role of Phosphatidylinositol$_{4,5}$-Bisphosphate in Neuron Activity

Simulation studies of neuromuscular incapacitation using high-intensity electric pulses (EPs) were previously reported. These studies hypothesized that a reversible action potential (AP) block can be achieved based on energy deposition causing neuronal electroporation. However, theoretical concepts were presented without elaboration of specific details on possible biological mechanisms. Recently, we discovered that nanosecond pulsed electrical field (nsPEF) could initiate phosphatidylinositol$_{4,5}$-bisphosphate ($PIP_2$) depletion in non-excitable cells, similar to activation of the $G_{q11}$ coupled receptors. $PIP_2$ is the precursor for important second messengers and is a key modulator of the neuronal ion channels involved in AP generation. By using primary hippocampal neurons (PHN) at different stages of development and the PLCδ-PH-EGFP optical probe of $PIP_2$ hydrolysis, GDIT investigators demonstrated that electric field (EF) exposure induced $PIP_2$ depletion in the PHN, and defined EF exposure parameters necessary to safely elicit reversible effects without neuronal damage. Results showed at five days after neuronal dissociation (D5) in mature neuronal cultures, the pre-exposure tonic $IP_3$ level is significantly higher than in one-day-old (D1) neurons. Such biological sensitization caused D5 neurons to respond intensely following a single 7.5 kV/cm 600 ns electric pulse (EP), while the D1 neurons did not show $PIP_2$ depletion after similar pulse. Despite the age of neuronal development, the stronger 15 kV/cm 600 ns or longer 7.5 kV/cm 12 µs EPs initiated profound $PIP_2$ depletion in all neurons studied, suggesting a direct impact on the neuronal plasma membrane during electroporation. Accordingly, D1 neurons exposed to such EPs had significant post-exposure propidium iodide (PI) uptake. In the more sensitive D5 neurons, $PIP_2$ recovery was achieved within 10 min after all 600 ns EPs exposures, but 12 µs EPs caused irreversible $PIP_2$ depletion. Thus, nsPEF-induced $PIP_2$ depletion in neurons could be the primary biological

mechanism responsible for both stimulation and latent inhibition of neuronal tissues. The results of this study were published in one abstract and one protocol submitted to 711 HPW/RHDR.

(b) (6) (2015, March). *New Insights into Biophysical Mechanisms of the nsPEF-induced Neuronal Response* (AFRL-RH-FS-AB-2015-0001, P.A. Case No. TSRL-PA-2015-0054, 24 Mar 15). Abstract for the 1st World Congress on Electroporation and Pulsed Electric Fields in Biology, Medicine, and Food & Environmental Technologies, 6-10 Sep 15 in Portorož, Slovenia. Submitted to 711 HPW/RHDR.

(b) (6) (2014, August). *Determining the Effect of Nanosecond Pulse Exposure on Primary Neurons Dissociated from Rat Central or Peripheral Nervous System* (RHDR-14-09). Protocol. Submitted to 711 HPW/RHDR.

### 2.6.4 Effects of Infrared Energy Exposure on Neurological Stimulation

Delivery of pulsed infrared (IR) laser energy has been shown to stimulate action potentials (APs) in neurons. However, the mechanism for this stimulation is not completely understood. Certain hypotheses suggest the rise in temperature from IR exposure could activate temperature- or pressure-sensitive channels, or create pores in the plasma membrane. Specifically, *in vitro* studies have found that pulsed IR exposure generates a nearly instant change in capacitance in the plasma membrane, characterized by inward rectification, a common feature in pore-forming exposures, such as electrical pulses and acoustic shock waves. Based on this similarity, we hypothesize that the mechanism of IR stimulation is the formation of short-lived nanopores in the plasma membrane. These transient, small-diameter pores allow the influx of extracellular ions that lead to AP generation, possibly through activation of secondary messenger pathways or depolarization of the cell membrane resulting in activation of voltage-gated ion channels. Studies using intensity-based calcium-responsive dyes show changes in intracellular calcium levels after various IR stimulation parameters; however, determination of the origin of the calcium proved difficult to determine. An influx of larger, typically plasma-membrane-impermeant ions (including YO-PRO-1 and PI) has been demonstrated, which suggests that calcium may originate from the external solution. To address this question, GDIT investigators assisted in quantifying the calcium mobilization in terms of influx from the external solution and efflux from intracellular organelles following IR stimulation by using Fura-2 (a calcium sensitive dye) and a high-speed ratiometric imaging system that rapidly alternates the excitation wavelengths. CHO-K1 cells, which lack voltage-gated $Ca^{2+}$ channels, and NG-108 neuroblastoma cells, which do not produce APs in an early undifferentiated state, were studied, in conjunction with pharmaceutical agents, to determine the origin of the $Ca^{2+}$ signals and investigate the role these mechanisms may play in IR neural stimulation. The results of this study greatly progress the fundamental understanding of IR stimulation of neurons. This study was published in two abstracts, one proceeding, and one manuscript submitted to 711 HPW/RHDR.

(b) (6) (2014, September). *Origins of Intracellular Calcium Mobilization Evoked by Infrared Laser Stimulation*

(AFRL-RH-FS-AB-2014-0033, P.A. Case No. TSRL-PA-14-0103, 17 Sep 14). Abstract for BIOS, Photonics West in San Francisco, CA (7-12 Feb 15). Submitted to 711 HPW/RHDR.

(b) (6) (2015, January). Origins of Intracellular Calcium Mobilization Evoked by Infrared Laser Stimulation (AFRL-RH-FS-PC-2015-0009, P.A. Case No. TSRL-PA-2015-0018, 28 Jan 15). *Proc. SPIE 9321, Optical Interactions with Tissue and Cells XXVI, 93210L*, SPIE Photonics West 2015 (7-12 Feb 15) in San Francisco, CA. Submitted to 711 HPW/RHDR.

(b) (6) (2014, October). Plasma Membrane Nanoporation as a Possible Mechanism Behind Infrared Excitation of Cells (AFRL-RH-FS-JA-2014-0010, P.A. Case No. TSRL-PA-2014-0082). *Journal of Neural Engineering*, *11*, 066006. Submitted to 711 HPW/RHDR.

(b) (6) (2015, July). *Short Infrared (IR) Laser Pulses Can Cause Nanoporation-Induced Activation of the $IP_3$ Pathway* (AFRL-RH-FS-AB-2015-0014, P.A. Case No. TSRL-PA-2015-0101, 31 Jul 15). SPIE Photonics West 2016, San Francisco, CA (13-18 Feb 2016). Submitted to 711 HPW/RHDR.

## 2.7 Electromagnetic Interference-Spine

In 2012, the Human Effects Advisory Panel concluded there were multiple risks of injury following extended duration electro-muscular incapacitation. A recent study conducted by Naval Medical Research Unit - San Antonio used the porcine model to evaluate untested electrical dosages and determined that pulse characteristics affect incident of spinal cord injury. Therefore, this study investigated repetition rate and amplitude as variables contributing to a higher incident of spinal cord injury. Tangentially, previous literature stated that a blood porcine model with a splenectomy procedure acted as a better human surrogate, thus subjects were exposed to stimulus charges with and without splenectomy procedures.

GDIT investigators initiated an experimental protocol to evaluate 40 Yorkshire swine that were tested at variable stimulus charge, repetition rate, and occurrence of splenectomy. The two stimulus charges tested were 80 µC and 60 µC, and the two repetition rates used were 40 Hz and 19 Hz. All data are still under review and any findings should be viewed as preliminary. At the time, it appears that removing the spleen does not provide a better model for the human conducted energy weapon exposure. X-ray imaging and animal dissection have confirmed 18 fractures in the vertebral column. Further analysis of current data is required to ascertain how the pulse characteristics affect incident of fracture. The data was presented in the following unpublished technical report submitted to 711 HPW/RHDR.

(b) (6) (2015). *Prolonged Electro-muscular Incapacitation (EMI) in a Porcine Model for Assessment of Spinal Injury*. Unpublished technical report. Submitted to 711 HPW/RHDR.

## 2.8 Quantum Biology

Propagation of solitons within biomembranes could be a significant biological mechanism for signal transduction. A soliton is a self-reinforcing solitary wave that maintains its wavelike shape as it propagates at a constant velocity. We have seen suggestions of the possibility for soliton-like transmission in our own research. Specifically, the nanopores that form in plasma membranes following exposure to nanosecond pulsed electrical field (nsPEF) can also be generated by thermal fluctuations in the plasma membrane (i.e., the basis for the soliton model) and may indicate the transmission of such a signal though the membrane upon nsPEF exposure. Additionally, based on previous high-speed imaging results from our laboratory, intracellular release of calcium has been demonstrated from nsPEF pulses theoretically too long to cause intracellular poration, but at more rapid speeds than calcium diffusion to these intracellular vesicles to cause calcium-induced calcium release. This observation could indicate a more rapid signaling mechanism from the cellular membrane to the interior structures. Finally, recent work has found that rapid change in temperature from the infrared laser stimulation reversibly alters the electrical capacitance of the plasma membrane to depolarize the cell and result in action potentials (APs). This capacitance is established by the spatial distribution of ions near the plasma membrane surface and underlies the mechanism responsible for the voltage wave in the soliton theory of APs.

GDIT investigators developed two models to accelerate the study of soliton propagation in biological membranes and to aid in collecting biologically relevant data. A physiological model of neurotransmission was established by isolating large neurons from earthworms. GDIT investigators documented specific solutions and methods for the isolation procedure and have performed preliminary studies showing the effectiveness of generating and recording APs on the isolated large neurons. As an alternative approach, GDIT investigators worked with government colleagues to design and implement optogenetic studies in primary and immortalized neurons. Optogenetic studies use a combination of genetics and optics to control well-defined events within cells. With respect to the research at AFRL, we were interested in using optogenetics to study neurotransmission following exposure to a stimulus. To achieve this goal, GDIT investigators established a protocol to reproducibly and efficiently transfect optogenetic plasmids into neurons (primary and the NG108 immortalized cell line). Both of the models developed by GDIT investigators will be critical in determining the propagation of energy on biologically relevant model systems. The results of this study were published in one abstract submitted to 711 HPW/RHDR.

(b) (6) (2015, July). *All Optical Experimental Design for Neuron Excitation, Inhibition, and Action Potential Detection* (AFRL-RH-FS-AB-2015-0011, P.A. Case No. TSRL-PA-2015-0095, 22 Jul 15). SPIE Photonics West, San Francisco, CA. Submitted to 711 HPW/RHDR.