AFRL-RH-FS-TR-2020-0003

General Dynamics Information Technology Directed Energy Bioeffects Research II Task Order 6 Final Report

Contract No. FA8650-13-D-6368

(b) (6)

General Dynamics Information Technology

February 2020

Final Report for October 2015 – February 2020

DESTRUCTION NOTICE – Destroy by any method that will prevent disclosure of contents or reconstruction of this document.

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Refer other requests for this document to 711 HPW/RHDR, (b) (6)

Air Force Research Laboratory
711th Human Performance Wing
Airman Systems Directorate
Bioeffects Division
Radio Frequency Bioeffects Branch
(b) (6)

NOTICE AND SIGNATURE PAGE

Using Government drawings, specifications, or other data included in this document for any purpose other than Government procurement does not in any way obligate the U.S. Government. The fact that the Government formulated or supplied the drawings, specifications, or other data does not license the holder or any other person or corporation; or convey any rights or permission to manufacture, use, or sell any patented invention that may relate to them.

Qualified requestors may obtain copies of this report from the Defense Technical Information Center (DTIC) (http://www.dtic.mil).

"General Dynamics Information Technology Directed Energy Bioeffects Research II Task Order 6 Final Report Contract No. FA8650-13-D-6368 (Final Report for October 2015 - February 2020)"

(AFRL-RH-FS-TR- 2020 - 0003) has been reviewed and is approved for publication in accordance with assigned distribution statement.

(b) (6)

(b) (6)
Contract Monitor
Radio Frequency Bioeffects Branch

(b) (6)

(b) (6)
Chief, Bioeffects Division
Airman Systems Directorate
711th Human Performance Wing
Air Force Research Laboratory

This report is published in the interest of scientific and technical information exchange, and its publication does not constitute an official position of the U.S. Government.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently 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)*
13-02-2020	Final	1 Oct 2015 – 13 Feb 2020

4. TITLE AND SUBTITLE

General Dynamics Information Technology Directed Energy Bioeffects Research II Task Order 6 Final Report Contract No. FA8650-13-D-6368 (Final Report for October 2015 – February 2020)

5a. CONTRACT NUMBER
FA8650-13-D-6368-0006

5b. GRANT NUMBER
N/A

5c. PROGRAM ELEMENT NUMBER
62202F

6. AUTHOR(S)

(b) (6)

5d. PROJECT NUMBER
N/A

5e. TASK NUMBER
N/A

5f. WORK UNIT NUMBER
HOEB

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

General Dynamics Information Technology
(b) (6)

8. PERFORMING ORGANIZATION REPORT NUMBER
N/A

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Air Force Materiel Command, Air Force Research Laboratory
711th Human Performance Wing, Airman Systems Directorate,
Bioeffects Division, Radio Frequency Bioeffects Branch
(b) (6)

10. SPONSOR/MONITOR'S ACRONYM(S)
711 HPW/RHDR

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
AFRL-RH-FS-TR-2020-0003

12. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Understanding the biological effects of directed energy (DE) is a primary goal of the Air Force Research Laboratory, 711th Human Performance Wing, Airman Systems 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 DE prior to or during mission operations that assisted in the prediction of health effects and establishment of accurate radio frequency (RF) safety standards. We completed research efforts directly aimed at identifying any biological impact from RF exposures ranging from direct current (DC) – terahertz (THz) frequencies, infrared (IR) irradiation, and to nanosecond duration pulses. Interestingly, each different type of 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 DE 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 6 (Novel Direct Energy Weapon Effects).

15. SUBJECT TERMS
Radiofrequency fields, biophysics, directed energy, electro-muscular incapacitation, millimeter wave, biomarkers, bioeffects, neurological effects, nanosecond pulsed electric field mechanisms

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			(b) (6)
Unclassified	Unclassified	Unclassified	SAR	49	19b. TELEPHONE NUMBER *(include area code)* N/A

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

THIS PAGE INTENTIONALLY LEFT BLANK

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

TABLE OF CONTENTS

Page

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6) [redacted]

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

LIST OF ACRONYMS

711 HPW/RHDR	Air Force Research Laboratory, 711th Human Performance Wing, Airman Systems Directorate (formerly 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
AFRL/RX	Air Force Research Laboratory, Materials and Manufacturing Directorate
AP	Action Potential
ATP	Adenosine Triphosphate
BEMS	Bioelectromagnetics Society
BH	Bulk Heating
Ca^{2+}	Calcium Ion
Cav1	Caveolin
CEW	Conducted Electrical Weapon
CHO	Chinese Hamster Ovary
CHO-hM_1	Chinese Hamster Ovarian Cell
CNS	Central Nervous System
DC	Direct Current
DE	Directed Energy
DNA	Deoxyribonucleic Acid
DoD	Department of Defense
DSPI	Digital Speckle Pattern Interferometry
EBEA	European BioElectromagnetics Association
ED_{50}	Effective Dose
EF	Electric Field
EH	Environmental Heating
E/I	Excitation/Inhibition
ELF-MF	Extremely Low-Frequency Magnetic Field
EM	Electromagnetic
EMI	Electro-muscular Incapacitation
EMP	Electromagnetic Pulses
ESPI	Electronic Speckle Pattern Interferometer
FDTD	Finite-Difference Time-Domain
FEM	Finite Element Model
FTG	Fast Thermal Gradient
GABA	Gamma-aminobutyric Acid
GDIT	General Dynamics Information Technology
Gd^{3+}	Gadolinium
GFAP	Glial Fibrillary Acidic Protein
GHz	Gigahertz
HEKa	Human Epidermal Keratinocytes
HPPM	High Peak Power Microwave
HPM	High Power Microwave

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6) [redacted]

HV	High Voltage
H2B	Histone-2B
IACUC	Institutional Animal Care and Use Committee
INS	Infrared Neural Stimulation
IOP	Intraocular Pressure
IP_3	$Inositol_{1,4,5}$-trisphosphate
IP_3R	Inositol Trisphosphate Receptor
IR	Infrared
IRL	Infrared Laser
IRLP	Infrared Laser Pulse
IRMMW	International Conference on Infrared, Millimeter, and Terahertz Waves
JBSA	Joint Base San Antonio
JNLWD	Joint Non-Lethal Weapons Directorate
mEPSC	Miniature Postsynaptic Excitatory Current
MD	Molecular Dynamics
MF	Magnetic Field
mIPSC	Miniature Postsynaptic Inhibitory Current
MMW	Millimeter Wave
MP	Membrane Potential
MPAS	Multiprobe Adapter System
MRI	Magnetic Resonance Imaging
mRNA	Messenger Ribonucleic Acid
MT	Microtubule
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Microwave
NG108	Neuroblastoma-Glioma
NLW	Non-lethal Weapon
NSEP	Nanosecond Electrical Pulse
nsPEF	Nanosecond Pulsed Electric Fields
Oxo-M	Oxotremorine M
PAS	Per-Arnt-Sim
PASK	Per-Arnt-Sim domain-containing Protein Kinase
PCNA	Proliferating Cell Nuclear Antigen
PEF	Pulsed Electric Fields
PHN	Postnatal Hippocampal Neuron
PI	Propidium Iodide
PIP_2	Phosphatidylinositol-$_{4,5}$-bisphosphate
PKA	Protein Kinase A
PLC	Phospholipase C
PM	Plasma Membrane
PRR	Pulse Repetition Rate
RF	Radio Frequency
RFR	Radio Frequency Radiation
RNA	Ribonucleic Acid
ROCE/SOCE	Receptor- and Store-Operated Calcium Entry

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6) [redacted]

SAR	Specific Absorption Rate
sEPSC	Spontaneous Excitatory Synaptic Currents
SILP	Short Infrared Laser Pulses
sIPSC	Spontaneous Inhibitory Synaptic Currents
Sub-MMWs	Submillimeter Waves
TBI	Traumatic Brain Injury
THz	Terahertz
TRPC	Transient Receptor Potential Channel
TRPC1	Transient Receptor Potential Cation Channel Subfamily C1
TRPV1	Transient Receptor Potential Vanilloid-1
TSRL	Tri-Service Research Laboratory
UWB	Ultrawideband
WBC	White Blood Cell
YP1	YO-PRO-1
3D	Three-Dimensional

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

EXECUTIVE SUMMARY

Understanding the biological effects of directed energy (DE) is a primary goal of the Air Force Research Laboratory, 711th Human Performance Wing, Airman Systems 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 DE prior to or during mission operations that assisted in the prediction of health effects and establishment of accurate radio frequency (RF) safety standards. We completed research efforts directly aimed at identifying any biological impact from RF exposures ranging from direct current (DC) – terahertz (THz) frequencies, infrared (IR) irradiation, and to nanosecond duration pulses. Interestingly, each different type of 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 and IR irradiation
- 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 DE technologies with biology at the cellular and organism levels
- Development of effective and state-of-the-art models of RFR exposures on biological systems
- Evolution of 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
- Exploitation of RF technologies in the design of non-lethal weapons for use on the battlefield

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 6 (Novel Direct Energy Weapon Effects).

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6) [redacted]

1.0 INTRODUCTION

The objective of the research conducted during the contract period was to perform exploratory and developmental studies and provide support relating to health and safety standards for human exposure to directed energies and human vulnerabilities to such exposures. This research included the impact of exposures to human performance. Studies conducted by General Dynamics Information Technology (GDIT) investigators assessed the physiological, behavioral, and long-term effects of radio frequency radiation (RFR) technologies.

All research projects involving human subjects have been approved for the use of human subjects 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 procured, maintained, and used according to an Institutional Animal Care and Use Committee (IACUC)-approved Animal Use Protocol and established animal welfare standards, compliant with: DoD Instruction 3216.01,[2] U.S. Department of Agriculture Animal Welfare Regulations;[3] The Guide for the Care and Use of Laboratory Animals, 8th Edition, National Research Council;[4] and AFMAN 40-401(1).[5] The Air Force Research Laboratory at Joint Base San Antonio (JBSA) Fort Sam Houston, Texas has been accredited by AAALAC, International since 1967.

Note: Citations with an asterisk denote a document submitted by a government author. GDIT will not provide a copy since document is already filed in the government case file.

[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] Department of Defense (DoD). (2010). *Use of Animals in DoD Programs* (DoD Instruction Number 3216.01). Retrieved February 21, 2017, from http://www.dtic.mil/whs/directives/corres/pdf/321601p.pdf

[3] U.S. Department of Agriculture. (2013). USDA Animal Welfare Regulations, 9 C.F.R. Subchapter A (2013). Retrieved February 21, 2017, from https://www.nal.usda.gov/awic/animal-welfare-act

[4] National Research Council (NRC). (2011). *Guide for the Care and Use of Laboratory Animals* (8th ed.). Washington, DC: National Academies Press.

[5] U.S. Air Force. (2005). The Care and Use of Laboratory Animals in DOD Programs, AFMAN 40-401(1) (2005). Retrieved February 21, 2017, from http://www.apd.army.mil/pdffiles/r40_33.pdf

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6) [redacted]

2.0 TASK ORDER 6: NOVEL DIRECT ENERGY WEAPON EFFECTS

The development of directed energy (DE) 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 DE. To assess the potential impact of a subset of DE on humans, GDIT scientists in conjunction with scientists at the Air Force Research Laboratory, 711th Human Performance Wing, Airman Systems Directorate, Bioeffects Division, Radio Frequency Bioeffects Branch (711 HPW/RHDR) engaged in determining the molecular bioeffects of four distinct types of DE including: millimeter wave (MMW) radiation, nanosecond electrical pulse (NSEP), infrared (IR) radiation, and terahertz (THz) radiation. The following sections will specifically detail the research performed with these types of DE. In summary, the GDIT bioeffects team was responsible for investigating processes and reporting results for the cellular and molecular responses of DE applications as they apply to providing knowledge on human health and understanding biomarkers of exposure. Applying this knowledge enables 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 NSEP/Air Force Office of Scientific Research (AFOSR)

Previous work from our laboratory and others demonstrated the formation of small, recoverable pores in cell membranes (i.e., nanopores) following exposure to nanosecond pulsed electric fields (nsPEF). Additionally, we observed differences in sensitivity to nsPEF in both acute membrane injury and 24 hr lethality across multiple cells lines. Despite much research in this field, direct, empirical evidence of and the mechanism(s) behind the formation of nanopores remain unclear. Additionally, the downstream effects of nanoporation on cellular physiology are largely unknown. Therefore, to address this gap in the knowledge, GDIT researchers studied the process of nanoporation in depth, including determining the biophysical effects of nsPEF on extracellular and intracellular membranes (including cell blebbing/swelling) and neurophysiological changes. The projects aimed at accomplishing these tasks are described in detail in the following subsections.

2.1.1 Biophysical Analysis of NSEP Exposure

Previous studies in the field show that NSEP exposure activates signaling pathways, induces oxidative stress, stimulates hormone secretion, causes cell swelling, and induces apoptotic and necrotic death. However, the underlying biophysical connection(s) between these diverse cellular reactions and NSEP remained unclear. Therefore, GDIT investigators participated in a study utilizing global genetic analysis to evaluate how two commonly studied cell types, U937 and Jurkat, respond to NSEP exposure. We hypothesized that by studying the genetic response of the cells following exposure we could gain direct insight into the stresses experienced by the cell and, in turn, achieve a better understanding of the biophysical interaction taking place during the exposure. Using Ingenuity Systems software, we found genes associated with cell growth, movement, and development to be significantly up-regulated in both cell types 4 hr post exposure

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

to NSEP. In agreement with our hypothesis, we also found that both cell lines exhibited significant biological changes consistent with mechanical stress induction. These results advance NSEP research by providing strong evidence that the interaction of NSEP with cells involves mechanical stress and were incorporated into one manuscript.

(b) (6) (2016, May). Evaluation of the Genetic Response of U937 and Jurkat Cells to 10-Nanosecond Electrical Pulses (NSEP). *PLoS One*, *11*(5), eD154555. Submitted to 711 HPW/RHDR.

2.1.2 Intracellular Membrane Permeabilization

Previous studies at the AFRL and at other institutions showed permeabilization of cell membranes following exposure to a threshold absorbed dose of nsPEF. The ultimate, physiological bioeffect of this exposure depends on the type of cultured cell and environment, indicating that cell-specific pathways and structures are stimulated. GDIT investigators participated in a study to investigate 10 and 600 ns duration pulsed electric field (PEF) effects on Chinese hamster ovary (CHO) cell nuclei. We hypothesized that pulse disruption of the nuclear envelope membrane would lead to previously observed cellular bioeffects, including increased cell death and decreased cell viability 24 hr post-exposure. To observe short-term responses to nsPEF exposure, CHO cells were stably transfected with two fluorescently-labeled proteins known to be sequestered for cellular chromosomal function within the nucleus: histone-2b (H2B) and proliferating cell nuclear antigen (PCNA). Results showed that H2B remained associated with chromatin after nsPEF exposure, whereas PCNA leaked out of nuclei permeabilized by a threshold absorbed dose of 10 and 600 ns PEF. A downturn in 24 hr viability, measured by MTT assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), was observed at the number of pulses required to induce permeabilization of the nucleus. In summary, the results of this study provided a link between intracellular membrane permeabilization and untoward cellular effects (i.e., cell death). The results of this study were included in one manuscript.

(b) (6) (2016). Permeabilization of the Nuclear Envelope Following Nanosecond Pulsed Electric Field Exposure (P.A. Case No. TSRL-PA-15-0110, August 2015). *Biochemical and Biophysical Research Communications*, *470*, 35-40. Submitted to 711 HPW/RHDR.

2.1.3 Membrane Properties Contributing to Nanoporation

Based on the aforementioned preliminary data from our group and others, we hypothesized that the biological response of cells to nsPEF is dependent on the physical properties of the plasma membrane (PM), including regional cholesterol content. Therefore, GDIT scientists felt it was essential to define the properties of the PM that are involved in the cellular response to nsPEF exposure. Our results showed that depletion of membrane cholesterol disrupts the PM and increases the permeability of cells to small molecules, including propidium iodide (PI) and calcium occurring after fewer nsPEF. Additionally, cholesterol depletion concurrently decreases the

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

"dose" of nsPEF required to induce lethality. In summary, the results of this study suggest that the PM cholesterol composition is an important determinant in the cellular response to nsPEF. The results of these studies were included in two white papers and one manuscript.

(b) (6) (2016, April). *White Paper on the Efficient Extraction and Measurement of Lipid Signaling Intermediates From Cells Exposed to Nanosecond Pulsed Electric Fields*. Submitted to 711 HPW/RHDR.

(b) (6) (2016, April). *White Paper on Experimental Design to Determine Changes in Caveolin Binding Partners Following Exposure to Nanosecond Pulsed Electric Fields.* Submitted to 711 HPW/RHDR.

(b) (6) (2016, February). *The Biological Response of Cells to Nanosecond Pulsed Electric Fields is Dependent on Plasma Membrane Cholesterol* (AFRL-RH-FS-JA-2016-115101, P.A. Case No. TSRL-PA-2016-0199, 16 Feb 16). *Biochimica et Biophysica Acta (BBA) Biomembranes, 1858*(11), 2636-2646 (November 2016). Submitted to 711 HPW/RHDR.

2.1.4 Cell Swelling and Blebbing

Cell swelling and blebbing has been frequently observed following nsPEF exposure. The postulated origin of these effects is nanoporation of the PM followed by diffusion of extracellular fluid across the cell membrane and disassembly of cortical actin structures. To expand on these initial observations, GDIT conducted an investigation to determine the connection between nanoporation and intracellular signaling. Specifically, the results of this study provided evidence that passive movement of fluid into the cell through nanopores and an increase in intracellular osmotic pressure are not solely responsible for this observed phenomena. We demonstrated that phosphatidylinositol-4,5-bisphosphate (PIP_2) depletion and hydrolysis are critical steps in the chain reaction leading to cellular blebbing and swelling. 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). Given the rather critical role that PIP_2 depletion appears to play in the response of cells to nsPEF exposure, it remains unclear how its downstream effects and, specifically, ion channel regulation may contribute to cellular swelling, blebbing, and unknown mechanisms of the lasting "permeabilization" of the PM. GDIT investigators continued these studies in experiments described later in this report (section 2.2.2). The results of this study were included in one journal article.

(b) (6) . (2016, November). *nsPEF-induced PIP_2 Depletion, PLC Activity and Actin Cytoskeletal Cortex Remodeling are Responsible for Post-exposure Cellular Swelling and Blebbing* (AFRL-RH-FS-JA-2016-115629, P.A. Case No. TSRL-PA-2016-0249, 10 Jun 16). *Biochemistry and Biophysics Reports*, *9*, 36-41. Submitted to 711 HPW/RHDR.

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

2.1.5 Multiple Pulse Effects on Membrane Permeabilization

Many of the aforementioned studies described within this report studied the effects of multiple NSEPs on cellular behavior and function. However, while these studies have taken place in which multiple pulses were delivered to cells, the effect of pulse repetition rate (PRR) is not well understood. To better understand the effects of PRR, a laser scanning confocal microscope was used to observe CHO-K1 cells exposed to ten 600 ns, 200 V pulses at varying repetition rates (5 Hz up to 500 KHz) in the presence of either FM 1-43, YO-PRO-1 (YP1), or PI fluorescent dyes, probes frequently used to indicate nanoporation or permeabilization of the PM. Dye uptake was monitored for 30 seconds after pulse application at a rate of 1 image/second. In addition, a single long pulse of equivalent energy (200 V, 6 µs duration) was applied to test the hypothesis that very fast PRR will approximate the biological effects of a single long pulse of equal energy. Upon examination of the data, we found strong variation in the relationship between PRR and uptake in each of the three dyes. In particular, PI uptake showed little frequency dependence, FM 1-43 showed a strong inverse relationship between frequency and internal cell fluorescence, and YP1 exhibited a "threshold" point of around 50 KHz, after which the inverse trend observed in FM 1-43 was seen to reverse itself. Further, a very high PRR of 500 KHz only approximated the biological effects of a single 6 µs pulse in cells stained with YP1, suggesting that uptake of different dyes may proceed by different physical mechanisms. The results of these studies were included in one abstract and one proceeding.

(b) (6) (2015, October). *High Frequency Application of Nanosecond Pulsed Electric Fields Dramatically Alters Cellular Membrane Disruption and Fluorescent Dye Uptake* (P.A. Case No. TSRL 15-0106, August 2015). SPIE Photonics West 2016, San Francisco, CA (13-18 Feb 2016). Submitted to 711 HPW/RHDR.

(b) (6) (2016, March). High Frequency Application of Nanosecond Pulsed Electric Fields Alters Cellular Membrane Disruption and Fluorescent Dye Uptake (P. A. Case No. TSRL-16-0232, April 2016). In E. Duco Jansen (Ed.). *Proc. SPIE 9706, Optical Interactions with Tissue and Cells XXVII*, 9706W. Submitted to 711 HPW/RHDR.

2.1.6 Neuronal Response to DE Exposure

The interaction of electromagnetic (EM) waves and neurological tissue is a topic of great interest to the U.S. Air Force and GDIT investigators. One objective of the U.S. Air Force research group is to examine whether high-energy sources affect neurological homeostasis and influence cognitive function. Specifically, the group seeks to understand how such exposures could affect neuronal excitability and, thus, learning, memory, and behavior of the military personnel involved.

It is unclear how overexposure to a DE field changes neurological function despite reported whole body physiological effects. We hypothesize that such changes occur within the brain hippocampal region due to excessive brain heating and/or nanoporation of somatic neuronal membranes, ultimately leading to deleterious neurological effects. GDIT investigators conducted

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6) .

a study to determine if we can detect neurophysiological changes within live brain slices or mature neuronal cultures after DE exposure. We 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 (Ca^{2+}) dynamics and electrophysiological conductance deep within live brain slices or dense neuronal cultures. Using these models and detection instruments, we were able to detect significant neurophysiological differences before and after DE exposure.

After completion, GDIT investigators proposed a study to explore the neurotransmitters and voltage-gated ion channels that contribute to neuronal activity using this new, state-of-the-art neurophysiological system. Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the human brain and KCNQ (Kv7.2, 7.3, 7.5) potassium channels act as an on/off switch for neuronal action potential (AP) firing. These channels are critically involved in most cognitive processes. Modulation of ligand-gated $GABA_A$ and voltage-gated KCNQ channels by intracellular Ca^{2+} (Ca^{2+}_i) are well documented in the literature. High-energy exposures, such as NSEPs or IR irradiation, have been shown to cause the formation of small pores in the PM of non-excitable and excitable cells allowing for unregulated ion flow into the cell, which leads to rises in intracellular Ca^{2+}_i. This suggests that NSEP or IR exposure could be responsible for modulation of KCNQ potassium channels leading to AP firing, consequent Ca^{2+}_i increases, and attenuation of inhibitory function of neuronal $GABA_A$ ion channels. Therefore, we detailed a series of studies with the goal of identifying the molecular mechanisms of enhanced neuronal excitability after NSEP or IR exposure. To address this goal, we investigated the mechanism/source of elevated intracellular calcium following NSEP and IR exposure using exogenously expressed probes and fura-2 intracellular calcium measurements. Additionally, we transitioned our experiments into a hippocampal neuron model to determine how intracellular calcium rises affect a complex system of naturally expressed ion channels. Future studies were proposed to evaluate post-exposure function of exogenously expressed human voltage-gated neuronal KCNQ potassium channels in CHO-K1 cells using patch clamp electrophysiology. Additionally, we proposed future studies to evaluate post exposure function of GABA channels in a CHO-K1 cell line expressing recombinant human $\alpha_{1\text{-}5}\beta_{2\text{-}3}\gamma_2$ GABA receptors.

The results of these studies were included in one technical report, one poster, and one white paper.

(b) (6) (2015, November). *Measure of Neurological Impact of Directed Energy Exposure* (AFRL-RH-FS-TR-2015-0034, 2 May 16). JBSA Fort Sam Houston, TX: Air Force Research Laboratory, 711th Human Performance Wing, Human Effectiveness Directorate, Bioeffects Division, Radio Frequency Bioeffects Branch. Submitted to 711 HPW/RHDR.

(b) (6) (2016, February). *New Insights into Biophysical Mechanisms of the nsPEF-induced Neuronal Response* (AFRL-RH-FS-PO-2016-115142, P.A. Case No. TSRL-PA-2016-0206, 23 Feb 16).

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

Poster for the 60th Annual Meeting of Biophysical Society in Los Angeles, CA on 27 Feb – 2 Mar 16. Submitted to 711 HPW/RHDR.

(b) (6) . (2016, April). *White Paper on Interaction of High-energy Sources and Neurological Tissue – Pathways of Enhanced Neuronal Excitability*. Submitted to 711 HPW/RHDR.

2.2 NSEP Long Term Effects

Given the Air Force Research Laboratory's success at defining critical biological effects following NSEP exposure, GDIT was tasked with expanding our studies to focus on the long-term and/or long-lasting effects of NSEP exposure. Studies of acute bioeffects show nanoporation immediately following exposure to NSEP. However, it is unclear if such bioeffects are quickly resolved by the cell or, rather, lead to long-lasting cellular effects including cell death and permanent changes in intracellular signaling. The following studies were conducted to explore the long-term effects of NSEP exposure.

2.2.1 Role of Membrane Proteins in Response to nsPEF Exposure

As mentioned previously, nsPEFs have been shown to induce changes in the PM, including PM permeabilization (termed nanoporation), which allows free passage of ions into the cell and, ultimately, cell death. Recent studies from our laboratory show that the composition of the PM is a critical determinant of PM nanoporation. Our group established a relationship between nanoporation and the cholesterol content of the PM (described in section 2.1.3). Specifically, depletion of membrane cholesterol increased the permeability of cells to small molecules, including PI and calcium, at shorter nsPEF exposures. Based on these results, we hypothesized that the biological response to nsPEF exposure could be influenced by lipid microdomains, including caveolae. Caveolae are specialized invaginations of the PM that are enriched in cholesterol and contain aggregates of important cell signaling proteins, including caveolin (Cav1), the major structural component of caveolae. Importantly, caveolae have been shown to play a significant role in cellular signal transduction, including control of calcium influx and cell death pathways. In the present study, results show that depletion of Cav1 rendered the cell less sensitive to nsPEF. We also observed that depletion of Cav1 increased the influx of calcium, while Cav1 overexpression produced the opposite effect. Additionally, imaging of the PM after nsPEF exposure showed localized depletion of PM Cav1. These results are consistent with previous publications showing that caveolae act as mechanical sensors by responding to localized changes in membrane dynamics (i.e., swelling), resulting in the release of Cav1 from the PM. Further, Cav1 is known to bind to and sequester important cell signaling proteins within caveolae, rendering the binding partners inactive. Results of co-immunoprecipitation studies showed dissociation of two critical Cav1 binding partners (transient receptor potential cation channel subfamily C1 (TRPC1) and inositol trisphosphate receptor (IP_3R)) after exposure to nsPEFs. Release of TRPC1 and IP_3R from Cav1 would activate downstream signaling cascades, including store-operated calcium entry, which could explain the influx in calcium after nsPEF exposure. In summary, the results of this study established a significant relationship between Cav1 and the activation of cell signaling pathways in response to nsPEFs.

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6) .

The results of this study were included in two abstracts, one poster, one oral presentation, and one journal article.

(b) (6) (2017, February). *Role of Membrane Lipid Microdomains in the Biological Response to Nanosecond Pulsed Electric Fields* (AFRL-RH-FS-AB-2017-116570, P.A. Case No. TSRL-PA-2017-0131, 6 Feb 17). Abstract for EMBO/EMBL Symposia 2017 (Molecular and Cell Biology of Membranes) in EMBL Heidelberg, Germany on 21-23 May 17. Submitted to 711 HPW/RHDR.

(b) (6). (2017, April). *Role of Membrane Lipid Microdomains in the Biological Response to Nanosecond Pulsed Electric Fields* (AFRL-RH-FS-PO-2017-117065, P.A. Case No. TSRL-PA-2017-0166, 27 Apr 17). Poster for EMBO/EMBL Symposia 2017 (Molecular and Cell Biology of Membranes) held in EMBL Heidelberg, Germany on 21-23 May 17. Submitted to 711 HPW/RHDR.

(b) (6) (2017, May). *Role of Membrane Lipid Microdomains in the Biological Response to Nanosecond Pulsed Electric Fields*. Oral Presentation for Lab Meeting held at Tri-Service Research Laboratory on 9 May 17. Submitted to 711 HPW/RHDR.

(b) (6) (2018, March). *Plasma Membrane Lipid Microdomains are Involved in Regulating Calcium Influx After Exposure to Nanosecond Pulsed Electric Fields* (AFRL-RH-FS-AB-2018-118114, P.A. Case No. TSRL-PA-2018-0137, 2 Mar 18). Abstract for 2018 Joint Meeting of the Bioelectromagnetics Society (BEMS) and the European BioElectromagnetics Association (EBEA) scheduled 25-29 Jun 18 in Piran, Portorož, Slovenia. Submitted to 711 HPW/RHDR.

(b) (6) (2018, June). *Caveolin-1 is Involved in Regulating the Biological Response of Cells to Nanosecond Pulsed Electric Fields* (AFRL-RH-FS-JA-2018-118476; P.A. Case No. TSRL-PA-2018-0161, 6 Jun 18). Unpublished manuscript. Submitted to 711 HPW/RHDR. The *Journal of Membrane Biology* requested additional data; author decided to submit paper to a different publication (*Cell Calcium*).

2.2.2 PM Lipid Changes Following nsPEF Exposure

Our previous results show that the nanoporation that occurs following nsPEF exposure affects phospholipids of the cell membrane, culminating in cascading PIP_2 intracellular signaling (described in section 2.1.4). To expand on these previous observations, we examined NSEP initiated PIP_2 hydrolysis and/or depletion from the PM. Our results showed an NSEP dose-dependent decrease in PIP_2 from the PM. This observation was confirmed using two fluorescent optical probes of PIP_2 hydrolysis: PLCδ-PH-EGFP and GFP-C1-PKCγ-C_{1a}. Results showed that a 50% maximum response occurs with a single 600 ns pulse achieving an effective dose (ED_{50}) of

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6).

electric field (EF) ~ 8 kV/cm within our model cell system. At 16.2 kV/cm, the ED_{50} for the pulse width was 484 ns. Reduction of the pulse width or EF amplitude gradually reduced the observed effect, but twenty 60 ns 16.2 kV/cm pulses produced an effect similar to a single 600 ns pulse of the same amplitude. PI uptake after the NSEP exposure confirmed a strong relationship between EF-induced PM impact and PIP_2 depletion. These results expanded our knowledge of NSEP-dependent cell physiological effects, and served as a basis for model development of new exposure standards, providing novel tools for drug independent stimulation and approaches to differential modulation of key cellular functions. The results of this study were included in one manuscript.

[redacted] (2016, June). Nanosecond Pulsed Electric Field Induced Dose Dependent Phosphatidylinositol-4,5-bisphosphate Signaling and Intracellular Electro-Sensitization (AFRL-RH-FS-JA-2016-115693, P.A. Case No. TSRL-PA-2016-0260, 30 Jun 16). *Biochimica et Biophysica Acta, 1859,* 438-445. Submitted to 711 HPW/RHDR.

2.2.3 Calcium Responses After nsPEF Exposure

Nanosecond electric pulses have been shown to open nanopores in the cell PM by imaging uptake of calcium ion (Ca^{2+}) and fluorescent dyes, including PI and YP1. Recently, we demonstrated that NSEPs induce the phosphoinositide intracellular signaling cascade causing PIP_2 depletion resulting in physiological responses similar to those observed following stimulation of G_{q11}-coupled receptors (described in section 2.1.5). Likewise, G_{q11} receptor agonists induced cellular swelling, blebbing, and intracellular Ca^{2+} rises, effects now commonly attributed to post-NSEP long-lasting "nanopermeabilization". Therefore, we measured uptake of YP1 following 15 min exposure to the hM_1 G_{q11}-coupled receptor agonist oxotremorine M (Oxo-M). This treatment resulted in small, but significant YP1 uptake in the Chinese hamster ovarian cell (CHO-hM_1) line. Increased intracellular YP1 fluorescence was not accompanied with changes in PI fluorescence, suggesting that YP1, but not PI, could pass through open ion channels as Oxo-M is not known to create nanopores. Products of PIP_2 signaling (inositol-1,4,5-trisphosphate and diacylglycerol) activate members of the canonical transient receptor potential channel (TRPC) family of channels, which are involved in receptor- and store-operated calcium entry (ROCE and SOCE). This suggests that nanoporation and activation of ROCE/SOCE channels could occur simultaneously after NSEP exposure. Indeed, the ROCE/SOCE/TRPC blocker gadolinium (Gd^{3+}, 300 μM) and the imidazole compound SKF-96365 (1-[2-(4-methoxyphenyl)-2-[3-(4-methoxyphenyl) propoxy] ethyl-1H-imidazole hydrochloride, 100 μM) significantly reduced intracellular Ca^{2+} rises after exposure to 1 and 20 NSEPs (16.2 kV/cm, 5 Hz). SKF-96365 reduced the Ca^{2+} response more significantly than Gd^{3+}. However, using similar NSEP exposure parameters, SKF-96365 was less effective on YP1 uptake compared to Gd^{3+}. Thus, it is possible that SKF-96365 could block stromal interaction molecule 1 interactions from inside of the cell, while Gd^{3+} could act on TRPC/nanopores from the outside of the cell. Our results present evidence of NSEP-induced ROCE and SOCE mechanisms and demonstrate that YP1 and Ca^{2+} cannot be used solely as markers of NSEP-induced nanoporation. This data is useful in planning and conducting future membrane biology studies of nanoporation and were included in one abstract, two oral presentations, and one journal article.

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, [redacted]

(b) (6) (2017, April). *Receptor- and Store-operated Mechanisms of Calcium Entry During the Nanosecond Electric Pulse-induced Cellular Response* (AFRL-RH-FS-AB-2017-116986, P.A. Case No. TSRL-PA-2017-0163, 3 Apr 17). Abstract for 2nd World Congress on Electroporation and Pulsed Electric Fields in Biology, Medicine, Food & Environmental Technologies held in Norfolk, VA on 24-28 Sep 17. Submitted to 711 HPW/RHDR.

(b) (6) (2017, June). *PPI Signaling and Ca^{2+} Entry Mechanisms in the Biological Response to Nanosecond Pulsed Electric Fields*. Oral Presentation for Lab Meeting held at Tri-Service Research Laboratory on 20 Jun 17. Submitted to 711 HPW/RHDR.

(b) (6) (2017, August). *PPIs and Receptor- / Store-Operated Mechanisms of Calcium During the Nanosecond Electric Pulse-Induced Cellular Response* (AFRL-RH-FS-OP-2017-117399, P.A. Case No. TSRL-PA-2017-0192, 18 Aug 17). Oral Presentation for 2nd World Congress on Electroporation and Pulsed Electric Fields in Biology, Medicine, Food & Environmental Technologies held in Norfolk, VA on 24-28 Sep 17. Submitted to 711 HPW/RHDR.

(b) (6) (2019, March). Receptor- and Store-operated Mechanisms of Calcium Entry During the Nanosecond Electric Pulse-induced Cellular Response. *BBA Biomembranes, 1861*(3), 685-696. Submitted to 711 HPW/RHDR.

2.3 NSEP White

GDIT investigators planned and executed a study to examine the effects of NSEP exposure on white blood cell (WBC) populations. WBCs, which include monocytes, lymphocytes, and granulocytes, are immune cells within the blood that are the first line of defense in the response to infection. GDIT scientists created a draft standard operating procedure for the isolation and detection of WBCs from whole blood, which is currently under review. Once isolated, different WBC populations were labeled with antibodies specific for individual cell types, including markers for B-cells, monocytes, and T-cells. These markers (and, as a result, the cell types they label) were differentiated using flow cytometry. After separation and labeling protocols were completed, we exposed blood samples to NSEP (10 NSEP, 150 kV/cm or 600 NSEP, 2.5 kV/cm) and measured differences in cell populations using flow cytometry. Results showed a separation of the granulocyte cells into two distinct populations at higher pulse counts. While the study was placed on hold due to biohazard restrictions, the preliminary results of the NSEP White study showed promising effects of NSEP on WBC populations.

2.4 Terahertz Bioeffects

Due to increased use of THz-based technologies, including those of relevance to the U.S. Air Force, more investigations are required to determine the biological effects associated with

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

exposure to THz radiation. To achieve this aim, GDIT investigators performed basic science studies examining the effect of THz exposure on cellular function, including mitochondrial effects (membrane permeability), cytoskeleton (microtubules (MTs) and tubulin), cell viability, and deoxyribonucleic acid (DNA) epigenetics. The results of these studies are outlined in the following section.

2.4.1 THz Effects on Macromolecules

Collective motions of water and of many biological macromolecules have characteristic time scales on the order of a picosecond. As a result, these biomolecules can strongly absorb THz radiation. Due to this 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 DNA and proteins. THz radiation can affect several gene expression pathways and, consequently, can alter various biochemical and physiological processes in cells. Furthermore, THz radiation has been shown to influence the expression of several genes within 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. In this study, GDIT investigators participated in a study to perform global profiling of transcripts in human cells exposed to 2.52 THz radiation and compare the exposure responses to a thermally-matched bulk-heating (BH) protocol. Our results show that both THz radiation and BH induce a significant change in the expression of numerous messenger ribonucleic acids (mRNAs) and microRNAs. The data also show that THz radiation triggers specific intracellular canonical pathways that are not affected in the BH-exposed cells. This study implies that THz radiation may be a useful, non-contact tool for the selective control of specific genes and cellular processes. The results of this study were included in one journal article.

(b) (6) (2016, January). Terahertz Radiation: A Non-contact Tool for the Selective Stimulation of Biological Responses in Human Cells. *IEEE Transactions on Terahertz Science and Technology*, *6*(1), 54-68. Submitted to 711 HPW/RHDR.

2.4.2 THz Effects on Mitochondria

Previous reports indicated that THz radiation can modulate membrane permeability and can cause a change in membrane potential (MP) in cells. However, it was unclear if THz frequencies could influence the permeabilization and polarization/depolarization of intracellular mitochondria. Thus, GDIT investigators examined how THz radiation at selected frequencies can affect mitochondrial activity. We initially examined the effect of THz irradiation on adenosine triphosphate (ATP) formation in mitochondria of exposed versus sham (unexposed) cells using an intracellular ATP concentration colorimetric assay. We next utilized a high-throughput oxygen and pH sensing system (Seahorse® XF24 extracellular flux analyzer) to assess the changes in mitochondrial bioenergetics. To monitor changes in mitochondrial MP post THz irradiation, we stained the exposed and sham cells with fluorescent dyes and then compared changes in the accumulation of the dyes using optical microscopy and flow cytometry. Results showed no significant changes in cell death following THz irradiation. However, we observed an increase in

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

mitochondrial respiration in cells exposed to both 1.04 THz and 1.27 THz frequencies compared to sham. GDIT investigators proposed future studies to perform live imaging of exposed cells to further elucidate the biological effects in response to THz irradiation, especially focused on mitochondrial respiration.

GDIT investigators sought to continue to examine changes in mitochondrial respiration using live cell imaging. Therefore, they initiated a study with the goal of using fluorescence imaging to study the bioeffects of 2.52 THz radiation on mitochondria and MTs. In order to perform imaging studies, the GDIT team built a custom, automated system to provide the ideal conditions for controlled THz radiation exposure to *in vivo* cell cultures and *in vitro* molecules. This unique system includes an optimized THz source, a multi-format tray exposure platform, and a motorized, upright fluorescence microscope for time-lapse imaging. A custom LabVIEW program precisely controls the location placement and exposure time of samples. A multi-format holder allows the user a variety of plates/dishes. Additionally, the program data logs the THz exposure time and power levels given to the cells, along with the incubator's environmental temperature, humidity, and CO_2 percentage. GDIT scientists performed a proof-of-concept imaging study with the new system to validate its use for future THz exposure experiments.

The results of these studies were included in one abstract, one poster, and one technical report.

(b) (6) (2015, August). *Determination of the Effects of Terahertz Radiation on Mitochondrial Activity* (AFRL-RH-FS-AB-2015-0019, P.A. Case No. TSRL-PA-2015-0108, 24 Aug 15). Abstract for SPIE Photonics West 2016 in San Francisco, CA on 13-18 Feb 16. Submitted to 711 HPW/RHDR.

(b) (6) 2016, February). *Determination of the Effects of Terahertz Radiation on Mitochondrial Activity* (AFRL-RH-FS-PO-16-115090, 11 Feb 16). Poster for SPIE Photonics West 2016 held in San Francisco, CA on 13-18 Feb 16. Submitted to 711 HPW/RHDR.

(b) (6) (2016, May). *Fluorescence Imaging of Terahertz Bioeffects*. Unpublished manuscript. Submitted to 711 HPW/RHDR.

2.4.3 THz Effects on Cytoskeleton

MTs are highly dynamic intracellular structures critical to many biological functions in eukaryotic cells. They grow and shrink via the addition or subtraction of tubulin heterodimers and, given their polar nature, are expected to interact with EM fields. In recent years, there has been growing interest in understanding how EM fields can interact with intracellular structures and how it can influence biological functions. Therefore, GDIT investigators participated in studies designed to define the interaction between THz frequency radiation and MT dynamics.

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

The dynamics of the low-frequency vibrational modes of MTs play a key role in many theoretical models regarding their biological function. We analyzed these dynamics through large scale, classical molecular dynamics (MD) simulations of a MT composed of 42 tubulin heterodimers to provide insights into the qualitative nature of vibrational energy absorption and dissipation mechanisms. Results of the computed MT absorption spectra and vibrational density of states in the THz regime were elucidated, along with an analysis of the vibrational dephasing rates of the tubulin center of mass dynamics, which are shown to be overdamped. Additionally, the presence of the MT modifies the vibrational properties of the solvation shell structure and dynamics within roughly 10 Å of the protein. These vibrational properties are similar to other globular proteins and indicate MTs are unlikely candidates for any large-scale collective vibrational processes in the terahertz regime such as Fröhlich condensates.

We expanded our studies from MD simulations to spectroscopic analysis using low frequency Raman spectroscopy, which is a highly sensitive and non-destructive technique used to investigate the vibrational and rotational modes of biological and non-biological materials. The Raman spectra measured provide information about the chemical structure and nature of these materials. GDIT investigators assisted in the design and construction of a low frequency Raman spectroscopy system that is able to measure signals <10 cm^{-1} to <400 cm^{-1}. The system consisted of a 514.5 nm monochromatic laser directed through a polarizing beam cube and half waveplate to adjust the intensity of the beam. The beam was expanded and reflected off a 514.5 nm high pass filter before passing through a 50x Mitutoyo objective, which focuses it onto the sample. The back scattered light was recollimated through the objective. The high pass filter and three 514.5 nm Bragg filters were used to reduce the Rayleigh signal. The remaining Raman signal was focused into a Shamrock 303i spectrometer with a cooled ANDOR CCD camera. Using high dynamic range data acquisition with background subtraction, this system allowed low frequency Raman spectroscopy of reduced cytochrome C, bovine serum albumin, MTs, and collagen in solution. The system has the advantage of enabling the measurement of the low frequency Raman signal without sacrificing the ability to perform traditional Raman spectroscopy. Once constructed, we utilized the low frequency Raman spectroscopy system to probe changes in MTs following EM field exposures. Recent studies have shown that MTs resonate at specific frequencies in the kHz to THz range; therefore, irradiating them at those specific EM frequencies could possibly induce protein conformational changes or morphogenetic perturbations, which would affect their normal function. We used our Raman spectroscopy system to identify conformational or morphological differences between tubulin and MTs, and to investigate the interactions between EM fields and MTs.

The results of these studies were included in four abstracts, one oral presentation, four poster presentations, and one manuscript.

(b) (6) 2016, February). *Impact of Terahertz Frequencies on Microtubule Polymerization and Dynamics* (AFRL-RH-FS-PO-2016-115048, P.A. Case No. TSRL-PA-2015-0188, 1 Feb 16). Poster for SPIE Photonics West in San Francisco, CA (7-12 Feb 16). Submitted to 711 HPW/RHDR.

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

(b) (6) (2015, November). *Terahertz (THz) Irradiation Effects on Microtubules*. Protocol. Submitted to 711 HPW/RHDR.

(b) (6) 2017, March). Qualitative Behavior of the Low-Frequency Vibrational Dynamics of Microtubules and the Surrounding Water (AFRL-RH-FS-JA-2016-116351, P.A. Case No. TSRL-PA-2016-0314, 2 Dec 16). *The Journal of Physical Chemistry B*, *121*(14), 3024-3031. Submitted to 711 HPW/RHDR.

(b) (6) (2017, January). *Dynamic Behavior of Microtubules Following Terahertz Excitation* (AFRL-RH-FS-PO-2017-116497, P.A. Case No. TSRL-PA-2017-0121, 20 Jan 17). Poster for SPIE Photonics West in San Francisco, CA on 28 Jan – 2 Feb 17. Submitted to 711 HPW/RHDR.

(b) (6) (2018, March). *Impact of Sub-Millimeter Waves on the Assembly Kinetics of Microtubules* (AFRL-RH-FS-AB-2018-118234; P.A. Case No. TSRL-PA-2018-0141, 29 Mar 18). Abstract for 43rd International Conference on Infrared, Millimeter, and Terahertz Waves (IRMMW-THz 2018) held at Nagoya Congress Center, Nagoya, Japan on 9-14 Sep 18. Submitted to 711 HPW/RHDR.

(b) (6) (2018, August). *Impact of Sub-Millimeter Waves on the Assembly Kinetics of Microtubules* (AFRL-RH-FS-PO-2018-118764; P.A. Case No. TSRL-PA-2018-0191, 24 Aug 18). Poster for 43rd International Conference on Infrared, Millimeter, and Terahertz Waves (IRMMW-THz 2018) held at Nagoya Congress Center, Nagoya, Japan on 9-14 Sep 18. Submitted to 711 HPW/RHDR.

(b) (6) (2018, August). *Implementing Low-Frequency Raman Spectroscopy to Study Biological Molecules* (AFRL-RH-FS-AB-2018-118821; P.A. Case No. TSRL-2018-0198, 30 Aug 18). Abstract for BiOS Photonics West 2019, Imaging, Manipulation, and Analysis of Biomolecules, Cells, and Tissues XVII (Conference BO500), to be held in San Francisco, CA on 2-7 Feb 19. Submitted to 711 HPW/RHDR.

(b) (6) 2018, August). *Low-Frequency Raman Spectra of Microtubules in Response to Electromagnetic Field Exposures* (AFRL-RH-FS-AB-2018-118772; P.A. Case No. TSRL-PA-2018-0188, 22 Aug 18). Abstract for BiOS Photonics West 2019, Optical Interactions with Tissue and Cells XXX (Conference BO400), held in San Francisco, CA on 2-7 Feb 19. Submitted to 711 HPW/RHDR.

(b) (6) (2019, January). *Low-Frequency Raman Spectra of Microtubules in*

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

Response to Electromagnetic Field Exposures (AFRL-RH-FS-PO-2019-119333, P.A. Case No. TSRL-PA-2019-0117, 15 Jan 19). Poster for SPIE Photonics West 2019 on 2-7 Feb 18.

(b) (6) (2019). *Qualitative Behavior of Microtubules Based Upon the Low-Frequency Vibrational Dynamics of the Protein and Surrounding Water* (AFRL-RH-FS-OP-2019-120395, P.A. Case No. TSRL-PA-2019-0211, 22 Oct 19). Oral presentation for Physics Graduate Seminar to be held at The University of Texas at San Antonio on 8 Nov 19. Submitted to 711 HPW/RHDR.

(b) (6) (2020, January). *Exposure to Microtubule Resonant Frequency Modulates Neuronal Activity* (AFRL-RH-FS-AB-2020-120705; P.A. Case No. TSRL-PA-2020-0115, 22 Jan 20). Abstract for 12th FENS Forum in Neuroscience scheduled to be held in Glasgow, UK on 11-15 Jul 2020. Submitted to 711 HPW/RHDR.

2.4.4 THz Effects on Epigenetics and Gene Expression

Recent reports have shown that exposure to non-ionizing EM waves, including submillimeter waves (sub-MMWs), can influence gene expression in skin tissues and various cell types. However, the mechanism(s) by which exposure to sub-MMWs alters gene expression has not been elucidated. In mammalian cells, DNA methylation is an epigenetic modification involving the addition of methyl groups to the cytosine in CpG dinucleotides by DNA methyltransferases. When it occurs in the promoter region of the gene, hypermethylation is typically associated with repression of gene expression while hypomethylation is associated with increased gene expression. DNA methylation is a dynamic process that is implicated in rapid cellular response to external stimuli. Accordingly, DNA methylation has been demonstrated as a control mechanism for the cellular response to ionizing radiation; however, its role in non-ionizing radiation-induced effects is not fully understood. To address this gap in the knowledge, GDIT scientists initiated studies to measure global DNA methylation in human skin cells (keratinocytes) exposed to sub-MMW radiation. We hypothesized that sub-MMW exposure induces epigenetic modifications that result in altered gene expression. Specifically, we explored the thermal and non-thermal effects of sub-MMW exposure on DNA methylation in human primary skin cell lines. Cells were exposed to sub-MMWs at high and low power intensities for different time durations, and changes in global DNA methylation were examined following each exposure. Results showed DNA demethylation following THz exposure (compared to non-exposed sham cells). GDIT investigators recommended future studies to determine the specific sites of demethylation in DNA from THz exposed cells and relate them to changes in gene expression, which could be critical in understanding biological effects of THz irradiation. In summary, the results of this study were essential for understanding the role DNA methylation plays in gene regulation in sub-MMW exposed cells.

The results of this study were included in two abstracts, one proceeding, and two poster presentations.

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

(b) (6) (2016, August). *Terahertz Radiation Effect on DNA Methylation* (AFRL-RH-FS-AB-2016-115914, P.A. Case No. TSRL-PA-2016-0277, 29 Aug 16). Abstract for SPIE Photonics West held in San Francisco, CA on 28 January – 2 February 2017. Submitted to 711 HPW/RHDR.

(b) (6) . (2017, January). *The Effect of Terahertz Radiation on DNA Methylation* (AFRL-RH-FS-PO-2017-116483, P. A. Case No. TSRL-PA-2017-0122, 20 Jan 17). Poster for SPIE Photonics West 2017 Conference on 28 Jan - 2 Feb 17. Submitted to 711 HPW/RHDR.

(b) (6) (2018, March). *Epigenetic Modifications Induced by Submillimeter Wave Exposure* (AFRL-RH-FS-AB-2018-118244, P.A. Case No. TSRL-PA-2018-0140, 29 Mar 18). Abstract for 43rd International Conference on Infrared, Millimeter, and Terahertz Waves (IRMMW-THz 2018) held in Nagoya Congress Center, Nagoya, Japan on 9-14 Sep 18. Submitted to 711 HPW/RHDR.

(b) (6) . (2018, June). *Epigenetic Modifications Induced by Submillimeter Wave Exposure* (AFRL-RH-FS-PC-2018-118592; P.A. Case No. TSRL-PA-2018-0177, 29 Jun 18). Proceeding for 43rd International Conference on Infrared, Millimeter, and Terahertz Waves (IRMMW-THz 2018) held at Nagoya Congress Center, Nagoya, Japan on 9-14 Sep 18. Submitted to 711 HPW/RHDR.

(b) (6) (2018, August). *Epigenetic Modifications Induced by Submillimeter Wave Exposure* (AFRL-RH-FS-PO-2018-118765; P.A. Case No. TSRL-PA-2018-0192, 24 Aug 18). Poster for 43rd International Conference on Infrared, Millimeter, and Terahertz Waves (IRMMW-THz 2018) held at Nagoya Congress Center, Nagoya, Japan on 9-14 Sep 18. Submitted to 711 HPW/RHDR.

2.5 DE Epigenetic

Exposures to non-ionizing EM waves in the radio frequency (RF) range have been shown to influence gene expression in various cell and tissue types. However, the specific mechanism(s) by which exposure to these waves alter gene expression is not completely clear. Recent studies have suggested changes in epigenetics as a plausible mechanism for the gene expression alterations observed in response to exposures to RF waves. In this study, we investigated if exposures to RF fields can influence epigenetics. Specifically, we examined modifications in DNA methylation patterns in response to exposures to 900 MHz RF fields in primary human keratinocytes. We assembled a custom system to allow the stable exposure of cell cultures to 900 MHz RF fields at a range of applied powers and resultant E fields. We used methylation sensitive restriction enzyme digestion and Global DNA Methylation ELISA assay to quantify the status of global DNA

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

methylation in cells exposed to 900 MHz RF fields for different time durations and power densities. Results showed significant global DNA demethylation in the RF exposed cells compared to the sham (unexposed) counterparts. Importantly, these changes occur in the absence of cell death and without a concomitant increase in temperature during exposures, suggesting that alterations in DNA methylation are not associated with toxic or thermal effects of the RF fields. This suggests that RF exposure changes DNA methylation patterns and can potentially alter gene expression.

The results of this study were included in two abstracts and one oral presentation.

(b) (6) (2018, August). *Changes in Epigenetics Following Exposure to Radiofrequency Fields* (AFRL-RH-FS-AB-2018-118760; P.A. Case No. TSRL-PA-2018-0189, 22 Aug 18). Abstract for SPIE Photonics West 2019, Optical Interactions with Tissue and Cells XXX (Conference BO400), held in San Francisco, CA on 2-7 Feb 19. Submitted to 711 HPW/RHDR.

(b) (6) (2019, March). *Epigenetics Changes Following Exposure to Radiofrequency Fields* (AFRL-RH-FS-AB-2019-119683, P.A. Case No. TSRL-PA-2019-0148, 27 Mar 19). Abstract for BioEM-BEMS held in Montpellier, France on 23-28 Jun 19. Submitted to 711 HPW/RHDR.

(b) (6) (2019, January). *Changes in Epigenetics Following Exposure to Radiofrequency Fields* (AFRL-RH-FS-OP-2019-119290, P.A. Case No. TSRL-PA-2019-0112, 14 Jan 19). Oral Presentation for BiOS Photonics West 2019, Optical Interactions with Tissue and Cells XXX (Conference BO400), held in San Francisco, CA on 2-7 Feb 19. Submitted to 711 HPW/RHDR.

2.6 Optogenetics

Recently, a major focus of 711 HPW/RHDR has been to develop novel sensors of RF, including biological sensors that can accurately measure RF exposure in human systems. GDIT investigators participated in a project to characterize novel RF sensing proteins that were developed by collaborators in the AFRL Materials and Manufacturing Directorate (AFRL/RX). These novel proteins consist of a protein-sensing element, Per-Arnt-Sim (PAS) domain-containing protein kinase (PASK), which is an evolutionary conserved kinase that coordinates cellular metabolism with metabolic demand in mammals. For RF sensing, PASK proteins were engineered to act as RF receivers to aid in the investigation of RF stimulation on neuronal function. GDIT investigators exposed the engineered protein, named BJMixL, to RF in a TEM cell (900 MHz, 15 W, 4 hr) and analyzed changes/damage in/to the protein using SDS-PAGE electrophoresis and flow cytometry. Results showed changes in the number of bands resolved on an SDS-PAGE gel after RF exposure and changes in the distribution of BJMixL via flow cytometry. Thus, GDIT investigators have characterized two methods (SDS-PAGE and flow cytometry) that can detect changes in BJMixL following RF exposure. Future studies were proposed using Western blot to detect changes in PASK activity after RF exposure.

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

2.7 Magnetic Literature Review

Studies exploring the biological effects of exposure to magnetic fields (MFs) are motivated by safety considerations of applications such as magnetic resonance imaging (MRI), as well as the search for therapeutic interventions. GDIT investigators broadly summarized the biological effects of MFs under the patchwork of experimental conditions and to provide current mechanistic explanations for observed bioeffects in a literature review. Key findings are as follows. First, the parameters of the field and the duration of exposure will vary the biological effects of MFs and, therefore, precludes the existence of any overarching mechanism. Second, the response of living tissue to strong static MFs is currently attributed to diamagnetic anisotropy while EM induction is likely responsible for effects observed in pulsed gradient fields. In response to the literature search and review, GDIT scientists suggest a more systematic approach to investigating the bioeffects of MFs to provide a more robust base of knowledge from which mechanisms could be elucidated. The findings of this study were included in one literature review.

(b) (6) (2017, October). *Bioeffects of Magnetic Fields – A Review of Literature*. Unpublished report. Submitted to 711 HPW/RHDR.

2.8 MF Brain

2.8.1 MF Exposure and Nanoparticles

Rapid development in nanomaterial synthesis and surface functionalization has led to advanced studies in actuation and manipulation of cellular functions for biomedical applications. One common actuation technique employs externally applied MFs to manipulate magnetic nanomaterials within cells in order to drive or trigger desired effects. While cellular interactions with low-frequency MFs and nanoparticles have been extensively studied, the fundamental mechanisms behind these interactions remain poorly understood. Additionally, modern investigations on these concurrent exposure conditions have been limited in scope, and difficult to reproduce. GDIT researchers participated in a study to define an easily reproducible method of investigating the biological impact of concurrent MF and nanoparticle exposure conditions using a well-defined *in vitro* CHO-K1 cell line model. This study aimed to establish grounds for in-depth fundamental studies of the mechanisms driving cellular-level interactions. Cells were cultured under various nanoparticle and MF exposure conditions singly or in combination from 0 to 500 µg/ml nanoparticle concentrations and direct current (DC), 50 Hz, or 100 Hz MFs with 2.0 mT flux density. Cells were then observed by confocal fluorescence microscopy, and subject to biological assays to determine the effects of concurrent extreme-low frequency MF and nanoparticle exposures on cell-nanoparticle interactions, such as particle uptake and cell viability by MTT assay. The results of this study indicated little to no variation in effect on cell cultures based on MF parameters alone; however, it is clear that deleterious synergistic effects of concurrent exposure conditions exist based on a significant decrease in cell viability when exposed to high concentrations of nanoparticles and concurrent MF. The results of this study were included in one proceeding.

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

(b) (6) (2016, April). Investigation of Superparamagnetic (Fe_3O_4) Nanoparticles and Magnetic Field Exposures on CHO-K1 Cell Line (AFRL-RH-FS-PC-2016-115407, P. A. Case No. TSRL-16-0236, 11 Apr 16) *Proc. SPIE 9706, Optical Interactions with Tissue and Cells XXVII*, 97061Y. Submitted to 711 HPW/RHDR.

2.8.2 MF Exposure and DNA Methylation in Cultured Cells

As discussed previously (section 2.4.4), DNA methylation is an epigenetic modification involving the addition of methyl groups to the cytosine in CpG dinucleotides by DNA methyltransferases. When it occurs in the promoter region of the gene, hypermethylation is typically associated with repression of gene expression while hypomethylation is associated with increased gene expression. DNA methylation is a dynamic process that is implicated in rapid cellular response to external stimuli. Accordingly, DNA methylation has been demonstrated as a control mechanism for the cellular response to ionizing radiation; however, its role in non-ionizing radiation-induced effects is not fully understood. GDIT investigators hypothesized that exposure to MFs or RF fields induce alterations in DNA methylation status. Specifically, we sought to capture the global cellular epigenetic profiles in response to exposure to specific MF and RF exposures to identify differentially expressed epigenetic markers that might serve as biomarkers of the given exposure. GDIT scientists exposed iCell® GABANeurons to MF (50 Hz, 2 milliTesla) or RF (900 MHz) and measured global DNA methylation using a commercially available kit (MethylFlash™ Global DNA Methylation Enzyme-Linked Immunosorbent Assay Kit, Epigentek). Results showed an increase in DNA methylation following exposure of neurons to MF. However, exposure to RF resulted in a decrease in methylation, which is consistent with experiments described in other sections of this report.

Given our findings indicating that exposure to extremely low-frequency magnetic fields (ELF-MFs) can alter epigenetic regulation in cultured neurons, we expanded our studies to investigate whether exposure to ELF-MFs can induce epigenetic changes in primary human keratinocytes. We investigated the effects on DNA methylation, in cells exposed to 50 Hz ELF-MFs at an intensity of 1 – 5 mTesla for various time durations using a set of 7-inch diameter Helmholtz coils placed inside an incubator. Exposed and unexposed (sham) cells were simultaneously placed into the incubator in which the environmental conditions were constant (37 °C, 5% CO_2, 95% humidity). We assessed differences in DNA methylation patterns in response to ELF-MF exposures using methylation sensitive restriction enzyme digestion and a Global DNA Methylation ELISA assay. Results showed significant changes in global DNA methylation in the ELF-MF exposed cells compared to sham counterparts. Our data highlight the potential of ELF-MFs to trigger epigenetic control of gene expression in keratinocytes, and provide novel insights to elucidate a potential molecular mechanism implicated in ELF-MF exposure in skin cells.

The results of these studies were published in one white paper, one abstract, and one poster presentation.

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

(b) (6) (2019, January). *A Preliminary Examination of Changes in DNA Methylation Following Exposure to Magnetic and Radiofrequency Fields*. White Paper. Submitted to 711 HPW/RHDR.

(b) (6) (2018, August). *Influence of Magnetic Field Exposure on Epigenetic Regulation in Human Keratinocytes* (AFRL-RH-FS-AB-2018-118778; P.A. Case No. TSRL-PA-2018-0193, 24 Aug 18). Abstract for upcoming BiOS Photonics West 2019, Optical Interactions with Tissue and Cells XXX (Conference BO400), held in San Francisco, CA on 2-7 Feb 19. Submitted to 711 HPW/RHDR.

(b) (6) (2019, January). *Influence of Magnetic Field Exposure on Epigenetic Regulation in Human Keratinocytes* (AFRL-RH-FS-PO-2019-119334, P.A. Case No. TSRL-PA-2019-0119, 22 Jan 19). Poster for SPIE Photonics West 2019 on 2-7 Feb 18. Submitted to 711 HPW/RHDR.

2.9 RF Neuro / AFOSR

The AFRL has a long-standing interest in the development of non-lethal, long-range weapons with the intent to incapacitate and/or control the behavior of target adversaries by manipulating neuronal function and physiology. In order to accomplish this long-term goal, it is necessary to understand the cellular and molecular interactions of RF with neurons. Thus, GDIT investigators (in collaboration with the 711 HPW/RHDR) conducted neurophysiology and molecular biology studies with the goal of identifying key cytoskeletal elements that respond to RF radiation.

Recent studies in the literature show that exposure to RF influences the central nervous system (CNS) by affecting concentration, memory, and cognitive performance. Specifically, RF exposure has been shown to reduce the number of neurites, alter the kinetics and activity of voltage-gated ion channels, alter AP firing rates, and change the properties of the neuronal PM. All of these biological impacts of RF exposure can lead to behavioral and cognitive outcomes including alterations in learning, memory, attention, and concentration. However, while promising, these studies are inconclusive and contradictory with some research describing benefits from RF exposure and others detailing harmful RF effects. To address these discrepancies, we sought to determine if the observed biological effects are the result of an intracellular RF antenna or sensor that absorbs and/or interacts with RF in order to mediate the specific biological outcomes. Previous studies in the field suggest that MTs could act as RF antennas within the cell, given their ability to receive and mediate the biological and neurophysiological response(s) to RF energy. Thus, in the present study, we hypothesized that exposing neurons at specific RF frequencies tuned to tubulin or MT resonance frequencies might disrupt natural signaling occurring in and around MTs, thus leading to neurophysiological changes. To address this hypothesis, we performed experiments to determine if exposing neuronal cells to RF fields that were previously shown to interact with MTs could mediate biological responses. First, we described the design and assembly of two, custom, environmentally controlled RF exposure systems. These state-of-the-art systems

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

are critical to the completion of the present and future studies of the bioeffects of RF on biological systems. Second, we performed RF exposures of neuronal cell cultures to define biological and neurophysiological effects following exposure at specific MT resonant frequencies.

Our results demonstrated the effects of MT resonant frequencies on neuronal cell lines and primary neurons. Specifically, we showed altered intracellular tubulin localization, increased basal calcium levels, and changes in neuronal physiology and function (reduced AP amplitude, depolarized resting MP, increased number of miniature synaptic current events, and increased spontaneous excitatory synaptic currents (sEPSC) and/or spontaneous inhibitory synaptic currents (sIPSC) amplitude and area) in cells exposed to MT resonant frequencies. Overall, our results showed that exposing neurons to MT and tubulin resonance frequencies might cause changes in biological cells that could affect their normal function and behavior. These results are essential to understanding the biomolecular response to RF and in shaping future experiments to explore this research further.

The results of these studies were included in two abstracts, one technical report, and one poster.

(b) (6) (2019, August). *Effect of Microtubule Resonant Frequencies on Neurons* (AFRL-RH-FS-AB-2019-120177, P.A. Case No. TSRL-PA-2019-0191, 28 Aug 19). Abstract for BiOS Photonics West 2020, Optical Interactions with Tissue and Cells XXXI (Conference BO400), held in San Francisco, CA on 1-6 Feb 2020. Submitted to 711 HPW/RHDR.

(b) (6) (2019, August). *Defining the Intracellular Mediators for Radiofrequency Wave Modulation of Neuronal Activity* (AFRL-RH-FS-TR-2019-XXXX). Unpublished manuscript. JBSA Fort Sam Houston, TX: Air Force Research Laboratory, 711th Human Performance Wing, Airman Systems Directorate, Bioeffects Division, Radio Frequency Bioeffects Branch. Submitted to 711 HPW/RHDR.

(b) (6) (2020, January). *Effect of Microtubule Resonant Frequencies on Neuronal Cells* (AFRL-RH-FS-PO-2020-120682; P.A. Case No. TSRL-PA-2020-0110, 15 Jan 20). Poster for SPIE Photonics West 2020 held in San Francisco, CA on 1-6 Feb 2020. Submitted to 711 HPW/RHDR.

(b) (6) (2020, January). *Modulation of Neuronal Activity by Electromagnetic Fields: Implication of Microtubules* (AFRL-RH-FS-AB-2020-120737; P.A. Case No. TSRL-PA-2020-0124, 23 Jan 20). Abstract for BioEM 2020 scheduled to be held in Oxford, UK on 21-26 Jun 2020. Submitted to 711 HPW/RHDR.

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

2.10 TRPV Expression

GDIT investigators participated in a pilot project to examine the effects of NSEPs on primary human epidermal keratinocytes (HEKa) cells. The goal of this study was to determine the biological response of HEKa cells after exposure to 600 NSEPs. Specifically, GDIT investigators examined cell viability, cell permeability, and TRPC expression following exposure to low (10 kV/cm) and high (20 kV/cm) power 600 NSEP exposures. Results showed increased cell death following exposure, which is consistent with previous NSEP cellular studies conducted at the AFRL. Additionally, exposed HEKa cells showed increased permeability, as evidenced by increased PI uptake (evaluated by flow cytometry). Results showed a change in TRPC1 and transient receptor potential vanilloid-1 (TRPV1) channel expression following NSEP exposure. Future studies were proposed to determine if the observations were due to thermal or non-thermal effects of NSEP delivery. The results of this study were included in one technical report that is currently under review.

(b) (6) (2019, May). *Bioeffects of Nanosecond Pulsed Electrical Field on Cell Survival, Permeabilization, and Protein Expression in Adult Human Epidermal Keratinocytes* (AFRL-RH-FS-TR-2019-XXXX). Unpublished manuscript. JBSA Fort Sam Houston, TX: Air Force Research Laboratory, 711th Human Performance Wing, Airman Systems Directorate, Bioeffects Division, Radio Frequency Bioeffects Branch. Submitted to 711 HPW/RHDR.

2.11 Quantum Biology (IR Stimulation)

2.11.1 Development of Optogenetic Methods to Study IR Effects on Neurons

Recently, IR light has been shown to both stimulate and inhibit excitatory cells. However, studies of IR light for excitatory cell inhibition have been constrained by the use of invasive and cumbersome electrodes for cell excitation and AP recording. Here, we present an all optical experimental design for neuronal excitation, inhibition, and AP detection. Primary rat neurons were transfected with plasmids containing the light sensitive ion channel CheRiff. CheRiff has a peak excitation around 450 nm, allowing excitation of transfected neurons with pulsed blue light. Additionally, primary neurons were transfected with QuasAr2, a fast and sensitive fluorescent voltage indicator. QuasAr2 is excited with yellow or red light and therefore does not spectrally overlap CheRiff, enabling imaging and AP activation, simultaneously. Using an optic fiber, neurons were exposed to blue light sequentially to generate controlled APs. A second optic fiber delivered a single pulse of 1869 nm light to the neuron causing inhibition of the evoked APs (by the blue light). When used in concert, these optical techniques enable electrode free neuron excitation, inhibition, and AP recording, allowing research into neuronal behaviors with high spatial fidelity. The results of this study were included in one proceeding.

(b) (6) (2016, March). All Optical Experimental Design for Neuron Excitation, Inhibition, and Action Potential Detection (AFRL-RH-FS-PC-2016-115008, P.A. Case No. TSRL-PA-2015-0183, 20 Jan 16) *Proc. SPIE 9690, Clinical and Translational*

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

Neurophotonics; Neural Imaging and Sensing; and Optogenetics and Optical Manipulation, 96901P, San Francisco, CA on 13-18 February 2016. Submitted to 711 HPW/RHDR.

2.11.2 IR Effects on Membrane Fluidity

The mechanism by which IR light affects cellular membranes is poorly understood. To clarify this gap in the knowledge, GDIT investigators participated in a study to test the hypothesis that IR light exposure causes a change in PM fluidity. To test this hypothesis, we used the membrane fluidity dye, di-4-ANEPPDHQ, to investigate membrane fluidity changes following IR light exposure. Results in CHO cells showed increased membrane fluidity with IR light pulse exposure and this increased fluidity scales with IR irradiance. Neuroblastoma-glioma (NG108) and primary neurons also exhibited increased membrane fluidity following IR exposure. Altogether, these results demonstrate that IR light induced a thermal gradient in cells that changed membrane fluidity. The results of this study were included in one abstract.

(b) (6) (2016, June). *Short Infrared Laser Pulses Increase Cell Membrane Fluidity* (AFRL-RH-FS-AB-2016-115659, P.A. Case No. TSRL-PA-2016-0257, 30 Jun 16). Abstract for SPIE 2017 held in San Francisco, CA on 28 January – 2 February 2017. Submitted to 711 HPW/RHDR.

2.11.3 IR Effects on Ion Channel Conductance

GDIT investigators conducted a pilot study to examine the effects of pulsed IR laser on ionic conductance across the PM of excitable and non-excitable cells. Specifically, we used direct ionic conductance measurements using patch clamp electrophysiology and advanced imaging techniques. Results show that PM ion channels in concert with intracellular regulatory mechanisms play a crucial role in previously reported post-IR exposure bioeffects. Furthermore, IR-induced pressure and thermal gradients act directly on the PM by changing the local environment, resulting in the generation of small, lipid ion channels in addition to modulation of protein ion channels in both excitable and non-excitable cells. The results of this study were included in one technical report.

(b) (6) (2016, October). *Infrared Laser Induced Alterations of Plasma Membrane Conductance in Excitable and Non-excitable Cells* (AFRL-RH-FS-TR-2017-0006, 10 Feb 17). JBSA Fort Sam Houston, TX: Air Force Research Laboratory, 711th Human Performance Wing, Airman Systems Directorate, Bioeffects Division, Radio Frequency Bioeffects Branch. Submitted to 711 HPW/RHDR.

2.11.4 IR Exposure Effects on APs in Neurons

Short infrared laser pulses (SILP) have many physiological effects on cells, including the ability to stimulate APs in neurons. GDIT investigators performed a study which revealed the opposite; specifically, the results of our study revealed that SILPs can also reversibly block APs.

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

A reversible AP block in hippocampal neurons was observed following SILP (0.26 to 0.96 J/cm^2; 1.37 to 5.01 ms; 1869 nm) with the block persisting for more than 1 s in cells exposed greater than 0.69 J/cm^2. The AP block was sustained for 30 s with SILPs pulsed at 1 to 7 Hz. Full recovery of neuronal activity was observed 5 to 30 s post SILP exposure. These results indicated that SILP can be used for noncontact, reversible AP block. Due to the high spatial precision and noncontact manner of IR light delivery, AP block by SILP (i.e., IR neural inhibition) has the potential to transform medical care for sustained pain inhibition and suppression of unwanted nerve activity. The results of this study were included in one manuscript.

(b) (6) (2016, December). Action Potential Block in Neurons by Infrared Light. *Neurophotonics, 3*(4), 040501-1 – 040501-4. Submitted to 711 HPW/RHDR.

2.11.5 IR Effects on Calcium Flux in Neurons

Pulsed IR laser energy has been shown to modulate neurological activity through both stimulation and inhibition of APs. While the mechanism(s) behind this phenomenon is (are) not completely understood, certain hypotheses suggest that the rise in temperature from IR exposure could activate temperature- or pressure-sensitive ion channels or create pores in the cellular outer membrane, allowing an influx of typically plasma-membrane-impermeant ions. Studies using fluorescent intensity-based Ca^{2+} sensitive dyes show changes in Ca^{2+} levels after various IR stimulation parameters, which suggests that Ca^{2+} may originate from the external solution. However, activation of intracellular signaling pathways has also been demonstrated, indicating a more complex mechanism of increasing intracellular Ca^{2+} concentration. GDIT investigators quantified the Ca^{2+} mobilization in terms of influx from the external solution and efflux from intracellular organelles using Fura-2 and a high-speed ratiometric imaging system that rapidly alternates the dye excitation wavelengths. Using non-excitable CHO-hM_1 cells and NG108 cells, we demonstrate that intracellular IP_3 receptors play an important role in the IR-induced Ca^{2+}, with the Ca^{2+} response augmented by ryanodine receptors in excitable cells. The results of this study were included in one journal article.

(b) (6) (2017, April). Ryanodine and IP_3 Receptor-mediated Calcium Signaling Play a Pivotal Role in Neurological Infrared Laser Modulation (AFRL-RH-FS-JA-2016-116288, P.A. Case No. TSRL-PA-2016-0309, 23 Nov 16). *Neurophotonics, 4*(2), 025001. Submitted to 711 HPW/RHDR.

2.11.6 IR Effects on Excitatory and Inhibitory Responses of Neurons

Infrared laser pulses (IRLP) of millisecond durations have been demonstrated to evoke and inhibit APs in excitable tissues; however, the mechanisms underlying IRLP's capability for both stimulation and inhibition are not completely understood. One possibility is that IRLP delivery generates a rapid temperature gradient that activates thermally mediated mechanisms within excitable cells. In these cell types, MP and AP waveforms are sensitive to temperature modulation, and this sensitivity manifests at individual channels. For this reason, temperature sensitivity of a

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

membrane is controlled by the unique composition of channel isoforms within it. Since different cell types express different populations of ion channels, we hypothesized that the IRLP's ability to modulate MPs and APs is dependent on the heterogeneity of ion channel subtypes that densely occupy the membrane, and their exclusive sensitivity to IRLP-induced rapid temperature gradients underlines the diversity of IRLP-induced neuromodulation. To test this hypothesis, GDIT investigators participated in a study collecting patch clamp recordings with simultaneous high-speed fluorescent Ca^{2+} imaging. We compared the impact of a single 1869 nm IRLP of different durations on MPs, APs, and intracellular Ca^{2+} (Ca^{2+}i) transients in motor neuron-like differentiated NG108-15 (dNG108) cells and postnatal hippocampal neurons (PHNs) (i.e., cell types that exhibit distinct voltage gated ion channel profiles). Results showed two major differences in dNG108 and PHN responses to IRLPs: the first is that 50% of dNG108 cells consistently fired APs in response to IRLPs, whereas the same IRLPs failed to evoke APs in 99% of PHNs. Furthermore, results showed that, in dNG108 cells, IRLP evoked Ca^{2+} transients over 5 times larger in magnitude than in PHN. Lastly, results showed the complete inhibition of APs (generated by current injections) in PHNs and dNG108s by IRLPs applied at the beginning of the voltage step. The results of this study indicated that AP inhibition in both dNG108 and PHN cells is likely due to IRLP-induced potentiation of K+ current as a response to its unique sensitivity to transient heat. Overall, our work suggests that IRLPs may act by temporally shifting the excitation/inhibition (E/I) balance within the membrane by modulating voltage gated ion channels that govern MP and comprise the AP waveform. Depending on the ratios of excitatory and inhibitory IRLP-susceptible channels, IRLPs of identical parameters could shift E/I balance in opposing directions in distinct cells and tissues, and that may underlie the diversity of neuromodulating effect of IRLPs. The results of this study were included in one manuscript.

(b) (6) (2019, August). *Dissecting the diversity of excitatory and inhibitory responses to a single short infrared laser pulse in postnatal hippocampal neurons and motor neuron-like differentiated NG108 cells* (P.A. Case No. TSRL-PA-2019-0192, cleared 28 Aug 19). Unpublished manuscript. Submitted to 711 HPW/RHDR.

2.12 Fast Thermal Gradient (FTG)

Infrared neural stimulation (INS) is the process by which the absorption of pulsed IR energy into neural tissue produces a FTG in the cell membrane, resulting in either depolarization (i.e., excitation) or hyperpolarization (i.e., inhibition) of the neuron. The intracellular mechanisms of this process are still largely unknown. We hypothesized that FTG-induced neural stimulation could also be produced by the absorption of pulses of RF energy. Pulses of RF may provide a novel, non-contact, non-invasive, long-range means of neural stimulation and could ultimately be used to manipulate or incapacitate neuronal function. To address this hypothesis, GDIT investigators conducted studies of FTG effects on cells and whole animal systems, as described in the following subsections.

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR (b) (6)

2.12.1 FTG Studies in Neuronal Cell Models

Infrared laser (IRL) exposure induces a rapid temperature change (i.e., a FTG) that is able to stimulate or inhibit neurons and, thereby, modify neurological functions. Despite extensive research, the fundamental mechanism(s) underlying how FTG causes neurological stimulation or inhibition remains unclear. While it is hypothesized that IRL-induced FTG acts directly on the neuronal PM, it is uncertain if the neurological effects observed in previous studies are mostly derived from presynaptic effects (i.e., modifications in AP firing) and/or from postsynaptic effects (i.e., alteration of the synaptic responses of the excitatory and inhibitory neuronal receptors). Therefore, GDIT investigators conducted experiments to study FTG-mediated changes in neuronal PM, AP firing rate, and miniature postsynaptic excitatory and inhibitory currents (mEPSCs and mIPSCs) following IRL-induced FTG exposure. Our results suggest FTG induced changes in both presynaptic and postsynaptic neurophysiological mechanisms. Specifically, we found that, after IRL pulse-induced FTG exposure, the amplitudes of APs were reduced, but the rate of APs was increased. In contrast, the quantities of both mEPSCs and mIPSCs were reduced, but the peak-to-peak frequency and peak amplitudes were increased. The results outlined in this study demonstrate the impact of FTG on neurons and neuronal network. This information is critical for understanding the complexity of the effects of FTG on neurological functions and for demonstrating how post-synaptic mechanisms might play a crucial role in neurological excitation or inhibition seen following IRL pulse exposure.

Furthermore, previous studies showed that exposure of cells to an infrared laser pulse (IRLP) can cause a rapid and transient rise in intracellular calcium levels in primary neurons and immortalized cells. The downstream effect activates ion channels, and induces second messenger signaling cascades. For example, the intracellular phosphoinositide signaling cascade, initiated by phospholipase C (PLC) dependent PIP_2 hydrolysis, can lead to activation of protein kinase A (PKA) and second messenger translocation within the cell. The mobilization of these messengers can modulate ion conductivity through channels, and coordinate cytoskeletal rearrangements that promote or suppress further downstream signaling. As a result, we hypothesized that exposure to a single IRLP with 2-4 ms duration could initiate a cellular effect that lasts for seconds to minutes. We evaluated the PIP_2 phosphoinositide signaling cascade from immortalized cells that exhibited genetically encoded reporters of PLC and PKA activity. Upon IRLP exposure, we observed a PIP_2 depletion, PLC and PKA translocation, and intracellular calcium release in motor neuron-like NG108 cells. Our data suggest that IRLP may induce second messenger systems at the membrane, and as a result modulate ionic signaling across the cell body.

The results of these studies were included in two oral presentations, one white paper, two technical reports, two proceedings, and three abstracts.

(b) (6) (2019, January). *Infrared Laser Induced Alterations of Plasma Membrane Conductance in Excitable and Non-excitable Cells*. Briefing presented on 25 Jan 19 to (b) (6)) (Tri-Service Research Laboratory) regarding status of current

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

projects to determine future goals for INS/FTG group. Submitted to 711 HPW/RHDR.

(b) (6) (2019, April). *A Pilot Study of Changes in Excitability of the Primary Hippocampal Neurons after Exposure to IR-Induced Fast Thermal Gradient*. White Paper. Submitted to 711 HPW/RHDR.

(b) (6) (2019, May). *Changes in Excitability of the Hippocampal Neurons After Exposure to Infrared Pulse-induced Fast Thermal Gradient* (AFRL-RH-FS-TR-2019-XXXX). Unpublished manuscript. JBSA Fort Sam Houston, TX: Air Force Research Laboratory, 711th Human Performance Wing, Airman Systems Directorate, Bioeffects Division, Radio Frequency Bioeffects Branch. Submitted to 711 HPW/RHDR.

(b) (6) (2019, December). *Electrophysiological Experiments to Study Synaptic Transmission and Plasticity Using Acute Hippocampal Slices* (AFRL-RH-FS-TR-2019-XXXX). Unpublished manuscript. JBSA Fort Sam Houston, TX: Air Force Research Laboratory, 711th Human Performance Wing, Airman Systems Directorate, Bioeffects Division, Radio Frequency Bioeffects Branch. Submitted to 711 HPW/RHDR.

(b) (6)
(2019, July). *Infrared Laser-Induced Fast Thermal Gradient Affects the Excitability of Primary Hippocampal Neurons* (AFRL-RH-FS-AB-2019-120085, P.A. Case No. TSRL-PA-2019-0184, 23 Jul 19). Abstract for BiOS Photonics West 2020, Optical Interactions with Tissue and Cells XXX (Conference BO400) held in San Francisco, CA on 1-6 Feb 20. Submitted to 711 HPW/RHDR.

(b) (6) (2019, July). *Pulsed Infrared Laser Activates Intracellular Signaling in NG108 Cells* (AFRL-RH-FS-AB-2019-120049, P.A. Case No. TSRL-PA-2019-0183, 9 Jul 19). Abstract for BiOS Photonics West 2020, Optical Interactions with Tissue and Cells XXX (Conference BO400) held in San Francisco, CA on 1-6 Feb 20. Submitted to 711 HPW/RHDR.

(b) (6) (2020, January). *Infrared Laser-induced Fast Thermal Gradient Affects the Excitability of Primary Hippocampal Neurons* (AFRL-RH-FS-OP-2020-120684; P.A. Case No. TSRL-PA-2020-0111, 17 Jan 20). Oral Presentation for general purpose for future briefings.

(b) (6)
2020, January). *Infrared Laser-Induced Fast Thermal Gradient Affects the Excitability of Primary Hippocampal Neurons* (AFRL-RH-FS-PC-2020-120677; P.A. Case No. TSRL-PA-2020-0108, 15 Jan 20). Proc. for SPIE BiOS

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

Photonics West 2020, Optical Interactions with Tissue and Cells XXXI (Conference BO400) held in San Francisco, CA on 1-6 Feb 20. Submitted to 711 HPW/RHDR.

(b) (6) (2020, January). *Induced Fast Thermal Gradient Alters the Excitability and Synaptic Activity of Hippocampal Neurons* (AFRL-RH-FS-AB-2020-120676; P.A. Case No. TSRL-PA-2020-0107, 15 Jan 20). Abstract for 12th FENS Forum in Neuroscience scheduled to be held in Glasgow, UK on 11-15 Jul 2020. Submitted to 711 HPW/RHDR.

(b) (6)
(2020, March). *Pulsed Infrared Laser Activates Intracellular Signaling in NG108 Cells* (AFRL-RH-FS-PC-2020-120923; P.A. Case No. TSRL-PA-2020-0147, 26 Mar 20). Proceeding for BiOS Photonics West 2020, Optical Interactions with Tissue and Cells XXXI (Conference BO400) held in San Francisco, CA on 1-6 Feb 2020. Submitted to 711 HPW/RHDR.

2.12.2 IR-Induced FTG Studies in Rats

To expand on our initial studies of IR-induced FTG in isolated cells, GDIT investigators performed a study to model the IR-induced change in temperature, *dT*, with depth into exposed neural tissue and to experimentally validate the results of the model with measurements of the IR-induced *dT* in exposed, *ex vivo*, neural tissue slices of varying thickness. Further, GDIT investigators established a rat animal model with known physiological and behavioral responses to "FTG-induced" neural manipulation in preparation for RF exposure studies. In this study, we conducted a series of experiments in which we exposed the sciatic nerve and the motor cortex of anesthetized rats *in vivo* with long-duration pulses of light (≥10 ms) from an IR laser source (λ=1470 nm). We measured IR-induced *dT* with depth into the exposed neural tissue, examined direct and indirect neuronal responses, and assessed thermal tissue damage histologically. The results of this work were essential in establishing conditions, parameters, and protocols for future FTG projects. The results of this study were included in two technical reports.

(b) (6) (2018, February). *Modeling of 1470 nm Laser-tissue Interaction in the Brain in Rats* (AFRL-RH-FS-TR-2018-0016, 9 Jul 18). JBSA Fort Sam Houston, TX: Air Force Research Laboratory, 711th Human Performance Wing, Airman Systems Directorate, Bioeffects Division, Radio Frequency Bioeffects Branch. Submitted to 711 HPW/RHDR.

(b) (6) (2018, March). *Evoked Responses from 1470 nm Laser-induced Fast Thermal Gradients in the Brain in Rats* (AFRL-RH-FS-TR-2019-0002, 19 Mar 19). JBSA Fort Sam Houston, TX: Air Force Research Laboratory, 711th Human Performance Wing, Airman Systems Directorate, Bioeffects Division, Radio Frequency Bioeffects Branch. Submitted to 711 HPW/RHDR.

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

2.12.3 RF-Induced FTG Studies in Rats

The Air Force Research Laboratory is interested in developing non-lethal weapons capable of long-range manipulation and/or incapacitation of neuronal function in human targets. As mentioned in the previous section, recent studies reported that neuronal tissue can be modulated during exposure to pulses of IR light in a process often referred to as INS. Since literature shows that neurons can be stimulated by the absorption of pulses of IR energy, we hypothesized that neurons could also be stimulated by the absorption of pulses of RF energy. Pulses of RF may provide a novel, non-contact, non-invasive, long-range means of neural stimulation and could ultimately be used to manipulate or incapacitate neuronal function. Thus, GDIT investigators conducted a study to examine the effect of an ultrashort (millisecond) RF pulse on physiological and behavioral responses of a *Rattus norvegicus* animal model. First, we determined the maximum pulse length that could be delivered to the animal without untoward effects (i.e., no evidence of lethality or extreme burn). After we determined the maximum pulse width, we measured temperature changes in carcasses to confirm the generation of an FTG under our exposure conditions. The information gained from the first aim of this study was necessary to establish the conditions for RF exposure in an awake, freely moving animal. Second, we applied the established RF pulse parameters to a conscious rat study. The goal of this portion of the study was to expose rats to RF (or sham exposure) and measure overt behavioral changes in a free field system. Importantly, results showed a reduction in locomotion (i.e., the distance moved) by animals exposed to RF (2.45 GHz, 10 ms, 500 kW peak power) compared to equivalent sham animals. The results of this study were included in one technical report.

(b) (6) (2020, January). *Thermal Wave Induced Transcranial Hyper-excitation (TWITCH) in 2.45 GHz-Exposed Rat (Rattus norvegicus)* (AFRL-RH-FS-TR-2020-XXXX). JBSA Fort Sam Houston, TX: Air Force Research Laboratory, 711th Human Performance Wing, Airman Systems Directorate, Bioeffects Division, Radio Frequency Bioeffects Branch. Submitted to 711 HPW/RHDR.

2.13 JNLWD NSEP2

2.13.1 NSEP Effects on the Spine

The Joint Non-Lethal Weapons Directorate (JNLWD) is interested in exploiting the use of NSEP as a means of inducing prolonged incapacitation. To support this effort, GDIT investigators performed simulations of NSEP using the method of Finite-Differences in the Time-Domain (FDTD) on a human model. Such modeling is essential to help guide the research and development of NSEP devices for neuromuscular incapacitation. Results showed that electrode configurations locally positioned around lumbar vertebrae gave field intensities that were stronger and more focused than corresponding fields delivered by electrodes placed near thoracic or cervical vertebrae. This research was expanded into large animal studies described elsewhere in this report (section 2.15) and was summarized in one technical report.

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR (b) (6)

(b) (6) (2015, September). *Nanosecond Electrical Pulse Bioeffects: Simulation of the Electric Field at the Spinal Cord in a Human Model* (AFRL-RH-FS-TR-2015-0036). JBSA Fort Sam Houston, TX: Air Force Research Laboratory, 711th Human Performance Wing, Airman Systems Directorate, Bioeffects Division, Radio Frequency Bioeffects Branch. Submitted to 711 HPW/RHDR.

2.13.2 NSEP Effects on Limb Movements

GDIT investigators participated in previous studies, which described temporary incapacitation of limbs in rats exposed to NSEPs. Based on this previous work, we hypothesize that the observed loss of neurological conduction to distal limbs is due to temporary disruption of signal transduction within the spinal cord. Short–duration, high–voltage pulse application to the rodent would generate an EF large enough to exceed PM poration thresholds determined through extensive *in vitro* studies on multiple cell types. Therefore, GDIT investigators wrote an animal protocol which sought to validate the observed effect, determine its repeatability, elucidate a dose response based on duration of the exposure, and determine the dependence of the effect on anatomical location. Modeling efforts using FDTD methods were completed in advance of studies in animal models to determine the field profile for each probe placement. The proposed studies were detailed in one animal protocol.

(b) (6) (2016, September). *Effects of Voltage Pulses Applied to Body Surface on Limb Movements in the Rat (Rattus norvegicus).* IACUC Protocol Number RHDR-16-11. Submitted to 711 HPW/RHDR.

2.14 NSEP Behavior

2.14.1 NSEP Sensory Effects

Recent research has provided evidence that NSEPs stop signals originating in the brain from reaching skeletal muscle. The possibility exists that signals from the body, transmitted by nerves, could also be disrupted from reaching the brain and would enable this technology to be useful in the context of a pain-relieving biomedical device. GDIT researchers proposed an investigation to determine the physiological effect nanosecond-duration high-voltage electrical pulses have on the transmission of signals from a peripheral nerve through the CNS. The altered characteristics of the signal, such as: amplitude, velocity, and wave profile, were measured. Additionally, this study would also determine the effect of NSEP stimulation directed at a peripheral nerve as opposed to current studies which have targeted the spinal cord. The results of this study were included in one protocol.

(b) (6) (2017, May). *Inhibitory Bioeffects of Nanosecond Electrical Pulses on the Sensory Pathways and Peripheral Nerves of Rats (Rattus norvegicus)* (Protocol Number RHDR-17-08). Submitted to 711 HPW/RHDR.

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

2.14.2 NSEP Incapacitation

High voltage (HV) NSEPs applied to cells and small aquatic organisms can cause a sudden alteration in intracellular ion concentrations, such as free calcium, and result in immobilization of the cell or organism. In a neuron, an HV NSEP can temporarily render the cell unable to produce an AP and, when applied to a nerve *in vivo*, can temporarily render the nerve unable to produce or propagate APs. Therefore, HV NSEPs could potentially be used in higher, vertebrate organisms (e.g., humans) to "stun" the central and/or peripheral nerves necessary in producing coordinated skeletal muscle movement (e.g., in the legs), resulting in temporary, yet prolonged, neuromuscular incapacitation. In this study, we investigated in rats the use of HV NSEPs in causing inhibition of the efferent, cortico-motor, neurological pathway, resulting in prolonged neuromuscular incapacitation of the hind-limbs. The work conducted within this study builds on the previous work primarily conducted by Old Dominion University and the AFRL. The results of this study were included in one technical report.

(b) (6) (2017, August). *Nanosecond Electrical Pulse Bioeffects in the Rat (Rattus norvegicus)* (AFRL-RH-FS-TR-2018-0005, 30 Mar 18). JBSA Fort Sam Houston, TX: Air Force Research Laboratory, 711th Human Performance Wing, Airman Systems Directorate, Bioeffects Division, Radio Frequency Bioeffects Branch. Submitted to 711 HPW/RHDR.

2.14.3 NSEP Stimulation and Post-Exposure Recovery

NSEP stimulation is currently being explored as a novel technology for the reversible inhibition of APs. Previous small animal studies have reported hindlimb incapacitation for upwards of ten minutes after nsEP stimulation. However, the previous studies were performed on anesthetized animals and the response of a conscious rat has not been reported. The long-term effects of nsEP stimulation were also unknown. Thus, GDIT investigators conducted a study to deliver nsEP stimulation to conscious rats and to evaluate recovery from the nsEP exposure with behavioral testing. Animals were randomly placed into one of four experimental groups: Negative Control, Sham Exposure, 15 kV nsEP, or 20 kV nsEP group (n=8/group). Prior to exposures, animals were trained on behavioral testing equipment in order to acclimate them to the protocol. Behavioral tests were selected to provide a broad assessment of motor and peripheral sensory function. Specifically, the cylinder test was used to test for hindlimb gross motor function; grid walk and narrow beam tests were used for assessment of balance, proprioception, and gross motor function; and the Von Frey filament and hot plate tests were used to test for peripheral sensory dysfunction at the hindlimb. Animals received nsEP stimulation in a clear, cylindrical enclosure and the time for them to rear, a normal exploratory behavior, was recorded. Results show that animals receiving 15 kV nsEP stimulation had a 6-fold increase in the mean time to rear when compared to Control and Sham animals; additionally, rats receiving 20 kV nsEP stimulation had an 11-fold increase in the mean time to rear when compared to Control and Sham animals. Animals were tested on the aforementioned behavioral exams on days 1, 2, and 5 post-exposure. Results showed no significant differences between groups on any of the behavioral exams, nor were there any significant differences within one group from one day to another. These findings provide evidence that nsEP stimulation does not have deleterious effects with respect to the

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

behaviors and movements examined within this study. Although initial incapacitation was observed to be voltage dose-dependent, GDIT investigators did not observe any long-term behavioral and locomotor effects that differed between the voltages tested. The results of this study provide evidence of the effectiveness of nsEP stimulation for robust hindlimb incapacitation and complete recovery without sensory or motor dysfunction as soon as 24 hr post-exposure.

(b) (6) (2019, June). *Physiological and Behavioral Response in Rats (Rattus norvegicus) following Application of Short-Duration, High-Voltage Electrical Pulse* (AFRL-RH-FS-TR-2019-XXXX). Unpublished manuscript. Submitted to 711 HPW/RHDR.

2.15 Thermal Eye

GDIT investigators initiated a project to investigate the feasibility of a non-lethal weapon (NLW) that selectively heats the eye with pulse-modulated EM RF exposure. Pain may be elicited in the eye by increasing temperature or intraocular pressure (IOP). Accordingly, under pulse-modulated RF exposure, brief rapid thermal expansion inside the eye may lead to sharp increases in the IOP while allowing the face to cool during the off-period. This project was supported and recommended by modeling studies of the eye. Specifically, using FDTD methods, the head of the Visible Man Model was exposed to RF plane waves of frequencies 100 MHz to 10 GHz. The relative specific absorption rates (SAR) inside the eye and facial skin were calculated to maximize eye heating. A finite element model (FEM) of the eye was further developed to simulate rapid thermal expansion of the vitreous fluid. Eye temperature, volume, and pressure were recorded during a pulse-modulated heating regime. Results showed an optimal exposure frequency near 2 GHz was found to maximize the SAR inside of the eye relative to the face. The FEM analysis indicated that temperature increase in the vitreous fluid by 2 K over 400 ms led to an increase in IOP by 17 mmHg. The periodic mean temperature increased monotonically throughout all pulses, while the periodic mean IOP reached a maximum after seven cycles (4.6 min) and returned to baseline after 18 min. The results suggest that the viscoelastic responses in the eye may be invoked to elicit temporary pain.

To expand upon modeling studies, GDIT researchers performed exposures on RF exposed porcine eyes. The goal of these studies was to measure changes in intraocular pressure and temperature following exposure to 915 MHz and/or 2.4 GHz RF in enucleated, porcine eyes. In these studies, the change in volume of the eye was measured using digital speckle pattern interferometry (DSPI). Additionally, we captured FLIR® thermal imaging and calorimetry measurements of temperature change in the eye during RF exposure and related temperature fluctuations to changes in intraocular pressure to define the mechanism of RF eye bioeffects. The results of these studies were included in one protocol, one technical report, four manuscripts, and one abstract.

(b) (6) (2016, June). *Thermoregulatory and Specific Absorption Rate in the Microwave-Exposed Rat (Rattus norvegicus*). Protocol. Submitted to 711 HPW/RHDR.

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6) .

(b) (6) (2017, May). *Simulated Eye Thermodynamics and Pressure Under Pulse-modulated Electromagnetic Exposure* (AFRL-RH-FS-TR-2017-0019, 14 Aug 17). JBSA Fort Sam Houston, TX: Air Force Research Laboratory, 711th Human Performance Wing, Airman Systems Directorate, Bioeffects Division, Radio Frequency Bioeffects Branch. Submitted to 711 HPW/RHDR. Document pulled from DTIC and will be replaced with two journal articles.

(b) (6) (2019, December). *Simulated Thermobiomechanics of the Eye During Pulse-modulated Radiofrequency Electromagnetic Exposure* (AFRL-RH-FS-JA-2019-XXXX). Unpublished manuscript. Delivered to government for review; not cleared as of 14 Feb 20.

(b) (6) (2020, January). *Simulated Specific Absorption Rate in the Eye Under 0.1 to 10 GHz Radiofrequency Electromagnetic Exposure* (AFRL-RH-FS-JA-2019-XXXX). Unpublished manuscript. Delivered to government for review; not cleared as of 14 Feb 20.

(b) (6) . (2020, February). *A Fiber-optic Digital Speckle Pattern Interferometer (DSPI) for the Remote, Long-range, Non-perturbing, and Non-invasive Assessment of the Thermomechanics of Materials within Strong Radiofrequency Electromagnetic Fields* (AFRL-RH-FS-JA-2020-XXXX). Unpublished manuscript. Incomplete as of 14 Feb 20.

(b) (6) 2020, February). *Volumetric Thermal Expansion of Enucleated Porcine Eyes Exposed to High Peak Power Radiofrequency* (AFRL-RH-FS-JA-2020-XXXX). Unpublished manuscript. Delivered to government for review; not cleared as of 14 Feb 20.

(b) (6) (2020, January). *Volumetric Thermal Expansion of Enucleated, Porcine Eyes Exposed to Pulsed Radiofrequency* (AFRL-RH-FS-AB-2020-120729; P.A. Case No. TSRL-PA-2020-0118, 23 Jan 20). Abstract for BioEM 2020 scheduled to be held in Oxford, UK on 21-26 Jun 2020. Submitted to 711 HPW/RHDR.

2.16 EMI Spine

Conducted electrical weapons (CEWs) are designed to cause temporary electro-muscular incapacitation (EMI) without significant injury. The typical discharge of an EMI device is a 5 s cycle of 19 Hz electrical pulses resulting in 5 to 30 s of incapacitation. To improve delivery and prolong incapacitation, novel, benchtop EMI devices have been tested in animal models. Importantly, while EMI studies have shown that electrical stimuli applied for a duration of 30 s

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

are successful at achieving prolonged incapacitation in a porcine model, they also show an increased risk of adverse side effects such as spinal compression and fractions, and increases in blood hematocrit and biochemical factors, when exposed to certain waveforms (particularly, waveforms with a pulse repetition rate, or pulse-to-pulse frequency, of 40 Hz) compared to the widely-used, commercially available, TASER® CEWs (waveform with a pulse-to-pulse frequency of 19 Hz). Therefore, GDIT investigators sought to assess the cause of the adverse effects in order to improve the safety of deployed EMI devices. To achieve this goal, GDIT scientists assessed the risk and cause of spinal compression fracture due to exposure to commercial-like and novel EMI devices in a porcine animal model. Porcine subjects were exposed to 19 Hz and 40 Hz electrical stimuli for 30 s. X-ray imaging and necropsy were used to assess EMI-induced spinal fracture. Accelerometers were attached to the limbs to measure the electro-muscular response during stimulation. X-ray imaging and dissection confirmed that spinal fractures occurred in the lumbosacral region of the spine in at least 89% of all subjects, regardless of the stimulus group. Imaging and accelerometry suggest that the electrical stimulations caused musculoskeletal fatigue-related stress fractures. From the results of this study, GDIT investigators concluded that spinal fractures occurred at an unusually high rate in the porcine model, as spinal fractures caused by commercial EMI devices are not common in humans. The high rate of spinal fracture may be due to both the prolonged duration of electrical stimulation (30 s) and the significant musculoskeletal differences between humans and pigs; the disparity between human and porcine EMI bioeffects suggests that the porcine model is not a good model of EMI-induced spinal fracture in humans. In summary, the results of this study were significant because of their importance in the risk assessment and development of EMI devices as non-lethal weapons. The results of the EMI spine studies were included in one technical report and one manuscript.

(b) (6) . (2015, December). *Prolonged Electro-muscular Incapacitation (EMI) in a Porcine Model for Assessment of Spinal Injury* (AFRL-RH-FS-TR-2016-0034, 15 Dec 16). JBSA Fort Sam Houston, TX: Air Force Research Laboratory, 711th Human Performance Wing, Airman Systems Directorate, Bioeffects Division, Radio Frequency Bioeffects Branch. Submitted to 711 HPW/RHDR.

(b) (6) (2019, August). Prolonged Electro-muscular Incapacitation in a Porcine Model Causes Spinal Injury (AFRL-RH-FS-JA-2018-119194, P.A. Case No. TSRL-PA-2018-0252, 25 Jan 19). *Journal of Forensic Sciences*, Advance online publication, doi:10.1111/1556-4029.14177 (to be published in January 2020 issue). Submitted to 711 HPW/RHDR.

2.17 Biomarkers of DE Overexposure

Previous studies in our laboratory of the effects of microwaves (MWs) on the CNS demonstrated that exposure to certain frequencies can produce non-homogeneous deposition of energy and thermal profiles in animal tissues. Therefore, GDIT investigators participated in a study to investigate possible genomic and proteomic biomarkers generated in rats after overexposure to high power microwaves (HPM). Exposure was performed using an S-band transmitter tuned to 2.07 gigahertz (GHz). Analysis of the maximum localized SAR in rat

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

carcasses exposed to an HPM density of 2.2 W/cm^2 in the far field, with the head facing and centered on the open waveguide. A dosage study determined the maximum amount of HPM under these conditions that could be given to male Sprague-Dawley rats within the range of 36-90 J/cm^2 for up to 40 s exposure with rat survival (recovery from thermal insult without need for analgesics and the animals' exhibition of normal behavior post treatment) three days post MW exposure. Transcriptomic and proteomic analysis, comparing sham and environmental heating (EH) controls to the MW conditions to identify significant, differential expression at endpoints of 6 and 24 hr post-exposure, was performed. Ribonucleic acid (RNA) microarray analysis of brain tissue identified 7 MW differential expression genes and 6 EH differential expression genes for the two endpoints. MicroRNA microarray analysis of 6 hr post-exposure plasma identified 4 MW differential expression microRNAs and 20 EH differential expression microRNAs, while two-dimensional differential gel electrophoresis of the plasma identified 18 differential expression MW proteins and 13 differential expression EH proteins for these two endpoints. The results of this study were included in one technical report.

(b) (6) . (2015, July). *Molecular Bioeffects of 2.06 GHz Microwave Exposure in the Laboratory Rat (Rattus norvegicus*) (AFRL-RH-FS-TR-2015-0033, 22 Apr 16). JBSA Fort Sam Houston, TX: Air Force Research Laboratory, 711th Human Performance Wing, Human Effectiveness Directorate, Bioeffects Division, Radio Frequency Bioeffects Branch. Submitted to 711 HPW/RHDR.

2.18 Thermal-Geometrical Targeting of Multi-band DE

Simulated time reversal of EM radiation can achieve focused energy at chosen locations in space, which is potentially useful in designing RF-based NLWs. The preliminary objective of this work was to develop a novel software tool that implements time reversal of the three-dimensional (3D) FDTD. The greater aim was to study the impact of different source frequencies and transmitter configurations on the total energy and focused field during time reversal. Specifically, GDIT investigators implemented an FDTD framework such that a modulated pulse was emitted from a point source and recorded at transmitter locations placed along a bounding box within the FDTD grid. Next, the time-reversed signals were re-emitted by selected transmitters chosen from combinations of faces on the sensing box. Simulations were performed with a Gaussian envelope pulse source function modulated by frequencies between 100 MHz and 1 GHz and with different transmitter configurations. Methods were repeated in free space and in the presence of a spherical lossless medium. Conservation of energy was confirmed in free space by comparing the radiated energy to the energy during time reversal. Time-reversed total energy and focusing both increased with source frequency and were greatest in transmitters located on faces perpendicular to the source polarization. In conclusion, a new software tool has shown that total field energy and focusing are similarly impacted by source frequency and transmitter placement. Future work will aim to reproduce these results in lossy media. The results of this study were included in one manuscript.

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

(b) (6) (2017, December). *3D Point Source Electromagnetic-Field Estimation Using Time Reversal*. Unpublished manuscript. Submitted to 711 HPW/RHDR.

2.19 EMP Amended

Electromagnetic pulses (EMPs) are short duration bursts of high intensity RF that are frequently observed following nuclear explosions and/or similar large scale blasts. 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 in our previous studies of the bioeffects of 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. At the conclusion of the study, GDIT scientists assisted with the composition of a report outlining the work completed towards understanding how EMPs transmit into tissue and impact eukaryotic cellular membranes. We describe the background of EMP, High Peak Power Microwave (HPPM), and Ultrawideband (UWB) research in brief and the research behind electric-pulse induced nanoporation of the PM. Specific chapters cover modeling the entrance of such pulses into tissue, cellular response to single biphasic pulses and theoretical explanation of effect, and lastly cell death and the impact of membrane constituents on the cell response to mono and biphasic electric pulses. Overall, this study provided a basis for understanding how directly applied fields may translate into free-field bioeffects. The results of our studies were published in one technical report.

(b) (6) (2015, July). *Cellular Effects of Bipolar Electric Pulses* (AFRL-RH-FS-TR-2016-0027, 18 Nov 16). JBSA Fort Sam Houston, TX: Air Force Research Laboratory, 711th Human Performance Wing, Airman Systems Directorate, Bioeffects Division, Radio Frequency Bioeffects Branch. Submitted to 711 HPW/RHDR.

2.20 IVIS

GDIT investigators participated in a study to determine if RF exposure can induce neurological damage in a manner similar to that observed in traumatic brain injury (TBI). Further, our goal was to perform these studies in the least invasive way possible, so that the methods used to evaluate RF neurological damage could be deployed to study damage in a military or clinical setting. To accomplish this goal, GDIT scientists used transgenic mice that emit light following neural injury. Thus, potential damage following RF exposure could be monitored using the IVIS® Spectrum In Vivo Imaging System. The IVIS® System utilizes the leading optical technology available for imaging research to provide a non-invasive platform for monitoring biological damage, cell trafficking, and gene expression patterns in live animals. Additionally, aside from being non-invasive, the benefit of using the IVIS® system is that it allows us to create 3D maps of neural damage over a specific timeline post-RF exposure. To date, the GDIT research team has used IVIS® to measure the level of glial fibrillary acidic protein (GFAP) in the transgenic mouse

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

model FVB/B-Tg(Gfap-luc)53Xen. When RF-induced TBI-like damage occurs, GFAP is upregulated and, in conjunction with the addition of exogenous luciferin D, bioluminescence is emitted from the animal. This bioluminescence is captured by the IVIS® system and can be quantified relative to the RF exposure parameters. The results of this study will be useful in the design and execution of future studies aimed at *in vivo* imaging of RF bioeffects in live subjects. Background summary for this project was submitted in one technical report.

(b) (6) (2020, February). *Neuropathology of Traumatic Brain Injury – A Mini-Review of Recent Literature*. Unpublished manuscript. Submitted to 711 HPW/RHDR.

2.21 EM Cell

GDIT investigators participated in a study to determine if EM waves are transmitted and/or received within or between cells. Specifically, this study sought to test the hypothesis that MTs coupled with mitochondrial energy generation form an antenna-source construct that creates a means by which the cell can use EM waves to communicate with other cells or its environment. The long-term goal of this study was to leverage the knowledge obtained by cellular EM generation to develop communication strategies in EM environments. GDIT investigators were involved in the development of a MD model of MTs to determine if they function as antenna-like structures within cells that are able to pass RF frequencies intracellularly. Preliminary results show that, using coarse grained MD simulations, MTs will not oscillate for more than a few nanoseconds following exposure to EM. However, the GDIT modeling team computed the magnitude of EM fields that could be generated by cells and the effect of these fields on neighboring cells via non-contact communication. Based on these results, GDIT investigators provided the EM cell team with suggestions and recommendations for future directions for the project.

2.22 Ubiome

GDIT procured equipment to establish a fully-functional microbiology facility to study the effects of NSEP on bacterial cultures.

2.23 Program Support (Team GDIT New Work Proposals)

2.23.1 Spinal Cord DE Exposure Proposal

GDIT investigators composed a proposal to determine the effect of DE exposures on the spinal cord. The proposed research would bridge the gap between *in vitro* and *in vivo* studies by determining the mechanisms of incapacitation in the spinal cord. Specifically, the goal of the proposed studies was to identify if NSEP or IR exposure could affect neurological conductance in the excised spinal cord. The spinal cord proposal described three major goals. First, GDIT scientists wanted to investigate the possibility of NSEP or IR exposure to induce neuronal conductance block on excised rat spinal cord. This direct approach outlined if previously observed "whole body" effects were related to direct alteration of neurological functions or neuro-muscular and muscular mechanisms. Second, GDIT scientists would transition their study into a spinal cord

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

slice model to determine if interconnectivity of the spinal cord neurons is affected by DE exposure. This aim would be achieved by using a convenient slice patch clamp technique. Third, GDIT scientists would investigate the spatial and temporal intracellular calcium wave distribution along a spinal cord slice after DE exposures. This goal would be achieved by calcium imaging during stimulation in the Fluo-4 AM loaded slices. The entirety of the proposal was included in one white paper.

(b) (6) (2016, June). *White Paper on Directed Energy Dependent Modulation of Neurological Conductance.* Submitted to 711 HPW/RHDR.

2.23.2 Alternate Organisms Proposal

Understanding the relationship between RF and neural circuits is a major research focus at 711 HPW/RHDR. To advance this mission, GDIT investigators proposed a project to adapt a recently developed zebrafish model designed for use in labeling and tracing active neural circuits *in vivo*. Use of an aquatic model, such as the zebrafish, will limit the use of more complex organisms (including mouse and rat models) while allowing investigators to study neural processes at the organismal level. The model can be easily obtained and utilized to study the effects of RF exposure on neural electrical activity. The project was proposed in one white paper.

(b) (6) . (2015, August). *White Paper on Adaptation of a Novel Zebrafish System to Explore Nanosecond Pulsed Electric Field (nsPEF)-Induced Neural Stimulation and Inhibition in Aquatic Species*. Submitted to 711 HPW/RHDR.

2.23.3 Burn Visualization Pilot Study

Visual diagnosis of second-degree burns has proven inadequate for determining the appropriate treatment regimen. Although multiple noninvasive imaging techniques have shown promise for providing information about burn wound severity, the ideal technology to aid burn wound excision would provide real-time readouts. Thus, GDIT scientists collaborated with researchers at the San Antonio Military Medical Center to examine a high-resolution IR camera (thermography) and a Multiprobe Adapter System® (MPAS-6; traNSEPidermal evaporative water loss, colorimetry) to assess their usefulness in predicting burn severity. Contact burn wounds of increasing severity were created in a porcine model. Wounds were assessed for 4 days with an IR camera and MPAS-6. In addition, each day, the burn wounds were biopsied for histological analysis to determine burn depth for correlation with noninvasive measures. Surface temperatures decreased with increasing burn severity, which was associated with increasing traNSEPidermal evaporative water loss. Melanin content correlated with the depth of collagen coagulation and was bimodal, with superficial and full-thickness burns having higher values than deep partial thickness wounds. Erythema content was highest in superficial burns and negatively correlated with necrosis (high-mobility group box protein 1 expression). Importantly, surface temperature taken on every single day after injury was predictive of all histologically determined measurements of burn depth (i.e., collagen coagulation, apoptosis, necrosis, vascular occlusion). The results of this study

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

indicated that IR imaging and skin quality probes can be used to support the diagnosis of burn severity. Most importantly, IR measurements gave insight into both the zone of coagulation and the zone of stasis on every postburn day studied. The results of this study were included in one manuscript.

(b) (6) . (2017, January/February). Noninvasive Techniques for the Determination of Burn Severity in Real Time. *Journal of Burn Care & Research*, *38*(1), e180-e191. Submitted to 711 HPW/RHDR.

2.23.4 Hematocrit Studies in *Sus scrofa* Subjects Exposed to a CEW

In laboratory studies of the pig *Sus scrofa*, hematocrit has consistently increased after CEW exposures, possibly due to contraction of the spleen. GDIT investigators performed studies to compare changes in the blood of splenectomized animals and intact sham control animals. After exposures to electrical waveforms (that may be used in developing new CEWs in the future), hematocrit increased significantly in both splenectomized and sham animals. There were no significant main-effect differences between values from the two groups. There were, however, significant interactive effects of time and splenectomy for hematocrit, red blood cell count, and hemoglobin. After peak values were reached for these variables, values returned toward baseline levels more slowly in splenectomized animals. This may have been due to the lack of a spleen to sequester red blood cells (thereby resulting in more cells remaining in the general circulation), unlike sham animals with intact spleens. Differences in hematocrit responses between animals and human subjects exposed to CEWs were discussed in a manuscript describing the experiments and results. The results of the study were included in one manuscript and one figure amendment.

(b) (6) (2018, August). Figures (OT) (AFRL-RH-FS-OT-2018-118820; P.A. Case No. TSRL-2018-0199, 30 Aug 18) for submission to the *Journal of Forensic Sciences* associated with the submitted paper titled, "Increased Hematocrit Due to Conducted-Electrical-Weapon Waveform Exposures in Splenectomized *Sus scrofa*" (AFRL-RH-FS-JA-2018-118456; P.A. Case No. TSRL-PA-2018-0157, 1 Jun 18) in response to reviewer comments. Submitted to 711 HPW/RHDR.

(b) (6) (2019, July). Increased Hematocrit Due to Electrical-Waveform Exposures in Splenectomized *Sus scrofa*. *Journal of Forensic Sciences*, *64*(4), 1196-1202. Submitted to 711 HPW/RHDR.

2.23.5 Maser Proposal

GDIT investigators submitted a proposal for research aimed to understand the biological effects of exposure to coherent MW photons emitted by masers (analogous to lasers). Masers emit MWs of a much narrower bandwidth and higher coherence than that of the transmitter systems currently in use at the AFRL. Dosimetry with existing transmitter systems is difficult due to the large, bulky machinery needed and the variations in power inherent in tube systems. Recent advancements in maser technology have led to the development of a small, table-top system that

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

can emit MWs of an extremely narrow bandwidth (50 Hz). Masers may improve our ability to perform dosimetry and improve the reliability and repeatability of our experiments. In addition, due to the currently limited and uncommon use of masers compared to lasers and to other types of MW sources, the bioeffects of masers remain largely unknown. By executing this proposal, we will decrease the gap in the knowledgebase on the bioeffects of masers and leverage the knowledge gained to improve and develop safety standards and DE weapons for the Air Force. This work is summarized in one white paper.

(b) (6) (2018, May). *Bioeffects of Coherent Radio Frequency (RF)*. White Paper. Submitted to 711 HPW/RHDR.

2.23.6 Electronic Speckle Pattern Inferferometer (ESPI) Design and Patent

GDIT investigators invented a fiber-optic-based ESPI, including the device design and methods of use. The ESPI is designed to measure the in-plane and out-of-plane displacement of a surface of a test material in three dimensions. The ESPI uses a laser beam of coherent radiation that is coupled into a single-mode fiber; the coherent radiation is divided within a single-mode fiber-optic beam splitter into two fiber-optically contained coherent beams of radiation known as the "test arm" and "reference arm." The beam of the test arm is terminated with a collimator and the beam outputted by the collimator is aimed at the surface of a test material; the radiation reflected from the test material is collected into a long-distance focus, large beam collimator (aimed normal to the surface of the test material) and is coupled into a multi-mode fiber that is terminated with another collimator aimed at a beam splitter/combiner cube. The beam splitter/combiner cube recombines the coherent radiation from the test arm and the coherent radiation from the reference arm (which is a single-mode fiber terminated with a collimator) and the recombined radiation is directed onto the sensor of a camera imaging system. The interference pattern fringes of the recombined coherent radiation beams and the number of fringes that move across the sensor of a camera correlates to, and can be used to calculate, the displacement of the test material. The incorporation of fiber optic cables to channel the coherent radiation of the test beam to and from a test material makes the ESPI capable of remotely sensing and measuring (with high spatial and temporal resolution) multi-dimensional displacement or movement of test materials within hazardous environments (such as high-power RF fields) from long-distance (i.e., with the device placed at a far distance from the test material) and with the operator at a remote location (i.e., outside the hazardous environment). The ESPI can produce measurements rapidly and in real-time and is non-contact, nondestructive, and non-perturbing to the test material. The ESPI design was described in one patent application.

(b) (6) (March 14, 2019). *Electronic Speckle Pattern Interferometer (ESPI) for Long-Range Measurement of Displacement of Materials within Hazardous Environments*. Submitted AF Form 1279 'Disclosure and Record of Invention' for Patent Application. Patent reviewed and given status "GO" for AF to pursue an application for the patent; awaiting assignment of a patent attorney 30 Aug 19; patent attorney assigned and (b) (6) submitted "Invention Election" form and background literature to attorney. Submitted to 711 HPW/RHDR.

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6)

2.23.7 Polytrauma Prairie Vole Injury Model

Psychological factors are known to influence long-term health; however, there is a shortage of evidence evaluating its impact upon biological and behavioral outcomes following injury. GDIT investigators participated in an experiment studying the effects of injury on prairie voles as a model of human cardiac function and regulation, referred to as the polytrauma prairie vole injury model. For this study, subjects (N=36) were housed in male/female pairs for 5 days, then half of the pairs were isolated (for 7 days) resulting in 4 injury study groups: (1) Paired-Female; (2) Paired-Male; (3) Isolated-Female; (4) Isolated-Male and (5) Anesthesia-Shams. On injury day, electrocardiogram was monitored and all animals received a TBI impact then ~30% hemorrhage. Animals were held for 1 hr, then resuscitated with 2x Lactated Ringers bolus (with 1 hr wait), allowed to regain consciousness (20 min stabilization period); the procedure ended with an open field behavior test and tissue collection. Results showed that isolated subjects displayed significantly higher mortality (p=0.050); specifically, isolated-Males were more likely to die than Paired-Males (p=0.080). Electrocardiogram data indicated significantly elevated heart rate for injury animals over Anesthesia-Shams and impaired cardiac regulation (i.e., decreased heart rate) during resuscitation for Isolated-Males (p=0.003). Further, isolated-Males were unable to complete the open field assessment and Isolated-Females tended to display elevated anxiety-like behavior (p=0.129). The results of this study suggest that the polytrauma prairie vole injury model can be used to further investigate the psychological factors that impact traumatic injury recovery. This model provides insight into the protective influence of social bonds on recovery from a physical injury, potentially informing future interventions and treatment strategies. The results of this study were included in one abstract.

(b) (6) [redacted]

(2019, October). *Social Stress Potentiates Negative Outcomes for Male Prairie Voles (Microtus ochrogaster) in Polytraumatic Injury*. Abstract for Neuroscience 2019 held in Chicago, IL on 19-23 October 19. Submitted to 711 HPW/RHDR.

Distribution B: Distribution authorized to U.S. Government agencies only (proprietary information, February 2020). Other requests for this document shall be referred to 711 HPW/RHDR, (b) (6) [redacted]