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RPPR Final Report
as of 28-Apr-2022

Agency Code: 21XD

Proposal Number: 77431ELRIP **Agreement Number: W911NF-21-1-0064**
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DUNS Number: 005421136 EIN: 362177139
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Begin Performance Period: 01-Jan-2021 **End Performance Period:** 31-Dec-2021
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Submitted By: Bozhi Tian Email: btian@uchicago.edu
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STEM Degrees: 1 **STEM Participants:** 1

Major Goals: In funded research, we have requested an ONI imager from Oxford Nanoimaging Inc. in order to assess neuron-glial interaction and extracellular vesicles (EV) dynamics. As one of the most sensitive and multifunctional microscopes, the ONI provides the capability of high-resolution imaging, cell tracking, and single-molecule imaging of biomolecular dynamics.

It has been demonstrated that glia-neuron interactions are important, but localized glia-mediated neuromodulation through physical processes (such as electrical stimulation, optical modulation, or thermal control) remains rare. In this study, we directly targeted glia with internalized nanostructures, creating subcellular glia/silicon interfaces - a hybrid cellular building block. Next, we explored several intracellular mechanisms for glial excitation using silicon. The outputs of the glia, which are endogenous chemical and bioelectric signals, were subsequently used to modulate neural activity through the natural interaction between the glia and the neurons. Currently, we are utilizing the acquired ONI system to observe the dynamics of organelles and extracellular vesicles (see below) during the triggered glia/neuron interactions. Unlike traditional neuromodulation methods, this approach differs fundamentally from them in two distinct ways. First, it is minimally invasive to neurons, since the silicon directly targets glia. Second, it explores the endogenous biological signals that traverse neurons via glia at various levels. At the same time, we are employing the ONI to study the dynamics of mitochondria in a range of mammalian cells, including glia and several cancer cell lines.

Additionally, the ONI imager is being used for nanoparticle tracking analysis (NTA), a procedure that calculates the diffusion coefficient and estimations of EV sizes to resolve the dynamics within live cells. Four different fluorescent markers can be used simultaneously to measure four different colors. By staining with fluorescence, organelles and EVs of interest can be specifically detected. Multicolor imaging can also characterize subpopulations and determine their size distribution. Using ONI super-resolution imaging in cultured cellular networks, we can not only determine organelle and EV structures, but also quantify organelle and EV dynamics under controlled electrochemical or photoelectrochemical stimulation.

Accomplishments: Our team has used the ONI nanoimager to study subcellular bioelectrical dynamics, ranging from studies of mitochondrial dynamics with machine vision to EV production for cardiac regeneration. Detailed information is provided below:

(1) A machine vision-assisted mitochondrial control for cardiac regeneration

The reproducibility of cell and tissue culture results is a challenge for many bioengineering subdisciplines. The

classical approach to achieving the desired cell phenotype involves delivering one set of conditions to the system. The researchers must then evaluate the results of the stimulation after a specific time period. For each batch of biological structures, this approach must be highly parallelized, consumes unnecessary materials, and is very slow. In this study, we created a machine-vision-assisted bioelectronic stimulation platform to solve this problem and enable new approaches to cell and tissue engineering. The system detects and understands the visual cues from phase-contrast microscopy and adjusts the stimulation conditions appropriately so that they are always just slightly challenging for the current state of the experiment. In this manner, the culture is always presented with the right set of conditions, making it possible to get the same result for batches of samples that require a different frequency or amplitude of stimulation.

Our previous studies found that porous materials had better coupling with biological structures and enabled more efficient and biocompatible interfaces. In order to learn more about the interactions between materials and cells, we have created gold electrode arrays with varying porosities. With such electrodes, we sequentially stimulate rat cardiomyocytes without experimenter oversight using a software that continuously monitors the cell culture, analyses the contraction frequency, the number of cells, and their morphology, then adjusts stimulation conditions in real-time to force the cells to contract at a faster rate in real-time. Whenever possible, the autonomous Agent targets a specified endpoint and tries to make the best decision it can. We aim to understand the differences in the subcellular response of cardiomyocytes during stimulation. Studying mitochondrial networks and investigating cell energy production will be carried out using dSTORM superresolution microscopy.

Combined with machine intelligence, automation has the potential to enable new types of experiments, enriching our understanding of materials, biological structures, and how they interact. This study will develop new tools for bioengineering and regeneration medicine. As of now, we have developed methods for electrode fabrication, computer software for stimulation, and dSTORM imaging. Next, we will use the platform to perform high-throughput in vitro stimulation experiments.

(2) EV production and myocardium regeneration therapy

Using model cell lines (e.g., HeLa, U2OS), we have shown that bioelectric stimulation increases exosome release. The cells were transfected with the pHluorin-CD63 reporter, an EV marker with a fluorescent tag, and cultured on gold electrodes. The transfection was performed using Fugene HD reagents and the pCMV-Sport6-CD63-pHluorin plasmid (Addgene # 130901). A bioelectrical potential was delivered at the biointerfaces using interdigitated gold electrodes. We adjusted the voltage and frequency of the alternating fields and optimized the electrical stimulation to activate the multivesicular body-plasma membrane (MVB–PM) fusion machinery and enhance exosome release without killing the cells.

To visualize EV production in cardiomyocytes (CMs), Lipofectamine MessengerMAXTM (Invitrogen) was used to transfect CMs with pHluorin-CD63. Lipofectamine MessengerMAXTM enables high-efficiency transfection and overexpression of targeted DNA in non-dividing primary cells, avoiding costly adenovirus and electroporation methods. Using the Nanoimager (ONI, Oxford, lens: Olympus, 1.4NA, 100X) or Nikon Ti2-E microscope, we examined the bioelectricity-induced exosome generation in CMs. A pH-sensitive reporter, pHluorin-CD63, facilitates the analysis of exosome lifecycles and dynamics. In response to bioelectronic stimulation, CM contraction rates synchronized to the frequency of the electrical signal. Calcium signals were observed during electrical stimulation and pHluorin-labelled exosome release events.

Our mission is to extend the application of EVs to myocardium regeneration therapy by engineering EVs with specific cell-type specificity and loading cardioprotective miRNAs into the generated EVs. We will conjugate EVs with the CM-specific peptide WLSEAGPVVVTVRALRGTGSW, which will enhance cardiac tropism. To minimize nonspecific uptake, the cells will be transfected with Lamp2b, an exosomal membrane protein, fused to CMP. In addition, CSTSMLKAC, a peptide known to target the ischemic myocardium, can be attached to EVs for in vivo cargo release in injured CMs. The role of miRNAs in cardiac development is increasingly evident. MiR-199a-3p, for instance, stimulates proliferation of mouse/rat CMs. In the Tian lab, we have successfully loaded cardioprotective miRNAs (e.g., miR-199a, miR-210) into EVs in 1 h using the Exo-Fect™ miRNA transfection kit.

Finally, we are currently evaluating the therapeutic effects of bioelectronic generated EVs in an myocardial infarction (MI) model. For instance, miR-210-loaded, CM-targeting EVs will be injected intramyocardially into a MI model generated in C57BL6 mice. The mice will be thoracoscopically segmented in the fourth intercostal space under general anesthesia in order to see the heart. To generate the MI model, the left anterior descending (LAD) coronary artery will be occluded for 45 minutes. EVs (1 mg/mL) in 30-40 mL PBS will be injected 3-4 times in 3-4

locations within 5 minutes of reperfusion in the peri-infarct area. By occluding the expiratory line, the lungs will be hyperinflated for a few respiratory cycles in order to prevent atelectasis and pneumothorax. We will evaluate mouse cardiac function using high-resolution echocardiography in order to determine regional ejection fraction (EF), fraction shortening (FS), and cardiac output (CO).

Training Opportunities: Although this is only an instrument grant, several members of the Tian lab have used the ONI nanoimager. Through a structured mentoring program, the researchers' development has been enhanced. The purpose of the mentoring program is to provide the researchers with the skills, knowledge, and experience they will need to excel in their careers. Some specific elements of this plan include:
University of Chicago Chemistry Department and Materials Research Science and Engineering Center (MRSEC) offer seminars and workshops on how to write competitive proposals.

The University of Chicago's Center for Teaching and Learning offers seminars and workshops on teaching and learning (http://teaching.uchicago.edu/).

PIs' annual research meetings provide training and mentoring in responsible research conduct. These meetings follow the online course at citiprogram.org. The course covers topics such as research misconduct, data management and sharing, publication practices and responsible authorship, peer review, mentor and trainee responsibilities, conflict of interest, and collaborative research. The exercises in the online course provide a starting point for discussing how to conduct proper research for the projects in this proposal.

Participation in workshops related to career development, such as how to apply for a faculty position, career paths outside of academia, tips for negotiating salary and start-up funds, and how to plan an independent research agenda. The workshops have been offered by the Physical Sciences Division at the University of Chicago.

Participation in the PI's weekly research group meetings during which the researchers presented their research regularly; feedback and coaching were provided to help them improve their communication and presentation skills.

Results Dissemination: We have disseminated our results in several ways to communities of interest.

During the past year of support, the PI has made four virtual visits to Universities, conferences, and symposiums, discussing some of the results of the ONI nanoimager.

Additionally, we are collecting additional data for at least four publications.

Honors and Awards: •Bozhi Tian was promoted to the full professor at the University of Chicago.

• Bernadette Miao, an undergraduate student who worked on the mitochondrial dynamics studies with ONR Nanoimager, has been selected for (1) the Goldwater Scholarship, (2) the Illinois Chemical Education Foundation Undergraduate Scholarship, (2) the Stamps scholarship, and (4) the Student Marshal in one year!

• Ellie Ostroff, an undergraduate student who worked on the EV production with bioelectronics, has received funding support from the 2021-2022 Quad Faculty Research Grant.

Protocol Activity Status:

Technology Transfer: Nothing to Report

PARTICIPANTS:

Participant Type: Undergraduate Student
Participant: Bernadette Miao
Person Months Worked: 2.00 **Funding Support:**
Project Contribution:
National Academy Member: N

RPPR Final Report
as of 28-Apr-2022

Participant Type: Undergraduate Student
Participant: Ellie Ostroff
Person Months Worked: 1.00 **Funding Support:**
Project Contribution:
National Academy Member: N

Participant Type: Graduate Student (research assistant)
Participant: Lingyuan Meng
Person Months Worked: 1.00 **Funding Support:**
Project Contribution:
National Academy Member: N

Participant Type: Graduate Student (research assistant)
Participant: Aleksander Prominski
Person Months Worked: 1.00 **Funding Support:**
Project Contribution:
National Academy Member: N

Partners

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I certify that the information in the report is complete and accurate:
Signature: Bozhi Tian
Signature Date: 4/14/22 11:45AM

DURIP: Super-resolution imaging of subcellular structures and dynamics during non-genetic biological modulation

Contract Number: W911NF2110064

Final progress report

Submitted to

ARO, USA

Attention: Dr. Albena Ivanisevic

Research Area: the Electronics Division

By

PI: Bozhi Tian

Department of Chemistry, the James Franck Institute, and the Institute for Biophysical Dynamics

The University of Chicago,

929 E 57th Street, IL 60637

A. What were the major goals and objectives of the project?

In funded research, we have requested an ONI imager from Oxford Nanoimaging Inc. in order to assess neuron-glial interaction and extracellular vesicles (EV) dynamics. As one of the most sensitive and multifunctional microscopes, the ONI provides the capability of high-resolution imaging, cell tracking, and single-molecule imaging of biomolecular dynamics.

It has been demonstrated that glia-neuron interactions are important, but localized glia-mediated neuromodulation through physical processes (such as electrical stimulation, optical modulation, or thermal control) remains rare. In this study, we directly targeted glia with internalized nanostructures, creating subcellular glia/silicon interfaces - a hybrid cellular building block. Next, we explored several intracellular mechanisms for glial excitation using silicon. The outputs of the glia, which are endogenous chemical and bioelectric signals, were subsequently used to modulate neural activity through the natural interaction between the glia and the neurons. Currently, we are utilizing the acquired ONI system to observe the dynamics of organelles and extracellular vesicles (see below) during the triggered glia/neuron interactions. Unlike traditional neuromodulation methods, this approach differs fundamentally from them in two distinct ways. First, it is minimally invasive to neurons, since the silicon directly targets glia. Second, it explores the endogenous biological signals that traverse neurons via glia at various levels. At the same time, we are employing the ONI to study the dynamics of mitochondria in a range of mammalian cells, including glia and several cancer cell lines.

Additionally, the ONI imager is being used for nanoparticle tracking analysis (NTA), a procedure that calculates the diffusion coefficient and estimations of EV sizes to resolve the dynamics within live cells. Four different fluorescent markers can be used simultaneously to measure four different colors. By staining with fluorescence, organelles and EVs of interest can be specifically detected. Multicolor imaging can also characterize subpopulations and determine their size distribution. Using ONI super-resolution imaging in cultured cellular networks, we can not only determine organelle and EV structures, but also quantify organelle and EV dynamics under controlled electrochemical or photoelectrochemical stimulation.

B. A description of what was accomplished under the goals during the total award period.

Our team has used the ONI nanoimager to study subcellular bioelectrical dynamics, ranging from studies of mitochondrial dynamics with machine vision to EV production for cardiac regeneration. Detailed information is provided below:

(1) A machine vision-assisted mitochondrial control for cardiac regeneration

The reproducibility of cell and tissue culture results is a challenge for many bioengineering subdisciplines. The classical approach to achieving the desired cell phenotype involves delivering one set of conditions to the system. The researchers must then evaluate the results of the stimulation after a specific time period. For each batch of biological structures, this approach must be highly parallelized, consumes unnecessary materials, and is very slow. In this study, we created a machine-vision-assisted bioelectronic stimulation platform to solve this problem and enable new approaches to cell and tissue engineering. (**Fig. 1A**) The system detects and understands the visual cues from phase-contrast microscopy and adjusts the stimulation conditions appropriately so that they are always just slightly challenging for the current state of the experiment. In this manner, the culture is always presented with the right set of conditions, making it possible to get the same result for batches of samples that require a different frequency or amplitude of stimulation.

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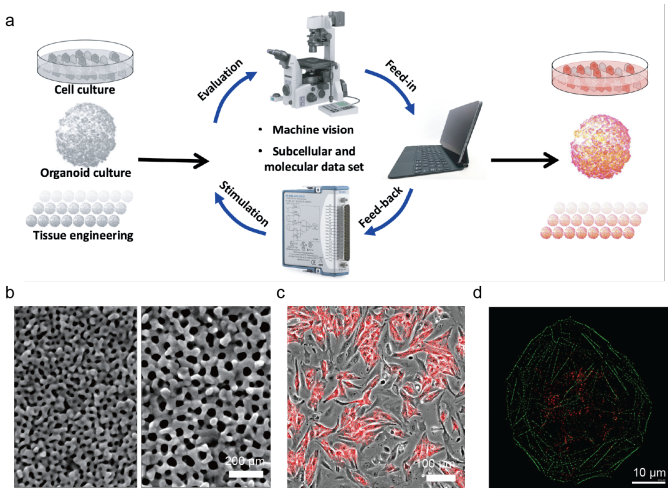

Figure 1. A machine vision-assisted mitochondrial control for cardiac regeneration. (**A**) Overview of active AI-controlled stimulation experiment. (**B**) Gold electrodes with different porosity. (**C**) AI-assisted analysis of cell culture. (**D**) dSTORM superresolution image of actin fibers (green) and mitochondria (red).

stimulation, and dSTORM imaging. Next, we will use the platform to perform high-throughput in vitro stimulation experiments.

(2) EV production and myocardium regeneration therapy

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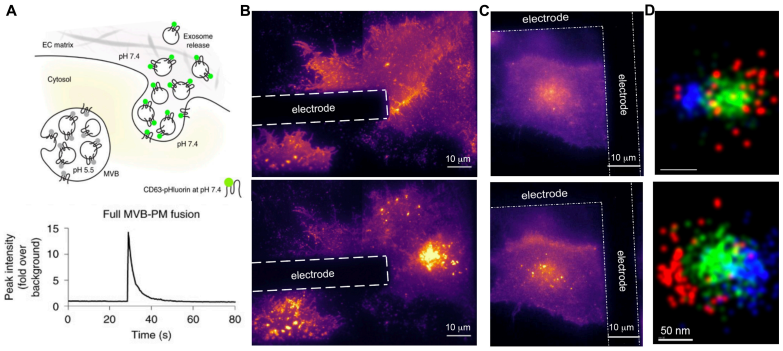

Figure 2. Real-time observation of bioelectronically induced exosome release. (**A**) pHluorin-CD63 reporter indicates the release of exosomes with a fluorescent flash. (**B**) Representative images of live HeLa cells before (top) and during (bottom) bioelectrical stimulation (interdigitated *parallel* electrodes, ± 1V, 2Hz). Scale bar, 10 µm. (**C**) Representative images of live HeLa cells before (top) and during (bottom) bioelectrical stimulation (interdigitated *branched* electrodes, ± 1V, 2Hz). Scale bar, 10 µm. (**D**) dSTORM super-resolution images of individual exosomes isolated from bioelectrically stimulated HeLa cells.

210) into EVs in 1 h using the Exo-Fect™ miRNA transfection kit.

Finally, we are currently evaluating the therapeutic effects of bioelectronic generated EVs in an myocardial infarction (MI) model. For instance, miR-210-loaded, CM-targeting EVs will be injected intramyocardially into a MI model generated in C57BL6 mice. The mice will be thoracoscopically segmented in the fourth intercostal space under general anesthesia in order to see the heart. To generate the MI model, the left anterior descending (LAD) coronary artery will be occluded for 45 minutes. EVs (1 mg/mL) in 30-40 mL PBS will be injected 3-4 times in 3-4 locations within 5 minutes of reperfusion in the peri-infarct area. By occluding the expiratory line, the lungs will be hyperinflated for a few respiratory cycles in order to prevent atelectasis and pneumothorax. We will evaluate mouse cardiac function using high-resolution echocardiography in order to determine regional ejection fraction (EF), fraction shortening (FS), and cardiac output (CO).

C. What opportunities for training and professional development did the project provide?

Although this is only an instrument grant, several members of the Tian lab have used the ONI nanoimager. Through a structured mentoring program, the researchers' development has been enhanced. The purpose of the mentoring program is to provide the researchers with the skills, knowledge, and experience they will need to excel in their careers. Some specific elements of this plan include:

- University of Chicago Chemistry Department and Materials Research Science and Engineering Center (MRSEC) offer seminars and workshops on how to write competitive proposals.
- The University of Chicago's Center for Teaching and Learning offers seminars and workshops on teaching and learning (http://teaching.uchicago.edu/).
- PIs' annual research meetings provide training and mentoring in responsible research conduct. These meetings follow the online course at citiprogram.org. The course covers topics such as research misconduct, data management and sharing, publication practices and responsible authorship, peer review, mentor and trainee responsibilities, conflict of interest, and collaborative research. The exercises in the online course provide a starting point for discussing how to conduct proper research for the projects in this proposal.
- Participation in workshops related to career development, such as how to apply for a faculty position, career paths outside of academia, tips for negotiating salary and start-up funds, and how to plan an independent research agenda. The workshops have been offered by the Physical Sciences Division at the University of Chicago.
- Participation in the PI's weekly research group meetings during which the researchers presented their research regularly; feedback and coaching were provided to help them improve their communication and presentation skills.

D. Results Dissemination

We have disseminated our results in several ways to communities of interest.

During the past year of support, the PI has made four virtual visits to Universities, conferences, and symposiums, discussing some of the results of the ONI nanoimager.

Additionally, we are collecting additional data for at least four publications.

E. Honors or awards received under this project in this reporting period?

- Bozhi Tian was promoted to the full professor at the University of Chicago.
- Bernadette Miao, an undergraduate student who worked on the mitochondrial dynamics studies with ONR Nanoimager, has been selected for (1) the Goldwater Scholarship, (2) the Illinois Chemical Education Foundation Undergraduate Scholarship, (2) the Stamps scholarship, and (4) the Student Marshal in one year!
- Ellie Ostroff, an undergraduate student who worked on the EV production with bioelectronics, has received funding support from the 2021-2022 Quad Faculty Research Grant.