AWARD NUMBER: **W81XWH-20-1-0113**

TITLE: A New In Situ Cryo-Electron Microscopy Approach to Directly Visualize Mutations in Mitochondrial Disease

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CONTRACTING ORGANIZATION: University of Pittsburgh

REPORT DATE: JUNE 2023

TYPE OF REPORT: Final Technical Report

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

V1-20210107

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
JUNE 2023	Final Report	01 March 2020 – 28 Feb 2023

4. TITLE AND SUBTITLE

A New In Situ Cryo-Electron Microscopy Approach to Directly Visualize Mutations in Mitochondrial Disease

5a. CONTRACT NUMBER
W81XWH-20-1-0113

5b. GRANT NUMBER
PR192466

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

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8. PERFORMING ORGANIZATION REPORT NUMBER

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

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

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

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Mitochondrial dysfunction is associated with several chronic, deployment-associated conditions in Veterans. *In situ* cryo-electron tomography (cryo-ET) imaging is a new 3D imaging approach that we have used to study patient cells with unprecedented detail. Our goal is to test the feasibility of cryo-ET to visualize mitochondrial structural changes in patient cells. We hypothesize *in situ* cryo-ET will resolve structural changes in the individual mitochondrial respiratory complexes and organization of these complexes into higher-order supercomplexes. We have now obtained primary fibroblast cells from patients with distinct mitochondrial complex I mutations including ND6, ACAD9, and NDUFV1 subunits and optimized growing these mutant cells on EM grids. We characterized effects of these mutations on mitochondrial morphology and dynamics in living patient cells. *In situ* cryo-ET revealed disruptions of mitochondrial inner membranes and crista morphology distinct to each mutant. Our results suggest these respiratory complex subunits are key regulators of overall mitochondrial structure and function and their disruption directly alters their function to produce profound human disease.

15. SUBJECT TERMS
Mitochondrial disease, Cryo-electron tomography, Respiratory complex, Supercomplex, Pesticide, Gulf War Illness

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
U	U	U	UU	16	19b. TELEPHONE NUMBER *(include area code)*

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1. INTRODUCTION

Mitochondrial diseases are often caused by mutations that disrupt structures of the mitochondrial respiratory complexes, significantly impairing mitochondrial function. Substantial numbers of people are also affected by mitochondrial dysfunction associated with prevalent diseases including diabetes, Alzheimer's disease, and Parkinson's disease. The combined impact of these mitochondrial impairments is enormous. Mitochondrial dysfunction is suspected in a variety of chronic, deployment-associated conditions in Veterans or in association with Gulf War Illness (GWI). For example, exposure to neurotoxicants including pesticides (*e.g.*, Agent Orange, permethrin, paraquat) and airborne hazards in military theater have been negatively associated with Veterans' health post-deployment. Yet, the precise mechanisms remain poorly understood. Our ability to accurately diagnose disturbances in mitochondrial function remains profoundly limited. Current diagnostic approaches test mitochondrial function (*i.e.*, respiration) and cannot resolve specific disease-causing mitochondrial structural defects despite evidence of broader mitochondrial dysfunction. Such approaches are often invasive and may alter the appearance of mitochondria, hindering accurate diagnosis. Thus, there is great clinical need for more accurate diagnostic approaches since better diagnosis will translate to improved patient outcomes. *In situ* cryo-electron tomography (cryo-ET) imaging is a new three-dimensional (3D) imaging approach to visualize disease-induced changes in mitochondrial structure directly in primary human patient cells for the first time. The unprecedented resolution provided by cryo-ET can visualize alterations in respiratory complex structures unique to the disease-causing mutations and/or acquired mitochondrial defects. We previously used *in situ* cryo-ET to identify profound structural changes in mitochondria within primary fibroblasts from a patient with Leigh Syndrome (LS), a debilitating mitochondrial disease. The structural alterations, produced by a novel mutation, have not been previously identified via conventional imaging, illustrating the power of this approach. We therefore hypothesize that: (**A**) *In situ* cryo-ET will visualize distinct structural changes both in the individual mitochondrial respiratory complexes and organization of the complexes into larger, higher-order assemblies termed supercomplexes. These visualized altered structures will explain the loss of functional efficiency and metabolic flux in complexes as evidenced in mitochondrial disease mutations, GWI, or exposures to pesticides and PB. (**B**) Drugs that improve respiratory chain function can correct structural abnormalities of supercomplex organization, providing the basis for novel therapies. To test these hypotheses, we aim to do the following: (**1**) To determine the effects of disease-causing mutations on individual mitochondrial respiratory complexes and higher-order supercomplex organization *in situ*; and (**2**) To identify effects of GWI and associated neurotoxicants on respiratory chain complex structure and higher-order organization *in situ* in patient cells. We have established new experimental systems where we have grown primary cells taken from patients directly on EM grids followed by imaging of the mitochondria within these cells via *in situ* cryo-ET to resolve disease-induced changes to mitochondrial structure and morphology. We have also developed novel drugs like JP4-039 that improve mitochondrial function in affected patient cells potentially through their actions on mitochondrial respiratory complex structure. In the short term, we expect that our proposed *in situ* cryo-EM studies will reveal mitochondrial structural alterations that disrupt mitochondrial respiratory complex structures in response to GWI or neurotoxicant exposures affecting Veterans. This may form the basis for new, highly accurate and non-invasive diagnostic approaches for mitochondrial disease. In the longer term, our studies will serve as a foundation for future work that leads to new, highly targeted personalized therapies for active military, Veterans and the general population that are directed at correcting structural changes responsible for mitochondrial dysfunction.

2. KEYWORDS

Keywords relevant to the work proposed here include:

1. Mitochondrial disease
2. Cryo-electron tomography
3. Respiratory complex
4. Supercomplex
5. Pesticide
6. Gulf War Illness

3. ACCOMPLISHMENTS

- **What were the major goals of the project?**
  The major goals of the project are as follows:

1) Sample preparation and imaging acquisition of three-dimensional tomograms from mitochondrial disease patient and control cells.
2) Analysis of imaged mitochondria and respiratory complexes from well-defined mitochondrial disease mutation-containing patient and control fibroblasts.
3) Determine whether the respective mitochondrial disease-causing mutations alter the 3D structures of the mitochondrial respiratory complexes *in situ.*
4) Determine whether improved respiratory chain function in patient cells following treatment with JP4-039 is via the drug's ability to correct respiratory complex structural abnormalities.
5) Visualize mitochondrial respiratory complex structure and supercomplex organization in healthy primary human fibroblasts after pesticide or pyridostigmine bromide (PB) treatment.
6) Determine whether JP4-039 corrects mitochondrial structural abnormalities in GWI or pesticide/PB-treated control cells.

- **What was accomplished under these goals?**

I. Sample preparation and imaging acquisition of three-dimensional tomograms from mitochondrial disease patient and control cells.

- We continued developing new approaches for sample preparation and imaging acquisition of three-dimensional tomograms. To date, most cryo-ET studies focus on vitreously frozen individual cells separated from their native tissue contexts. This reliance on imaging of single cells is primarily due to technical challenges associated with preparing fresh tissue sections at a thinness sufficient for visualization via cryo-ET. Moreover, such challenges are especially the case with highly heterogenous and specialized tissues, such as brain. Indeed, brain tissue is especially affected by this limitation as the subcellular and synaptic milieu can significantly vary across neuroanatomical locations. To address this, we established new instrumentation and a workflow that consists of: 1) high-pressure freezing of fresh brain tissue; 2) tissue trimming followed by cryo-focused ion beam milling via the H-bar approach to generate ultrathin lamellae; and 3) tomographic imaging in a cryo-transmission electron microscope. We applied this workflow to visualize the fine ultrastructural details of organelles, as well as cytoskeletal and synaptic elements that comprise the cortical neuropil within fresh, unfixed mouse brain tissue. Consequently, we now have an optimized set of conditions for high pressure freezing of mouse brain tissue (Fig.1). Additionally, we imaged this frozen mouse

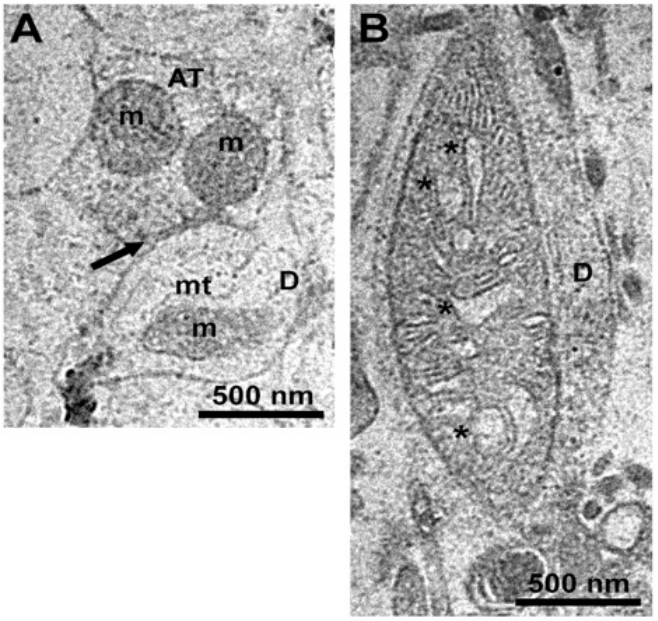

Figure 1. Successful ultrastructural preservation of brain tissue via high-pressure freezing. (A) Electron micrograph image from high-pressure frozen mouse cortex illustrating a neuron axon terminal (AT) containing two mitochondria (m) and forming a synapse (arrow) onto a dendritic shaft (D) containing a mitochondrion and microtubules (mt). **(B)** Electron micrograph of mouse cortex demonstrates a condensed mitochondrion with large open cristae (*). Condensed mitochondria are indicative of active ATP synthesis at time of preservation, further demonstrating our ability to capture cells and organelles in biologically-relevant functional states.

brain tissue via cryo-ET, revealing details of the neuronal cytoskeleton and synaptic architecture under near-native conditions within intact brain tissue (Fig. 2). Future studies will apply principles of the above workflow to the analysis of human tissues including brain tissue from patients. Overall, our work integrates the strengths of cryo-electron microscopy and tissue-based approaches to produce a generalizable workflow capable of visualizing subcellular structures within complex tissue environments.

- In parallel, we have continued developing focused ion beam/scanning electron microscopy (FiB/SEM) technology to capture the detailed three-dimensional (3D) structures of mitochondria directly in native tissues, including in brain cortical tissue. Our 3D reconstructions provide new views of the brain cytoarchitecture in ultrafine detail. Our FiB/SEM imaging of brain cortical tissue reveals mitochondria which make numerous contacts with the rough endoplasmic reticulum (RER) (Fig. 3). These findings provide a putative bioenergetic mechanism by which the mitochondria contacting the RER may fuel local protein translation and presumably lipid biosynthesis. Future work will examine the functional relevance of these mitochondrial-RER contacts in the context of mitochondrial disease by imaging these contacts directly in the cells and tissues of affected subjects.

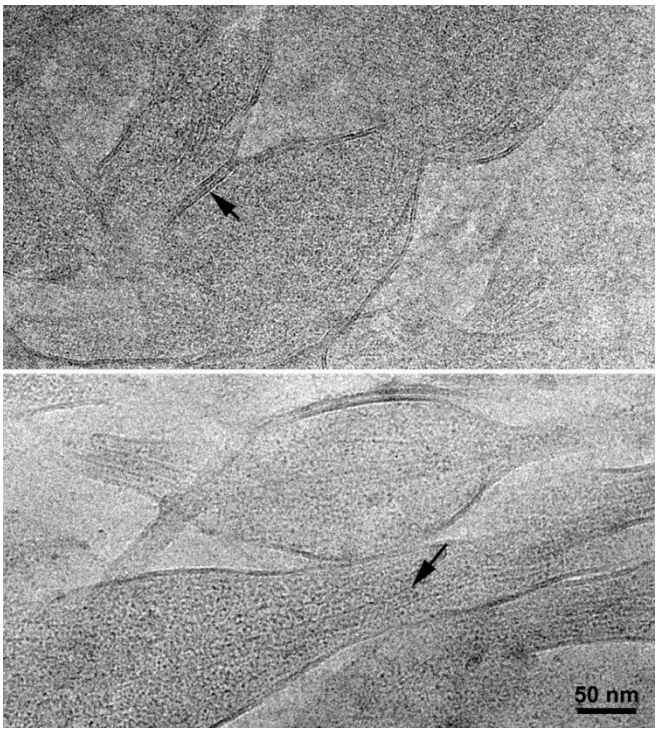

Figure 2. Micrographs of a FIB-milled lamella of mouse brain tissue. Untilted micrographs from a 3000 nm-thick cryo-FIB milled lamella of untreated, high-pressure frozen brain tissue acquired from wildtype C57BL/6J mice. Images were recorded with a pixel size of 0.33 nm, 300 kV, zero-loss energy filtering (20 eV slit), and with a 4 µm underfocus dose of 100 $e^-/Å^2$. **(A)** Micrograph of a putative synapse between two adjacent neurons as indicated by the arrow. **(B)** Image featuring axonal varicosities with a prominent microtubule traversing the axon, as indicated by the arrow. Scale bar = 50 nm.

II. Analysis of imaged mitochondria and respiratory complexes from well-defined mitochondrial disease mutation-containing patient and control fibroblasts.

- Building on our work investigating complex V mutant cells, we have made significant progress in visualizing mitochondria affected by mitochondrial disease-causing mutations in additional respiratory complexes including complex I directly in the primary skin fibroblast cells of patients as well as in unaffected controls. As noted above, we focused on the following mitochondrial disease-causing complex I mutants:

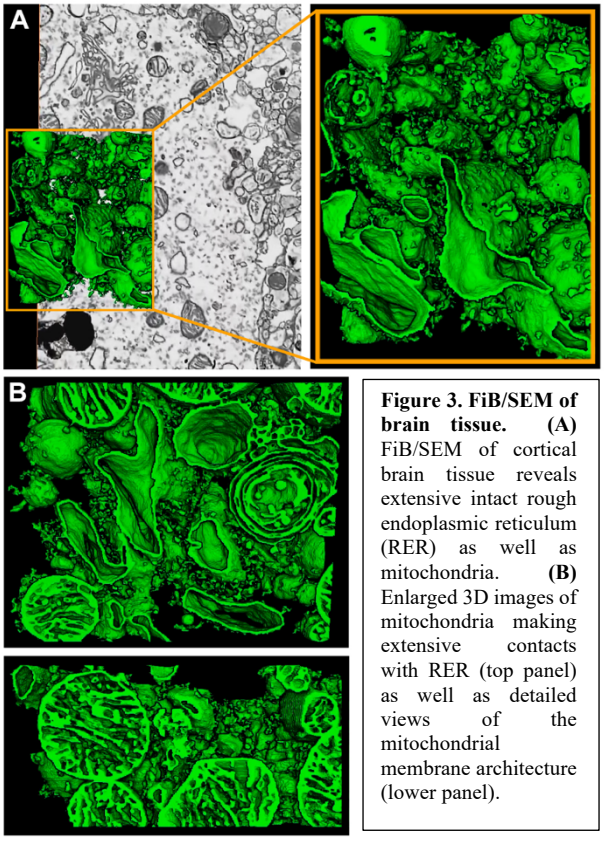

Figure 3. FiB/SEM of brain tissue. (A) FiB/SEM of cortical brain tissue reveals extensive intact rough endoplasmic reticulum (RER) as well as mitochondria. **(B)** Enlarged 3D images of mitochondria making extensive contacts with RER (top panel) as well as detailed views of the mitochondrial membrane architecture (lower panel).

- **ND6 mutant:** ND6 is a mitochondrial chromosome-encoded structural subunit of mitochondrial respiratory complex I embedded in the inner mitochondrial membrane.
- **NDUFV1 mutant:** NDUFV1 is a nuclear-encoded subunit of respiratory complex I that forms part of the NADH binding domain that extends into the mitochondrial matrix.
- **ACAD9 mutant:** ACAD9 is a respiratory complex I assembly factor, and mutations within this protein lead to a complete deficiency of fully assembled respiratory complex I.

Following additional rounds of cryo-ET imaging, we now possess an extensive library of cryo-electron tomograms of each of the 3 mutant fibroblast cells, alongside cells from the age-matched unaffected control. Using these datasets, we have begun developing quantitative measures of mitochondrial ultrastructure in both two- and three dimensions. Foremost, our two-dimensional (2D) analyses demonstrate significant differences that are distinct between the respective mitochondrial disease mutants (Fig. 4). We find that the mitochondria in both ND6 and ACAD9 mutant cells have significantly smaller distances between the mitochondrial outer membrane and inner membrane (MOM-MIM distance) compared to the control; in contrast no significant difference in MOM-MIM distance is observed in NDUFV1 mutant mitochondria (Fig. 4A). We similarly find alterations in the mitochondrial crista morphology amongst the mutants, as reflected in diminished cristae tip angles in ND6 and ACAD9 mutants (Fig. 4C) or in cristae width in ND6 mutant mitochondria (Fig. 4D). Nevertheless, the widths of the junctions from which cristae emerge from the mitochondrial inner membrane do not differ between the various mutants versus the controls (Fig. 4B). This suggests that the distinct disease-causing mutations in respiratory complex I that characterize these mutants do not impact other aspects of mitochondrial biology such as mitochondrial cristae junctions – the sites on the mitochondrial inner membrane from which cristae emerge.

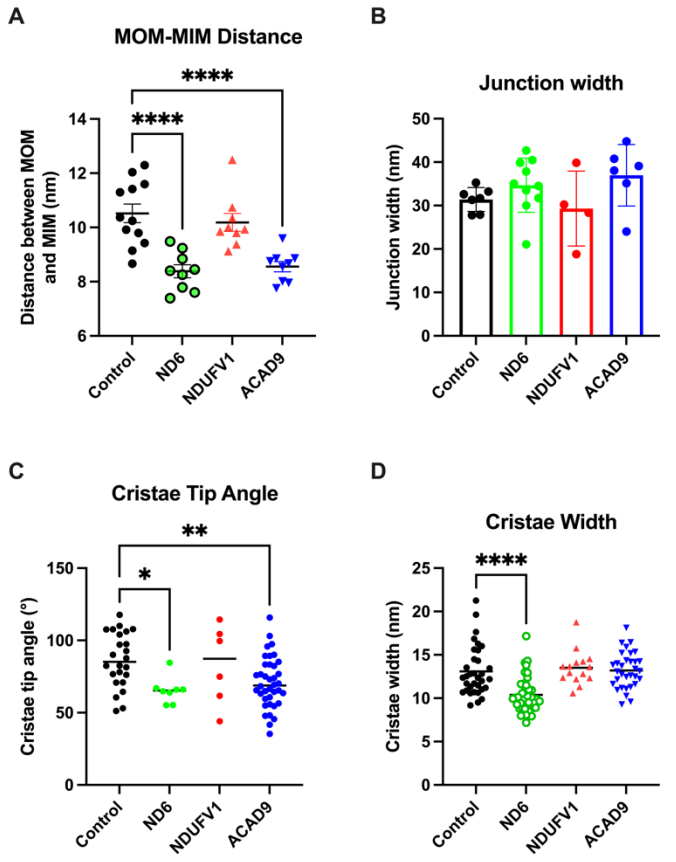

Figure 4. Quantification of mitochondrial ultrastructure in the mitochondrial mutant cells. We quantified a series of key ultrastructural parameters using two-dimensional analyses of mitochondria within our cryo-ET imaging data of primary patient cells and compared to primary cells from a matched unaffected control subject. **(A)** The distance between mitochondrial outer membranes (MOM) and mitochondrial inner membrane (MIM) were significantly decreased in both ND6 and ACAD9 mutant cells ($p<0.0001$). **(B)** There were no significant differences in the width of junctions where cristae emerged from the MIM ($p>0.05$). **(C)** ND6 mutant cells exhibited a significantly decreased cristae tip angle ($p<0.01$), as did ACAD9 cells ($p<0.001$) compared to controls. **(D)** ND6 cells also exhibited a decreased cristae width versus controls ($p<0.0001$).

III. Determine whether the respective mitochondrial disease-causing mutations alter the 3D structures of the mitochondrial respiratory complexes *in situ.*

- We have continued developing subtomogram averaging approaches to resolve the structures of the individual respiratory complexes as well as the higher-order supercomplexes *in situ* within the patient and control mitochondria. To achieve this, in addition to our ongoing work with Dr. Min

Xu at Carnegie Mellon University, we have also begun a collaboration with Dr. Martyn Winn a at the Collaborative Computational Project for electron cryo-microscopy (CCP-EM). Dr. Winn and CCP-EM are leaders in the development of computational approaches in analyses of cryo-EM and cryo-ET data. Together, we are working on applying the computational toolkits and workflows to resolve the individual respiratory complexes and supercomplexes directly in patient cells using our *in situ* cryo-ET data.

IV. Determine whether improved respiratory chain function in patient cells following treatment with JP4-039 is via the drug's ability to correct respiratory complex structural abnormalities.

- As detailed in our previous year's progress report, we have now comprehensively metabolically characterized the effects of antioxidant JP4-039 on mitochondrial function in the respective patient cells containing mitochondrial disease-causing mutations (*i.e.*, ND6, ACAD9, NDUFV1 mutants). Using Seahorse metabolic analyses, we found that treatment of the control cells with JP4-039 significantly increased all measures of mitochondrial respiration including basal and maximal respiration as well as spare respiratory capacity ($p<0.0001$). Our data therefore suggest that JP4-039 can have therapeutic value by positively impacting mitochondria function in the context of specific mitochondrial defects. Future work can explore the development of compounds that specifically target and repair the structural disruptions produced by each of the mitochondrial disease-causing mutations studied here.

V. Visualize mitochondrial respiratory complex structure and supercomplex organization in healthy primary human fibroblasts after pesticide or pyridostigmine bromide treatment.

- In the coming months, we plan to co-administer pesticide and pyridostigmine to our healthy human fibroblasts followed by cryo-ET imaging to resolve effects of the drugs on respiratory complex structure and organization.

VI. Determine whether JP4-039 corrects mitochondrial structural abnormalities in GWI or pesticide/ pyridostigmine bromide-treated control cells.

- Our recent progress in resolving effects of the mitochondrial disease-causing mutations on mitochondrial structure and morphology in patient cells will lay the groundwork for visualizing effects of JP4-039 directly on the respiratory complex structures via cryo-ET. We expect to conduct these experiments in the next 12 months.

- **What opportunities for training and professional development has the project provided?**
  Nothing to Report.

- **How were the results disseminated to communities of interest?**
  Our results have been disseminated to communities of interest at local, national, and international scientific meetings (see below). Collectively, presenting recent findings stemming from this project were instrumental in advancing the idea that *in situ* cryo-ET offers a new approach towards better understanding and treating mitochondrial diseases by identifying how these illnesses alter mitochondrial structure and morphology. In presenting this work through papers, talks, and abstracts, our findings were broadly disseminated to a broad scientific audience whose expertise spans multiple disciplines including neuroscience, cell biology, structural biology, imaging, biophysics, and clinical medicine. Furthermore, we have also published our results extensively. Our findings were published in impactful journals including *Structure*, *Frontiers in Physiology*, and *Journal of Computational Biology*, with three manuscripts under preparation based on work directly based on this award. We expect to submit these manuscripts in the next 6-12 months.

- **What do you plan to do during the next reporting period to accomplish the goals?**
  This is no longer applicable as this is the final report for this award.

4. IMPACT

- **What was the impact on the development of the principal discipline(s) of the project?**
  Our results have been widely disseminated to communities of interest at local, national, and international scientific meetings. These meetings have included the Annual Meeting of the American Society of Cell Biology (2020), Annual American Physical Society Meeting (2021), International Symposium on Endoplasmic Reticulum (2021), the United States Army Research Lab Conference on Soldier Protection Against Evolving Threats (2021), and the Biomolecular Dynamics and Cryo-EM meeting (2022). Additionally, results have been presented as an invited speaker at Weill Cornell Medical College's Department of Biochemistry (2020) an invited speaker at Carnegie Mellon University's Division of Biological Science's Seminar Series (2022), as well as an invited speaker at the Van Andel Institute's Metabolism/Nutrition seminar series (2022). Collectively, presenting recent findings stemming from this project were instrumental in advancing the idea that *in situ* cryo-ET offers a new approach towards better understanding and treating mitochondrial diseases by identifying how these illnesses alter mitochondrial structure and morphology. This has begun generating considerable interest within the greater scientific community.

- **What was the impact on other disciplines?**
  In presenting results from this project, our findings have been broadly disseminated to a diverse scientific audience whose expertise spans multiple disciplines including neuroscience, cell biology, microscopy, and clinical medicine. In the longer-term, appealing to a broader audience fosters new knowledge that leads to development of new, highly targeted therapies for active military, Veterans, beneficiaries, and the general population that are directed at correcting structural changes responsible for mitochondrial dysfunction. Ultimately, such a development could significantly reduce serious morbidity and mortality from mitochondrial diseases and its resulting neurological and cardiovascular consequences. Since current treatments only address disease symptoms, our project's development of methods to directly visualize mitochondrial disease-causing mutations and their effects on the mitochondrial architecture may produce new personalized interventions to treat underlying causes unique to each patient, significantly improving patient care for mitochondrial disorders. Such treatments may also boost mitochondrial function in healthy individuals and thus could be used as tools to increase fitness of active military personnel especially in combat situations. Moreover, our personalized approaches to visualizing mitochondrial disorders can lead to fundamental insights into the mechanisms for development of these illnesses and may even be applied to the study of other diseases.

- **What was the impact on technology transfer?**
  Nothing to Report.

- **What was the impact on society beyond science and technology?**
  Nothing to Report.

5. CHANGES/PROBLEMS

There have been no changes in the scope of work since the last reporting periods and therefore the SOW remains the same as originally defined.

6. PRODUCTS

- **Publications, conference papers, and presentations**

Journal publications

Data based on the studies proposed for this award were published in the following papers:

1. Zeng X, Lin Z, Uddin MR, Zhou B, Cheng C, Zhang J, **Freyberg Z**, Xu M. Structure detection in 3D cellular cryo-electron tomograms by reconstructing 2D annotated tilt-series. J Computational Biology 2022; 29(8):932-941. doi: 10.1089/cmb.2021.0606. PMID: 35862434.

2. Gupta T, He X, Uddin MR, Zeng X, Zhou A, Zhang J, **Freyberg Z**, Xu M. Self-supervised learning for macromolecular structure classification based on cryo-electron tomograms. Front Physiol. 2022; 13:957484. doi:10.3389/fphys.2022.957484. PMID: 36111160.

We have 3 additional manuscripts in preparation based on the work resulting from this award and expect to submit them in the next 6-12 months.

Books or other non-periodical, one-time publications
Nothing to report.

Other publications, conference papers, and presentations
Data based on the studies originally proposed for this award were presented at the following meetings:

1. A top-down perspective on structural dynamics in cryo-EM. Invited talk presented at the Biomolecular Dynamics and Cryo-EM meeting (2022), Leeds, UK/Hybrid meeting

Additionally, Dr. Freyberg was an invited speaker at the following seminars where he presented the work produced from this funded work:

1. Invited speaker, Metabolism/Nutrition invited seminar series, Van Andel Institute, Grand Rapids, MI; 2022

- **Website(s) or other Internet site(s)**
  Nothing to Report.

- **Technologies or techniques**
  Nothing to Report.

- **Inventions, patent applications, and/or licenses**
  Nothing to Report.

- **Other Products**
  Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

• Name:	Zachary Freyberg M.D., Ph.D.
• Project Role:	Principal Investigator
• Researcher Identifier (*e.g.* ORCID ID):	ORCID ID: 0000-0001-6460-0118
• Nearest person month worked:	1.8
• Contribution to Project:	Dr. Freyberg has designed and analyzed all experimental data concerning both the live-imaging and *in situ* cryo-ET microscopy of mitochondria from cells taken from

	patients with mitochondrial disease and associated controls.
• Funding Support:	DoD Peer Reviewed Medical Research Program Discovery Award (PR192466); PA Tobacco Formula Fund, NIH/NIA R21 Award (R21AG068607), NIH NIDA CEBRA Award (R21DA052419), NIH/NIGMS R01 (R01GM134020), NIH/NIDDK R01 (R01DK109907), DoD Expansion Award (PR210207), Pittsburgh Foundation Award (FPG00043-01)

• Name:	Jill Glausier, Ph.D.
• Project Role:	Co-Investigator
• Researcher Identifier (*e.g.* ORCID ID):	ORCID ID: 0000-0001-9838-3414
• Nearest person month worked:	0.6
• Contribution to Project:	Dr. Glausier has analyzed the tomographic data to successfully build three-dimensional maps of the imaged cells. She will use her expertise in mitochondrial assays to validate the structural findings concerning complex organization with biochemical assays on patient mitochondria including blue native gels and clinical electron transport chain analysis.
• Funding Support:	DoD Peer Reviewed Medical Research Program Investigator-Initiated Research (PR192466); NIH/NIDA (N0175N95019C00047), NIH/NIDA (DA051390), NIH/NIMH (R21MH125012)

• Name:	James Conway, Ph.D.
• Project Role:	Co-Investigator
• Researcher Identifier (*e.g.* ORCID ID):	ORCID ID: 0000-0002-6581-4748
• Nearest person month worked:	0.6
• Contribution to Project:	Dr. Conway has provided technical expertise and assistance with imaging and data analysis.
• Funding Support:	DoD Peer Reviewed Medical Research Program Investigator-Initiated Research (PR141292), NIH/NIAID (R01AI089803)

• Name:	Jiying Ning, Ph.D.
• Project Role:	Research Associate
• Researcher Identifier (*e.g.* ORCID ID):	N/A
• Nearest person month worked:	3.3
• Contribution to Project:	Dr. Ning has maintained the patient and control primary human cells (*e.g.*, primary fibroblasts) used in the study as well as optimized the sample preparation methodologies.
• Funding Support:	Department of Defense Peer Reviewed Medical Research Program Discovery Award (PR192466), PA Tobacco Formula Fund, NIH/NIGMS R01 (R01GM134020), NIH/NIDDK R01 (R01DK109907)

• Name:	Alexander Makhov, Ph.D.
• Project Role:	Facility Manager
• Researcher Identifier (*e.g.* ORCID ID):	N/A
• Nearest person month worked:	0.6
• Contribution to Project:	Dr. Makhov has assisted with data collection for the proposed cryo-electron microscopy and tomography data. He maintains the cryo-electron microscopes in an ongoing manner and conduct microscope repairs.
• Funding Support:	Department of Defense Peer Reviewed Medical Research Program Discovery Award (PR192466)

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

The PI of this award, Dr. Freyberg, has been awarded the following awards since the last reporting period:

Grant Number	Grant Title	Role in Project and Percentage of Effort	Years Inclusive	Source $ Amount
PR210207	DoD Expansion Award, "New dopaminergic mechanisms of pancreatic hormone secretion and therapeutics in diabetes"	PI 20% Effort 2.40 Calendar Months	2022-2025	DoD Total: Direct: Indirect:
FPG00043-01	Direct visualization of the molecular mechanisms of cell plasticity in healthy and neurodegenerative disease states	PI 5% Effort 0.60 Calendar Months	2022-2024	The Pittsburgh Foundation Total: Direct:

- **What other organizations were involved as partners?**
  Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

- **Collaborative Awards**
  Not Applicable.

9. APPENDICES

Award Expiration Transition Plan Questionnaire, Report of Inventions and Subcontracts, Award Chart

Transition Plan Questionnaire

Directions: Please answer all questions that apply for each product under development. Please fill out one document per product.
This is not an application for funding; however, answers will help us understand the outcomes and products from your award.

1. After the award closes, would you be willing to periodically provide voluntary information (via email) regarding the project status (i.e. where the research is headed)? **Yes** [X] or **No** [ ]
These responses will help CDMRP demonstrate the return on its investments and will help demonstrate that the CDMRP is a responsible and successful steward of federal research funding.

2. What **conclusion(s)** does your final data support?

Our final data support the conclusions that we are capable of utilizing in situ cryo-electron tomography (cryo-ET) approaches to visualize effects of human disease processes on subcellular structures directly in the cells of affected individuals. Our focus on human mitochondrial disease enabled us to directly determine the impacts of specific disease-causing mutations on mitochondrial structure. Specifically, we revealed that mutations within individual subunits of a single mitochondrial respiratory complex, Complex I, are sufficient to cause substantial derangements of mitochondrial ultrastructure including loss of the mitochondrial cristae. Overall, our studies allowed us to link the biochemical perturbations caused by disease mutations directly to changes in organelle structure, leading to novel insights into the pathophysiology of mitochondrial dysfunction. Just as importantly, the application of cryo-ET to human mitochondrial disease suggests the promise of a novel precision medicine-focused approach, where the study of pathogenic mutations in readily accessible and minimally invasive patient samples may reveal important, yet subtle changes visible under near-native conditions. This work also serves as a foundation for the development of new, personalized therapies that specifically target and correct the structural disturbances caused by disease-causing mutations.

3. Will you/have you applied for/obtained follow-on-funding for this project? **If yes**, please list (a) funding organization, (b) total budget requested/obtained, and (c) title of the funded proposal. *This information will be recorded as an outcome to this award*.

I have not yet applied for or obtained follow-on funding for this project.

4. What will be **the next step(s)** for this project?

The next steps will be to use in situ cryo-ET to determine the effects of metabolic activity on mitochondrial respiratory complex structures in primary cells from both healthy human subjects as well as from individuals with mitochondrial diseases using the in situ cryo-ET methodologies that we have developed. We will also examine potential disease-induced changes in the ssembly of supercomplexes which represent the higher-order organization of the individual respiratory complexes within the mitochondrial membrane. Our longer-term goal will be to advance the emerging concept of “mitochondrial typing”, linking signature classifications of respiratory complexes within mitochondria to activity- or disease-driven alterations in metabolic function. This typing can be applied beyond primary mitochondrial disorders to other diseases caused by mitochondrial dysfunction including Alzheimer’s disease, Parkinson’s disease or diabetes. Discovering disease-induced changes in respiratory complex structures and their higher-order organization within supercomplexes may lead to new perspectives on pathobiology and provide a framework for new treatment development.

5. How would you classify your **lead candidate product**? Please choose best option

(a) Therapeutic (Small Molecule, Biologic, Cell/Gene Therapy): Please choose, if applicable
(b) Diagnostic
(c) Device
(d) Research Tool to Address a Research Bottleneck
(e) Knowledge Product (Non-material product such as a compound library, database, something that improves clinical practice, education, etc.)
(f) Other - Please Specify:

6. How does your candidate product aid the Warfighter, Veteran, Beneficiary, and/or General Population?

Our ability to directly visualize the impacts of mitochondrial disease-causing mutations on mitochondria directly in the cells of affected individuals will significantly contribute to a better fundamental understanding of mitochondrial disease, and lead to development of a better, highly accurate diagnostic approaches for identifying mitochondrial disease and/or dysfunction in affected individuals. Ultimately, by elucidating the structural mechanisms underlying mitochondrial disease, our work may also produce better, more effective treatments which will directly impact the health of military Service members, Veterans, their beneficiaries as well as the general population. The development of interventions personalized to the structural abnormalities unique to each patient, may lead to significant improvements or even cures in patient care. Additionally, these treatments may also be applied to boost mitochondrial function in healthy individuals and increase fitness of active military personnel especially in combat situations.

7. Therapy / Product Development, Transition Strategies, and Intellectual Property

Describe the steps and relevant strategies required to move the candidate product (knowledge or tangible) to the next phase of development and/or commercialization. Please address any issues with intellectual property.

PIs are encouraged to explore the technical requirements and the current regulatory strategies involved in product development as well as to work with their organization's Technology Transfer Office (or equivalent regulatory/legal office), federal/international regulatory experts, to develop the transition plan and to explore developing relationships with industry, DoD advanced developers (e.g. USAMMDA), and/or other funding agencies to facilitate moving the product into the next phase.

The steps and relevant strategies required to move the knowledge that has resulted from the funded studies to the next phase of development include the establishment of robust computational pipelines to resolve alterations to the structures of mitochondrial respiratory complexes and supercomplexes produced by mitochondrial disease-causing mutations at subnanometer resolution. Once we can reliably visualize these structural changes, this will pave the way for rationally designed small therapeutic molecules intended to directly target and therefore correct these disease-induced structural alterations. Moreover, we can categorize the structural changes to mitochondrial respiratory complexes in order to find commonly shared features among different individuals with mitochondrial disease. This will lead to the development of reproducible biomarkers for mitochondrial disease. We will work closely with the Technology Transfer Office at the University of Pittsburgh and do not anticipate any issues with intellectual property.

REPORT OF INVENTIONS AND SUBCONTRACTS

(Pursuant to "Patent Rights" Contract Clause) (See Instructions on back)

Form Approved
OMB No. 9000-0095
Expires Aug 31, 2001

PLEASE DO NOT RETURN YOUR COMPLETED FORM TO THIS ADDRESS. RETURN COMPLETED FORM TO THE CONTRACTING OFFICER.

1.a. NAME OF CONTRACTOR/SUBCONTRACTOR	c. CONTRACT NUMBER	2.a. NAME OF GOVERNMENT PRIME CONTRACTOR	c. CONTRACT NUMBER	3. TYPE OF REPORT *(X one)*
UNIVERSITY OF PITTSBURGH THE	W81XWH-20-1-0113			a. INTERIM [X] b. FINAL

b. ADDRESS *(Include ZIP Code)*	d. AWARD DATE *(YYYYMMDD)*	b. ADDRESS *(Include ZIP Code)*	d. AWARD DATE *(YYYYMMDD)*	4. REPORTING PERIOD *(YYYYMMDD)*
3420 Forbes Avenue, Pittsburgh, PA 15260-3203	20200301			a. FROM 20200301 b. TO 20230228

SECTION I - SUBJECT INVENTIONS

5. "SUBJECT INVENTIONS" REQUIRED TO BE REPORTED BY CONTRACTOR/SUBCONTRACTOR *(If "None," so state)*

NAME(S) OF INVENTOR(S) *(Last, First, Middle Initial)* a.	TITLE OF INVENTION(S) b.	DISCLOSURE NUMBER, PATENT APPLICATION SERIAL NUMBER OR PATENT NUMBER c.	ELECTION TO FILE PATENT APPLICATIONS *(X)* d.				CONFIRMATORY INSTRUMENT OR ASSIGNMENT FORWARDED TO CONTRACTING OFFICER *(X)* e.	
			(1) UNITED STATES		(2) FOREIGN			
			(a) YES	(b) NO	(a) YES	(b) NO	(a) YES	(b) NO
None	None	None						

f. EMPLOYER OF INVENTOR(S) NOT EMPLOYED BY CONTRACTOR/SUBCONTRACTOR		g. ELECTED FOREIGN COUNTRIES IN WHICH A PATENT APPLICATION WILL BE FILED	
(1) (a) NAME OF INVENTOR *(Last, First, Middle Initial)*	(2) (a) NAME OF INVENTOR *(Last, First, Middle Initial)*	(1) TITLE OF INVENTION	(2) FOREIGN COUNTRIES OF PATENT APPLICATION
(b) NAME OF EMPLOYER	(b) NAME OF EMPLOYER		
(c) ADDRESS OF EMPLOYER *(Include ZIP Code)*	(c) ADDRESS OF EMPLOYER *(Include ZIP Code)*		

SECTION II - SUBCONTRACTS *(Containing a "Patent Rights" clause)*

6. SUBCONTRACTS AWARDED BY CONTRACTOR/SUBCONTRACTOR *(If "None," so state)*

NAME OF SUBCONTRACTOR(S) a.	ADDRESS *(Include ZIP Code)* b.	SUBCONTRACT NUMBER(S) c.	FAR "PATENT RIGHTS" d.		DESCRIPTION OF WORK TO BE PERFORMED UNDER SUBCONTRACT(S) e.	SUBCONTRACT DATES *(YYYYMMDD)* f.	
			(1) CLAUSE NUMBER	(2) DATE *(YYYYMM)*		(1) AWARD	(2) ESTIMATED COMPLETION

SECTION III - CERTIFICATION

7. CERTIFICATION OF REPORT BY CONTRACTOR/SUBCONTRACTOR *(Not required if: (X as appropriate))* [ ] SMALL BUSINESS or [X] NONPROFIT ORGANIZATION

I certify that the reporting party has procedures for prompt identification and timely disclosure of "Subject Inventions," that such procedures have been followed and that all "Subject Inventions" have been reported.

a. NAME OF AUTHORIZED CONTRACTOR/SUBCONTRACTOR OFFICIAL *(Last, First, Middle Initial)*	b. TITLE	c. SIGNATURE	d. DATE SIGNED
Byrnes, Zachary J.	Associate Director, Grants Management & Contracting Office of Sponsored Programs, University of Pittsburgh	DocuSigned by: Zachary Byrnes C43C8A9CA61648A...	05-12-2023 | 1:52 PM EDT

PR192466: A new *in situ* cryo-electron microscopy approach to directly visualize mutations in mitochondrial disease

PI: Zachary Freyberg, MD, PhD, University of Pittsburgh, PA **Budget:** Total Award Cost

Topic Area: Mitochondrial Disease **Mechanism:** DoD Discovery Award

Research Area(s): 0100 (Cell Biology), 0200 (Genetics and Molecular Biology). **Award Status: 03/01/2020-02/28/2023**

Study Goals: 1) Sample preparation and imaging acquisition of 3D tomograms from mitochondrial disease patient and control cells; 2) Analysis of imaged mitochondria and respiratory complexes from well-defined mitochondrial disease mutation-containing patient and control fibroblasts; 3) Determine if respective mitochondrial disease-causing mutations alter the 3D structures of the mitochondrial respiratory complexes *in situ;* 4) Determine whether improved respiratory chain function in patient cells following treatment with JP4-039 is via the drug's ability to correct respiratory complex structural abnormalities; 5) Visualize mitochondrial respiratory complex structure and supercomplex organization in healthy primary human fibroblasts after pesticide or pyridostigmine bromide (PB) treatment; 6) Determine whether JP4-039 corrects mitochondrial structural abnormalities in GWI or pesticide/PB-treated control cells.

Specific Aims: (1) To determine the effects of disease-causing mutations on individual mitochondrial respiratory complexes and higher-order supercomplex organization in situ; and (2) To identify effects of GWI and associated neurotoxicants on respiratory chain complex structure and higher-order organization in situ in patient cells.

Key Accomplishments and Outcomes:

Publications:

1) Zeng X, Lin Z, Uddin MR, Zhou B, Cheng C, Zhang J, **Freyberg Z**, Xu M. Structure detection in 3D cellular cryo-electron tomograms by reconstructing 2D annotated tilt-series. J Computational Biology 2022; 29(8):932-941. doi: 10.1089/cmb.2021.0606. PMID: 35862434.

2) Gupta T, He X, Uddin MR, Zeng X, Zhou A, Zhang J, **Freyberg Z**, Xu M. Self-supervised learning for macromolecular structure classification based on cryo-electron tomograms. Front Physiol. 2022; 13:957484. doi:10.3389/fphys.2022.957484. PMID: 36111160.

3) Ning J, Glausier JR, Hsieh C, Schmelzer T, Buck SA, Franks J, Hampton CM, Lewis DA, Marko M, **Freyberg Z**. Cryo-FIB workflow for imaging brain tissue via *in situ* cryo-electron microscopy. BioRxiv 2023; 2023.02.11.528064; doi: https://doi.org/10.1101/2023.02.11.528064.

Patents: none to date

Funding Obtained: none to date