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14. ABSTRACT

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RPPR Final Report
as of 17-Jun-2022

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Final Report for Period Beginning 18-Dec-2020 and Ending 31-Dec-2021

Title: DURIP: Multi-Mode Microplate Reader for Research and Education

Begin Performance Period: 18-Dec-2020

End Performance Period: 31-Dec-2021

Report Term: 0-Other

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Distribution Statement: 1-Approved for public release; distribution is unlimited.

STEM Degrees:

STEM Participants:

Major Goals: We requested funds for a state-of-the-art SpectraMax iD5 Multi-Mode Microplate Reader with a StakMax plate handling system. This five-mode platform can measure absorbance, fluorescence, luminescence, time-resolved fluorescence (TRF), and tunable fluorescence polarization (FP) in samples. The platform uses a xenon flash lamp and an ultra-cooled photomultiplier tube (PMT) that reduces background noise for excellent sensitivity and a wide dynamic detection range. The system includes 2 injectors for flash kinetics measurements and the StakMax plate handling system enabled the automated reading of up to 50 microwell plates. The system dramatically upgrades and enhances the research activities of several projects at Columbia University that have been funded by, or will be submitted for funding to, the Department of Defense. The PI of this proposal, Scott Banta, is currently funded as PI on two ARO grants (W911NF-19-1-0325, Program Officer Dr. Dan Cole) and W911NF-18-1-0239, Program Officer Dr. Bob Kokoska). The PI is also funded as a co-PI on an ARO MURI grant (W911NF-14-1-0263, Program Officers Dr. David Stepp and Dr. Robert Mantz). The PI has had a Molecular Devices SpectraMax M2 Microplate Reader in continuous operation for over 15 years, and this system has been used to support the research activities of at least 10 DoD-funded grants and has been used for the collection of data in at least 50 peer-reviewed manuscripts. The new SpectraMax 1D5 is a transformative upgrade for this essential research tool. In addition to supporting multiple Defense-related projects at Columbia University, the SpectraMax iD5 Microplate Reader will be incorporated into the educational activities within the Department of Chemical Engineering at Columbia University. Enzymatic reaction measurement techniques will be incorporated into two relevant courses. The Microplate reader will also be used by postdoctoral, graduate and undergraduate researchers as well as local high school students through the Columbia University Inside Engineering program and the HYPOTHEkids (HK) Maker Lab summer program. Therefore, the funding of this DURIP proposal significantly enhances both research and research-related education at Columbia University.

Accomplishments: See Attached PDF Document

Training Opportunities: All students in the laboratory were trained to use the equipment.

Results Dissemination: Nothing to Report

Honors and Awards: Nothing to Report

Protocol Activity Status:

Technology Transfer: Nothing to Report

Partners

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I certify that the information in the report is complete and accurate:
Signature: Scott Banta
Signature Date: 6/13/22 10:58AM

Project Status

The requested funds ($86,846.33) were used for the purchase of state-of-the-art SpectraMax iD5 Multi-Mode Microplate Reader with a StakMax plate handling system as well as two SpectraDrop Micro-Volume Spectrometers and a Sorvall Legend Refrigerated Microcentrifuge.

Molecular Devices is a world leader in the development of microplate reading systems with over 30 years of experience. The following is an itemized list of the equipment received:

SpectraMax iD5 Multi-Mode Plate Reader (id5-INJ)	$44,627.60
StakMax Plate Handling System (Stakmax)	$17,480.56
SpectraMax iD5 ScanLater Set (6590-0147)	$4,011.25
Includes Enhanced TRF Module and Membrane Holder for Western Blots	
SpectraDrop Quick Drop Micro-Volume Spectrophotometer (2 Units)	$11,888.88
SpectraDrop Micro-Volume Starter Kit (0200-6282)	$2,890.63
SpectraMax Adapter – 24-well sample slides, Sample covers, Storage Case	
Sorvall Legend Refrigerated Microcentrifuge (Fisher 75 002 446)	$5,947.41
Total:	**$86,846.33**

The Microplate reader, Plate handling system, Spectrophotometers, and Centrifuge were received in 2021 and early 2022 are all located in the new Banta Lab (Room 440) located in the Mary Woodard Lasker Biomedical Research Building located at 3960 Broadway, New York, NY 10032

Supporting Information

Microplate readers are workhorse instruments in all biomolecular engineering laboratories[3-5]. These systems allow optical measurements to be taken in small samples in disposable plastic plates. Over the last 18 years, the Banta Lab has used a Molecular Devices SpectraMax M2 Microplate Reader (purchased in 2005) to perform a wide range of critical experiments including: cell quantification, DNA quantification, protein quantification, colorimetric kinetic assays, fluorescent kinetic assays, and luminescent kinetic assays. The system is able to shake a single plate and incubate the plate at temperatures up to ~50°C. This system has been used by every postdoctoral fellow, graduate student, and undergraduate student that has been in the Banta Lab at Columbia University. The old M2 Microplate reader is still functional and will serve as a back-up for as long as it is operational.

Fig 1. SpectraMax iD5 Multi-Mode Microplate Reader. StakMax plate handing system not shown.

With the DURIP award, we purchased a new SpectraMax iD5 Multi-Mode Microplate Reader (**Fig 1**) with a StakMax plate handling system. **This substantially upgrades our capabilities in numerous ways which will dramatically increase our research activities including those supported by three current DoD-funded grants.** The new microplate reader has introduced **four** significant new capabilities that will specifically advance our research productivity.

First, the SpectraMax iD5 has a StakMax plate handling system so that up to **50 microplates** can be automatically fed into the system and measured optically. We routinely read one 96-welled plate at a time, changing them into the machine by hand. The new microplate reader can read 6 to 384-well microtiter plates and the StakMax will automatically feed and read up to 50 plates. Thus 19,200 different measurements could potentially be made in one day. This will lead to a game-changing increase in throughput.

Second, the SpectraMax iD5 will have **internal injectors** for delivering reagents to the plate wells. Previously we have used pipettes to quickly inject a reagent and then the plate is inserted and the door is closed before measurements could be made. This is acceptable for slow reactions; however, faster reactions could not be monitored using this method. For example, as part of our **ARO-funded MURI award** we were exploring the development of tethered ATP-swing arms for substrate channeling applications. ATP concentrations are routinely measured using the luminescence produced by the luciferin/luciferase reaction[7]. The addition of the enzyme or the substrate creates a burst of light that is proportional to the ATP concentration. Generally, this reaction was too fast, and the students had to use a plate reader with injector capabilities in a Chemistry laboratory on the other side of the campus. The ability to automatically inject a reagent or enzyme into the plate wells to start a reaction will dramatically improve the quality of the data we can collect and allow us to perform assays in our lab that were previously not possible.

Third, the SpectraMax iD5 will allow us to perform **Time-Resolved Fluorescence (TRF)** measurements. A challenge in fluorescence intensity measurements is that the fluorescence emission times are short and there can be background signals from the excitation light and from other materials in the samples. In TRF, a lanthanide fluorophore (terbium or europium) is used, which has a long-lived fluorescence emission. Thus dramatically better signal-to-noise ratios can be obtained by exciting the sample and measuring just the longer-lifetime lanthanide emissions[8]. We have previously used fluorescent proteins to measure distances in synthetic biomaterials via fluorescent resonance energy transfer (FRET)[9]. The new SpectraMax 1D5 will enable time resolved FRET (TR-FRET) which will be a tremendous new capability allowing for sensitive measurement of distances in our protein and DNA samples.

Finally, the SpectraMax iD5 will allow us to perform **Fluorescence Polarization (FP)** measurements. When samples are excited with polarized light, the emitted light can be polarized depending on the mobility of the fluorophore. Large proteins and complexes produce polarized signals while small, fast moving molecules such as cofactors are depolarized when free in solution. This effect allows for binding interactions to be monitored in solution and is especially valuable for monitoring the freedom of intrinsically fluorescent molecules such as the nicotinamide cofactors[10]. We perform extensive enzyme kinetics in the Banta Lab and methods to determine the dissociation constants of cofactors reduces the uncertainty in the determination of other kinetic rate constants[11] and can elucidate the impacts of mutations on the formation of the enzyme/cofactor complexes. We previously made these types of measurements one at a time using a circular dichroism spectrometer and the ability to do these measurements in a microplate would substantially enhance our productivity and the accuracy of our kinetic measurements.

With price changes and discount from Molecular Devices, we were able to purchase additional equipment with the same budget price. In addition to performing kinetic assays with the microplate reader, we routinely use the platereader to measure biomolecule concentrations in cuvettes. DNA has a characteristic absorbance at 260 nm and proteins have a characteristic absorbance at 280 nm. Quantification of DNA and protein concentrations is performed routinely by every member of the laboratory, and this can be challenging when the microplate reader is being used for longer experiments. Therefore we were able to purchase 2 **SpectraMax QuickDrop Micro-Volume Spectrophotometers**. These systems can quantify very small amounts of DNA, RNA, and proteins with sample volumes as low as 0.5 µL. The systems have a small footprint, 4 sec read times and no moving parts. In addition, funds were available to purchase a **Sorvall Legend Micro R21 Microcentrifuge**. This is a workhorse instrument for centrifuging up to 24 microcentrifuge tubes under refrigeration at up to 20,100xg. This capability is essential for all DNA and protein isolation protocols and will dramatically increase the experimental throughput in the laboratory.

The SpectraMax iD5 Multi-Mode Microplate Reader is being used at Columbia University in several Defense-related research projects including two different ARO-funded grants as well as an ARO-sponsored Multi University Research Initiative (MURI) grant. The last SpectraMax Microplate reader we purchased in 2005 is still functional and is used routinely. **We expect the new SpectraMax iD5 will have a similar longevity and will be enabling cutting-edge research discoveries in the Banta Lab for at least the next 15 years.** The specific application of the Microplate Reader for the currently funded ARO projects is outlined below.

ARO Grant: Modulation of Enzymatic Reaction Trajectories via Applied Mechanical Forces (W911NF-19-1-0325)

Enzymes have evolved with remarkable activities, and selectivities. This is accomplished through the intricate formation and stabilization of catalytic transition states. In many cases enzyme structures are dynamic, with subtle conformational changes potentially occurring during reactant binding, catalysis and product release[12-15]. There is a growing appreciation that mechanical forces are involved in catalytic pathway trajectories[16-18] and **we hypothesize that that addition of applied external mechanical forces could be exploited to influence kinetic steps thus directing alternative catalytic reaction trajectories.** This hypothesis will be explored using both simple DNA springs**[19]** and DNA tweezers**[20, 21]** as well as more complex DNA origami tweezers**[22]** and DNA origami force clamps**[23]**. These tools will be used to apply tension and compression forces in and around the active site of the thermostable alcohol dehydrogenase D (AdhD) enzyme from *Pyrococcus furiosus***[24, 25]**. These efforts will further elucidate the importance of applied mechanical forces on the biocatalytic mechanisms and will help inform efforts focused on the design and development of synthetic and biomimetic enzymes and biocatalysts.

The PI on this DURIP proposal, Scott Banta, is the PI of this recently funded grant from ARO (W911NF-19-1-0325, Program Officer Dr. Dan Cole). The new Multi-Mode Plate Reader will significantly augment all of the Specific Aims of the project.

Specific Aim 1: *To introduce DNA springs and small DNA tweezers to multiple sites across several loops over the active site of AdhD to identify hot spots and to begin to explore the impact of mechanical stresses (tension and compression) on the steady state kinetic behavior of the enzyme.* This high throughput approach will allow us to identify locations in the enzyme where applied forces can deform the active site, leading to altered activities, selectivities, and stabilities.

Specific Aim 2: *To use DNA origami tweezers and DNA origami force clamps to apply varying directional forces between key sites near the AdhD active site to characterize the impact of calibrated forces on the steady state kinetics behavior of the enzyme.* The use of DNA origami tweezers and force clamps will allow for the introduction of varying and calibrated forces at a single location, enabling the probing of the effects of increasing force on reaction activity, selectivity, and stability.

Specific Aim 3: *To demonstrate dynamic and controllable changes in catalytic activity that can be achieved by altering applied forces to the enzyme during catalysis.* An advantage of the DNA force approach is that the forces could be applied or released during the catalytic turnover of the enzyme. The activity of the enzyme will be monitored in real time, and the effect of force application or removal on the reaction trajectory will be quantified.

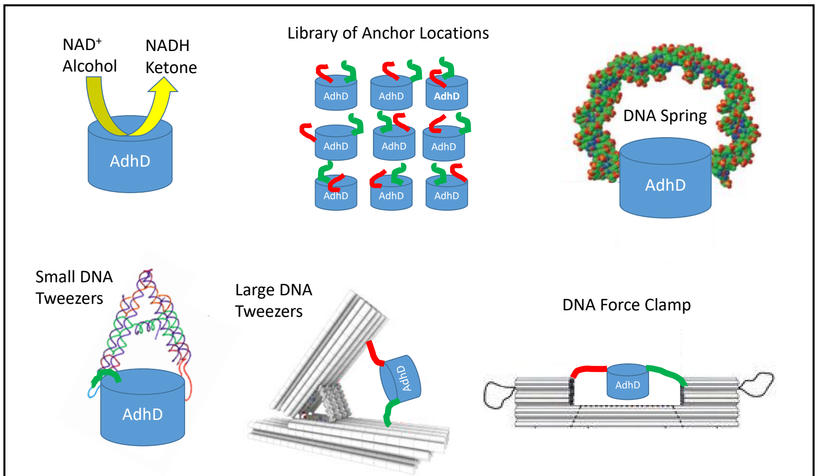

Fig 2. Overview of experimental approaches that will be explored in the research. The kinetic activity of the AdhD enzyme has been extensive investigated, and a library of AdhD mutants with fused short ssDNA oligonucleotides will be created so that different anchoring points can be explored. DNA springs and small DNA tweezers will be used to apply tension and compression forces on the anchoring points to identify "hot spots" that lead to altered kinetic activities. More elaborate DNA origami structures (tweezers and force clamps) will be explored with better calibrated distances and applied forces to explore how forces in and around the enzymatic active site can be used to drive enzymatic specificities and reaction trajectories.

All 3 of the Specific Aims of this proposal will involve the collection of enzyme kinetic data to assess the impacts of the applied forces on the performance of the enzymes. We routinely performed these assays in our existing SpectraMax M2 microplate reader, however time on the machine must be shared between the existing projects in the Banta Laboratory, including other ARO-funded grants. The new iD5 Microplate Reader allows more time to be devoted to this project**, increasing the number of kinetic assays** that can be performed during the lifetime of the grant. And the addition of the fluorescence polarization capability will allow us to measure **cofactor dissociation constants of the enzymes**[10] which will significantly improve our ability to quantify the kinetic parameters for the enzymes, which is a capability we have not had in a 96-well plate format.

A central goal of the project will be to characterize the impact that small applied forces have on the kinetics of a model enzyme (**Fig 2**). Currently we are working on site-specifically attaching single stranded DNA oligonucleotides to unique positions on the enzyme. This will allow us to build DNA springs, tweezers, and force clamps with DNA origami and apply forces across the enzymes in different configurations. **A critical challenge in this work will be the determination of whether the DNA origami structures have been properly assembled and if they are in a configuration that will produced the desired applied forces.** We will attach fluorophores to the DNA origamis and the enzymes which can participate in non-radiative dipole-dipole coupling[26]. This will allow us to make FRET and TR-FRET measurements, allowing us to characterize spatial arrangements and estimate the distances between the ends of the DNA origamis[27] from which we can calculate the magnitude of mechanical stress applied on the enzyme. **Thus, we will be able**

to characterize the applied mechanical stress on the enzymatic activity of AdhD before and after performing kinetic experiments. The ability to perform kinetic assays and TR-FRET on the same samples was not possible in the old Microplate reader. And the StakMax system will allow us to measure the TR-FRET in a large number of samples, which cannot be done currently. In addition, we will be able to vary the temperature from ambient up to 66°C would which will allow us to take advantage of the extreme thermostability of the AdhD enzyme.

In summary, the new SpectraMax iD5 microplate reader **dramatically improves the research capabilities** that can be brought to bear on this funded project. We will be able to substantially increase our ability to perform the kinetic characterization of the enzymes and will be able to estimate the applied mechanical forces in a high-throughput manor that is not possible with our existing equipment.

ARO Grant: Characterizing and Enhancing Biological Corrosion of Metals (W911NF-18-1-0239)

Metal oxidizing bacteria are able to use the oxidation of metals as an energy source for growth, and they play an important role in mining operations as they can help to liberate valuable metals from lean ores[28, 29]. These cells can also contribute to microbiologically influenced corrosion (MIC)[30] and this has led to interest in understanding metal/microbial interactions to ensure the protection of metallic materiel. We have been interested in characterizing and engineering the iron-oxidizing bacterium *Acidithiobacillus ferrooxidans* for the improved performance in mining operations and for the production of biochemicals from CO_2*[31-36]*. **The overarching goal of this grant was to develop *A. ferrooxidans* strains and culture conditions to enhance the MIC activity for a wide range of metals and alloys.** This will be accomplished through the fundamental investigation of the MIC capabilities of the wild type cells, the investigation of culture conditions and additives to improve MIC, and finally the genetic modification of the cells to produce additives that will enhance MIC. The completion of his project will be used to demonstrate that cells and culture conditions can be developed that will lead to enhanced corrosion abilities which could be used to reduce the environmental impact of military activities and could be used to rapidly oxidize various metallic objects. In the future, engineered organisms could eventually be used for the recycling of electronic waste, for the rapid degradation of unwanted material, and eventually to obtain useful chemical or electrical energy from scrap metal resources. **Therefore, there are potential new opportunities to alter the life cycle of metal resources using biotechnology approaches**.

The PI on this DURIP proposal, Scott Banta, is the PI of this funded grant from ARO (W911NF-18-1-0239, Program Officer Dr. Bob Kokoska) which ended May 2021. Professor Alan West in the Department of Chemical Engineering at Columbia University is the co-PI. A new Multi-Mode Plate Reader will significantly augmented all of the Specific Aims of the project.

Specific Aim 1: *To fundamentally characterize with electrochemical and surface-science methods the MIC of metals and alloys by wild type A. ferrooxidans cells.* There has been significant prior work in the mining space to understand the oxidation of certain metal ores and we will expand this research to better understand the oxidation of military-relevant metals and alloys.

Specific Aim 2: *To evaluate chemical additions that would enhance the MIC of metals and alloys that are recalcitrant to oxidation by the bacteria alone*. Chemicals (such as acetone, glycerol, ethylene glycol, etc.) that can enhance MIC by disrupting passivation layers will increase the efficiency of the microbial oxidation of metals and alloys.

Specific Aim 3: *To explore the feasibility of genetically modifying A. ferrooxidans cells to produce additive compounds that would enhance metal and alloy microbial oxidation.* Potential toxicity of the additives will be evaluated and metabolic engineering approaches will be explored to engineer *A. ferrooxidans* cells to produce chemical additives, such as acetone, glycerol or ethylene glycol. The ability of these cells to oxide a range of metals and alloys beyond the capabilities of wild type cells will be evaluated.

All 3 of the specific Aims in this proposal rely on our ability to quantify the optical properties of samples for the evaluation of cell densities, DNA concentrations, protein concentrations, and solution turbidities. We have recently discovered a new aerobic corrosion mechanism where the introduction of pyrite, chloride, and *A. ferrooxidans* cells can lead to thiosulfate production in a low pH environment that can cause the rapid corrosion of stainless steels[2] (**Fig 3**.)

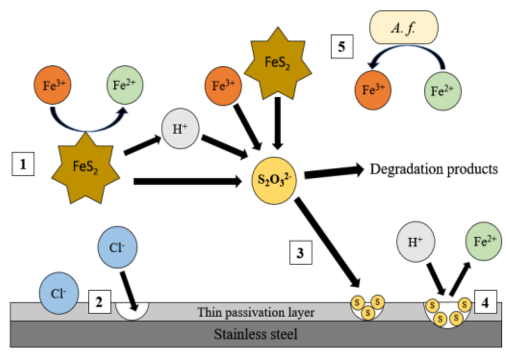

Fig 3: Newly discovered mechanism for the aerobic corrosion of stainless steel[2]. 1. Pyrite is oxidized by ferric ions. 2. chloride can pit the surface. 3. Thiosulfate can oxidize the surface leading to sulfur deposition. 4. Protons can corrode the steel. 5. *A. ferrooxidans* cells regenerate the ferric iron.

Currently many of these measurements are made using a NanoDrop system which allows for small volumes (1-2 μL) and these measurements are typically made one at a time. The two new SpectraMax Quick Drop systems will accelerate this work as well as the new iD5 Microplate Reader, which can use SpectraDrop Micro-volume plates which allow for 64 samples as small as 2 μL to be measured and these Micro-volume plates are compatible with the StakMax automated plate reader. This allows us to **automate the tedious reading of microscale samples**, dramatically increasing research productivity.

In addition, the iD5 Microplate Reader can be used to scan Western blots when the secondary antibody is tagged with a lanthanide. This exploits the TRF capabilities of the system enabling sensitive measurements of proteins in samples. We routinely run Western blots in the Banta Lab and we have scanned them using a system available in another laboratory. **This will allow Western blots to be scanned in the laboratory**, saving time and streamlining our research.

In summary the SpectraMax iD5 Microplate Reader will **dramatically improve our productivity** on this research project as we will be able to automate the reading of small volume samples and we will gain the ability to scan our own Western blots. This is in addition to increasing the access to the microtiter plate for other enzyme kinetic and endpoint measurements.

ARO MURI Project: Bio-inspired Design of Adaptive Catalysis Cascades (W911NF-14-1-0263)

A key feature of cellular metabolism is the use of spatial organization of enzymes in self-assembling complexes termed metabolons[37]. These structures limit distances between active sites and enable substrate channeling, which results in enhanced local substrate concentrations and the protection of liable intermediates[38]. Many groups have begun to explore spatial organization of enzymatic active sites, and thus far it has been clear that spatial organization can lead to enhanced pathway throughput in both natural and synthetic multi-step cascades[39-41]. For example, we have performed **the first protein engineering study** in the citrate synthase/malate dehydrogenase metabolon complex[1] and shown that a single mutation can dramatically decrease the substrate channeling effect[6] (**Fig 4)**. However, these promising results are just the "tip of the iceberg", as further catalytic improvements will be gained with increasing organizational complexity, including the incorporation of novel substrate channeling pathways, electron transfer relays, and metabolons into advanced materials.

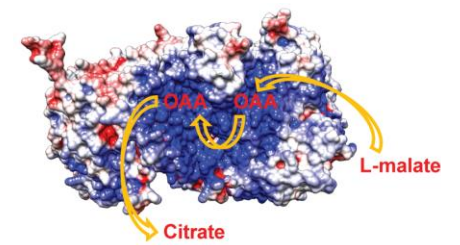

Fig 4. Structure of metabolon formed by citrate synthase and malate dehydrogenase[1]. The structure was determined via cross-linking and mass spectrometry. A single mutations at the protein interface has been shown to dramatically reduce substrate channeling between these enzymes[6].

The PI on this DURIP proposal is a Co-PI and Thrust Leader on an ARO MURI Award "Bio-inspired Design of Adaptive Catalysis Cascades (W911NF-14-0263) (Program Officers, Dr. David Stepp and Dr. Robert Mantz). The PI of the collaborative team is Dr. Shelley Minteer, University of Utah. The other team members involved in this proposal are Dr. Plamen Atanassov from the University of New Mexico, Dr. Scott Calabrese Barton from Michigan State University, Dr. Matthew Sigman from the University of Utah, and Dr. Ian Wheeldon from the University of California, Riverside.

Analysis of natural metabolons provides clear evidence of the importance of spatial organization, in terms of inter-catalyst distance and ratio of catalyst activities, for effective multi-step catalysis. **The focus of Thrust I, led by Prof. Scott Banta, is to develop new nanoscale and biomolecular engineering tools to precisely control the spatial organization of hybrid cascades of inorganic, organo-, and enzyme catalysts and to use these novel catalysts to understand the effects of spatial proximity on cascade catalysis.** The working hypothesis is that effective coupling of a sequential reaction along a cascade requires precise catalyst co-localization.

One of the projects within in the highly successful MURI program is the development of substrate channeling mechanisms that resemble the "swing arms" found in the pyruvate dehydrogenase complex (PDC)[42] which catalyzes the oxidative decarboxylation of pyruvate to acetyl-CoA. We have developed a synthetic analog for cofactor regeneration, where two dehydrogenase enzymes (formate dehydrogenase and malate dehydrogenase, for example) are tethered by the spycatcher/spytag protein capture platform[43, 44]. PEG chains are then used to tether the NAD(H) cofactor to the enzymes[45]. This allows for successive oxidation and reduction reactions to occur without the need to add soluble cofactors, and instead electrons are transferred by the PEG swing arms (**Fig 5**).

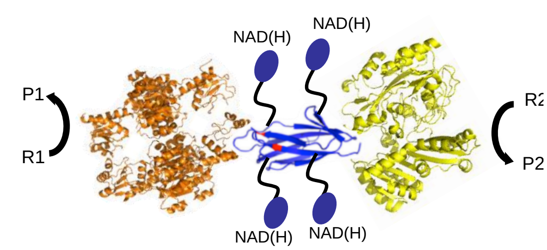

Fig 5: Substrate channeling by synthetic swing arms. NAD(H) is tethered to a designed fusion protein complex containing two different dehydrogenases. When combined electrons can be transferred between active sites without the need for addition cofactor.

We have performed extensive characterization of the steady state kinetics of this newly designed formate:malate oxidoreductase[46]. We found that the enzyme follows a bi bi ping pong mechanism and that the K_M values for the substrates match the K_M values of the parent dehydrogenases. Unfortunately, the k_{cat} value was very low, likely due to limited electron transfer between activite sites due to limited number of cofactor-modified swing arms. We also found that the fusion protein degraded over time leading to a modest total turnover number.

We have **recently greatly improved this result by increasing the number of NAD(H) swing arms** on the protein and localizing them to the middle of the fusion protein where cleavage may be occurring. We increased the potential number of swing arms from 4 up to 7 and found a 6-fold increase in the k_{cat} value and a 50% increase in the total turnover number.

In the coming year, we have applied for Add-On funding to our MURI award to explore native cofactor swing arms. It has been discovered that RNA molecules can be occasionally capped with NAD(H) on their 5' ends[47, 48], and this can be enhanced by some genetic modification[49]. NAD(H)-capped RNAs will be evaluated as potential swing arms and these swing arms could eventually be captured and tethered to fusion proteins in vivo. The SpectraMax iD5 Microplate reader will accelerate this project as we will need to perform extensive enzyme kinetics with the NAD(H)-capped RNA molecules and we will rely on the fluorescence polarization of the cofactors to characterize NAD(H)-capped RNA binding by the proteins. This is a capability we currently do not have in the laboratory.

In summary, the new iD5 Microplate reader **substantially improves our experimental capabilities on this project** as we work to explore whether cofactor-capped RNA molecules could be used to engineer novel substrate channeling mechanisms in living cells. The fluorescence polarization capabilities will be essential for this effort and will dramatically improve our ability to advance this research.

Research Related Education at Columbia University

The Fu Foundation School of Engineering and Applied Science at Columbia University aims to prepare engineering leaders who will solve the problems of the new century, while fostering scientific inquiry but never losing sight of its human implications. The School's programs are designed to produce well-educated engineers who can put their knowledge to work for society. **Through a synergy of teaching and research, the school seeks to educate a distinguished cadre of leaders in engineering and applied science who will thrive in an atmosphere of recently emerging technologies.**

This mission is accomplished in the Department of Chemical Engineering through a curriculum that is grounded in core engineering fundamentals, and augmented with elective courses that offer exposure to cutting-edge technologies and techniques. The new iD5 Miroplate Reader requested in this DURIP proposal will be incorporated into multiple courses including the required Undergraduate Introduction to Chemical Engineering course (CHEN2100), Undergraduate Independent Research courses (CHEN 3900), as well as the Graduate-level Protein Engineering (CHEN4800). In addition, the Microbial Growth and Characterization Suite will be utilized by dozens of undergraduate, graduate, and postdoctoral scholars in the Banta laboratory.

Incorporation of Protein Purification Concepts into the Undergraduate Curriculum

The first required course in the Chemical Engineering Department curriculum is the Introduction to Chemical Engineering course for undergraduate students. The goals of the course include an introduction Chemical Engineering, introduction to engineering calculations, and introduction to material and energy balances. One of the distinguishing features of Chemical Engineering as compared to other related Engineering disciplines is the study of chemical reactions, chemical reactors, and other topics in applied chemistry. The expertise of the PI lies in the Bioengineering area and thus Bioengineering concepts are introduced throughout the semester. For the last several years, a few example problems have been used to guide discussions throughout the semester, and one of the main examples has involved a process where seaweed (kelp) is converted to a biofuel (jet diesel). These discussions include a focus on biological reactions and enzyme kinetics. This includes an explanation of modern laboratory experiments including enzyme assays. The PI has taught this course every Fall semester since 2016, including a remote version in Fall 2020 and a hybrid version in Fall 2021. **Undergraduates in the department of Chemical Engineering benefit from exposure to the SpectraMax iD5 Microplate Reader in the Introduction to Chemical Engineering course.**

Characterization of Recombinant Proteins in the Protein Engineering Graduate Elective

The Protein Engineering graduate elective was offered for the first time by PI at Columbia University in the fall semester of 2005. In the fall of 2006, the course was offered through the Columbia Video Network, which allowed students from around the world to enroll and participate in the class. The course has been offered frequently since then using CVN to broaden the audience for the material. The course is aimed at senior undergraduates and graduate students and it introduces fundamental tools and techniques currently used to engineer protein molecules. The course covers the methods used to analyze the impact of protein mutations on different protein functions, with a specific emphasis on enzymatic catalysis. Throughout the semester specific case studies are used to reinforce the concepts covered in the course, as well as to demonstrate the wide impact of protein engineering research. A significant portion of the course is devoted to the understanding of genetic engineering techniques including recombinant protein expression and characterization. The course was last offered remotely during the Summer of 2021. **The capabilities of the SpectraMax iD5 Microplate Reader are covered in the Protein Engineering class and students will have the opportunity to visit the lab in the future and observe the functions of the equipment in the laboratory thus increasing their exposure to modern protein engineering methodologies.**

Postdoctoral, Graduate, and Undergraduate Research Experiences

Columbia is a world-renowned institute of research excellence. Training opportunities at the postdoctoral, graduate, and undergraduate levels are available in the labs of the PI on this proposal. Due to some recent graduations and a hiring freeze implemented due to the Covid-19 pandemic, the lab group is smaller than normal. Currently, the group includes 2 postdoctoral fellows, 6 graduate students, 1 masters student and 4 undergraduate students, and this will expand further in the future. All of the students in the laboratory will use the SpectraMax iD5 Micorplate Reader, QuickDrop systems, and the microcentrifuge. Many of the projects will be directly related to the grants described above that are of direct interest to the DoD, as well as, other ongoing and future research projects that the new equipment will enable. **These activities will help to train the next generation of experimentally adept and quantitatively oriented investigators.**

Local Middle and High School Students

Over the summers prior to the pandemic, the PI has participated in a Columbia Engineering Outreach lab visitation program called **Inside Engineering**, which is an exciting opportunity both for local schools and faculty members to reinforce and reinvigorate the K12 science curriculum. The program is aligned with Common Core State standards and meets a critical need for more math and science field trips, especially relevant for NYC public schools serving under-represented minorities and other high need communities. Inside Engineering is partnering with *Explorable Places*, an educational technology start-up company dedicated to connecting educators with meaningful experiential learning opportunities to take the program to scale and reach more under-represented minority students around New York City. Every June, for the last four years, 6th grade students from Mott Hall II visited the PI's lab and participated in DNA extraction experiments. The students are shown how DNA is quantified in the laboratory using a microplate reader. **This program will be expanded to include additional schools in the year going forward.**

The PIs will also participate in the **HYPOTHEkids (HK) Maker Lab** summer program at Columbia University. The Hk Maker Lab is an intensive six-week summer program to learn the foundations of design. Focusing on addressing a health problem, participants design, prototype and test a biomedical device and develop an associated business plan. The program culminates in a presentation to leading executives from the biomedical community. The ultimate goal of the Hk Maker Lab projects is to have them be incubated using the state-of-the-art facilities at Harlem Biospace. In previous summers, the PI hosted two local female minority high school students in his lab as part of the HK Maker Lab program. **The PI plans to host additional students through this program going forward and the students will use the SpectraMax 1D5 microplate reader as part of their summer research.**

References

1. Wu, F. and S. Minteer, *Krebs cycle metabolon: structural evidence of substrate channeling revealed by cross-linking and mass spectrometry.* Angew Chem Int Ed Engl, 2015. **54**(6): p. 1851-4.
2. Inaba, Y., et al., *Microbially Influenced Corrosion of Stainless Steel by Acidithiobacillus ferrooxidans Supplemented with Pyrite: Importance of Thiosulfate.* Appl Environ Microbiol, 2019. **85**(21).
3. Powers, J.L., et al., *Lactate dehydrogenase kinetics and inhibition using a microplate reader.* Biochemistry and Molecular Biology Education, 2007. **35**(4): p. 287-292.
4. Rogers, A., *Enzyme Kinetics: Theory and Practice*. 2009, Springer. p. 71-103.
5. Crouch, S.R., et al., *Kinetic Determinations and Some Kinetic Aspects of Analytical Chemistry.* Analytical Chemistry, 1998. **70**(12): p. 53-106.
6. Bulutoglu, B., et al., *Direct Evidence for Metabolon Formation and Substrate Channeling in Recombinant TCA Cycle Enzymes.* ACS Chem Biol, 2016. **11**(10): p. 2847-2853.
7. Ford, S.R., *Improvements in the Application of Firefly Luciferase Assays*. 1998, Humana Press. p. 3-20.
8. Ergin, E., et al., *Time-Resolved Fluorescence Resonance Energy Transfer [TR-FRET] Assays for Biochemical Processes.* Current Pharmaceutical Biotechnology, 2016. **17**(14): p. 1222-1230.
9. Wheeldon, I.R., S.C. Barton, and S. Banta, *Bioactive proteinaceous hydrogels from designed bifunctional building blocks.* Biomacromolecules, 2007. **8**(10): p. 2990-4.
10. Shore, J.D., et al., *NADH binding to porcine mitochondrial malate dehydrogenase.* Journal of Biological Chemistry, 1979. **254**(18): p. 9059-9062.

11. Campbell, E., I.R. Wheeldon, and S. Banta, *Broadening the cofactor specificity of a thermostable alcohol dehydrogenase using rational protein design introduces novel kinetic transient behavior.* Biotechnol Bioeng, 2010. **107**(5): p. 763-74.
12. Kull, F.J. and S.A. Endow, *Force generation by kinesin and myosin cytoskeletal motor proteins.* J Cell Sci, 2013. **126**(Pt 1): p. 9-19.
13. Hess, H., *Engineering applications of biomolecular motors.* Annu Rev Biomed Eng, 2011. **13**: p. 429-50.
14. Butler, P.J., K.K. Dey, and A. Sen, *Impulsive Enzymes: A New Force in Mechanobiology*. Cellular and molecular bioengineering, 2015. **8**(1): p. 106-118.
15. Zhao, X., et al., *Powering Motion with Enzymes.* Accounts of Chemical Research, 2018. **51**(10): p. 2373-2381.
16. Wang, N., *Review of Cellular Mechanotransduction.* Journal of physics D: Applied physics, 2017. **50**(23): p. 233002.
17. Ingber, D.E., N. Wang, and D. Stamenovic, *Tensegrity, cellular biophysics, and the mechanics of living systems.* Rep Prog Phys, 2014. **77**(4): p. 046603.
18. Hu, X., et al., *Molecular stretching modulates mechanosensing pathways.* Protein Sci, 2017. **26**(7): p. 1337-1351.
19. Zocchi, G., *Controlling Proteins Through Molecular Springs.* Annual Review of Biophysics, 2009. **38**(1): p. 75-88.
20. Liu, M., et al., *A DNA tweezer-actuated enzyme nanoreactor.* Nature Communications, 2013. **4**: p. 2127.
21. Zhou, C., Z. Yang, and D. Liu, *Reversible Regulation of Protein Binding Affinity by a DNA Machine.* Journal of the American Chemical Society, 2012. **134**(3): p. 1416-1418.
22. Funke, J.J. and H. Dietz, *Placing molecules with Bohr radius resolution using DNA origami.* Nature Nanotechnology, 2015. **11**: p. 47.
23. Nickels, P.C., et al., *Molecular force spectroscopy with a DNA origami-based nanoscopic force clamp.* Science, 2016. **354**(6310): p. 305-307.
24. Machielsen, R., et al., *Production and characterization of a thermostable alcohol dehydrogenase that belongs to the aldo-keto reductase superfamily.* Appl Environ Microbiol, 2006. **72**(1): p. 233-8.
25. Solanki, K., W. Abdallah, and S. Banta, *Extreme makeover: Engineering the activity of a thermostable alcohol dehydrogenase (AdhD) from Pyrococcus furiosus.* Biotechnol J, 2016. **11**(12): p. 1483-1497.
26. Choi, Y., et al., *DNA Origami-Based Forster Resonance Energy-Transfer Nanoarrays and Their Application as Ratiometric Sensors.* ACS Appl Mater Interfaces, 2018. **10**(27): p. 23295-23302.
27. Müller, B.K., et al., *Single-Pair FRET Characterization of DNA Tweezers.* Nano Letters, 2006. **6**(12): p. 2814-2820.
28. Rawlings, D.E. and S. Silver, *Mining with Microbes.* Nat Biotech, 1995. **13**(8): p. 773-778.
29. Rawlings, D.E., *Heavy metal mining using microbes.* Annu Rev Microbiol, 2002. **56**: p. 65-91.
30. Li, K., M. Whitfield, and J. Van Vliet Krystyn, *Beating the bugs: roles of microbial biofilms in corrosion*, in *Corrosion Reviews*. 2013. p. 73.

31. Li, X., et al., *Addition of citrate to Acidithiobacillus ferrooxidans cultures enables precipitate-free growth at elevated pH and reduces ferric inhibition.* Biotechnol Bioeng, 2014. **111**(10): p. 1940-8.
32. Li, X., et al., *Engineering Acidithiobacillus ferrooxidansgrowth media for enhanced electrochemical processing.* AIChE Journal, 2014. **60**(12): p. 4008-4013.
33. Kernan, T., et al., *Engineering the iron-oxidizing chemolithoautotroph Acidithiobacillus ferrooxidans for biochemical production.* Biotechnol Bioeng, 2016. **113**(1): p. 189-97.
34. Li, X., A.C. West, and S. Banta, *Enhancing isobutyric acid production from engineered Acidithiobacillus ferrooxidans cells via media optimization.* Biotechnol Bioeng, 2016. **113**(4): p. 790-6.
35. Kernan, T., A.C. West, and S. Banta, *Characterization of endogenous promoters for control of recombinant gene expression in A. ferrooxidans.* Biotechnol Appl Biochem, 2017: p. (In Press).
36. Guan, J., et al., *Development of Reactor Configurations for an Electrofuels Platform Utilizing Genetically Modified Iron Oxidizing Bacteria for the Reduction of CO2 to Biochemicals.* Journal of Biotechnology, 2017: p. (In Press).
37. Srere, P.A., *The Metabolon.* Trends Biochem. Sci., 1985. **10**(3): p. 109-110.
38. Wheeldon, I., et al., *Substrate channelling as an approach to cascade reactions.* Nat Chem, 2016. **8**(4): p. 299-309.
39. Moehlenbrock, M.J., et al., *Metabolon catalyzed pyruvate/air biofuel cell.* J Am Chem Soc, 2010. **132**(18): p. 6288-9.
40. Liu, F., S. Banta, and W. Chen, *Functional assembly of a multi-enzyme methanol oxidation cascade on a surface-displayed trifunctional scaffold for enhanced NADH production.* Chem Commun (Camb), 2013. **49**(36): p. 3766-8.
41. Lin, J.-L. and I. Wheeldon, *Kinetic Enhancements in DNA–Enzyme Nanostructures Mimic the Sabatier Principle.* ACS Catalysis, 2013. **3**(4): p. 560-564.
42. Zhou, Z.H., et al., *The remarkable structural and functional organization of the eukaryotic pyruvate dehydrogenase complexes.* Proceedings of the National Academy of Sciences, 2001. **98**(26): p. 14802-14807.
43. Reddington, S.C. and M. Howarth, *Secrets of a covalent interaction for biomaterials and biotechnology: SpyTag and SpyCatcher.* Curr Opin Chem Biol, 2015. **29**: p. 94-9.
44. Zakeri, B., et al., *Peptide tag forming a rapid covalent bond to a protein, through engineering a bacterial adhesin.* Proc Natl Acad Sci U S A, 2012. **109**(12): p. E690-7.
45. Ozbakir, H.F. and S. Banta, *Kinetic and transport effects on enzymatic biocatalysis resulting from the PEGylation of cofactors.* AIChE Journal, 2018. **64**(1): p. 12-17.
46. Ozbakir, H.F., K.E. Garcia, and S. Banta, *Creation of a formate: malate oxidoreductase by fusion of dehydrogenase enzymes with PEGylated cofactor swing arms.* Protein Eng Des Sel, 2018. **31**(4): p. 103-108.
47. Bird, J.G., et al., *The mechanism of RNA 5′ capping with NAD+, NADH and desphospho-CoA.* Nature, 2016. **535**(7612): p. 444-447.
48. Chen, Y.G., et al., *LC/MS analysis of cellular RNA reveals NAD-linked RNA.* Nat Chem Biol, 2009. **5**(12): p. 879-881.
49. Cahova, H., et al., *NAD captureSeq indicates NAD as a bacterial cap for a subset of regulatory RNAs.* Nature, 2015. **519**(7543): p. 374-377.

FEDERAL FINANCIAL REPORT

(Follow form instructions)

1. Federal Agency and Organizational Element to Which Report is Submitted OFC OF NAVAL RESEARCH BOSTON REGIONAL OFFICE 495 SUMMER ST ROOM 617 BOSTON, MA 02210-2109	2. Federal Grant or Other Identifying Number Assigned by Federal Agency (To report multiple grants, use FFR Attachment) W911NF2110039	Page 1	of pages

3. Recipient Organization (Name and complete address including Zip code)
THE TRUSTEES OF COLUMBIA UNIVERSITY OFFICE OF THE CONTOLLLER, SPF
615 WEST 131ST STREET, 4TH FLOOR
NEW YORK, NY 10027

4a. DUNS Number	4b. EIN	5. Recipient Account Number or Identifying Number	6. Report Type	7. Basis of Accounting
04-917-9401	13-5598093	GG017048	Final	X Cash ☐ Accrual

8. Project/Grant Period From: (Month, Day, Year)	To: (Month, Day, Year)	9. Reporting Period End Date (Month, Day, Year)
'01/01/2021	12/31/2021	12/31/2021

10. **Transactions**	Cumulative
(Use lines a-c for single or multiple grant reporting)	
Federal Cash (To report multiple grants, also use FFR Attachment):	
a. Cash Receipts	$86,846.53
b. Cash Disbursements	$86,846.53
c. Cash on Hand (line a minus b)	$0.00
(Use lines d-o for single grant reporting)	
Federal Expenditures and Unobligated Balance:	
d. Total Federal funds authorized	$88,319.00
e. Federal share of expenditures	$86,846.53
f. Federal share of unliquidated obligations	$0.00
g. Total Federal share (sum of lines e and f)	$86,846.53
h. Unobligated balance of Federal funds (line d minus g)	$1,472.47
Recipient Share:	
i. Total recipient share required	$0.00
j. Recipient share of expenditures	$0.00
k. Remaining recipient share to be provided (line i minus j)	$0.00
Program Income:	
l. Total Federal program income earned	$0.00
m. Program income expended in accordance with the deduction alternative	$0.00
n. Program income expended in accordance with the addition alternative	$0.00
o. Unexpended program income (line l minus line m or line n)	$0.00

11. Indirect Expense	a. Type	b. Rate	c. Period From	Period To	d. Base	e. Amount Charged	f. Federal Share
	N/A	0.00%				$0.00	$0.00
				g. Totals:			

12. *Remarks: Attach any explanations deemed necessary or information required by Federal sponsoring agency in compliance with governing legislation:*

IC was not charged to project. Prepared by: Raquel Ferrer - If you have any questions email rf2316@columbia.edu or call 212 854-1038.

13. Certification: By signing this report, I certify that it is true, complete, and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent information may subject me to criminal, civil, or administrative penalities. (U.S. Code, Title 218, Section 1001)

a. Typed or Printed Name and Title of Authorized Certifying Official Lorraine Ramos Associate Director SPF	c. Telephone (Area code, number and extension) (212) 854-1038 d. Email address rf2316@columbia.edu
b. Signature of Authorized Certifying Official ST Lorraine Ramos Digitally signed by Lorraine Ramos Date: 2022.03.28 17:12:20 -04'00'	e. Date Report Submitted (Month, Day, Year) 03/25/2022

Standard Form 425
OMB Approval Number: 0348-0061
Expiration Date: 10/31/2011