Methylene Blue as a Means to Bypass the Compromised Complex I in Mitochondrial Disease

Grant Proposal

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Author Note

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Abstract (RQ) or Executive Summary (Eng)

Perioperative treatment of mitochondrial disease (MD) patients is a delicate matter. Pre-existing symptoms due to the nature of the disease can make otherwise moderately dangerous situations, like anesthetic infusion, particularly hazardous. For MD patients with a Complex I mutation, Complex I-inhibiting anesthetics like propofol can further compromise the Electron Transport Chain (ETC), thereby impairing the system by which ATP is produced; this can lead to acute tissue necrosis, organ systems failure, and even death in more severe cases. In this study, a *C. elegans* model of the patient population was treated with a Complex I inhibitor, Rotenone, to simulate anesthetic infusion. Half of the worms were treated with an alternative electron carrier, methylene blue (MB), prior to being exposed to Rotenone in order to determine MB's potential as a preventative treatment for MD patients forced to undergo surgery. In MB samples, the reduction of cytochrome c oxidase was significantly greater than in non-MB samples, thus suggesting that the methylene blue effectively bypassed the damaged Complex I and allowed the ETC to function normally. The reduction reaction of cytochrome c oxidase was compared to two baselines: untreated C. elegans mitochondria, and a reduction reaction prepared with dithiothreitol (DTT). In Rotenone-treated samples of C. elegans, those preemptively infused with MB showed greater peaks at the Soret band, alpha-band, and beta-band, indicating greater proportions of successfully reduced cytochrome c as a result of methylene blue.

Keywords: Electron Transport Chain, mitochondrial disease, methylene blue, anesthetics, surgery

Full Title

Mitochondrial Diseases

Mitochondrial diseases are a symptomatically diverse group of disorders characterized by mitochondrial dysfunction. The mitochondria are primarily responsible for energy production through oxidative phosphorylation and are instrumental to many of the metabolic pathways in the human body. Mitochondrial diseases are most commonly caused by genetic mutations in either mitochondrial DNA (mtDNA) or nuclear DNA (nDNA), consequently impairing the mitochondrial respiratory chain, which generates ATP (Gorman & Chinnery, 2016). Bodily functions, ranging from the conduction of nerve impulses to the regeneration of damaged tissue, all rely upon ATP, so it is understandable that a lack thereof would be detrimental to a patient's health. Mitochondrial diseases

affect virtually every tissue in the body, particularly attacking those with high energy demands, such as the brain, heart, and muscle (Sasano, 2007). Clinical manifestations vary greatly depending on the type and severity of the condition but can range from mild muscle weakness to neurodegeneration and multi-organ failure. Effective therapeutic strategies remain limited, highlighting the urgent need for new approaches to treatment and management despite advances in understanding the genetic and biochemical bases of these diseases (Gorman & Chinnery, 2016).

Complex I Inhibition & Anesthetics

Inhibition of Complex I of the Electron Transport Chain is a hallmark of several mitochondrial diseases. Complex I catalyzes the oxidation of NADH oxidoreductase, reducing ubiquinone to ubiquinol and generating a proton gradient across the inner mitochondrial membrane (Sharma, et al., 2009). This gradient allows protons to flow back through the mitochondrial matrix via ATP synthase, which provides the energy necessary to combine ADP and inorganic phosphate (Pi) into ATP in later complexes (Pereira, et al., 2023). Inhibition of Complex I not only compromises the rest of the process of mitochondrial respiration but enhances the generation of reactive oxygen species (ROS), known proponents of tissue damage (Sharma, 2007). Leaving Complex II as the only functional gateway to the rest of the Electron Transport Chain reduces the number of electrons eventually transported to Complex III.

Certain substances, such as rotenone and paraquat, can integrate easily with the mitochondrial membrane, granting them increased access to ubiquinone binding sites. These compounds interfere with electron transport by either directly blocking electron flow or prematurely diverting electrons, effectively truncating the pathway. As a result, oxidative phosphorylation is disrupted, directly leading to decreased ATP production. Additionally, since electrons are forced out of the Electron Transport Chain, they can interact prematurely with oxygen outside of the system and lead to an accumulation of reactive oxygen species (ROS). The buildup of ROS further contributes to cellular damage and oxidative stress, which are closely linked to various neurodegenerative disorders and other metabolic diseases. The presence of foreign bodies at this crucial point of the Electron Transport Chain has the potential to disrupt ATP production, leading to symptoms that mimic mitochondrial dysfunction, including fatigue, neurodegeneration, and metabolic imbalances (Pereira, 2023).

Popular general anesthetics such as isoflurane have been shown to inhibit Complex I in a similar manner, raising a concern about surgical practices with regard to mitochondrial disease patients (Vanlander, 2012). The anesthetic's inhibitory properties may exacerbate existing symptoms in those with pre-existing mitochondrial dysfunction, leading to prolonged post-operation recovery time, worsened neurological symptoms, organ failure, and even sudden death. Understanding the interactions between anesthetic agents and the Electron Transport Chain is crucial for improving surgical protocol for mitochondrial disease patients (Sasano, 2007).

Anti-Inhibitory Properties of Methylene blue

Methylene blue (MB), a popular tissue stain, has been at the forefront of mitochondrial disease research in recent years. MB can interact with components of the mitochondrial electron transport chain by donating electrons; it has been shown to bypass Complex I by acting as an alternative electron carrier (Gureev, 2022). If Complex I was already damaged from a pre-existing disease, treatment with MB would be able to restore some degree of mitochondrial function (Lee & Boelsterli, 2014). This property renders MB a strong candidate for clinical application in mitochondrial diseases, and as a preventative measure for such patients exposed to Complex I inhibitory substances. Recent studies have also indicated that MB offers neuroprotective, cardioprotective, and cytoprotective benefits, making it even more appealing an option to treat mitochondrial disease, which so often damages the brain, heart, and cells due to their high energy demands (Lee & Boelsterli, 2014).

Study

If proven effective, MB therapy has the potential to offer a robust preventative measure for mitochondrial disease patients in the perioperative setting. This proposal aims to investigate the inhibitory properties of isoflurane on Complex I activity and the potential of MB as a therapeutic drug for Complex I-related mitochondrial diseases. Rotenone, which inhibits Complex I via the ubiquinone-binding sites, mimics the behavior of isoflurane in isolated *C. elegans* mitochondria, and will therefore be used as the inhibitory treatment. For two treatment groups – with versus without MB – spectrophotometric analysis of enzyme absorption in Complex III will be conducted. In Complex III, cytochrome c is reduced by electrons carried by ubiquinol. If electrons are successfully diverted from the compromised Complex I, Complex III activity in the MB group will likely respond with increased activity as measured through reduced cytochrome c. Greater proportions of cytochrome c reductase in inhibited samples treated

with MB are anticipated when measured at 550 nm for maximized absorbance (Vanlander, 2012). Given additional time, the enzymatic assay will also be performed on samples of mitochondria extracted from *C. elegans* mutated for mitochondrial myoencephalopathy, strain LB10, to provide more realistic insight into MB's uses in the specific patient population.

Section II: Specific Aims

This proposal's objective is to evaluate whether methylene blue has the potential to prevent severe metabolic side-effects in mitochondrial disease patients treated with volatile anesthetics.

Specific Aim 1: to establish the baseline absorption of reduced cytochrome c in unmutated, untreated *C. elegans* mitochondria.

Specific Aim 2: to observe the effects of methylene blue on unmutated *C. elegans* mitochondria through the absorption of reduced cytochrome c.

Specific Aim 3: to determine the difference between Complex I inhibited *C. elegans* mitochondria treated with MB versus Complex I inhibited mitochondria treated without MB.

Treatment with methylene blue is intended as a replacement for Complex I-mediated electron transport. For this reason, we expect that absorption of cytochrome c reductase will stay roughly the same in MB-treated mitochondria when compared to the baseline absorption of reduced cytochrome c in untreated mitochondria. Because rotenone disables the function of Complex I and MB is expected to compensate for this loss, the absorption of reduced cytochrome c in the MB-treated rotenone samples at benchmark absorption peaks are expected to be greater than the absorption in the rotenone samples without MB. Over a 12-minute reaction observed at 3 minute intervals, the following should be observed if cytochrome c oxidase is fully reduced: The Soret band should shift positively, from 409 nm to 414 nm, and increase in magnitude; the α-band should shift negatively, from 530 nm to 520 nm; the β-band should increase greatly in magnitude.

Section III: Project Goals and Methodology

Methodology

Mitochondrial Isolation

In order for the cytochrome c assay to accurately reflect the activity of the mitochondrial Electron Transport Chain, purified mitochondria must first be isolated from the required samples of *C. elegans*. This is done through harvesting the worms, centrifuging them, and lysing the resultant pellet in a mitochondrial isolation buffer. Using a Dounce homogenizer or a similar implement, the nematodes are mechanically lysed. The cellular material undergoes differential centrifugation in order to purify the mitochondrial pellet.

Spectrophotometric Analysis

In order to determine the cytochrome c reductase activity in each treatment group, a spectrophotometric assay is performed on purified samples of mitochondria. Purified mitochondrial pellets are suspended in a reaction buffer containing cytochrome c oxidase (Sigma-Aldrich, 2019). Reduction of cytochrome c is observed over a 12-minute reaction, with measurements taken every 3 minutes. Special attention is paid to benchmark absorption points, such as the Soret band, α-band, and β-band.

Specific Aim #1: to establish the baseline absorption of reduced cytochrome c in unmutated, untreated *C. elegans* mitochondria.

Justification and Feasibility. Cytochrome c, the protein reduced in Complex III of the electron transport chain, displays distinct absorption peaks in its reduced state, which can be reliably measured using spectrophotometry at 550 nm (Hollis, 2003). Measuring these peaks in the unmutated and untreated *C. elegans* mitochondria will provide a baseline for understanding how various treatments and mutations affect the interactions between components of the electron transport chain.

Summary of Preliminary Data.

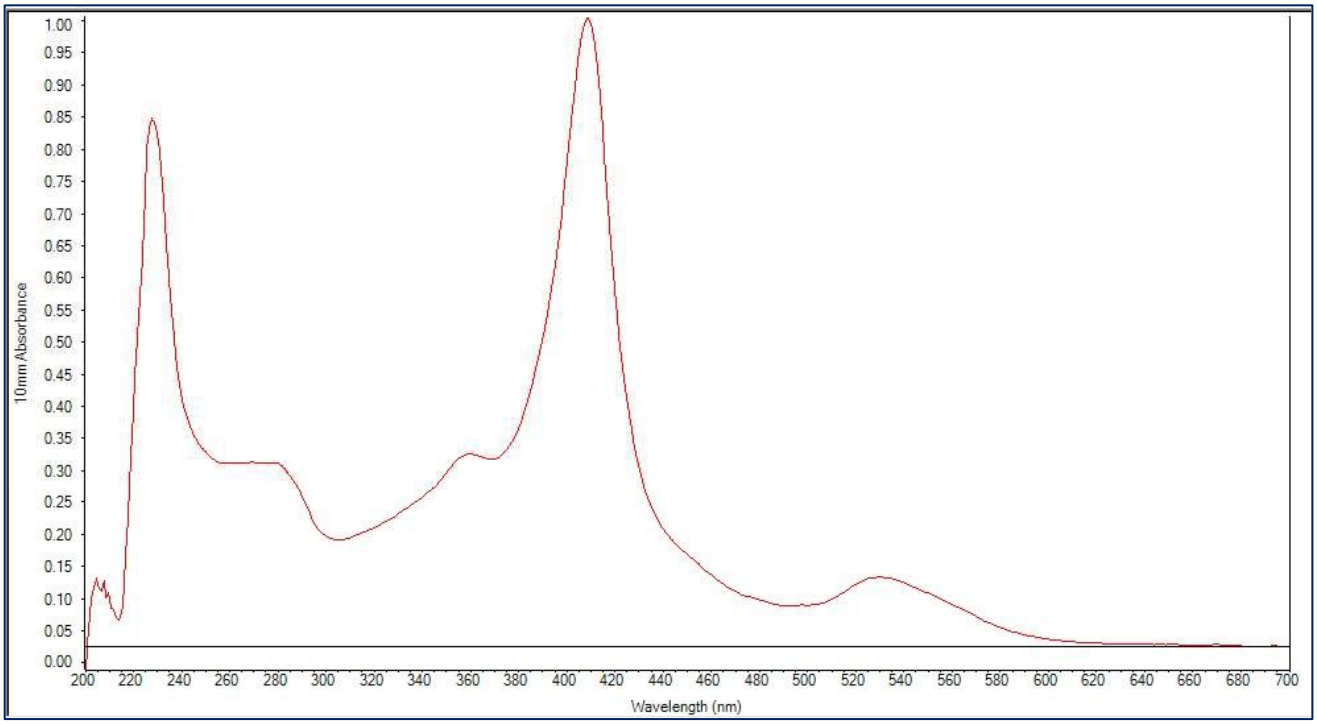


Figure 1: The full reduction reaction observed on untreated and unmutated *C. elegans*

Figure 1 shows cytochrome c oxidase in its original form, meaning that no reduction took place over the 12-minute observation period. Note the Soret band at 409 nm, α-band at 530 nm, and weak β-band at 550 nm, all measurements conducive with the absorption peaks of cytochrome c oxidase. These results suggest a lack of mitochondrial material in the sample used.

Expected Outcomes. While this outcome failed to establish a baseline as intended, it verified a previous hypothesis that using a Fly Smasher is an ineffective proxy for a Dounce homogenizer in the mitochondrial purification step. Running the cytochrome c assay with dithiothreitol to observe a full reduction reaction, however, verified that the assay itself is effective and that the errors were due to the sample (Appendix I & II).

Potential Pitfalls and Alternative Strategies. We expect that running the same conditions but with a proper Dounce homogenizer will yield more useful results in the future. Isolation of mitochondria is a crucial step in the experimental process, so it is highly likely that the failure to execute it effectively is responsible for the inconclusive data.

Specific Aim #2: to observe the effects of methylene blue on unmutated *C. elegans* mitochondria through the absorption of reduced cytochrome c.

Justification and Feasibility. Methylene blue (MB) has been shown to influence mitochondrial function through its ability to receive electrons from NADH and carry them to Complex III; it can thus be inferred that MB has the potential to directly affect the amount of cytochrome c that is reduced, particularly in mitochondrial disease patients, by increasing the flow of electrons to Complex III (Yang, et al., 2017). Observing the absorption of reduced cytochrome c after MB treatment in unmutated *C. elegans* mitochondria provides insights into how MB modulates mitochondrial redox states. This aim is justified by prior studies that establish MB as a redox agent capable of bypassing Complex I and influencing the electron flow.

Summary of Preliminary Data.

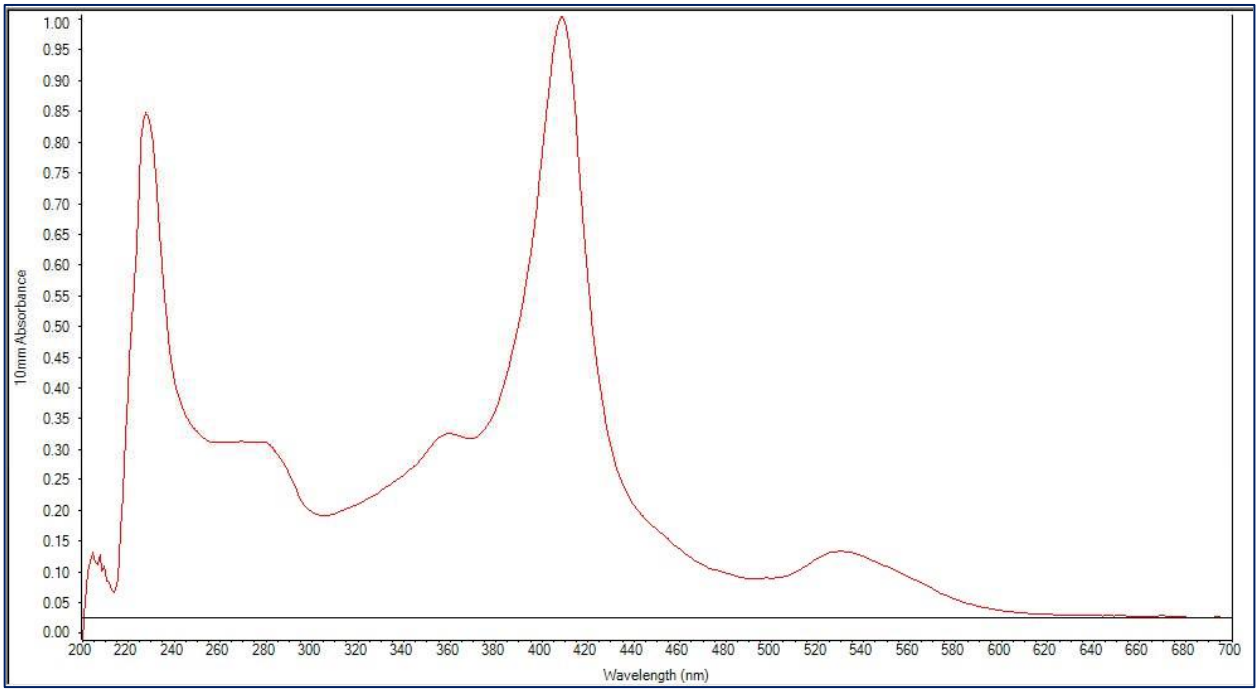


Figure 2: The full reduction reaction observed on MB-treated, unmutated *C. elegans*

Expected Outcomes. Similar to Specific Aim #1, this outcome failed to establish a baseline as intended. Running the cytochrome c assay with dithiothreitol to observe a full reduction reaction, however, verified that the assay itself is effective and that the errors were due to the sample (Appendix I & II).

Potential Pitfalls and Alternative Strategies. We expect that running the same conditions but with a proper Dounce homogenizer will yield more useful results in the future. Isolation of mitochondria is a crucial step in the experimental process, so it is highly likely that the failure to execute it effectively is responsible for the inconclusive data.

Specific Aim #3: to determine the difference between Complex I inhibited *C. elegans* mitochondria treated with MB versus Complex I inhibited mitochondria treated without MB.

Justification and Feasibility. Comparing the effects of MB on Complex I-inhibited *C. elegans* mitochondria versus untreated mitochondria requires examining how MB compensates for impaired electron flow. Complex I inhibitors, such as rotenone and isoflurane, disrupt ubiquinone-binding sites and causing electron leakage, leading to increased reactive oxygen species (ROS) and reduced mitochondrial efficiency (Pereira, et al., 2023). MB, as a redox-active compound, can bypass Complex I inhibition by accepting electrons and facilitating their transfer directly to downstream components like cytochrome c.

Summary of Preliminary Data. This data is yet to be obtained.

Expected Outcomes. We anticipate that the baseline absorption index for mutated *C. elegans* samples will yield lesser absorption than the unmutated samples. Due to MB's properties as an electron carrier, we expect that MB-treated samples will show greater absorption than untreated samples in the mutated nematode population.

Potential Pitfalls and Alternative Strategies. Equipment procurement is a critical step in this experiment, as shown through the faulty data obtained by using a Fly Smasher in lieu of a Dounce homogenizer. Additionally, it is important to ensure that sample sizes are large enough to transfer from the centrifuge tubes to the spectrophotometer cuvettes without losing any organic material.

Section III: Resources/Equipment

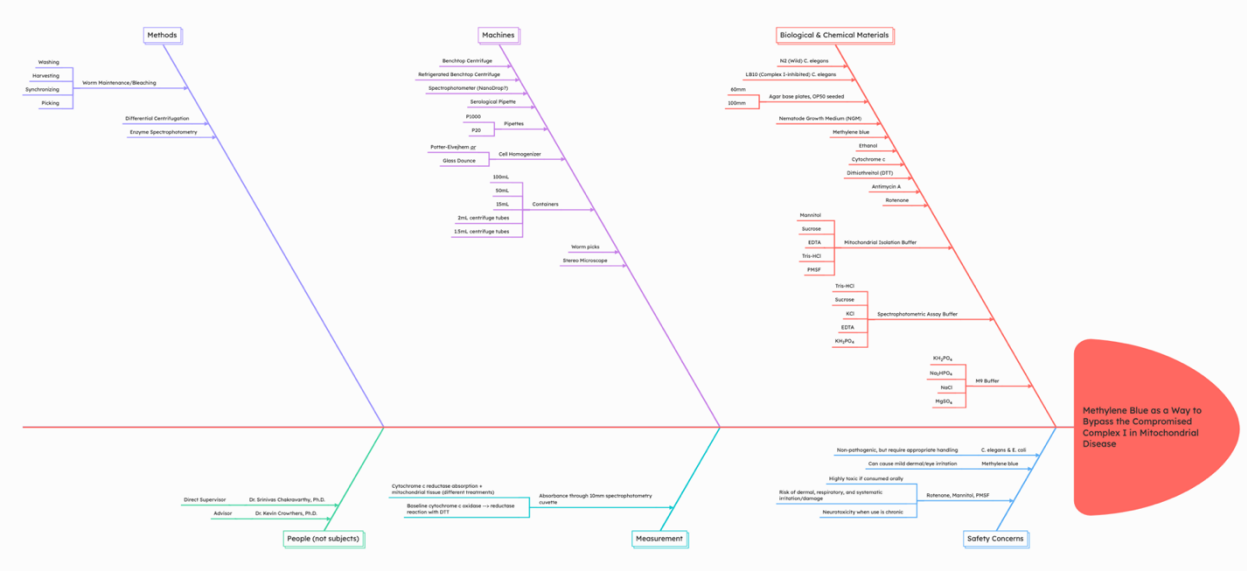


Figure 3: Full mindmap of preliminary data procedure, including all resources/equipment used

Link to webview of mindmap

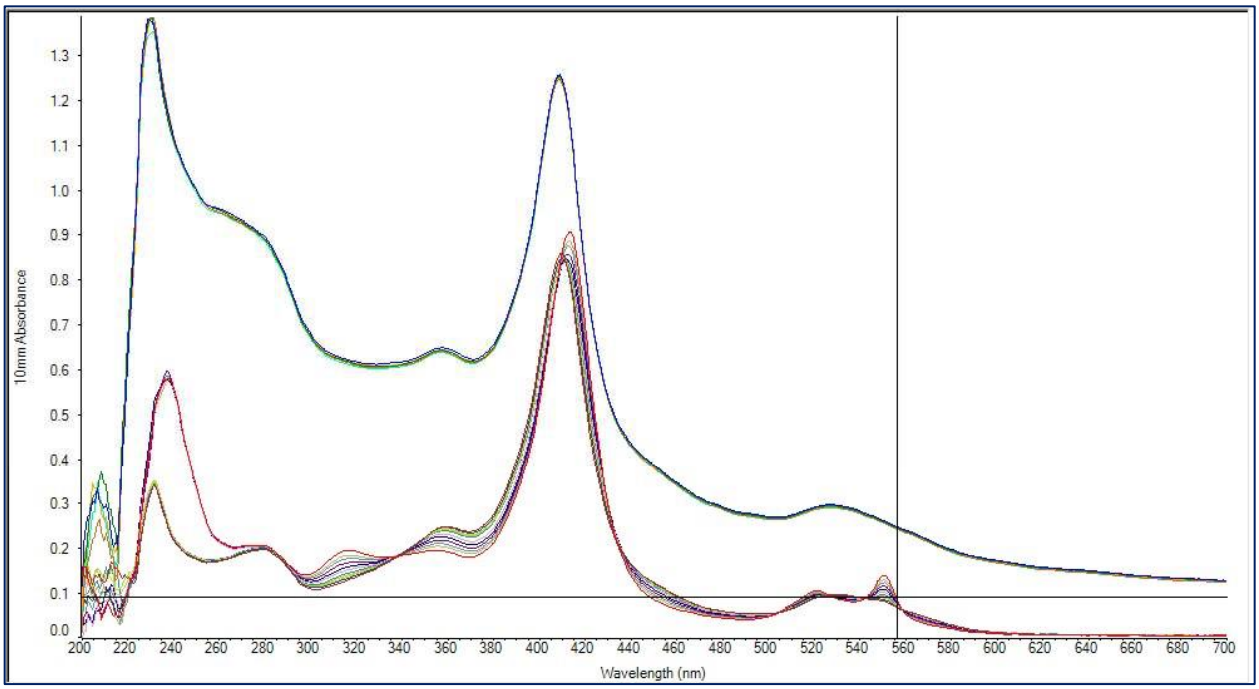
Section V: Ethical Considerations

No special ethical considerations were made in light of this project.

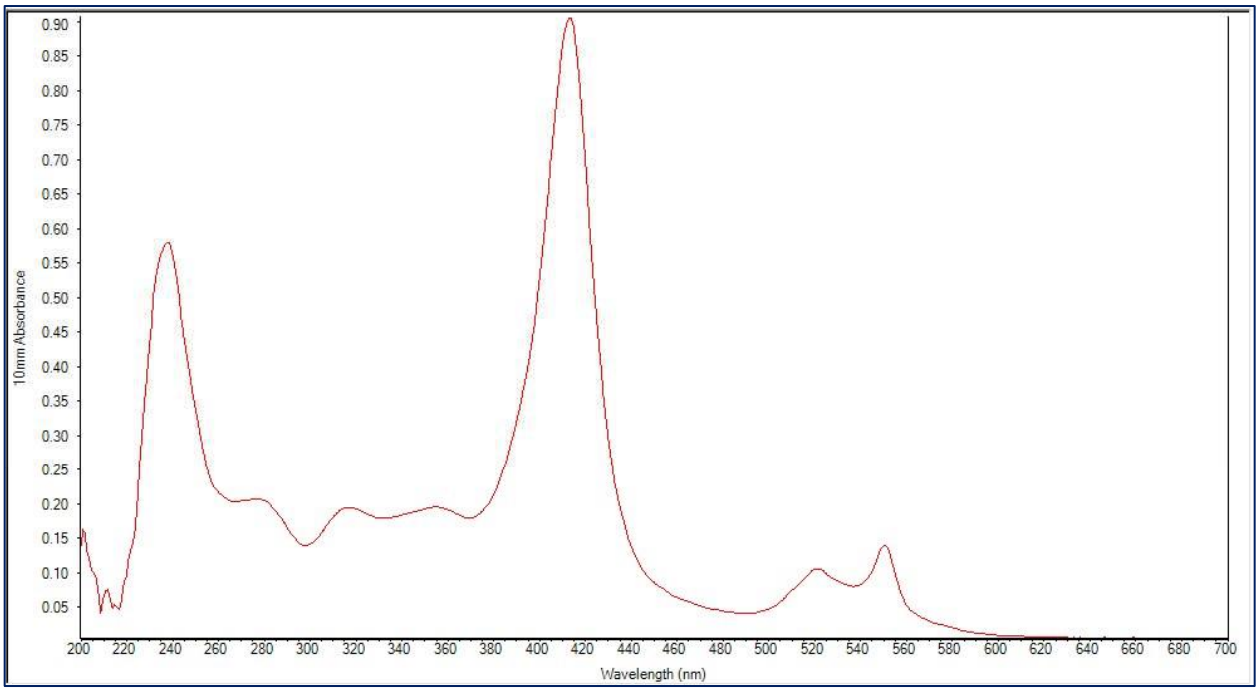
Section VI: Timeline

Link to full Timeline in Gantt Chart form

Section VII: Appendix



Appendix I: The full, 12-minute reduction reaction of cytochrome c oxidase to cytochrome c reductase as a result of dithiothreitol (DTT)



Appendix II: The final measurement of the cytochrome c oxidase to cytochrome c reductase reaction from DTT, taken at minute 12. Note that the Soret band has shifted to 414 nm, the α-band to 520 nm, β-band at 550 nm has increased in magnitude.

Section VIII: References

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