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How mtDNA mutations cause mitochondrial disease

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**14. ABSTRACT**

We sought to develop *C. elegans* as a model system to study how mutant mtDNA levels can vary across different cell types. We have overcome technical hurdles and have optimized a protocol pipeline to isolate cells, FACS sort them, and perform ddPCR. We have optimized this pipeline for muscle cells, neurons, and intestinal cells, across three different heteroplasmic mutant mtDNA. Our pipeline demonstrates proof of concept. It can be applied to measure mutant mtDNA levels from any cell type that can be labeled with GFP. While our work in done using *C. elegans* as a model system, theoretically, our pipeline can be used in other systems including mammalian systems. Additionally, we observe lower mutant mtDNA levels in neurons and muscles compared to the rest of the somatic tissues, suggesting active regulation of mutant mtDNA levels in somatic cells. Finally, we also observe a small but significant decrease in mutant mtDNA levels in neurons as a function of age. Overall, our data suggest that mutant mtDNA levels change in a dynamic manner in *C. elegans*.

**15. SUBJECT TERMS**
mtDNA, mitochondria, heteroplasmy, cell type, FACS, ddPCR

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## INTRODUCTION

Mutations in the mitochondrial genome (mtDNA) cause devastating diseases with a myriad of clinical features. Tissues with high energetic demands including the brain, liver, muscle, and heart are particularly vulnerable to mtDNA mutations. Majority of mtDNA diseases are caused by heteroplasmic mutations, Heteroplasmy refers to a state in which mutant mtDNA coexists with wildtype mtDNA in the same cell. Heteroplasmic mtDNA mutations cause disease then their level exceeds a critical threshold (typically 60-80%). While it is known that mutant mtDNA levels can vary between tissues, the underlying mechanisms are not known. In this project, we seek to develop *C. elegans* as a model system to study how mutant mtDNA levels can vary across different cell types. Below I detail the progress we have made on this project.


## KEYWORDS

mtDNA, mitochondria, heteroplasmy, cell types, FACS, droplet digital PCR (ddPCR)


## ACCOMPLISHMENTS

### Specific Aim 1: Determine cell type specific differences in mutant mtDNA heteroplasmy levels

*Subtask 1: Cross three heteroplasmic strains (uaDf5, mptDf2, mpt1) into transgenic lines in which specific cell types (neurons, muscles, epithelial like seam cells, phagocytic coelomocytes, and intestinal cells) are fluorescently labeled with GFP. Timeline: Months 1-2.*

We have successfully crossed three heteroplasmic strains into transgenic lines in which neurons, muscles and intestinal cells are fluorescently labeled. Furthermore, we discovered that our downstream protocols work better in animals without the germline. We believe this is because the germline has a lot of organellar debris that interfere with cell sorting. Consequently, we crossed in *glp-1* mutation into our strains, which prevents the development of the germline. We decided against crossing heteroplasmic strains into transgenic lines labeled with epithelial like seam cells and phagocytic coelomocytes because we realized that we would not be able to do the downstream work in all five cell types in a timely manner. Thus, we are focusing on characterizing mutant mtDNA levels from three cell types.

*Subtask 2: Perform fluorescence activated cell sorting (FACS) across 4 developmental stages (L1, L2, L3, and L4) and different ages of adulthood (Day 1, Day 4, and Day 10). Timeline: Months 3-7.*

We encountered a fair amount of technical hurdles that we had to overcome to develop a protocol for cell isolation and FACS that worked consistently and reliably. Specifically, to ensure that our sorted cells were enriched for GFP, we imaged our sorted cells. However, initially we failed to detect reliably high counts of GFP positive cells in our sorted samples. We were able to overcome this limitation by culturing the sorted cells for 24 hours. By giving 24 hours for the cells to recover, we saw a dramatic increase in the number of GFP positive cells we were

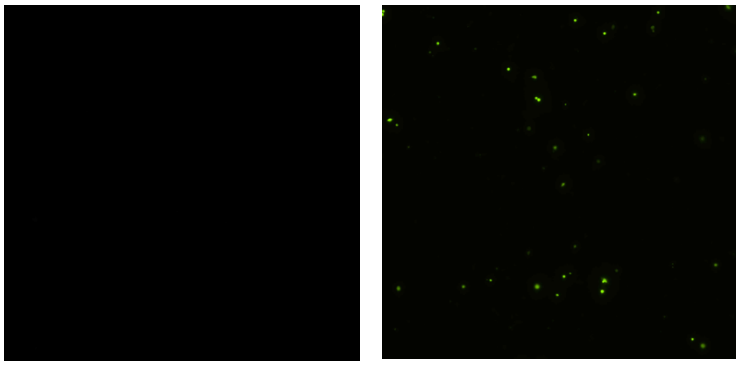

**Figure 1. FACS enriches for GFP labeled muscle cells.** Image on the left is of sorted GFP negative cells and image on the right is of sorted GFP positive cells. Muscle cells are labeled with GFP in these samples. Cells were cultured for 24 hours prior to imaging.

able to observe (**Figure 1**). For neurons, we could even see growth of neural processes, thus confirming that we are enriching for the correct cell population (**Figure 2**). Overall, we are now able to reliably perform cell isolation and sort GFP positive cell population from neurons, muscles and intestinal cells (**Figure 3**). Our preliminary data from neurons shows that the mutant mtDNA *uaDf5* levels do not change dramatically from L1 stage of development to Day 1 stage of adulthood (**Figure 4**). Consequently, we decided against measuring mutant mtDNA levels in the larval stages of L2, L3 and L4, as they would not be very informative. Instead, we will focus on determining mutant mtDNA level changes during adulthood as a function of age. We have discovered that we can obtain sufficient number of Day 8 animals to be able to perform cell isolation. Consequently, we are currently performing FACS on L1 stage, Day 1, and Day 8 of adulthood, which we believe will provide us with informative data.

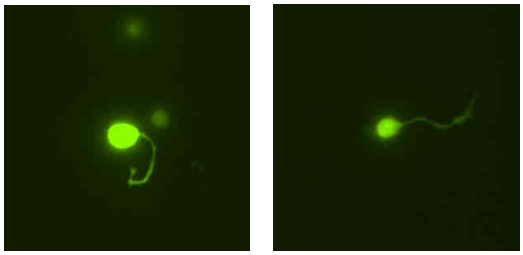

**Figure 2. GFP positive neurons grow processes in cell culture.** Representative images of sorted GFP positive neuronal cells growing in cell culture. Demonstrates enrichment of the correct cell types.

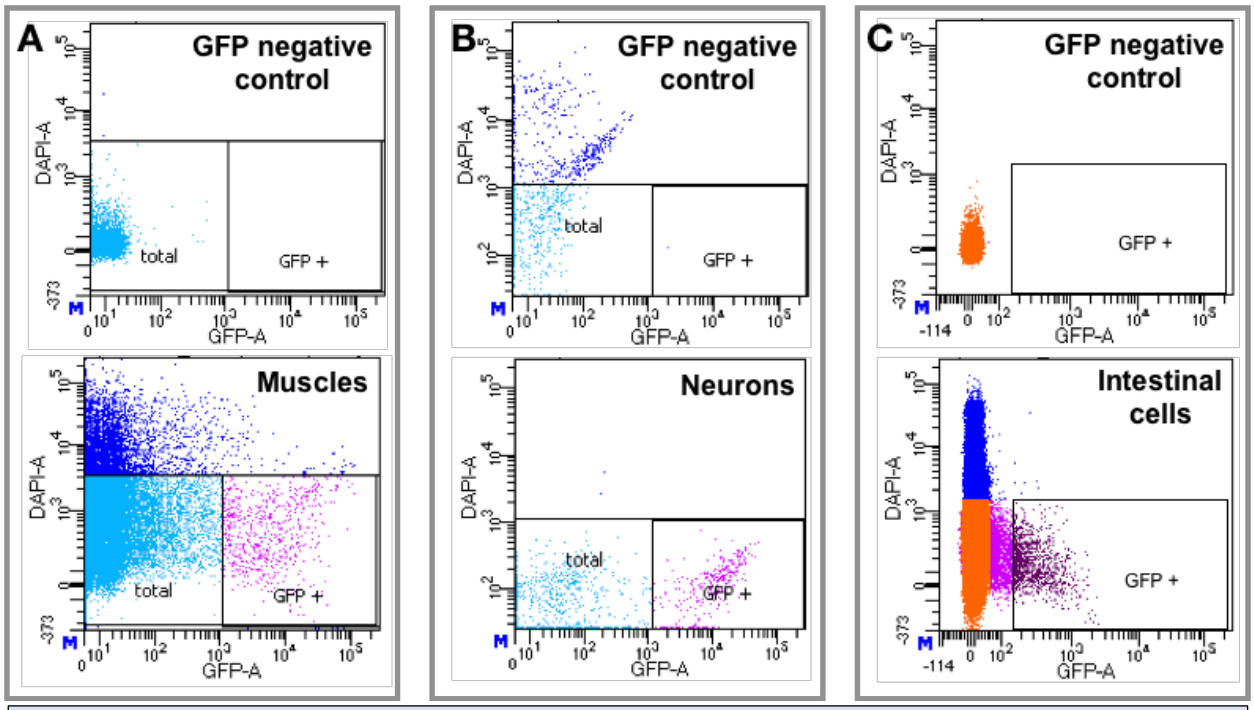


**Figure 3. GFP labeled cell populations can be effectively sorted.** The GFP signal intensity is indicated on the X-axis. DAPI stain intensity is on the Y-axis. Viable cells fail to take up DAPI. FACS profiles of cells isolated from strains in which (**A**) Muscles, (**B**) Neurons, or (**C**) Intestinal cells are labeled with GFP. Top profiles are of cells isolated from strains without any GFP labeled cells. Note the presence of the GFP positive but DAPI negative population of cells in the bottom profiles but their absence from the top profiles.

*Subtask 3: Perform droplet digital PCR (ddPCR) on the flow sorted GFP positive and negative fractions to determine mutant mtDNA levels across cell types. Timeline: Months 8-9.*

Although we had to overcome some technical hurdles, we have been able to successfully perform ddPCR on sorted samples to measure mutant mtDNA levels from muscle cells and neurons for all three mutant heteroplasmies. The first difficulty we encountered was not getting good separation of the positive ddPCR droplet population from the negative population. This made it

difficult to accurately measure mutant mtDNA levels. We were able to determine that this lack of separation is a product of high lysis buffer concentration. Consequently, by lysing our sorted cells in 2X diluted lysis buffer, we were able to achieve sufficient separation between positive and negative droplet populations. We have now optimized ddPCR protocol for all three heteroplasmic mtDNA mutations (**Figure 5**). Next, we first performed experiments to determine how many sorted cells we needed to pool into a single sample. We determined that pooling 200 cells per well provides us with sufficient template to be able to accurately measure mutant mtDNA levels with ddPCR. Third, from our pilot experiments, we noticed that the mutant mtDNA levels in both our GFP positive and GFP negative cells are lower than in the homogenate (isolated cells before cell sorting) (**Figure 6A**). Given that the homogenate has much higher concentration of mtDNA template, we wanted to ensure that the difference in mutant mtDNA levels between the homogenate and the sorted cells is not an artifact of differences in mtDNA template. We therefore performed an experiment in which we serially diluted the template and then measured mutant mtDNA levels using ddPCR. Our data show that the mutant mtDNA levels do not correlate with mtDNA template concentration, thus ruling out any technical artifacts as a source for the difference (**Figure 6B**). Instead, our data suggest that the homogenate contains unsortable cells that have high mutant mtDNA levels. In light of these findings, to ensure that we are comparing mutant mtDNA levels to the right control, we now compare mutant mtDNA levels from the GFP positive samples to both GFP negative samples as well as the homogenate. Overall, we have a working ddPCR protocol that allows us to accurately measure mutant mtDNA levels. We have begun the process of collecting ddCPR data from our samples.

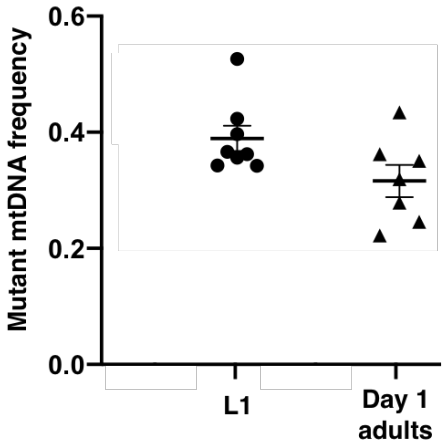


**Figure 4. Change in mutant mtDNA levels with age.** There is a significant but small change in mutant mtDNA *uadf5* levels from L1 stage to Day 1 of adulthood.

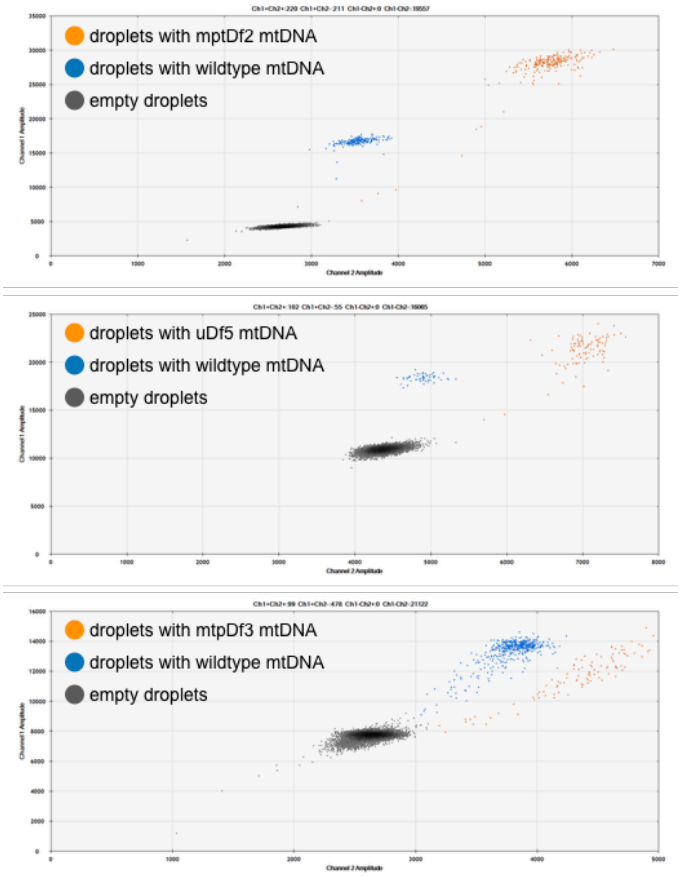


**Figure 5. Reliable measurement of mutant mtDNA levels with droplet digital PCR (ddPCR).** Representative ddPCR profiles of single samples for each of the three mutant mtDNA heteroplasmies. Empty droplets do not contains any mtDNA molecules. Each positive droplet corresponds to a single mtDNA molecule.

*Milestone(s) achieved: Determined how heteroplasmy dynamics change during development and aging across different cell types for 3 different mutant mtDNA.*

We had originally anticipated being able to achieve these milestones in 9 months. Based on the progress so far, while we still believe that we can achieve all of these milestones, given the technical hurdles we

had to overcome, we anticipate it taking us an additional 4-6 months.

**Specific Aim 2: Investigate mitochondrial mechanisms that regulate cell type specific differences in mtDNA heteroplasmy levels.**

*Subtask 1: Cross heteroplasmic strains with GFP labeled cells generated in Aim 1 into different mutant backgrounds that affect mitophagy (pink-1 and parkin mutants), mitochondrial biogenesis (atfs-1 mutant), and fusion/fission (fzo-1 and drp-1 mutants). Timeline: Months 3-4.*

We have finished crossing our heteroplasmic strains with GFP labeled cells into *parkin* and *drp-1* mutant backgrounds. We have not been able to cross our strains into other mutants he had originally proposed because they are on the same chromosome as the GFP transgenes. Consequently, we will have to identify recombination events between the transgene and the mutants as a way to generate these lines. Since it requires a significant amount of effort to obtain products of recombination, we will do these experiments after we have finished collecting data for all Aim 1 subtasks as well as subtasks 2 and 3 for Aim 2.

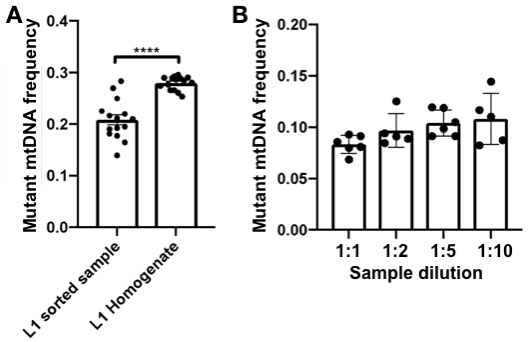


**Figure 6. Unsortable cells have higher mutant mtDNA levels.** (**A**) Homogenate represents isolated cells before FACS. Mutant mtDNA *uaDf5* levels are higher in homogenate compared to sorted cells. (**B**) Mutant mtDNA levels are not dependent on mtDNA template concentration.

*Subtask 2: As in Aim 1 subtask 2, perform FACS across developmental stages and different ages of adulthood from the different mutants. Timeline: Months 10-14.*

We are still on track to perform these experiments, although they will be delayed by about 4-6 months. We will focus our efforts on collecting data from muscle cells and neurons in the *parkin* and *drp-1* mutant backgrounds since we already have preliminary data that we can successfully measure mutant mtDNA levels using ddPCR from these cell types.

*Subtask 3: As in Aim 1 subtask 3, perform ddPCR on the flow sorted GFP positive and negative fractions to determine mutant mtDNA levels across cell types. Timeline: Months 15-16.*

We plan to finish this subtask along with subtask 2. Since we have now acquired sufficient experience in doing ddPCR on sorted cells so we should not encounter any hurdles in being able to accomplish this task.

Subtask 4: Write manuscript describing findings from the study on cell type specific heteroplasmy dynamics. Timeline: Months 17-18.

Our plans to write a manuscript describing our findings from this project remain unchanged. We plan to publish one paper from this work. It will represent an important milestone in the study of cell type specific differences in mutant mtDNA levels.

*Milestone(s) achieved: Determined the role that mitochondrial physiology (mitophagy, biogenesis, and fusion/fission) plays in regulation of cell type specific heteroplasmy dynamics.*

We original milestone of determining the role of mitochondrial physiology in the regulation of heteroplasmic mutant mtDNA dyanamics in cell type specific manner remains unchanged.

**IMPACT**

We have overcome technical hurdles and have optimized a protocol pipeline to isolate cells, FACS sort them, and perform ddPCR. This pipeline has to be optimized for each cell type and

each heteroplasmic mutant mtDNA. We have optimized it for muscle cells, neurons, and intestinal cells, and for *uaDf5*, *mptDf2* and *mptDf3* heteroplasmic mutant mtDNA. Our pipeline demonstrates proof of concept. It can be applied to measure mutant mtDNA levels from any cell type that can be labeled with GFP. While our work in done using *C. elegans* as a model system, theoretically, our pipeline can be used in other systems including mammalian systems. Additionally, we observe lower mutant mtDNA levels in neurons and muscles compared to the rest of the somatic tissues (comprises the homogenate). These data suggest existence of active molecular mechanisms that either decrease mutant mtDNA levels in these tissues, or increase mutant mtDNA levels in rest of the soma. Finally, we also observe a small but significant decrease in mutant mtDNA levels in neurons as a function of age. Overall, our data suggest that mutant mtDNA levels change in a dynamic manner in *C. elegans*.

**CHANGES/PROBLEMS**

While we encountered a few technical challenges, we were able to overcome all of them and none of these change the overall direction and scope of the proposed work. Most of the challenges that we encountered and resolved have already been described above in the accomplishments section. However, they are briefly summarized here. First, relevant to subtask 1 of Aim 1, we discovered that debris from the germline of animals interferes with cell isolation using FACS. Consequently, we crossed all our strains into the *glp-1* mutant background, which prevents germline development. Having to do this additional cross delayed our timeline. To remain on track to be able to accomplish all other tasks, we decided to focus on measuring mutant mtDNA levels from three of the five proposed cell types. Second, relating to subtask 2 of Aim 1, it was important for us to confirm that FACS is enriching for GFP-positive cells. We had difficulties visualizing GFP positive cells but we overcame this difficulty by first culturing isolated cells for 24 hours before imaging (**Figures 1 and 2**). Third, relating to subtask 3 of Aim 1, we initially encountered difficulties in reliably measuring mutant mtDNA levels using ddPCR. However, we figured out that the lysis buffer was interfering with the ddPCR. Consequently, by diluting the lysis buffer, we were able to overcome this technical hurdle and obtain reliable measurement of mtDNA with ddPCR (**Figure 5**). For task 1 in Aim 2, we could not cross all the heteroplasmic strains with GFP-labeled cells generated in Aim 1 into different mutant backgrounds that affect mitophagy (pink-1 and parkin mutants), mitochondrial biogenesis (atfs-1 mutant), and fusion/fission (fzo-1 and drp-1 mutants). This is because some of these mutants are on the same chromosome as *glp-1* mutations, which we need to prevent germline development. Consequently, our current plan is to first focus on acquiring data from the rest of the mutant backgrounds. We are still on track to accomplish the remaining tasks 2 and 3 for Aim 2, although they might take us 4-6 months longer than originally anticipated. We do not however anticipate this having a significant impact on expenditures.

**PRODUCTS**

2019 Oral presentation at the Single Cell Biology Symposium at Vanderbilt University.
2019 Oral presentation at the 21st International *C. elegans* Conference in Los Angeles.
2019 Oral presentation at the MRC's Mitochondrial Biology Unit in Cambridge, UK

**PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

Name: Nikita Tsyba
Project role: Graduate student
Nearest person month worked: 12

Contribution to the project: Mr. Tsyba has performed all the experiments for this project. He has worked on optimizing the protocol to isolate cells from *C. elegans* to be used for FACS. He also developed ddPCR protocol to quantify mutant mtDNA levels from flow sorted cells.

Name: Benjamin Saunders
Project role: Research Assistant I
Nearest person month worked: 12
Contribution to the project: Mr. Saunders is aiding Mr. Tsyba in their efforts to carry out fluorescence activated cell sorting (FACS) coupled droplet digital PCR (ddPCR) in *C. elegans* and downstream analysis of the data. Duties performed by Mr. Saunders include maintaining worm stocks, cleaning bacterial contamination from worm strains, growing large scale worm cultures, performing PCR to verify presence of heteroplsamic mutant mtDNA and qualitatively checking mutant mtDNA levels. Additional duties include preparing worm plates, making aliquots of reagents, preparing media, autoclaving, dishwashing, and maintenance of relevant equipment.

**SPECIAL REPORTING REQUIREMENTS**
Nothing to report

## Appendices
Nothing to report