THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF CHEMISTRY

Proteostasis of *E. coli* and Human Embryonic Kidney (HEK 293T) Cells Under Heavy Metal Stress

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ABSTRACT

Heavy metals are known to have toxic effects in both microbes and humans. Some, such as silver, copper and gold, have been used as antimicrobial agents for decades, while others, such as lead and mercury, have been the cause of severe environmental toxicity in many notable instances. While the general effects of such heavy metals have been well studied in both microbes and humans and the mechanism of toxicity of many are understood, this work introduces a new method of detecting and visualizing heavy metal-induced proteome stress in both prokaryotic and eukaryotic cells using novel fluorogenic sensors. AgHalo, protein-based sensor, was used to detect and measure protein aggregation induced by the environmental presence of heavy metals while CHW-128, a small-molecule sensor, was used to detect environmental heavy metal-induced protein aggregation in mammalian cells. The use of these sensors facilitates explicit detection and visualization of proteome stress in both prokaryotic and eukaryotic cells.

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ACKNOWLEDGEMENTS

I would like to thank Professor Xin Zhang for providing me the resources to carry out my thesis research, the guidance to plan and structure my work and the trust to let me head my own project as an undergraduate. I would also like to thank Matthew Fares for his consultation and guidance throughout my research, Charlie Wolstenholme for his assistance and guidance in my mammalian cell culture work, and Leeann Grainger for gathering and processing confocal images for me.

Chapter 1

Introduction

Heavy metals, defined by Sutton et. al as naturally occurring metals that have a high atomic weight and a density at least 5 times greater than that of water, have widespread industrial use.[10] They are also, in most cases, toxic to both prokaryotes and eukaryotes. In the instance of bacteria, this toxicity can and has been utilized by humans in the form of implementing heavy metals as antimicrobial agents. Inorganic mercury compounds have been used in treatment of STD's and skin infections for centuries, silver nitrate has been used to treat infected burns and wounds, and copper has been used as an antibacterial agent in seeds and plants.[5]

Industrial use of these toxic heavy metals has also posed substantial problems for humans throughout history. The issue of heavy metal toxicity has been particularly relevant in recent years with the water crisis in Flint, Michigan, in which tens of thousands of citizens were exposed to toxic levels of lead as the town switched to the contaminated Flint River as a source of drinking water.[4] Another notable historical instance of heavy metal toxicity posing widespread problems is the use of lead-based paints in the United States prior to their being banned by the federal government in 1978.[8] Despite this ban, however, lead paint has continued to pose health problems for those exposed to houses containing it, particularly for young children who ingest the paint.

The mechanism of toxicity of many heavy metals is relatively well understood. Most heavy metals exert oxidative stress on cells induced by the formation of free radicals resulting oftentimes in disrupted metabolism.[2] This oxidative stress and metabolic malfunction leads to protein aggregation, as damage to DNA, ribosomal machinery and proteins themselves cause the production of mutant and misfolded proteins.[7] The Zhang chemistry laboratory at The Pennsylvania State University specializes in the synthesis and use of fluorogenic sensors that detect a variety of proteome conditions in the cell, one of which being protein aggregation. Therefore, my work involves utilizing two such fluorogenic sensors-- AgHalo, a protein-based sensor that indicates proteostasis of the metastable portion of the proteome, and CHW-128, a small-molecule sensor that detects the formation of protein oligomers and aggresomes-- to detect heavy-metal induced proteome stress in prokaryotic cells (*E. coli)* and eukaryotic cells (HEK-293T).

The predominant existing method of detecting protein aggregation in mammalian cells is Proteostat®, a fluorescent assay produced by Enzo Life Sciences, Inc. Though Proteostat® has been proven to be effective in detecting protein aggregation induced by a variety of stress conditions and could have easily been used in my own work, there are several advantages of using our own probe (CHW-128) instead of Proteostat®.[9] First, obviously, the CHW-128 was synthesized in-house and did not need to be ordered from an outside source. More importantly, however, are two fundamental advantages that CHW-128 provides over Proteostat®, one of which is the requirement of cell membrane permeabilization in order to carry out the Proteostat® assay, which results in the death of the cells being analyzed prior to fluorescent imaging. CHW-128 is membrane permeable and therefore does not require this extra step and also does not

disrupt normal cell function when used. This huge advantage allows for the analysis of live, growing cells throughout the entire procedure using CHW-128. The second advantage of CHW-128 is that it is able to detect small, insoluble oligomers, whereas Proteostat® detects only large aggresomes. A comparison of fluorescence microscopic images generated using Proteostat® (Figure 1) and CHW-128 (Figure 2) to detect protein aggregation in mammalian cells in the presence of MG-132, a protease inhibitor that prevents degradation of improperly-folded proteins and induces the formation of aggregates, reveals the equal if not superior effectiveness of CHW-128 in accomplishing the task of detecting protein aggregation in mammalian cells.[9] In both assays, cells were incubated in the presence of the protease inhibitor, stained with the respective aggregation-detecting probe as well as nuclear stain, and imaged. The blue portions of the images depict cell nuclei, while the red depicts protein aggregates. The control samples were not treated with protease inhibitor, hence there is no detectable protein aggregation in either.

Several basic concepts should first be understood in order to accurately interpret the results and data presented in this work. Primarily, optical density is an excellent indicator of overall *E. coli* growth; as the bacteria grow and divide, they take up more and more of the media, which results in greater absorbance of light and an increased optical density. Increasing optical density over time corresponds to growing cells, while constant optical density over time indicates cell growth has halted. Many of the subsequent figures are graphs displaying optical density vs. time, hence they essentially depict whether the presence of metal kills, stresses, or has no effect on cell growth. Secondly, fluorescent cell imaging does not provide quantitative data regarding

the extent of protein heavy metal-induced protein aggregation, but rather an explicit visualization of the protein aggregation caused in cells by the environmental presence of heavy metal.

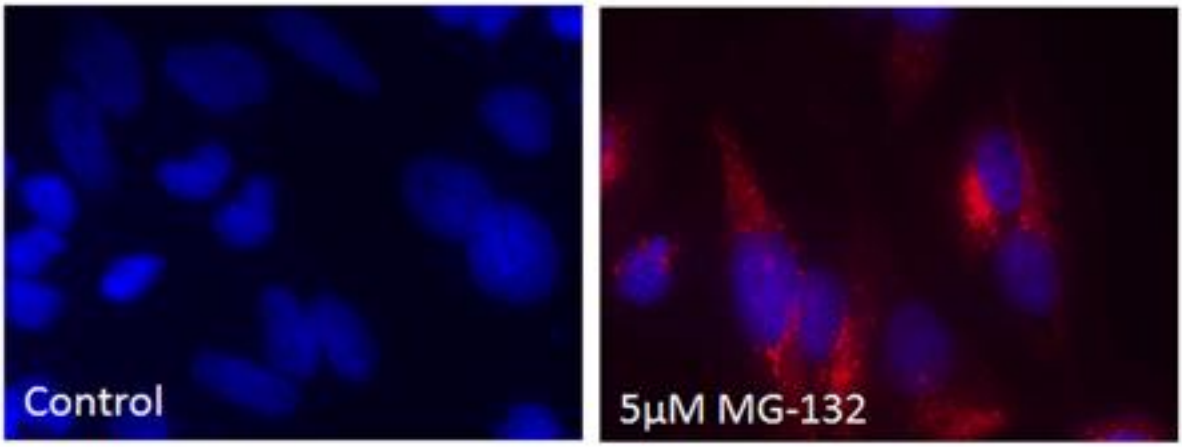


Figure 2: Protein Aggregation in HeLa Cells Grown in Presence of 5 uM MG-132 Protease Inhibitor Detected Using Proteostat®

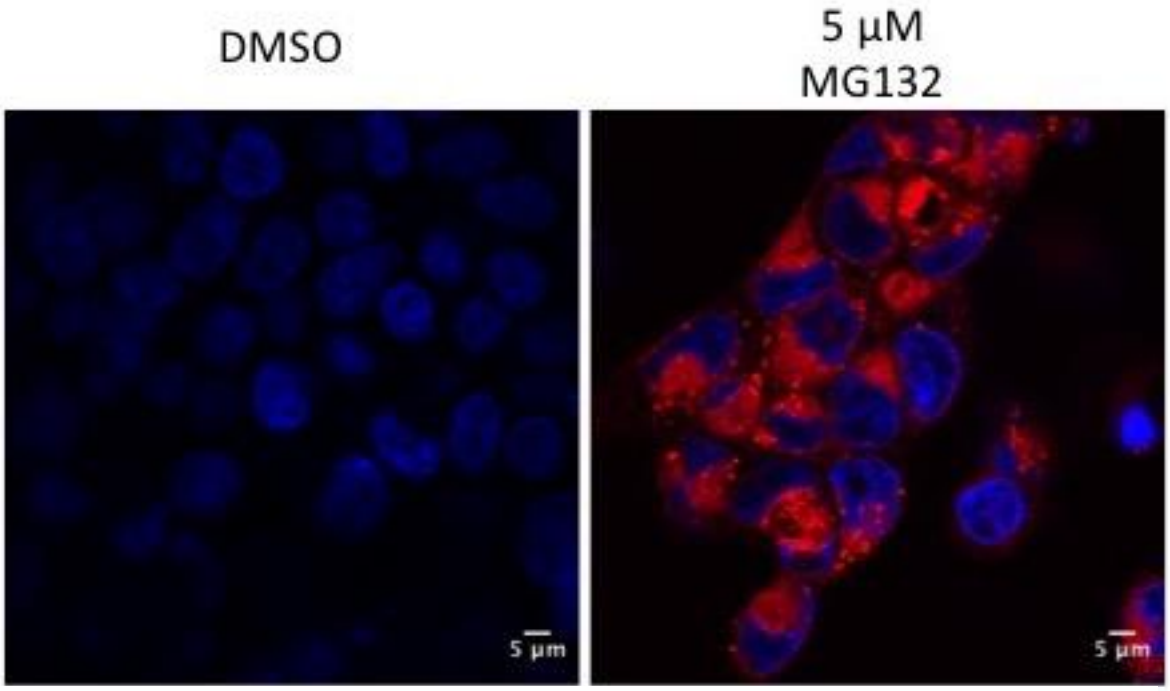


Figure 1: Protein Aggregation in HEK-293T Cells in Presence of 5 uM MG-132 Protease Inhibitor Detected Using CHW-128

Chapter 2

Materials and Methods

Determining Stressful/Toxic Ranges of Metal Concentrations in *E. coli*

Before fluorescence assays could be run using AgHalo, appropriate concentrations of metal had to first be determined to ensure that the *E. coli* were, in fact, undergoing heavy metal-induced stress that would cause protein aggregation. BL21 competent *E. coli* were transformed with a plasmid encoding the AgHalo protein according to the transformation protocol listed in Appendix A. Although fluorescence was not being directly monitored in these preliminary assays, it was important to keep all cellular conditions the same throughout the course of the work (hence, transformation of the AgHalo plasmid as opposed to just growing BL21 competent *E. coli*). The cells were then plated and one colony was inoculated into 15 mL M9 minimal media (prepared according to protocol listed in Appendix A) and grown for 12-16 hours. 0.5 mL of this overnight culture was transferred into 14.5 mL of fresh M9 minimal media to continue growing. It is important to note that Luria Broth was initially tested as a cell-growing media, however it demonstrated a protective effect on the *E. coli* against the heavy metal as compared to M9 minimal media (seen in Supplemental Figure 1), meaning that the cells grew in LB in the presence of equal concentrations of heavy metal that caused the cells to die when grown in M9. For the sake of using smaller amounts of heavy metal (both as a safety and economic concern), M9 was chosen as the media in which to carry out subsequent assays.

In initial growth assays, metal was added simultaneously to the overnight culture being transferred into fresh media. This procedure resulted in little to no cell growth at even low

concentrations of $CdCl_2$ (Figure 3). Several assays were then run in which metal was added when the samples reached and optical density of 0.4 in order to allow the cells to adapt to fresh media prior to being introduced to the stress condition. The results of these assays can be seen in Figures 4-10.

Fluorescent Detection of Heavy Metal Stress in *E. coli* Cells Using AgHalo

Once stressful/toxic concentrations of each metal were determined via the growth defect assays previously described, fluorescence measurements were taken to get a quantitative measurement of the degree of cellular proteome stress induced by each metal. This was difficult to accomplish, considering we desired to detect proteome stress caused directly by the heavy metal in growing, healthy cells and producing and fluorescently labeling the AgHalo takes time. The following protocol was designed and tested preliminarily without the addition of small molecule in order to ensure the cells continued growing throughout the procedure. First, an overnight culture of BL21 competent *E. coli* cells transformed with the plasmid encoding for AgHalo was grown for 12-16 hours. 1 mL of this culture was transferred into 19 mL of fresh M9 minimal media. These samples were grown to an optical density of 0.3 at 37°C, at which point they were induced to overproduce AgHalo with 50 μM IPTG and transferred into a 30°C shaker. The cells were grown to an optical density of 0.6 at 30°C, spun down in a centrifuge at 4000 rpm for 7 minutes, and the media was removed from the cell pellet. The pellet was resuspended in 2 mL of fresh M9 minimal media and divided into two 1 mL aliquots. A small molecule (P1) was added to one of these two aliquots at a concentration of 40 μM which conjugates to AgHalo and fluoresces only under misfolding or aggregation conditions.[12] The other sample served as a

control to measure background fluorescence without the addition of the fluorogenic probe (P1). The samples were incubated at 30°C for 10 minutes to allow for P1 conjugation to AgHalo, at which point the cells were again spun down at 4000 rpm for 7 minutes and the media was removed from the cell pellets. Each pellet was resuspended in 10 mL of fresh M9 media with the appropriate concentration of the designated heavy metal. The samples were then incubated at 30°C and fluorescence measurements were taken every 15 minutes. Taking these measurements involved removing 1 mL of sample, normalizing the optical density to 0.5, transferring 100 μL of the sample into a 96-well plate. The sample was excited at 440 nm and emission was measured at 545 nm.[12]

Fluorogenic Detection of Heavy Metal Stress in HEK-293T Cells Using CHW-128

HEK-293T cells grown to a confluency of approximately 50% were incubated for 6 or 24 hours in the presence of a designated concentration of heavy metal and 5 μM CHW-128 in Delbucco's Modified Eagle Media. The metal-containing media was then removed and their nuclei were stained using Hoechst 33342 nuclear stain in DMEM (incubate for 30 minutes). At this point, the nuclear stain-containing media was removed and replaced with Fluorobrite DMEM and the cells were imaged using a ZOE Fluorescent Cell Imager.

An assay was also performed in which the cells were incubated for 6 hours in the presence of heavy metal ($AgNO_3$) only and the CHW-128 was added simultaneously with the Hoechst nuclear stain in order to reduce fluorescent labeling of undesired cellular components. Confocal microscopic images were taken of the cells from this assay.

Chapter 3

Results and Discussion

Growth Defect and Determining Toxic Concentration Ranges of Metals in *E. coli*

The results of the growth defect assays can be seen below in Figures 3-7.. Adding the metal immediately upon transferring the overnight culture into fresh M9 media produced little to no cell growth at even low concentrations of $CdCl_2$.

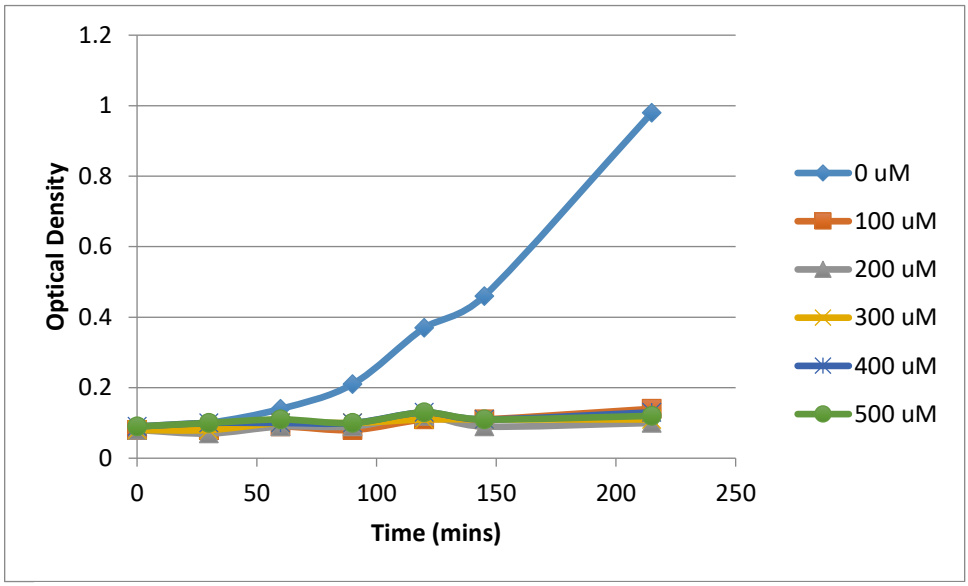


Figure 3: *E coli.* Growth in Presence of Heavy Metal Added Upon Transfer of Overnight Culture to Fresh Media

When given time to adapt to fresh media, the toxic effect of $CdCl_2$ was less severe. Metal was added at optical densities of either 0.4 or 0.6 in the assays illustrated in Figures 4-10.

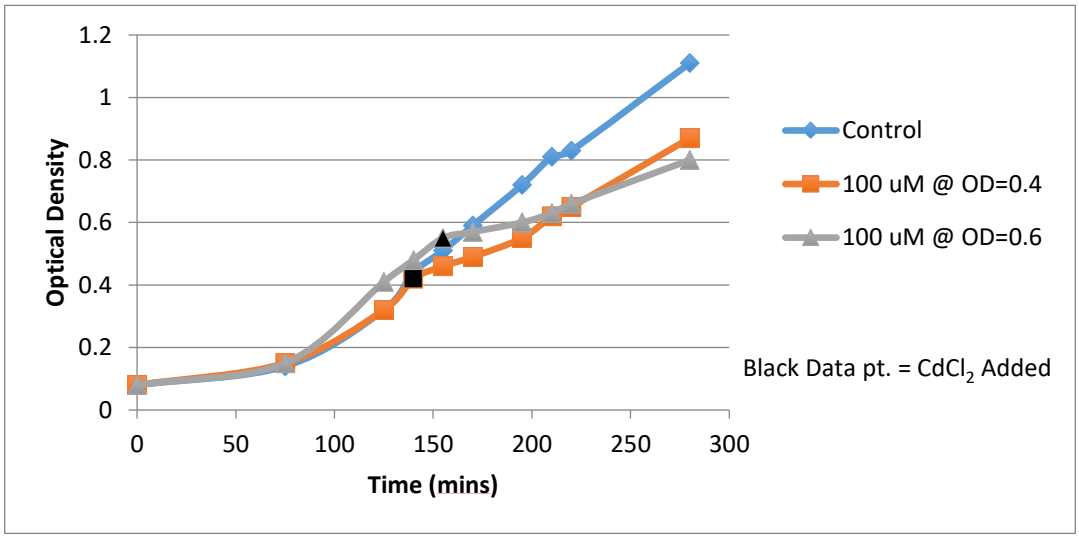


Figure 4: 100 uM $CdCl_2$ Added at Optical Densities of 0.4 and 0.6

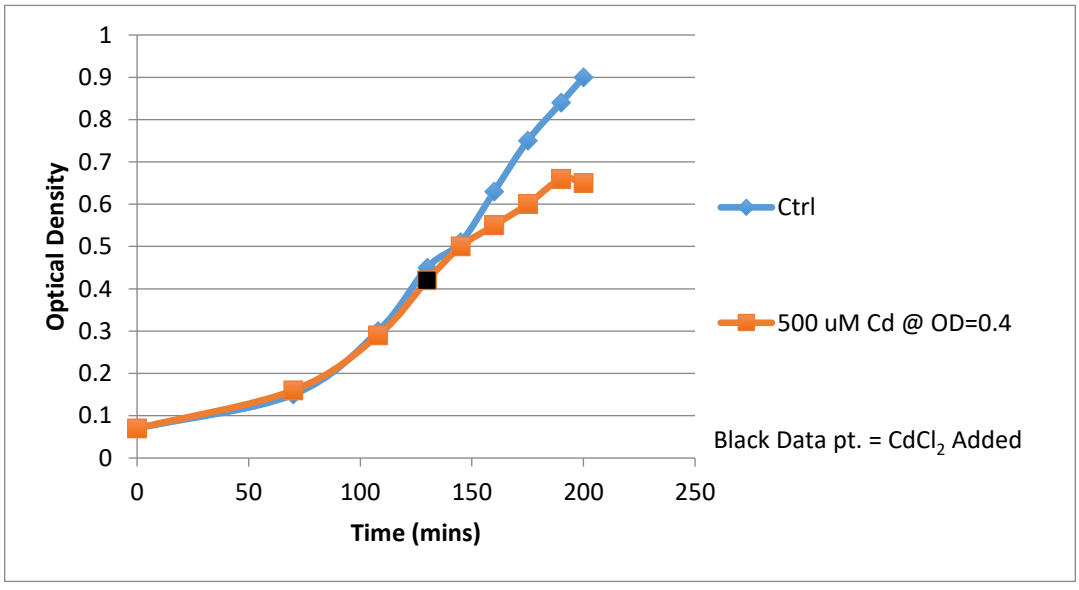


Figure 5: 500 uM $CdCl_2$ Added at Optical Density of 0.4

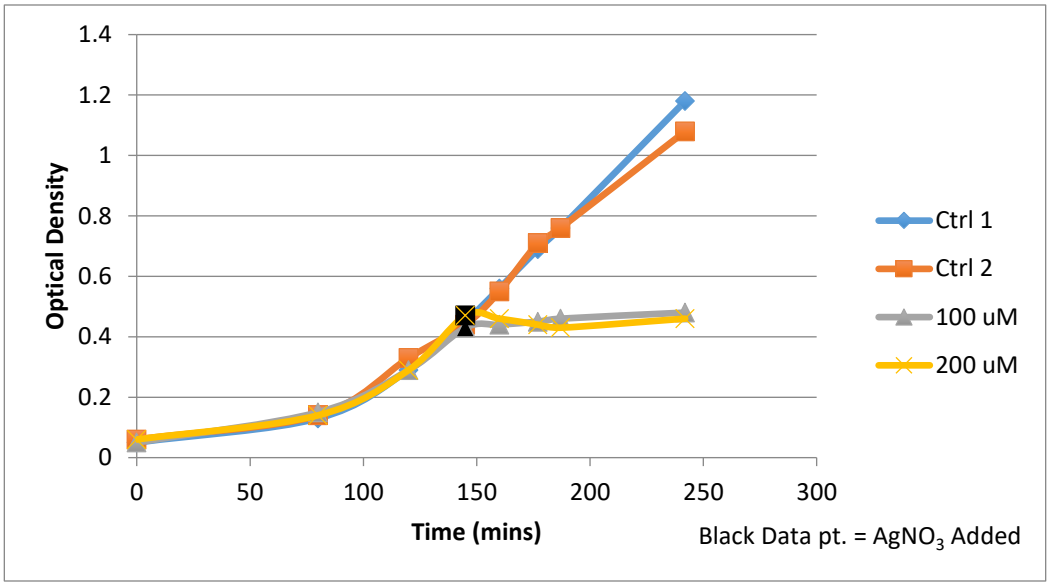


Figure 4: $AgNO_3$ Added at Optical Density of 0.4

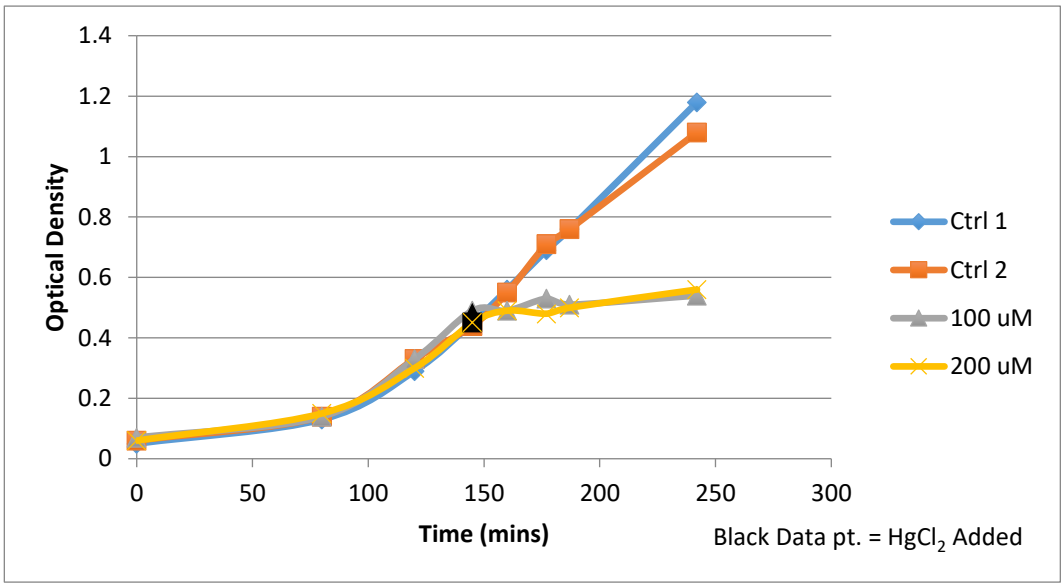


Figure 5: $HgCl_2$ Added at Optical Density of 0.4

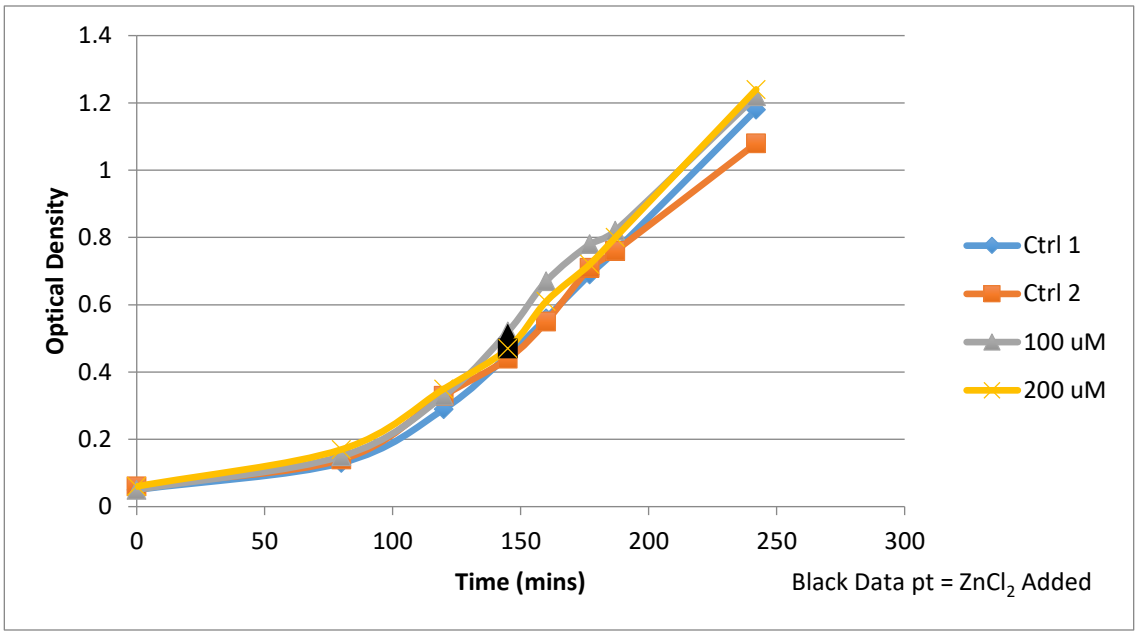


Figure 6: $ZnCl_2$ Added at Optical Density of 0.4

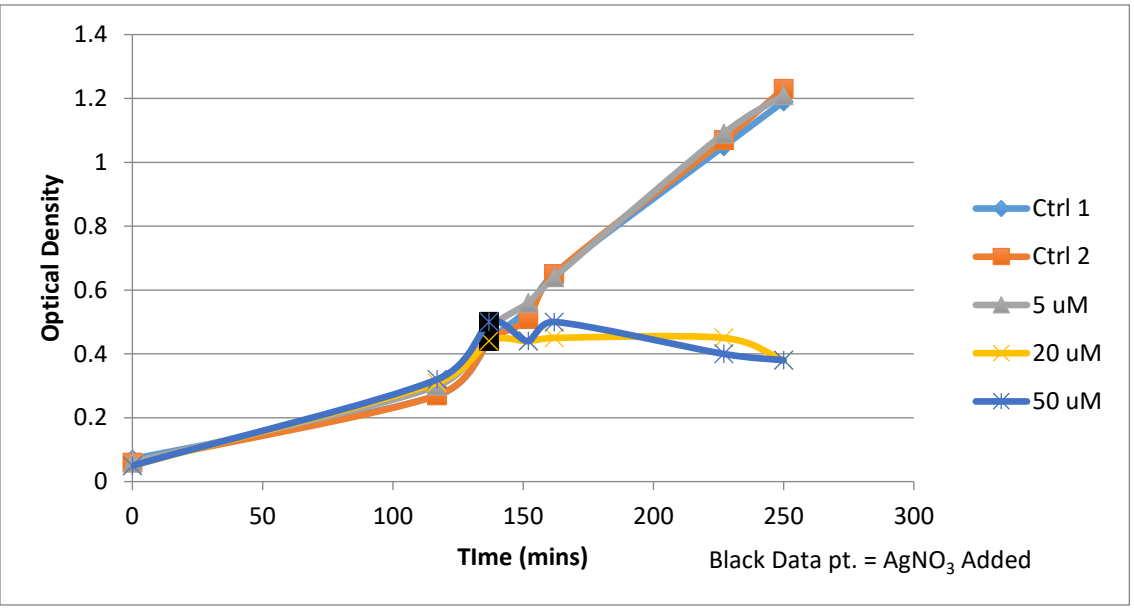


Figure 7: $AgNO_3$ Added at Optical Density of 0.4

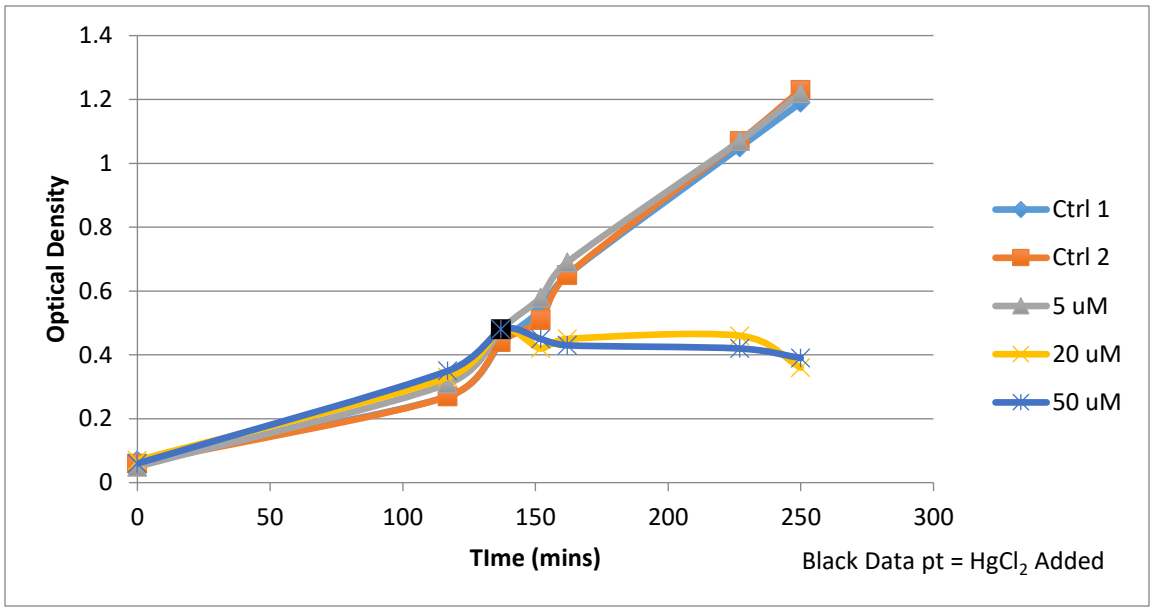


Figure 8: $HgCl_2$ Added at Optical Density of 0.4

Fluorescent Detection of $AgNO_3$-induced Protein Aggregation in *E. coli* Using AgHalo

The fluorescent detection of protein aggregation in *E. coli* induced by the presence of $AgNO_3$ did not produce the desired results. Though there were slight differences in the

wavelength of emission corresponding to small molecule P1 conjugated to the AgHalo protein between samples exposed to metal and samples not exposed to metal, there was not a statistically significant enough difference to conclude that it resulted from heavy metal-induced protein aggregation. It is conjectured that the failure of this assay was due to insufficient incubation time of the cells in the presence of small molecule. This assay will be repeated at a later date, however the cells will be incubated in the presence of P1 for 30 minutes.

Fluorogenic Detection of Heavy Metal Stress in HEK-293 T Cells Using CHW-128

Figures 11-20 were collected using a ZOE Fluorescent Cell Imager. The blue regions of each image depict the nuclei of the cell, while the surrounding red spots depict protein aggregation as detected by CHW-128. The oblong, irregular shape of the cells grown in the presence of heavy metal indicates illness. A sample grown in the presence of 50 μM $AgNO_3$ died completely so no image was taken. Cells grown in the presence of $CdCl_2$ were imaged after 24 hours, while cells grown in the presence of $AgNO_3$ and $HgCl_2$ were imaged after 6 hours. These time points were decided upon based on the much more severe toxic effect of $AgNO_3$ and $HgCl_2$ compared to $CdCl_2$ observed in *E. coli*.

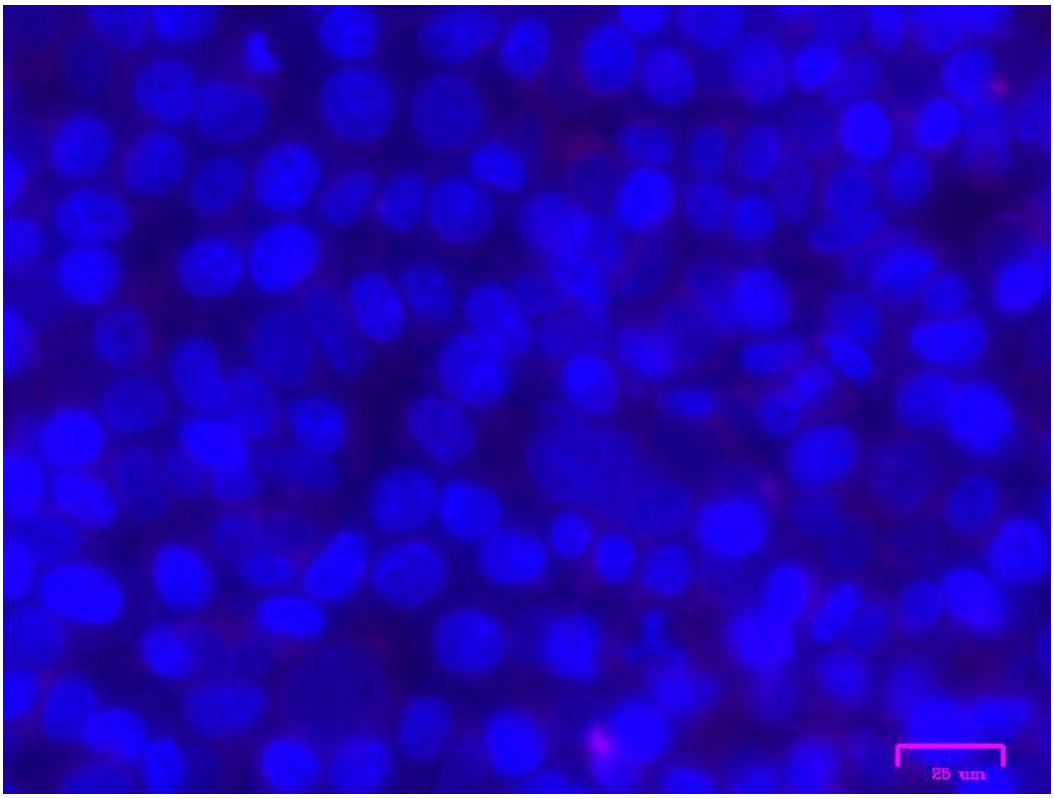


Figure 9: Control Sample for HEK-293T Cells Grown in Presence of $CdCl_2$ (No Metal Added)

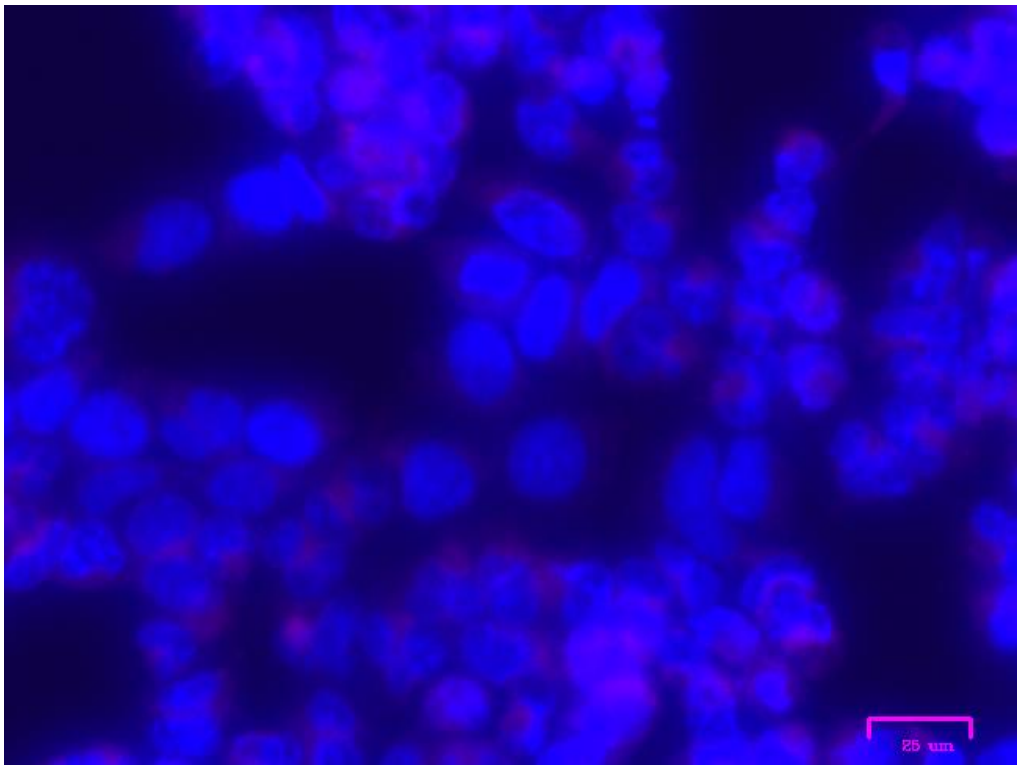


Figure 10: HEK-293T Cells Grown in Presence of 10 uM $CdCl_2$ for 24 Hours

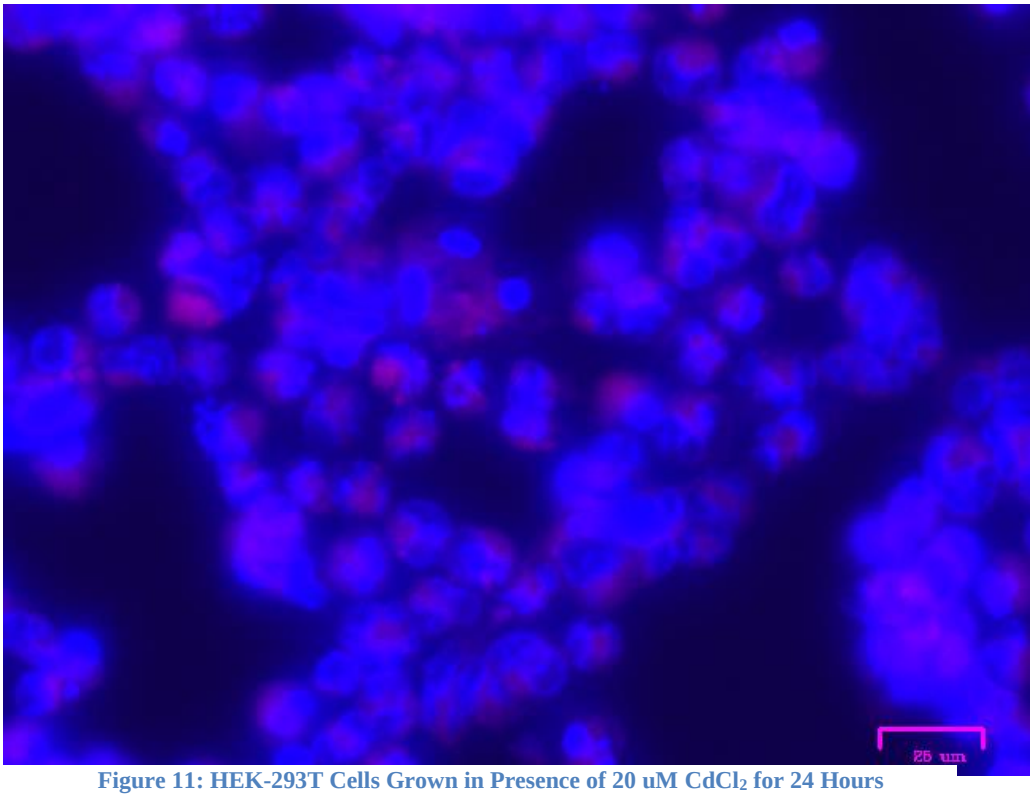


Figure 11: HEK-293T Cells Grown in Presence of 20 uM $CdCl_2$ for 24 Hours

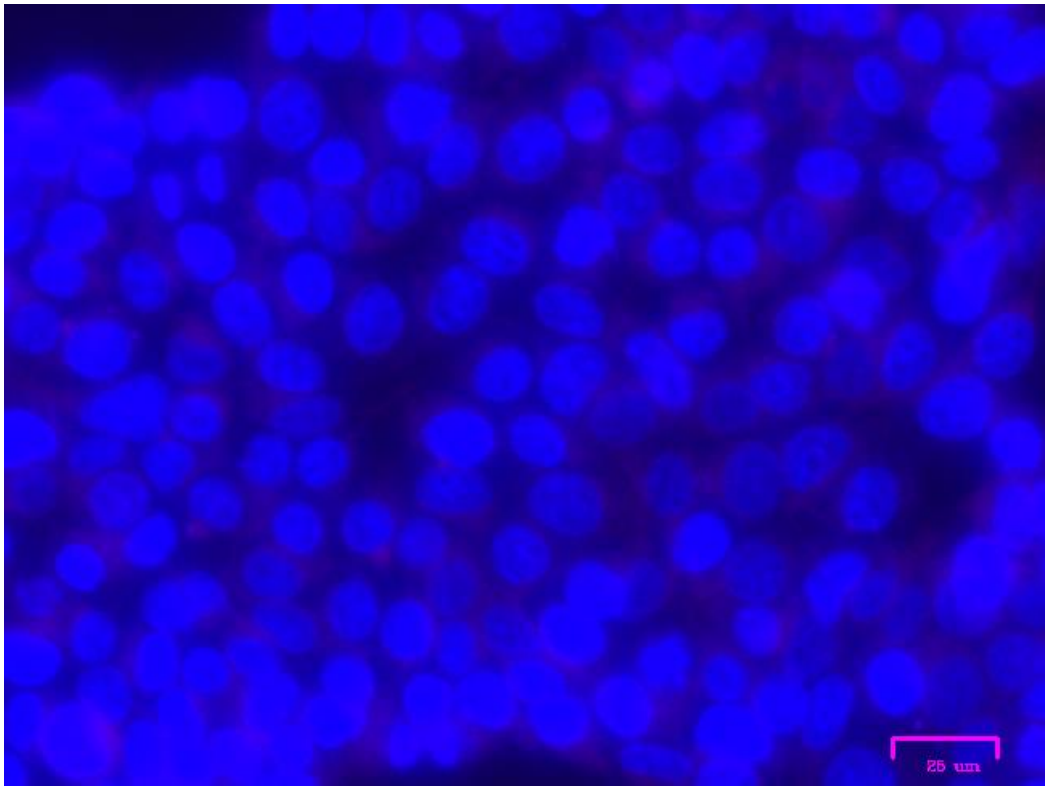


Figure 12: Control Sample for HEK-293T Cells Grown in Presence of $AgNO_3$ (No Metal Added)

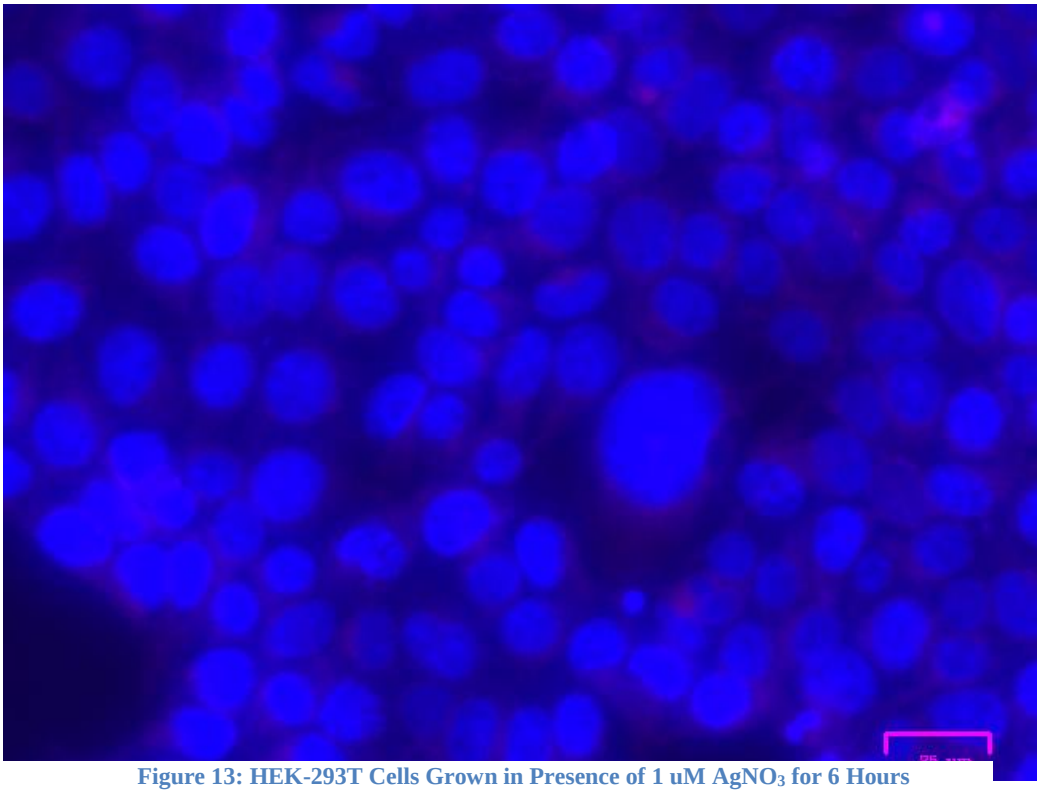


Figure 13: HEK-293T Cells Grown in Presence of 1 uM $AgNO_3$ for 6 Hours

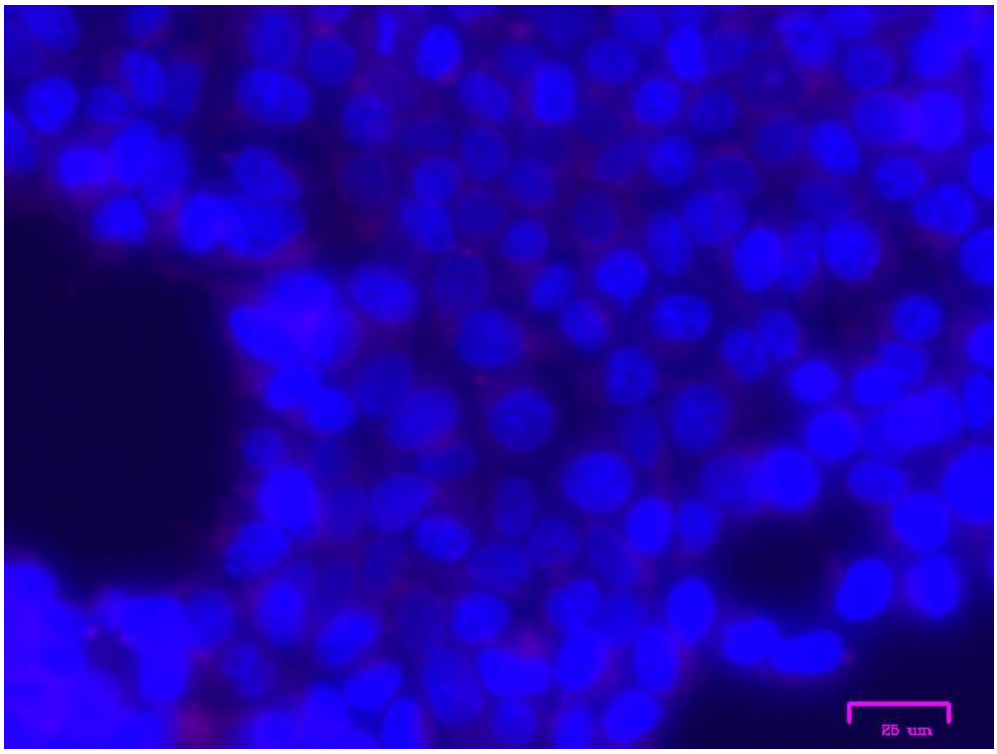


Figure 14: HEK-293T Cells Grown in Presence of 10 uM $AgNO_3$ for 6 Hours

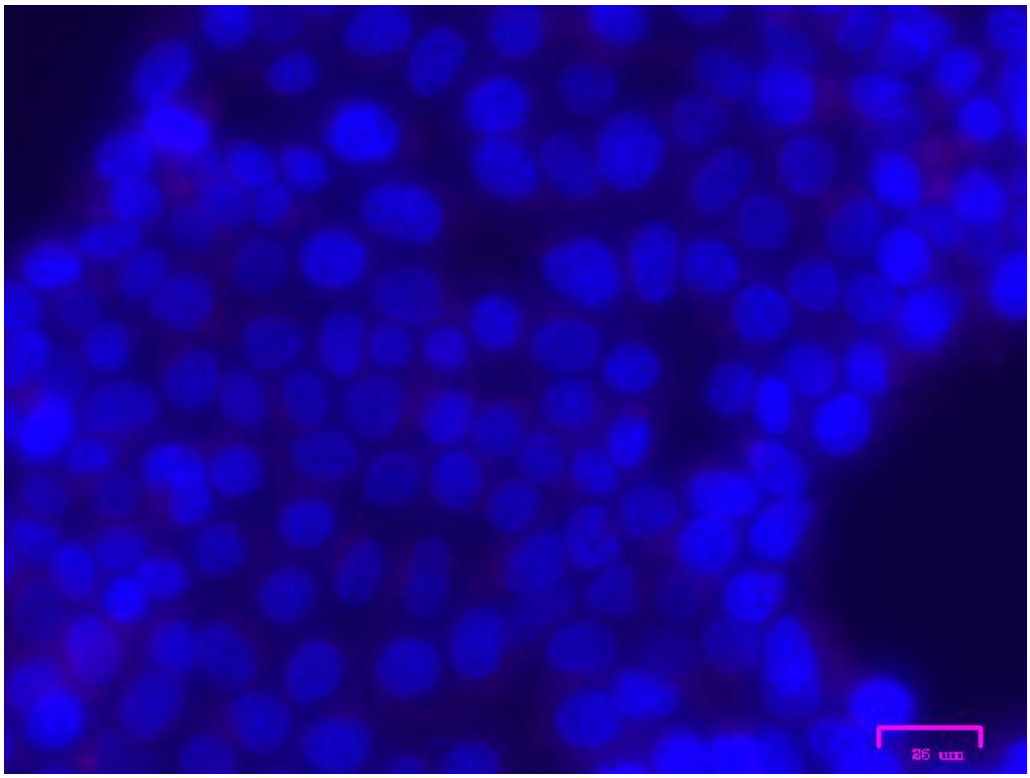


Figure 15: Control Sample for HEK-293T Cells Grown in Presence of $HgCl_2$ (No Metal Added)

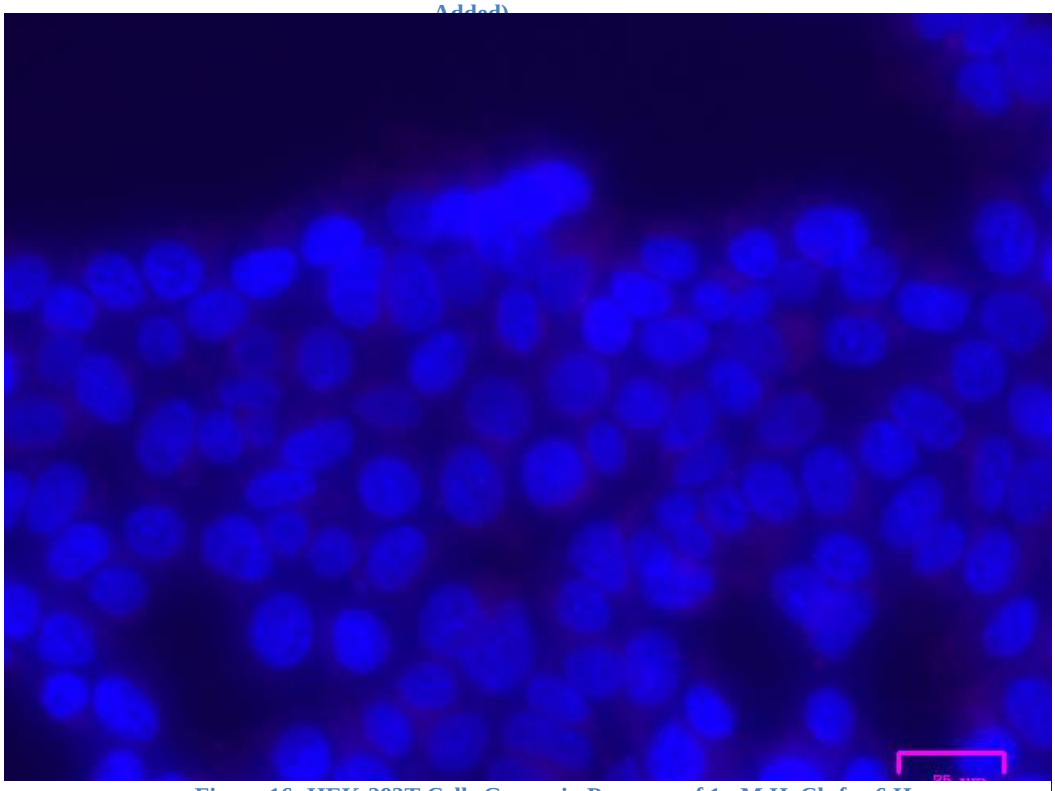


Figure 16: HEK-293T Cells Grown in Presence of 1 uM $HgCl_2$ for 6 Hours

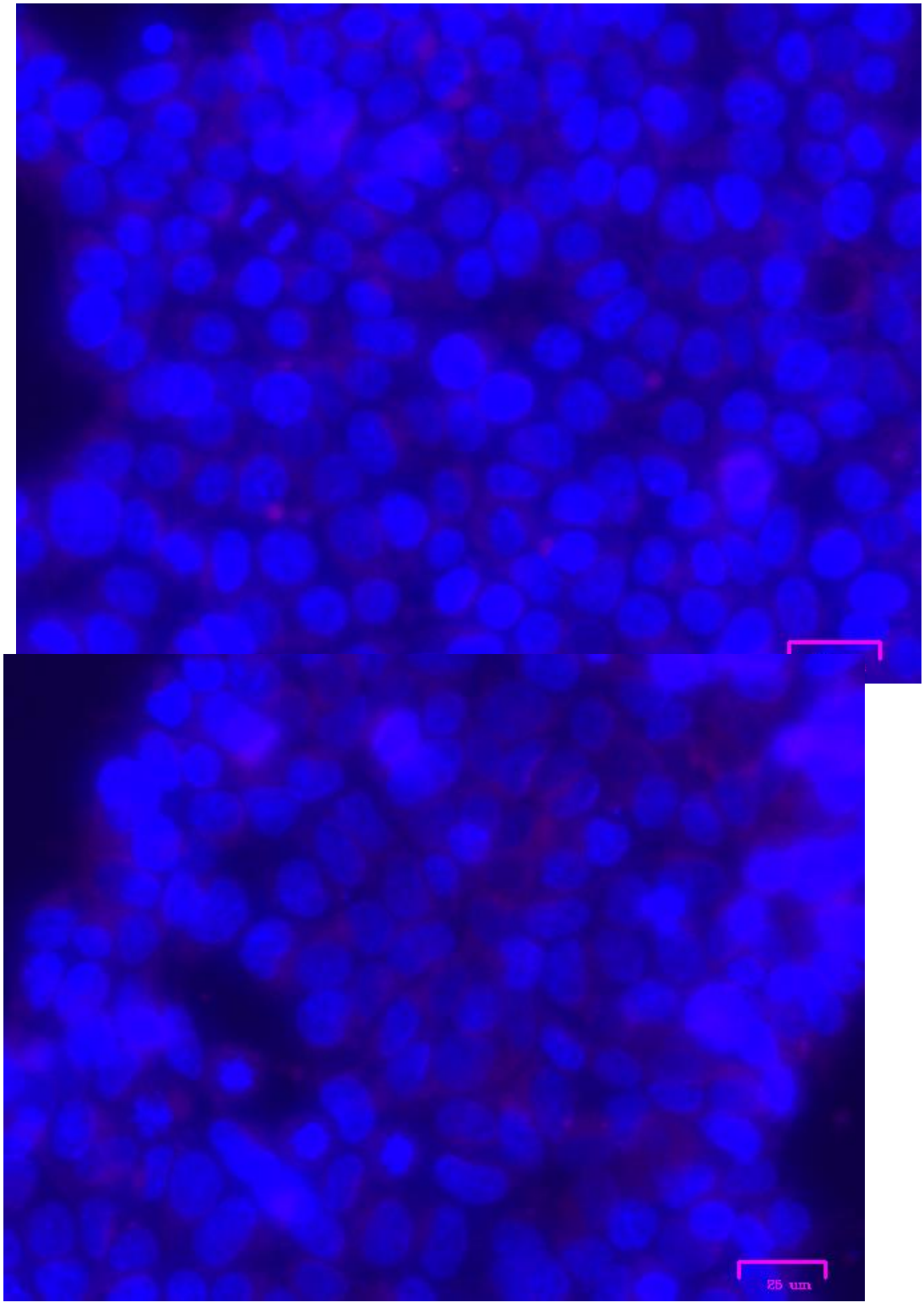

25 um

Figure 18: HEK-293T Cells Grown in Presence of 50 uM $HgCl_2$ for 6 Hours

The following images were taken using a confocal microscope, which provides a much higher resolution image than the ZOE Fluorescent Cell Imager. The assay was carried out following the same protocol as that used to generate the previous images, however CHW-128 was not added until the nuclear stain step, as opposed to growing the cells in the presence of both CHW-128 and metal. This modification was made in an effort to reduce fluorescent staining of undesired portions of the cell. As before, the blue regions correspond to cell nuclei, while the red regions correspond to protein aggregates.

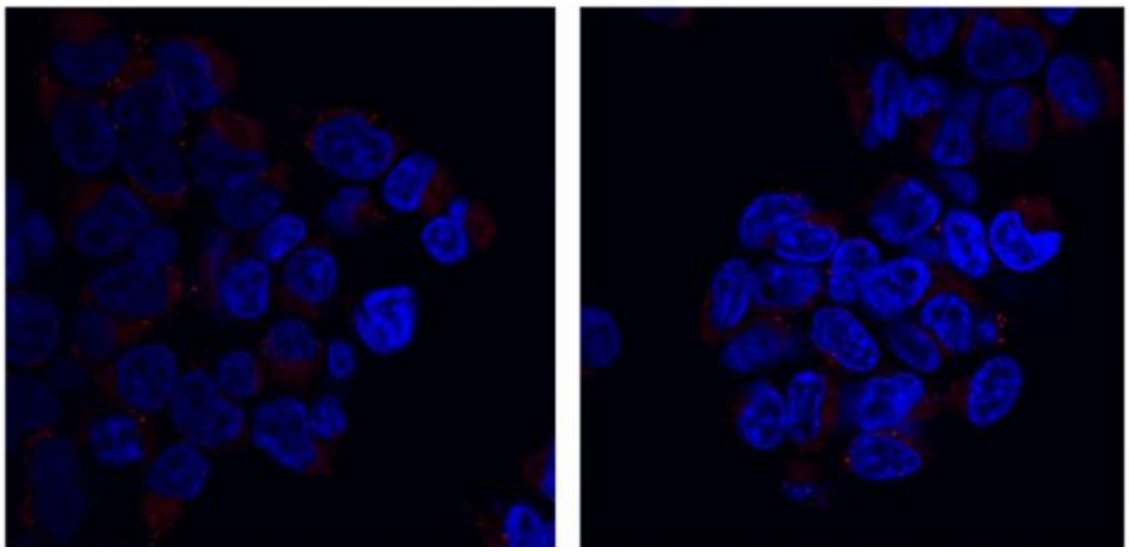


Figure 21: Control Sample for HEK-293T Cells Grown in $AgNO_3$ (No Metal Added) and Imaged Using Confocal Microscope

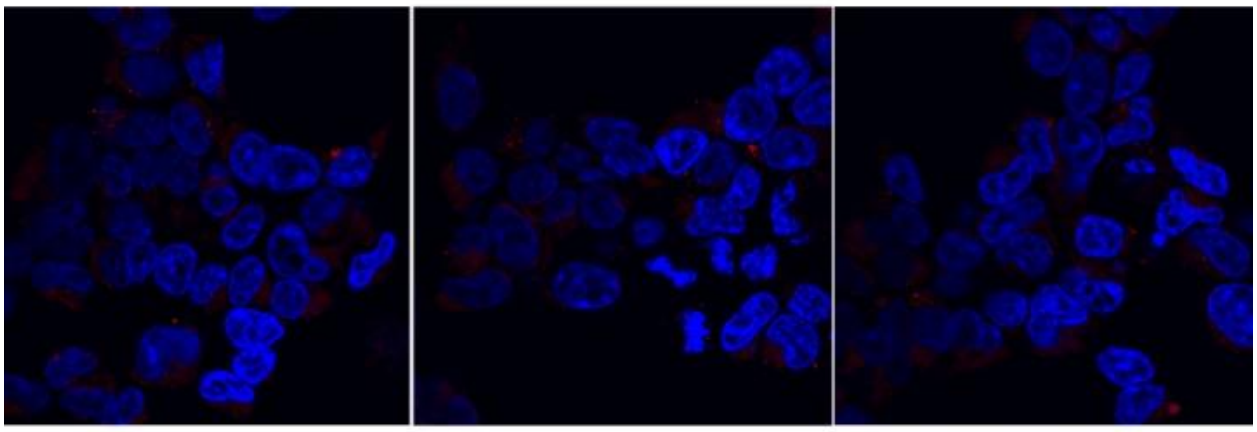


Figure 22: HEK-293T Cells Grown in Presence of 1 uM $AgNO_3$ For 6 Hours and Imaged Using Confocal Microscope

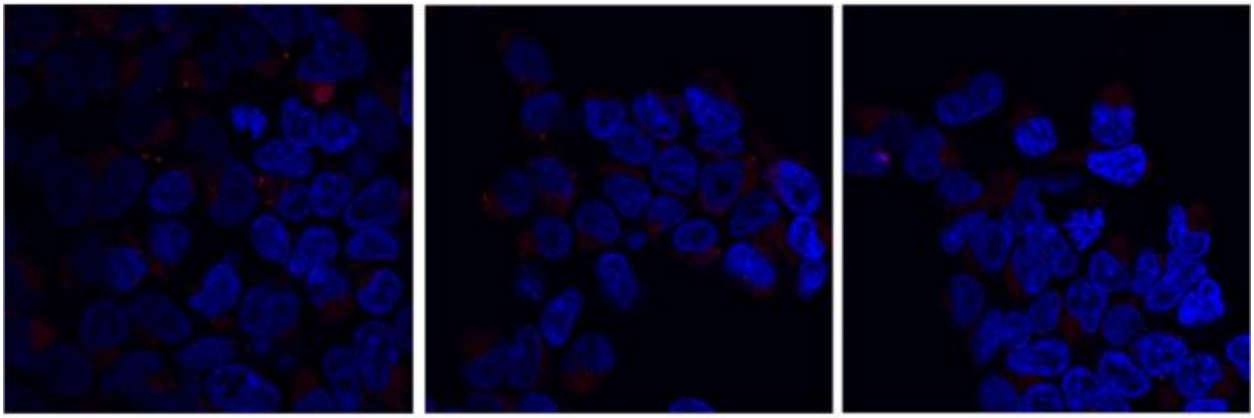


Figure 23: HEK-293T Cells Grown in Presence of 10 uM $AgNO_3$ For 6 Hours and Imaged Using Confocal Microscope

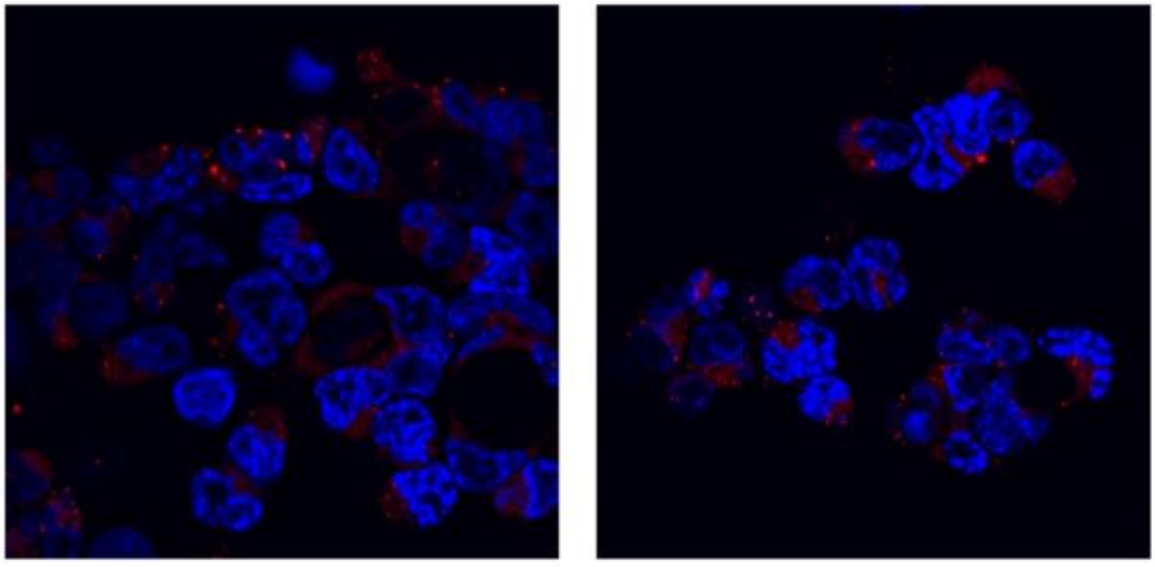


Figure 24: HEK-293T Cells Grown in Presence of 50 uM $AgNO_3$ For 6 Hours and Imaged Using Confocal Microscope

Chapter 4

Conclusion and Future Work

From the growth defect assays, a general conception of the concentration range at which each heavy metal tested becomes toxic to *E. coli* (except for $NaAsO_2$ and $ZnCl_2$, which showed no toxic effect even at high concentrations) was obtained. $AgNO_3$ and $HgCl_2$ exhibited lethality at much lower concentrations than did $CdCl_2$. These observations were used to guide the mammalian cell work and help determine how long to incubate the cells and what concentrations to test for each heavy metal. The fluorescent cell images taken of the mammalian cells (HEK-293T cells) reveal very clearly protein aggregation induced by the presence of heavy metal, increasing in severity at increased metal concentrations.

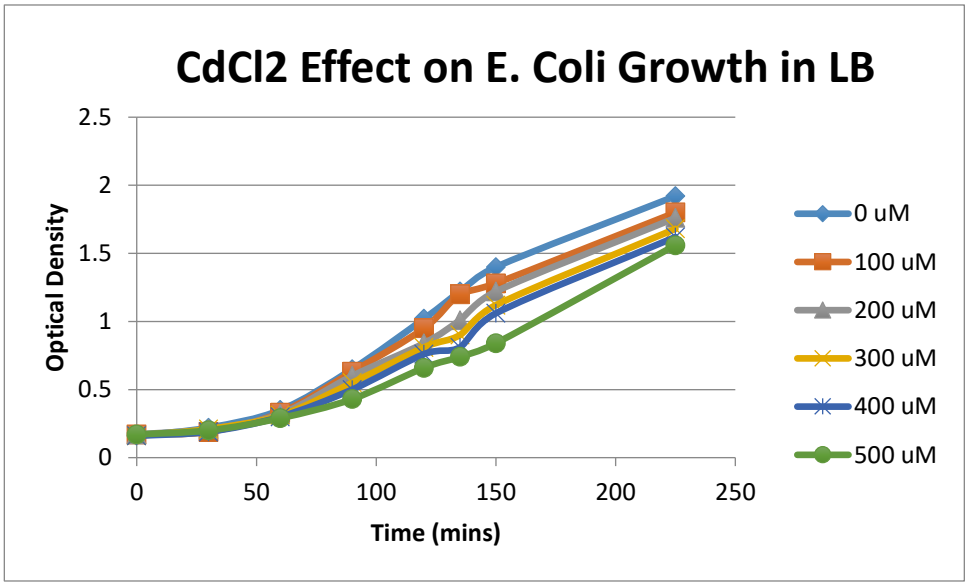
Due to the extent of time taken to select an appropriate media in which to grow the *E. coli*, optimize heavy metal concentrations, and establish a satisfactory mammalian cell line, limited data was collected using the experimental techniques that actually provide the best visualization and characterization of the effect of heavy metal on the cellular proteome in both prokaryotes and eukaryotes. Only $AgNO_3$, which was observed to have the most noticeable toxic effect on cells, was used in the AgHalo fluorescence assay and to generate confocal microscopic images in HEK-293T cells, which provide the best visualization of protein aggregation. If I stay to continue my work next semester, I intend to collect fluorescence readings and confocal images of cells exposed to a wider array of heavy metals at a range of concentrations in order to establish a more complete picture of the effect of heavy metal on proteostasis

Appendix A

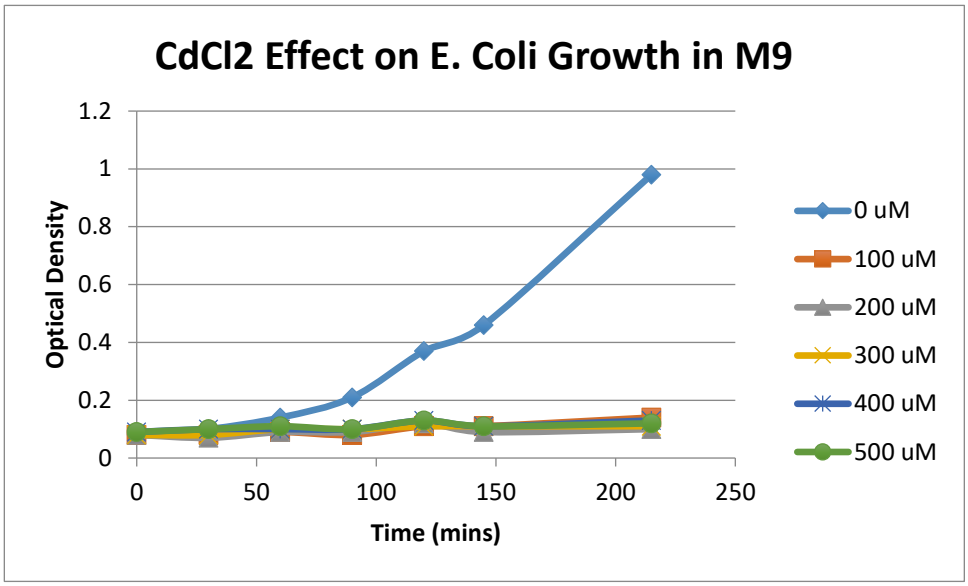
Supplemental Information

Heavy Metal Tested	Toxic Range
$CdCl_2$	100-500 µM
$NaAsO_2$	No Apparent Toxic Effect
$AgNO_3$	20-200 µM
$HgCl_2$	20-200 µM
$ZnCl_2$	No Apparent Toxic Effect

Table 1: List of Heavy Metals Used and Their Toxic Ranges (defined as the range between the lowest concentration of metal that resulted in noticeably decreased *E. coli* growth as determined by monitoring optical density and the highest concentration tested)



M9 Media



Minimal Recipe

Supplemental Figure 1: Protective Effect of LB on *E. coli* Growth in Presence of Heavy Metal Compared to M9 Minimal Media

from Spring (adapted Cold Harbor protocols-

doi:10.1101/pdb.rec12295 Cold Spring Harb Protoc 2010.)

To make 1 L of 5x M9 salt solution aliquot 800 mL H2O and add

64 g Sodium phosphate (dibasic, 7 hydrate) Na2HPO4•7H2O

15 g Potassium phosphate (monobasic) KH2PO4

2.5 g NaCl

5.0 g NH4Cl

Stir until dissolved

Adjust to 1000 mL with ddH2O

Aliquot into 200 mL portions

Sterilize by autoclaving and store at 4C

To Make 1 L of M9 Medium

Measure ~700 mL of ddH2O (sterile)

Add 200 mL of 5x M9 salt solution (see above)

Add 2 mL of 1M MgSO4 (Filter sterilize before use and store excess at room temperature)

Add 10 mL of 20% (w/v) glucose (2mg/mL) (Filter sterilize before use and store excess at 4C)

Add 100 uL of 1 M CaCl2 (Filter sterilize before use and store excess at room temperature)

**Add 10 mL of 10% (w/v) Cas amino acids (1 mg/mL)

Adjust to 1000 mL with ddH2O

Transformation Protocol

1. Add 2 uL of desired plasmid to 5 uL BL21 competent *E. coli* cells
    a. Thaw cells on ice
2. Incubate at room temperature for 30 minutes
3. Heat shock cells at 42°C for 45 seconds
4. Recover in ice for 2-3 minutes
5. Add cells to 500 uL Super Optimal Broth (SOB)
6. Shake cells at 37°C for 60 minutes
7. Plate cells w/ kanamycin
8. Incubate 12-16 hours

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