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2D Gel Protein Analysis of Recovered and Damaged Copper (II) Nitrate Treated *S. cerevisiae* Cells

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BIOL 8888

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08 December 2021

Introduction

Reactive oxidative species (ROS) are highly reactive chemicals formed from $\cdot O_2^-$ (Hayyan, Maan, et al.), and are normally made during natural processes of the mitochondrial oxidative phosphorylation, as well as a response to foreign bodies (Ray et. al). These ROS species may imbalance the interior of the cell by overwhelming the antioxidative response from a cell (Ray et. al). Furthermore, excessive amounts of certain types of metal ions will cause ROS inside the cell (Cervantes-Cervantes et. al). For example, the application of toxic amounts of Copper II allows the copper ions to pull electrons from surrounding molecules, such as oxygen, forming RO species (Cervantes-Cervantes et. al). This is because Copper II is a readily redox-active metal ion and reacts to make a variety of different ROS (Houghton et. al) by undergoing the Fenton Reaction, seen in Figure 1 (Barbusinkski).

The application of overwhelming ROS on the cell results in DNA damage, interruption in biosynthetic pathways, disruption in homeostasis (Cervantes-Cervantes et. al), and ultimately can lead to cell apoptosis (Liang et. al). A simple mechanism of apoptotic action is detailed in Figure 2; ROS can directly damage *S. cerevisiae* DNA in the nucleus, and ROS can go into the mitochondria, inducing cytochrome c expression, which results in apoptosis. Of course, the cell has natural defenses against ROS, such as antioxidant metabolites, enzymes, metal-binding proteins, transcription factors, and regulators (Perrone, Gabriel G., et al.). Some of these defenses, such as glutathione (GSH), an antioxidant metabolite, directly acts on ROS by reducing the ROS; 2 GSH molecules are oxidized to form glutathione disulfide (GSSG) (Wu, Guoyao, et al.).

Furthermore, the cell has other specific defenses when in the presence of toxic amounts of metals. For copper, specific defenses are CUP1/CUP1P, CUP2, SOD1, and CTR1. CUP1P, a

metallothionein protein, directly binds to copper ions and removes superoxide radicals (Cherry, J. M., et al.). CUP1 expression is induced upon addition of extra copper into the cell, which suggests a direct mechanism against copper poisoning (Jeyaprakash, Ayyamperumal, et al). The CUP2, also known as ACE1, locus when expressed transcribes a transcription factor (TF) that acts as an enhancer for the CUP1 gene (Buchman, C, et al.). CUP2 TF has been shown to have increased expression in high copper concentrations (Buchman, C, et al.). SOD1 is a stress response factor that transcribes SOD1P, which acts on free radical oxygens and converts them into a less toxic form (Schmidt, Martin, et al.) as seen in Figure 2 (Perrone, Gabriel G., et al). Lastly, CTR1 is directly involved with copper by facilitating the uptake of copper from the environment by making specific plasma membrane proteins (Dancis, Andrew, et al.).

The main focus of this experiment is on the apoptotic effect and protein expression differences in *S. cerevisiae* cell populations when treated with Copper (II) Nitrate. *S. cerevisiae* is chosen as the subject of this study as it is a model organism, and since its full genome has been mapped, any variance of protein expression can be analyzed. By examining the potential changes in protein expression, we hope to find a quantifiable difference of the amount of proteins expressed when a cell is subjected to ROS from Copper (II) Nitrate. Previous research has concluded that copper uptake, distribution, and detoxification is a delicate balance due to the toxic yet necessary properties of copper (Peña Maria Marjorette, et al). Furthermore, the regulation of copper related proteins must also be controlled to ensure proper survival (Peña Maria Marjorette, et al). Therefore, if the cell fails to maintain balance or regulation, then it may ultimately undergo apoptosis.

Previous research in the lab has indicated that during an acute metal treatment on *S. cerevisiae*, the apoptotic rate can vary between different metals due to their individual properties.

Previous research in the lab has indicated that 80uL of 100mM Copper (II) Nitrate results in a roughly 20% apoptotic subpopulation indicated by FAM-FLICA, a fluorescent that binds to cleaved Caspase-1. Similarly, the same fluorescent subpopulation can be seen when using propidium iodide (PI) (Figure 4), a fluorescent that binds to DNA, so, PI is shown to be a significant marker for apoptotic cells. This is because for PI to enter the cell and bind to DNA, the plasma membrane must be porous for the fluorescent to enter.

When Copper (II) Nitrate is added to media with *S. cerevisiae*, it obviously affects the entire cellular population, however only a subset of that population is damaged enough to be able to bind to PI. The larger population must still be affected by the metal and receive cellular damage, but they are able to recover from the resulting oxidative stress. Interestingly enough, the recovered and damaged cell population have consistent population percentages when analyzed on the Fortessa. For example, the Copper (II) Nitrate treated damaged cells make up roughly 17-20% of the overall population, while the non-fluorescent recovered cells are the remaining 83-80% majority.

General protein expression differences between Copper (II) Nitrate treated damaged *S. cerevisiae*, and Copper (II) Nitrate treated recovered *S. cerevisiae* were examined and quantified. Because there are differences in protein expression when a cell undergoes apoptosis, we expect there to be differences in protein expression in the entire treated population, as the recovered may express more damage related proteins and genes, as well as proteins and genes related to cell survival and damage control, while the damaged population may express more cell death proteins.

Materials and Methods

1. Starter Culture
    a. 4-5 colonies of yeast are chosen from YEPD agar plate. These colonies are scooped with a sterilized inoculating loop, then placed into 5mL of YEPD medium in a 15mL falcon tube. This falcon tube is vortexed and then placed in a rotating shaker set at 30C at 250RPM overnight. This culture will serve as the basis for the future water bath experiment.
    b. This step serves, to start the growth of the yeast, and to have a weekly population to run experiments. It is important to make a new starter culture on a weekly basis because the cells will inevitably age after a couple of days, which may affect their response to Copper (II) Nitrate. Additionally, glycerol stocks are made from these samples as necessary. Lastly, we assume that we are working without a mutant population, and always will work with the same cell lineage, which is maintained through the use of plating and glycerol stocks.
2. Growth of Starter Culture via Water Bath
    a. 10uL of the starter culture is pipetted into 100mL of YEPD media in an Erlenmeyer flask. The flask is placed into a water bath set at 30C and set to shake at 250RPM overnight until media turbidity has reached an OD600 of 2.0-2.2, which is expected at 16 hours of growth.
    b. This step is to allow the starter culture to grow in a fresh medium, where it can grow and be used for further testing. We do this step to have a larger volume of viable cells to work with, because for some experiments, we would need 60mL of cells, which is unobtainable from a 15mL falcon tube. An OD of 2.0-2.2 is a

required reading as that is the start of the exponential phase of yeast, which is its highest energy phase.

3. Determination of OD via Biospectrometer
    a. Biospectrometer is turned on at least 30 minutes prior to first reading to warm up the bulb. 600nm wavelength is selected. To blank, 1mL of YEPD media is pipetted into a cuvette and then used to blank. To read OD of media, 100uL of media culture is pipetted into a clean cuvette. 900uL of YEPD is then pipetted in with the 100uL of culture, then the blend is mixed via pipetting 5 times to ensure dispersion of media. Sample is placed into biospectrometer and read at 600nm. Resulting OD is then multiplied by 10, as the blend was a dilution of 10.
    b. YEPD, not YEP or H_20, is used to blank to maintain consistency between flasks as YEPD naturally has a yellowish hue and added dextrose.
4. Sample Treatment of Copper (II) Nitrate and Untreated
    a. 10mL of the media culture is aliquoted into a 50mL falcon tube. Repeat to have 2 sample tubes, where one will be Untreated (UT) and the other will be treated with Copper (II) Nitrate (Cu). Add 80uL of 100mMol Copper II Nitrate to the Cu sample tube. Vortex to mix. Immediately put both Cu and UT samples into shaker set at 30C at 250RPM for 1 hour.
    b. 80uL of 100mMol Copper II Nitrate is used as it was previously determined by the lab in a copper growth curve experiment. Additionally, 30C is used as that is optimal temperature for yeast growth. 250RPM is standard for yeast growth. 1 hour of metal treatment was chosen as previous research in the lab has indicated that as the preferred time for apoptotic pathways to be expressed.

5. Washing Out of Metals and Resting Cells
    a. After 1 hour of metal treatment, samples are placed into a centrifuge set at 5000RPM at 4C, and let spin for 2 minutes. Immediately decant liquid, pouring away from the cell pellet. Resuspend the pellets in 5mL of ddH_2O and vortex. Centrifuge the sample again at 5000RPM at 4C for 2 minutes. Decant liquid again, pouring away from the cell pellet. Resuspend the pellet with 10mL of YEPD and vortex. Place into the shaker set at 30C at 250RPM for 2 hours for rest.
    b. Cells should be washed to ensure the removal of any remaining metal after pouring off.
6. Addition of Propidium Iodine
    a. At 1 hour into the resting phase, the tubes are removed and 100uL of 1mg/mL PI is added into each of the 10mL samples. The tubes are then wrapped completely in aluminum foil to shield the fluorescent from light, and then placed back into the shaker set at 30C at 250RPM for the remaining hour to complete rest.
    b. While previous research in the lab has used 10uL of [250ug/mL] PI for 50uL of cells, a dilution experiment (Figure 6) was performed to find the minimum amount of PI to effectively stain cells. This is because when following the previous research's amounts, to stain 10mL of cell media, 2mL of [250ug/mL] or 500uL of [1mg/ml] would have to be used per sample, which means a minimum of 1mL of [1mg/ml] would be used per experiment as Cu and UT both have to be stained. Considering that the largest vial of PI we could find online was a 10mL [1mg/mL], to save time and money, we performed a dilution experiment (Figure 6) of PI and tested on 3 yeast samples: UT, treated with Cu, and treated with

Cadmium (II) Nitrate (Cd). The samples must be shielded from light to avoid bleaching of the fluorophore.

7. FacsARIA Cell Sorting
    a. After the resting phase is completed, the samples are removed from the aluminum foil and placed into a centrifuge set at 5000RPM at 4C for 1 minute. The media is discarded, and the cell pellets are resuspended in 5mL of phosphate buffered solution (PBS) and then vortexed. The media is then handed to the FacsARIA CORE operator (thanks Sandy!) where FLOW cytometry is ran at 338nm to assess the Cu sample and compared to the UT. FLOW gates are made to identify subpopulations (Figure 4) as well as the percentage of subpopulations to the total population by using. The ARIA then sorts the Cu sample into two populations, apoptotic and recovering, which are fluorescent and non-fluorescent cells respectively (Figure 4).
    b. The cells are resuspended in PBS because YEPD has a yellowish hue and PI adds a reddish color to the media, which may affect the accuracy of sorting with the ARIA laser. We theorized that flash freezing the cells prior to sorting would rupture the cells, causing protein leakage and may cause the expression of different stress proteins than the ROS we wanted to focus on. Additionally, we thought to use a formaldehyde to essentially arrest the cell, but we figured there were too many biological and experimental variables to account for as we would have to wash out the formaldehyde prior to cell sorting as it would naturally clog the ARIA. Therefore, we chose the best course of action for our limited resources,

which is to start the cell sorting immediately after the apoptosis pathway expression take place.

8. Sample storage
    a. Apoptotic and recovering samples are centrifuged at 7000RPM at 4C for 15 minutes. The liquid is poured out away from the resulting pellet with any leftover liquid being pipetted out, and the pellet is resuspended with cold 1mL PBS and mixed via pipetting. The samples are then centrifuged again under the same previous conditions, and the liquid is then carefully pipetted out. Samples are then flash frozen with zinc beads and placed into the -80C freezer for later use.
9. Protein Isolation
    a. Samples are taken out of the -80 freezer and put on ice. After thawing, pellets are resuspended with Lysis buffer, and collectively transferred to a mini screw cap containing .5mm silica beads. Cell membranes are broken with a mini bead beater for 30 seconds, and then the samples are immediately put on ice for 30 seconds. Repeat this process 3-5 times. After the last icing, screw caps are centrifuged at 15,000 RCF at 4C for 30 minutes. Resulting supernatant is pipetted out and placed into a clean microcentrifuge tube, and then placed on dry ice to flash freeze. Samples can be stored in the -80 freezer afterwards.
    b. For every 13mL of samples (roughly $6.0x10^6$ cells sorted), 200uL of lysis buffer is used, but to achieve a more concentrated protein sample, a lower volume of lysis buffer can be used. The .5mm silica beads are filled to the lower indicated line on the mini screw cap. Samples must be kept on ice to prevent proteins from denaturing, and it is important to use cold lysis buffer to maintain temperature.

10. Protein Concentration Determination via BioRad (BSA) Assay

    a. First, a Bradford standard curve must be established using a BSA protein assay kit. First, 20mg/mL BSA must be diluted into 1.5mg/mL, 1.0mg/mL, 0.5mg/mL, and 0.25mg/mL concentrations. This is done with lysis buffer as the diluent. 30uL of 20mg/mL BSA is mixed with 370uL lysis buffer to make the 1.5mg/mL. 20uL BSA is mixed with 380uL lysis buffer to make the 1.0mg/mL concentration. 200uL of the 1.0 concentration is separately mixed with 200uL of lysis buffer to make the 0.5mg/mL concentration. Finally, 150uL of the 0.5mg/mL concentration is mixed with 150uL lysis buffer to make the 0.25mg/mL concentration. To create a readable sample for the biospectrometer, 20uL of a concentration or unknown protein is pipetted into a clean microcentrifuge tube, then 100uL of reagent A is added to each tube, then 800uL of reagent B is added in that order. Each sample must be completed in triplicate. A blanking sample is prepared by using 20uL of lysis buffer. Protein samples are tested by putting 10uL of sample into 90uL of PBS prior to aliquoting and adding reagent A and B, essentially diluting by 10-fold. Samples are read at 750nm. A sample BSA curve can be seen in Figure 8.

    b. The BSA curve follows the protocol for a Bradford standard curve. Once the absorbance values have been found with the biospectrometer, taking the average or best value from each will allow a standard curve. It is expected that each concentration's absorbance increases linearly, i.e., if the 0.25mg/mL concentration average absorbance is .05, then the 0.5mg/mL concentration average should be absorbance of 0.10. Plotting these absorbance values on a plane, we can calculate a standard curve equation in the format of y=mx+b. Then

we can input the absorbance value of our unknown protein sample for the y value and solve for x.

11. 2D Cleanup/Protein Purification

    a. Protein purification is achieved through the use of the GE 2D protein clean up kit. First, 100ug of protein must be placed into 3 microcentrifuge tubes; this can be calculated by dividing 100ug by the concentration of the protein in ug/uL, which will solve for the uL amount to pipette per tube (e.g., 100ug ÷ 20ug/uL = 5uL). 300uL of Precipitant is added, and the solution is mixed via vortex. Solution is placed on ice for 15 minutes to incubate. 300uL of co-precipitant is added, and vortexed to mix. The sample is centrifuged for 5 minutes at 13,000RPM or greater. Supernatant is then removed without disturbing pellet. Sample is centrifuged again for 30 seconds with the pellet facing outwards. Supernatant is removed with pipette. 40uL of co-precipitant is layered on top of pellet and let to sit for 5 minutes. Samples are then centrifuged again for 5 minutes at 13,000RPM or greater, and the wash is pipetted out. 25uL of DI H_20 is pipetted on each pellet, and then vortexed for 10 seconds to disperse pellet. 1mL of cold wash buffer and 5uL of wash additive is added to each sample, and then vortexed until proteins are fully dispersed. Samples are incubated at -20C for 1 hour. Every 20 minutes, samples are vortexed for 30 seconds. Samples from 2D clean up are centrifuged for 5 minutes at maximum RPM. Wash buffer is decanted, and the samples are left to air dry inverted. When the pellet has visibly started to dry, 15uL of S/R buffer is added directly to the pellet. Once the pellet has dispersed, the 15uL of the third sample is transferred to the first sample. Repeat with the second sample,

which will make the first sample's volume now be 45uL. 15uL of S/R buffer is added to the third sample. This volume is then transferred to the second sample, and finally from the second sample to the first sample, resulting in 60uL of total volume, and theoretically, 5ug/uL.

b. For our 2D clean up, we used 21.88uL of our apoptotic proteins and 12.91uL of our recovering proteins.

12. SDS Page Gel

a. Preparing Clean and Unclean Samples

i. Cleaned samples are prepared by diluting the concentration to 1ug/uL. 4uL (20ug) of cleaned sample is pipetted into fresh microcentrifuge tube. 12uL of S/R buffer is added. 4uL 5X Sample Buffer is added. Sample is placed on a heat block for 5 minutes at 95C.

ii. Unclean samples are prepared by diluting concentration to 1ug/uL. E.g., 1uL of 20ug/uL is added to a clean microcentrifuge tube. 15uL of lysis buffer is added. 4uL of 5X Sample Buffer is added. Sample is placed on a heat block for 5 minutes at 95C

b. Preparing Glass Plate

i. Glass plates, one with spacer and one without spacer must be washed with 70% ethanol and H_2O before use. Glass plates are assembled by placing the non-spacer plate on top of the spacer plate, and then clamped together to form a seal. Plates and clamp are placed into holding apparatus, and leaks are checked with 70% ethanol. Gel comb is placed in between plates,

and then a pen mark is made 1cm below the length of the comb. This will serve as the fill line for the discontinuous gel.

c. Preparing 12% Discontinuous and Stacking PA Gel

   i. 15mL of 12% Discontinuous PA is prepared by adding 4.95mL ddH_2O, 3.75mL 8.8pH 1.5M Tris-HCl, 6mL 30% Protogel (polyacrylamide), 150uL 10% SDS, and then is mixed via inversion. When ready to pour gel, 150uL of 10% APS and 6uL of TEMED is added to start the polymerization. Only about 9mL of solution will be used to pour to the previously made mark. Isopropanol can be pipetted on top of the gel to prevent drying.

   ii. 15mL of 12% Stacking PA is prepared by adding 10.4mL ddH_2O, 1.88mL 0.5M Tris-HCl, 2.5mL 30% Protogel, 150uL of 10% SDS, and then is mixed via inversion. When ready to pour, 40uL of 10% APS and 20uL of TEMED is added to the solution to start polymerization. Only about 5mL of solution will be used. After pouring, immediately place comb in between glass plates to form wells.

d. Loading Samples

   i. Wells are written down with the sample that will be pipetted into it. The ladder will always be well 1. An example well chart:

Well	1	2	3	4	5
Sample	Ladder	Clean	Clean	Unclean	Unclean
Amount	4uL	10uL	7uL	10uL	7uL

Amounts are varied to ensure proper technique. Since each sample, besides the ladder, has a 1ug/uL concentration, then comparing the 10uL to 7uL band results should result in a 30% decrease from 10uL to 7uL. Furthermore, comparing unclean to clean band intensity will determine the amount of protein loss after 2D cleanup.

e. Running SDS Page Gel
    i. Gel apparatus is filled with 1X running buffer. Gel is then ran at 60V for 20 minutes. Once ladder has reached discontinuous gel line, voltage is increased to 100V for at least 30 minutes. Once ladder reaches halfway through the discontinuous gel, voltage is increased to 150V until completion.

f. Analyzing SDS Page Gel
    i. After running is complete, gel is removed and placed into a light shielded container on a rotating shaker and washed with DI H_2O for 5 minutes. Water is poured out, and then Coomassie brilliant blue is added to the container. Gel is left gently rotating overnight to stain.
    ii. After the stain has completed, gel is washed with DI H_2O for 5 minutes 3 times. After the last wash, gel can be scanned on the GE Typhoon.

13. IEF

a. Untreated (A) and treated (B) samples are removed from the -80C and put on ice. 25ug of each sample is placed into a fresh collection tube labeled sample A and sample B. 1uL of 500mM TrisHCL at 8.8pH is added to each tube. 1uL of Cy3 is

added to sample A, and 1uL of Cy5 is added to sample B. Solutions are mixed via vortex and then spun down. Samples are shielded from light and incubated on ice for 30 minutes. Cy3 and Cy5 labeling is stopped by adding 1.2uL of 10mM lysine. Solutions are briefly mixed via vortex and spun down. Samples are left covered on ice for 10 minutes. 2X Sample Buffer is added to each tube to the equal volume of the total reaction volume, e.g., if there is 5uL sample, 1uL TrisHCL, 1uL Cy3, and 1.2uL lysine, the total volume is 8.2uL, so 8.2uL of 2XSB must be added, making the final volume 16.4uL. Combine both samples together by moving one sample into the other tube. Add rehydration buffer to the combined sample to reach 360uL of total volume. Sample is loaded into a loading boat. An 18cm 4-11pH gel strip is removed from its package, and the gel side is loaded into the boat, gel side facing down. Remove air bubbles with pipette tip, and then cover the gel strip with 700uL of cover fluid solution. Boat is placed into IEF machine and run for 24 hours.

b. Two sets of samples must be made with inverse Cy3 and Cy5 labeling for two gels, this is because it is important to be able to confirm the validity of a gel; see Figure 10. While it would be far better to run triplicates of a gel, the time of obtaining enough apoptotic cells makes triplicates extremely difficult to achieve for the scope of this experiment.

c. IEF Program:

    i. 75uAMP Rehydration for 16 hours

    ii. Step, 500V, 500V/hr for 1 hour

    iii. Grad, 1000V, 800V/hr for 1 hour

iv. Grad, 10000V, 16500V/hr for 1 hour

v. Step, 10000V, 13700V/hr for 1 hour

vi. Step, 300V for 3 hours

14. 2D Gel Electrophoresis

a. Plate Cleaning

i. 2D Spacer plates and plates without spacers are washed with LiquidX to remove any debris, then are inspected for any residue, and then left to dry. After plates dry, the gel side is washed with ethanol and water.

b. Gel Casting

i. Plates without spacers are placed onto spacer plates gel side facing inwards. Plates are placed into a clamp apparatus with other plates with plastic separators. Cover plate is secured using screws and clamps. Gel mixture is poured directly over the apparatus, air bubbles are removed, and then 2mL of isopropanol is added on top of the gel to prevent drying. After 1 hour of casting, isopropanol is poured off, and the gel is lightly washed with DI H_2O. Multiple gels are cast, and the best are selected depending on the perfection of the gel line.

c. IEF Preparation

i. Using tweezer, IEF strip is removed from the boat, and excess cover fluid is dripped off. IEF is placed into a petri dish, gel side facing inwards. IEF is equilibrated with EB1 for 20 minutes covered with low agitation via a shaker. EB1 is then poured off, and then gel is equilibrated with EB2 and bromophenol, and covered for 20 minutes with agitation via a shaker. EB2

is then poured off, and the gel strip is washed by dipping into 1X SDS running buffer. IEF strip is placed in between the 2D plates at the lip, with the IEF gel side contacting the gel in the 2D plate. IEF is overlaid with 0.5% low melting agarose, 1 to 1.5mL.

d. Gel running
    i. Gel plate is placed into a holder, and holder is loaded into 2D gel electrophoresis container. Container is filled with 1X running buffer to fill line. Electrodes are attached, and the container and gel are shielded from light. The gel is ran at 2-10Amp overnight, and then increased up to 30AMP the next day, depending on the progress of the gel.
e. 2D Gel Scanning via Typhoon
    i. 2D gel is analyzed using the GE Typhoon. Cy3 and Cy5 are scanned, resulting in different fluorescence values on the gel. A ratio is established to ensure consistency.

15. 2D Gel Analyzation
    a. ImageQuant Analyzation
        i. 2G gel picture is saved and then is placed into ImageQuant analyzation software. 4 bands are selected, and their values are recorded; see Figure 11. Because gel is scanned for Cy3 and Cy5, a ratio must be established between the two fluorescents by selecting the same 4 bands per fluorescent overlay. This is repeated for the second gel with the same 4 bands to confirm that the gels are equal.
    b. DeCyder

i. After gel ratios have been established, gel images are uploaded into DeCyder, a gel imaging software. Gel A protein bands are compared using the fluorescent amount of Cy3 and Cy5, and their volume ratios are compared for each protein of interest. Each protein of interest volume ratio must be confirmed with Gel B, and the volume amount between the two gels are averaged together. Average volume ratio is calculated by obtaining a ratio for a protein from its Cy3 and Cy5 volume for both Gel A and Gel B. The ratios are then averaged together. Average volume ratio example:

	Gel A	Gel A	Ratio	Gel B	Gel B	Ratio	AVG
	Cy3 Volume	Cy5 Volume		Cy3 Volume	Cy5 Volume		
Protein 1	1.0	2.0	0.5	3.0	1.0	3.0	1.75

Results

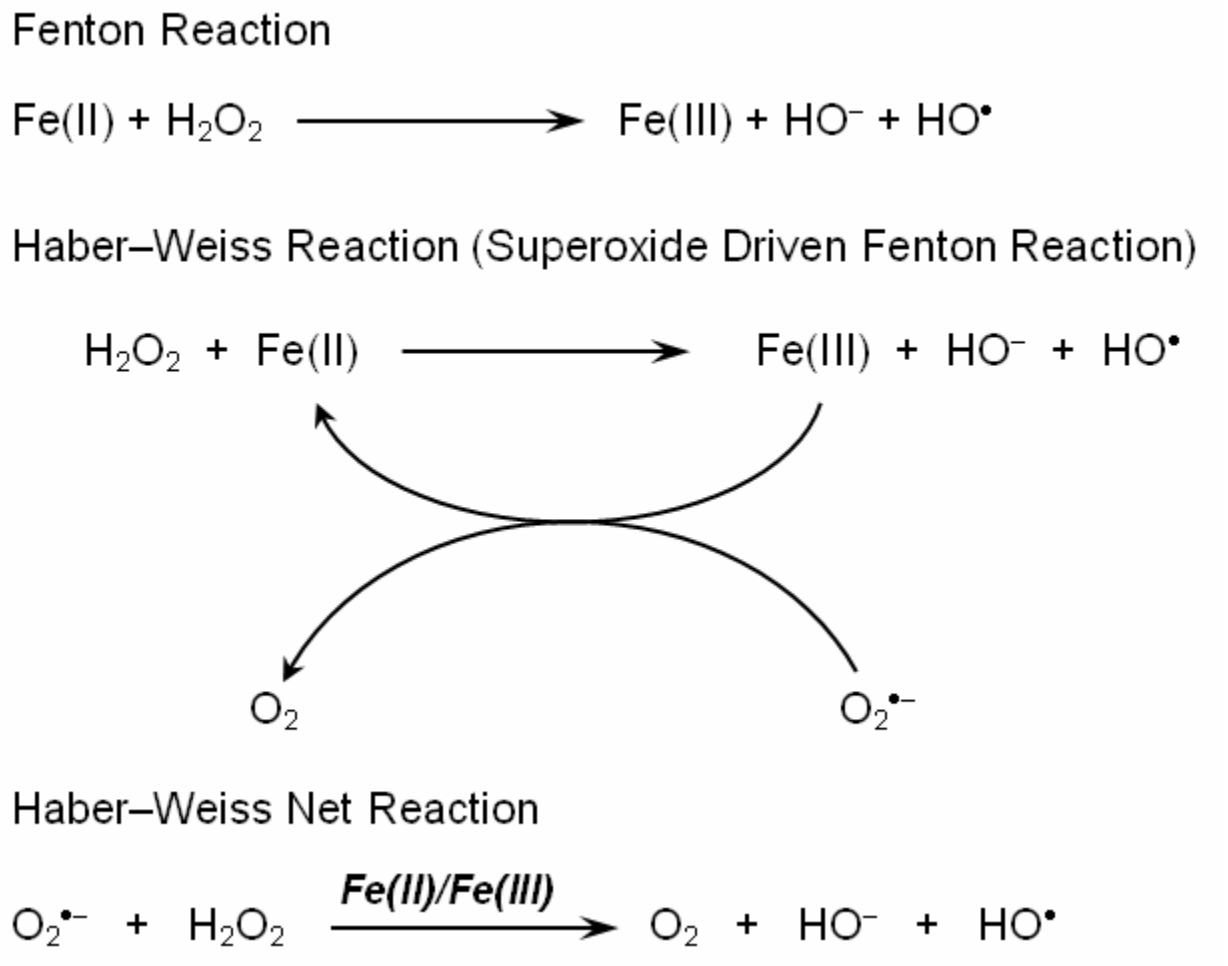


Figure 1: Fenton Reaction (Barbusinski, Krzysztof. (2009). Fenton reaction - Controversy concerning the chemistry. Ecological Chemistry and Engineering S. 16. 347-358.)

This figure shows the Fenton Reaction. While Fe(II) is used as an example, it's important to note that Cu(II) follows the same reaction. The metal interacts with hydrogen peroxide, ripping off an electron to become Fe(III) and splitting the hydrogen peroxide molecule into HO- and HO radical. Furthermore, Fe(III) can interact with $\cdot O_2$- free radical to donate its electron, returning to Fe(II). This allows Fe(II) to undergo the Fenton Reaction repeatedly. Finally, the net reaction shows Fe(II) or Fe(III) catalyzing the formation of O_2, HO-, and ·HO from an $\cdot O_2$- and H_2O_2.

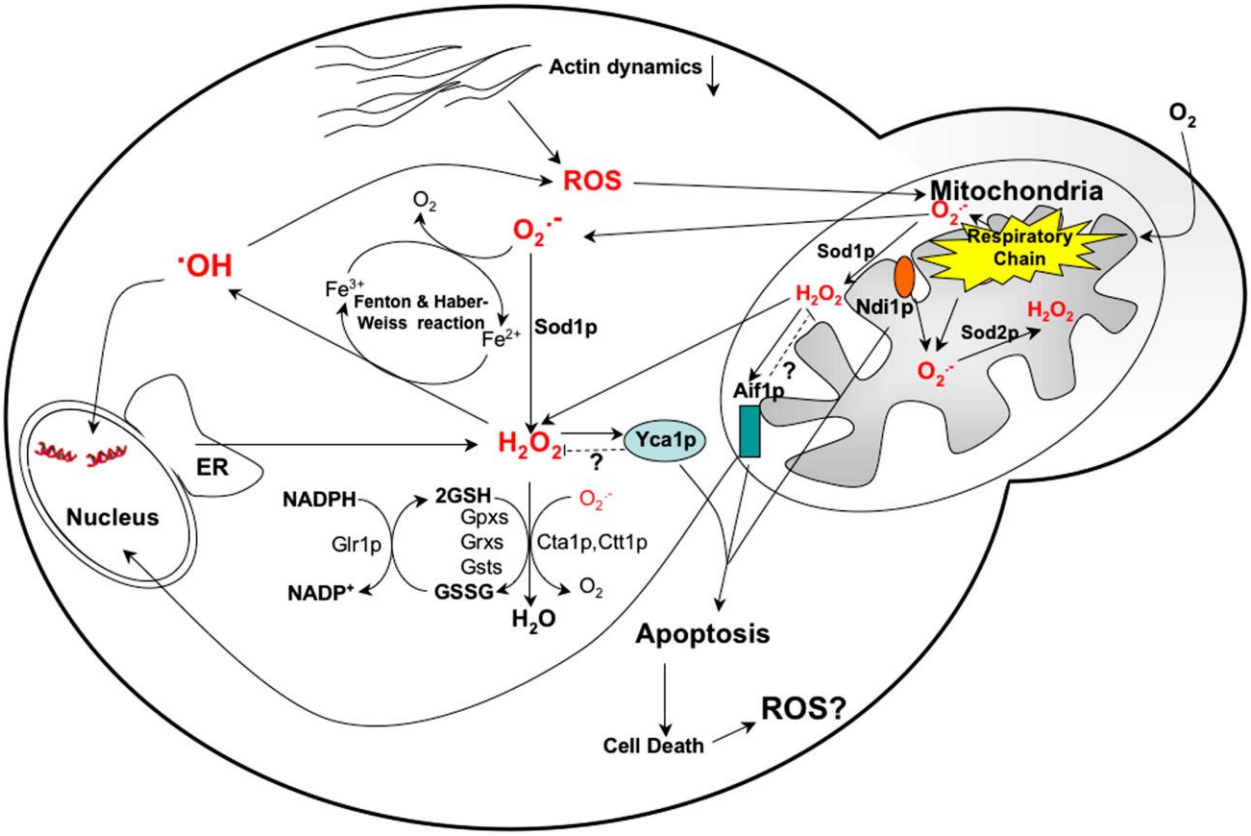


Figure 2: Potential sources of ROS relevant to apoptosis in yeast (Perrone, Gabriel G., et al. "Reactive Oxygen Species and Yeast Apoptosis." Biochimica Et Biophysica Acta (BBA) - Molecular Cell Research, vol. 1783, no. 7, July 2008, pp. 1354–1368., https://doi.org/10.1016/j.bbamcr.2008.01.023)

Figure 2 shows a simplistic and straightforward depiction of ROS' effect on the cell. After the Fenton & Harber-Weiss Reaction with copper, $\cdot O_2$- directly acts on H_2O_2, creating an ·OH that will enter the nucleus or mitochondria. In the nucleus, DNA damage occurs, and multiple pathways cascade which can lead to apoptosis and cellular death.

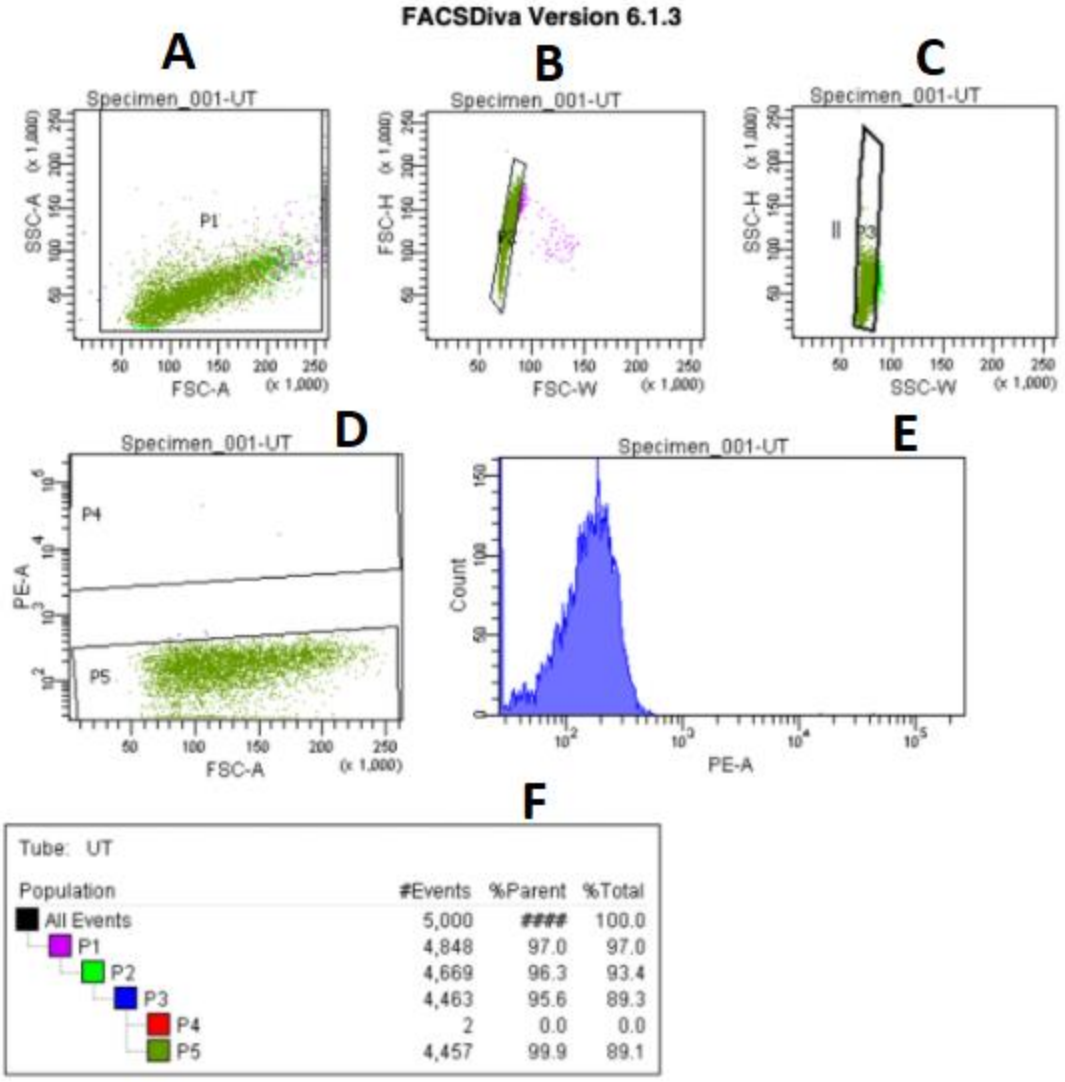


Figure 3: FacsARIA Flow Cytometry for UT sample

Figure 3 shows an example of the FacsARIA flow cytometry for a UT sample. **A** shows Forward Scatter Area (FSC-A) on the X-axis, and Side Scatter Area (SSC-A) on the Y-axis. The population was gated to exclude any abnormalities or debris that did not coincide with the larger population. **B** shows FSC-Width compared to FSC-height. A subpopulation is shown to be within the parent population of FSC-H, but these cells are nearly doubling the parent population in width. These cells are most likely undergoing the budding process, and are excluded from selection. **C** shows the comparison of SSC-H to SSC-W of the gated population from B. Cells are gated to exclude any abnormalities. **D** shows the FSC-A to recovering A of the population gated in C. Here we can see two populations gated, P4 and P5, where P4 are events that have a higher fluorescent value than P5. These higher fluorescent events indicate the staining of PI on the cell. **E** shows a histogram of PE-A, which shows the number of events per value of PE-A. Most events lie between 10^2 and 10^3, which coincides with D as most of the population did not have a fluorescence. Finally, **F** shows a population hierarchy chart with each gated population

listed as well as number of events, the percent of its parent population, and the percent of the total population.

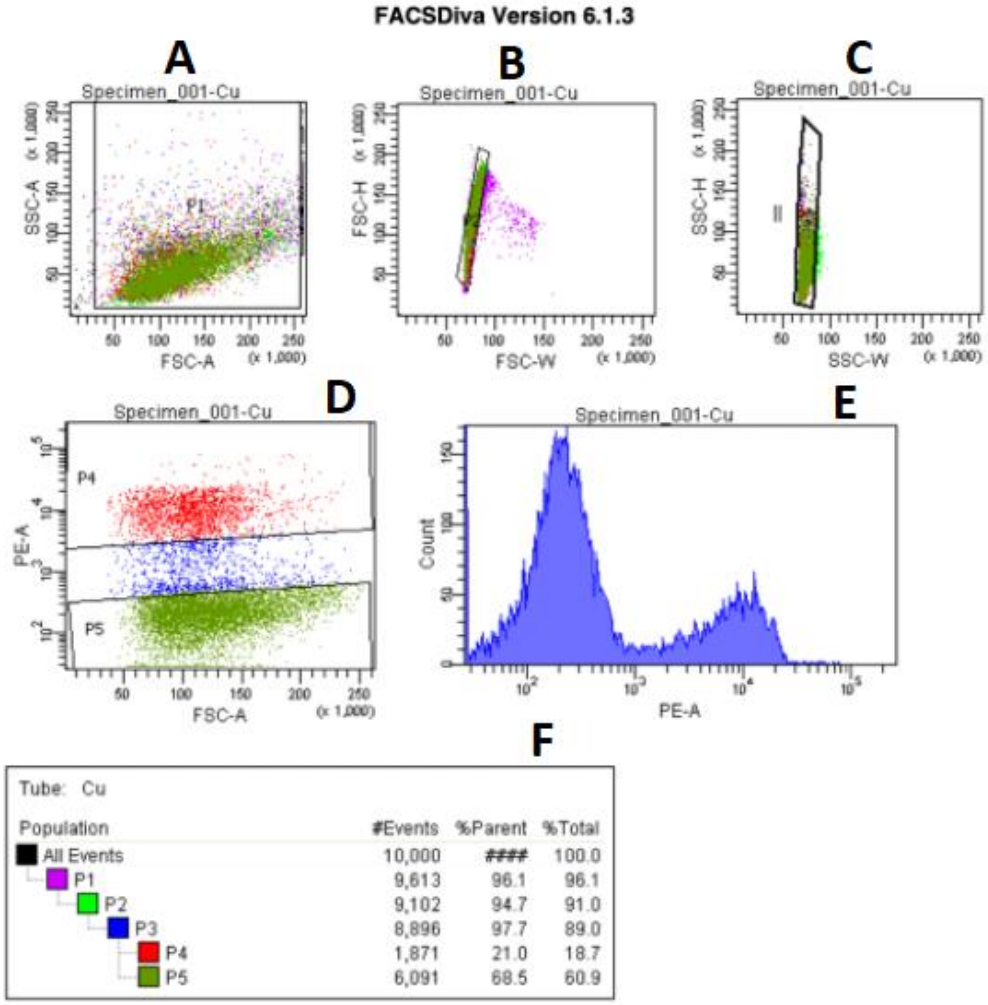


Figure 4: FacsARIA Cu Flow Cytometry

Figure 4 shows an example of the FacsARIA flow cytometry for cells treated with Cu. The parameters, i.e., the gating for **A-C**, throughout the experiment remains the same logic as Figure 3, where cells are excluded from sorting based on abnormalities identified by FSC-A by SSC-A, FSC-H by FSC-W, and SSC-H by SSC-W. **D** shows the gates of two populations, P4 and P5, where P4 are highly fluorescent events, meaning that PI has bound effectively, and P5 are the events that did not fluoresce and are similar to the UT P5 events from Figure 3. Events not within P4 or P5 are not included to sort as they do not exhibit neither high nor low enough fluorescence and could flaw the resulting sample collections. **E** shows the histogram of PE-A,

indicating two main populations at fluorescent values 10^2 and 10^4. **F** shows the population hierarchy table, and P4 accounts for 18.7% of all events, and 21% of the vetted population.

Experiment : 08092021_001 | Sort Report | Report Date : 2021.08.10 at 13:38:03
Specimen : Specimen_001 | Device : 2 Tube
Tube : Global Sheet1 | User ID : Administrator
Sort Layout : Sort Layout_001 | Cytometer : FACSAriaII (1)
Application : FACSDiva Version 6.1.3

Sort Settings

Sort Setup	70 micron	Phase Mask	0
Frequency	87.0	Single Cell	Off
Amplitude	8.0	Sweet Spot	On
Phase	0.00	First Drop	138
Drop Delay	41.00	Target Gap	9
Attenuation	Off	Plates Voltage	4,500
Precision	Purity	Voltage Centering	0
Yield Mask	32	Sheath Pressure	70.00
Purity Mask	32		

Side Stream Voltage (%)

Far Left	Left	Right	Far Right
0.00	52.00	40.00	0.00

Neighboring Drop Charge (%)

2nd	3rd	4th
15.00	10.00	0.00

Acquisition Counters

Threshold Count	32214590
Processed Events Count(evt)	23814225
Electronic Aborts Count(evt)	261183
Sort Elapsed Time(hh:mm:ss)	01:53:42

Sort Counters

	Left	Right
Sort Rate(evt/s)	889	2726
Conflicts Count(evt)	291587	421991
Conflicts Rate(evt/s)	42	61
Efficiency(%)	95	97

Sort Layout

Left	Right
P4 : 6068332	P5 : 18599115

Figure 5: FacsARIA Sort Report for Cu

Figure 5 shows an example of the sort report the FacsARIA gives at the end of a sorting session. The Sort Settings, who's results may fluctuate by +/-0.5, shows the size of the tube in which cells are processed through, the frequency and amplitude of the machine, the type of precision, as well as pressure and voltage. Furthermore, the machine is able to display the exact number of cells that are dispersed to both sides, where left is the apoptotic and right is the recovering events. For this run, the FacsARIA sorted roughly 24 million cells, giving 6.06 million apoptotic and 18.6 million recovering cells.

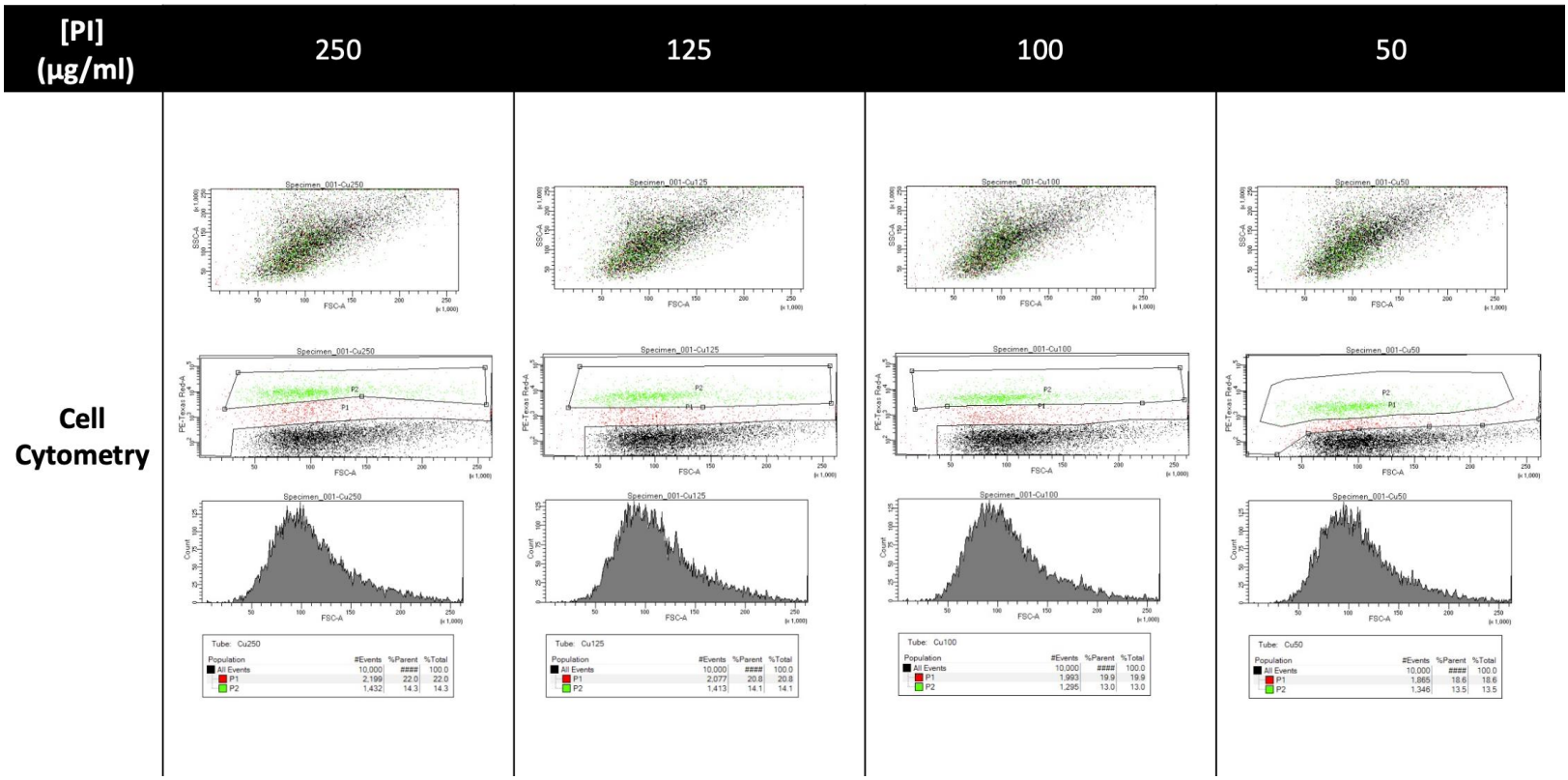


Figure 6: Copper (II) Nitrate sample Dilution of PI

Figure 6 shows FLOW cytometry of cells treated with Cu and stained with different concentrations of PI at 640nm. From left to right, the PI concentration decreases, from [250ug/mL] to [50ug/mL]. 2 populations were gated, P1 and P2, where P1 is the entirety of all fluorescent events, and P2 is only the most fluorescent events. As the concentration of PI decreases, there is only a marginal decrease. At half concentration, P1 is only 0.2 smaller than original. At the lowest concentration, P1 is 0.7 smaller than the original. This could be an operator error of gating, as excluding a couple of events might result in this drop. Overall, we are confident with our data and decided to stain with a 50ug/mL concentration.

Month	Day	Apoptotic in millions	Recovering in millions
July	8	2.6	11
	15	8.3	27
	22	5.3	34
	23	4.9	25
	26	7.3	30
	30	6.5	23
August	2	2.7	21
	9	6.8	29
	10	6	18
	7	6	19
September	27	2.2	20
October	6	2.2	21
	12	3.7	24
	12	3.3	21
	19	6.8	46
	21	6.1	19
	21	3.3	19
Total	17 Sessions	84	407

Figure 7: FACSARIA Session Table

Figure 7 shows the number of ARIA sessions from July to October. A total of 17 sessions resulted in 84 million apoptotic cells, and 407 million recovering cells. Each day's cellular sorted amount was rounded to two significant figures. Overall, there are a total of 491 million cells were sorted in approximately 25.5 hours, resulting in 17% being apoptotic and 83% being recovering cells.

	Blank	0.25	0.5	1.0	1.5	Apoptotic	Recovering
Sample 1	0.000	.049	.099	.190	.246	.101	.157
Sample 2	-0.011	**.041**	**.098**	**.201**	**.286**	.083	**.149**
Sample 3	**-0.003**	.041	.040	.194	.273	**.087**	.148

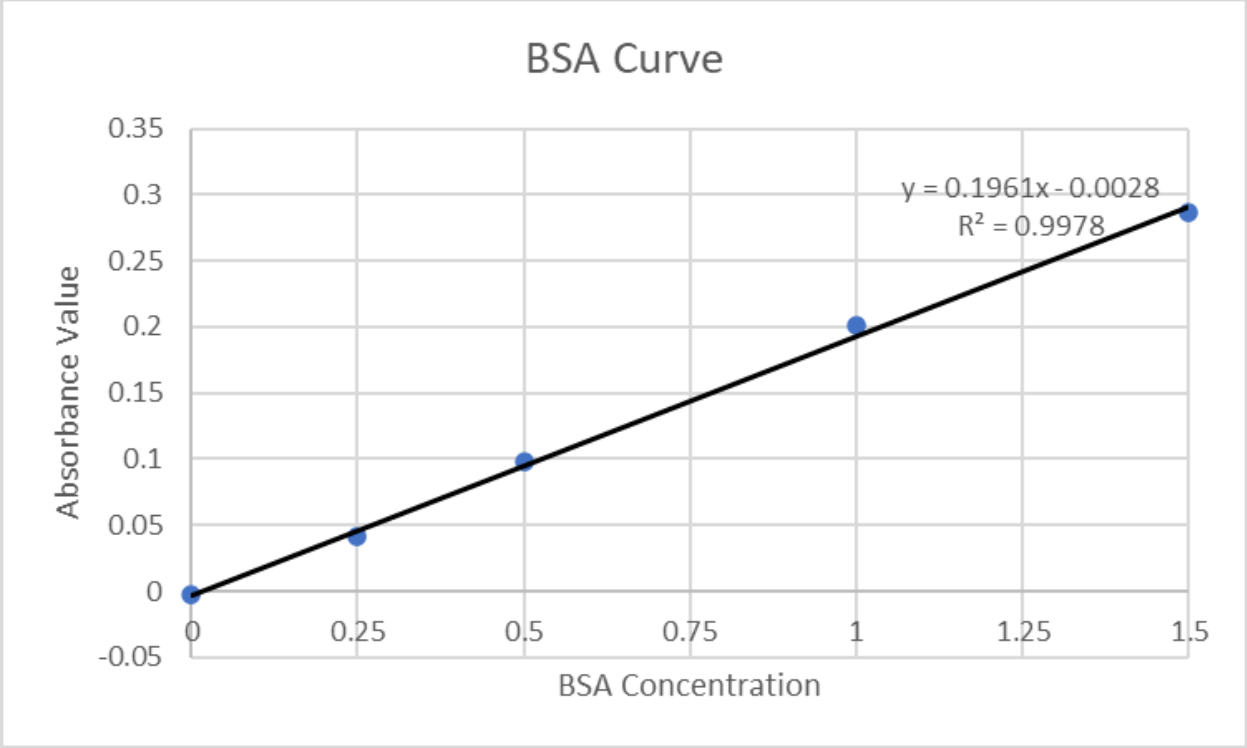
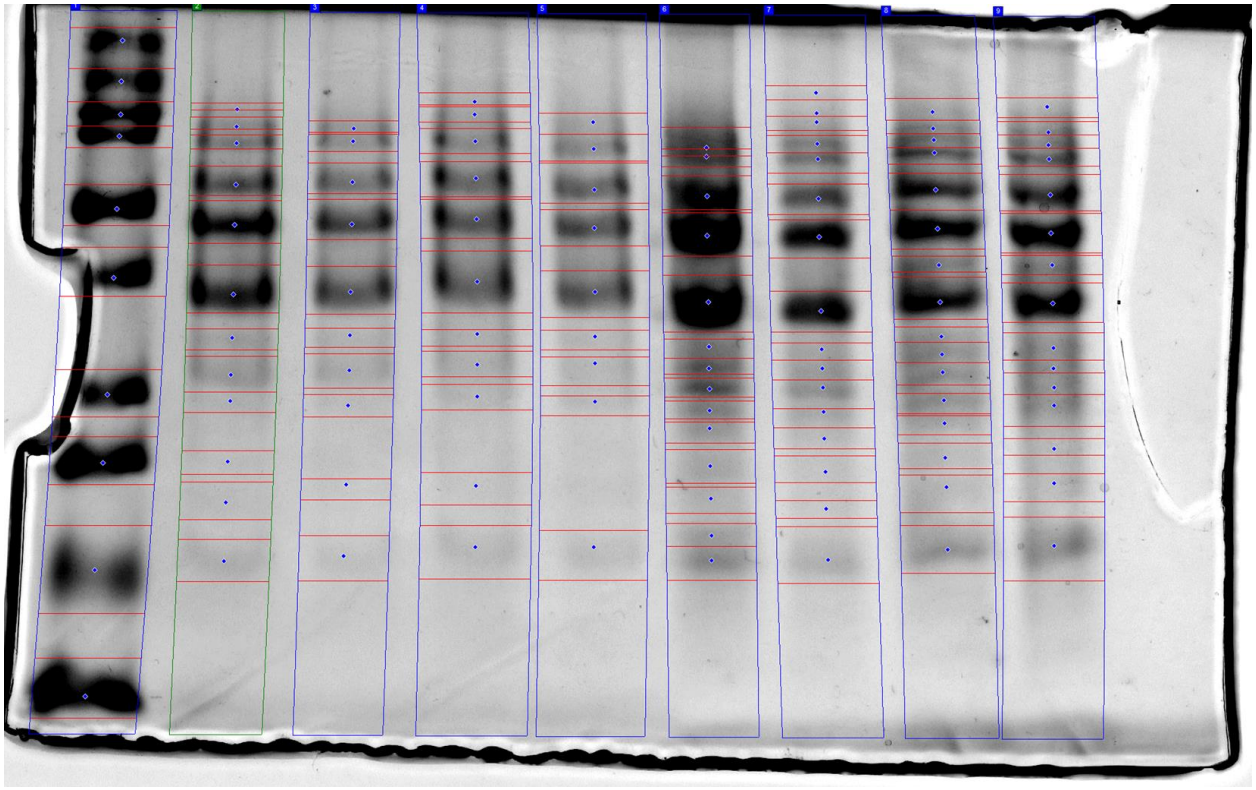


Figure 8: Biorad Assay

Figure 8 table shows the BioRad Assay for concentrations 0.25 mg/mL, 0.5 mg/mL, 1.0 mg/mL, 1.5 mg/mL, and the unknown apoptotic and recovering protein concentrations. Each concentration was read in triplicates at 750nm on the biospectrometer. The bold values for Blank through 1.5 mg/mL were chosen to be the values that created the BSA Curve graph. A line of best fit was calculated, resulting in $y = 0.1961x - 0.0028$ with an R^2 value of 0.9978. The bold apoptotic and recovering values were input into the equation, resulting in 0.457mg/mL and 0.774 mg/mL respectively. Because these samples were diluted by 10-fold, the original samples are therefore 4.57mg/mL and 7.74mg/mL.



Lane	1	2	3	4	5	6	7	8	9
Type	Ladder	Clean	Clean	Clean	Clean	Unclean	Unclean	Unclean	Unclean
Cell		Apoptotic	Apoptotic	Recovering	Recovering	Apoptotic	Apoptotic	Recovering	Recovering
Amount		10uL	7uL	10uL	7uL	10uL	7uL	10uL	7uL

Figure 9: SDS Page Gel Electrophoresis

Figure 9 shows the resulting gel from SDS Page gel electrophoresis performed with 2D Cleanup protein samples. The table shows the lane number, lane type, cell type, and volume amounts loaded into each well. Lane 1 was unfortunately slightly chipped when removing the gel from its container, but does not interfere with the interpretation. Each lane is signified by the blue or green column; the column colors are unnoteworthy. Each band is represented by a blue dot, with the red rows to outline the size of the band. Lanes 6-9 have darker and higher band intensity than Lane 2-5, which is to be expected due to inevitable protein loss during the previous cleanup step. Lanes were selected to have 10uL and 7uL of protein volume placed into its well to check for consistency; therefore, when compared, the 7uL lane should have 70% intensity compared to the 10uL lane.

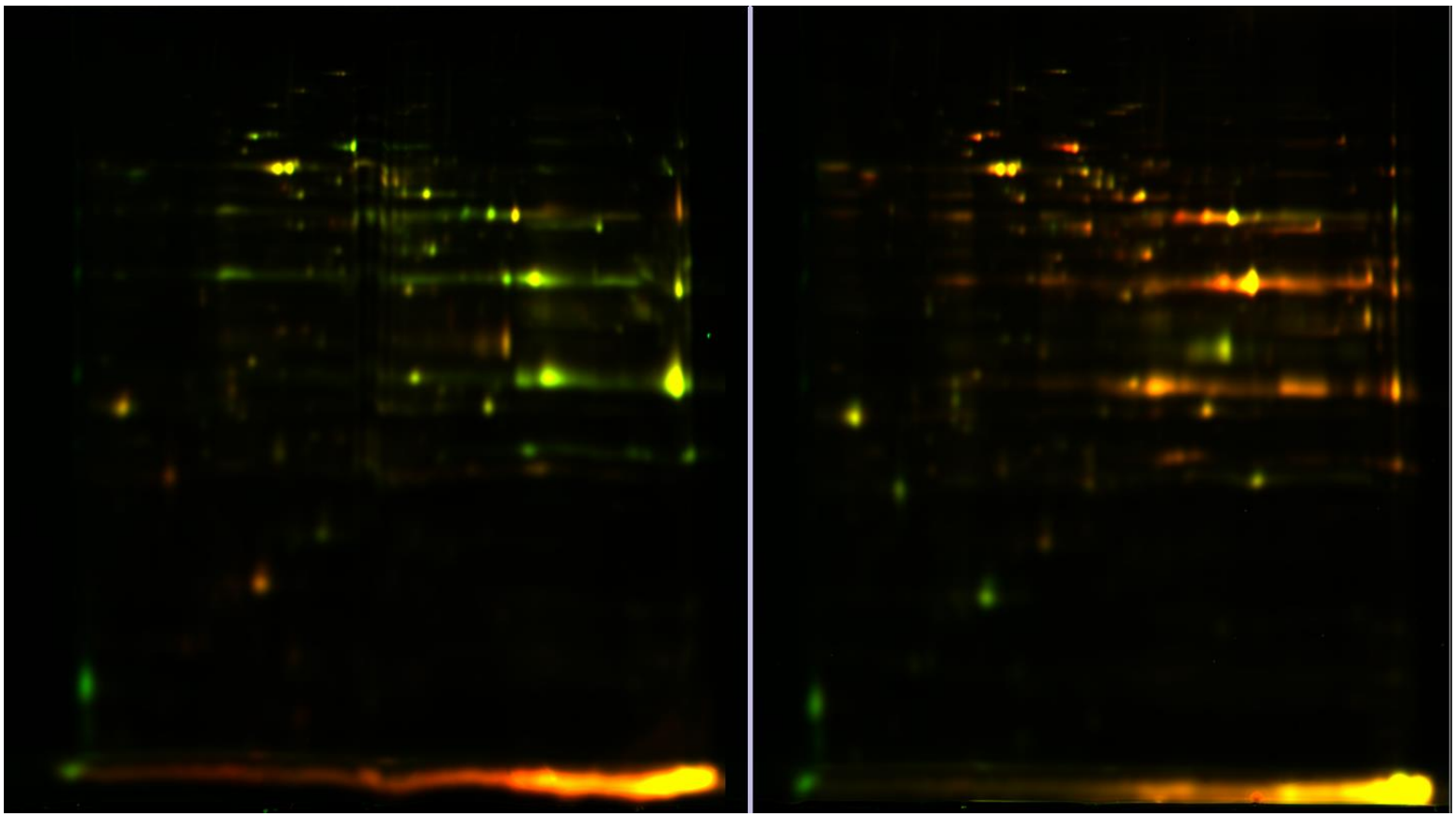
ImageQuant Table

Lane 1	1	2	2	3	3	4	4	5	5	6	6	7	7	8	8	9	9
Band #	V	B #	V	B #	V	B #	V	B #	V	B #	V	B #	V	B #	V	B #	V
1	228	**1**	1	**1**	19	**1**	1	**1**	2	**1**	77	**1**	1	**1**	1	**1**	1
2	204	**2**	23	**2**	47	**2**	3	**2**	45	**2**	31	**2**	1	**2**	4	**2**	2
3	338	**3**	62	**3**	117	**3**	44	**3**	162	**3**	144	**3**	1	**3**	8	**3**	1
4	369	**4**	187	**4**	261	**4**	144	**4**	189	**4**	531	**4**	11	**4**	46	**4**	24
5	934	**5**	427	**5**	327	**5**	189	**5**	215	**5**	792	**5**	31	**5**	149	**5**	63
6	614	**6**	579	**6**	1	**6**	271	**6**	2	**6**	2	**6**	102	**6**	318	**6**	246
7	569	**7**	10	**7**	24	**7**	2	**7**	3	**7**	22	**7**	289	**7**	1	**7**	1
8	843	**8**	59	**8**	1	**8**	2	**8**	6	**8**	47	**8**	315	**8**	199	**8**	166
9	267	**9**	2	**9**	2	**9**	2	**9**	5	**9**	13	**9**	1	**9**	1	**9**	3
10	1425	**10**	4	**10**	3	**10**	2			**10**	2	**10**	1	**10**	2	**10**	1
		11	3			**11**	4			**11**	3	**11**	12	**11**	2	**11**	1
		12	27							**12**	2	**12**	1	**12**	1	**12**	2
										13	7	**13**	1	**13**	1	**13**	1
										14	48	**14**	2	**14**	1	**14**	2
												15	1	**15**	2	**15**	4
												16	4	**16**	4		

TV	5791		1384		802		664		629		1721		769		734		514
7:10	NA				.58				.95				.44				.70
C:UC	NA		.80				.90										

Figure 10: ImageQuant Table

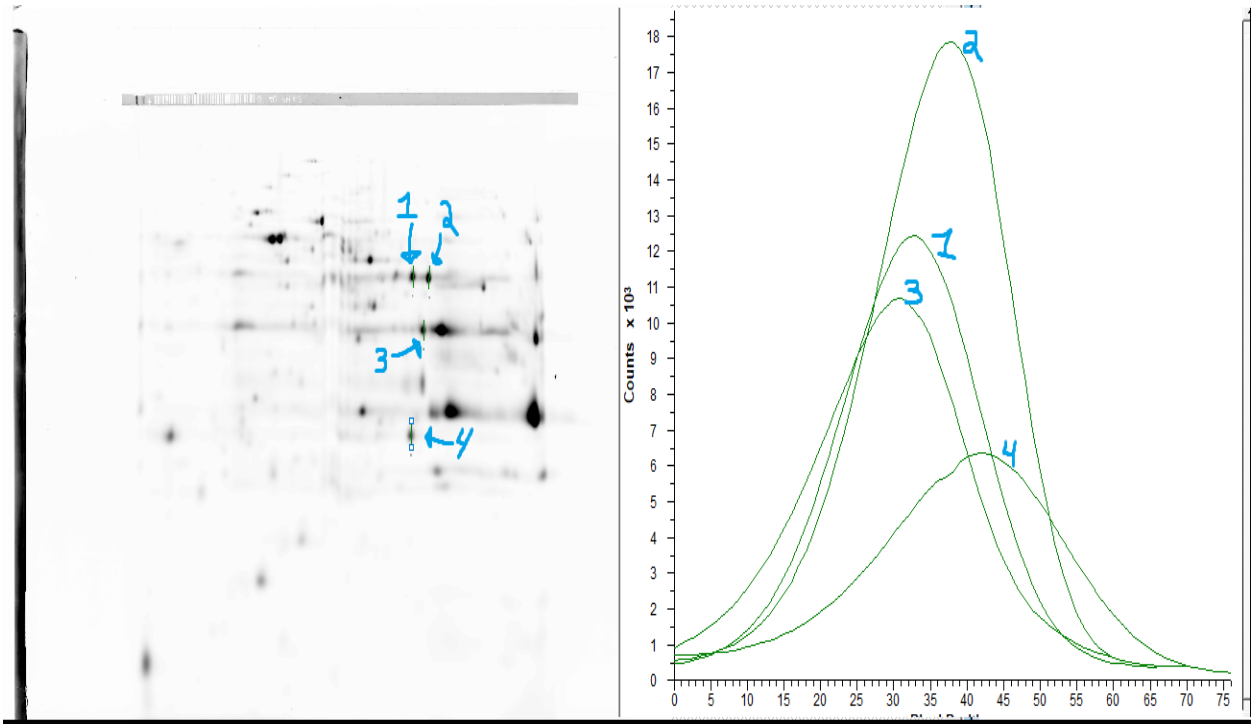
Figure 10 shows the computer analysis of the SDS Page gel from Figure 9. Lane 1-9 corresponds with lanes 1-9 of Figure 9. Each lane has its band number (B #) with its band volume (V) rounded to the nearest whole number. The bottom rows are the total volume (TV) of all bands per lane rounded to the nearest whole number, a 7:10 ratio (7:10), which was calculated by dividing the 7uL lanes by their 10uL pair, and the clean:unclean ratio (C:UC), which was calculated by dividing the total volumes of the 10uL clean lane by its corresponding 10uL unclean lane. From the data set, it shows that most of the 7:10 ratio did not have corresponding accuracy most likely to technical errors; we would expect to see closer to a 70% ratio for each comparison, like lanes 8 and 9. However, the C:UC ratio shows that we recovered .80 (80%) and .90 (90%) of apoptotic and recovering cells respectively from 2D cleanup.



	Cy3	Cy5
Gel A	APOPTOTIC	RECOVERING
Gel B	RECOVERING	APOPTOTIC

Figure 11: 2D Gel Electrophoresis Constellation

Figure 11 shows the scanning results from the Typhoon. Left gel is Gel A, the right gel is Gel B. The table shows the different Cy labeling for apoptotic and recovering cells per gel. For Gel A, multiple green bands (apoptotic) can be observed, with fewer yellow and red bands (recovering). For Gel B, many red bands (apoptotic) can be observed, with fewer yellow and green bands (recovering); this is to be expected since the dye is inverse between Gel A and Gel B, so the fluorescence per band should be similar in intensity and band formation. However, Gel B has larger streaks and is not as tight as Gel A, and this is because of general differences when the gels were cast.



	A	A	Ratio	B	B	Ratio
	Cy3	Cy5	Cy3:Cy5	Cy3	Cy5	Cy3:Cy5
1	12500	6500	2:1	6600	16000	1:2
2	18000	19500	1:1	25500	27000	1:1
3	10600	5000	2:1	5500	17000	1:3
4	6500	5000	1:1	4500	6000	1:1

Figure 12: ImageQuant Analysis of Gel Band Volume

Figure 12 shows an example of ImageQuant analysis on Gel A Cy3 fluorescence. The left image shows the gel with band selection, and the right image shows the band fluorescent values. 4 bands were selected from the gel and their corresponding values were put into the table. Values were read at the corresponding peak. After Gel A Cy3 was analyzed, Gel A Cy5 was analyzed with the same band selection, and their ratios are listed in the table. Gel B Cy3 and Cy5 were then analyzed with the same 4 band selection from Gel A Cy3, and their ratios were calculated and rounded to the nearest whole number. The Gel A and Gel B Cy3:Cy5 ratios resulted in close correspondence with each other, showing that the two gels have equal fluorescence.

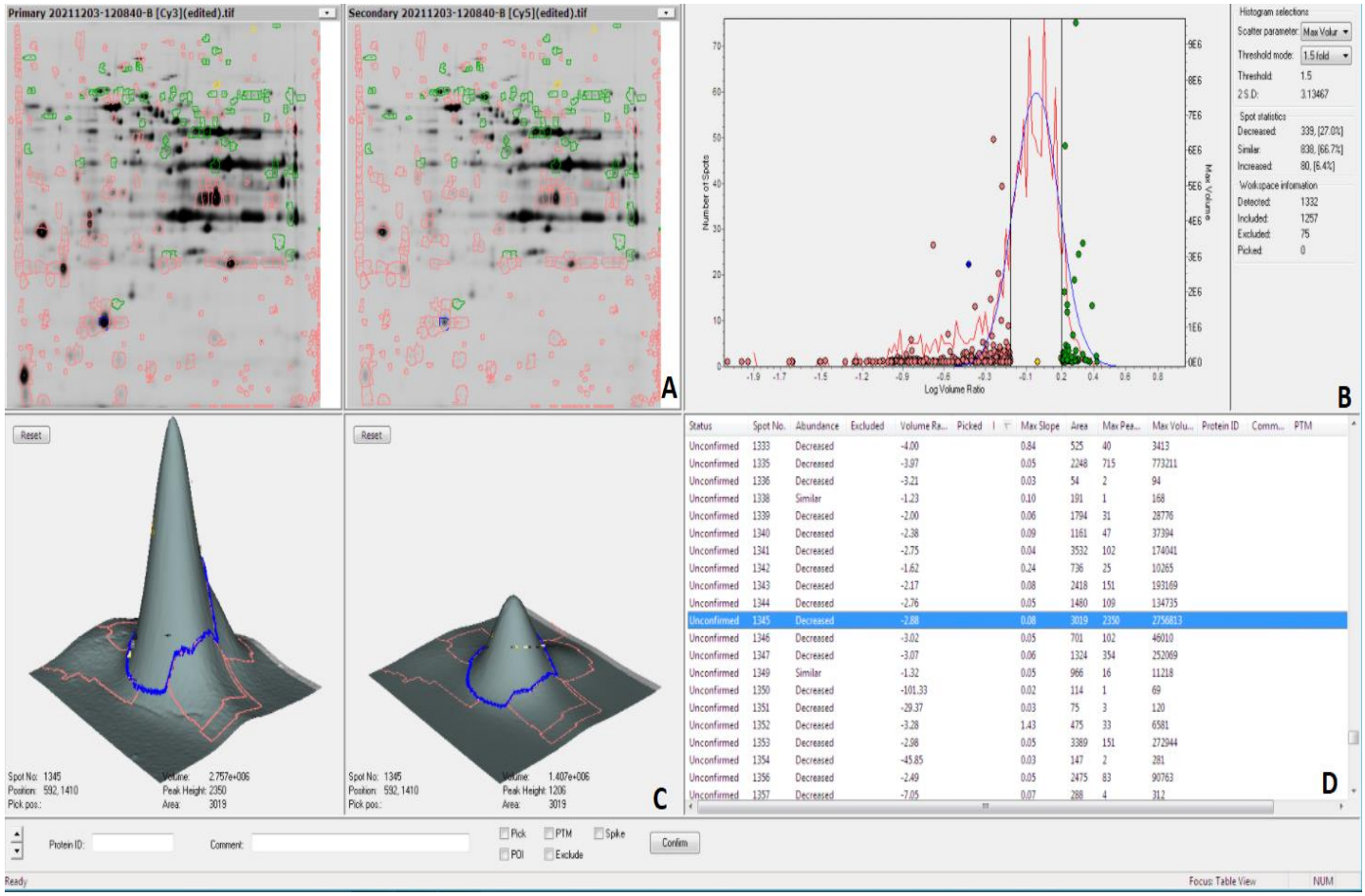
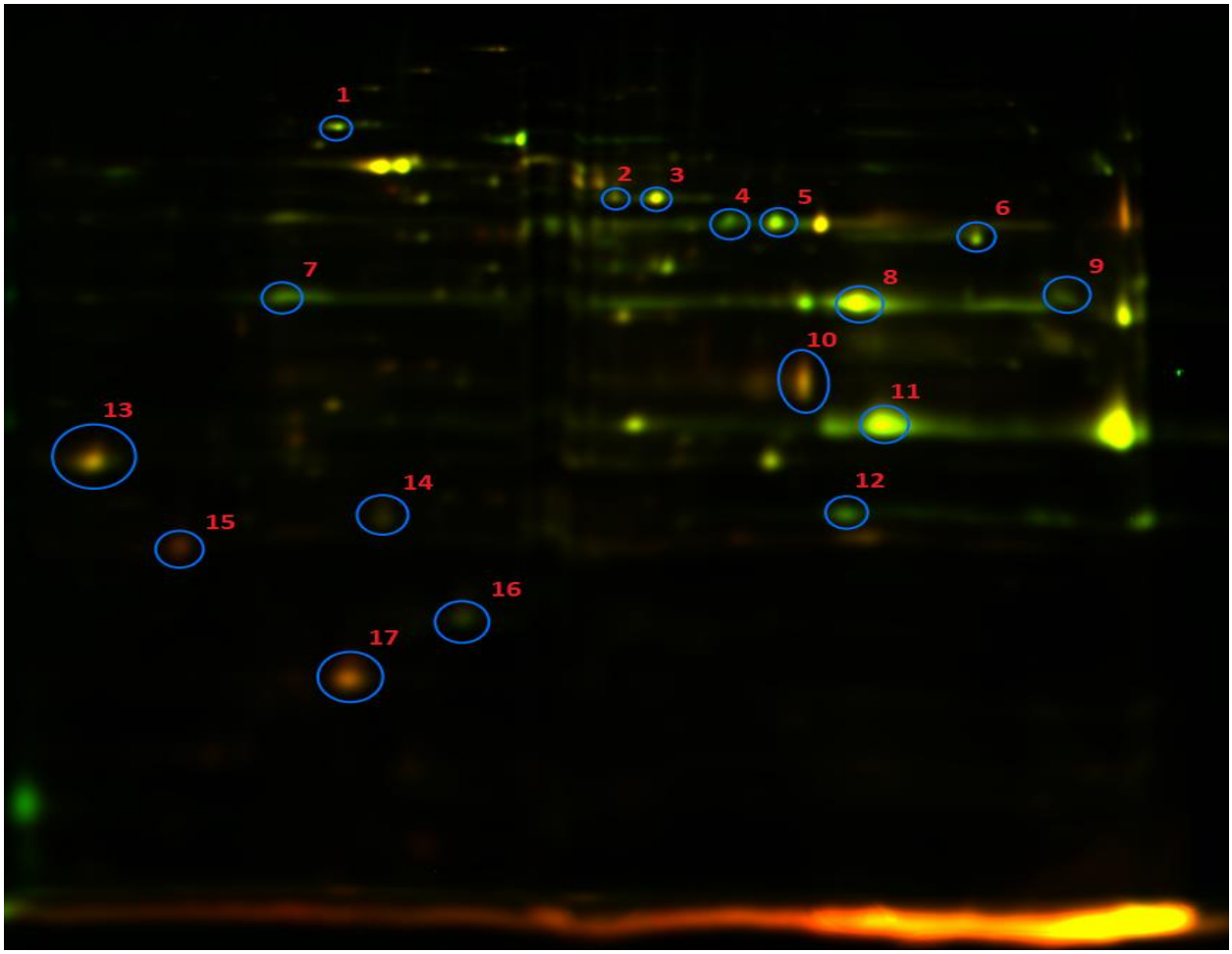


Figure 13: DeCyder Analysis

Figure 13 shows DeCyder analysis of Gel B. **A** shows Cy3 and Cy5 images. The software automatically highlights band differences, with red showing a negative volume ratio, green showing a positive volume ratio, and blue is the selected protein. Similar volume ratios are normally highlighted in yellow, but are excluded here for analysis. **B** shows a volume ratio compared to the number of spots for both Cy3 and Cy5. Number of spots peak from a log volume ratio of -0.2 to 0.2 ratio, and those spots are excluded for having a low volume ratio difference. **C** shows a blue highlighted single protein selected from the gel, with the left protein peak being from Cy3 and the right from Cy5. The peak height corresponds to the overall volume of that protein. **D** shows the spot number, abundance type, and volume ratio of the selected protein compared from Cy3 to Cy5. For the protein selected in C, it has decreased abundance and a volume ratio of -2.88, which means that this protein labeled with Cy5 has less volume than the Cy3 labeled one.



	AVR	Identified Protein	Cell Type
1	1.35	No Match	Apoptotic
2	0.03	SSB1	N/A
3	0.09	SSB2	N/A
4	1.82	No Match	Apoptotic
5	1.56	No Match	Apoptotic
6	1.15	No Match	N/A
7	1.5	No Match	Apoptotic
8	1.66	No Match	Apoptotic
9	1.35	Enolase 1	Apoptotic
10	1.59	No Match	Recovering
11	1.3	GAPDH3	Apoptotic
12	1.73	No Match	Apoptotic
13	1.54	EFB1	Recovering
14	0.09	EGD2	N/A
15	2.5	DDR48	Recovering
16	0.22	Phosphomannomutase	Apoptotic
17	2.5	APH1	Recovering

Figure 14: Constellation Map for Gel A

Figure 14 shows selected proteins of interest after DeCyder analysis. 17 proteins of interest were chosen, and their average volume ratio (AVR), identification, and corresponding cell type are placed in the table. AVR is the volume ratio of a protein between Cy3 and Cy5, and is calculated by averaging the ratioed volume amount of the proteins of interest between Gel A and Gel B (see Method 15 table). AVR is either green or red; AVR in red are averages that do not exhibit high enough average difference (<1.5) to be significant for a protein, but are still worthy of noting. AVR in green are significant (≥1.5) averages. Proteins of interest were identified using previous 2D gels from past experiments and the Yeast Protein Map (http://www.ibgc.u-bordeaux2.fr/YPM/index.html). Proteins that were unable to be identified are listed as "no match". Cell types are defined as Apoptotic, Recovering, or N/A, which correspond to green, red, and yellow bands respectively. While many significant proteins for apoptotic cells have no match, recovering cells had greater significant expression of APH1, DDR48, and EFB1.

Discussion

Wildtype *S. cerevisiae* cells were grown to their logarithmic phase in fresh YEPD medium. As soon as this growth phase was reached, media was aliquoted, and 80uL of 100mM Copper (II) Nitrate was added for 1 hour. After this treatment, metal was washed out, and cells were resuspended and allowed to rest for 2 hours. After the first hour of rest, PI was added into the media to start the staining of DNA, which would signify that apoptosis is taking place. After the end of the rest phase, cells were resuspended in PBS, and then cell sorted by the FacsARIA into two populations, apoptotic and recovering cells. Cells were separately lysed, and their proteins were extracted. Proteins were then purified via a 2D Cleanup kit, then an SDS Page Gel was ran to ascertain the amount of cleaned proteins. Proteins were then labeled with either Cy3 of Cy5 fluorescent, and then placed on an IEF strip. After strip was ran, it was loaded into a 2D Gel, and 2D Gel electrophoresis was ran. Gels were then scanned with the Typhoon, ratios established, and then images are uploaded on the DeCyder. Proteins of interest were selected, identified, and an AVR was calculated.

Constellation Map

Out of all proteins of interest, 17 were selected and circled on Gel A. These proteins were compared to previously analyzed gels to find identification, and then the average volume ratio between Gel A and Gel B was calculated. Because Gel A had apoptotic cells labeled with Cy3, the apoptotic proteins of interest that are seen in the green band have a higher volume of proteins compared to recovering cells, which is confirmed through the average volume ratio. Conversely, any red bad seen in Gel A are recovering cell proteins that have a higher volume than the apoptotic cells. While many significant apoptotic proteins did not have a match found, recovering had greater expression of EFB1, DDR48, and APH1 by a ratio of 1.54, 2.5, and 2.5

respectively. Interestingly enough, DDR48 is a stress response gene that is from the DNA-Damage-responsive family, and is transcribed when there is DNA damage in the cell (McClanahan, T, and K McEntee). Since recovering cells are expressing more of DDR48, then this suggests that DDR48 is a significant protein related to cell survival when under oxidative stress. APH1, also known as HNT2, is a dinucleoside triphosphate hydrolase (Brevet, A, et al.), which is a type of enzyme involved in catalyzing nucleoside triphosphates into nucleotides and diphosphates (Davies, Oluwafemi, et al.). This allows for free nucleotides to be made, which may in turn be used in DNA repair (Davies, Oluwafemi, et al.). This also points towards an important difference between the apoptotic and recovering cells; since the recovering cells are exhibiting more DNA repair proteins, then they are more likely going to survive. Finally, EFB1 is translation elongation factor 1 beta, which is responsible for facilitating RNA translation by binding to aminoacylated tRNA (Hiraga, Kazumi, et al.). EFB1 therefore has importance for the ribosomal A site, so greater expression of EFB1 could mean that there is higher amounts of protein synthesis in the cell.

2D Gel Electrophoresis and Gel Analysis

After 2D gel electrophoresis was performed, the two gels, A and B, were scanned with the Typhoon (Figure 11). To confirm that the gels had similar amounts of band volumes, ratios were established for 4 bands (Figure 12), which resulted in similar ratios between the gels. For example, the band 2 value for Gel A was 18,000 and 19,500 for Cy3 and Cy5 respectively, which is a 1:1.08 ratio, and the corresponding band for Gel B was 25,500 and 27,000, which is a 1.05 ratio. While band 3 was somewhat of an outlier, we are confident in the analysis to proceed to protein analysis on the DeCyder. We analyzed 1,500 proteins from the DeCyder analysis, and manually selected proteins of interest that had high average volume ratios which are defined as

greater than or equal to 1.5. These proteins were then checked on Gel B for their average volume ratio, and finally the average volume ratio of a protein of interest were averaged together.

BSA and 2D Cleanup

To be able to successfully run a 2D gel electrophoresis, each protein concentration sample must be at the minimum of 50ug after 2D cleanup. During 2D protein cleanup, it is inevitable to lose some of your sample's concentration. We have been successful at recovering 75% after cleanup when analyzing with an SDS Page gel. These proteins were isolated, and a BSA was performed (Figure 8) on a biospectrometer. We can see that we ended with 0.457mg/mL (4.57ug/uL) and 0.774 mg/mL (7.74ug/uL) respectively of apoptotic and recovering proteins. This was calculated by selecting values from the BioRad samples to create a graph and line of best fit, and then plugging the absorbance value of our protein sample into the y-value of the calculated curve. The total volume of the unknown samples after the BSA is 110uL each. After the ImageQuant analysis of the 2D cleanup via a SDS Page (Figure 10), the C:UC ratio shows a recovery rate of 80% and 90% of apoptotic and recovering respectively, which resulted in a final concentration of 402.16ug/uL and 766.26ug/uL for our apoptotic and recovering samples.

FacsARIA

Prior to 2D, we use the FacsARIA to sort the two treated populations, into those that fluoresce (apoptotic) and those that do not (recovering). These populations are shown in Figure 4, and are gated in a way to select against cells that are not strong candidates for either apoptotic or recovering population. By selecting the top and bottom values of the fluorescence, we are confident that the cells in P4 and P5 are only cells that have strong fluorescence or cells that have no fluorescence respectively, and there is no accidental machine mixing. Because yeast generations are every 90 minutes, this limits the amount of time that the ARIA can be used to

sort our sample, since both the damaged and recovered population may still be undergoing mitotic division. Analyzing the proteins of the subsequent generations post metal treatment may mislead results as they are not the population that was subjected to reactive oxidative species, and may have different protein expression. Continuing, the ARIA is limited in the speed at which it sorts; over multiple cell sorting sessions, the ARIA, on average, can sort through roughly 35 million cells in total per session. When we apply the assumption that Copper (II) Nitrate results in a maximum of a 20% damaged population, 7 million apoptotic cells are acquired per sorting session. From protein isolation, we have achieved, on average, from 6 billion WT cells (100mL of WT *S. cerevisiae* at 2OD read at 600nm) 9,750ug in total (19.5ug/uL in 500uL). Therefore, we can obtain 1.62×10^{-6}ug of protein from a single WT cell. 7 million apoptotic cells results in 11.37ug, resulting in 14 cell sorting sessions. However, the ARIA does not necessarily sort the average number of apoptotic cells every time. We have seen as high as 8.5 million apoptotic cells, and as low as 2.6 million apoptotic cells. Furthermore, we have seen a lower amount of proteins from apoptotic cells, which calls into question if the protein amount differs between untreated, damaged, and recovered cells, which is not examined in this report.

Propidium Iodine

Propidium Iodine was originally used at 250ug/mL concentrations and added in 10uLs to stain 50uLs (3×10^5) of cells. Because we intend to stain a 10mL volume (6×10^7 cells), we would therefore need 2mL of PI at 250ug/mL. We proposed that this amount of PI is excessive, so we designed a short experiment to account for cellular staining. 250ug/mL, 125ug/mL, 100ug/mL, and 50ug/mL were chosen for our concentration variables, and all were tested on the same copper treated sample and results were visualized on the Fortessa. As Figure 6 shows, between all concentrations, there was minimal difference, suggesting that a 50ug/mL concentration stains

equally to a 250ug/mL concentration. This also suggests that 250ug/mL does not overstain cells, which maintains the validity of previous experiments' fluorescence results that this project's fluorescence logic is based on. Furthermore, by reducing the concentration from 250ug/mL to 50ug/mL, an 80% deduction, this results in a reduction from the calculated 2mL to 400uL of PI addition for 10mL volume at 250ug/mL PI. We then decided to increase the concentration of PI from 250ug/mL to 1mg/mL by making this new concentration from our stock solution, which further reduces the amount of PI addition from 400uL to 100uL.

Final Remarks

While more experiments, mainly the 2D Gel electrophoresis, need to be repeated, we are confident that the results from this experiment are invaluable and a great preliminary result that will allow for more in-depth analysis in the future. For the future tasks, identifying the "No Match" proteins for the apoptotic cells will give us greater insight to the differences between the recovered and damaged populations; since the recovering cells express more protein synthesis and DNA repair proteins, we suspect that the apoptotic cells will express more proteins involved with apoptosis or necrosis. Hopefully in the future, we can identify the CUP1/CUP1P, CUP2, SOD1, and CTR1 proteins mentioned previously; the difference in protein volume expression would give us valuable insight to the mechanisms that yeast have to defend themselves against ROS and heavy metals. Furthermore, we can obtain more apoptotic cells with the FacsARIA to perform RNA isolation for a nanostring panel, and we could screen for greater RNA expression of suspected genes. In conclusion, *S. cerevisiae* cells subjected to ROS from Copper (II) Nitrate become two distinct populations: recovered or damaged, which is visualized through PI staining. These populations express different types of proteins in different amounts; the *S. cerevisiae* cells

that are able to recover express more proteins involved in DNA damage repair and protein synthesis.

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