The Effects of Flow Cytometry Based Cell Sorting: A Metabolomics Study

The Effects of Flow Cytometry Based Cell Sorting: A Metabolomics Study

Zachary Niziolek

A Thesis in the Field of Bioengineering

for the Degree of Master of Liberal Arts in Extension Studies

Harvard University

May 2022

Abstract

Cell sorting is a common technique that is used to create a highly purified population for downstream analysis. As cells are isolated for multiple other processes, evaluating the effects of sorting is crucial. Prior studies have indicated there are no genomic or transcriptomic immediate of the cells but there may be metabolomic changes. In this study cells were sorted into two collection tubes, methanol, and PBS in order to stimulate immediate and post spin fixation. After sorting the cells displayed no differences in cell cycle state, showing that cellular division does not change post sorting. An immediate quenching of the metabolism by sorting into methanol is necessary to capture the better biological representation of aldehydes present within the cell. A more delayed quenching like sorting into PBS is useful when measuring non aldehyde based metabolomics, such as amino acids or energy cycles.

Dedication

I dedicate this thesis work to my wife, Haley, who has been a constant source of encouragement and inspiration during the challenges of school and life. Thank you for all the support and providing me with an example of achieving your goals. I am truly grateful for having you in my life.

Acknowledgments

Thank you to my advisors, Bogdan and Charles. The insight and technical expertise allowed me to complete this thesis and grow as a scientist. This project would not have been possible without their help. Thank you to all my professors during my time at HES for teaching and educating me. Thank you to my parents, Tom and Judy, for all the support throughout the years. Thank you to my family, Charlie and Kimmie for the love and compassion.

Table of Contents

List of Tables

List of Figures

Chapter I.

Introduction

Cytometry has been a tool that has been used since the 1600s and has allowed scientists to visualize and characterize both physical and chemical properties of cells (Murphy 2002). During the 1960s, Flow Cytometry, which is a method for measuring properties of cells as they move in a fluid past a detection source, was developed. This method is extremely useful as it is capable to measure thousands of single cells in a short time frame. This allowed researchers to analyze cell behavior, morphology, lineage, and DNA content. The base technology was utilized to eventually create a function for isolation of particular cells based on criteria of one's choosing. This was coined by Becton Dickinson as Fluorescent Activated Cell sorting (FACs) and commercialized as such, but generally referred to as just flow cytometry cell sorting (Herzenberg et al 2002).

Cell sorting gives an opportunity to further analyze cells separated from their natural environment, for example separating a cytotoxic T cell from a tumor. This allows for a homogenous population to be isolated from a heterogenous population, providing access to analysis of particular cell types or even single cell levels through genomic and metabolomic means. This isolation process however causes the cells to undergo pressure, shearing forces, and electrostatic forces (Shaprio 2005). These processes can induce cellular stress and possibly lead to a misinterpretation of results based on the assumptions of the research projects (Groh et al 1996). The goal of this work is to determine the possible effects of sorting can have on cellular metabolism, particularly at what level does it cause cellular stress.

Stress is a common part of life and is a typical response to particular stimuli that generate both a mental and physical response. Stress is a widespread phenomenon also affecting individual cells and the response to that stress is a widely studied field. Cellular stress can be caused by many different factors, such as lack of nutrients, physical or oxidative damage. These stressful stimuli have different pathways which cause a slew of reactions that create changes in cellular behavior and ultimately can lead to cellular death (Fulda et al 2010). Cellular stress plays an important role with many diseases and ailments, and understanding these stressors is crucial for potential treatments. Since sorting is a powerful way to isolate cells to discover how these particular diseases stress cells, it is crucial to ensure that sorting is not causing a bias towards that information. With these matters in mind the stress of cells must be measured after isolation, by determining how their cell cycle and general metabolism are affected.

Basis of Cellular Stress

Cellular stress is important to manage because it can lead to changes in the cell's transcriptome or, in extreme cases, cellular death. Apoptosis, or programmed cellular death, is a key pathway that is known to be activated in cases of immense cellular stress. Regulation of this pathway is extremely important in biology, and crucial in understanding cancer. As cells undergo cellular stress it may be a more appealing avenue to undergo apoptosis than it is to risk a potential incorrect recovery of the cells (Lowe & Lin 2000). This pathway has been highly detailed, and information on particular genes and metabolites involved in it have been outlined on the KEGG database (Meers and

Mealy 1994). While cellular stress can be measured through those genes, other ways to measure it are through the Heat Shock Response.

Heat or oxidative stress may cause cells to have damaged or misfolded proteins. This can cause issues in cellular processes and result in the halting of protein translation. To combat this effect, cells have chaperones, in particular heat shock proteins that limit protein aggerates and help to re fold proteins. This can be measured through the presence of the cytokines and metabolites which helps promote those actions (Fulda et al 2010). Measurement of these molecules post sorting would be beneficial to see how stressful cell sorting is. In addition to protein issues arising from cellular stress, DNA damage can occur as well as a result from oxidative stress (Silva et al 2015).

DNA damage takes the form of single or double stranded breaks in the DNA and is a common effect of UV damage. If the damage is extensive enough and cannot be repaired, various molecules and genetic pathways will be activated and start to initiate apoptosis. Damages to DNA have been linked to aging, cancer, mutagenesis, and many other diseases (Friedberg 2003). DNA changes can also result from oxidative stress, which is typically caused by reactive oxygen species (ROS). Commonly found in the form of H2O2, O2-, OH, OOH-, NO and OONO- these small compounds are difficult to measure directly, but the products of the reaction with other compounds present in a cell can be measured (Trachootham et al 2008). Commonly found in cases of abundant ROS, aldehydes are also known to increase from cellular stress particularly in DNA damage cases (Bruce et al 2011).

Aldehydes are compounds that are classified by a carbon double bonded to oxygen and a single bond to hydrogen (-CHO). These compounds are highly reactive and

also play a role in the damage of DNA. They are found commonly in cases of oxidative stress, as products of cellular lipid peroxidation and are key to glycation's reactions and have been linked to Parkinson's Disease (Chen et al 2014). They are extremely reactive and electrophilic compounds that can cause protein crossing linking, preventing the proteins from functioning properly. When these compounds are present in high amounts it causes instability in cellular homeostasis and may result in DNA damage (Voulgaridou et al 2011). Aldehydes are more easily analyzed than ROS, and so can be used as a good proxy of oxidative stress.

In addition to ROS and aldehydes, the cellular metabolism more generally is affected by the stress level. All of these stress responses are highly studied and many of the pathways and associated compounds have been discovered. This can provide a solid base for comparison between the cells that have been sorted and the unsorted ones. Typical cell functions occur extremely fast and the response to stimuli is a quick process. In order to assess this rapid response to cell sorting, the cellular metabolism, or what is currently occurring within a cell, must be measured. Cellular metabolism is a broad term and can be measured through multiple techniques but one of the most accurate, highly analytical, and quantitative is through mass spectrometry (MS).

Mass spectrometry is a technique that measures type and abundance of the molecules and proteins in the solution. This technique is typically combined with either gas chromatography or liquid chromatography to further separate compounds (Domon & Aebersold 2006). Through a combination of both techniques, it allows for a precise analysis and accurate representation of cellular phenotype as this will demonstrate the end result of both genomic and proteomic changes (Dunn et al 2005). This tool will be

instrumental in the real time effects of cell sorting, as the metabolic changes can be quenched, and those changes can be analyzed.

Possible Cell Sorting Stressors

Flow Cytometry based cell sorting is a technique that can use many instrumentation types, from various vendors. However, all flow cytometers are able to produce a streamline flow of cells through the machine by properties of laminar flow and hydrodynamic focusing. With the addition of a nozzle, a piezo electric crystal and electrically charged deflection plates, alongside a salt-based buffer (sheath buffer), the flow cytometer is able to sort cells. This whole process is known as flow cytometry based electrostatic cell sorting.

Cells are carried through a ceramic nozzle which vibrates at a given frequency to create droplets. These droplets are charged, which enables the machine to direct them into different positions. This process is precisely timed and allows the knowledge of which cell is in which droplet. Then using the electrostatically charged plates, the droplet with the desired cell is separated and directed into a collection container. This is an extremely useful tool because it allows the isolation of particular cell types from another to properly study how those cells, in that particular environment, may behave, or what their transcriptional state is like (Ibrahim et al 2007).

Cell sorting is an accurate method to separate and quantify specific cells, however it can be a stressful process. The cells are under pressure, ranging from 20-70 psi, as they are brought into laminar flow and then forced through a small orifice and then deflected into a collection tube by electrostatic forces. This has the potential to cause a lot of

physical stress and possibly cause the death of the cells. Isolation of cells through this flow cytometry based electrostatic cell sorting introduces them to osmotic pressures, electrostatic charges, shear forces and possible laser irradiation. All aspects of flow cytometry based cell sorting stressors are highlighted in figure 1. The shear force is caused by the sheath or carrier fluid moving in parallel to the cells, pushing them through the machine. Shear forces have been shown to already cause stress of cells and could possibly lead to cellular death (Hua et al 1993). In addition, the cells must be in a single cell suspension to be optimally cell sorted. In order to achieve this, they have to be outside of their traditional environment and this has a chance to lower the threshold for cellular stress. This information is useful to understand and evaluate as it can affect the interpretation of other results, be it sequencing data, drug responses or clinical applications (Franke et al 2010). All these steps are crucial to allow for cell sorting, however each step is conducive to the stressing of the cells.

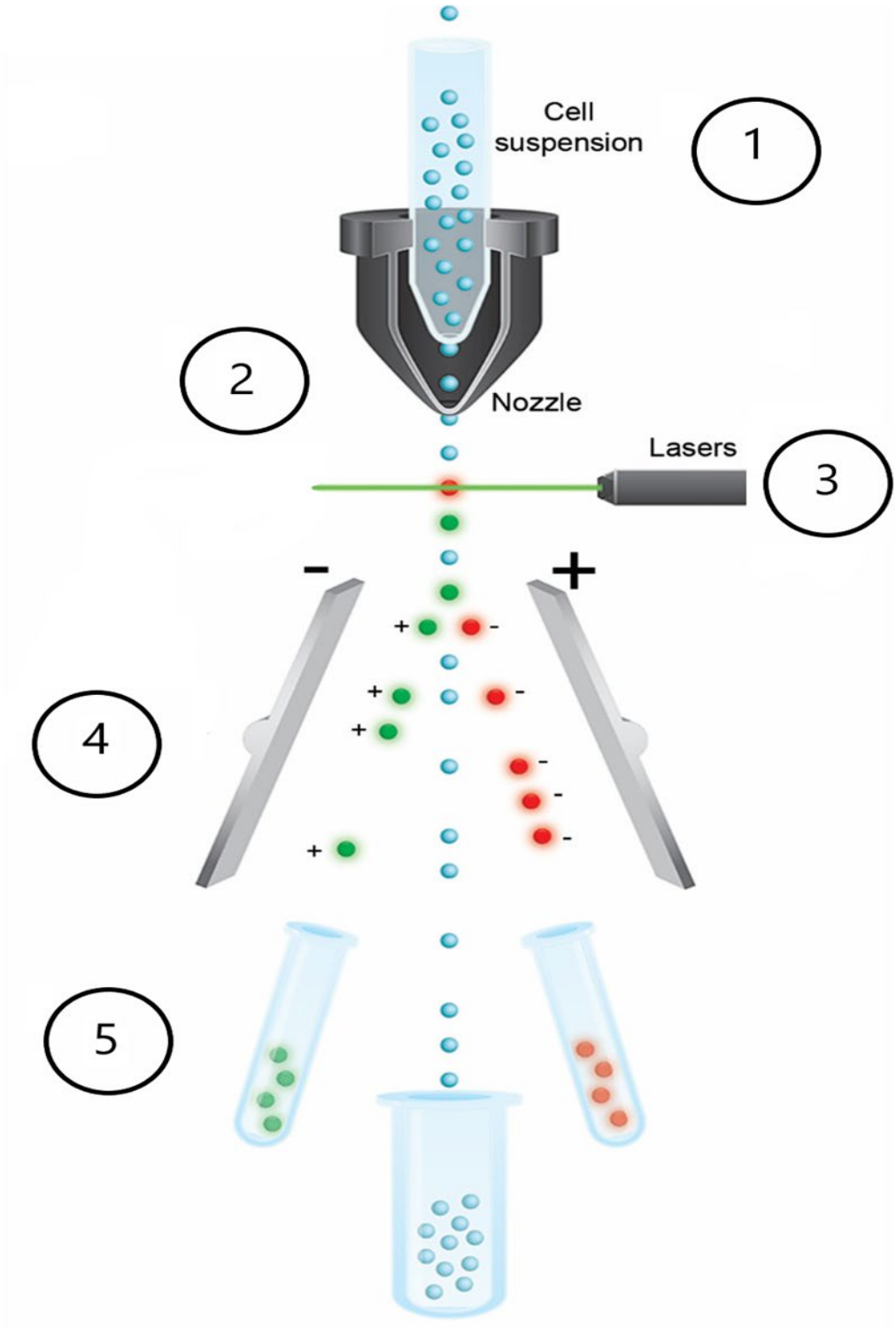


Figure 1. Sorting Overview.

Sorting steps that have potential spots for causing stress. 1) Sample injection and laminar flow 2) Shear Stress 3) Laser irradiation 4) Electrostatic Forces 5) Osmotic Pressures. Figure edited from (Hodne & Weltzien 2015).

Evaluations of Cell Sorting

With the increase in usage of cell sorting for purification of cells for downstream analysis, specifically gene expression levels through RNA sequencing, there has been an increased interest in evaluating the effects of cell sorting. These studies have shown that there have been minimal if any changes of gene expression when looking at leukocytes (Beliakova-Bethell et al 2014). A pool of flow cytometry core facilities extensively studied multiple cell types and demonstrated that minimal or no gene expression changes occurred post sorting. (Box et al 2020). With multiple studies confirming there is no transcriptional changes to cells by sorting them, it is prudent to look at the more immediate effects of sorting.

Even though the changes to gene expression are minimal it is still important to measure other outcomes sorting may have upon the cells. Sorting has been shown to possibly affect astrocytes and caused an increase in oxidative stress which changed their redox state (Llufrio et al 2018). They showed a fifty percent increase of reactive oxidative species when comparing the sorting to the control. They did note that there was a decrease in effect when sorting into serum, as it may allow the cells to slightly recover post sorting. Sorting was also said to cause changes in the metabolome when looking at mouse macrophages (Binek et al 2019). Binek stated that sorting the cells caused a signaling cascade which resulted in an inflammation-like stress. Sorting also displayed differences when comparing analytes involved with energy consumption and cellular damage between the control and the sorted samples. Though sorting is an extremely powerful tool for isolation of cells for a deeper analysis, it does open the

possibility to creating a different cell phenotype. How cells are behaving, and their general health, are crucial to determine the effects of various drugs and disease treatments. Because both of these studies state these metabolomic changes, it is then important to validate these findings and to determine what effects sorting has on cellular metabolism and behavior. These finding will be important because it will allow a distinguishable difference between the artifacts of sorting versus actual biological relevance.

In order to determine the effects that cell sorting has on cells, cellular division and the immediate metabolism of the cells must be measured. Since there was no or minimal genomic and transcriptome changes it is an assumption that protein abundances haven't been altered. Though there maybe changes to the proteome through various phosphorylations, all of those effects will be able to be measured through metabolomics. The general metabolomics combined with targeted aldehyde measurement will allow for an analysis of those potential changes. In addition, analyzing the cell cycle state will display crucial information of whether or not sorting has halted cellular division which is the final determinant for cellular fate.

These effects will be measured by sorting K-562 cells, which are a leukemia based suspension cell line. K-562 cells were used as they are an immortalized cell line that has been highly studied for its role of apoptosis in control of cell cycle, making it useful to measure stress (Klein et al 1976). The samples will be sorted into methanol and PBS in order to measure the effects of sorting. Methanol will quench the metabolism immediately post sorting, while the PBS sorted samples will be spun down and resuspended which is the traditional method for post sorting workflows. The sorting

effects will be compared to unsorted controls, hydrogen peroxide (H2O2) treated samples and hard spun samples as indicated in figure 2. The H2O2 treated samples will be used to stimulate oxidative stress and the hard spun samples will mimic mechanical stress. All of these samples will be analyzed through untargeted metabolomics and targeted aldehyde analysis. In addition, they will be compared across cell cycle state, determining their divisional status (figure 3). These experiments will demonstrate the effects of cell sorting and determine how impactful it may be on biasing post sorting results.

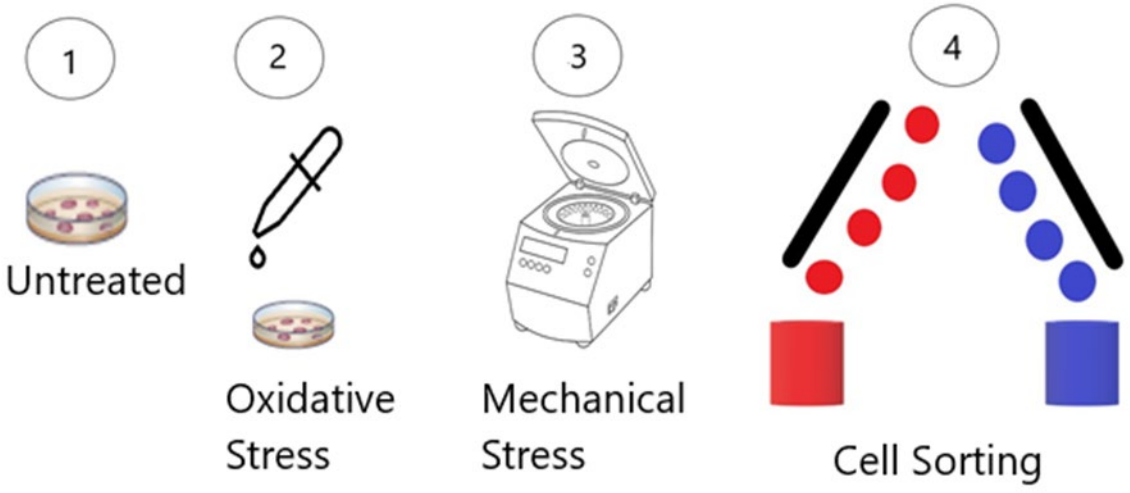


Figure 2: Experimental Samples.

Samples refer to 1) Unsorted control 2) Hydrogen Peroxide treated sample 3) Hard spun at 1,000xg 4) Sorted samples, directly into methanol or into phosphate buffer saline (PBS)

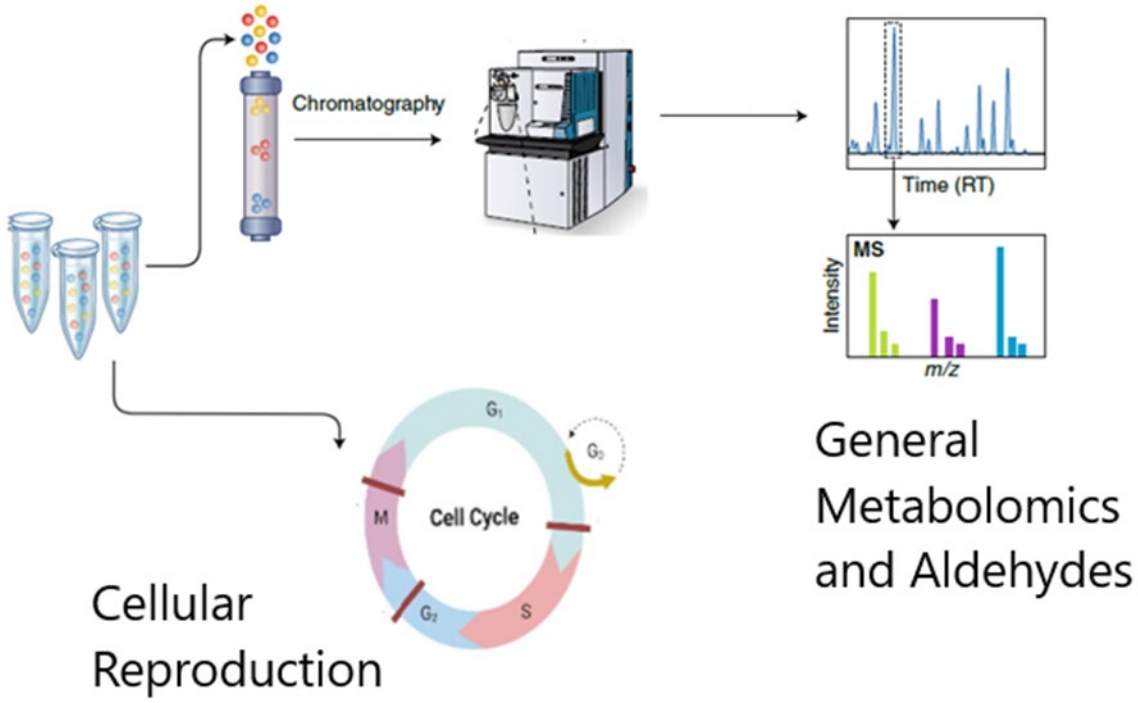


Figure 3: Experimental Workflow.

All treatment groups will be analyzed by LCMS and GCMS to measure both general metabolomics and aldehydes respectively. They will also be used to measure cell cycle analysis

Chapter II.

Materials and Methods

K-562 (ATCC #CCL-243) human cells were used for the cell sorting and as the control samples. All experiments were performed within the Bauer Core and the Harvard Center for Mass Spectrometry. All solvents used were HPLC or higher grade. All samples were handled with lab coat and gloves as samples are BL2.

Cell Culture

All cells were grown at 37°C at 5% CO2 in T-75 flasks(Corning # 430641U) until confluency (approximately 8 million cells) then split into different T-75 flasks. Cells were cultured in Iscove's Modified Dulbecco's Medium (ATCC #30-2005) with 10% Fetal Bovine Serum and 1% penicillin G and streptomycin.

Cell Preparation and Treatment

Cells were spun down in multiple 15mL tubes at 250xg for 6 minutes and resuspended at a density of 10 million cells per mL in 1xPBS. Samples were 95% viable after spin down and resuspended and aliquots of 500 thousand cells were transferred into microcentrifuge tubes for positive and untreated controls. All sorting and controls ran in triplicate and were created three times; one for cell cycle analysis, one for aldehyde

analysis and one for general untargeted metabolomics. The hard spun control was spun down at 1000xg for 10 minutes at room temperature. The hydrogen peroxide treated samples were incubated in 1mM H2O2+PBS for 2 hours. Immediately following treatment, samples were fixed in 1mL of methanol.

Cell Sorting

The sorting was done on a Beckman Coulter MoFlo Astrios with a 100µm nozzle at 26 PSI. The frequency was set to 42,000 with an amplitude of 50.0. All of the sorting was done at room temperature to help determine the maximum effects of the sorting. Cells were sorted based on side scatter (SSC) and forward scatter (FSC) properties, alongside Sytox Red (Thermo #S34859) negativity (see Figure 4). As cells die, their membrane becomes compromised and starts to break apart. Sytox Red is a nucleic acid stain that will stain the nucleic acids of dead cells since it is only able to pass the broken cellular membrane. Sytox Red negativity indicated an intact cellular membrane and was used as a tool to select for live cells.

Sorted cells were deposited into two types groups of collection tubes, ones containing PBS and ones Methanol. The sorting deposited the cells with a residual of 2nL of buffer per cell, leading to 1mL of extra sort buffer into the tubes. In order to properly halt cellular metabolism, they were sorted into 2.5 mL methanol. The volume of methanol combined with sheath buffer resulted in an 80% methanol sample. The PBS sorted samples were incubating in PBS for 5 minutes as they waited for sorting to complete. The cells sorted into PBS were spun done immediately post sorting at 250xg

for 6 minutes and resuspended in 1mL of 100% methanol.

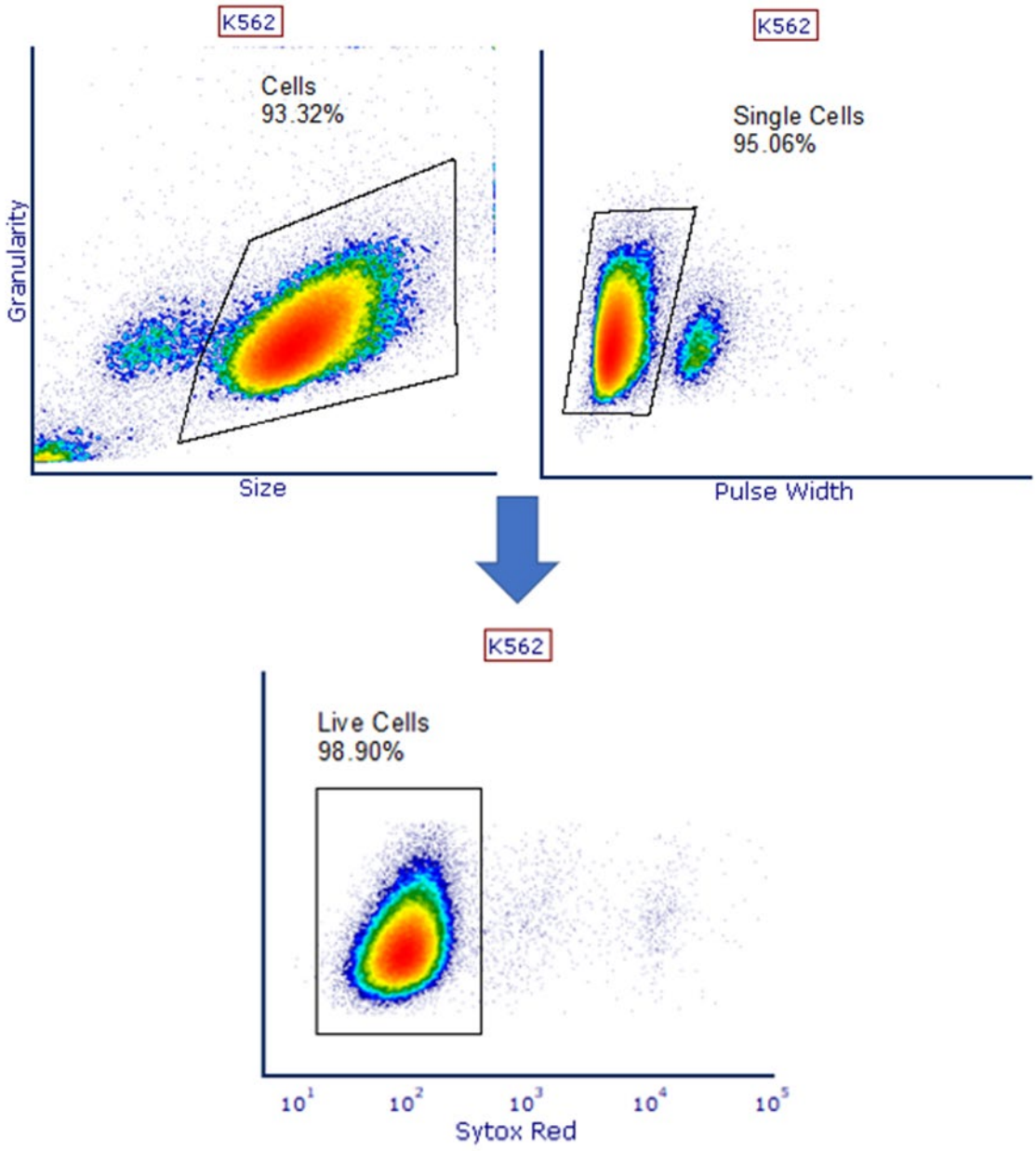


Figure 4: Cell Sorting Plots.

Cells were selected based on size and granularity and then single cells were selected against side scatter width. Live cells were selected by sorting negative on Sytox Red levels.

Cell Cycle Analysis

For the cell cycle analysis, all the non-sorting samples were resuspended in 200µL of PBS. For the sorted samples, the sorting was done to collect 500 thousand cells in 7.5mL of 100% methanol to get to 80% methanol. 800µL of ice cold methanol was added drop wise while continuingly mixing the samples to ensure thorough fixation. After an incubation on ice for 30 minutes, the samples were spun down at 200xg for 10 minutes and washed with PBS. This spinning and washing was performed twice. Once washing was complete they were stained with 500µL of DAPI buffer (10µg/mL DAPI in PBS) and incubated at 4°C for one hour. After completion, samples were run on a BD Symphony A3 (Becton Dickinson). All samples were run on low and the DAPI signal from the 405nm laser on the BV421 channel was measured to ensure small CVs for the DNA peaks. Data was analyzed via FCS Express 7 (De Novo).

Aldehyde Measurements

All samples were handled within a chemical safety hood with proper personal protective equipment. A Stock solution of 10mL of methanol and 0.06 grams of (2,3,4,5,6-Pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA) was created. 500µL of methanol + PFBHA solution was used to fix each of the samples. 5µL of benzaldehyde was spiked into all the samples to use as an internal standard. All samples had 800µL of sample transferred to new glass vials. 500µL of methanol was added to all the samples and incubated for 30 minutes at room temperature. One milliliter of hexane and 6 drops

of sulfuric acid were added. Samples were mixed by vortexing for a minute and then spun down at 2,500 x g. The supernatant was transferred to smaller autosampler tubes. Samples were dried under nitrogen flow and then resuspended in 50µL of hexane and transferred to glass microinserts. All samples were run on a Thermo GC – QE Obritrap mass spectrometer with an Agilent DB5-MS 30m analytical column. One microliter was injected. The injector was kept at 300°C and operated in splitless mode for the first 2 minutes before being purged at 5ml min-1 for 2 minutes. The GC oven was kept at 50°C for 4 minutes, then heated to 300°C at 15°C min-1, and finally kept at 300°C for 24 min. The mass spectrometer source was operated in electron impact mode at 70eV. Analysis was carried out in positive mode, with a resolution of 60,000, an AGC target of 1e6, and a scan range of 66.7 to 1,000 m/z. All transfer lines were kept at 310°C. A seven points standard curve was prepared for quantifying malondialdehyde, methylglyoxal and 4-hydroxy-nonenal. The standards were prepared as a 100uM solution for the most concentrated point of the curve and standards 6-1 were prepared as a 1/5 dilutions series, in the same methanol+ IS solution as used for the samples. 800µL of each standard points were then prepared in the same way as the samples. All data was analyzed in Tracefinder (Thermo Fisher).

Untargeted Metabolomics

Samples were first extracted using a modified Bligh and Dyer biphasic extraction (Bligh & Dyer 1959). In short, 4mL chloroform and an appropriate volume of methanol were added to each sample to reach a 2:1 ratio (chloroform:methanol). After a 10 min

incubation in an ultrasound bath, 2mL of water was added to induce phase separations. Samples were vortexed and centrifuged at 2500xg. The supernatants, containing the hydrophilic compounds, were transferred to new vials, and dried under nitrogen flow. Samples were then resuspended in 40µL of 30% acetonitrile in water. 15µL aliquots of each sample were then transferred to glass microinserts. The remaining of the sample volumes were pooled together for MS/MS data acquisition. All samples were run on a Thermofisher ID-X orbitrap mass spectrometer coupled to a Vanquish LC. 5µL of each sample was injected on a HILICON iHILIC-P Classic column (150x2.1mm 5micron particles). The LC conditions were as follow: mobile phase A, 20mM ammonium carbonate, 0.1% ammonium hydroxide in water. Mobile phase B, Acetonitrile 97% in water. The gradient started at 93% B and a flow rate of 0.05ml per min. The flow rate was increased to 0.15mL per min in 30 seconds, while keeping B at 93%. The gradient then changed to 40% B over 18.5min, and then to 0% B in 7min. After 5min at 0% B, the initial conditions were restored (93% B) in 3min and the column was re-equilibrated at 93%B for 9 min. the column was maintained at 40°C. For MS1 runs (for all individual samples) the mass. Spectrometer was acquiring in switching positive and negative ion mode, with a 120000 resolution and a mass range of 65 to 1000 m/z. For MS/MS data acquisition, the pool sample was analyzed in positive and negative mode separately, using each time an AquireX deepscan with 5 reinjections of the pool sample. All the data was analyzed using Compound Discoverer (Thermo Fisher, version 3.2).

Compounds identification was performed using either a local MSMS database (constructed by running pure standards in the same conditions as the samples) or an online database (MzCloud). Each match was manually inspected. Compounds for which

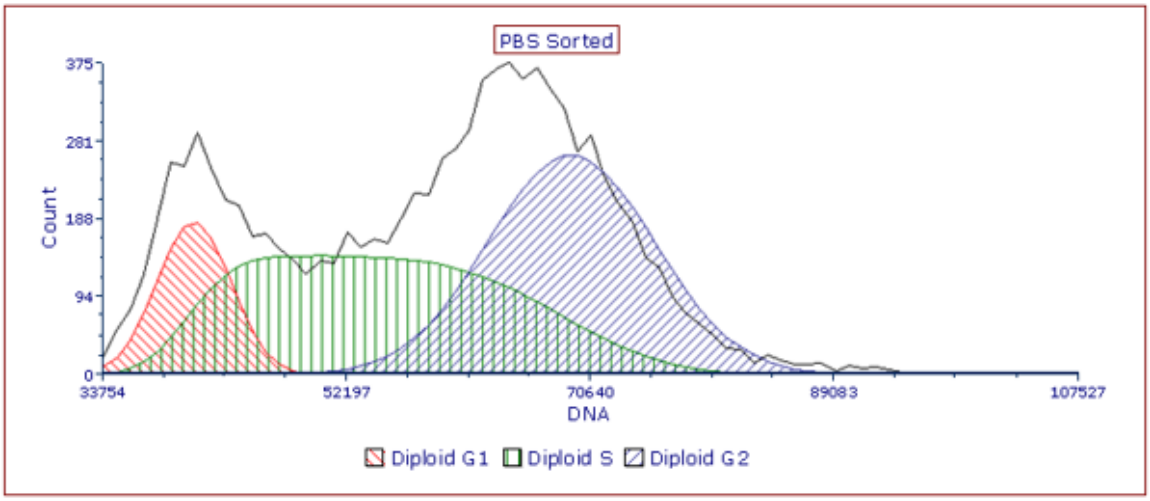
no match were found to be satisfactory are assigned with their most probable formula (based on accurate mass, isotopic distribution, and fragments probable formula). Principal component analysis and hierarchical clustering were all done in Compound Discover (Thermo Fisher).

Chapter III.

Results

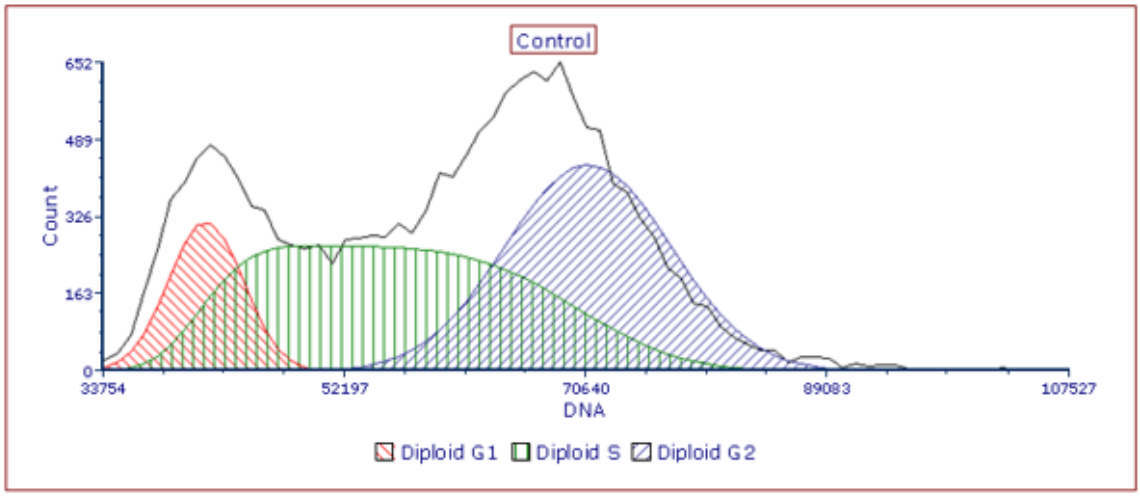
K562 cells were successfully sorted, aldehydes were quantified, and metabolomic data was acquired. Cells were successfully fixed, and the DNA measured to compare their cell cycle states. These experiments helped determine the effect of cell sorting on cellular stress.

A



Cycle	G1 Mean	G1 CV	%G1	G2 Mean	G2 CV	%G2	%S	G2/G1	%Total
Diploid	40807.73	6.90	13.66	69413.99	9.09	43.87	42.47	1.70	100.00

B



Cycle	G1 Mean	G1 CV	%G1	G2 Mean	G2 CV	%G2	%S	G2/G1	%Total
Diploid	41753.40	6.84	13.44	71022.49	8.93	41.13	45.44	1.70	100.00

Figure 5: Cell Cycle Analysis.

A) PBS sorted sample B) Unsorted Control. Plots represent DNA count (x-axis) vs count of cells (y-axis).

Cell Cycle analysis is done by measuring the amount of DNA within the samples. The 4′,6-diamidino-2-phenylindole (DAPI) stain is used as it binds to the adenine-thymine regions of DNA. This dye stains the samples stoichiometrically allowing for the direct translation of fluorescent intensity to DNA amount. These values are plotted linearly and through a modeling software the G0/G1, S and G2 phase are able to be determined (Nunez 2001). These regions help recognize which status the cells are in, either actively dividing or halting reproduction.

All the samples generated a similar profile of DNA content, using the PBS vs unsorted as the representative plots shown in figure 5. This was consistent across all of the treatments (data not shown). There were no significant differences in the percentage of cells within the different stages of cell cycle. A diploid analysis was performed in FCS Express 7 (De Novo); this mathematically plotted the cells based on the DNA content and measured the values across all different cell cycle stages. Because there is no significant increase in G0/G1 observed, there is no cell cycle arrest occurring. This demonstrates that there is not enough stress to immediately halt the cellular division for all the cells present. This is consistent with previous studies at different institutes, confirming that the sorting was similar to those previous studies. Though there are still possibilities for the sorted cells to be stressed, it is not enough to influence a crucial cellular process. There is a possibility that the effects of cell sorting were not measured immediately through cell cycle differences and needs more cellular divisions to truly display changes. As there are no significant differences measured through cell cycle, metabolic activity will be compared across all treatment groups.

Metabolomics : Aldehydes

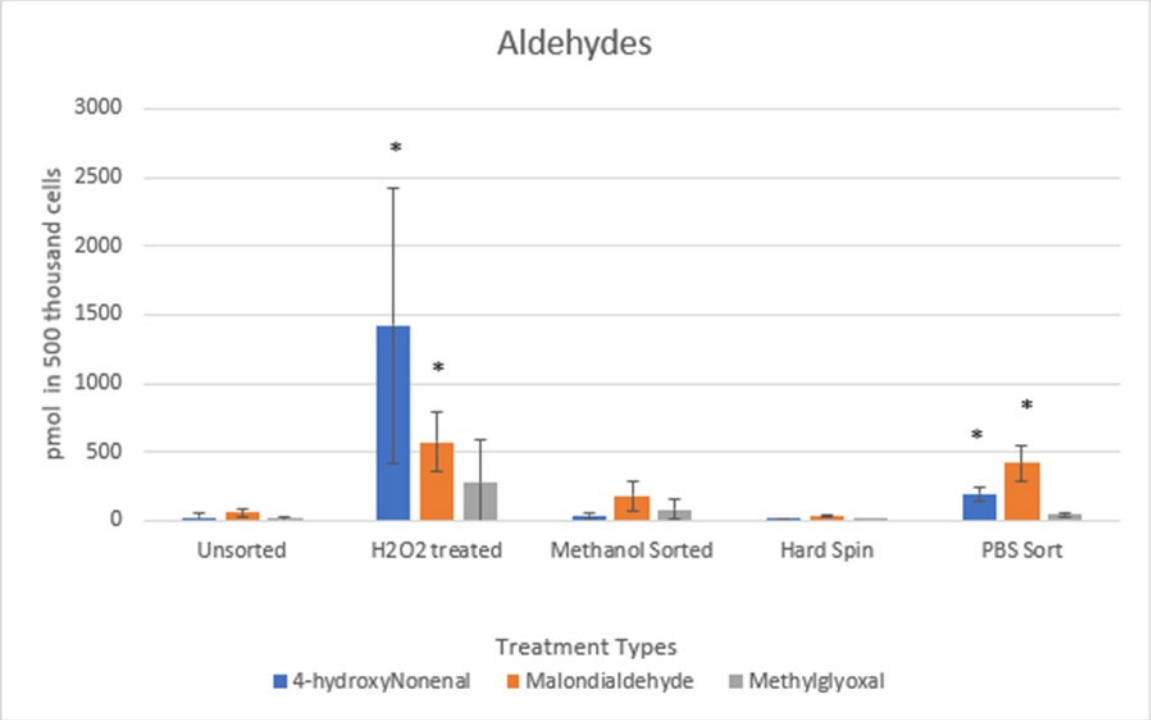


Figure 6: Aldehyde Measurements.

*Average aldehyde measurements of each sample within the treatments. * Indicates student's t-test significance versus unsorted control based on alpha level of 0.05. Error bars standard deviations.*

The aldehyde levels were measured across all the samples and averaged together in each treatment group. When comparing the aldehyde concentrations to then unsorted control, there is an increase among all the conditions. The hard spin samples however did not have any differences that were statistically significant from the unsorted sample. This would imply that this kind of physical stressors does not cause the stress pathways leading to aldehyde generation to be activated.

The hydrogen peroxide treated samples, however, showed at a minimum a 10-fold and up to 50-fold increase when compared to the unsorted samples (when comparing the different aldehydes). A student's t-test was used to determine the significance of the values between unsorted and hydrogen peroxide treated samples, and there was significant difference for both 4-hydroxyNonenal and Malondialdehyde, with 0.03 and 0.007 p-values respectively. Though the Methylglyoxal levels were higher than the control it did not show any statistical differences, (p-value of 0.1) even with an average 10-fold increase.

Though the Methanol sort had an 8-fold increase in methylglyoxal compared to the unsorted, it did not show any statistical differences between the two sample types. In addition, there was statistical difference (p-values of 0.037 and 0.047) between the methanol sort and the H2O2 treated samples, except for the Methylglyoxal levels (p-value of 0.36). The immediate quenching of metabolism through sorting into methanol demonstrates a difference in aldehyde production from sorting into PBS. When comparing the two sorted sets they show statistical differences between 4-hydroxyNonenal and Malondialdehyde (0.007 and 0.035) while methylglyoxal did not pass significance testing as the p-value was 0.44.

The PBS sorted samples had significantly increased levels of 4-hydroxyNonenal and Malondialdehyde compared to the unsorted control (7 fold increase, p-value 0.03 and 3 fold increase, p-value 0.04 respectively). Though there was on average increase of 4 fold in the Methylglyoxal levels compared to the control, this was not statistically significant. When comparing between the PBS sorted and the H2O2 treated samples there were no statistical differences, even though there was a 7, 1, and 6-fold increase for 4-

hydroxyNonenal and Malondialdehyde and Methylglyoxal respectively. This information highlights a difference between the methanol and PBS sorting.

With this information it is confirmed that hydrogen peroxide treated samples have a large amount of oxidative stress and cause an increase in aldehyde production. With both sorting conditions being lower than the H2O2 but higher than the unsorted, there is up to a 7 fold increase of aldehydes from cellular sorting. These values are still well below the threshold set by the hydrogen peroxide treated samples.

Metabolomics: Unbiased Clustering

To further explore the effects of cell sorting on cellular metabolism, an untargeted metabolomics analysis was performed. Over 6,000 metabolites were measured and compared across all of the samples. Figure 7 shows that the overall signals measured were comparable across all the samples. This is crucial, because the untargeted analysis is conducted on median normalized values (overall signal is corrected so that each sample as the same median intensity), and that normalization could introduce bias if the biomass were not relatively similar. In addition, because the number of cells used was identical, if large variation had been observed, it would have then indicated that there was some cell lysis prior to methanol quenching.

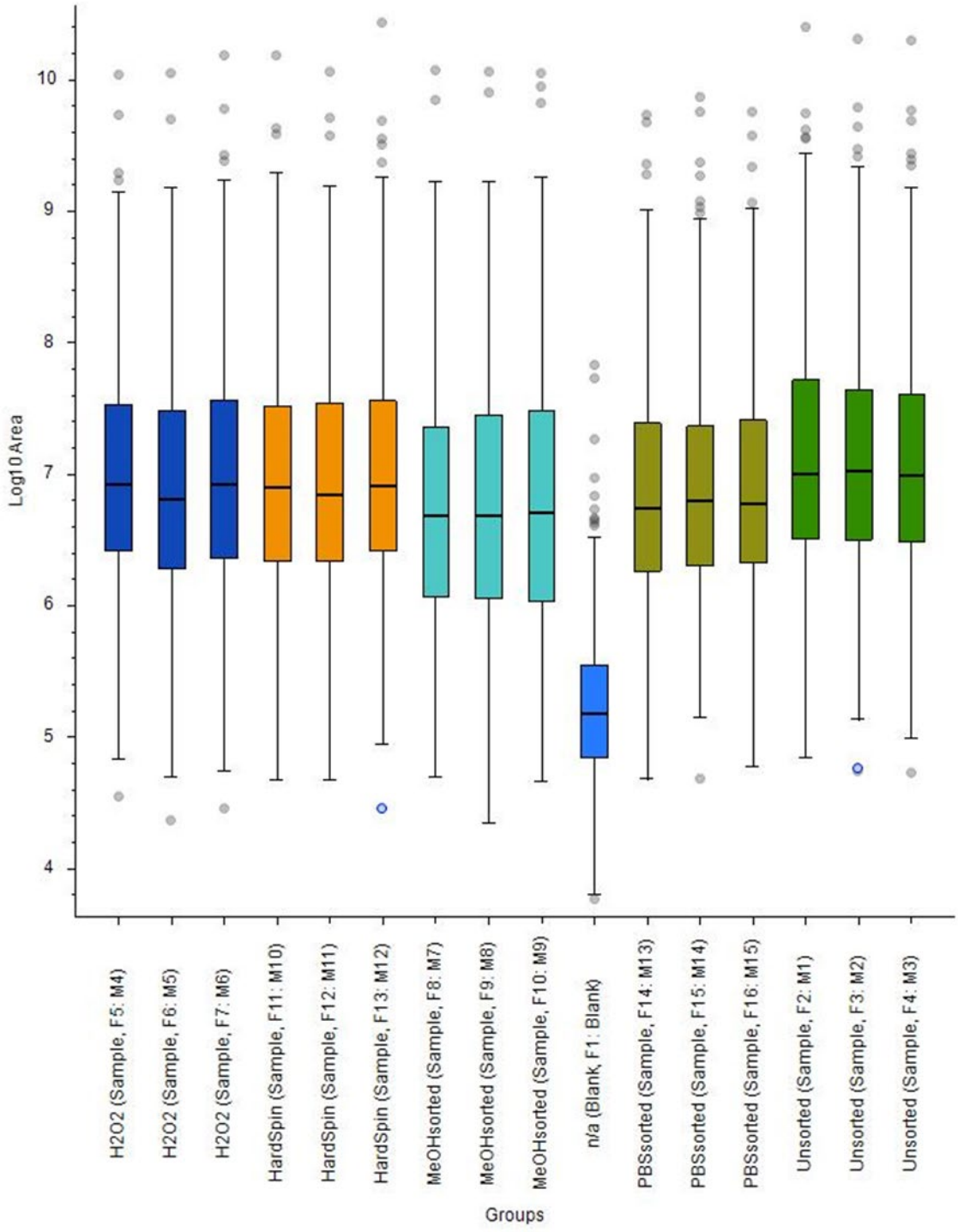


Figure 7: Signal Distribution.

Overall signal distribution measured in all samples and blank control.

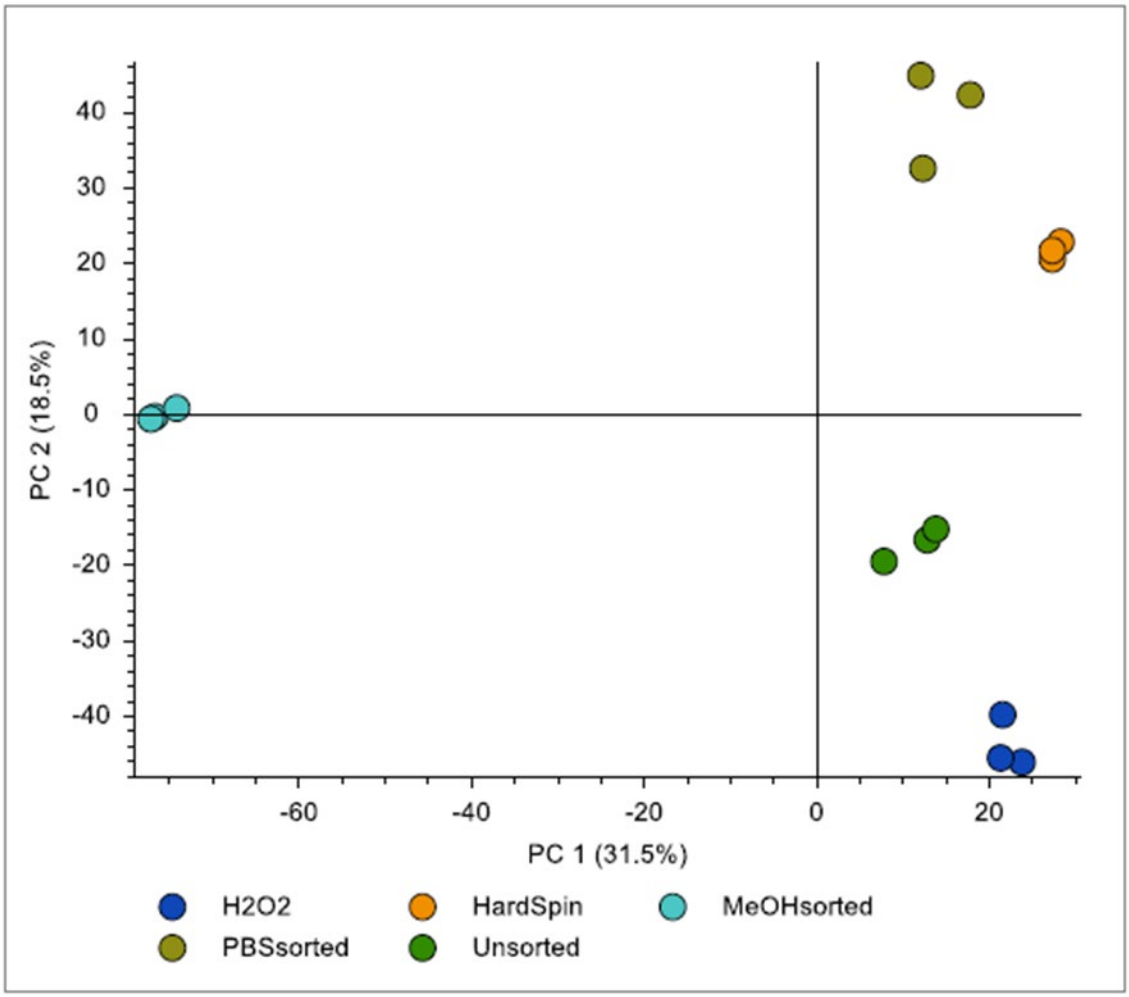


Figure 8 PCA.

PCA of all groups in the metabolomic data set produced by Compound Discover software.

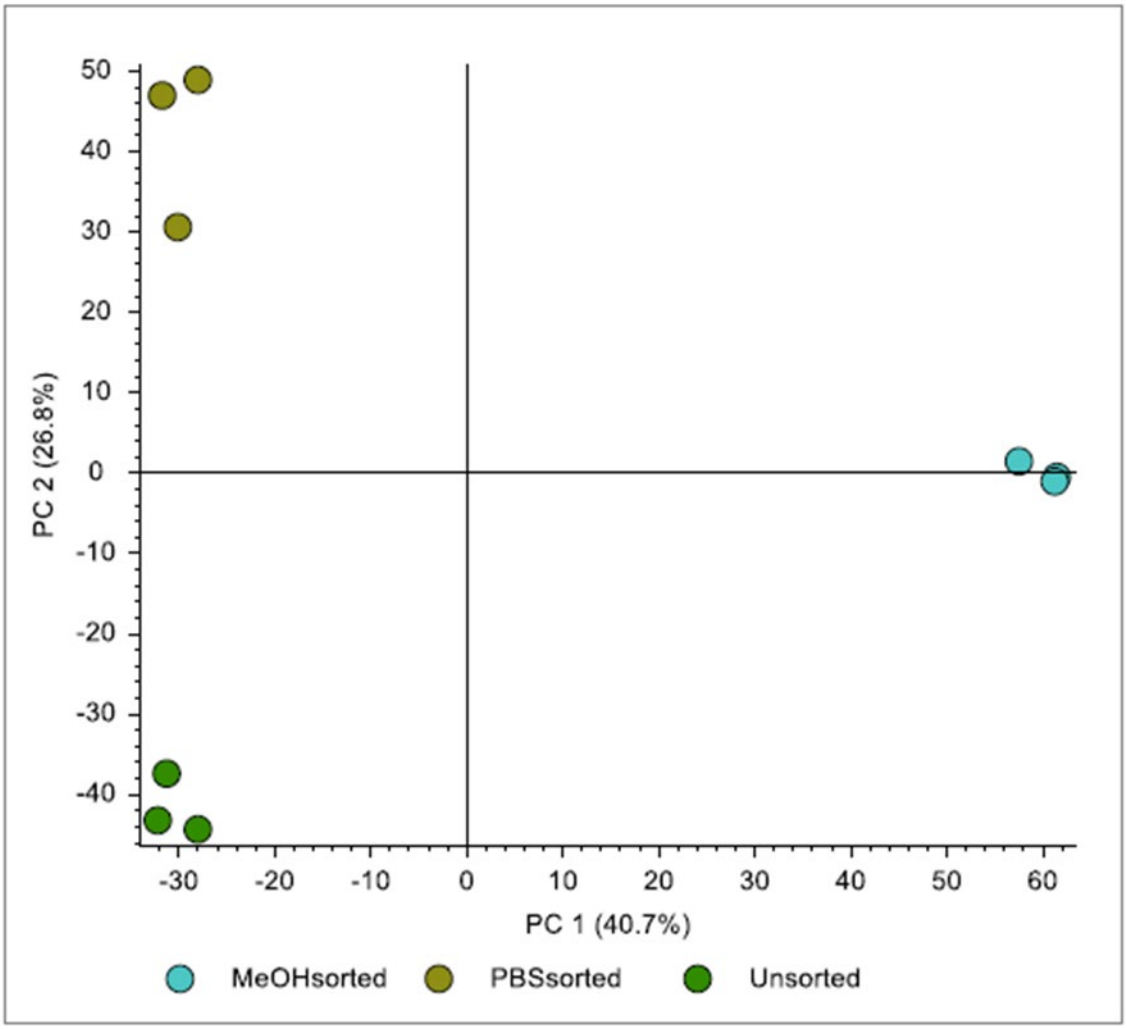


Figure 9 Sorted vs Unsorted PCA.

PCA of sorted samples versus unsorted produced by Compound Discover software.

PCA allows for an easier comparison of the samples across all compounds as it keeps the trends of the dataset while decreasing the complexity. This is a very powerful tool as it is unsupervised and finds patterns and clusters without knowing any of the prior biological or sample information (Lever et al 2017). This allows for clustering of samples based on all on the compounds with minimal computational power required. With the PCA it is important to note that it takes all metabolites into account when clustering the

sample. Even if there are significant differences within particular key metabolites, if there are minimal differences across more metabolites it will normalize to display that in the clustering. These potential compounds can be greatly involved in important pathways and will be important to be separately examined.

The PCA (Figure 8) shows that all the replicates for a group cluster near each other, demonstrating the reliability of the replicates. As shown in Figure 8 there is clustering of cells sorted into methanol on the left of axis PC1. This represents 40% variance between the samples, indicating many differences between the methanol sorted samples and other groups. The PBS sorted samples are separated by axis PC2 only, but this is still a major source of variance, approximately 26%. In figure 9 just the sorted sets and the unsorted control are represented. This highlights differences in the samples from the unsorted as they are clustered on the left side of axis PC1. The unsorted cells are situated between the methanol and the PBS sorted samples on the second principal component axis, showing that the global variance is not just due to sorting effects.

These results as a whole are interesting as they show a clear difference between the immediate quenching of the metabolism compared to the other four groups. The methanol sorted samples demonstrate the biggest difference from all the other sets. With the PBS sorted and hard spun sample, it can highlight an avenue for how potential mechanical stress affects cellular metabolism.

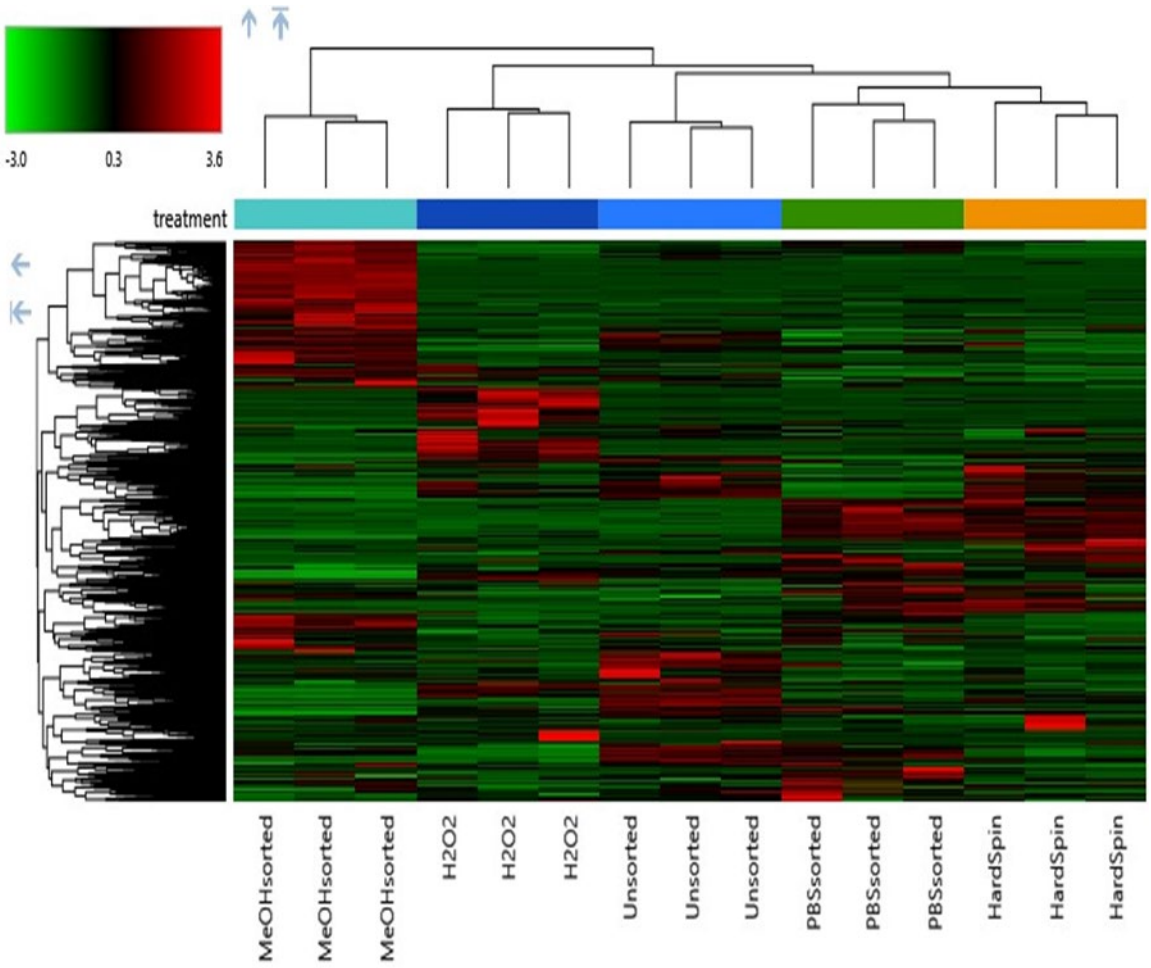


Figure 10 Hierarchical Cluster Analysis.

Hierarchical Cluster Analysis of all compounds across treatments. The distance function was called using a Euclidean method and a complete linkage was used.

Hierarchical clustering (Figure 10) can be used to have a better overview of the samples' variations than the PCAs. Similar to the PCA, all the samples within each sample group cluster close to one another, indicating that the replicates are similar to one another. Similarly, to what was observed in the PCAs, there is close clustering of the PBS sorted and hard spin groups, representing a high similarity in the levels of metabolites.

This could be the effect of the final spin before methanol fixation instead of the sorting itself.

The methanol sorted samples are furthest away from the other samples showing the most variance between the groups. Table 1 is a list of all the named compounds that are found to be in major differences in methanol sorted versus the other samples. Some of the compounds are found to be statistically different and the majority of the compounds listed are found in higher concentrations then the other sample groups.

3-(tert-butyl)-1-methyl-4,5-dihydro-1H-pyrazol-5-one *	Lauric acid *	Sorbic acid
3,3-Dimethylglutaric acid *	Levulinic acid *	Stachydrine *
5-(6-hydroxy-6-methyloctyl)-2,5-dihydrofuran-2-one *	N-Isovalerylglycine	Tyrosine methyl ester *
Bis(methylbenzylidene)sorbitol	N-Methyl-2-pyrrolidone *	
Camphanic acid *	NP-007909 *	
Cyclo(leucyloprolyl) *	NP-018817 *	
Dipropyleneglycol dibenzoate	NP-020205 *	
EPK *	Penicillin G *	
Ethylhexanoic acid *	Perillartine *	
Heptanoic acid *	Phosphoenolpyruvic acid	
Hexanoic acid *	Platyphyllenone	
Hydroxyglutaric acid *	Salicylhydroxamic acid	

Table 1 Methanol Sorted Compound List.

*Compound list of the zoomed-in differential hierarchical clustering of methanol sorted samples. Samples highlighted in red color are lower in amount compared to the unsorted sample. * Indicates statistical significance based on p-value < 0.05*

Metabolomics: Amino Acids

Amino acids are crucial building blocks for the creation of proteins and are extremely important for homeostasis. They control many aspects of cellular activity, and mis-regulation, or absence of these molecules, can cause stress and potentially lead to apoptosis. Amino acids also play a vital role in immune health, neurological stability and are used as intermediates for many compounds (Li et al 2007). Their levels in the various treatments were therefore examined.

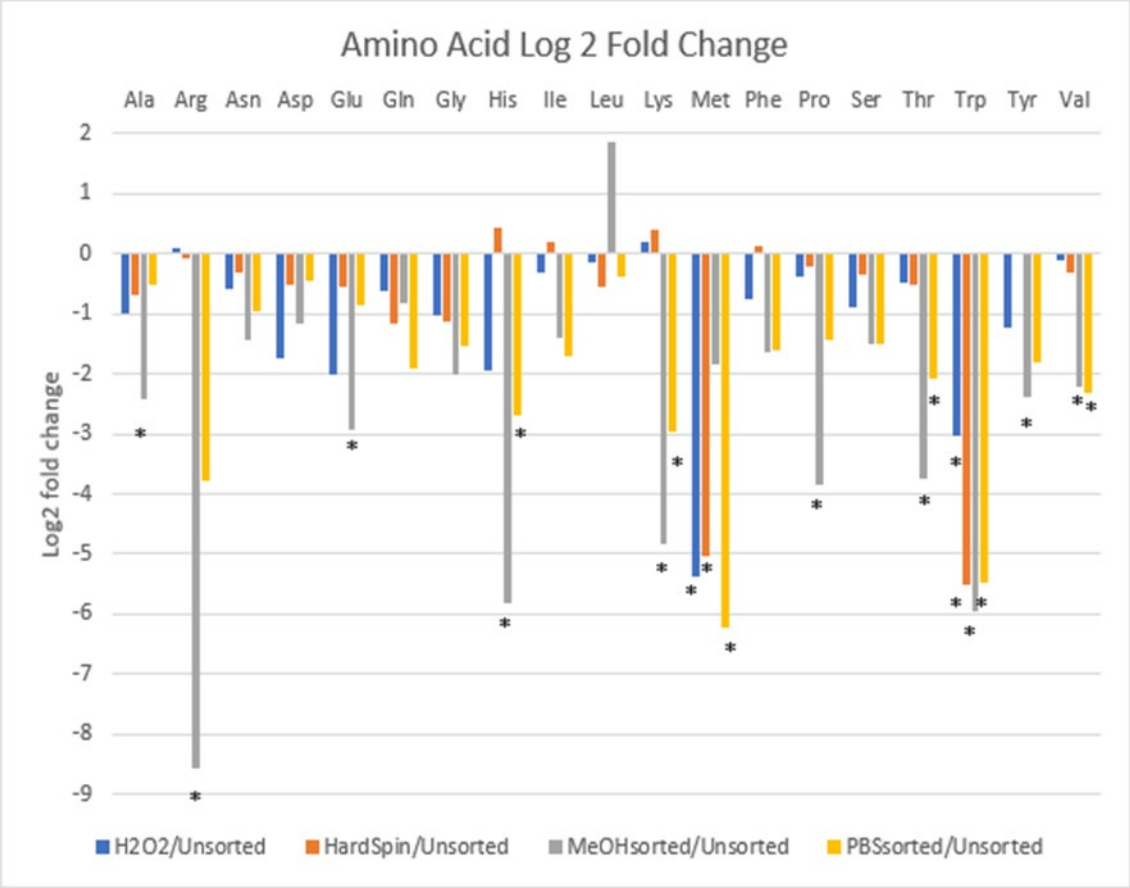


Figure 11: Amino Acid Log 2 Fold Change.

*Log 2 Fold Change of amino acids levels in all samples groups compared to unsorted control. * Indicates p-value < 0.05 and greater than 2 fold change.*

As shown in figure 11, 19 of the 20 standard amino acids were identified and measured (only cysteine was missing). The Log2 fold change was used to compare the values from all the treatments against the unsorted control. As the majority of values were lower than unsorted it leads to negative Log2 fold changes. This demonstrates across the board that there are lower abundances in the treated and sorted samples. The interesting compound to consider is Leucine as it is increased in the methanol sorted

sample, however that value is not statistically significant compared to the unsorted controls.

After doing an Analysis of Variance (ANOVA) post-hoc testing to determine the significance of the amino acids' intensity compared to the unsorted, the majority of the sorted sets had significant differences. In the methanol sorted samples only two amino acids had a p-value lower than 0.05 and did not pass the variance tests: leucine and glutamine. All the other amino acids had significant differences and had much lower values across all of those compounds. In figure 11 asterisks were applied to indicate significance and show more than a 2 fold change, showing major changes of amino acid quantities. The amino acids that satisfied both criteria were arginine, glutamate, histidine, lysine, methionine, threonine, tryptophan, tyrosine, and valine.

Both the hydrogen peroxide and hard spun samples had differences between each other and the unsorted controls. However, only methionine and tryptophan had statistical differences and passed the log2 change threshold. The PBS sorting, however, was more similar to the methanol sorted samples and had a majority of those differences that were significant when compared to the unsorted. Of the amino acids found and compared only histidine, lysine, methionine, threonine, tryptophan, and valine were significant and passed the 2-fold threshold.

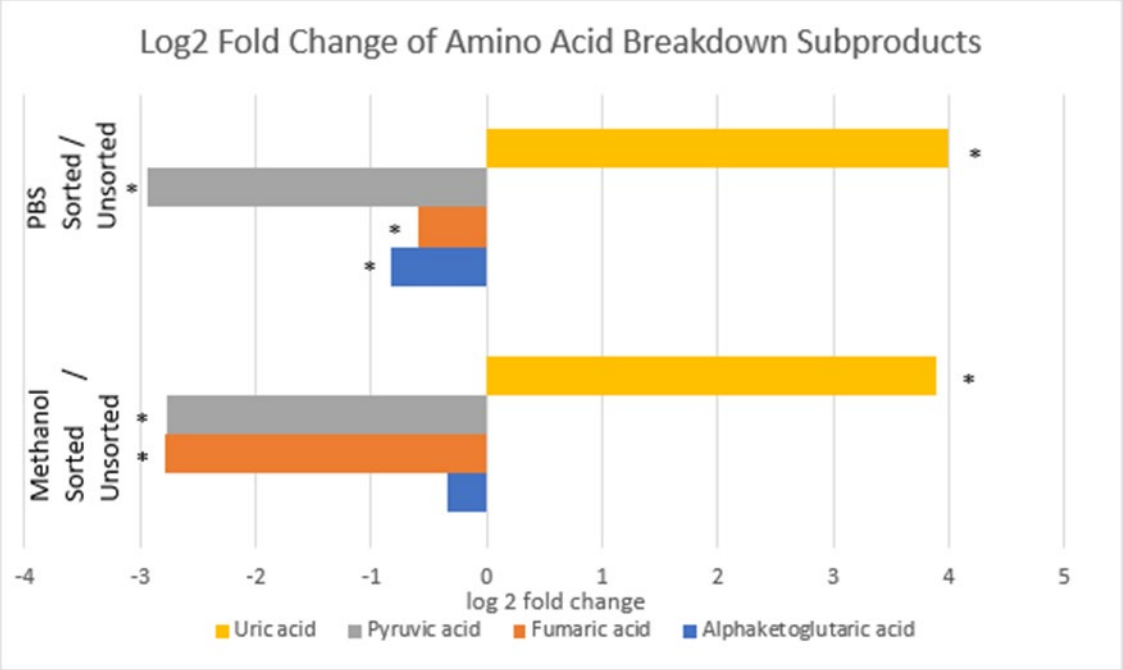


Figure 12: Amino Acid Subproducts Log 2 Fold Change.

*Amino acid breakdown products for the sorted samples. * Indicates significance as the p-value is less than the alpha level of 0.05.*

Amino acids break down into pyruvate, oxaloacetate, fumarate, acetoacetate, α-ketoglutarate, succinyl-CoA, and acetyl-CoA (Felig 1975). These compounds are components of the TCA cycle and can be an indication of either a low supply or a heightened demand of those compounds. Of those only α-ketoglutarate, pyruvate and fumarate were identified within the data set and, as shown in figure 12, had a lower amount in the sorted samples versus the unsorted. The pyruvate for PBS sorted samples and fumarate for the methanol sorted samples did not pass the alpha level of 0.05, and the results were not significantly different than the control. The values were all much lower than the unsorted controls which is interesting as it demonstrates that even with the lower amounts of amino acids, the break down products of those amino acids are also lower.

As the amino acids are broken down, in addition to particular subsets on the particular amino acids, there is also a byproduct of nitrogen. This nitrogen is typically disposed of from cells as either urea or uric acid. Both of the sorted groups have significantly more uric acid than in the control and is found to be almost 4 fold higher in intensity. The same significant difference was found for all the intermediates and nitrogen excess when comparing the H2O2 treated samples and the unsorted; however, the increase of nitrogen was only a 2-fold (data not shown). The hard spin samples had no statistically different results for both the amino acid intermediates and the excess nitrogen between the unsorted samples. This helps demonstrate that the amino acids were actually broken down as there was an increase of nitrogen by products and also a decrease of amino acids and their derivatives. The breakdown of amino acids is a useful process that cells can undergo when more energy is needed, potentially due to the effects of sorting.

Metabolomics: Energy

Cells need energy to perform their responsibilities and creation of it is a highly regulated pathway. This pathway is driven by the mitochondria and an electron transport chain. Disruptions in this pathway from either energy deprivation or an abundance of reactive oxygen species can lead to apoptosis (Huttemann et al 2012). As outlined in figure 13, each step is identified and many of the components involved in those steps have been discovered within the dataset. These processes require an input of glucose and then pyruvate and result in energy in the form of ATP. Also occurring during

these processes is the production of reduced Nicotinamide adenine dinucleotide (NADH) which is a key component of the final energy production step, chemiosmosis.

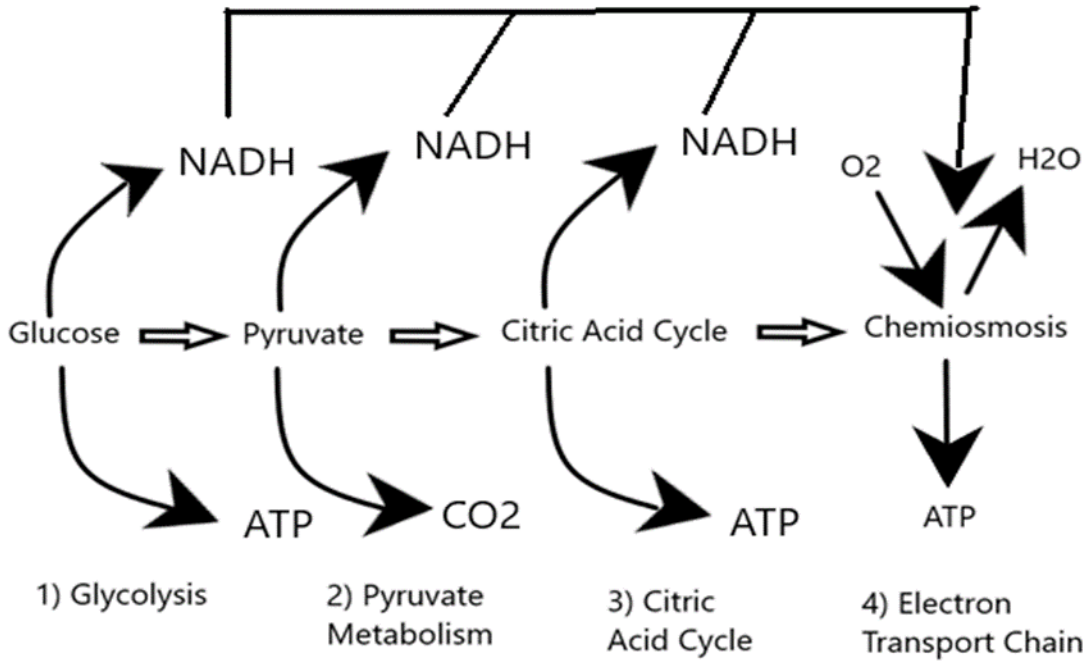


Figure 13 Overview of Energy Production.

Cellular energy production overview.

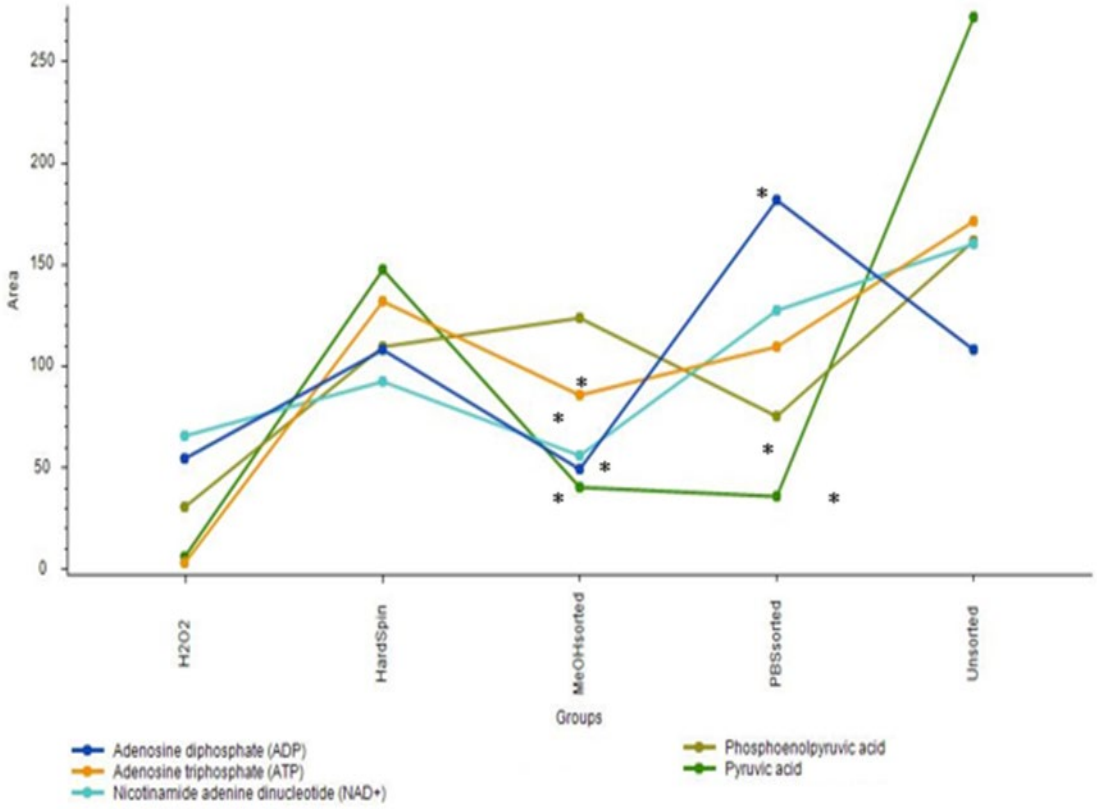


Figure 14: Glycolysis Products.

*Glycolysis Products Area that has been scaled. * Indicates significance as the p-value is less than the alpha level of 0.05 (when compared to the control samples).*

Energy creation in cells typically results in adenosine triphosphate (ATP) and can be used to release usable energy for cells to use. ATP can be created through the process of glycolysis which breaks down glucose, one of the most common compounds found in cells. The breakdown of glucose is a multistep process that involves many components that creates ATP in an anaerobic environment (Gatenby & Gillies 2004). Inputs of glucose, Adenosine diphosphate (ADP) and ATP are required for glycolysis and then result in an output of pyruvate and an excess of ATP.

As displayed within figure 14, ADP had significant differences for both sorted sets when compared to the unsorted, however in the PBS sorted samples it had an increase in ADP. The ATP amount in the PBS sorted samples did not have any statistical differences from the unsorted control. With the methanol sorted samples there is significant decreases of ATP and ADP compared to the unsorted set. Another key component of glycolysis is the creation of NADH from NAD. NAD was found to be statistically lower in the methanol sorted samples, but not more than a log-2 difference. The PBS sorted samples had lower amounts, but these values were not found to be statistically significant. These differences between the two sorted groups are important to consider when designing projects for looking at energy output of glycolysis.

The final output of glycolysis is pyruvate, in addition to the ATP. When looking at the methanol sorted samples, they have overall a statistically significant lower amount of glycolysis output related compounds compared to the unsorted samples. Phosphoenolpyruvate is the step immediately before the creation of pyruvate and found though to be lower than the unsorted, it is not statistically significantly different. Pyruvate itself is found to be in much lower quantity and those values are statistically significant for both the methanol and PBS sorted samples.

The Citric Acid Cycle is another cycle important in energy production as it creates NADH and then via oxidative phosphorylation ATP is produced. Whereas glycolysis was an anerobic reaction, the TCA cycle is aerobic. This cycle has many intermediates, but the major output is ATP via the oxidative phosphorylation (Akram 2015). As shown in figure 15 there are many compounds involved in the steps of TCA cycle and the majority of them are found to be in lower quantities compared to the

unsorted samples. Of the rest of the compounds found in the data set, only cis-Aconitate, citric acid, and fumaric acid were found not to be significantly changed as they did not pass the 0.05 alpha level. They were all found to be in lower concentrations, yet only pyruvate passed the 2-fold change as it was 2.9-fold lower than the unsorted.

The methanol sorted samples had significant differences in the samples except for alpha-ketoglutarate and citric acid as they were also above of the 0.05 alpha level. All of the compounds were found to be decreased in the methanol set and cis-aconitate, malic acid, and pyruvic acid passed the 2-fold change threshold (-2.75, -2.84, -4,58, -2.76 respectively). Demonstrating that if glycolysis and TCA cycle is a downstream analysis, it is important to recover the cells in a PBS or media buffer then fixate the cells.

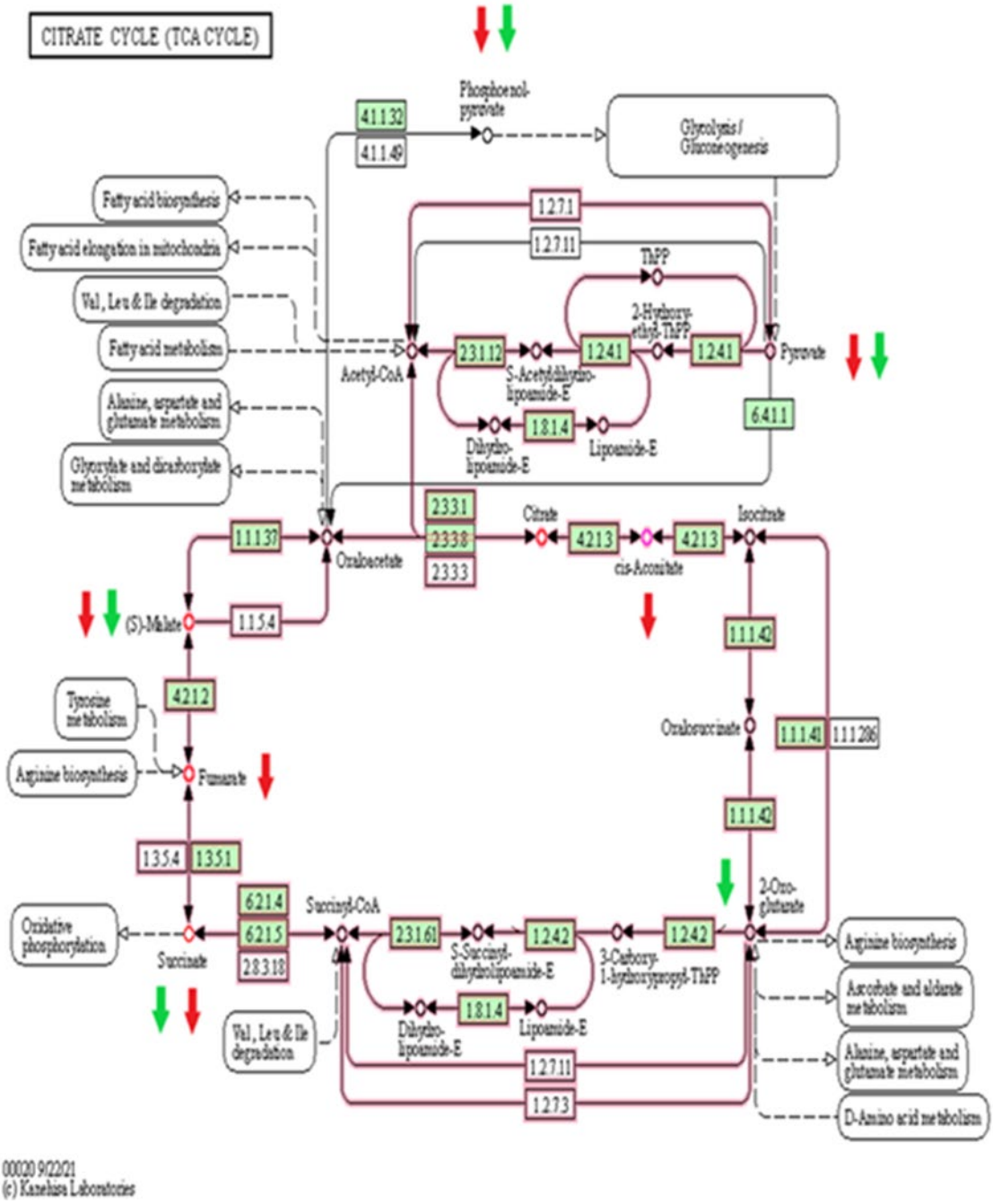


Figure 15 Citric Acid Cycle.

Green arrow indicates PBS sorted. Red arrow indicates methanol sorted. Direction of arrow indicates significantly (p-value < 0.05) higher or lower than unsorted control. Red outline indicates immediate TCA. Schematic was original from KEGG database and edited to fit criteria of my experiment.

Chapter IV.

Discussion

The aim of this study was to determine the effects of cell sorting on cellular metabolism. Cell sorting is a useful tool for isolation of particular cells of interest for downstream applications. With the use of cell sorting, the cells have to be prepared into a single cell suspension and then ran through a machine where they are electrostatically isolated into different tubes. This process has the potential to introduce stress and change how the downstream analysis is interpreted.

Prior studies have demonstrated that there is minimal effect of gene expression upon cells after cell sorting (Puttaswamy et al 2010). Others have shown that there are some metabolic effects upon sorted samples and have shown increases in oxidative stress and cause an inflammation like status. These studies have looked at specific compounds, in particular NADPH and other energy related compounds (Klinc & Rath 2007). With this information it is important to look at broad metabolic study to see all potential metabolites changing, to understand the full effects of cellular sorting.

This study isolated cells by sorting them, alongside having controls to help represent other stressors that are involved in the sorting process but not a direct response of sorting. The first check to determine the stress of cell sorting was to measure the DNA content of each sample and determine what state of cell cycle they are in. All of the samples had no statistical differences in their division states, which shows that cellular division was still continuing post sorting. This indicates that the stressors of cell sorting are not enough to halt division and cells are still able to function normally and have no lasting effects on this process. Because the cells were fixed immediately after sorting, it is

possible the full effect of the sorting stress on cell cycle was not yet observable. A follow up study to test this would be to allow time for regrowing of the cells, to determine if they expand slower, indicating that their cell divisional state had in fact changed.

When looking at the metabolites analyzed, there were two major sets of data created: one analyzing the aldehydes and another looking at a broader range of metabolites. The aldehydes are important group to analyze for this study, because they have been found to be increased in response to cellular stress in previous studies. Aldehydes are formed as a result to oxidative stress and are impactful in various diseases (Hoshi et al 2012).

The aldehydes that were detected in the samples were 4-hydroxyNonenal, Malondialdehyde and Methylglyoxal, all of which were found in higher concentrations in all of the treated and sorted samples compared to the unsorted control. However, the H2O2 had a significant increase in the 4-hydroxyNonenal and Malondialdehyde compounds but not the methylglyoxal. The methanol sorted samples did not show any statistical difference between the unsorted control but did show a difference in malondialdehyde in comparing the H2O2 control. The PBS sorted did show a statistical difference in all compounds except for methylglyoxal from the unsorted control but had no statistical differences when compared to the H2O2 samples. The hard spun samples did not have much an increase and no significant differences compared to the unsorted samples, showing that, in those conditions, mechanical stress does not cause oxidative stress. As oxidative stress is a kinetic activity there is a possibility that the fixation immediately after the mechanical stress halted metabolism not giving the cells enough time to react.

These results demonstrate that the H2O2 treated samples are a good positive control for a representation of oxidative stress. The immediate fixation of the samples through methanol has higher levels of aldehydes but the increase is not significant versus the unsorted samples. Whereas the PBS sorted samples do have significant changes, showing that there is a stress as a result of sorting the cells. Since metabolism is not halted immediately post sorting in the PBS sorted samples, the stressful effects of sorting had more time to be expressed in the production of aldehydes. This demonstrates that production of the stress related aldehydes is increased in the response from sorting and possibly could affect other downstream processing. These increases in aldehydes are not as high as the positive control of H2O2, demonstrating some stressful effects but not a large increase in oxidative stress. These results combined with the cell cycle show that there is some stress due to cellular sorting. It causes some changes but not enough to immediately alter cell cycle nor to harbor the hallmarks of a full oxidative stress.

Metabolomics is a relatively new field of study in biology that measures the compounds in samples, giving the most accurate representation of what is currently happening within a cell and showing the phenotype of the cells (Dettmer et al 2007). When analyzing the dataset, the first step was to confirm that input signal distribution is the same across all samples for each treatment type. The overall signal produced was similar across all samples and groups, showing there were no biomass differences for sample input. This eliminated the possibility for flow sorting to have killed or lysed the majority of cells. This also allows for an accurate comparison of the analytes found in the data.

When analyzing a large dataset, a global overview is a useful start, and that is why clustering is such a useful tool. Two types of clusters were implemented, a PCA and a hierarchical analysis. The PCA allows for a decreasing of the dataset and aggregates like samples near each other by their values across all metabolites. The hierarchical clustering allows for grouping and shows close relations by proximity alongside linkages. Both clustering methods show that all samples are similar to one another within the same treatment group, indicating no major outliers.

The PCA and hierarchical clustering both show that the methanol sorted samples have the greatest differences compared to the other sample groups. The methanol sorted samples showed a large about of up-regulated compounds in the hierarchical clustering. Those compounds are of large interest as they were not observed to be increased upon the PBS sorting This indicates that they are detected because of the immediate quenching. These overall differences between the two sorted sets show that some recovery activity can occur if the cells are allowed to incubate in PBS as they exit the sorter. Though this incubation was short (about 5 min), it was long enough to allow for a global recovery.

Many different compounds were determined to be up regulated for the methanol sorted samples compared to the unsorted. Of the compounds listed in table 1, there is an increase of fatty acids such as 10-HDA, 12-Aminododecanoic acid, Lauric acid, 3,3-Dimethylglutaric acid and Hexanoic acid. These fatty acids and other lipid based derivatives are indicative that there is destabilization of the membrane. This may be due to the sorting directly into methanol, the cells have a semi permeabilized membrane from the stressors and don’t have time to recover like the PBS based sorting.

Sphingosine has been found to increase in early apoptotic pathways which has also been up-regulated (Abrahan et al 2010). While Stachydrine also up regulated is the opposite, it is involved in reducing apoptosis and inflammation (Li et al 2020). 3-Phosphoglyceric acid is another important compound of interest as it is involved with glycolysis and can indicate that glycolysis was starting to ramp up before being immediately halted.

After the separation of the methanol sorted samples the H2O2 group has the most distance to the control in the clustering, indicating the next largest sets of changes. This demonstrates that the H2O2 treatment of cells causes both increases in aldehydes but also other metabolic changes. As the hard spin and PBS sorted sample are clustering near one another, this could potentially highlight more the effect of centrifugation of the cells rather than the actual sorting. These mechanical stressors can also be due to the mechanical nature of the stress applied by sorting, such as shear stress and electrostatic deflection into collection tube. This does, however, have cause for debate about which method of sorting is providing the most accurate biological representation. Depending on the downstream application of the experiment the immediate quenching maybe more desired, such is the case for measuring aldehydes post sorting. However, if the aim of the experiment is more untargeted metabolomics, then sorting into PBS or another buffer would be more desirable as it causes more similarity to in the non-sorted fraction.

As there were over 6,000 metabolites identified and compared across the groups, specific crucial biological processes were identified. Amino acids are important in maintaining homeostasis and creating proteins for cellular functions. These amounts were depleted across all the samples when compared to the controls. This result explains that the amino acids were utilized for recuperating from the stress of cell sorting or the

treatments rather than protein construction. Amino acid decrease can be explained by using amino acids for creating new proteins such has the heat shock response or glucose related proteins (Lee 1992). However, since it has been shown that transcriptional changes don't occur after sorting then it is more likely that the amino acids are being utilized for other biological processes. The breakdown metabolites of amino acids which are found when they are broken apart was also found to be decreased in both sorted sets of samples. However, an increase of nitrogen waste was found in both sorted sets, which is a by-product of breaking down the amino acids. Taken together, this indicate that amino acids were likely broken down for additional energy usage (Tokunaga et al 2004). This may highlight a stress related attribute of the sorting, as the amino acids could be decreased to allow for a larger creation of energy needed to recover from the stress of sorting. The immediate fixation of the cells sorted into methanol demonstrated a larger change from the unsorted fraction. Showing that if amino acid measurements is the goal of future studies then sorting into PBS or another buffer would be more advantageous.

Energy creation is one of the most crucial activities of cells and allows for other cellular functions to occur. Both anaerobic and aerobic steps of energy creation have been widely studied and many of the compounds found in the cycles have been identified in the metabolic dataset. ATP is a crucial energy source that cells utilize for many biological processes and is a major factor in determining cellular health. Both sets of sorted samples had lower amounts of ATP when compared to the unsorted. However, the PBS sorted sample had a higher amount of ADP compared to both the unsorted and methanol sorted samples, indicating a lower ATP/ADP ratio. This indicates that energy

was used more intensely in those cells, these results show that the energy cycles have ramped up post sorting, displaying the response to the sorting.

The first process in energy production is glycolysis which breaks down glucose with the assistance of NAD and ADP to pyruvate, NADH, and ATP. There are multiple steps within the cycle that create different compounds which are utilized to create pyruvate (Lunt & Vander Heiden 2011). There is a stoppage of the cycle at the point of PEP as there is abundance of the compound and the glycolysis cycle cannot continue until it is lowered. It is possible that the PBS sorted samples had their metabolism stopped at the point where the cells were ramping up energy production and did not have a chance to go through the full cycle, leaving a surplus of PEP. The reason the methanol showed differently was that there was more of an immediate fixation and the cells did not get to go through the energy creation process via glycolysis. These results are in line with the lower amino acids, indicating that the breakdowns of the amino acids are being used to create energy in the citric acid cycle.

After glycolysis the cells typically continue with energy production and use the newly created pyruvate to start the TCA. This cycle is important as it is used to create more NADH which will be utilized during chemiosmosis for creation of more ATP (Wan et al 1989). As in the case of glycolysis, the methanol sorted samples also appeared to have been halted before the majority of energy cycling could occur. The difference between glycolysis is there are no key points within the cycle that have been up regulated compared to the unsorted control. The methanol sorted samples overall also have lower levels of the components involved compared to the PBS sorted samples. Again,

demonstrating that by sorting into PBS there is some recovery of the cells, as energy production has ramped up.

All of these differences highlight some effects of cell sorting on downstream analysis of the cells. This can be further studied and many of the upregulated metabolites can be analyzed to determine the full role of each of the compounds, alongside the potential limiting of these effects. An interesting follow up study would be to sort the cells and allow them to recover by growing them back in some media for varying amounts of time (hours), possibly demonstrating alleviating effects of cell sorting. This could also demonstrate the proper way to collect cells after isolation by sorting, to minimize the stressful effects of sorting.

As found in aldehyde measurements, there is an increase in oxidative stress of the cells. A recent study looks at the role of sheath fluid and the metabolic response of cells (Ryan et al 2021). This would be another avenue to test, to ensure that a more viable sheath fluid with potential anti-oxidants could be used. Another follow up would be focusing on the upregulated compounds and trying to build a genetic system were those compounds are limited to see how they behave in a more typical environment. Measuring the viability, cell cycle and metabolomics of those depleted samples would give insight to how the differences of the compounds found in my dataset affect cells. These studies should also be compared across multiple cell types and tissues to identify if the effects are cell type specific.

Across all the treatments and sorted sample sets multiple metabolites were identified and compared. These results show that there are major differences between the sorted and unsorted sets, resulting in differences in concentrations of multiple different

types of metabolites. This demonstrates that there is some stress caused by cell sorting, resulting in differences of key metabolic activities. This stress causes changes in amino acid concentration and amount of energy related molecules. However, these differences do not lead to a halt of cellular division or a major decrease of amount of cellular material.

With all of this data it displays some effects of cellular sorting on the immediate metabolic activity of cells. However, with the other studies discussed these effects do not carry over to gene expression changes or even proteomic changes. These effects should be taken into consideration however when analyzing downstream metabolic studies of other samples. It is important to determine what application the cells will be used for after sorting, because that determines when to quench the metabolism. When measuring aldehydes or oxidative stress post sorting, it will be important to immediately quench the metabolism by sorting into methanol. If downstream untargeted metabolomics or specific metabolomics such as amino acids or energy cycling, it may be more beneficial to wait to halt cellular metabolism by sorting into a buffer and then quenching. For all these reasons flow cytometry based cell sorting can be continued to be used but these effects should be heavily weighed as it may bias future results.

References

Abrahan, C. E., Miranda, G. E., Agnolazza, D. L., Politi, L. E., & Rotstein, N. P. (2010). Synthesis of sphingosine is essential for oxidative stress-induced apoptosis of photoreceptors. Investigative ophthalmology & visual science, 51(2), 1171-1180.

Alseekh, S., Aharoni, A., Brotman, Y., Contrepois, K., D'Auria, J., Ewald, J., ... & Fernie, A. R. (2021). Mass spectrometry-based metabolomics: A guide for annotation, quantification and best reporting practices. Nature methods, 18(7), 747-756.

Akram, M. (2014). Citric acid cycle and role of its intermediates in metabolism. Cell biochemistry and biophysics, 68(3), 475-478.

Beliakova-Bethell, N., Massanella, M., White, C., Lada, S. M., Du, P., Vaida, F., ... & Woelk, C. H. (2014). The effect of cell subset isolation method on gene expression in leukocytes. Cytometry Part A, 85(1), 94-104.

Binek, A., Rojo, D., Godzien, J., Rupérez, F. J., Nuñez, V., Jorge, I., ... & Barbas, C. (2018). Flow cytometry has a significant impact on the cellular metabolome. Journal of proteome research, 18(1), 169-181.

Box, A., DeLay, M., Tighe, S., Chittur, S. V., Bergeron, A., Cochran, M., ... & Brundage, K. (2020). Evaluating the Effects of Cell Sorting on Gene Expression. Journal of biomolecular techniques: JBT, 31(3), 100.

Dettmer, K., Aronov, P. A., & Hammock, B. D. (2007). Mass spectrometry-based metabolomics. Mass spectrometry reviews, 26(1), 51-78.

Domon, B., & Aebersold, R. (2006). Mass spectrometry and protein analysis. science, 312(5771), 212-217.

Felig, P. (1975). Amino acid metabolism in man. Annual review of biochemistry, 44(1), 933-955.

Franke, A., McGovern, D. P., Barrett, J. C., Wang, K., Radford-Smith, G. L., Ahmad, T., ... & Parkes, M. (2010). Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. Nature genetics, 42(12), 1118-1125.

Friedberg, E. C. (2003). DNA damage and repair. Nature, 421(6921), 436-440.

Fulda, S., Gorman, A. M., Hori, O., & Samali, A. (2010). Cellular stress responses: cell survival and cell death. International journal of cell biology, 2010.

Gatenby, R. A., & Gillies, R. J. (2004). Why do cancers have high aerobic glycolysis?. Nature reviews cancer, 4(11), 891-899.

Groh, V., Bahram, S., Bauer, S., Herman, A., Beauchamp, M., & Spies, T. (1996). Cell stress-regulated human major histocompatibility complex class I gene expressed in gastrointestinal epithelium. Proceedings of the National Academy of Sciences, 93(22), 12445-12450.

Herzenberg, L. A., Parks, D., Sahaf, B., Perez, O., Roederer, M., & Herzenberg, L. A. (2002). The history and future of the fluorescence activated cell sorter and flow cytometry: a view from Stanford. Clinical chemistry, 48(10), 1819-1827.

Hoshi, H., Hao, W., Fujita, Y., Funayama, A., Miyauchi, Y., Hashimoto, K., ... & Miyamoto, T. (2012). Aldehyde-stress resulting from Aldh2 mutation promotes osteoporosis due to impaired osteoblastogenesis. Journal of Bone and Mineral Research, 27(9), 2015-2023.

Hua, J., Erickson, L. E., Yiin, T. Y., & Glasgow, L. A. (1993). A review of the effects of shear and interfacial phenomena on cell viability. Critical Reviews in biotechnology, 13(4), 305-328.

Hüttemann, M., Helling, S., Sanderson, T. H., Sinkler, C., Samavati, L., Mahapatra, G., ... & Lee, I. (2012). Regulation of mitochondrial respiration and apoptosis through cell signaling: cytochrome c oxidase and cytochrome c in ischemia/reperfusion injury and inflammation. Biochimica et Biophysica Acta (BBA)-Bioenergetics, 1817(4), 598-609.

Klinc, P., & Rath, D. (2007). Reduction of oxidative stress in bovine spermatozoa during flow cytometric sorting. Reproduction in Domestic Animals, 42(1), 63-67.

Lee, A. S. (1992). Mammalian stress response: induction of the glucose-regulated protein family. Current opinion in cell biology, 4(2), 267-273.

Lever, J., Krzywinski, M., & Altman, N. (2017). Points of significance: Principal component analysis. Nature methods, 14(7), 641-643.

Li, L., Sun, L., Qiu, Y., Zhu, W., Hu, K., & Mao, J. (2020). Protective effect of stachydrine against cerebral ischemia-reperfusion injury by reducing inflammation and apoptosis through P65 and JAK2/STAT3 signaling pathway. Frontiers in Pharmacology, 11, 64.

Li, P., Yin, Y. L., Li, D., Kim, S. W., & Wu, G. (2007). Amino acids and immune function. British Journal of Nutrition, 98(2), 237-252.

Llufrio, E. M., Wang, L., Naser, F. J., & Patti, G. J. (2018). Sorting cells alters their redox state and cellular metabolome. Redox biology, 16, 381-387.

Lunt, S. Y., & Vander Heiden, M. G. (2011). Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. Annual review of cell and developmental biology, 27, 441-464.

Murphy, D. B. (2002). Fundamentals of light microscopy and electronic imaging. John Wiley & Sons.