Developing a flow cytometry based viability assay for post-oxidative stress survival in yeast species

Introduction

Yeasts display an acquired stress resistance (ASR) response where a mild dose of primary stress helps the cells to survive a secondary dose of severe stress. The mild primary stress may be the same or different from the secondary severe stress. The overall goal of the project is to understand ASR traits in evolutionary related yeasts species. Specifically, previous work in the lab has identified an increased magnitude of ASR response in C. glabrata when exposed to phosphate starvation as a primary stress and hydrogen peroxide treatment as a secondary stress. This increased magnitude of ASR is not seen in the closely related S. cerevisiae. Downstream investigations suggest the ASR phenotype is closely related to the nutrient sensing TOR pathway.

Motivated by these previous discoveries, the current project seeks to understand the evolutionary trajectory of ASR. While C. glabrata and S. cerevisiae are closely related, the magnitude of their ASR phenotype does not provide information on the ancestral state of the trait. Therefore, the magnitude of ASR in an evolutionarily related outgroup species needs to be accessed and compared. Accessing ASR in multiple species requires a high-throughput method and data analysis pipeline.

The post-oxidative stress survival rate in ASR is traditionally accessed using the gold standard colony forming units (CFU) assay. CFU assay is labor intensive and the small sample plated may induce variance in the output. In addition, CFU assay only gauges cell viability and depends on cells' ability to proliferate on an agar plate. The hallmark of cell death in yeast is the permanent loss of membrane permeability. CFU assay cannot reliably access this hallmark of death.

My project aims to develop a flow cytometry based high-throughput viability assay using yeast viability dyes. The specific viability dyes used include FUN-1, Fungalite 1, and Fungalite 2. FUN-1 and its analogs are transported from cytosol to the vacuole in metabolically active yeast. The stains then give rise to the formation of CIVS structures in the vacuoles of metabolically active cells. The CIVS emits bright, concentrated red fluorescence. Dead and permeabilized cells do take up the dye. Proteins and nucleic acids in dead, permeabilized cells stain brightly and have a broad fluorescence emission spectrum. They show appreciable signals in both the green and red regions of the spectrum. This is because free thiols of proteins and peptides may react spontaneously with FUN-1 stain, generating red-fluorescence in permeabilized cells. Fungalite 1 and Fungalite 2 are membrane permeability based dyes where the red-fluorescent nucleic acid stain, propidium iodide, stains dead cells with compromised cell membrane. If this high throughput flow cytometry based viability assay can be well estabilshed and verified as a CFU replacement, then it could increase

the work flow and allow efficient examination of post-oxidative stress survival in multiple yeast species. By applying this method to various evolutionarily related yeast species and comparing the magnitude of ASR in these species, we can gain insights regarding the evolution trajectory of the ASR trait.

In this bioinformatics course project, I hope to build a pipeline for processing and analyzing flow cytometry based FCS data and use R to construct informative figures that help in evaluating the results. FCS stands for Flow Cytometry Standard, and it provides a standard format for flow cytometer output across different machine brands and models. FCS is a binary file with three major segments. A header segment records instrument settings and keywords, a data segment, and an analysis segment. The data segment contains all recorded parameters for each event in flow cytometry.

Figure

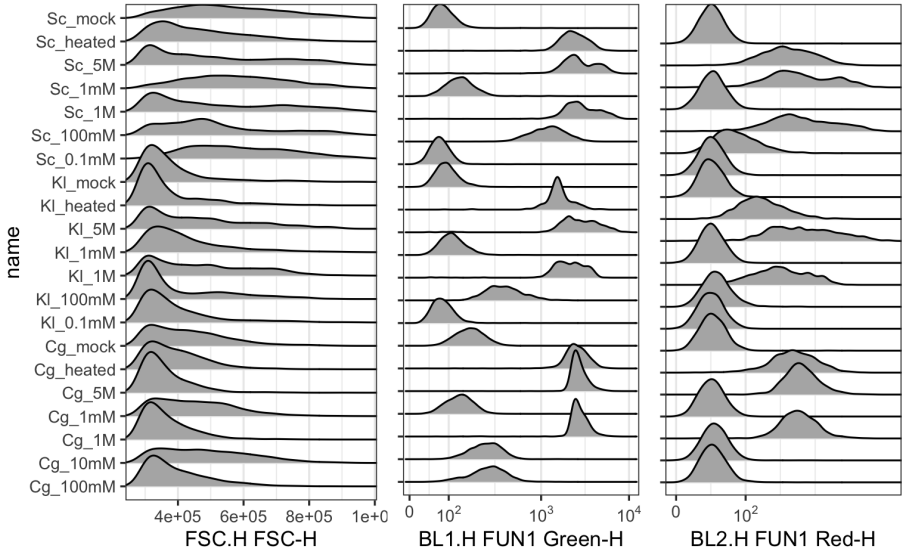


Figure 1. Ridges plot for flow cytometry results in post-oxidative stress yeast cells. Intensity of the FSC, BLH1, and BLH2 channels are recorded. FUN-1 fluorescence level appears to be significantly altered after 5M and 1M H2O2 treatments.

An example of the figure I want to generate is the ridge plot. This figure has species and experimental conditions on the y-axis. The intensity of flow cytometer channels are on the x-axis. The figure shows low FUN-1 fluorescence in mock treated cells, high fluorescence in stock concentration [H2O2] treated cells, and some potential shifts in the intermediate [H2O2] conditions. Using this figure, I hope to compare fluorescence level of FUN-1 stained post-oxidative stress cells, and eventually decide if FUN-1 is useful for reliability accessing viability.

This figure effectively compares the fluorescence intensity of each sample. With the overlayed ridges, the comparison between each sample is easy and even slight shifts in intensity can be spotted. From this plot, it can be concluded that mock treated (thus mostly live) Cg cells display low fluorescence intesnity in both the green and red channels. Heated and 5M H2O2 treated (thus dead) cells have high fluorescent intensity in both channels. While the intermediate conditions' fluorescent intensity seems to differ, no conclusion can be drawn yet. This figure is a representation of the type of figure I hope to produce with my data. I do not intend to reproduce this figure with its oringinal data. I hope to plot my data in this form and thus achieve my goal of comparing fluorescence level of different treatment and also potentially compare utility of different viability stains.

Materials and Methods

Data Data for analysis is obtained through my own experimental work. The data is stored on the lab RDSS drive. I have copied and pasted the data folder through the GUI. The folder is titled with experiment date and each FCS file is labeled with experimental conditions. These labels were generated during the experiment. If you would like to recieve a copy of the data, please contact me at hanxi-tang@uiowa.edu.

Methods *Experimental Techniques* Post-oxidative stress yeast cells were collected and stained with FUN-1 or Fungalight viability dyes (detailed experimental protocols are avaliable upon request). Stained cells were run through Attune flow cytometry. Two filter settings,the seperate red and green setting and the long pass filter setting, were applied in the FUN1 experiment. A single filter setting was applied in the Fungalite experiment. Data was exported in forms of FCS files through Attune's interface and stored on a shared RDSS drive. For Fungalight flow cytometry experiments, an additional set of CFU assays were also conducted and data was appended to the corresponding flow cytometry data.

Data Annotation and Metadata For the FUN1 staining data, FCS files are imported in R. The Attune generated file names have a few common phrases that hinder the interpretation of the graphs. Thus,the longest substring function from the PTXQC package is used to remove the common phrases in the file names, while the unique sample labels are kept intact. The write.table function is used to write data into .tsv files and thus linking it to its metadata. For the Fungalite data, cell populations are gated on the Attune software based on its fluorescence intensity. Cells with low green and low red fluorescence is gated in the "unstained" gate. Cells with high green but low red is gated in the "live" gate. Cells with high green and high red is gated in the "dead" gate. Cell count within each gate is exported from Attune software. Cell counts for each condition is correlated with their CFU count in Excel.

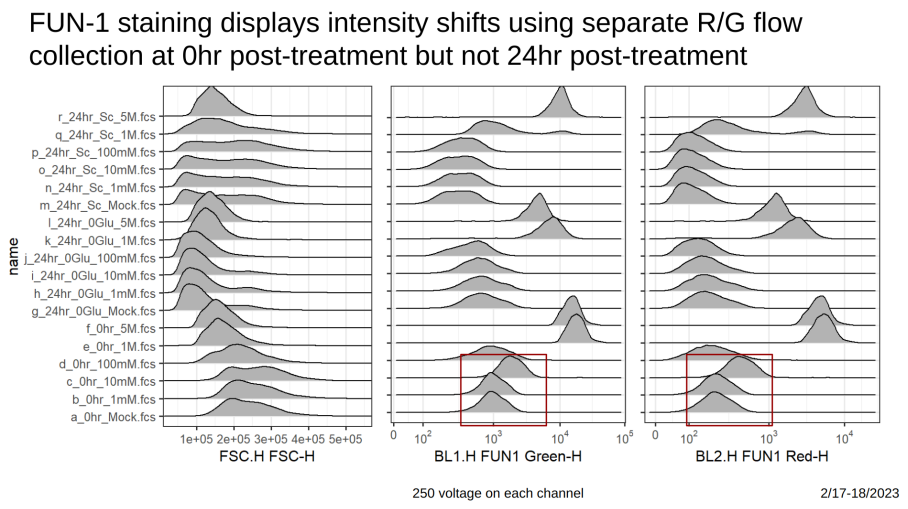
Graphing In order to analyze the FUN1 staining data, the "flow core" package from Bioconductor is installed and used to read the FCS files. All needed

packages need to be loaded before analysis is run. The goal graph is generated using ggplot2, ggcyto, and ggridges. Graph axes and order of experimental conditions are adjusted as needed. The intensity (height) of BLH1 (green), BLH2 (red), and FSC channel are graphed seperately in the FUN1 seperate red and green setting. The intensity (height) of the BLH2 and the FSC channels are graphed seperately in the FUN1 long pass filter setting. When graphing the Fungalite data, a percent variable was generated for each cell population using the equation: percentage = cell count in the population / total cell count. The percent variable is graphed on the x-axis and the CFU survival rate was graphed on the y-axis. A scatterplot was generated. Color of data points indicates different treatment conditions.

Statistical Analysis After previous graphing, Fungalite appeared to have superior distinguishing power in predicting CFU survival results. Therefore, data analysis was focused on Fungalite results. Outliers due to documented technical errors or arbituary settings are filtered out of analysis. The linear regression model function was used to build a model where Y is the CFU survival rate and X is the percent gated for each population. The predcited linear regression line is then added onto the scatter plot. The next step in statistical analysis would be to add multiple variables to the model in order to increase the predicting power.

Result

FUN-1 Graphing



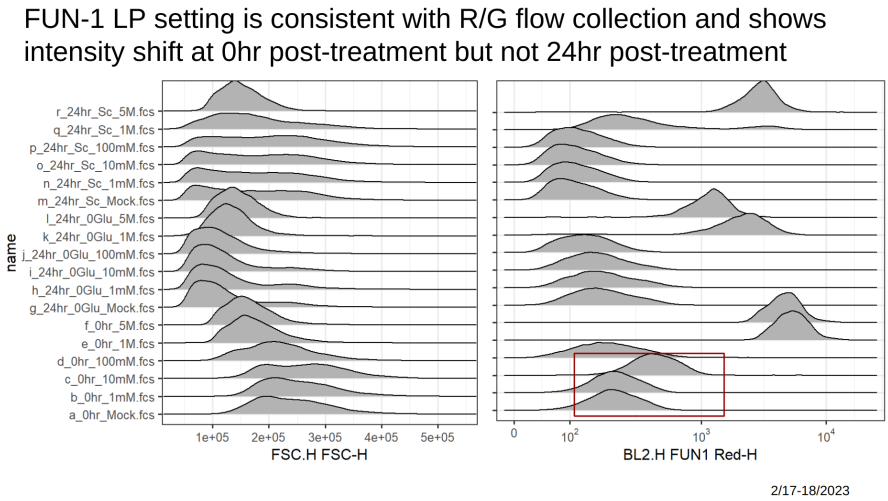


Figure 2. Ridges plots for flow cytometry of FUN1 stained post-oxidative stress C. glabrata cells. A. Intensity of FSC, FUN1 Green and FUN1 Red channels in C. glabrata at 0 hour post treatment and 24 hour post treatment. B. Intensity of the FSC and long pass channel in post-oxidative stress C. glabrata at 0 hour and 24 hour post treatment.

C. glabrata experiencing various oxidative stress levels were plotted on the same scale (Figure 2). Yeast experiencing severe oxidative stress (1M and 5M H2O2) display bright red and green fluoresnce. Yeasts treated with lower oxidative stress (10mM, 1mM H2O2) display low red and green fluorescence, and appears similar to the mock treated cells. The 100mM H2O2 treated cells appear to have a shifted red and green fluorescence, however, the shift is minimal and disappears after 24 hour incubation. The seperate red and green filter setting (Figure 2A) and the long pass filter setting (Figure 2B) display consistent fluorescence patterns.

Fungalite Graphing

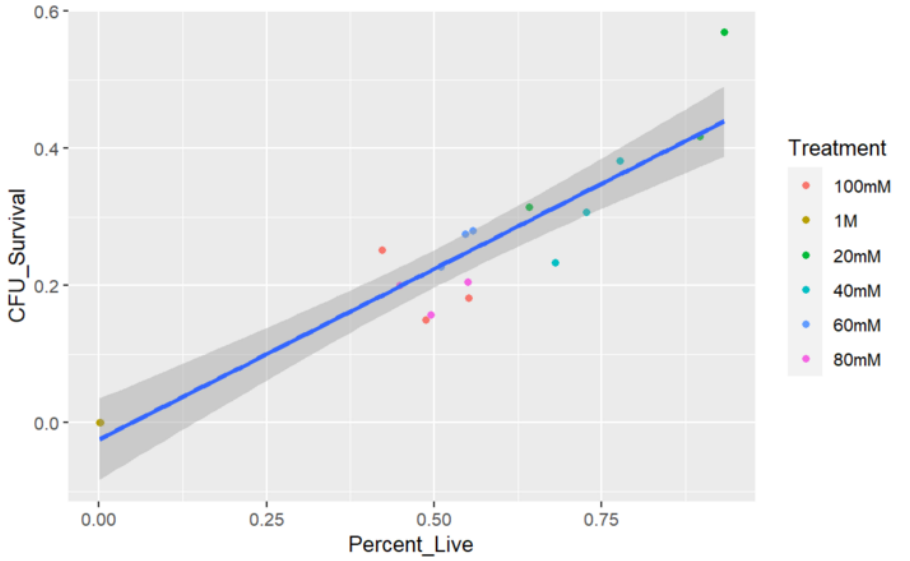


Figure 3. Scatter plot dipicting variation in percent live and CFU survival rate. The line of best fit (blue) from a linear regression model and its 95% confidence intervals (shades) are also graphed.

C. glabrata treated with various oxidative stress levles were stained with Fungalite and ran through flow cytometry. The live cell population (cells with high green and low red fluorescence) is gated and its percentage is plotted against CFU survival rate. Low oxidative stress levels resulted in high live population percentage. High live population percentage is correlated with high CFU survival rate. The plot and the linear regression model shows that the percent live is highly correlated with CFU survival rate. The r-squared value of the regression is 0.86, with a p-value of 1.83e-8.

Discussion

Overall, the findings are consistent with the paper in the reference. Fungalite is a superior viability dye and it can predict CFU survival rate at 0 hour post treatment. However, the correlation r-sqaure value can be further improved. Specifically, the current dataset used for analysis has various technical flaws. If a more carefully collected dataset can be applied, the correlation may be higher. In addition, the unstained population in these samples are worth investigating. A model accounting for the unstained population may better predict CFU survival rate.

Reference

David B. Berry,Qiaoning Guan,James Hose,Suraiya Haroon,Marinella Gebbia,Lawrence E. Heisler,Corey Nislow,Guri Giaever,Audrey P. Gasch. “Mul-

tiple Means to the Same End: The Genetic Basis of Acquired Stress Resistance in Yeast." PLOS Genetics, November 10, 2011 https://doi.org/10.1371/journal.pgen.1002353