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

Introduction

The unicellular fungus *Saccharomyces cerevisiae* and its related yeasts species have been extensively studied as a model organism in genetics and pathogenesis studies. Yeast cells undergo either accidental cell death or regulated cell death pathways in response to environmental stress. Determining the precise point of cell death and assessing the survival state of yeast cells are crucial in stress and pathogenesis studies. The widely accepted hallmark of yeast cell death is the irreversible loss of plasma membrane integrity.

Several techniques and tools have been developed to evaluate the level of yeast cell death, viability, and vitality. For instance, Propidium Iodide, a membrane permeability-based dye, can be employed to assess cell death. Spot dilution and colony forming unit (CFU) assays are used to test cell culture viability. Metabolic activity-based dyes, such as FUN-1 (also know as ViaVac Red/Green), provide information on cell culture vitality.

Assessing yeasts cell death, viability, and vitality is an integral part of understanding stress responses. Yeasts exhibit an acquired stress resistance (ASR) response, where a mild dose of primary stress helps cells to survive a secondary dose of severe stress. The mild primary stress may be the same or different from the secondary severe stress. Previous studies have identified an increased magnitude of ASR response in *Candida glabrata* when exposed to phosphate starvation as a primary stress and hydrogen peroxide treatment as a secondary stress. However, this increased magnitude of ASR is not observed in the closely related *S. cerevisiae*. Subsequent investigations suggest that the ASR phenotype is closely related to the nutrient sensing TOR pathway.

Motivated by these findings, the next natural step is to understand the evolutionary trajectory of ASR. Although *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. Thus, it is necessary to assess and compare the magnitude of ASR in an evolutionarily related outgroup species.

Traditionally, the gold standard colony forming unit (CFU) assay is employed to assess the post-oxidative stress survival rate in ASR. However, this assay is labor-intensive, and the small sample plated may induce variance in the output. Moreover, CFU assay can only gauge cell viability and relies on the cells' ability to proliferate on an agar plate. It cannot reliably access the loss of membrane integrity, which is regarded as the hallmark of yeast cell death. Therefore, a new viability assessment method is needed to properly assess post-oxidative stress cell death, increase throughput, and decrease data variation.

The goal of this project is to develop a flow cytometry-based, high-throughput cell death assay using fungal staining dyes, such as FUN-1 (Thermo Fischer) and FungaLight 1 (Thermo Fischer). FUN-1 and its analogs are transported from the 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 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. FungaLight 1 is a membrane permeability-based dye composed of SYTO9 and Propidium Iodide (PI). The green SYTO9 stain is cell membrane permeable and labels both live and dead cells. PI is a red-fluorescent nucleic acid stain that stains dead cells with compromised cell membranes. When PI diffuses into cells with compromised membranes, its fluorescence level is increased by FRET from interaction with SYTO9. Therefore, live cells are expected to be fluorescently green while dead cells are expected to fluoresce highly in both red and green channels.

If this high throughput flow cytometry based viability assay can be well established 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, insights can be gained regarding the evolution trajectory of the ASR trait. In order to address this biological question, this bioinformatics course project aims to build a pipeline for processing and analyzing flow cytometry based FCS data and use R to construct informative figures that help in evaluating staining 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.

Methods

Experimental Techniques

Cell Culture & Oxidative Stress Treatment

Wild type *C. glabrata* (yH 181), *S. cerevisiae* (yH 154), and *K. lactis* (yH 149) were grown to mid-log phase in rich media. In oxidative stress conditions, *C. glabrata*, *S. cerevisiae*, and *K. lactis* were exposed to 0-1M hydrogen peroxide for 2 hours at a cell density of approximately $1x10^7$ cells per mL. In heat treatment conditions, yeasts were heated for 2min at 75°C.

Staining and Flow Cytometry

Post-treatment yeast cells were collected, treatment media was removed, and yeasts were stained with 6.3uM FUN-1 for 30min at 30°C in the FUN-1 conditions. Alternatively, yeasts were stained with 3.34uM SYTO9 and 20uM PI for 30min at 25°C in the FungaLight conditions. Stained cells were diluted and ran through Attune flow cytometry. FUN-1 stained samples were excited with a 488nm laser. Green fluorescence was collected with a 530±30nm bandpass filter and red fluorescence was collected with a 590±40nm bandpass filter. FungaLight stained samples were excited with a 488nm laser. Green fluorescence was collected with a 530±30nm bandpass filter and red fluorescence was collected with a ≥600nm long pass filter.

Colony Forming Units (c.f.u.) Assay

Post-treatment yeast samples were diluted to $0.25x10^7$ cells/mL with ddH_2O and plated on yeast synthetic complete agar medium. Colony forming units were counted after 48 hours at 30°C incubation.

Data Processing

Obtaining Raw Data

Flow cytometry data files were generated via Attune NxT Flow Cytometer in forms of FCS files. For FungaLight flow cytometry experiments, live population gate were drawn based on the mock treated samples, and applied to all samples in the experiment. Gated event count was exported via Attune software's GUI in the form of a tsv file. Counts from CFU assays were stored in forms of tables and later manually appended to the corresponding flow cytometry data. All relevant raw data are provided in the project's GitHub repository.

Data Annotation and Metadata

For the FUN-1 staining data, FCS files were imported into R using the Flow Core package. The Attune generated file names contain a few common phrases that hinder the interpretation of the graphs. Thus, the longest substring function from the PTXQC package was used to remove the common phrases in the file names, while the unique sample labels were kept intact. The FCS files were then separated into species. The write.table function was used to write wrangled data into .tsv files.

For the FungaLight data, event count within the live population gate, total events, and CFU counts were written into a table using the Cbind function. Variables of interest such as "percent live" were added into the table. Specifically, percent live variable was calculated as (events in live population gate) / (total events). CFU Survival was calculated as (colonies in treatment condition) / (colonies in mock treated).

Data Analysis

Data Graphing

Data was graphed using R packages ggplot2, ggcyto, and ggridges. Graph size, axes, and order of experimental conditions were adjusted as needed. The intensity (height) of BL1 (green), BL2 (red), and FSC channel are graphed separately in the FUN-1 flow cytometry panels (Fig. 1A, Fig. 1B). The mean intensity of BL1 and BL2 were calculated and graphed as independent variables. The CFU survival was graphed as the dependent variable (Fig. 1C, Fig. 1D). A two-way scatter plot was generated.

Fore FungaLight data, percent live was graphed as the independent variable and the CFU survival rate was graphed as a dependent variable. A two-way scatterplot was generated. Colors of data points indicate different H2O2 treatment conditions.

Statistical Analysis

Data analysis in Fig. 1C and Fig. 1D was done using a linear regression model and the LM function in base R. The adjusted R-squared value was calculated for the corresponding variables and reported in the graph.

In Fig. 1E, outliers due to documented technical errors or arbitrary 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 predicted linear regression line is then added onto the scatter plot. The adjusted R-squared values and p-value

was also reported in the graph. (Detailed scripts for data processing and data analysis are available in the project's GitHub repository under the "Analysis&Script" sub-directory.)

Results and Discussion

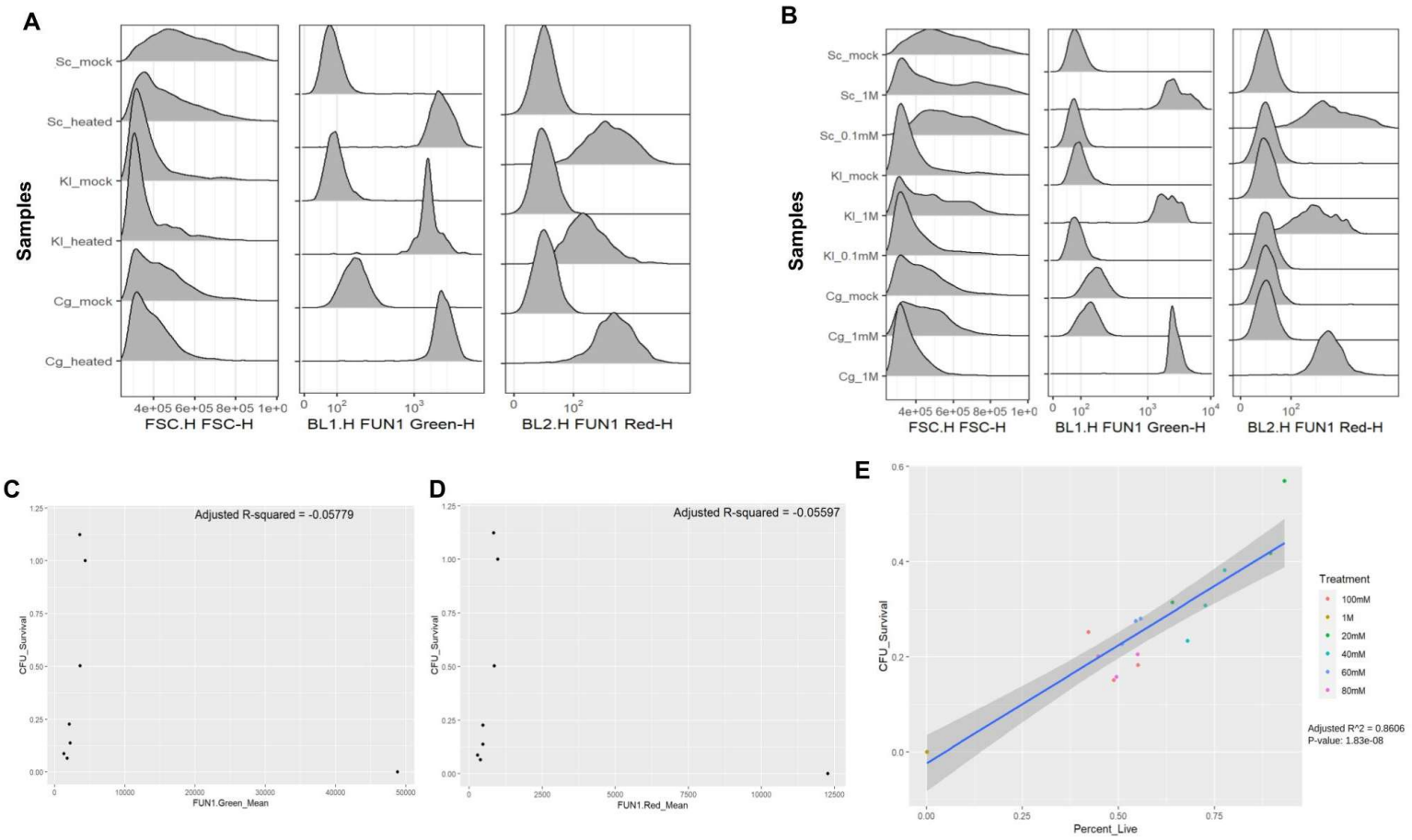

Figure 1. Analysis of flow cytometry results from yeast samples stained with viability dyes. **(A)** Ridges plots for flow cytometry of FUN-1 stained post heat stress yeasts. Sample names indicating yeast species and treatment conditions are shown on the vertical axis. Intensity of FSC, FUN-1 Green and FUN-1 Red channels are plotted on the horizontal axis. **(B)** Ridges plots for flow cytometry of FUN-1 stained post-oxidative stress yeasts. Sample names indicating yeast species and treatment conditions are shown on the vertical axis. Intensity of FSC, FUN-1 Green and FUN-1 Red channels are plotted on the horizontal axis. **(C)** Scatter plot of FUN-1 stained post-oxidative stress *C. glabrata* cells' mean green fluorescence and CFU survival rate. Adjusted R-squared value is shown. **(D)** Scatter plot of FUN-1 stained post-oxidative stress *C. glabrata* cells' mean red fluorescence and CFU survival rate. Adjusted R-squared value is shown. **(E)** Scatter plot of FungaLight stained post-oxidative stress *C. glabrata*. The flow cytometry result was gated by population. Percentage of live population is plotted against CFU survival rate. The line of best fit (blue) from a linear regression model and its 95% confidence intervals (shades) are also graphed. Adjusted R^2 and p-value are shown. Colors depict various treatment conditions.

This project aims to develop a flow cytometry based high throughput yeast viability assay and verify its accuracy against the gold standard CFU assay, and thereby provide a method to potentially quantify the magnitude of ASR trait in various species.

The initial approach to this method development utilized the FUN-1 vitality dye. FUN-1 distinguishes live and severe heat killed yeast cells in *C. glabrata*, *S. cerevisiae*, and *K. lactis* (Fig. 1A). Mock treated cells show a spectrum of low fluorescence signals in both the FUN-1 green ($0\sim10^3$) and the FUN-1 red channels ($0\sim10^2$). Heat killed cells show high intensity fluorescence in the green (above 10^3) and red channels (above 10^2). The live and dead cell fluorescence patterns are distinct from each other. In this staining process, live cells were stained with the dye and actively transported the dye to form CVIS structure in its vacuole. Heat killed cells are unable to actively transport and metabolize FUN-1. Therefore, FUN-1 diffused into heat killed cells are accumulating and emits bright green and red fluorescence. It is clear that FUN-1 is capable of distinguishing live and heat killed yeasts in flow cytometry.

FUN-1 is also capable of distinguishing live and severe hydrogen peroxide treated cells in *C. glabrata*, *S. cerevisiae*, and *K. lactis* (Fig. 1B). Mock treated cells gave low signals in both the red and green channels. Yeasts treated with severe oxidative stress (1M H_2O_2) show high intensity fluorescence in the green (above 10^3) and red channels (above 10^2). However, in yeasts treated with mild oxidative stress (0.1mM-1mM H_2O_2), the staining pattern is similar to mock treated cells. Severe oxidative stress is known to immediately kill yeasts. Yeasts treated with a high concentration of H2O2 will become immediately metabolically inactive, and passively accumulate FUN-1 dye. In mild oxidative stress treated conditions, the reactive oxygen species level remain low and do not cause threat to survival. The yeasts remain metabolically active in these mild treatment conditions, and actively metabolize FUN-1 to give low florescence in both channels. These data indicates that FUN-1 is capable of distinguishing live and severe oxidative stress treated yeasts.

FUN-1's post-oxidative stress survival prediction power was compared to the traditional CFU assays. *C. glabrata* was exposed to a range of various oxidative stress (0-1M H_2O_2), then stained with FUN-1 and also plated for CFU. The mean fluorescence signals is then plotted against CFU survival rate (Fig. 1C, Fig.1D). The mean green fluorescence level is clustered around 0-5000 for a varied range (0%-100%) of CFU survival rates. The mean red fluorescence level is clustered around 0-1000 for a varied range (0%-100%) of CFU survival rates. The adjusted R^2 values are all around 5% for both correlations. These results suggest that FUN-1 staining is not as sensitive as CFU in assessing post-oxidative stress survival. FUN-1 cannot distinguish intermediate levels of post-oxidative stress survival.

A yeasts cell death assessment dye, FungaLight, was then used to develop the flow cytometry assay (Fig. 1E). In this approach, *C. glabrata* was exposed to a range of various oxidative stress (0-1M H_2O_2), then stained with FungaLight and also plated for CFU. In intermediate H2O2 treated conditions, FungaLight stained cells separate into clear live and dead populations with distinct staining patterns (graph not shown here, FCS files available in repository). The live cell population was gated based on staining patterns of mock treated cells. The percentages of live cells correlate strongly with CFU survival rates in various post-oxidative stress conditions. With an adjusted R^2 value of 86%, FungaLight flow cytometry data can be used as a good predictor of CFU survival rate.

Comparing the two dyes, FungaLight is a superior option for developing this flow cytometry based viability assay. Even though FungaLight as been used as a method of verification in previous papers, a pure flow cytometry based viability have not been established. This project aims at developing such assay and verifying its prediction power against the CFU assays.

Even though the current data showed FungaLight's prediction power in post-oxidative stress *C. glabrata*, FungaLight's usage in other yeasts species have not yet been explored. A plausible next step is to establish FungaLight's usage in other yeasts, and verify its prediction power against CFU survival results. In addition, FungaLight's prediction power may be further improved by using an alternative survival assessment timepoint. Yeasts membrane integrity is further compromised at 24 hour post oxidative stress treatment. Therefore, an incubation may help in improving FungaLight's accuracy and sensitivity. However, the incubation timepoints and incubation media need to be carefully chosen.

Conclusion

The main insight from the data presented can be concluded as the percentage of live population in FungaLight stained *C. glabrata* is a useful predictor of CFU survival rate.

The robustness and reproducibility issues of this project partially lies within establishing good experimental techniques. FungaLight staining accuracy is highly dependent upon appropriate usage. Each usage parameter may change across different yeast species, growth media, and treatment conditions. In particular, a parameter that may present an obstacle to reproducing this project is the percentage of unstained cells in the population. When used with inappropriate staining cell density, FungaLight may show a population of unstained cells. A potential remedy to this would be to optimize staining cell density using microscopy and flow cytometry.

In data analysis, one potential barrier to reproducing the figures is graphing the ridges plot. Even though the ridges format is extremely useful for visualizing these flow data and comparing across conditions, producing the ridges plot requires additional codes and ggridges graphing package. The detailed codes in the repository may help in producing these graphs. In the case that the exact ridges plots cannot be reproduced, a remedy may be to use the autograph function of the flow core package and produce traditional density plots of each condition. This graphing method will lead to the same conclusion regarding this flow cytometry method.

Reflection

Throughout this project, I learned that reproducibility goes beyond copying and pasting existing codes. Getting codes to work properly requires a foundational understanding of the analysis software, understanding of data structure, and understanding of the analysis logic. Simply copying and pasting codes do not lead to reproducible figures.

This course taught me various strategies that I have incorporated into data analysis and research. First, I learned to organize data folders and electronic lab notebook efficiently. I learned to also use annotated methods to integrate code chunks, graphs, and comments in the process of figure production. Version control and building a repository via GitHub is also a useful practice that I hope to continue in the future.

After my first year of experience in graduate research, I feel that research in the field of biology and basic science is already pretty hard to reproduce even without a bioinformatics component. When this data analysis and bioinformatics component is thrown into the mix, it became an upgraded challenge. It is the researcher's responsibility to find a way to reduce the complication of the bioinformatics component, and improve the reproducibility of their research.

References

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

Rego, A., Ribeiro, A., Côrte-Real, M. et al. "Monitoring yeast regulated cell death: trespassing the point of no return to loss of plasma membrane integrity." *Apoptosis*, July 07, 2022. https://doi.org/10.1007/s10495-022-01748-7