UNIVERSITY OF CALIFORNIA SAN DIEGO

Investigation of Heterogeneity in Yeast Cells Under Oxidative Stress H_2O_2 and Diamide

A Thesis submitted in partial satisfaction of the
requirements for the degree Master of Science

in

Biology

by

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The Thesis of Yu-Chieh Lee is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

Abbreviations	Definition
ROS	Reactive Oxygen Species
H_2O_2	Hydrogen peroxide
glyceraldehyde-3-phosphate dehydrogenase gene promoter	pGPD
green fluorescence protein	GFP
mRNA-decapping enzyme 2	DCP2
peroxiredoxins	Prx
plasma membrane ATPase 1	Pma1

LIST OF FIGURES

LIST OF TABLES

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ABSTRACT OF THE THESIS

Investigation of Heterogeneity in Yeast Cells Under Oxidative Stress H_2O_2 and Diamide

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Master of Science in Biology

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Professor Nan Hao, Chair
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Oxidative stress has been a widely studied topic owing to its hazardous characteristic to biological functioning and involvement in the pathogenesis of multiple diseases. It is crucial to understand the regulation of oxidative stress as well as how homeostasis is maintained. To further realize cell responses to oxidative stress, we used microfluidic technology to investigate heterogeneity in yeast cells when affected by oxidative stressors hydrogen peroxide and diamide. We have identified that under both H_2O_2 and diamide stress, a constitutive GFP in yeast cells will respond with three types of intensity fluctuations: one with continuous maintenance of the GFP intensity, another with a drastic drop in intensity

when the stress is added but followed with intensity recovery after removal of the stress, and lastly the type that also has a drastic drop in intensity but no recovery is seen even after the stress is removed. This heterogeneity in the yeast cells can be seen while under 0.5mM to 1mM H_2O_2 stress and 4mM to 7mM diamide stress; however, in comparison to diamide, the cells under H_2O_2 stress respond with a more defined distinction among the three cell types that better corresponds with the stress concentration. Building off these results, we integrated a superfolder pHluorin gene into the yeast genome and were able to conclude that the GFP intensity fluctuations are indeed correlated to intracellular pH alterations upon H_2O_2 addition but not diamide addition. Alternative possibilities of the heterogeneity under H2O2 and diamide stress are discussed in this paper.

CHAPTER 1 INTRODUCTION

1.1 The Role of *Saccharomyces cerevisiae* in Research

Saccharomyces cerevisiae (*S. cerevisiae*), also known as the budding yeast, is an unicellular eukaryotic organism used commonly in biology research due to its ability to serve as a eukaryotic model system (Mell & Burgess, 2003). This unique characteristic of *S. cerevisiae* allows studies to be done in much more simplicity and significantly lower cost, yet still provide invaluable understandings into basic cell biology such as "aging, regulation of gene expression, signal transduction, cell cycle, metabolism, apoptosis, neurodegenerative disorders" (Karathia et al., 2011), these insights can also be extended to treatment of human diseases including cancer (Hanson, 2018).

Several factors contribute to *S. cerevisiae* being a commonplace powerful genetic model system. First of all, *S. cerevisiae* was the first eukaryote to be fully sequenced. This was completed in 1996 with the combined effort of scientists from numerous countries, and this genome sequencing has since then provided remarkable information for the entire biological community (Hanson, 2018). It is now known that the budding yeast possesses roughly around 6000 genes, with about 5570 speculated to be protein-coding genes. In result, "nearly all biological functions found in eukaryotes are also present and well conserved in *S. cerevisiae*" (Parapouli et al., 2020); moreover, "up to 30% of genes implicated in human disease may have orthologs in the yeast proteome" (Karathia et al., 2011).

Secondly, *S. cerevisiae* possesses an important trait which grants us more convenient genetic manipulation when compared to other possible model organisms: yeast meiosis. The fact that yeast is capable of reproducing through meiosis and since "all four products of meiosis can be isolated and propagated as haploid organisms", this has enabled researchers to efficiently edit the yeast genome and further on explore the relationships between genotype and phenotype (Burgess et al., 2017).

Last but not the least, modification of the yeast genome is relatively effortless and straightforward since DNA integration at intended sites is exceptionally effective through homologous recombination (Hanson, 2018). Homologous recombination is when genetic material is exchanged through single or double-stranded nucleic acids, such as the crossovers during meiosis. Likewise, it is also a crucial mechanism used for DNA repair, DNA replication-fork rescue, chromosome maintenance, etc. (Sung & Klein, 2006). The nature of how homologous recombination happens allows it to be an efficient method for the integration of DNA at targeted sites of the genome as long as the two ends of the DNA strand are homologous to the destined chromosomal sequences. More astonishingly, efficient integration of DNA can be done with only 40bp of homology flanked at both sides of the inserted DNA strand. Homologous recombination can be utilized in various cell types but is especially effective in yeast; therefore, permitting *S. cerevisiae* as a powerful model organism (Hanson, 2018).

Other than the hallmarks mentioned above, *S. cerevisiae* holds other features that makes it a great model system. For example, the rapid proliferation nature of yeast permits researchers to obtain large populations of cells in a matter of short time, or that the low pH and high sugar concentration in yeast media is selective for yeast which makes avoiding contamination notably easier than tissue culture (Hanson, 2018). In short, even though *S. cerevisiae* is an unicellular organism it is still an extraordinary eukaryotic model system containing extreme convenience in genetic manipulation and analysis, with an essential role in the research field.

1.2 Significance of Reactive Oxygen Species and Oxidative Stress Regulation

Reactive oxygen species (ROS) are byproducts of normal cell activity and it is a term for oxygen radicals or some non-radical derivatives of oxygen (Costantini, 2019). Some of the most frequently mentioned and important ROS in biological functioning are O_2^-

(superoxide anions), H_2O_2 (hydrogen peroxide) and OH• (hydroxyl radicals) (Ye et al., 2015).

ROS can act as either beneficial or costly to cellular processes. At low or moderate concentrations, ROS often serves as secondary messengers and takes an important part in "signaling pathways, gene expression, regulation of immune responses, and the maintaining of cellular homeostasis" (Kruk et al., 2019). In reverse, ROS at high concentrations may cause damage to cell components such as DNA, RNA, proteins, lipids, and carbohydrates. (Kruk et al., 2019). An example of this dual characteristic can be found in cancer cells; while playing the role of secondary messengers in signaling cascades, ROS can "induce and maintain the oncogenic phenotype of cancer cells", nevertheless, it also holds the ability to "induce cellular senescence and apoptosis and can therefore function as anti-tumorigenic species" (Matés et al., 2008). Therefore, it is not difficult to see the significance of ROS in biological systems.

As said in Manda et al., "ROS are able to regulate life and death"(Manda et al., 2009), hence unsurprisingly, aerobic organisms have developed multiple mechanisms for maintaining cellular redox homeostasis that regulates ROS. The regulation on ROS accumulation is controlled by two defense systems: enzymatic and non-enzymatic. Non-enzymatic antioxidants include glutathione, vitamin C, vitamin E, coenzyme Q10, flavonoids, phenolic acids, etc.; this system protects cells from excessive ROS by interrupting the free radical chain reaction (Nimse & Pal, 2015). On the other hand, the main enzymatic antioxidants are the superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), which either break down or remove ROS (Kruk et al., 2019).

When these oxidant defense systems fail, it results in an imbalance in intracellular redox; this is known as oxidative stress. The most common definition for oxidative stress is "a state where oxidative forces exceed the antioxidant systems due to loss of the balance

between them" (Yoshikawa & Naito, 2002). Oxidative stress was brought up in 1985 in the book 'Oxidative Stress', while at the same time the review 'Biochemistry of Oxidative Stress' introduced information on pro-oxidants and antioxidants. From then on, redox biology has been a widely focused topic for the past 30 years among extensive varieties of areas, including chemistry, biochemistry, cell physiology, general biology, medicine, etc. (Sies, 2015)

When oxidative stress is induced, the excessive ROS produced causes great damage to the cells. Oxidative stress is known to be hazardous to biological functioning and could result in DNA/RNA damage, protein damage, lipid peroxidation, and carbohydrate degradation. Moreover, oxidative stress is not only involved in the pathogenesis of several diseases including cancer, neurodegenerative diseases hypertension, diabetes mellitus, etc. but also the aging process (Kruk et al., 2019).

To sum up, ROS plays a crucial role in cellular signaling and functioning; it can either be favorable or deleterious depending on the concentration of ROS present. Hence the proper regulating mechanisms on ROS is the exceptional key in maintaining cellular redox homeostasis. Without appropriate balance leads to oxidative stress, which is destructive to biological molecules and is associated with various diseases. Therefore, research on ROS and oxidative stress is unquestionably necessary as it provides us useful insight on understandings of intracellular redox stability and further on, even possibilities of recognizing and treating diseases. (Yoshikawa & Naito, 2002)

1.3 Project Background: Variation of Yeast Cell Types in Response to H_2O_2

This thesis project is based on results from previous microfluidics experiments that were run by other lab members. Initially, the lab was intending to get a glimpse of whether there is a correlation between Tau protein and mRNA-decapping enzyme 2 (DCP2) proteins during oxidative stress conditions. Therefore, a microfluidics experiment with H_2O_2 as the

oxidative stress were run with a BY4741 yeast strain (Brachmann et al., 1998) with the genomic alterations of glyceraldehyde-3-phosphate dehydrogenase gene promoter (pGPD) tagged with a green fluorescence protein (GFP) and the Tau protein, while the DCP2 protein was tagged with a mCherry fluorescent protein. In this experiment, two interesting observations were made.

The first observation was that after H_2O_2 stress (0.5mM /1mM /1.5mm /2mM) was added to the cells, the yeast cells responded with three different cell types. The three different cell types include continuous maintenance in the GFP intensity, a drastic drop in GFP intensity but followed with recovery after the H_2O_2 stress was removed, and cells that did not recover from the stress even after H_2O_2 removal. The second observation was a correlation between DCP2 protein aggregation and stress response in the yeast cells. As mentioned above, three cell types were seen when H_2O_2 stress was added. From the microfluidic experiments performed, we noticed that most of the aggregation of DCP2 proteins were seen among the cells that either maintained their GFP intensity even after the stress was added, or in those cells that showed GFP intensity recovery.

In order to further confirm that the three cell types seen are not due to artificial errors or the Tau protein itself, another microfluidics experiment was then conducted. This follow up microfluidic experiment was performed with a yeast strain made by other Hao lab members in the past and was already available; it is a BY4741 yeast strain with the genomic alterations of having pGPD tagged with the GFP. In other words, this yeast strain contains the same genome as the strain previously used, but without the Tau protein or the mCherry tagged onto DCP2 protein, therefore allowing us to ensure whether the three cell types observed from the previous experiment was not due to other factors but simply stress response of the cells to H_2O_2. From this experiment, the same three cell types still existed when H_2O_2 (0.5mM /1mM /1.5mm /2mM) was added as the oxidative stress. (Figure 1.1)

This result excluded the possibility that the Tau proteins played a factor, moreover, also validated that the distinct differentiation of GFP intensity in the cells was not an error.

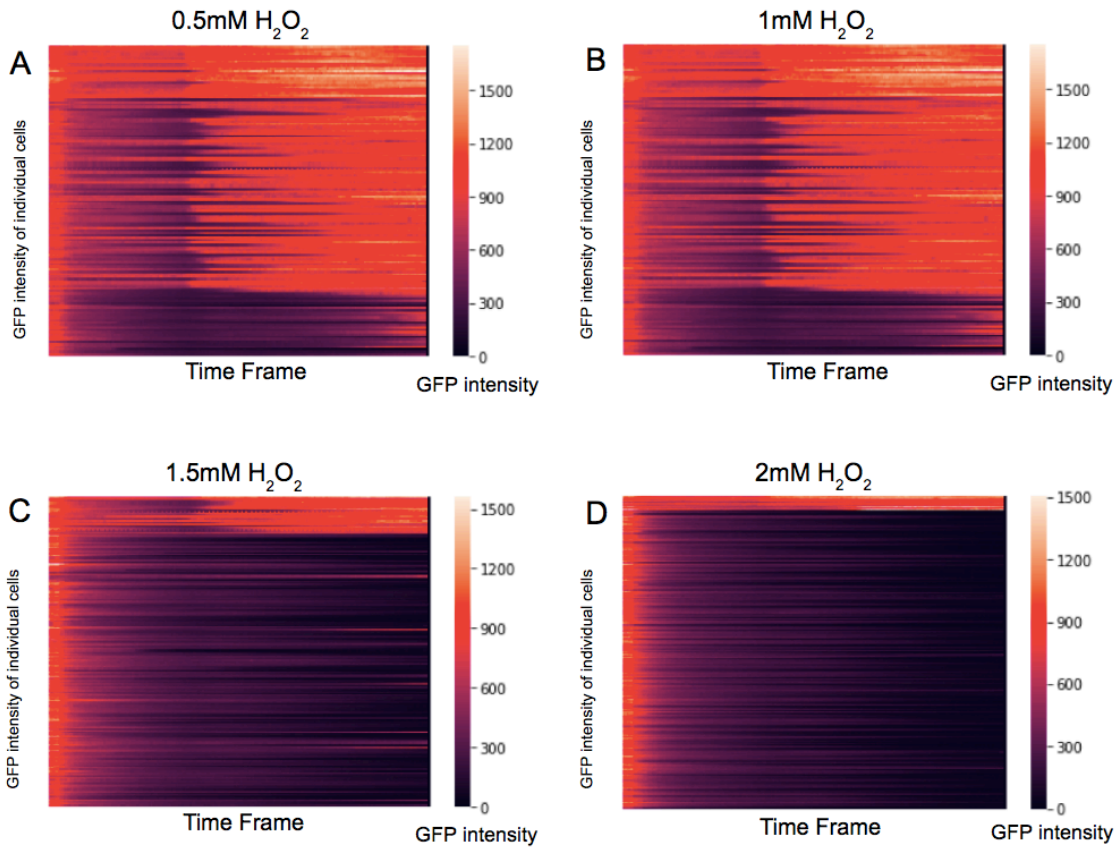

Figure 1.1 Heat map displaying GFP intensity over time in NH1189 under H_2O_2. Microfluidics experiment setup: 10 minutes baseline with SD media → 2 hours hydrogen peroxide stress (A) 0.5mM H_2O_2 (B) 1mM H_2O_2 (C) 1.5mM H_2O_2 (D) 2mM H_2O_2 → 4 hours recovery with SD media. Snapshots of cells were taken every 2 minutes over the 6 hours and 10 minutes experiment period.

1.4 Hypothesis and Experimental Approach

H_2O_2 as a ROS creates fluctuation in the cellular redox status, and these differences in the redox status could lead to apparent subpopulations among even isogenic cells (Radzinski et al., 2018). Therefore, the emergence of the three cell types under H_2O_2 stress from previous microfluidics experiments is considered as one of the strategies for cells to cope with environmental stress. The heterogeneity observed under H_2O_2 stress in the previous experiments led us to wonder whether yeast cells under other oxidative stress will show the same response; therefore, we decided to also take a deeper look into heterogeneity in cells under diamide, which is another commonplace reagent in oxidative stress research. In addition to comparing the cell responses when under varied oxidative stress, we will be discussing a possible reason leading to the phenotypic heterogeneity among the genetically homogenous yeast cell population as well. If the mechanism behind this phenomenon is found, it can give us more insight on what differentiates between the cells capability to deal with oxidative stress.

Based on the earlier experiment results, we hypothesized that the disparate fluctuations seen in the GFP intensity among the isogenic cells is due to pH change in the yeast cytosol after addition of H_2O_2 stress since it is already known that GFP is sensitive to the environmental pH (Remington, 2011). If this assumption is correct, then pH regulation in yeast cells determines the three variations of H_2O_2 stress responses. In other words, if the pH is maintained, then the GFP intensity remains the same; if the pH fluctuates due to addition of H_2O_2 but then reaches pH homeostasis after regulation, the GFP intensity will drastically drop at first but then recover after the H_2O_2 is removed. However, if regulation of pH in the yeast cells failed, then the GFP intensity would not be able to recover even after the removal of H_2O_2 stress.

In order to verify whether pH fluctuation in yeast cytosol is the underlying reason of the three distinct cell types, a pHluorin is integrated into the yeast genome. pHluorin is a pH sensitive GFP variant, therefore allowing us to detect the pH changes in the yeast cytosol during the microfluidics experiments. Microfluidic experiments are performed with the pHluorin integrated yeast strain; the first 10 minutes is conducted with SD media serving as the baseline, followed with 2 hours of H_2O_2 stress, and then lastly 4 hours of recovery period with SD media after the removal of H_2O_2.

CHAPTER 2 MATERIALS AND METHODS

2.1 Genomic DNA Extraction

Scrape off some cell colonies to 100uL of Lysis buffer (200mM LiAC, 1%SDS) and ressuspend by pipetting. Place the mixture at a 70°C heat block for 15 minutes, and add 300uL of ethanol; the solution is mixed well through vortexing. After centrifuging at 14,000rpm for 3 minutes, the supernatant is vacuumed out and the tube is left to air dry at room temperature. The extent of dryness is visually inspected. 100uL of TE buffer (10mM Tris 8.0, 1mM EDTA) is used for resuspending, the solution is then centrifuged at 14,000 rpm for 30 seconds. The DNA will be in the supernatant ready for use.

2.2 pHluorin Plasmid Reconstruction

To construct the new plasmid pYTK096_pTDH3_MRV55, PCR fragments were combined through Gibson Assembly (New England BioLabs). The PCR fragments included vector backbone pYTK096, the TDH3 promoter fragment from NH268 yeast genomic DNA extraction, and the superfolder pHluorin gene which is amplified from the vector (MRV55) p426MET25_sfpHluorin (a gift from Eckhard Boles; Addgene plasmid # 115697; http://n2t.net/addgene:115697; RRID: Addgene_115697). PCRs were performed with Phusion polymerase and cleaned up with the PCR purification kit (New England BioLabs) according to the manufacturer's instructions. All the primers used are listed below.

Prior to the Gibson Assembly, the vector backbone pYTK096 was digested with restriction enzymes HF-EcoR1 and HF-Pst1 (New England BioLabs). The digestion reaction consisted of 5uL of 10x Cutsmart buffer, 1ug of plasmid DNA, 1uL of HF-EcoR1, 1uL of HF-Pst1, and nuclease-free water to a total volume of 50uL. The reaction mixture was incubated at 37℃ for an hour and the digestion reaction products were directly loaded and run on a 1% agarose gel at 120V for 25 minutes. The band at 3900bp is carefully cut down

and the DNA is extracted with a gel extraction kit (Qiagen) according to the manufacturer's instructions.

Gibson assembly was performed with the protocol and buffer provided by New England BioLabs. The gibson assembly reaction mixture consists of 0.1 pmol of digested plasmid pYTK096, 0.2 pmol of pTDH3 PCR fragment, 0.2 pmol of MRV55 pHluorin PCR fragment, 10uL of 2x Gibson Assembly Mastermix, and deionized water to a total volume up to 20uL. The reaction mixture is incubated in a thermocycler at 50°C for 15 minutes, and placed on ice while waiting to perform bacteria transformation.

2uL of plasmid from Gibson Assembly was added to 50uL of NEB 5-alpha competent E. coli cells, and mixed gently by tapping the eppendorf tube. The mixture is then first placed on ice for 30 minutes, followed by one minute of heat shock in a 42℃ water bath, then placed on ice again for 2 minutes. 1mL of LB media was added to the tube and shook at 37℃ for an hour. Cells were centrifuged at 4000rpm for 5 minutes, the supernatant was vacuumed out until only 100uL left in the tube. The remaining supernatant was vortexed well with the cells and plated on a LB+Kanamycin plate, then incubated at 37℃ overnight. Eight single cell colonies were picked from the plate the following day and inoculated in 3mL of LB+Kanamycin (1:1000) media in a 15mL round tube, the round tubes are placed in a 37℃ shaker and shook for 15-16 hours. Plasmids were then extracted with a miniprep kit (Qiagen), and the correct sequences were confirmed through Sanger sequencing.

2.3 pHluorin Yeast Strain Construction

The pHluorin yeast strain NH1387 is constructed with the LiAC transformation method. Prior to the day of transformation, NH268 was inoculated in 2mL of YPD medium and shook at 30℃ overnight for approximately 16 hours. After 16 hours of cell growth,

150uL of the cell culture was diluted with 15mL of YPD medium and again shook at 30℃ for approximately 4 hours.

Plasmid pYTK096_pTDH3_MRV55 was digested with the restriction enzyme HF-Not1 (New England BioLabs). The digestion reaction consisted of 5uL of 10x Cutsmart buffer, 1ug of plasmid DNA, 1uL of HF-Not1, and nuclease-free water to a total volume of 50uL. The reaction mixture was incubated at 37℃ for an hour; 30uL of deionized water was added to the digestion reaction products and 5uL were loaded and run on a 1% agarose gel at 120V for 25 minutes to confirm success of digestion. The other 75uL of reaction mixture is directly used for transformation.

10uL of ssDNA was heated up at a 100℃ heat block for 5 minutes and then stored on ice. After 4 hours of cell growth, the cells were centrifuged at 3000rpm, 25℃ for 2 minutes and the supernatant was poured out. 1mL of TE/LiAC (10mM Tris 8.0, 1mM EDTA, 100mM LiAC) was used to resuspend the cells and moved to an eppendorf tube. The cells were then centrifuged at 3000rpm for 30 seconds and the supernatant was vacuumed out. 100uL of TE/LiAC, 10uL of ssDNA, and the 75uL reaction mixture after digestion was added and vortexed. 600uL of PEG-TE/LiAC (50% PEG, 10mM Tris 8.0, 1mM EDTA, 100mM LiAC) was added to the tube and mixed by pipetting and gentle vortex. The mixture was rotated in a 30℃ incubator for 30 minutes, followed by 15 minutes in a 42℃ water bath, and then placed on ice for 5 minutes. The mixture was centrifuged at 5000rpm for 5 minutes; the solution mixture is carefully vacuumed out without disturbing the cells on the side of the tube. 100uL of TE buffer (10mM Tris 8.0, 1mM EDTA) was used to resuspend the cells; the cells are centrifuged at 5000rpm for 2 minutes, and the solution is again carefully vacuumed out. 70uL of TE buffer is used to resuspend the cells, which were then plated onto a SD -URA plated and placed in a 30℃ incubator for at least two days.

Eight single colonies were picked from the SD -URA plate and replated again on a new SD -URA plate and a YPD plate. The plates were placed in a 30℃ incubator overnight. Colonies from the SD -URA plate were used for genomic DNA extraction, and the DNA extracted are used in the PCR reaction for confirmation of correct genomic insertion. PCRs were performed with Phusion polymerase; the primers used for confirmation are listed below. Colonies that were confirmed positive through PCR were picked from the YPD plate and replated to a new YPD plate, the new YPD plate is incubated at 30℃ overnight. Colonies from the new YPD plate are used for double confirmation of correct genomic insertion through PCR, using the same primers and procedure as previously described.

From the colonies that are positive, one of them was randomly picked from the newest YPD plate, inoculated in 2mL of YPD medium, and shook at 30℃ for approximately 16 hours. 750uL of cell culture is mixed with 750uL of 50% glycerol and stored at -80℃ as stock.

2.4 Tagging DCP2 with mCherry

DCP2 was tagged with mCherry in both yeast strains NH1189 and NH1387, producing strains NH1284 and NH1435 correspondingly. The DNA fragment containing DCP2-mCherry-Leu was used for transformation and was amplified through PCR from another strain NH539 made by other lab members in the past. PCRs were performed with Phusion polymerase; the primers used are listed below.

The PCR product is cleaned up with the PCR purification kit (Qiagen) according to the manufacturer's instructions. Approximately 800ng of PCR product was used for the LiAC transformation method as mentioned in the above section and plated on a SD -Leu plate and replated on a new SD -Leu plate and YPD plate. Genomic DNA extraction and colony PCRs

were performed to confirm whether the DNA fragment is correctly inserted into the genome or not. The primers used to confirm are listed below.

2.5 Microfluidics

Microfluidics setup is conducted as described previously (Hao & O'Shea, 2011). "The microfluidics device was constructed with polydimethylsiloxane using standard techniques of soft lithography and replica molding. Yeast cells were grown at 30℃ to A_{600nm} of 0.4. The cells were quickly concentrated, loaded into the microfluidic device and immobilized by incubation for 5 min in the device, which was pretreated with 2 mg ml^{-1} concanavalin A solution. Two Falcon test tubes filled with 40 ml of medium were connected to two inlets using soft polyethylene tubing (Intramedic, inner diameter, 0.86 mm; outer diameter, 1.27 mm). One Falcon test tube with medium was connected to the outlet. The flow of medium in the device was maintained by gravity, generated by a 15-cm height difference between the Falcon test tubes connected to the inlets and outlet. Exchange of media in the device could be triggered in seconds by manually changing the connectivity of the two inlets." (Hao & O'Shea, 2011)

2.6 Time-lapse microscopy

Time-lapse microscopy experiments were performed as described previously (Jiang et al., 2020). A Nikon Ti-E inverted fluorescence microscope with an Andor iXon X3 DU897 electron multiplying charge-coupled device camera and a Spectra X light-emitting diode light source. A CFI Plan Apochromat Lambda DM 60x oil immersion objective [numerical aperture, 1.40; working distance (WD), 0.13mm] was used for all experiments. Three positions were chosen for each microfluidics channel. For each position, phase contrast, YFP, mCherry, and infrared red fluorescent protein (iRFP) images were taken sequentially every 2

minutes. When the acquisition of the image series started, cells loaded in the microfluidic device were maintained in synthetic defined (SD) medium (2% dextrose) for the first 10 minutes before stress was introduced. After 2 hours of stress, media input was switched manually back to SD medium for another 4 hours before ending the experiments (Jiang et al., 2020).

2.7 Image Analysis

The images were processed using custom MATLAB code for single-cell tracking and fluorescence quantification.

2.6 Comparison of fluorescence intensity at pH5.2 and pH 7.88

pH of the SD medium is adjusted with Tris pH8.0 buffer to pH 7.88.

Yeast cells were grown at 30℃ in original pH 5.2 SD media to A_{600nm} of approximately 0.5-1.0. Cells were centrifuged at 3000 rpm for 2 minutes, and the supernatant was poured out. For measuring fluorescence intensity at pH 5.2, 10uL of the cells were pipetted to a microscope slide after a brief vortex, and the intensity was measured. To measure fluorescence intensity at pH 7.88, cells were suspended with the adjusted pH 7.88 SD media and then shaken again at 30℃ for another hour before the intensity measurement.

For each measurement, five snapshots were taken at various positions. The snapshots were taken using the same microscope as mentioned in section 2.6. For all snapshots, phase contrast at 60ms, infrared red fluorescent protein (iRFP) at 640 nm, 300ms, 10%, and the corresponding fluorescence were taken. GFP at 470 nm, 10ms, 10% were taken for both strain NH1284 and pHluorin NH1435, while mCherry at 555 nm, 10ms, 25% were taken for NH874.

2.7 Genetic Information of Yeast Strains

Table 2.1 Summary of yeast strains used in this project

Strain Name	Strain Information	Genotype	Source of Reference
NH268	BY4741	Nhp6a-iRFP:Kan	Hao Lab
NH874	BY4741	Nhp6a-iRFP:Kan, pTDH3::pTDH3-mCh-URA3	Hao Lab
NH1168	BY4741	Nhp6a-iRFP:Kan, pGPD::pGPD-GFP-Tau301 single copy, Dcp2-mCherry-URA	Hao Lab
NH1189	BY4741	Nhp6a-iRFP:Kan; TDH3-pRS306-pGPD-GFP	Hao Lab
NH1284	BY4741	Nhp6a-iRFP:Kan; TDH3-pRS306-pGPD-GFP; DCP2-mCherry-Leu	this project
NH1387	BY4741	Nhp6a-iRFP:Kan, pTDH3-sfpHluorin-CYC1 terminator-URA	this project
NH1435	BY4741	Nhp6a-iRFP:Kan, pTDH3-sfpHluorin-CYC1 terminator-URA; DCP2-mCherry-Leu	this project

2.8 Primers Information

Table 2.2 Summary of primers used in this project

PCR fragment	**DNA template**	**primers**
pTDH3	NH268 genomic DNA extraction	Forward Primer: 5'ATAAAGCCCACATGGATAACATTACCCC TGCAGTTCGAGTTTATCATTATCAATACTG CC 3' Reverse Primer: 5'TTCTTCTCCTTTGCTCATTTCTAGATTTGT TTGTTTATGTGTGTTTATTCGAAAC 3'
MRV55 pHluorin	p426MET25_sfpHluorin	Forward Primer F: 5'GAATAAACACACATAAACAAACAAATCT AGAAATGAGCAAAGGAGAAGAAC 3' Reverse Primer: 5'AAAAAAATTTCAAGGAAACCGTGTACTG CAGCAAATTAAAGCCTTCGAGCG 3'
DCP2-mCherry-Leu	NH539 genomic DNA extraction	Forward Primer: 5' AAGCCCAAGCCTCTTAATGATG 3' Reverse Primer: 5'CGATGTTGAAGACGGTGATGTCA 3'
Yeast Strain to Confirm	**Expected PCR product length**	**primers**
NH1387	810bp	Forward primer: 5' AAGCTTTGGCACATCAATGC 3' Reverse primer: 5' TATTTTGGGCATGTACGGGT 3'
NH1284, NH1435	2.3kb	Forward Primer: 5' AAGCCCAAGCCTCTTAATGATG 3' Reverse Primer: 5' CGATGTTGAAGACGGTGATGTCA 3'
Sanger Sequencing sample		**primers**
pYTK096_pTDH3_MRV55		primer 1: 5' TGCGAGGCATATTTATGGTG 3' primer 2: 5' GGTTGAAACCAGTTCCCTGA 3' primer 3: 5' TCCTGCACGTTTTTGTTCTG 3'

CHAPTER 3 Experiments and Results

3.1 Overview of experiments

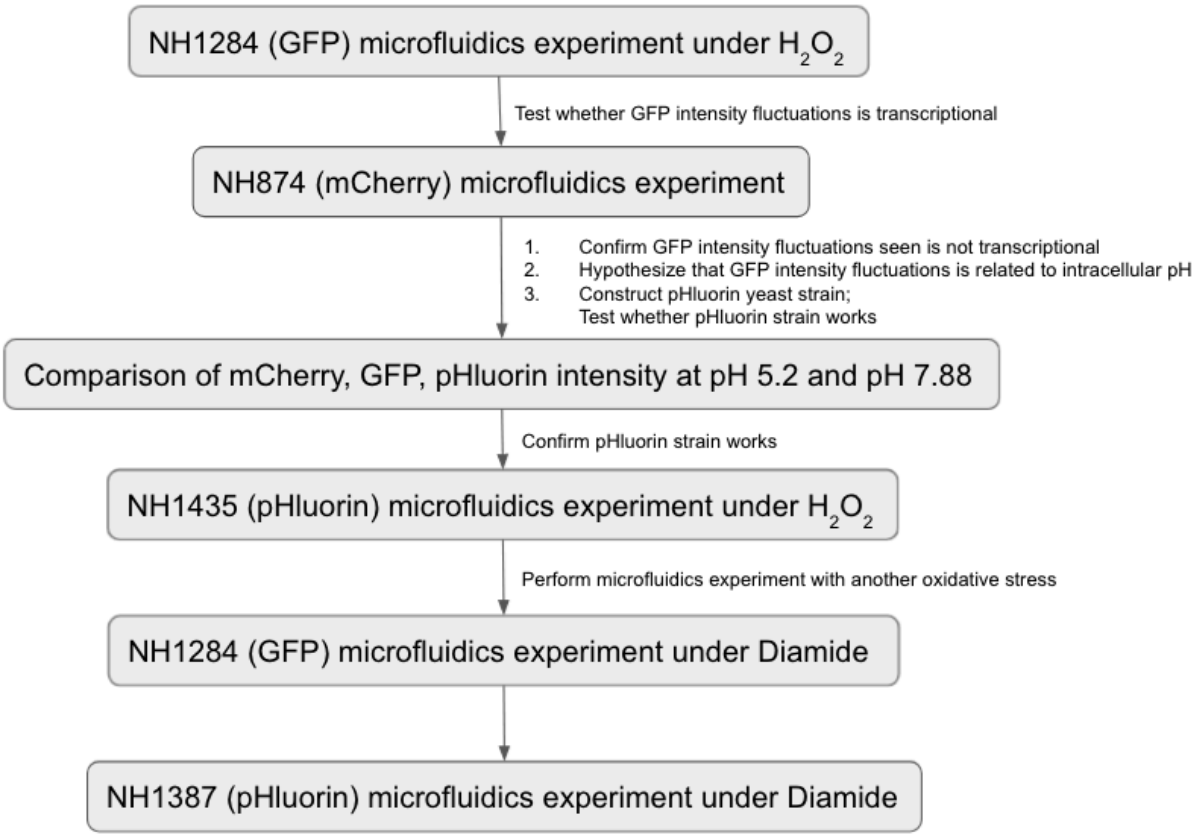

Figure 3.1 Flowchart of experiments performed and reasoning

3.2 mCherry intensity fluctuations under H2O2 stress

3.2.1 Rationale

From previous experiments performed by other lab members we see that the GFP intensity in the cells appear to have varied fluctuations when under hydrogen peroxide stress at the concentration range 0.5mM to 2mM. On that account, it is necessary to understand whether the intensity fluctuations is related to changes in transcription strength, or if it is related to some GFP specific characteristics.

This goal can be achieved by replacing the GFP with mCherry. We ran a microfluidics experiment under the same hydrogen peroxide stress conditions to observe whether mCherry fluorescence intensity fluctuates in the same way as GFP does. If the intensity fluctuations we see in GFP is actually due to transcriptional reasons, then we expect to see similar fluctuations in mCherry intensity. However, if our assumption is correct that the fluctuations are due to the characteristics of GFP itself and is not affected by transcription strength, then we would expect to see an overall maintenance in mCherry fluorescence intensity.

A microfluidics experiment was conducted with strain NH874 (pTDH3-mCherry) at 0.5mM and 0.75mM H_2O_2. Details can be seen in the materials and methods section.

3.2.2 Results

Results from the microfluidics experiment indicated that the mCherry intensity maintained throughout the 6 hour experiment time span, and was not affected by the addition of hydrogen peroxide stress as GFP intensity was. In Figure 3.1, it is seen that mCherry intensity varies at different H_2O_2 concentration; the overall intensity is higher at 0.5mM and lower at 0.75 mM. However, the mCherry fluorescence intensity stayed at approximately the same level and no fluctuations were observed. The three GFP fluorescence fluctuation cell types seen previously in strain NH1189 were absent.

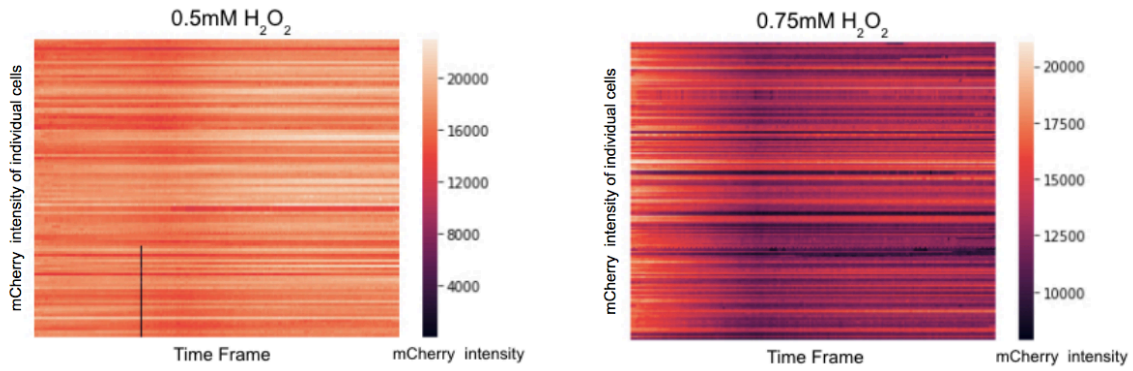

Figure 3.2 Heat map displaying mCherry intensity over time in yeast cells under H_2O_2. (A) 0.5mM H_2O_2 stress (B) 0.75mM H_2O_2 stress was added to the cells after 10 minutes of SD medium flow-through and removed after 2 hours.

3.3 Investigation of heterogeneity in cells under H_2O_2 stress

3.3.1 Rationale

NH1284 was constructed by tagging DCP2 with mCherry in the strain NH1189 (pGPD-GFP), which is the strain that confirmed the existence of three cell types upon addition of H_2O_2 stress. Moreover, in previous microfluidic experiments done with strain NH1168 (pGPD-GFP-Tau301, Dcp2-mCherry), a correlation between DCP2 aggregation and GFP fluorescence intensity maintenance was found. Since those experiments were performed more than half a year ago, it was necessary to construct a strain that contains both pGPD-GFP and DCP2-mCherry in order to validate the results.

Details of constructing NH1284 can be found in section 2.4. Microfluidic experiments were performed with the newly made strain NH1284, using hydrogen peroxide as the oxidative stress. If the results from past experiments still stands true, we expect to see three types of GFP fluorescence intensity fluctuation: GFP intensity maintained overall, a drop in the GFP intensity after addition of hydrogen peroxide but followed by intensity recovery after the stress is removed, and a drop in GFP intensity after addition of hydrogen peroxide with no recovery even after the stress is removed. Besides the three cell types, we also expect to see DCP2 aggregation in the cells that maintained their GFP intensity overall during the 6 hour experiment span.

After corroborating the two results from the microfluidics experiments using NH1284, we proposed the idea that the GFP intensity fluctuations were affected by pH variation in the cytosol. Our hypothesis was that the addition of hydrogen peroxide stress could alter the cytosol pH. In detail, if the cytosol pH was not affected then the GFP intensity will be maintained; however, if the cytosol pH was affected and then regulated to reach homeostasis, it would result in a drastic drop in the GFP intensity but a recovery later on.

Lastly, if the cytosol pH was affected but homeostasis was not reached, it would result in the type of cells in which their GFP intensity never recovered.

With the interest of testing out our hypothesis, we would have to track the pH of the yeast cell cytosol in real time. Hence we introduced the superfolder pHluorin (MRV55 / p426MET25_sfpHluorin; a gift from Eckhard Boles; Addgene plasmid # 115697; http://n2t.net/addgene:115697 ; RRID:Addgene_115697) into the yeast genome. Since the MRV55 vector is a 2 micron plasmid, this means that under normal conditions there will be about 40-60 copies per cell after transformation. Yet, considering the fact that we will be measuring and comparing the fluorescence of the pHluorin between single cells, we need to ensure that each cell only has one copy of the superfolder pHluorin. A problem that emerges from this is that with only one copy of the pHluorin gene in each cell, the fluorescence intensity will be greatly decreased as originally there would have been on average 50 copies per cell. In hope to solve this issue, we decided to switch the original CYC1 promoter in the MRV55 vector to a much stronger TDH3 promoter.

We constructed a new plasmid pYTK096_pTDH3_MRV55 using pYTK096 as the vector backbone, pTDH3 as the new promoter, and the pHluorin gene from the original MRV55 plasmid. Details on the construction pYTK096_pTDH3_MRV55 and strain NH1387 containing this new plasmid is described in section 2.2 and 2.3. (Following strain NH1387, strain NH1435 was made by tagging mCherry onto DCP2 in the strain NH1387 for continued observation of the correspondence between DCP2 aggregation and GFP intensity maintenance in cells.) Details of tagging DCP2 with mCherry can be seen in section 2.4.

Microfluidic experiments were conducted using strain NH1435 under H_2O_2 stress at 0.25mM, 0.5mM, 0.75mM and 1mM , which are the concentrations which the three cell types were observed in NH1284 during the microfluidic experiments. If our hypothesis, the GFP intensity fluctuations are caused by pH alteration and regulation in the cell cytosol, is correct,

then we expect to see similar results as the microfluidics experiment performed with strain NH1284. Three types of pHluorin GFP intensity fluctuations should be seen, one type with continuous high pHluorin GFP intensity, another type with an intensity drop after addition of H_2O_2 but intensity recovery after removal of H_2O_2, and lastly, the type that shows no pHluorin GFP intensity recovery even after removal of H_2O_2. The pHluorin intensity magnitudes are expected to be greater than the original GFP's since pHluorin itself is even more sensitive to pH compared to the original GFP sensitivity.

3.3.2 Results of GFP intensity fluctuation under H2O2 stress

Using the elbow method to identify our optimal K and then using K-means clustering, results from the microfluidic experiments using strain NH1284 confirms the existence of the three types of GFP intensity fluctuations under the H_2O_2 concentration range of 0.25mM, 0.5mM, 0.75mM, and 1mM. As seen in figure 3.2, when at lower hydrogen peroxide concentrations, cell type that can maintain GFP intensity is the majority. As the concentration increases, the GFP intensity drops when hydrogen peroxide stress is added; at the highest concentration 1mM H_2O_2, mostly all the cells are those that the GFP intensity does not recover even after removal of hydrogen peroxide stress.

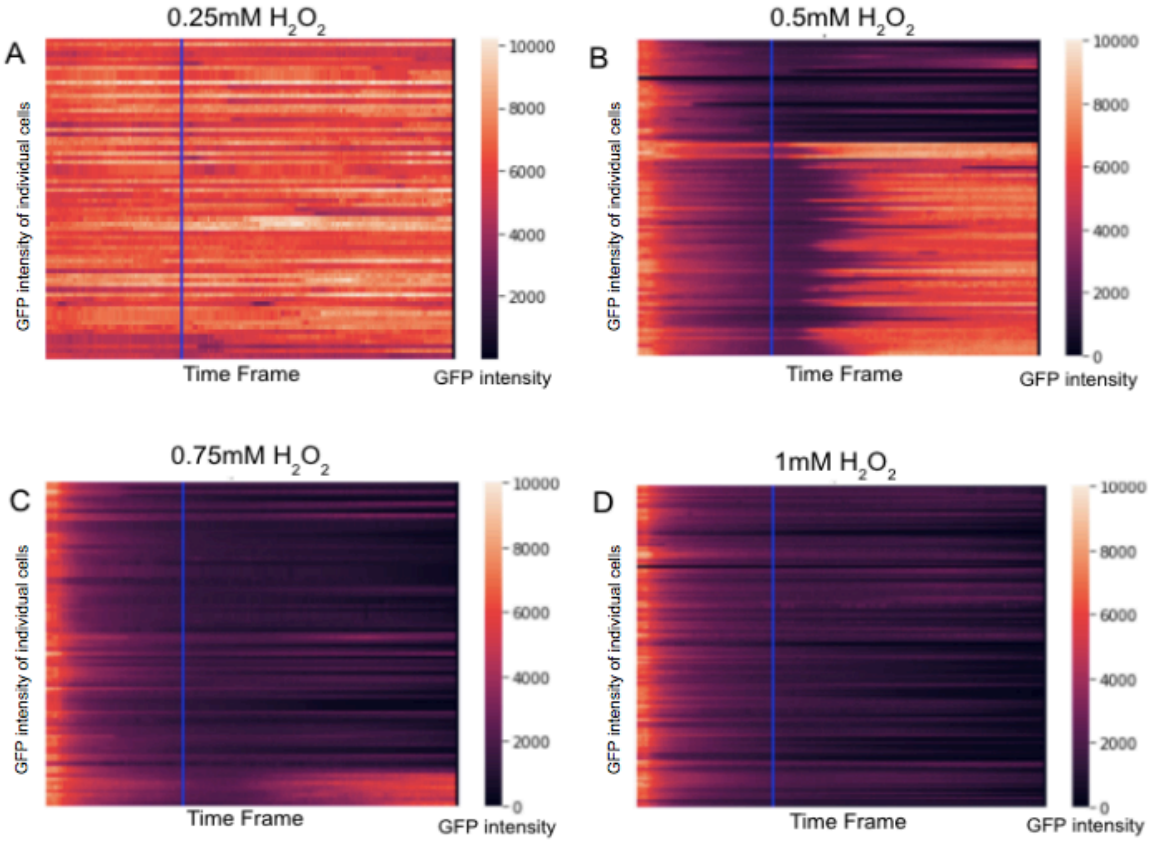

Figure 3.3 Heat map displaying GFP intensity time trace in NH1284 under H_2O_2. Microfluidics experiment setup: 10 minutes baseline with SD media → 2 hours hydrogen peroxide stress (A) 0.25mM H_2O_2 (B) 0.5mM H_2O_2 (C) 0.75mM H_2O_2 (D) 1mM H_2O_2 → 4 hours recovery with SD media. Snapshots of cells were taken every 2 minutes over the 6 hours and 10 minutes experiment period. The blue vertical line indicates when the stress was removed.

3.3.3 Results of pHluorin intensity fluctuation under H_2O_2 stress

The pHluroin intensity fluctuations indicate intercellular pH fluctuations in NH1435 while under H_2O_2 stress and are depicted as in the heat map below. The integrated superfolder pHluorin emission intensity at 580nm should peak at pH 9.0 and is lowest when at pH 5.0 (Reifenrath & Boles, 2018). Based on our assumption, the pHluorin intensity fluctuations should be similar to the GFP intensity fluctuations seen in NH1284 and also express the three types as described earlier. Once more using the elbow method to select the optimal k and then utilizing the K means clustering method, the pHluorin intensity fluctuations in NH1435 are indeed sorted into three types as the GFP intensity fluctuations in NH1284 did. When looking at the heat map, we can see that the pHluorin intensity fluctuations correspond well with the GFP intensity fluctuations in NH1284 surprisingly well. Under 0.25mM H_2O_2, pHluorin intensity remained the same and was not affected by the addition of H_2O_2 stress, under 0.5mM H_2O_2, majority of the cells demonstrated a drop in pHluorin intensity after addition of H_2O_2 stress and intensity recovery after removal of the stress. Under 0.75mM and 1mM H_2O_2, the cells showed no recovery in the pHluorin intensity even after removal of the H_2O_2 stress. All of these patterns are in agreement with the GFP intensity fluctuations seen in figure 3.2, indicating that the GFP intensity is indeed related to the intracellular pH fluctuations caused by addition of H_2O_2.

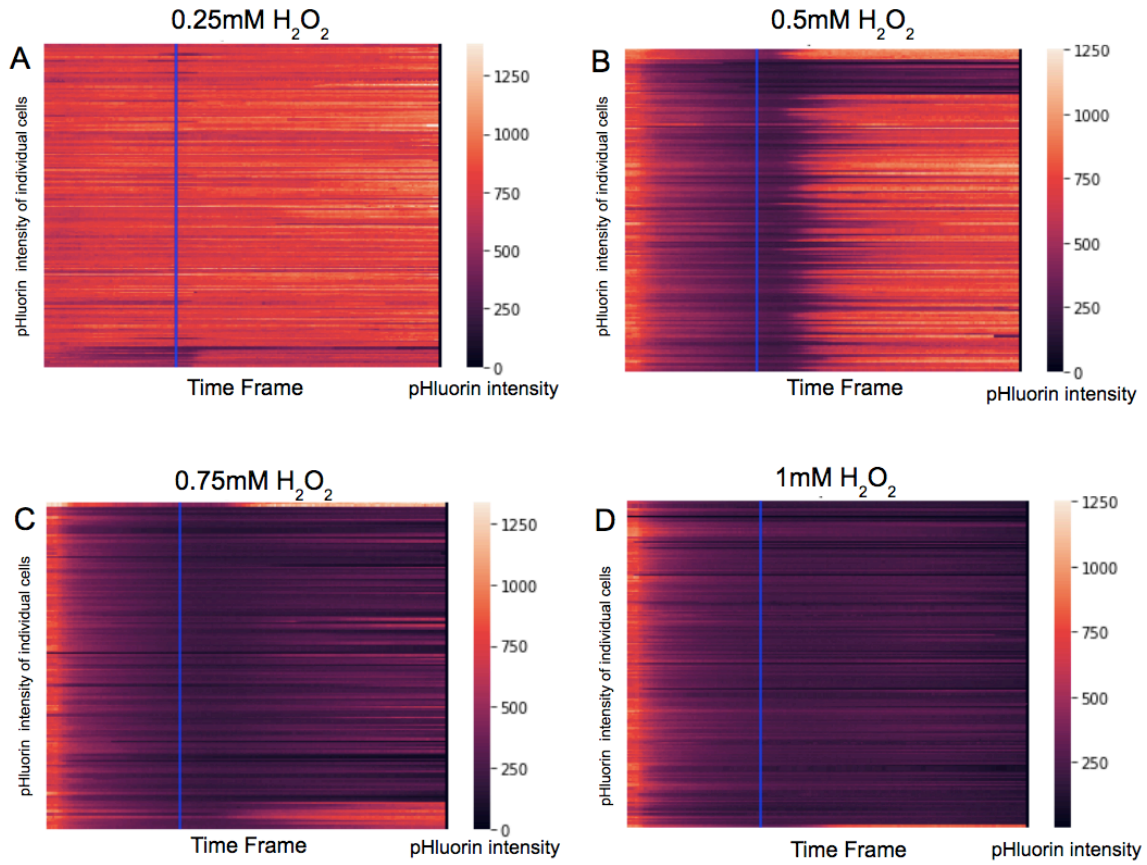

Figure 3.4 Heat map displaying pHluorin intensity time trace in NH1435 under H_2O_2. Microfluidics experiment setup: 10 minutes baseline with SD media → 2 hours hydrogen peroxide stress (A) 0.25mM H_2O_2 (B) 0.5mM H_2O_2 (C) 0.75mM H_2O_2 (D) 1mM H_2O_2 → 4 hours recovery with SD media. Snapshots of cells were taken every 2 minutes over the 6 hours and 10 minutes experiment period. The blue vertical line indicates when the stress was removed.

3.3.4 Results of DCP2 aggregation in correspondence to cell types

From the microfluidic experiments conducted with strain NH1284 under H_2O_2 stress, it can be seen that there are DCP2 aggregation in those cells that either maintained the GFP intensity throughout the entire experiment time frame, or the cells that showed intensity recovery despite the initial intensity drop after addition of H_2O_2 stress. Even though DCP2 aggregation can also be found in some of the cells that did not show GFP intensity recovery even after removal of stress, the amount is much less and the aggregation is not as obvious when compared to the aggregation observed in the other two type of cells. This is demonstrated by figure 3.4. The left snapshot is when the cells are under 0.5mM H_2O_2 where the majority of type 1 and type 2 GFP intensity fluctuations exists. The right snapshot is when the cells are under 1mM H_2O_2 where the cells are mainly those that the GFP intensity does not recover. The little white dots in the cells indicate DCP2 aggregation, which are abundant in the cells from the left snapshot but not the right one.

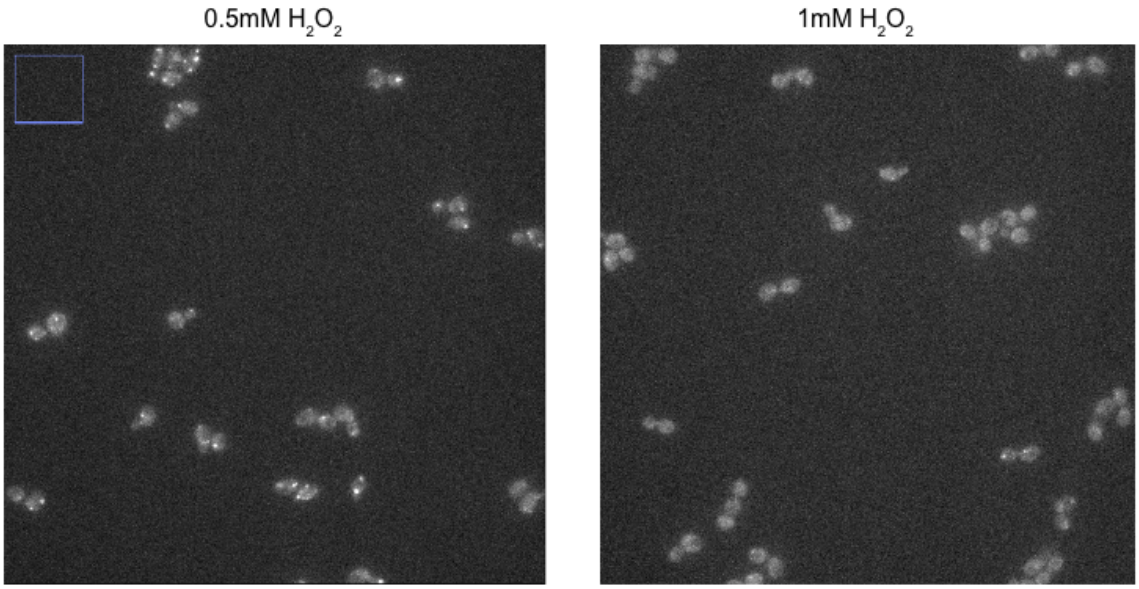

Figure 3.5 Snapshot of strain NH1284 cells at 555nm (mCherry) under H_2O_2 stress showing DCP2 aggregation.

3.4 Investigation of heterogeneity in cells under diamide stress

3.4.1 Rationale

Since we have observed a variation of GFP intensity fluctuations under hydrogen peroxide stress, we wondered whether this phenomenon can also be seen in other types of oxidative stress or if it is specific to hydrogen peroxide. Diamide is also a common oxidative stress used in studies; therefore, we decided to use it as another source of stress and perform microfluidic experiments with the same NH1284 strain. We also performed microfluidic experiments with the pHluorin strain NH1387 in order to examine whether a pH correlation with the GFP intensity exists when diamide acts as the stress source.

3.4.2 Results of GFP intensity fluctuation under diamide stress

Using the K means clustering method, results from microfluidic experiment with diamide as the oxidative stress still shows that the cells' GFP intensity fluctuations are split into 3 types, which can be seen in figure3.5. The three types of fluctuations remain the same as mentioned using hydrogen peroxide as the stress. However, contrary to the results from cells under hydrogen peroxide stress, the GFP intensity fluctuations under diamide stress does not show a clear cut among the concentration 4mM to 7mM. The differentiation between the three types of fluctuations are not as obvious, moreover, a mixture of the types can be seen no matter at low or high diamide concentration.

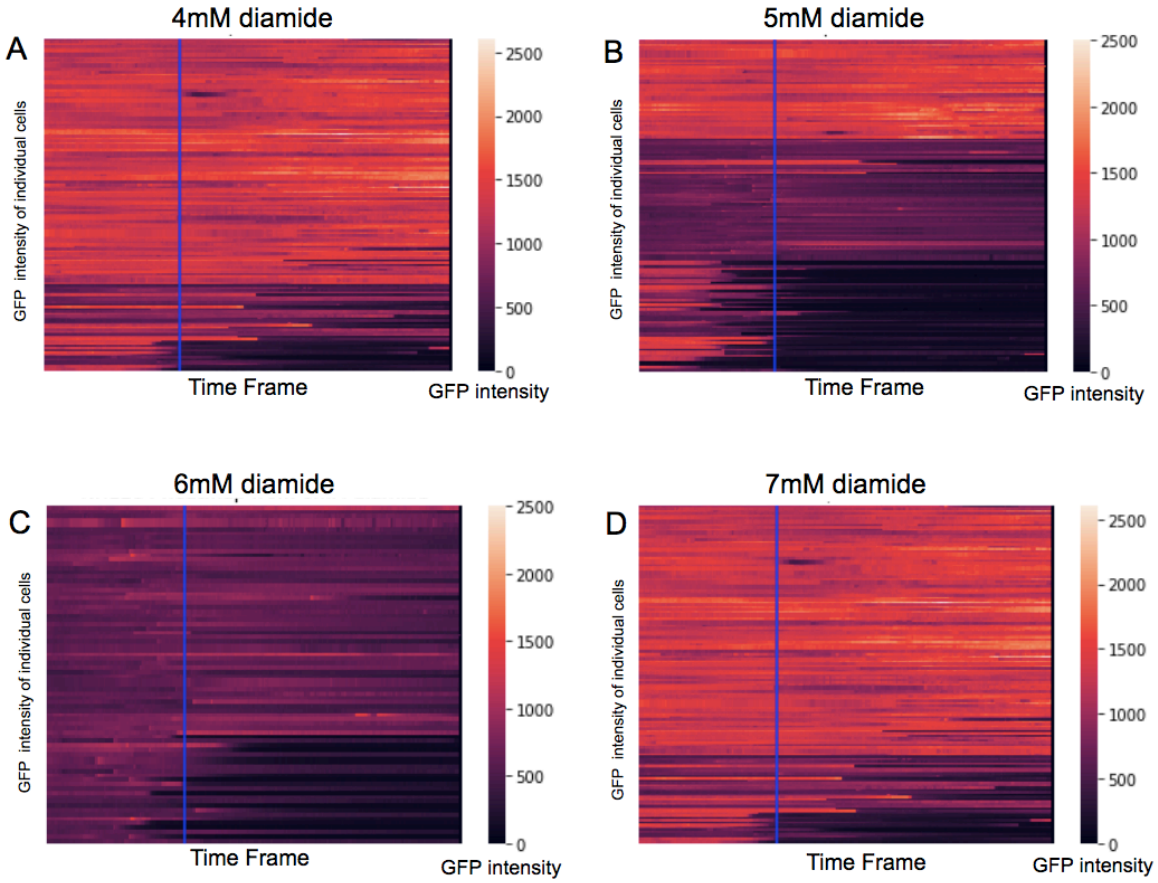

Figure 3.6 Heat map displaying GFP intensity time trace in NH1284 under diamide. Microfluidics experiment setup: 10 minutes baseline with SD media → 2 hours diamide stress (A) 4mM diamide (B) 5mM diamide (C) 6mM diamide (D) 7mM diamide→ 4 hours recovery with SD media. Snapshots of cells were taken every 2 minutes over the 6 hours and 10 minutes experiment period. The blue vertical line indicates when the stress was removed.

3.4.3 Results of pHluorin intensity fluctuation in under diamide stress

The pHluorin intensity under diamide stress appeared to have no patterns or any features that are somewhat relatable to the GFP intensity fluctuations when also under diamide stress. In NH1284 microfluidic results, there are still some variations between the GFP intensity fluctuations and the heterogeneity exists. However, the NH1387 microfluidic results did not show any of those. The pHluorin intensity fluctuations remained similar among all cells while under 4mM and 5mM diamide stress. Moreover, it can be seen from figure 3.6 that the pHluorin intensity stands at the same level throughout the time frame of the entire experiment, indicating the intacellular pH was also not affected by the presence of dimaide.

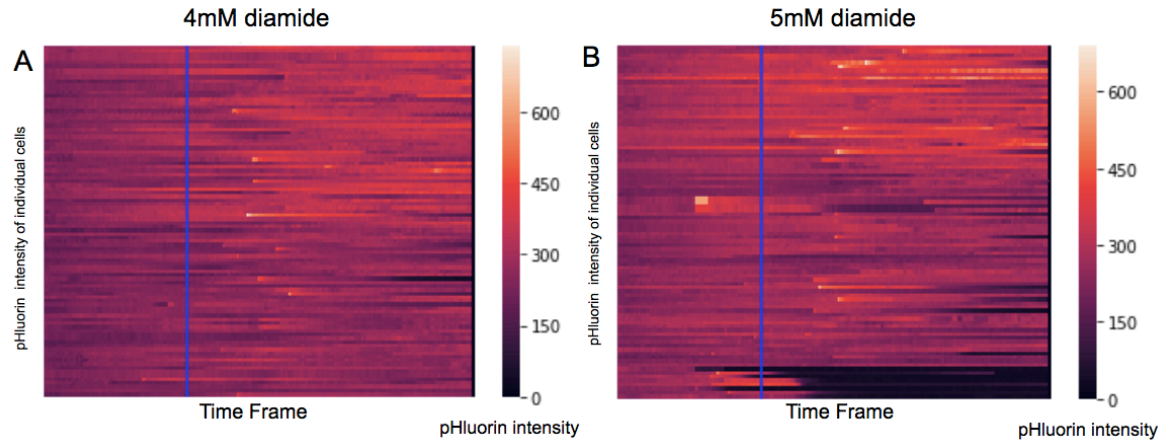

Figure 3.7 Heat map displaying pHluorin intensity time trace in NH1387 under diamide. Microfluidics experiment setup: 10 minutes baseline with SD media → 2 hours diamide stress (A) 4mM diamide (B) 5mM diamide → 4 hours recovery with SD media. Snapshots of cells were taken every 2 minutes over the 6 hours and 10 minutes experiment period. The blue vertical line indicates when the stress was removed.

3.4.4 Results of DCP2 aggregation in correspondence to cell types

DCP2 aggregation can also be seen in the cells while under diamide stress at 4mM, 5mM, 6mM, and 7mM. Figure 3.7 is a display of the aggregation observed in the cells. There are no transparent distinctions between the type of cells show DCP2 aggregation and those that do not since the GFP intensity fluctuations seen in NH1284 are also not apparent. We can only assure that DCP2 does aggregate when the yeast cells are under diamide stress.

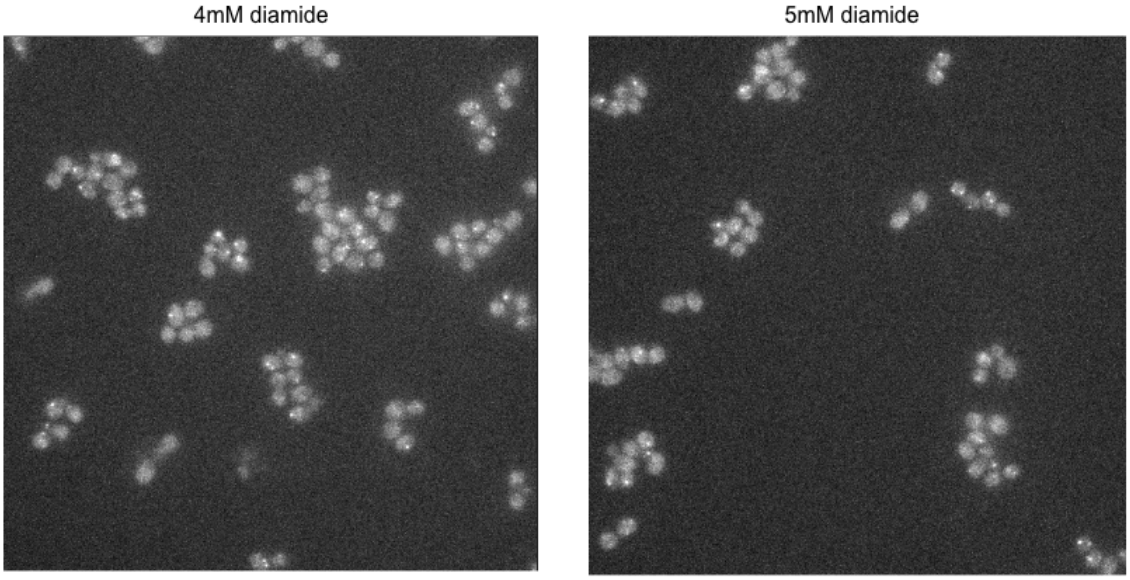

Figure 3.8 Snapshot of strain NH1284 cells at 555nm (mCherry) under diamide stress showing DCP2 aggregation.

3.5 Comparison of GFP, superfolder pHluorin, and mCherry intensity at different pH

3.5.1 Rationale

After constructing the pHluorin strain NH1387 and NH1435, we decided to conduct a control experiment to ensure the constructed pHluorin strain does actually reflect changes of the environmental pH. The emission intensity at 508 nm of the MRV55 superfolder pHluorin peaks at pH9.0 and is lowest when at pH 5.0 (Reifenrath & Boles, 2018). We decided to measure the pHluorin intensity at approximately two pH levels: pH 5.2, which is the original SD media pH, and at pH 7.88, which was SD media adjusted with Tris pH8.0.

In order to compare the pH sensitivity, we would measure the fluorescence intensity of the original GFP, pHluorin, and mCherry, using strain NH1284, NH1435, and NH874 respectively. We expected that the fluorescence intensity measured from the NH1435 pHluorin and NH1284 original GFP will both show a difference between pH 5.2 and pH 7.88, yet the NH1435 pHluorin intensity will display a greater gap since the pHluorin is more sensitive to pH. On the other hand, considering mCherry isn't sensitive to pH as GFP or pHluorin is, the NH874 mCherry should show approximately similar fluorescence intensities at either pH 5.2 and pH 7.88, or the least disparity among all three original GFP, pHluorin, and mCherry.

3.5.2 Results

The snapshots taken indicated that the superfolder pHluorin in our constructed strain NH1435 does indeed work as expected. As seen in figure 3.8(C), the pHluorin GFP has a higher intensity at pH7.88 when compared to the intensity when at pH5.2, with about 200AU difference in the median. Furthermore, in figure 3.8(B), it can be seen that the original GFP also has a higher intensity when at pH7.88; however, the median difference between intensity at pH7.88 and pH5.2 is only around 50AU, which is much lower than that of the pHluorin

GFP. This result also indicates that even though GFP is sensitive to pH originally, the pHluorin is much more sensitive to pH, which is why it is necessary to use the pHluorin gene instead of just a GFP. On the other hand, since mCherry is not sensitive to pH as GFP is, mCherry intensity shows basically no variation at either pH5.2 and pH7.88; this can be seen at figure 3.8(A).

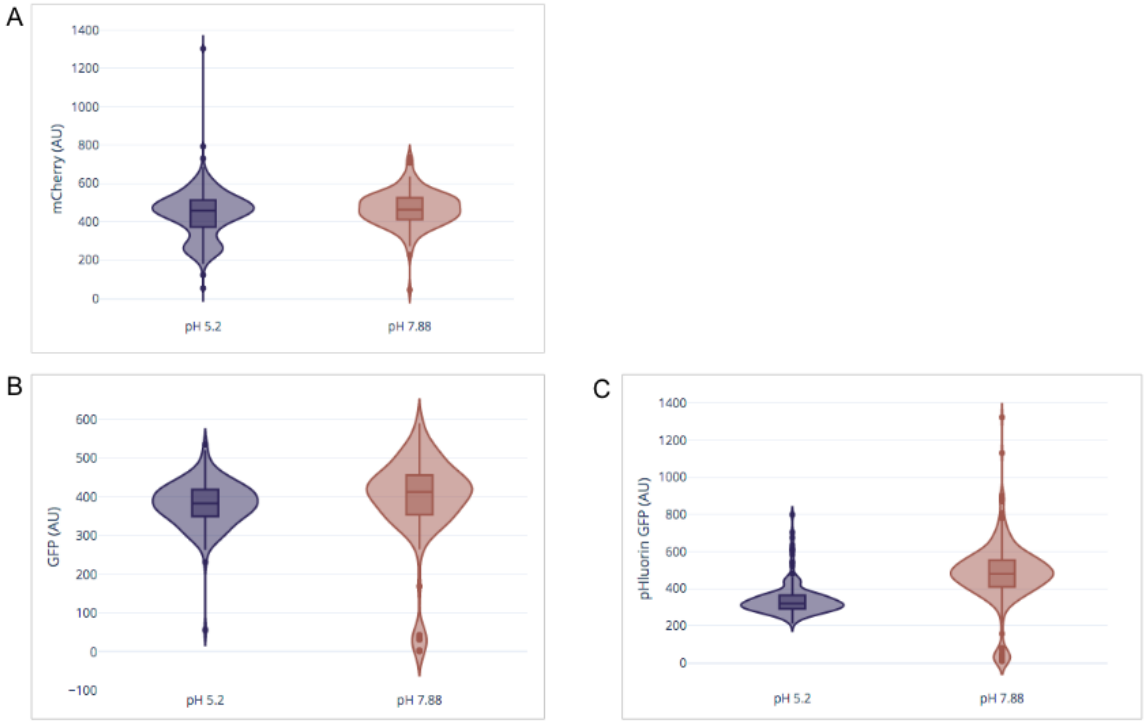

Figure 3.9 Violin maps comparing various fluorescence intensities at pH 5.2 and pH 7.88. (A) mCherry in NH874 (B) GFP in NH1284 (C) pHluorin GFP in NH1435

CHAPTER 4 Discussion and Future Direction

4.1 Discussion

In this project, we utilized yeast genetics and microfluidics technology with live-cell imaging to investigate the relationship between intracellular pH and heterogeneity in the budding yeast under two types of oxidative stress, hydrogen peroxide and diamide. Experiment results with NH1284 has shown that under hydrogen peroxide stress at the concentration range of 0.25mM to 1mM, the intensity fluctuations of a constitutive GFP in the budding yeast will diverge into three types: one with continuous high GFP intensity, another with an intensity drop after addition of H_2O_2 but intensity recovery after removal of H_2O_2, and lastly, the type that shows no GFP intensity recovery even after removal of H_2O_2. On the other hand, even though our experiment results show that these three types are still observable when the budding yeast is placed under 4mM to 7mM of diamide stress, contrary to the distinct separation of cell types dependent on the H_2O_2 stress concentration, this defined difference was not seen in the results from the diamide stress.

In addition, microfluidic experiments with the superfolder pHluorin have demonstrated the correlation between intracellular pH and the heterogeneity mentioned above. The pHluorin GFP intensity fluctuations comply with the three types of GFP intensity fluctuations observed in strain NH1284, indicating a high possibility that the GFP intensity variations we have observed is the result of H_2O_2 induced pH alterations in the cytosol. It is not a great surprise that the GFP intensity is affected by the environmental pH; it is know from studies that GFP is pH sensitive. As stated in studies, the GFP chromophore can be in two forms, A form being neutral protonated which absorbs light at 395nm and B form being anionic, absorbing light at 475nm. A and B forms exist at an intensity ratio of 6:1 while at equilibrium, and this ratio is extremely sensitive to the environmental pH (Remington, 2011). The fluorescence of wild type GFP decreases when pH is less than 6 (Campbell & Choy,

2001); this further supports our result since hydrogen peroxide is a weak acid and should decrease intracellular pH, hence lowering GFP intensity.

On the other hand, we cannot fully conclude that intracellular pH is the one and only factor affecting the GFP intensity fluctuations, therefore here we discuss another prospect. In previous studies they have revealed that GFP fluorescence is tightly associated to the structure of the protein, and when denatured the fluorescence is lost (Alnuami et al., 2008). Oxidative stress is known to target and damage proteins, hence it is not a surprise that the presence of hydrogen peroxide and diamide impacts the GFP fluorescence. In another study that has concluded that GFP expression is indeed affected by hydrogen peroxide concentration, they noted that GFP expression decreases as hydrogen peroxide concentration increases, and the fluorescence is eventually quenched. A possible explanation provided by the study was the oxidation molecules that occurred at high hydrogen peroxide concentrations (Umakoshi et al., 2011). The heterogeneity we have observed could be a result of H_2O_2 homeostasis, which is mainly determined by peroxiredoxins (Prx) activity. Prx are proteins that scavenge for H_2O_2; studies have shown that the "Prx expression increases up to fivefold upon exposure to H_2O_2", and it is also possible that the scavenging rate is limited by the internal H_2O_2 concentration (Goulev et al., 2017). These assumptions are not definite since current research remains unclear on this topic, the details would require continued research in future studies to validate and expand on.

4.2 Future Directions

From our results we have identified the relationship between intracellular pH and the heterogeneity in GFP intensity, there are a couple of approaches we can take in our future studies. First of all, we can take a closer look at the heterogeneity confirmed in strain NH1284. Among the three cell types observed, it is reasonable to extend the experiment time period in order to ensure that there are no drastic GFP intensity fluctuations after our original

6-hour experiment period. One major point is for us to look at the cells that did not show GFP intensity recovery even after the removal of oxidative stress, and to verify whether the cells are slow in recovery or the fluorescence is quenched in the long term. This was not performed in the original experiments due to technical difficulties with our microfluidics device. The device and methods we used can only last for about 6 hours without the cells being washed away, hence to be able to observe the cells over an even longer time period requires different microfluidics devices such as the one used in Li et al. (Li et al., 2017)

Secondly, we can also examine the relationship between DCP2 aggregation and the GFP intensity fluctuations. It was noticed from the experiments that most of the DCP2 aggregation happens in the cells that could maintain their GFP intensity or recover from the intensity drop. We assume that it could be due to the fact that DCP2 plays an important role in p-bodies which are related to stress regulation in cells; however, this requires more background research to pursue in this direction.

Thirdly, we could advance on studying how pH homeostasis is maintained in thc cells while under H_2O_2 stress, and moreover, study whether variation in pH regulation is the underlying reason heterogeneity is observed. To do this, we would have to identify which genes play a part in this pH regulation, and the best scenario would be to be able to pinpoint the gene that affects this heterogeneity. That is, the transcriptional activity of the gene would be able to correspond with the GFP intensity fluctuations when under hydrogen peroxide stress. The plasma membrane ATPase 1 (Pma1) could be a place to start; since Pma1 is an essential proton pump and a major intracellular pH regulator, it would be a crucial gene for us to look at. More research on past studies and papers will need to be done to recognize other genes that might play a part in the pH regulation pathway.

Another direction for us to approach would be to examine the possibility of oxidation molecules affecting the GFP intensity. We would have to consider if the oxidative stress

hydrogen peroxide and diamide are affecting the cellular redox states and therefore altering the GFP intensity. A potential method would be to introduce a redox-sensitive GFP (roGFP) into the genome of the budding yeast, which should allow us to gain a better understanding of the cellular redox states upon addition and removal of H_2O_2 and diamide.

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