A Systematic Experiment for Studying the Role of Chromatin Remodelers in Transcription Regulation

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by

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Abstract

The regulation of transcription is crucial to establish cellular responses to changing environments. This regulation involves a complex combination of signaling pathways, transcription factors, and the generic transcription machineries to initiate and maintain proper response. While the main signal carriers for yeast stress responses are well characterized, there are many additional mechanisms involved in modulating the response that are poorly understood. Many of these components are opaque to traditional genetic screen due their subtle effect.

In this project we establish an experimental system combining a genetic screen with high-throughput time-lapse microscopy to allow us to use the trajectory of induction in individual cells as a phenotype. We use this strategy to dissect the role of chromatin modifiers in establishing the dynamics of transcriptional response to an acute stress event (high osmolarity induced by KCl). To do so, we are using a large ($\sim$ 380) subset of the GFP-tagged protein library that contains proteins whose expression is induced in response to osmotic shock. We follow the induction of these proteins in population of cells in both wild type strains and strains with deletion of key chromatin remodelers and the main transcriptional factors involved in this stress response. Using this strategy we quantify the effect of different remodelers in the dynamics and variability of the response of each of the target proteins.

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Contents

Chapter 1

Introduction

We are interested in understanding regulation and in using yeast as a model for change in response to stimulus. I will now give a brief background on these issues.

1.1 Gene expression

DNA contains all of the hereditary information needed for the cell function and development. The central dogma of molecular biology states that genetic material is transcribed into RNA and then translated into protein which will perform variety of functions in the cell. All the cells in an organism share the exact same DNA sequence, but we can still observe diversity in the protein and mRNA contents between cells, in different tissues, and under different conditions [7]. Similarly, there are differences in gene expression between individuals in a population of single-cell organisms. This diversity in gene expression is possible due to extensive regulatory system at multiple layers: transcription, exportation, translation and post-translation [16]. Every step in the process of transcription and translation is regulated dynamically. Mechanisms that underline the control of gene expression are subject to extensive research.

The cell's transcriptional regulation plan regulates the beginning of this process, and thus has an important role in determining the cell's protein content. The promoter is a regulatory segment of the gene located upstream to the transcription start site (Figure 1.1). To initiate the gene transcription the RNA polymerase complex binds to the DNA at the promoter. Regulation of the binding efficiency of transcription factors and transcriptional machinery to the promoter can regulate the transcription of a gene. [27]

The TATA box is a DNA sequence found in the promoter region of genes in eukaryotes. It is normally bound by the *TATA binding protein* (TBP) in the process of transcription [13]. While some genes contain a TATA box core promoter element, the majority of core promoters fall into various TATA-less categories. The recruitment of the TATA binding protein (TBP) to promoters is achieved via the action of two related complexes, transcription factor IID (TFIID) and Spt-Ada-Gcn5 acetyltransferase (SAGA) complex [25]. TFIID and SAGA represent two global transcription regulatory pathways. The SAGA pathway favors TATA-containing genes. The TFIID pathway, on the other hand, predominates at TATA-less genes and appears to involve less regulation-of-stress related genes than SAGA-dominated genes [29] . Most yeast genes are not regulated exclusively by one pathway or the other.

1.2 Chromatin

In eukaryotic cells, DNA is located within the nucleus, where it is wrapped around histone proteins to form nucleosomes [12]. This complex is tightly bonded by attraction of the negatively charged DNA to the positively charged histones. This structure of DNA and proteins is called chromatin. Chromatin serves as a way to condense DNA within the cellular nucleus, but also as a way to control gene expression. The packaging of DNA into chromatin generates a barrier to the transcription machinery. In its default state, the tight coiling that characterizes chromatin structure limits the access of these substances to eukaryotic DNA. Therefore, a cell's chromatin must "open" in order for gene expression to take place. Various changes in chromatin structure can inhibit access of transcription factors to the DNA and therefore can regulate gene expression [24] [21]. Histone variants, post translational histone modifications and ATP-dependent chromatin remodeling events, all contribute to create distinct structural and functional chromatin domains. Chromatin remodelers are large, multisubunit, biochemically diverse protein complexes that play a central role in nucleosome dynamics. Interactions of chromatin with chromatin remodeling proteins can result in domains of chromatin that are opened, closed, or poised for activation. Chromatin structure contributes also to the complexity in gene expression regulation [22]. It allows simultaneous regulation of groups of related genes. [23]

1.3 Osmotic Stress Response

Because the yeast cells cannot physically escape from the stress environment they have to sense and adapt to changes in their environment condition using internal mechanisms [9]. A complex signaling network governs the response to different environmental conditions. Most are sensed by plasma membrane receptors and relayed via signaling pathways to the expression machinery, adjusting the gene expression pattern to the environmental condition. Often, different signaling pathways converge on promoters of key target genes. There is also an overlap between the osmotic stress pathway and the general stress response pathway [9]. The underlying molecular mechanisms of this cellular response demonstrates the interplay of signaling events, regulation of gene expression and control of metabolism that occurs in any living cell.

1.4 Yeast as a model

The budding yeast is considered an excellent model organism due to many properties, among them the ease of performing genetic manipulations. The yeast is a single celled organism with a short generation time, and can be easily cultured. Unlike most other microorganisms, the yeast lab strain exists in both diploid and haploid states. It allows the use of a mating protocol and a relative control of the genetic background of the cell. This property also allows convenient isolation of recessive mutations in haploid strains, and complementation tests in diploid strains.

The yeast is a simple model organism which includes ∼6000 genes. Using this small set of genetic information it is capable to sense and adapt to changes in its environment. As a eukaryote, the *S. cerevisiae* shares the complex internal cell structure of plants and animals without the high percentage of non-coding DNA that can confound research in higher eukaryotes. The basic mechanisms of gene expression and its regulation the basic signaling pathways are highly conserved in eukaryotes from yeast to human. The availability of the *S. cerevisiae* genome sequence and the complete set of deletion mutants have further enhanced its power as a model for understanding the regulation of eukaryotic cells [2]. This simplicity of the system is also a disadvantage since some mechanisms

such as splicing cannot be investigated in this model.

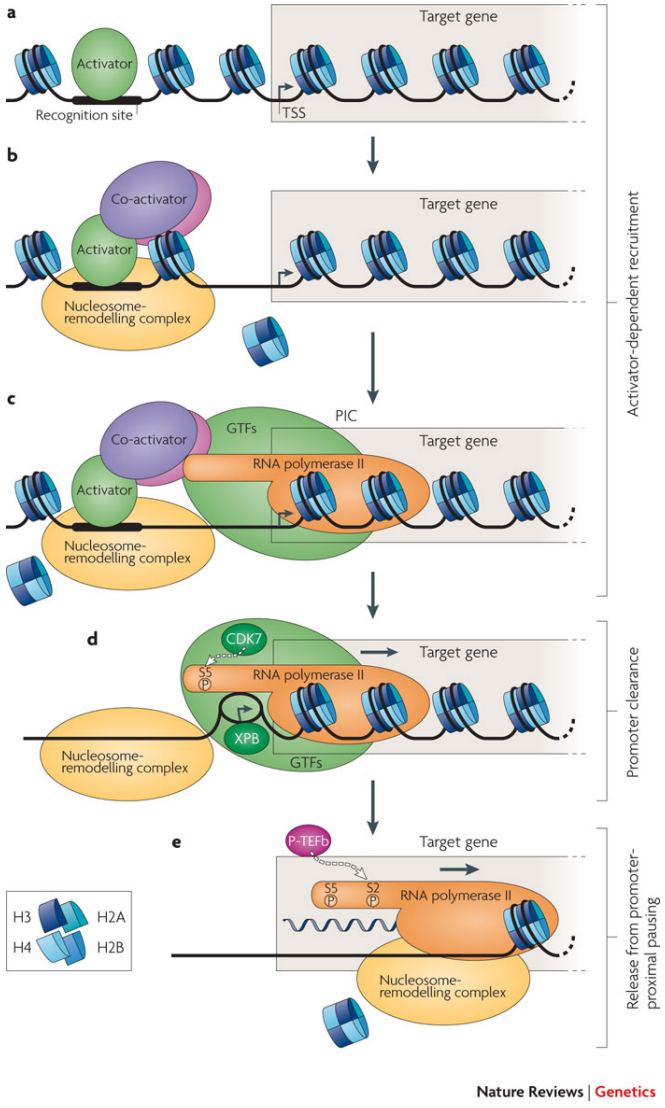


Figure 1.1: The transcription initiation machinery [27]

Chapter 2

Motivation and Goals

2.1 Experimental System Motivation

Gene expression regulation is a complex process that involves a complex combination of signaling pathways, transcription factors and other factors. Traditional researches in this field have established some of the main relationships in this complex network, but the detailed specific and global mechanism behind the control of gene expression is still unclear. We aim to establish a system that will enable studying of the regulatory network using several approaches. We aim for a system that will meet the following desiderata.

- **Single Cell Level**
  Gene expression involves biochemical reactions. Thus, quantitative relation between the involved transcription-factors concentrations resulting in dynamic fluctuations in expression levels in downstream genes. Variation between cells is present in genetically identical cell populations, even when the cells are exposed to the same environmental condition [20] [11] . One reason is that the living cells possess very low copy numbers of many components, including DNA and important regulatory molecules. Thus, stochastic effects in gene expression may account for the large amounts of cell-cell variation observed in populations [17]. In every individual cell a combination of biochemical parameters, noise, and slowly varying cellular states together determine the effective level of expression of each gene.
  Gene expression and regulation have traditionally been investigated by measure-

ments of the average values for a population of cells. Such measures give information about the mean expressional behavior in the cells population, but mask the behavior of the specific cell and biological processes in which cellular heterogeneity plays a role (Figure 2.1a).

- **Dynamic Response in Living Cells**

  Cellular dynamics is based on the expression of specific genes at specific times. The dynamic response and specifically the timing and rate of expressional response are the main characteristics of the stress response pattern. For example, the delay in timing of the expression may imply that the pathway leading to the gene transcription is changed.

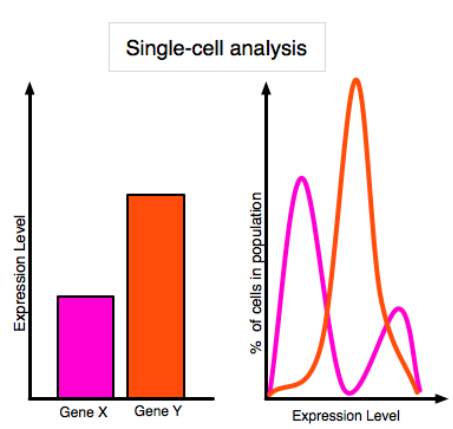
  Many studies of gene expression in stress response have measured the level of expression before the stress induction and at a specific time point after the stress [18]. Such experiments only take into account the change in protein or mRNA level and ignore the time factor of the response (Figure 2.1b). In recent years, many microarray and sequencing experiments are used to generate time course data [15]. These methods, however, are not in single-cell level.

  We aim to collect a series of time-course measurement that will give us the profile of expression over time. We also measure the same culture of cells through the whole experiment to get the time course measurement of the exact same cells population. The combination of single-cell scale and dynamic time course measurement of living cell population will provide us the ability to study the regulatory mechanism of gene expression in more accurate and detailed way.

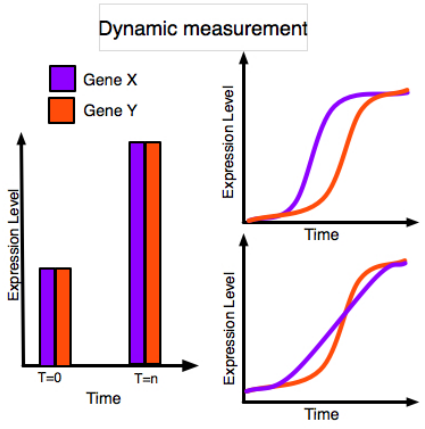
- **Systematic**

  In the past years, large-scale experiments are used vastly in the field gene expression regulation. The advantage of large scale experiments in this area is the ability to investigate a system. Unlike classic methods, high throughput experiments do not concentrate only on a specific gene or protein. Therefore, they may lead to the identification of new factors in the regulatory machinary. In addition, the experiment design in high throughput experiments enables to test different drug derivatives, multiple dose ranges, multiple time points, or cell cultures with unique genetic backgrounds, in a single experiment (Figure 2.1c).

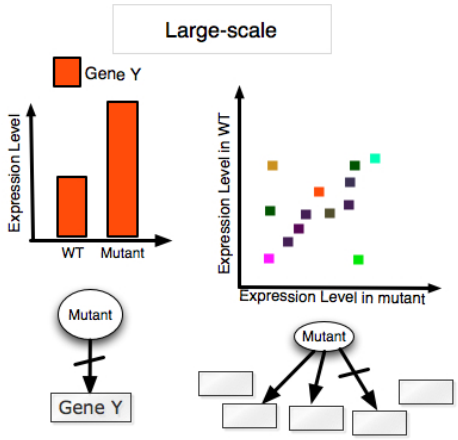
These three requirements motivate us toward an experimental strategy that will enable gene expression investigation with the specificity of single-cell dynamic quantitative measurement and the ability to imply general conclusions of the system and the regulatory network of large-scale experiments.



(a) Single cell measurement



(b) Dynamic measurement



(c) Large scale

Figure 2.1: **An example of the advantage of the system we aim to use** (a) dynamic measurements vs. measurements in 2 single time points (b) measurement in single cell level vs. whole population level. (c) large scale experiments vs. small scale with specific TFs.

2.2 General Strategy

The general strategy we use to carry out large-scale time-coarse gene expression measurement consists of a simple concept of reporter fluorescent proteins and deletion mutations. To measure the effect of a transcription factor on a specific gene, we used strains with a reporter gene and specific knockout mutations. We use automated microscopy system to measure and track the expression level of the gene of interest.

Fluorescent reporter is a gene encoding a fluorescent protein, attached to a gene or reporter of interest and transcribed with it. It enables direct visualization of structures and dynamic processes in living cells [10] (Figure 2.2a). The diversity of fluorescent proteins covers a wide visible spectrum, providing alternative possibilities for multicolor labeling. Fluorescence is detectable after protein folding and chromophore maturation. Many factors can influence the apparent expression level of a fusion construct, including efficiency of transcription, mRNA stability, efficiency of translation, maturation rate, and stability of the protein. We use fluorescent proteins as a protein labels, adding a fluorescent protein in frame with a gene of interest. Genetic construct can highlight localization and expression level of the expressed protein of interest (Figure 2.2b).

Specific knockout mutations are used broadly in genetics. Phenotypic analysis of a strain carrying a deletion mutation of a gene is a powerful way to determine the gene function [28] [6] (Figure 2.2b).

Fluorescent microscopy is an important technique for visualization of cellular organella and macromolecules in the yeast cell. Such microscopic imaging is a challenge in yeast due to the small cell size. Two filters are normally used in this technique; an illumination (or excitation) filter which ensures the illumination is near monochromatic and at the correct wavelength, and a second emission (or detection) filter which ensures none of the excitation light source reaches the detector (Figure 2.2c). We use images in 2 different colors - red fluorescence and green fluorescence. Time-lapse microscopy is a repeated microscopic imaging collection of a specific field of view at discrete time intervals. The resolution is the duration of the time interval between the images. A motorized stage combined with a microscope enables to perform microscopy measurements in high-throughput level.

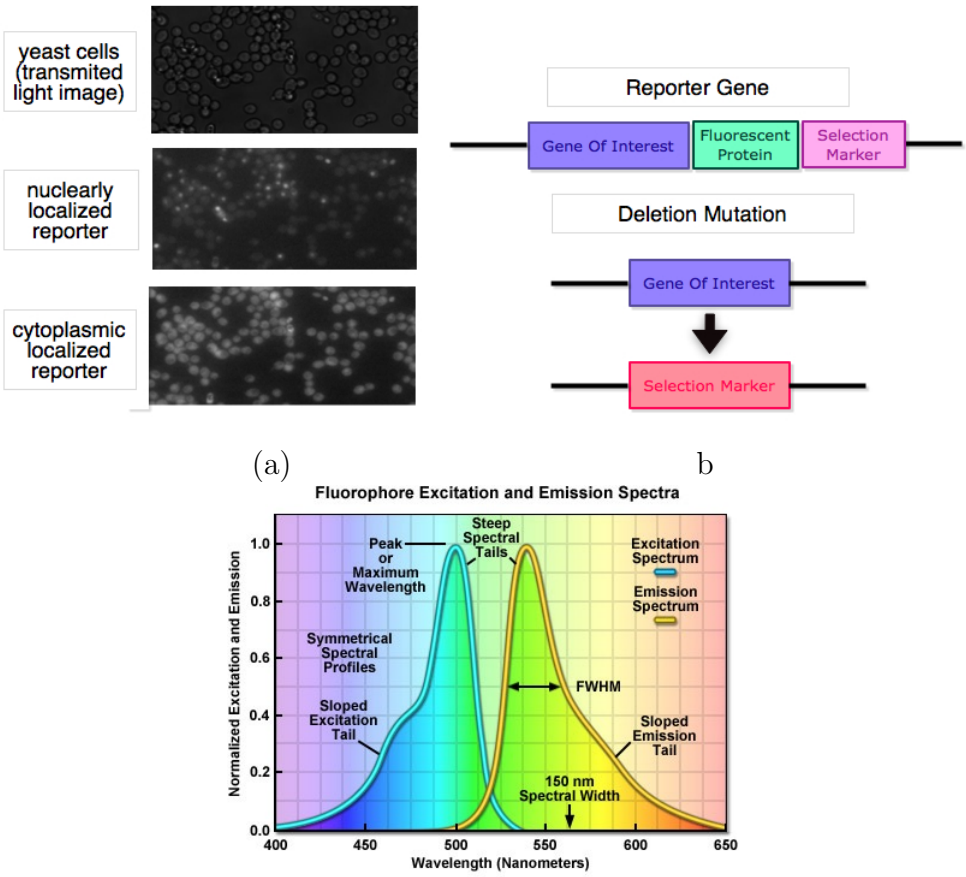


Figure 2.2: (a)localization in images taken with fluorescent microscope. (b) scheme of the genetic design of the reporters and knockouts. (c) excitation and emission spectrum.

2.2.1 Protein level and mRNA level

Gene expression process is a series of biological amplification steps. The first amplification step is transcription, producing many mRNA molecules from a single gene [30]. The second amplification step is translation, producing many protein molecules from a single mRNA molecule (Figure 2.3) [14]. Most of the regulation on this process is done on the transcription step, but its effect is increased when we measure the protein levels. A stochastic and variable behavior in the beginning of the process will generate big variability in the protein level at the cells [8]. For example, a gene whose DNA opening step at the promoter is unstable, but produces many mRNA copies when it is

activated, might have a dramatic diversity in the amount of protein molecules in different individual cells. The disadvantage in protein level measurement is that a new level of regulation is measured and regulatory loops might mask some of the mRNA expression phenotype [16]. Another disadvantage comes from the use of fluorescent proteins as reporters attached to the proteins. This attachment might bother a functional sub unit of the protein or change it's localization pattern. This change in the reporter protein's behavior might have a major effect on the cell's behavior and on the expression level of the reporter protein.

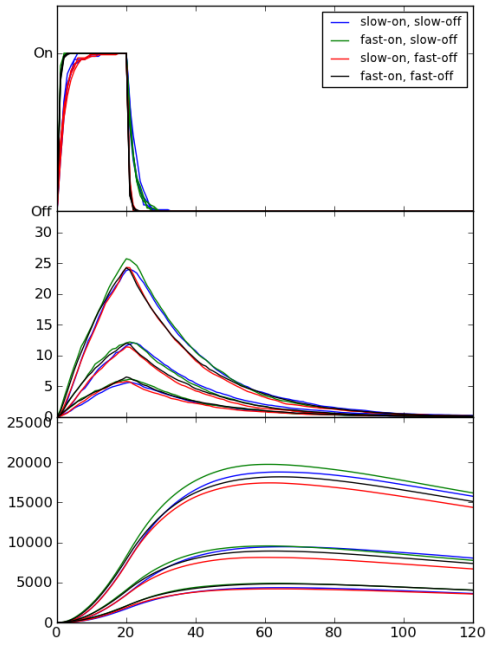


Figure 2.3: Average trajectories of promoter state, mRNA and protein variables (simulation, courtesy of Marek Strajbl). Trajectories are clustered into three groups that correspond to different rates of mRNA production. The color coding indicates the rates of opening and closing of the promoter.

2.3 Goals

The transcription machinery involves a complex combination of signaling pathways, transcription factors, and the generic transcription machineries to initiate and maintain a proper response. There are many additional mechanisms involved in modulating the response that are poorly understood. Many of these components are opaque to traditional genetic screen due to their subtle effect.

Our goal is first to produce a basic time-lapse experiment in single-cell level based on the general strategy of reporter gene and deletion mutation. Next, we aim to establish a system for large-scale measurement of gene expression based on this concept. We try to combine a genetic screen with high-throughput time-lapse microscopy to allow us to use the trajectory of induction in individual cells as a quantitative phenotype. We use this strategy to dissect the role of chromatin modifiers in establishing the dynamics of transcriptional response to an acute stress event (high osmolarity induced by KCl).

Our long-term goal is to dissect the effect of each regulator and examine the effect of combination of regulators in this complex regulatory system.

Chapter 3

Basic Experiments

The pilot experiment aims to prove the concept of the general motivation and strategy of the experimental system, and demonstrate the possible output we can obtain from it. When we first started to establish the protocol for this pilot experimental design at the new laboratory, we had to start from the very basic details. The first task of collecting and calibrating the basic protocols was an inevitable task that had to be done carefully, since many future experiments at the lab will be based on it.

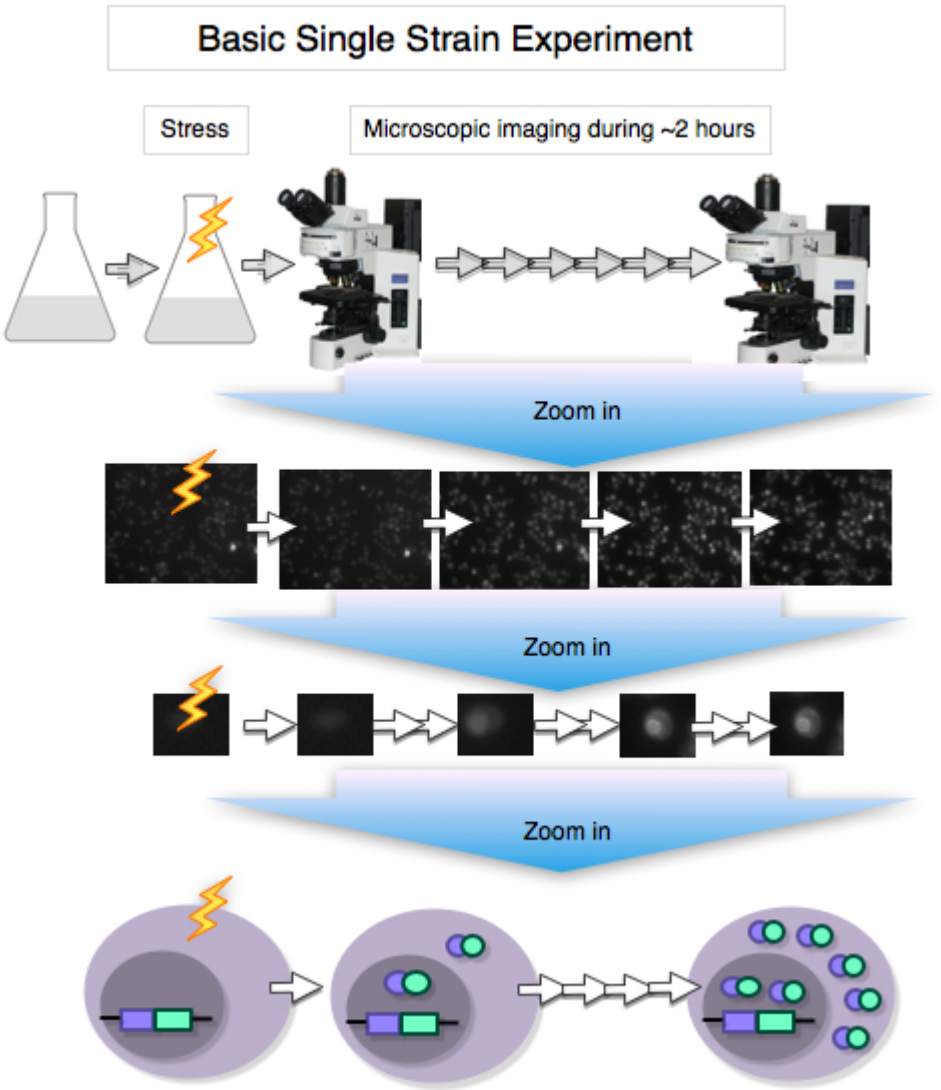


Figure 3.1: Time course microscopy generates measure of the reporter protein level at a single cell level

3.1 Single Strain Experiment

In a basic experiment, to obtain gene expression measurements of a specific gene in response to osmotic stress, we would take a cells culture with a fluorescent protein attached to the gene of interest, bring its media to a 0.4M KCl concentration, and then measure the fluorescence every few minutes during the next two hours. The output of such experiment in our system will be a collection of quantitative traces. A trace describes the dynamics of the reporter gene level in a single cell over time following the stress induction(Figure 3.1).

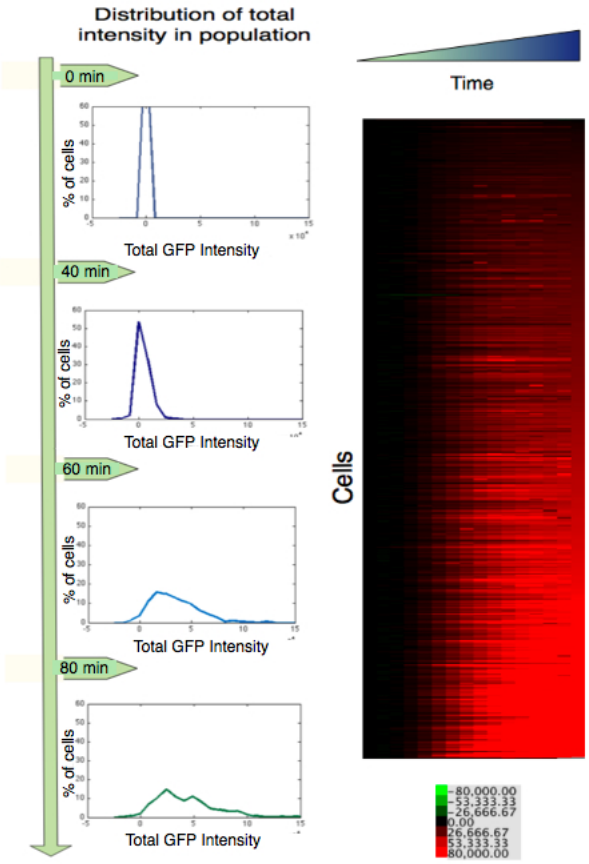


Figure 3.2: Output of single strain experiment with Msc1 as a reporter: the change in reporter intensity distribution over time ,and the traces detected from the image analysis. Each row represents a trace of a single cell.

In Figure 3.2a we see the population in each time point as it is extracted from the images. After the tracking process of each cell we have more accurate information about the cells population. In Figure 3.2b we can see the variability of the expression level and the timing in the measured strain over time. The reporter protein level is lower in a sub-population of the cells. The variability in the response timing is seen in all the levels of expression.

3.2 Comparison Experiment - Theoretic discussion

After we characterize the stress response in a single strain, the next step is to use the results to compare multiple strains (Figure 3.3).

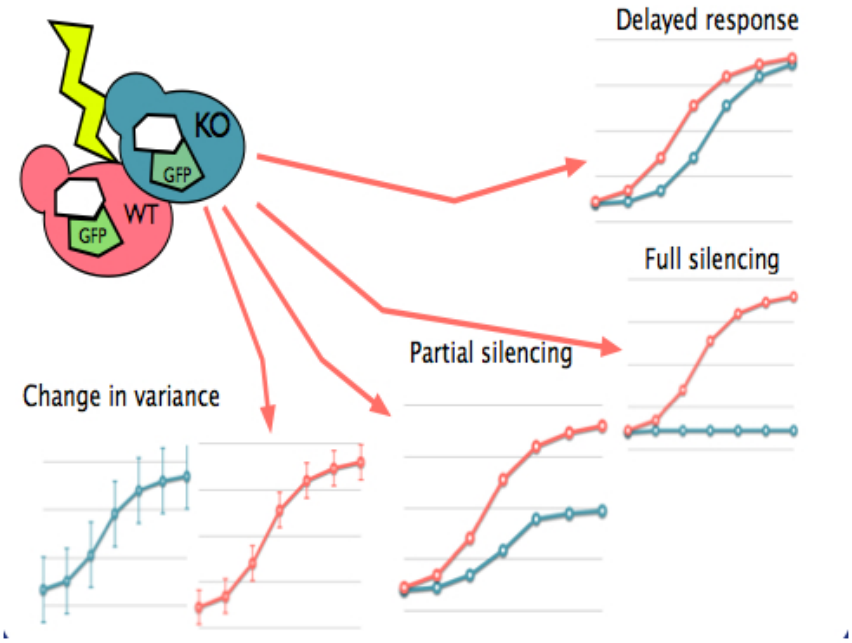


Figure 3.3: Possible outcomes from a comparison experiment

The comparison can be done in two manners:

- Different reporters with a common genetic background - a comparison of this type examines the global influence of the common genetic background.

- Different genetic deletions with a common reporter protein - a comparison of this type examines the effects of different factors on the same target gene.

We wish to compare the time of the response , the level of response, and the variability in both parameters. Some of the possible outcomes are summarized in Figure 3.3. The outcomes can lead to conclusions regarding to the role of the deleted gene or the reporter. For example, in a full silencing outcome, where the XXXΔ mutation silences the WT expression, a derived conclusion can be made saying that XXX is essential for the expression of the reporter gene in osmotic stress condition. The exact role of XXX could be direct activation, repression of another repressor or other. In partial silencing outcome, the derived conclusion can be that the protein XXX has a role in one of the parallel pathways affecting the expression of the reporter gene. Further experiments are needed to conclude the exact mechanism in both cases.

3.3 Example of Comparison between Two Experiments

In a simple experiment, we tested the expression level in a small data set of 9 strains: 3 reporter genes with 3 different genetic backgrounds.

The genetic variants we used in this test are WT (no additional deletion mutation), Hog1Δ strain and Swr1Δ strain. Hog1 is a mitogen- activated protein kinase (MAPK) required for the response to osmotic stress, whose signaling pathway originates at the membrane [3]. The SWR1 complex replaces the histone H2A with the variant H2A.Z at specific chromatin regions. The substitution of core histones with the histone variants can generate a structurally and functionally distinct region in the chromatin [1].

The reporter genes used in this experiment are EMI2 - Non-essential protein of unknown function required for transcriptional induction of an early meiotic-specific transcription factor; TPS2 - Phosphatase subunit of the TPS complex, whose expression is known to be induced by stress conditions; and EFT1 - Elongation factor 2, catalyzes ribosomal translocation during protein synthesis. This choice contains several genetic backgrounds and several reporter genes, thus it allows the examination of the measurements in two dimensions. I note that this experiment was done before the growth conditions calibration. Therefore, I use it only as a test case for our system analysis.

When we compare the response of the three reporters in WT strains under osmotic

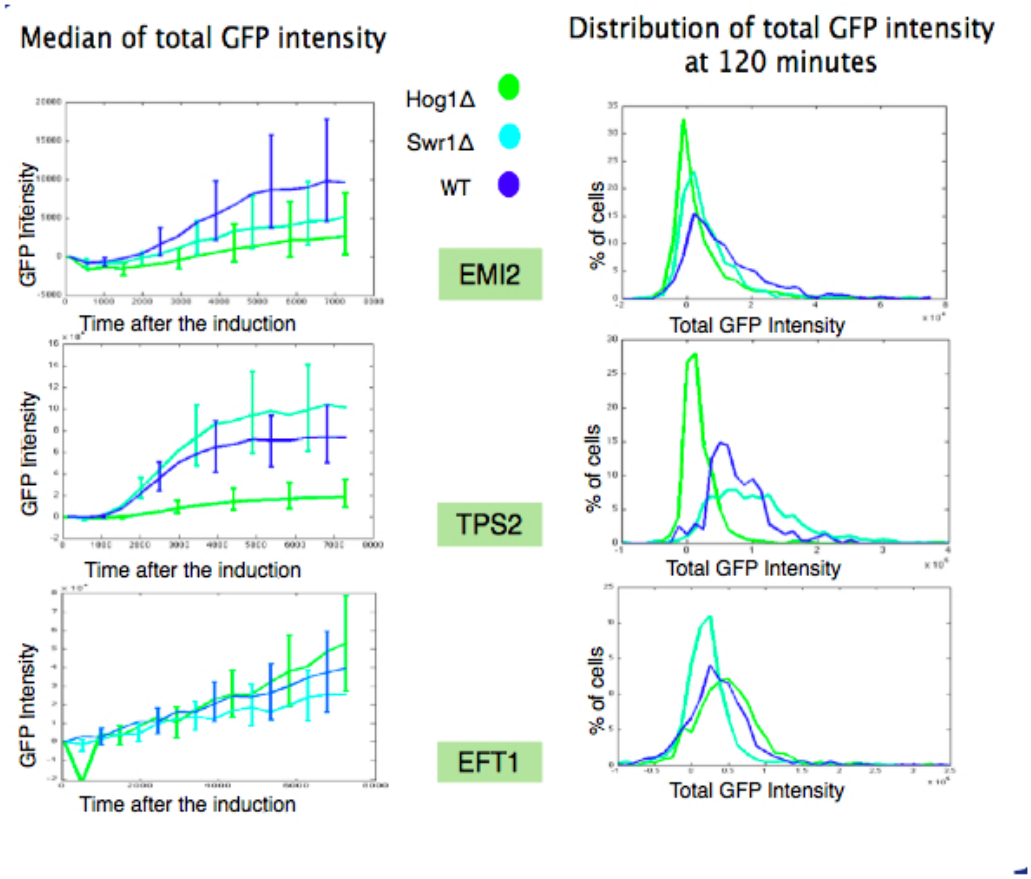


Figure 3.4: The median and population distribution of fluorescence in WT, Hog1Δ and Swr1Δ with 3 reporters.

stress (Figure 3.4) we can conclude that EFT1 does not have a significant role in the osmotic stress response. Therefore, its expression is unaffected by the stress (we see a constant increase in protein level, regardless of the shift to stress conditions), while the induction of TPS2 is stronger and faster then the induction of EMI2. If we examine the level of expression in the mutant strains, we can see that the influence of swr1 deletion on the expression level can be positive or negative. Swr1 has a role in the regulation of expression of two of the reporters but it is not the same role. This kind of result could be due to several scenarios - indirect influence, competition in the cell, or gene specific behavior of the swr protein. This outcome is expected in chromatin remodeling

proteins that change the accessibility of the genes, and does not serve as a typical activator/repressor of a group of target genes. Another clear result is that hog1 deletion silences the reporter gene TPS2. This suggests that Hog1 has a role in the signal transduction process that eventually leads to the high expression of the TPS2 reporter genes under stress induction. We can also notice the difference in distribution of the intensity in the population in the different response patterns: in Swr1Δ and WT strains, with TPS2 reporter, the main difference between the strains is in the variation in the population. In Swr1Δ and HogΔ with EMI2 reporter the main difference is in the part of non-responding cells in the population. To get to a clear conclusion, we need to perform further experiments - using larger reporters and deletions set, double knockouts or other molecular and structural methods.

Chapter 4

System

The main goal of this work is to establish an experimental system that will enable lerge-scale experiments of gene expression measurement based on the simple concept of reporter proteins and deletion mutations. In this chapter I describe the establishment of a systematic experimental protocol and the calibration process toward it. The main steps in this process are strain construction, growth of the strain cultures for measurement, the actual microscopic measurement and the image analysis. I will explain in detail the requirements and the methods used in order to execute each of the experimental steps and to adapt it to high-throughput format (Figure 4.1).

4.1 Strains Construction

In this experimental system we use the basic concept of knockout mutation and a reporter gene to investigate the relationship between a regulatory protein and the expression of the reporter gene. In practice, to carry out this concept we decided to construct a collection of strains, each strain contains a green fluorescent protein (GFP) connected to a reporter gene and a knockout of a potential regulator.

The construction of a new strain with a reporter-protein and a mutation is usually done using a molecular process such as mating and homologous recombination. The constructed strain, originated from a single cell, was identified and verified using a selectable marker. These classic methods are still used for a low throughput strain construction, but they are not feasible when we need to construct a library with a large amount of strains. To overcome this challenge and construct a collection of strains we use a sys-

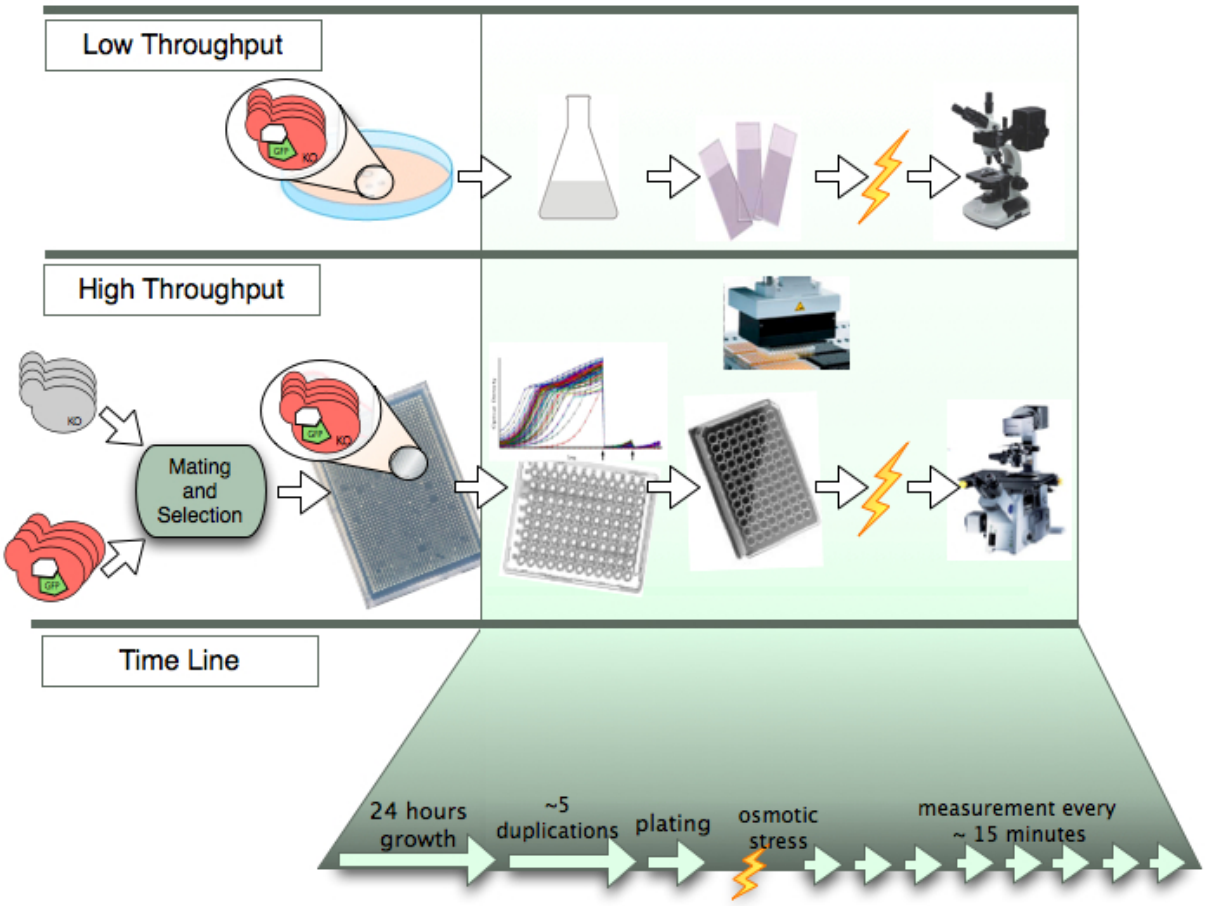


Figure 4.1: The experiment flow and timeline in low throughput and in high throughput

tematic mating and selection process. This process was first introduced in a method called Synthetic genetic array (SGA). [26]

SGA methodology is a systematic method that enables the construction of double mutants and the mapping of the synthetic genetic interactions in large scale. A typical SGA analysis involves two main steps - a library construction step and a phenotype measurement step. The collection construction step usually involves a cross of a query strain to the array of viable deletion mutants, and through a series of replica-pinning procedures, haploid double mutant colonies are selected. In the original SGA experiments, the phenotypic measurement step consisted of the scoring of each colony according to its growth defects. Applying SGA analysis to several query mutations enabled learning of

genetic interaction network with functional information associated with the position and connectivity of a gene on the network . The SGA methodology can be used in variety of experimental designs, with various strains in the construction step and various phenotypic properties in the measurement step. Any genetic element marked by a selectable marker can be crossed into any ordered array of strains, to construct a new collection.

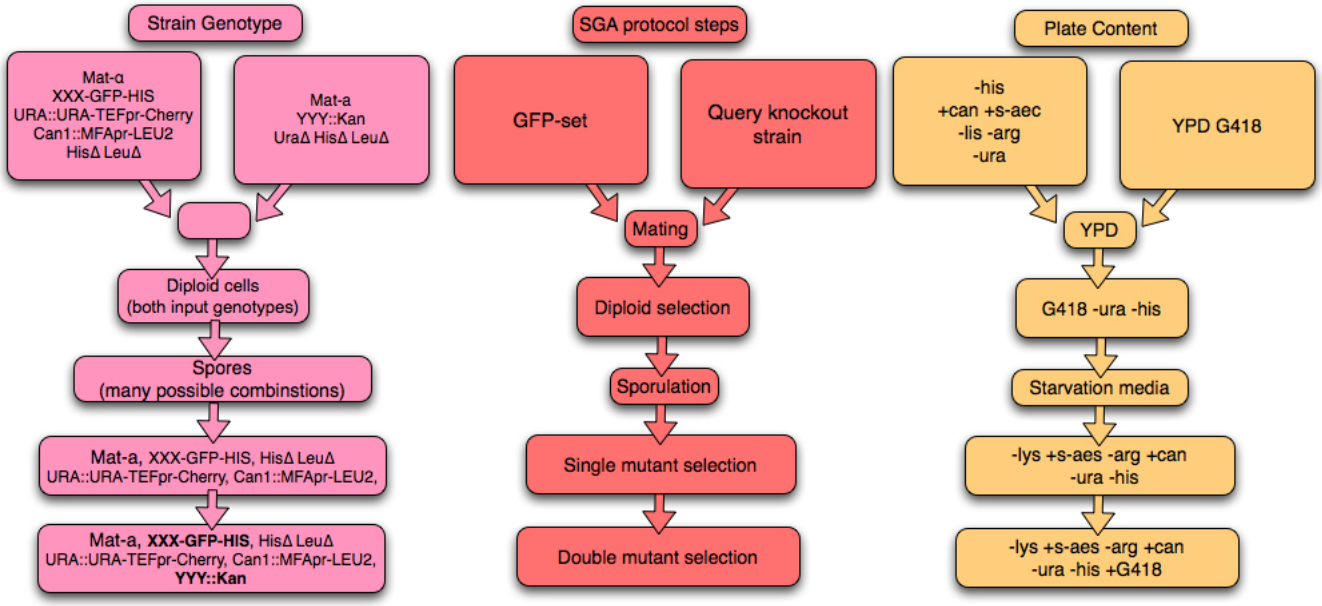


Figure 4.2: **The SGA protocol in our mutation-reporter experiments:** The mating and selection steps, the appropriate selection plate and the selected cells genotypes.

I used mating and selection process in a similar way to the process used in the SGA protocol with several changes (Figure 4.2). The construction of the strains collection with the SGA protocol requires a distinct design of the strains that will be mated in the beginning of the process - a query strain and a source library. As a query strain, I constructed a strain with a precise deletion mutation. In the past, several approaches have been proposed to generate mutations including genetic footprinting and random mutagenesis. Genetic footprinting has the advantage that all genes can be tested for their contribution to fitness under a particular condition relatively quickly, but the tested strains cannot be recovered. Random mutagenesis is relatively rapid, but the subsequent matching of phenotypes and mutant genes is slow. These limitations can be overcome

by deleting each gene in the genome in a directed fashion. The deletion mutation for this system was generated using PCR-mediated strategy that exploits the high rate of homologous recombination in yeast. For this method, short regions of yeast sequence identical to those found upstream and downstream of a targeted gene are placed at each end of a selectable marker gene through PCR. The resulting PCR product, when introduced into yeast cells, can replace the targeted gene by homologous recombination. I used a PCR reaction to verify the mutants and to make sure that the strains will not contain any copy of the knocked out gene. As a source library we decided to use a library based on the yeast GFP collection. This collection contain ∼6000 yeast strains. In each strain a single ORF is tagged in its 3' end in the chromosomal location with GFP. For our experiment we used a library constructed by Michal Breker (Berker & Schuldiner, Personal Communications), by mating the GFP library with a strain expressing constitutive cytoplasmic mCherry. The genotype of the GFP-cherry library will be used for selection with the SGA protocol:

- xxx-GFP::HIS3 - where xxx is the reporter gene, His3 is the selection marker
- Tef2-cherry::URA3 - The cytoplasmic mCherry protein, Ura3 is the selection marker
- his3Δ1 leu2Δ0 met15Δ0 ura3Δ0
- lyp1Δ can1Δ::MFA1pr-LEU2 - can1 and lyp1 deletion will be used for selection of haploids.
- The MFA1pr-LEU2 will be used for selection of a mating type cells

According to the SGA protocol, we used a series of selection steps, each step requires replica pinning on an appropriate plate.

- The diploid selection was done on plates containing G418 as a selection for the deletion and without histidine nor uracil as a selection to the GFP reporter with mCherry background.
- The haploid selection step was done with the same selections for deletion, GFP marker and mCherry background, with additional selection for haploid cells: canavanine as a selection for haploids without arginine, s-aec as a selection for haploids without lysine, and without leucine as a selection for mating type.

4.2 Growth Protocol

In the laboratory, yeast can be grown in either liquid media or on solid agar plates. Yeast cells grow differently under different conditions. The cells density in a culture over time can be described as growth curves. The growth curve on glucose media has four major phases (Figure 4.3): lag phase, log phase, early stationary phase, and late stationary phase. At the lag phase, the cell density increases slowly. The lag phase duration and rate depends on the source of the culture. At the log phase, the exponential growth phase, cells duplicate exponentially. They start to grow on glucose, and they use this rich source of energy to grow as fast as possible by fermenting it to ethanol. The duplication time depends on the strain and on the growth conditions (temperature, growth media). After a while the yeast will start exhausting the sugar and they shift from using glucose to ethanol in a *diauxic shift*. At The early stationary phase, the saturation phase, the cells still grow exponentially but the doubling time is much longer. Cells are stressed at this phase. At The late stationary phase, after the ethanol reserves are consumed, the yeast stops growing. The cells prepare for nutritional hardship and reduce their activity.

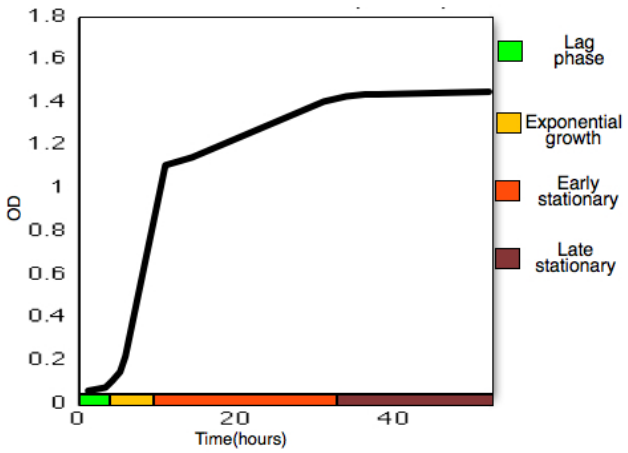


Figure 4.3: growth cure scheme

As yeast cells adapt to their environment, the expression pattern at the cell is different in every phase of the growth. The expression pattern in the cell in response to stress also depends on the state of the cell when the stress is induced. As a first step I did a simple experiment to check weather the time passed since the cells were in the stationary

culture has an affect on gene expression and on the stress response. I performed this experiment with several strains, including:

- Msc1- Protein of unknown function;
- Hxk1- a cytosolic protein that catalyzes phosphorylation of glucose during glucose metabolism; the expression is highest during growth on non-glucose carbon sources.

I diluted a stationary culture of each of the tested strains in different time points before the salt induction time. The induction ratio was calculated so the final OD will be ∼0.5 in all the diluted cultures.

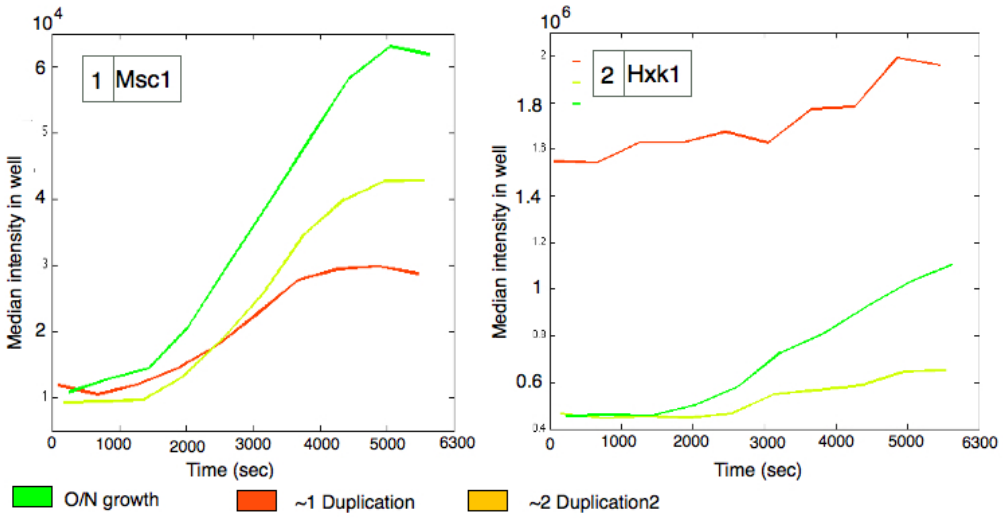


Figure 4.4: **growth test results - Median intensity over time after salt induction** (1) in Mcs1 (2) in Hxk1

Figure 4.4 describes the median GFP level in the cells population over time. In Msc1(Figure 4.41) , a reporter with a dramatic stress response, the most significant effect was seen in the overnight culture that had more than 12 hours since the stationary stress. The same phenomenon was seen in other reporter genes that I tested. In the single duplication culture, 30 minutes after the induction a main part of the population was still not expressing GFP while in the overnight culture all the cells were expressing GFP at this point (Figure 4.5 2). The difference between the populations is not only in the level of expression but also in the timing. In Hxk1 (Figure 4.42), the population that had only 1 duplication still expresses proteins that were produced during earlier

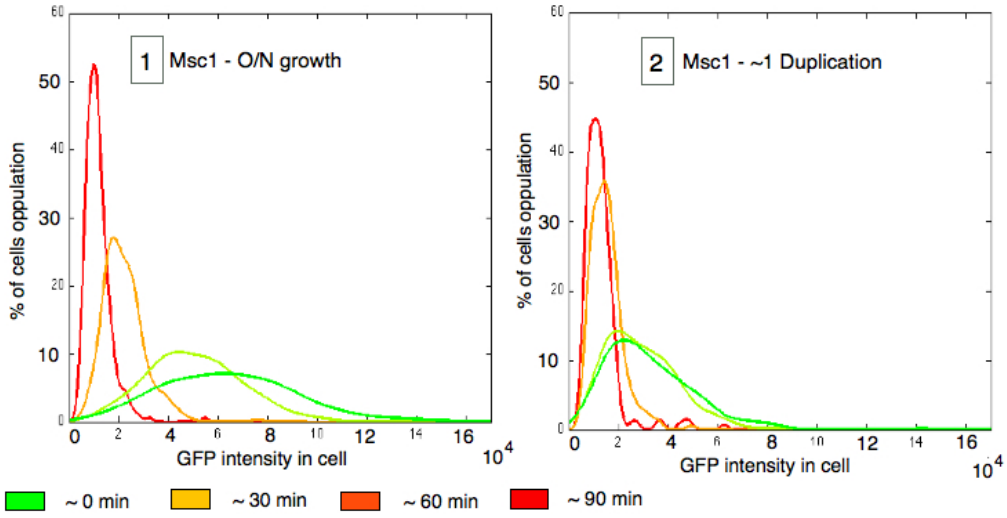


Figure 4.5: **growth test results - Histogram of the population in time points after the salt induction** (1) in a culture with O/N growth since the saturation (2) in a culture with 1 duplication since the saturation

stationary growth on non-glucose media.

The practical conclusion from this test is that we want for our system to use cells in the middle of the fast growing exponential phase. Moreover, to make sure that the yeast culture forgot its history, we want to make sure that there were several cell divisions since the lag phase. We also want to make sure the yeasts do not go through another diauxic shift. Another requirement is to bring the culture to a specific cells density that will fit to the microscopy protocol. Meeting both requirements is extremely challenging when dealing with different strains that differ in their duplication rate.

The task of bringing the culture to a specific range of density is further complicated when we move to large-scale experiments. In our system, we work with a plate containing 96 or 384 different strains at a time, each with different growth rate. In some cases the doubling time of strains ranges from 90 min to 120 min, implying that in 6 hours the fast strain will multiply by 16 while the slow strain only by 8. To overcome this challenge we use a robotic platform. This platform has 3 robotic arms. The robotic movement arm is responsible for moving plates from one place to another (incubator, plate reader, table). The 2 liquid handling arms are able to perform pipette operations of media or yeast culture in parallel for a plate or in single well. The strategy we decided to use in order

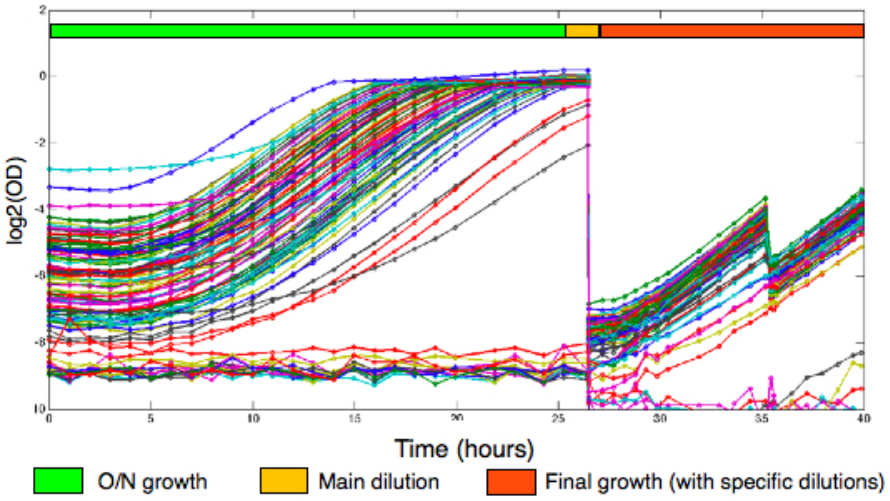
to follow the requirements in the large scale system consists of 3 steps (Figure 4.62):

- The first step - Overnight growth
  During ∼24 hours the strains are grown in a 96 well plate, and the OD in each well is measured once in ∼2 hours. At the end of this step we calculate the growth rate of each strain in its exponential growth phase. This step ensures that all strains are in an early stationary phase and thus will recover relatively quickly when inoculated into fresh media. In addition most strains reach similar densities after this step (see below).

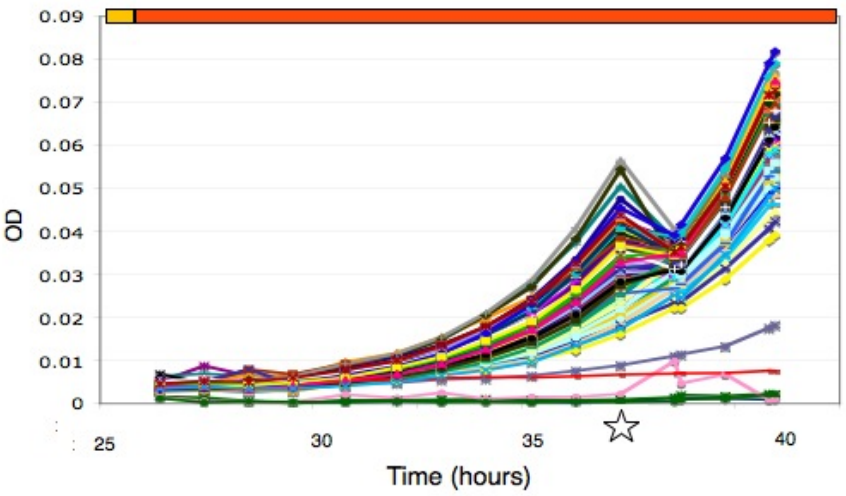
- The second step - Main dilution
  All the strains are diluted into fresh media through several dilutions cycles in a ratio of ∼1/400. Following this step the culture is starting to get out of the saturated condition.

- The third step - Growth to mid-log
  At this step we perform OD measurement every ∼60 minutes. For each well, we use the growth curve from the first step to calculate its growth rate at the exponential growth phase. We estimate the density of the culture in the well at the end-time according to the calculated rate and the current OD. We decide to dilute the well if the culture started to grow in exponential rate and the estimated end-time OD is above the target range that we allow. The dilution ratio will be the ratio between the estimated end-time OD and the desired end-time OD. We perform this decision and dilution to make sure that at the end of the process the culture will be in log-phase and within the wanted range of OD, and that after the main dilution it will not get saturated (Figure 4.62).

This strategy is problematic since the rate of growth in the third step might be different from the rate calculated in the first step, leading to a lack of accuracy of the estimated end-time OD. A direct consequence will be a wrong dilution of wells in the third step, and those wells will not get to the desired OD. The change in growth rate comes from the temperature at which the plate is staying during the frequent measurements and dilutions, and from the initial OD in the current growth curve (Figure 4.7) . To overcome this challenge, we can make an adaptive estimation of the growth rate that using the O/N measure as prior.



(a) Growth process

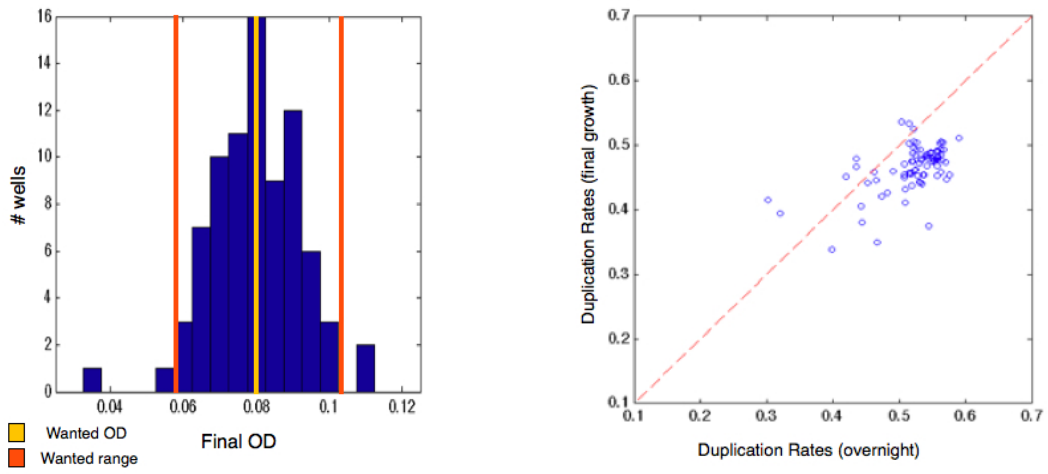


(b) Final growth step - between the main dilution and the end of the process

Figure 4.6: **OD measurement of single 96-well plate during the growth protocol** (1) The whole growth process (2) The final step of the protocol. The star indicates the specific dilutions time.

4.3 Plating and Time-Course Imaging

The time-lapse microscopy of large set of strains requires a whole new protocol and considerations, from technical motorized system, through special microscope plates to



(a) (b)

Figure 4.7: (a) The OD histogram of the plate at the end of the protocol (b) The duplication rates at the O/N step relative to the rates at the final step

imaging time optimization. Time-lapse microscopy is a the repeated microscopic imaging collection of a specific field of view at discrete time intervals. The resolution is the duration of the time interval between the images. We aim to have measurement each ∼15 minutes, over 2 hours. A motorized stage combined with a microscope enables to test different drug derivatives, multiple dose ranges, multiple time points, or cell cultures with unique genetic backgrounds.

After bringing the yeast cultures to the requested cells density, we plate them on a multi-well microscopic plate to start the plate preparation process. The microscope plate we use is a glass-bottom multi-well plate with homogenous in flatness and thickness. The microscopy protocol parameters and details were tested and determined to produce reliable measurement. The goal of this protocol is to produce microscopic plates with several restrictions. They were defined to guarantee 3 parameters: Time lapse imaging, object detection and cells condition.

To enable time lapse imaging we need to keep the cells in the same location through the whole experiment. To do so we coat the microscopy glass-bottom plate and let it dry. The concanavalin A (conA, carbohydrate-binding protein) coating protocol was calibrated to get an even level of conA. Figure 4.3 is part of an image taken on a plate covered with conA before the calibration. The clouds in the image are cells in a different

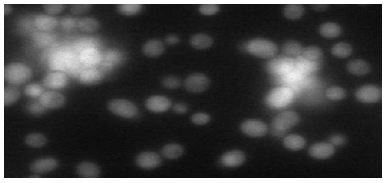
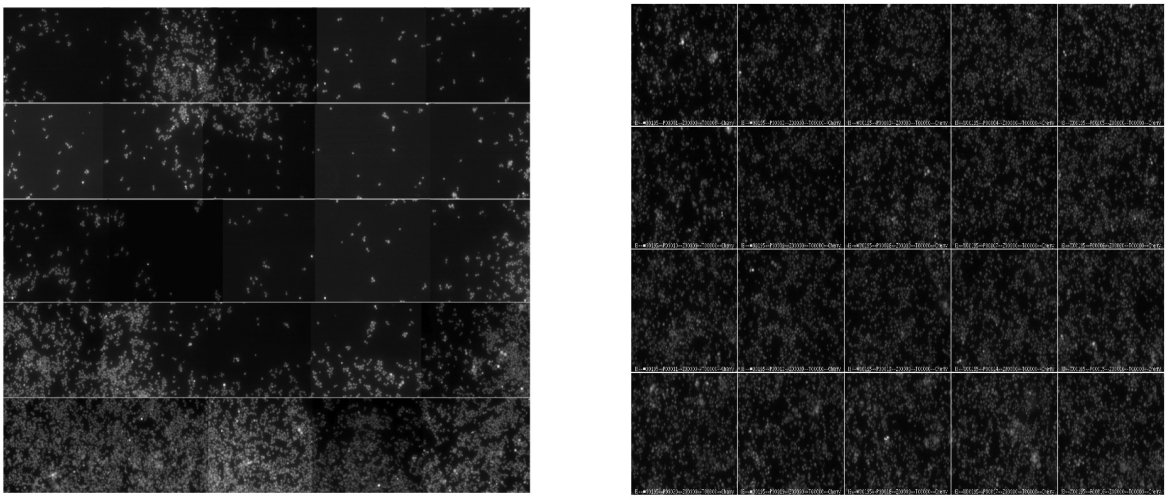


Figure 4.8: Image taken before the coating protocol calibration

focus plane. We add the yeast culture on the dried plate and let them descend on the conA and attach to the plate bottom. To prevent landing of cells in the field of interest during the experiment, we apply several washing steps with specific parameters, in which we dispense and aspirate new media in the cells. The wash process should be accurate and slow- to wash the wells properly, but prevent displacement of the cells on the plate. A displacement caused by plating with the wrong parameters (Figure 4.9) will cause the release of the cells from the plate, which will lead to an heterogenous number of cells over the well and a decrease in the cells number in the measured population. During the process, we minimize the time during which the cells are not located in a liquid media environment, so the only stress condition will be the stress we will induce later.

Reliable autofocusing methods are also indispensable for the routine use of microscopes



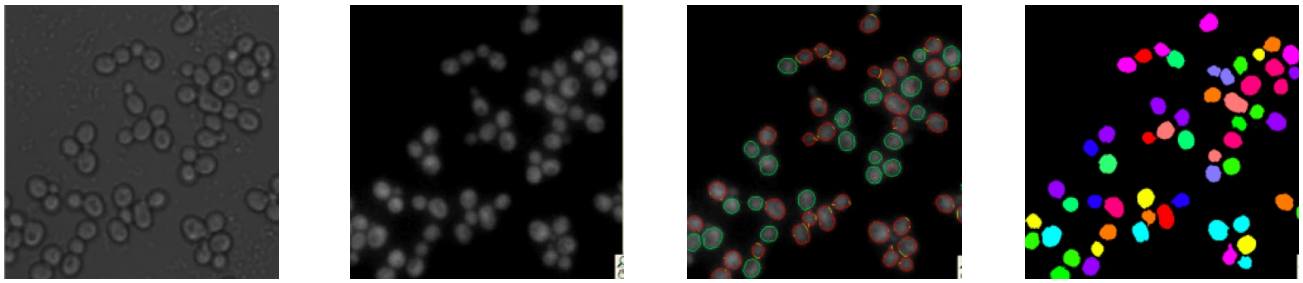
(a) before (b) after

Figure 4.9: **The surface of the plate in adjacent fields** (a) before the calibration of plate preparation protocol (b) after the calibration

on a large scale. This may be a challenge because of many reasons, including the mechanical instability of the microscope and the irregularity of glass slide surfaces. The autofocusing time should be optimized to enable microscopy of a plate in 15 minutes, to provide a sufficient number of time points after the induction.

4.4 Image Analysis and Object Detection

The complex experimental process described above is calibrated to produce a large set of images of good quality that will enable the derivation of quantitative measurements of the intensity level in each cell. The goal of the image analysis step is to provide the reliable quantitative time-coarse measurement that will represent the expression level in the cell. The detection of the cells in the images was done by the ScanR Analysis program (Figure 4.10). We used a library expressing a cytoplasmic mCherry fluorescent protein to enable a better cell detection with the image analysis program. With the images of mCherry (Figure 4.10b) it is more convenient to define the cell shape and edge line, and to separate the interface between neighbor cells. The intensity of the mCherry protein also gives us information about the cell state.



(a) Transmitted light (b) mCherry (c) edge detection (d) final objects

Figure 4.10: **The steps of object detection**

The image analysis process produces several measures for each cell object, such as cell area, cell elongation, GFP intensity and mCherry intensity. There are several ways to define the expression level of a reporter protein in the cell using these measures, each way having a different biological meaning:

- Total intensity- The total intensity of GFP in the cell gives us information about the amount of the reporter GFP molecules in the cell. Therefore, from the difference in the values of the total intensity in the cell before and after the induction

we can learn about the amount of reporter-GFP molecules transcribed since the induction.

- Mean intensity - The total GFP intensity in the cell devided by its size. This is actually a normalization of the intensity to the cell size.
- Ratio of GFP and Cherry intensity - If the levels of mCherry in the cells indicates the size and the transcriptional state of the cell, this measure gives us a normalization of the GFP levels with both cell size and cell state.

The problem with measures based on cell size comes from the change in the cell size during the experiment. When the cells are exposed to the salt media, they first shrink and then swell slowly. In different strains there might be a different profile of this change of cell size in response to the salt environment. The cell state normalization is also problematic, since it is not clear if the expression Tef2-mCherry promoter is informative for the purpose of cell state normalization. We can use each intensity measure as a fold change relative to the level before the induction or as the difference in the intensity between the time points. The reduction of the GFP level before the induction gives us a sort of normalization in the cell transcriptional state, but it ignores the initial level and we will get difference of X if the initial level was X or if it was 10X. The ratio of expression is informative and it provide ratio relative to the initial measurement, but it is problematic especially for stress proteins with very low basal expression. The question of which parameter is the right parameter to use in order to demonstrate the level of a reporter protein in the cell is critical for the accuracy of the expression levels we produce and their biological meaning.

4.5 Gating

Clearly, not all the cells on the plate are well-detected, and among the detected objects there are objects that we want to drop out. In the gating step we aim to select only the population of objects that we wish to use for the experiment result. The gating process is done according to the cell area, the cell elongation factor (a measure of the circular shape of the object), the cell circularity factor (a measure of the quality of the object closure during the detection) and the mCherry intensity. There are few types of objects that we wish to eliminate from the data:

- A dividing cells will have different expression patterns because of the duplication process.
- Unidentified object - dust or a cell in a different focus plane.
- Neighboring cells that the image analysis program detected as a single object
- A trace that is actually a combination of the traces of two cells, caused because of the movement of cells on the plate during the experiment.

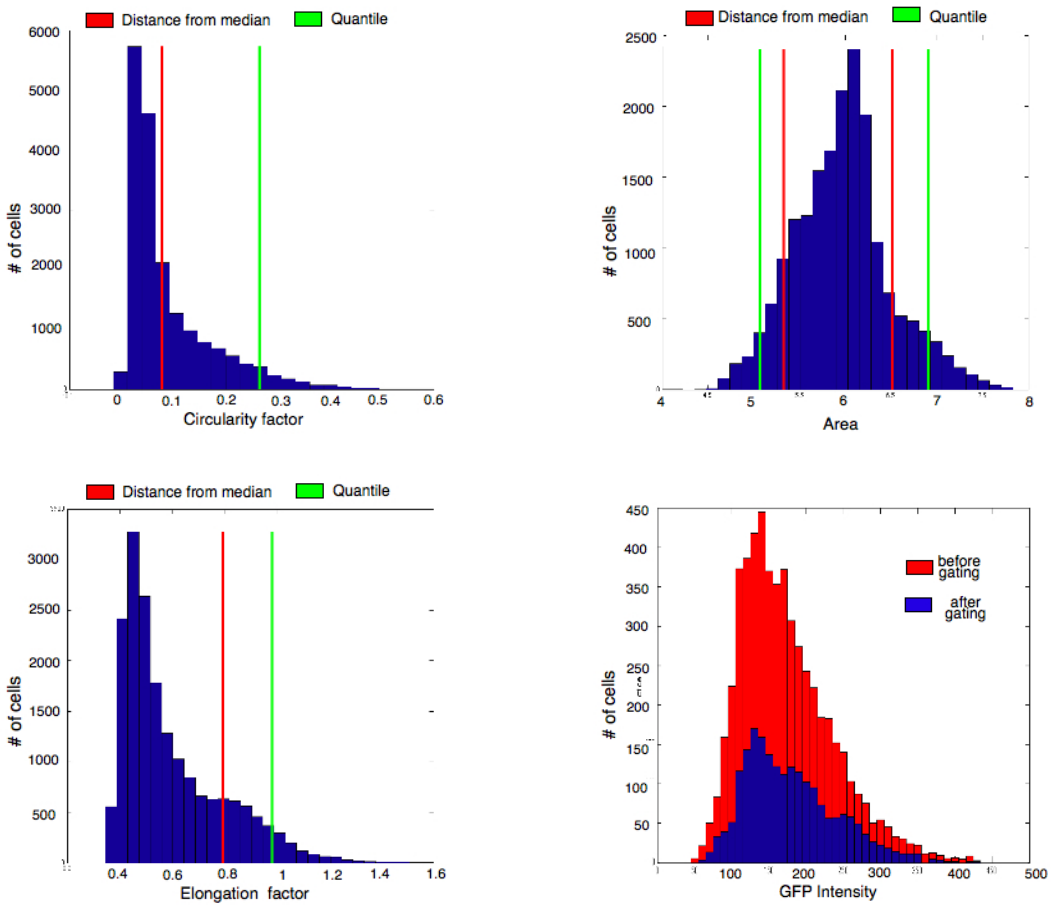
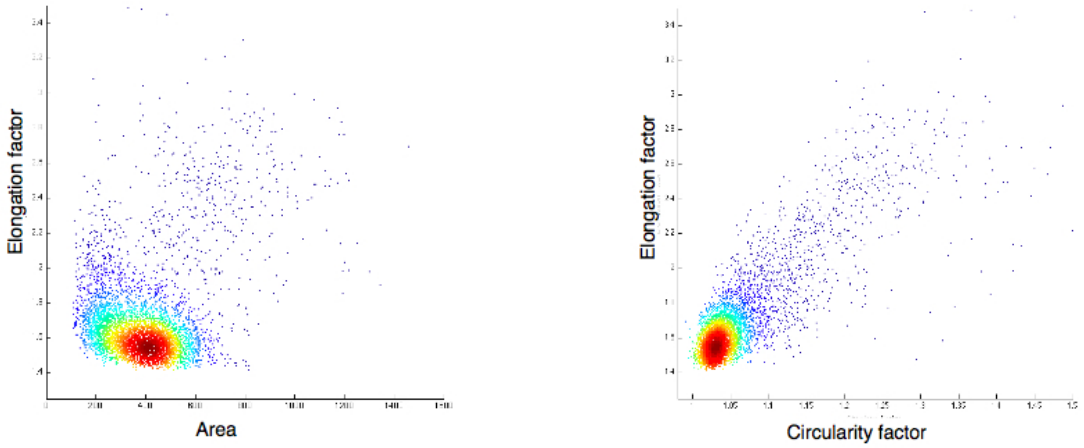


Figure 4.11: **The distribution of the parameters in the population: The gating parameters** (a)circularity factor (b) area (c)elongation, **And the measurement parameter:** (d) the GFP distribution before and after the gating

There are two main strategies to identify and eliminate these objects. The first strategy is a single time point gating. We gate the cells population in each image



(a) area vs. elongation (b) circularity vs. elongation

Figure 4.12: **Comparison of parameters distribution and density** (a) area vs. elongation (b) circularity vs. elongation

according to the distribution of each of the parameters. We will remove the trace of a cell if it has the gated property in one of the time points. We tested two ways to gate the population in a single time point:

- Median - gate the cells according to the difference between their parameters and the median value of the population.
- Quantile - gate a fixed quantile of the population.

In the area distribution (Figure 4.11b) the two strategies are similar, and we would like to gate according to the quantile since we want to enable cells of variable sizes. The elongation and Circularity factors (Figure 4.11a,c) are scores given to the cell and in the non-symmetric distributions we will choose only the cells which are close enough to the score of most of the population. A lower gate is not relevant in this case. The gating by circularity and gating by elongation is usually redundant, while the gating by area is different and require a different condition (Figure 4.12a,b). The distribution of the GFP intensity (Figure 4.11d) can be used to verify that the gated cells were not an interesting sub-population in this well. The second strategy is time-lapse strategy. In this strategy we examine the change of each of the gating parameters through time. We will find objects that are not necessarily eliminated according to the gating decision

in each single time point but from the trace we conclude that they are not part of the population we would like to measure. Obviously, we want to enable some level of change in the cell properties, but tracking these changes can help in identifying problematic traces.

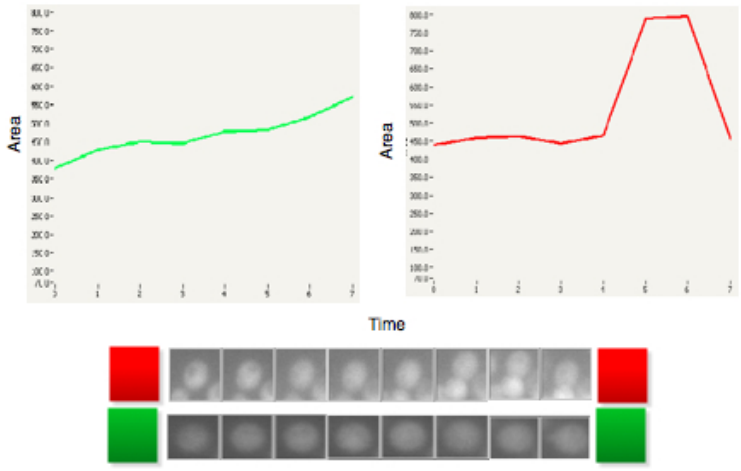


Figure 4.13: **Images gallery and a plot of single cell area over time**

Chapter 5

Osmotic Shock

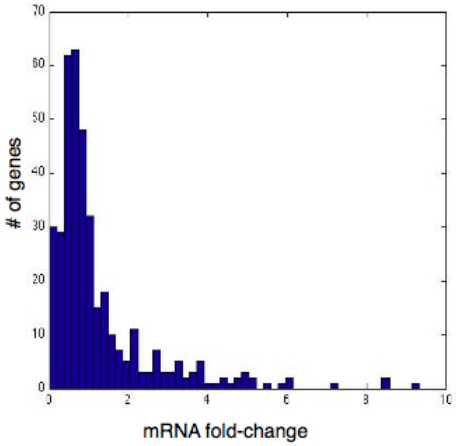
5.1 Osmotic Stress Response and Signal Transduction

Yeast cells sense their external environment condition and adapt to it. A complex signaling network governs the response to osmotic stress condition. MAP kinase cascades are important components in cellular adaptation programs. Each MAPK cascade is composed of three sequentially activating kinases. The budding yeast has at least five signal pathways containing a MAP kinase cascade. The high osmolarity glycerol (HOG) MAPK pathway is essential for yeast survival in osmotic stress condition [3]. In unstressed cells, Hog1 localizes evenly throughout the cell. Following osmotic stress, activated Hog1 accumulates in the nuclear compartment, where it participates in a modification of the transcriptional pattern. Hot1, for example, is a transcription factor that targets Hog1p to osmostress responsive promoters. It is required for the transient induction of glycerol biosynthetic genes in response to high osmolarity. The Msn2 and Msn4 transcription factors also play major roles in the yeast general stress response by mediating the transcription of hundreds of genes [4] [5].

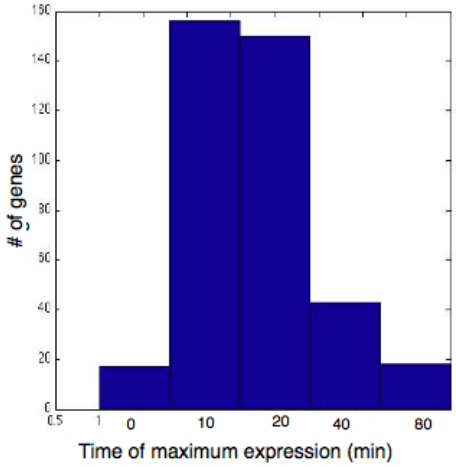
5.2 Reporters Collection

In order to examine the influence of chromatin remodelers on osmotic stress response, we choose a set of reporter genes. The set was chosen to contain strains that showed

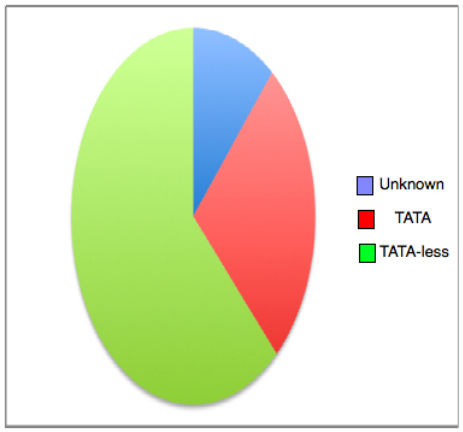
a variety of expression levels in stress in previous experiments. It also contains control proteins that are not predicted to change their expression level in response to the stress. Figure 5.1a describes the RNA expression of the genes in our set as measured in previous mRNA sequencing experiment. Figure 5.1b describes the timepoint after the salt induction when the maximum expression was measured.



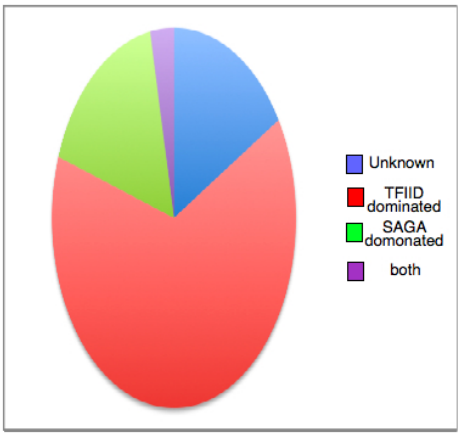
(a) mRNA expression



(b) mRNA timing



(a) area vs. elongation



(b) circularity vs. elongation

Figure 5.1: **Characteristics of the reporters set** (a) mRNA expression level (b) time of maximum mRNA expression (c) TATA/TATA-less promoters (d) SAGA/TFIID

Obviously, we don't expect the same timing and level output from our experiments, since the measurements here are of population RNA measurement and in system we measure protein level. Yet, in future experiments we will be interested in the relations

between the two measures.
We will also notice some mechanistic characters of the set (Figure 5.1c,d). These characters will help us to check the experimental system later and affect the choice of knockout genes.

5.3 Single Plate Analysis

The following examples were generated in experiments that were carried out with the wrong microscopic parameters, and therefore they show informative but noisy measurements.

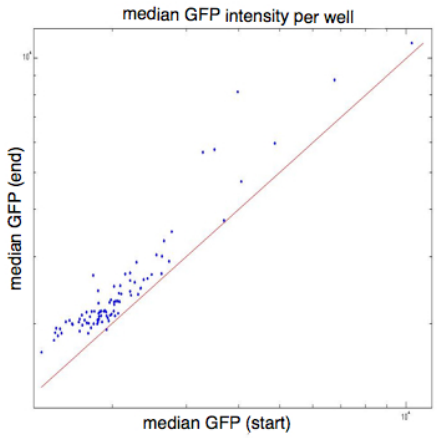
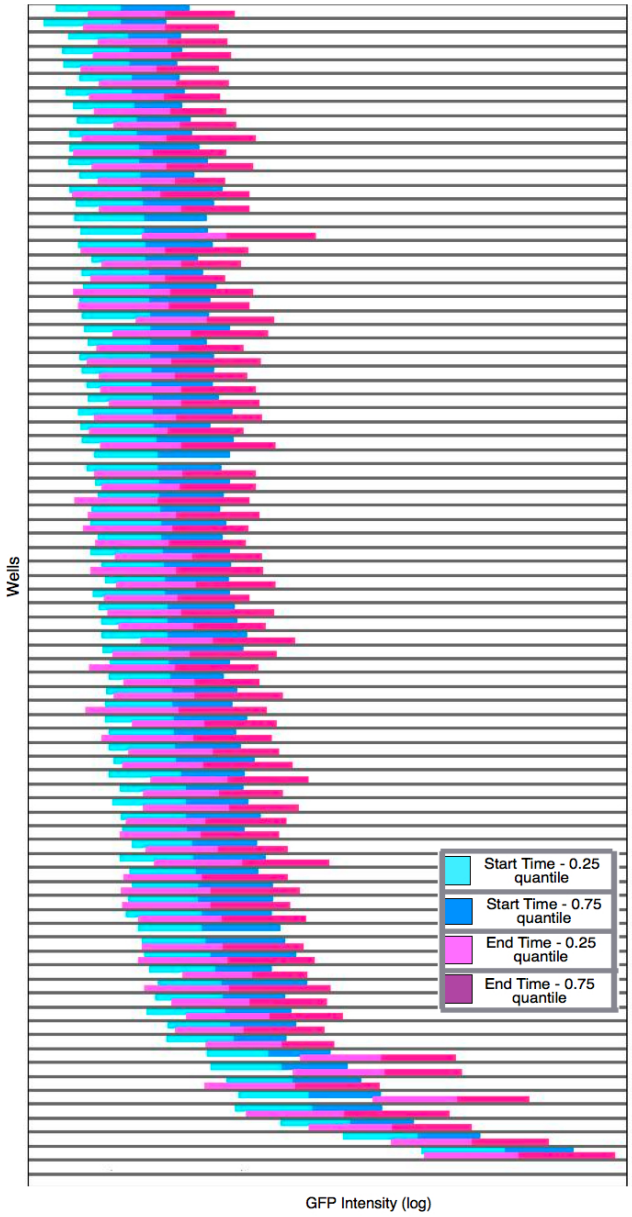


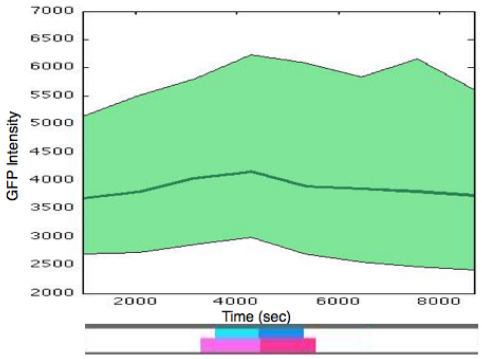
Figure 5.2: median GFP intensity, last vs. first measurement

When we examine the stress effect on a single plate, we actually examine the variation between the reporter genes under common genetic background. We compare the expression in each strain to the expression before the stress induction. The comparison of the median expression in each well in a WT plate (Figure 5.2) shows that after the induction almost all the reporter genes are expressed in higher level than before the induction. This observation might be due to a change in the cell detection caused by photo bleaching, a change in the actual cell size or additional stress caused to the cells during the imaging and plating process. Yet, we clearly notice that some of the strains

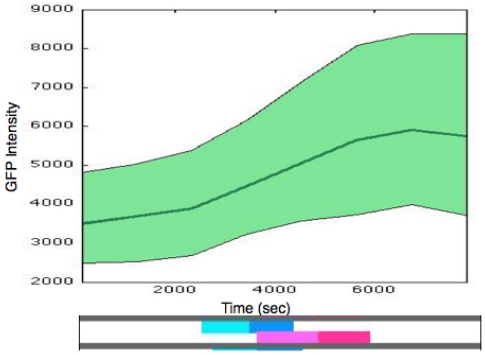
show higher induction then others.



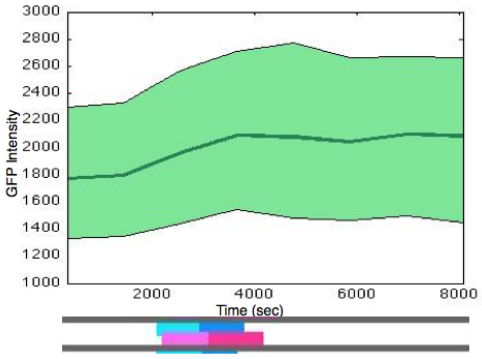
(a)



(b) reporter strain #86



(c) reporter strain #87



(d) reporter strain #82

Figure 5.3: (a) the medians and quantiles of a plate in start time and end time, sorted by the median expression in start time (b)(c)(d) GFP intensity over time

To get a more detailed image of the stress affect we examine the median and the variability of each strain population in the plate. Figure 5.3 provides a general view of the stress effect in both variation and expression-level manners. It enables to examine the distribution of the expression levels in the population relative to the median value. There is a clear shift at the end-time populations (in pink) relative to the start-time populations (in blue), at all levels of expression. The shift intensity is different between the wells. We will look closer at a few strains that demonstrate different stress effects in this plate. In well #86 (Figure 5.3b) we see in the bars plot that the median before and after the stress induction is similar, but the variability in the population is getting higher over time. In wells #87 (Figure 5.3c) and #82 (Figure 5.3d), there is an induction of the reporter gene. In well #87 the whole population is induced while in well #82 the lower quantile size is almost fixed during the measurement time and only the upper quantile is changed. This indicates that only sub-population of the cells was induced in response to the stress.A single cell analysis of the response in this well could indicate if the induced sub-population is exactly the sub-population with the higher initial expression and if there is a correlation between the expression values in this sub-population before and after the induction (I don't have the measurements in single-cell level in this plate due to a technical problem in the experiment).

5.4 Mutants Choice

To examine the role of chromatin remodelers and transcription factors on the expression of the reporters set, we follow the induction of these proteins in population of cells in both wild type strains and strains with deletion of key chromatin remodelers and the main transcriptional factors involved in this stress response. Using this strategy we quantify the effect of different remodelers in the dynamics and variability of the response of each of the target proteins. I used mating and selection methods to create 8 different sets, each set contains the set of $\sim$ 380 reporters and one of the knockouts. All the strains are haploids and contain a single KO and a reporter gene. The chosen genes should be non-essential genes that don't have critical role in the mating process. For example, bdf1, a protein involved in transcription initiation at TATA-containing promoters and associates with the basal transcription factor TFIID, was first chosen to the mutants set. Unfortunately, bdf1 mutants are unable to undergo the meiotic divisions required

for sporulation. Therefore, we cannot use them in our strain construction protocol. We chose a small set of genes that we want to test their effect on the expression under hyper osmotic stress. This group contains proteins from three groups (Figure 5.4):

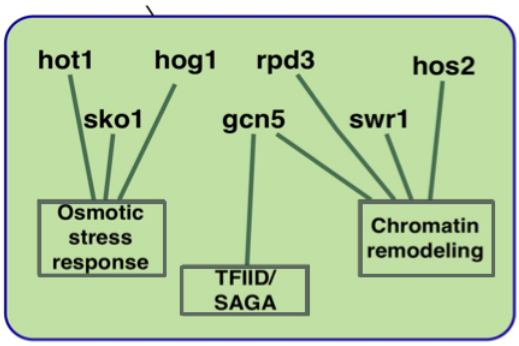


Figure 5.4: The genes chosen as mutants

- Chromatin remodelers - to examine the effect of chromatin remodeling under stress.
- Osmostress response - we chose a few proteins related to the osmotic stress response pathway as a check for our system. We will compare the target genes of these factors with the targets of the remodeling factors to examine the relation between the osmotic stress pathway and chromatin remodeling.
- TFIID/TATA - we chose TFs related to TFIID/TATA as a check to our system. We can test wether the deletion of these factors influence the reporter genes being expected to be their target genes in our reporters set .

5.5 Examples of Plate Comparison

Hog1 is a main factor in the signal transduction pathway in response to salt induction. The comparison between the expression pattern in a plate of Hog1Δ mutants and a plate of WT reporter strains highlights the alternative pathways to hog, and its group of unique target genes. In section 3.3 we saw a full silencing of TPS2 reporter under Hog1Δ. The full silencing was detected also in the full-plate experiment in TPS2 and other reporters. Yet, in most of the reporters we measured a delayed higher expression

in Hog1Δ relative to WT (Figure 5.4a). Previous experiments have shown that the Hog1 and general stress (Msn2/4) pathways interact, at both the signaling and promoter level (Figure 5.4b) [4]. According to this fact we can conclude that the unique targets of hog1p will show full silencing in Hog1Δ strains, but the common targets with Msn2/4 will be expressed by it in Hog1Δ cells. A possible explanation to the higher and delayed expression in this sub-group is that since the Hog1 pathway is not active and the cell does not adapt properly to the stress condition, the cell will sense a harder stress condition and response to it with higher expression levels of the stress response genes.
The gcn5 protein, an acetyltransferase, modifies N-terminal lysines on histones H2B and H3. It is a subunit of the RSC chromatin-remodeling complex, altering replication stress tolerance. The rpd3 protein is a Histone deacetylase. It regulates transcription, silencing, and other processes by influencing chromatin remodeling. The following example highlights the role of chromatin structure in the stress response (Figure 5.6). CV (coefficient of variation) is a normalized measure of dispersion of a probability distribution. The CV is defined as the ratio of the standard deviation to the mean. It is useful because the standard deviation of data must always be understood in the context of the mean of the data. In Figure 5.6a, most of the reporters have the same median in wt and knockout strains, but some of those strains have digh/low CV indicating that the variation is higher/lower relative to the mean in the mutant strain. The same thing happens also in gcn5 mutant, but in Hog1 mutants we don't see this phenomenon. This leads to the conclusion that the two chromatin remodeling factors affect the variability in expression under stress (it was shown in steady state before [19]).

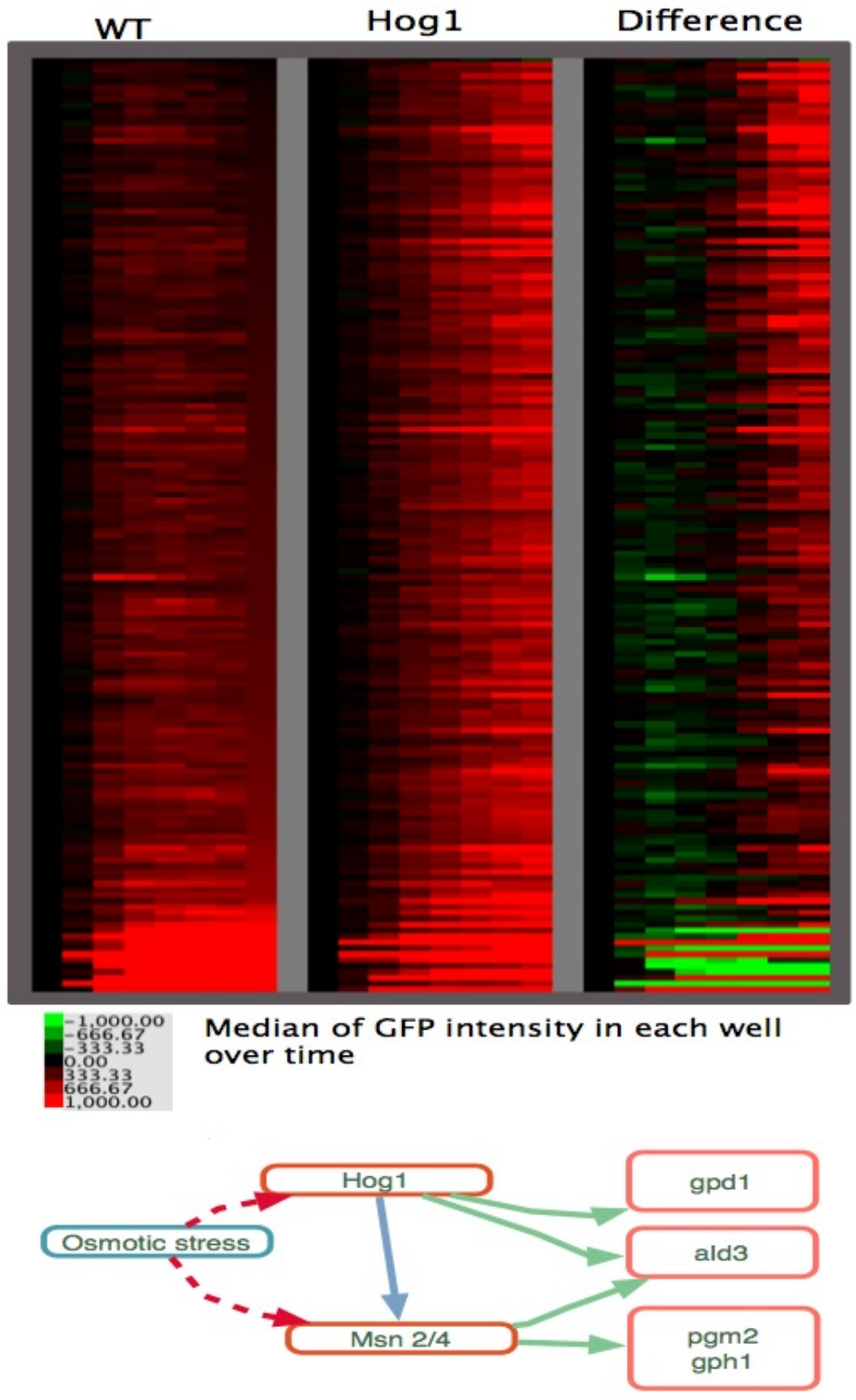


Figure 5.5: The median intensity of the reporter genes in a plate with hog1 deletion and in wt plate, sorted according to the expression level at the last measurement

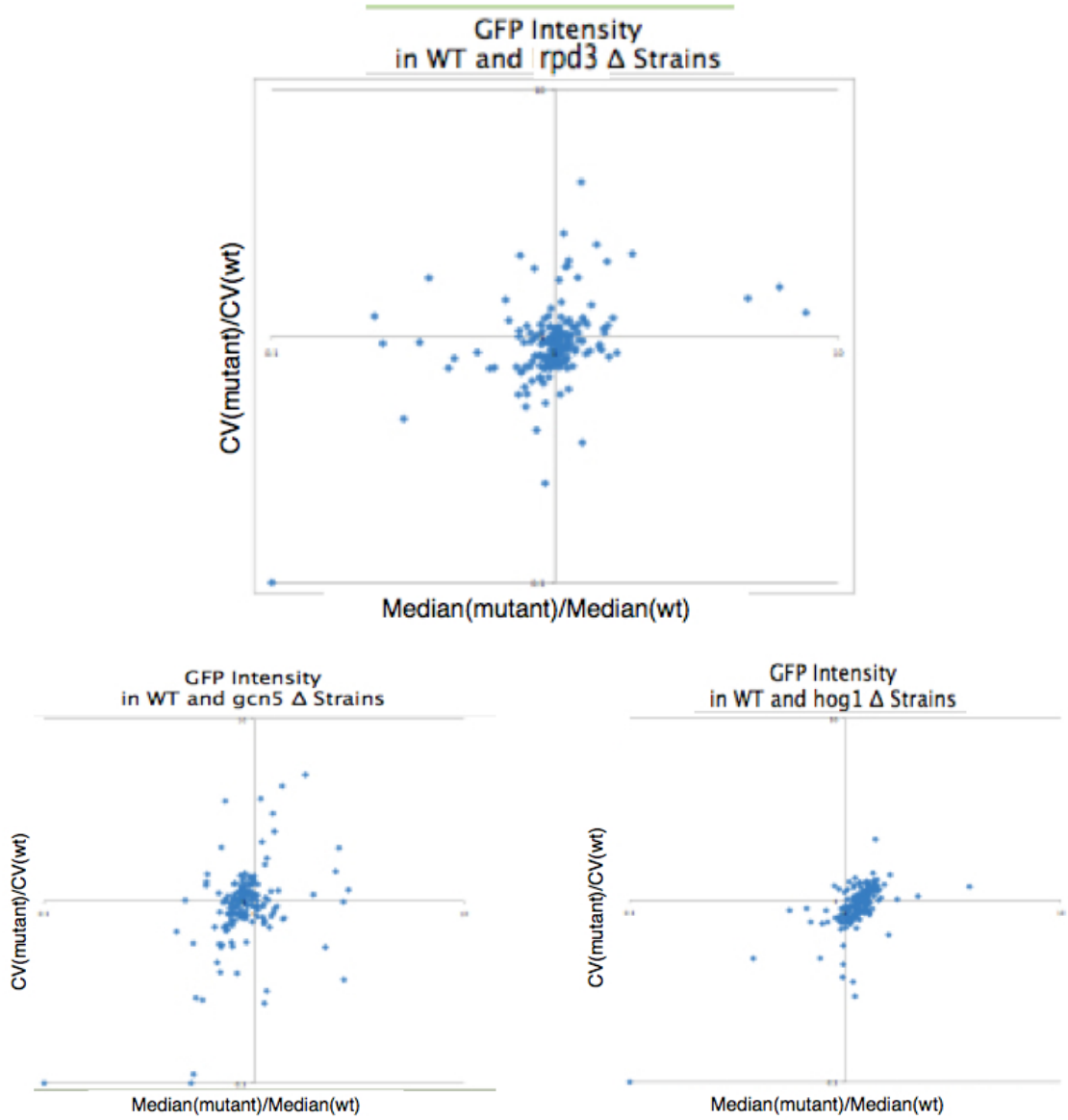


Figure 5.6: f ratio of the CV in mutants and WT 2 hours after the salt induction

Chapter 6

Conclusion and Discussion

The motivation of this project was to systematically produce time-course gene expression measurements at a single-cell level. We implemented these three criteria in the experimental system, and demonstrated their importance in my experiment design. In addition, I performed a pilot experiment on a small data-set to demonstrate that the general concept of the experimental system (knockout mutations and reporter genes) combined with dynamic single-cell measurements allows investigation of gene expression regulation under stress.

The system establishment process highlights the importance of system calibration and the challenges we are still facing. The first task of collecting and calibrating the basic protocols was an inevitable task that had to be done carefully, since many future experiments at the lab will be based on it. The transformation from this level of basic protocols to the systematic level was also a main challenge in integration of the components to a working system. During the calibration process we tried to identify the external factors that might affect gene-expression, to analyze the effects of each factor and to decide on strategies for coping. Eventually, the main timeline protocol was adapted to high-throughput format and its advantages were described. We are now able to construct libraries, to perform systematic and directed culture growth, to plate the cell cultures on microscope plate and to analyze of microscope images. Yet, we are facing some technical challenges in two main areas.

- The microscopy protocol is a critic step in the whole process. The autofocus and timing of the imaging should be improved to enable a more detailed, accurate and redundant expression pattern extraction. The extraction of the measurements

from the images can also improve the output quality. We will deal with this challenge by optimizing the pre-imaging parameters (autofocus parameters, plate shift, protocols) and post-imaging improvements (image analysis and object detection).

- Each step in the timeline from the beginning of the growth until the end of the microscopic imaging requires various resources. These resources include clean tips for each plate, fresh media - including selection media and salt media, plates for the dilutions and more. The systematic performance of the experiment will require parallel preparations of several plates at the same time, which requires the establishment of resource administration protocols.

Through the specialization in the plate construction protocol we learned much about the possibilities coming from SGA strategy and the variety of experiments it enables. The adaptation to high-throughput raised some conclusions regarding the plate design and experiment design. The plate design should take into account the control and validation of the data. Some levels of control are general to the experimental system - such as the haploidity of the cells and the plate control (an identical strain present in all plates). Other levels of control are specific to the specific effect that we aim to test. In our case, for example, we will add stress-control reporters that are not related to stress response and their expression level is expected to be homogenous. The effect of the plating and fluorescent imaging on the cells expression pattern will require an additional control.
The computational conclusion from the comparison experiments is that a more systematic method is needed to produce robust analysis scheme. To achieve this, we will define parameters that will characterize the gene expression profiles and use them to compare the expression in the large set of strains. A clear definition of the possible relations between parameter characterizations will help us to examine the connection between the deleted proteins.
The several experiments that were already performed imply that chromatin remodeling proteins have an affect on the variability in expression under stress (it was shown in a steady state before [19]), and that factors in the signaling pathway may affect the level and timing of the response. These conclusions convince me that further examination of data of this kind, with the set of knockout genes and reporters I chose, may lead to interesting inputs regarding the role of transcription factors and chromatin remodelers in osmotic stress response.

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