Demonstration of the utility of a dual-color luciferase reporter in *S. cerevisiae*

by

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I dedicate this thesis to my mom and dad for their encouragement and support. I also dedicate this work to Aravind for his love and laptop, without both of which this work would have been impossible.

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ABSTRACT

A reporter gene helps determine the regulation of a gene of interest by producing a protein product that can be easily detected when the gene of interest is active. The luciferase gene from click beetles can be used as a reporter because its protein product emits light that can be measured. Monitoring transcription by luciferase activity is hampered in fast growing organisms due to sudden changes in oxygen concentration and cell number as these variables also affect light emission. This study uses green luciferase to report transcription of a gene of interest and red luciferase to simultaneously report activity of a reference gene. The ratio of these colors can (i) mitigate sudden changes in rapid growth conditions, and (ii) be used to predict productivity of the cell population during processes such as the Yeast Respiratory Oscillation. We envision this tool to provide valuable insights into genetic control in *S. cerevisiae*.

TABLE OF CONTENTS

Page

LIST OF FIGURES

LIST OF TABLES

Chapter I

GENERAL INTRODUCTION AND FOCUS

1.1 Introduction

A eukaryotic cell is akin to a complex, orchestrated machine. This microscopic, molecular machine is regulated by the expression of genes. Genes encode proteins that directly or indirectly dictate a cell's function. Therefore, studying gene expression patterns will provide valuable insights into the transcriptional mechanism of a cell. Assessing the transcriptional behavior of a cell is an exceptionally powerful tool for exploring basic biology in organisms with simple and complex genetic profiles, drug discovery, and even generating datasets with information about genetic processes.

Prior to the development of reporter gene technology, assays such as northern and western blots were used to reveal levels of RNA and proteins respectively but they proved to be time consuming while being insufficient for measuring real-time changes in gene expression. Advances in genetics led to the discovery of the first genetic reporter, lacZ, in 1980[8]. Since then, several reporter genes have been discovered to suit the nature of the experiments. The central idea of a reporter gene is simple: The gene product can be easily detected and measured. By attaching a regulatory sequence from the gene of interest upstream of the reporter gene, a measurable output is obtained when the gene of interest is actively transcribed and translated. One of the examples of a well-studied reporter system is luciferase. Luciferases are proteins that catalyze light production in bioluminescent organisms such as fireflies, click beetles, and some bacteria[16,53]. For certain systems, this reporter is advantageous over other available reporters due to its relatively short half-life, high sensitivity, continuous output, and its ability to be monitored over sustained periods of time using a photo detector. Luciferase also provides an attractive benefit to study transcriptional control in organisms that alter their

transcription patterns in the presence of light since it does not require an external light excitation[65,82].

Since the firefly luciferase gene was first cloned in 1985[15], extensive research has enabled scientists to use this reporter to observe temporal changes in gene expression, study circadian rhythms and even develop biosensors[18,69]. Exploration of these fundamental molecular mechanisms is often done in organisms that are genetically tractable and those that possess simple nutritional requirements. The eukaryotic model organism, *Saccharomyces cerevisiae* , also known as baker's yeast, is often the preferred choice of organism for scientists due to its single-celled nature, small genome size, short generation time, and our expansive understanding of *Saccharomyces* genetics[25,64]. In this study, I demonstrate the use of a luciferase reporter to elucidate the complex and dynamic nature of gene expression during times of rapid metabolic activity in *S.cerevisiae*. The following sections elaborate on concepts that will aid the reader to understand this material.

1.2 Luciferase as a reporter gene

The bioluminescent protein luciferase is derived from a variety of organisms that include fireflies (*Lampyridae*), click beetles (*Elateridae*), certain species of glowworms (*Phengodidae*), and aquatic organisms such as sea pansies (*Renillidae*) and copepods (*Metridinidae*)[83,86]. While all of the luciferase reactions catalyze light production, the requirements for the reaction vary widely. For instance, the luciferase reaction in the three Coleoptera families (including fireflies, click beetles, and glow worms) requires substrates luciferin in addition to O_2, Mg^{2+}, and ATP. In case of marine organisms (like sea pansies and copepods), however, the reaction requires the substrate coelenterazine in addition to O_2 and Mg^{2+} (does not require ATP)[28,75].

Since the luciferases from marine organisms are less sensitive than firefly luciferases and require a substrate (coelenterazine) that is considered more unstable

than luciferin due to auto-oxidation[76,86,95,100], this study only deals with variants of firefly and click beetle luciferase. The following table provides a comprehensive outline of the features of luciferases used in the study.

Table 1: Features of luciferases used in the study

Luciferase	**Source**	**Peak emission wavelength**	**Molecular weight**	**pH sensitive?**
Fluc(Firefly luciferase)	*Photinus pyralis*	550-570nm[49,86]	∼62kDa	Yes[49]
PpyRE8(*P.pyralis* Red Emitting)	*Photinus pyralis*	618nm[51]	∼62kDa	No[5]
PpyRE9(*P.pyralis* Red Emitting)	*Photinus pyralis*	617nm[6]	∼62kDa	No
CBG99 (Click Beetle Green)	*Pyrophorus plagiophthalamus*	537nm[52,86]	∼60kDa	No
CBR (Click Beetle Red)	*Pyrophorus plagiophthalamus*	615nm[91]	∼60kDa	No

Branchini *et al.*, first reported the development of red-emitting PpyRE variants by introducing random mutations in the firefly luciferase coding sequence[5]. These reporters showed promise with improved properties such as increased thermostability and pH insensitivity[5], thereby making these luciferases an attractive alternative to the existing reporters. The naïve click beetle luciferase (that emits a yellow-green color), on the other hand, is extracted from the dorsal anterior organs of the Caribbean click beetle, which is engineered genetically to yield the green shifted CBG99 and red shifted CBR. These luciferases have been successfully expressed in mammalian tissues and used in in vivo bioluminescence imaging[51,91].

For gene expression studies, the luciferase gene is cloned downstream of a promoter of interest and is used as a non-invasive, real time reporter of promoter activity. An ultrasensitive, cooled charge-coupled device (CCD) camera fitted with appropriate filters detects the light output simultaneously; the output is measured in arbitrary units called relative luminescence units (RLUs). A schematic of the process is as shown below.

Besides reporting gene regulation, this reporter is found to be extremely useful to detect bacterial contamination and environmental toxins[1,5] while also being

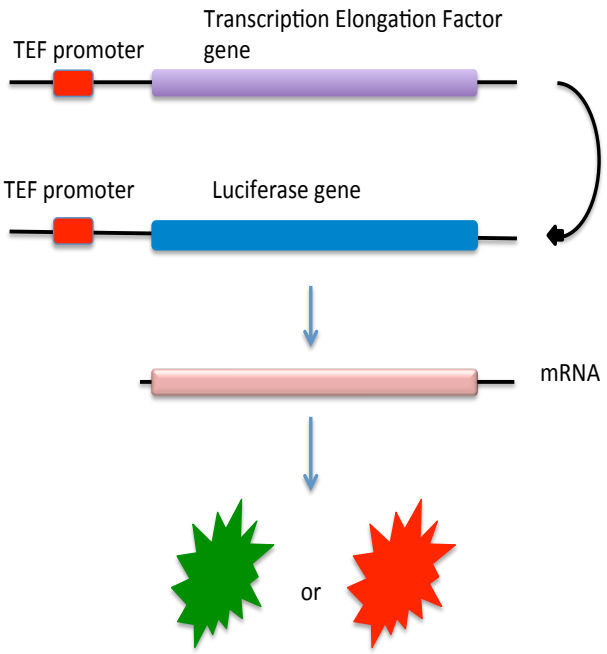

Figure 1.1: Schematic of a reporter gene. The Transcription Elongation Factor (*TEF1*) promoter and gene are used as an example to illustrate the working of a luciferase reporter in the figure. The promoter from the native *TEF1* gene is copied upstream of the luciferase gene and the reporter is transcribed and translated when the native *TEF1* gene is actively expressed. The output of the luciferase reaction is luminescence (expressed in arbitrary units called Relative Light Units (RLU)).

used to test the antibiotic susceptibility in pathogenic bacteria[84]. Furthermore, the reporter is used in in vivo bioluminescence imaging, an emerging tool to visualize tissue depth, tumors and to track cell receptor modulation in cancers[43,101]. The reporter also finds its use in whole cell and cell extract based biosensors to trace mercury and aromatic compound toxicity[36,63].

However, for specific applications, as detailed in the next section, a single color firefly luciferase is insufficient as a reporter to accurately reflect temporal changes in gene regulation. Here, I demonstrate the applicability of a dual color luciferase system in *S.cerevisiae* to investigate real time genetic control under rapid growth conditions that have traditionally been inaccessible.

1.3 Dual-color rationale

During appropriate nutrient conditions, *S. cerevisiae* undergoes rapid metabolic changes and exhibits increased growth rate. Using luciferase to report the activity of a single gene becomes insufficient since light emission is also obscured by constant changes in gene expression, cell number and oxygen demand. To circumvent this issue, we hypothesize that two luciferases (using the same substrate) engineered to report the activity of two separate genes would be a more sensitive indicator of gene activity than a single luciferase alone.

A reference gene (also known as a housekeeping gene) is a constitutively expressed gene regardless of variations in experimental conditions, making it an excellent normalizing control for gene expression studies[46]. This study uses the same principle of a reference gene to normalize gene expression during times of rapid metabolic activity in *S.cerevisiae*. The system is designed to employ one color of luciferase to report the activity of a gene of interest while a separate color is used to simultaneously report the activity of a stably expressed reference gene. I hypothesize that the constitutively expressed reference gene will provide a baseline reference for cell number and substrate availability thereby compensating for changes in these variables.

To illustrate the figure below, consider the data from a reporter that is unable to distinguish colors (i.e., a single color luciferase reporter). Initially, it would appear that the total luminescence is 200 RLUs and the luminescence increased to 300 RLUs in case 1 while in case 2, the luminescence decreased to 130 RLUs. However, a dual color reporter reveals subtle changes in luminescence output. For instance, let us consider the green/red ratio in both cases: Initially, the ratio is 1 (100 RLUs/100 RLUs). In case 1, the ratio has changed to 2 (200/100), thus indicating that the promoter of interest has been upregulated. In case 2, the ratio has changed to 1.6 (80/50), again indicating upregulation in the promoter of interest. Therefore, a dual color reporter helps parse out differences between

promoter regulation and changes in variables such as number of viable cells and oxygen levels. As outlined above, the dual color reporter system will allow us to simultaneously monitor two genes when the luciferases used emit light of different wavelengths. The mixed emission spectra can be quantified by splitting them with filters. The concept of using dual luciferases to monitor cellular activity has been previously used in cultured mammalian cells[56,58,99], bacterial cells (and cellular extracts)[5], plant tissues[60], and even whole organisms[59]. However, most of these systems employ luciferases that require two different substrates that are insufficient to compensate for variables such as substrate loss during continuous recording.

1.4 Continuous culture and the Yeast Respiratory Oscillation

Continuous culture is a technique to grow cells at a near steady state where the specific growth rate is governed by the concentration of a limiting component in the medium[26]. The system is maintained in a vessel known as chemostat (also known as a bioreactor or fermentor) in which culture (in the form of cells, media, and byproducts) is continuously removed at the same rate as fresh media is added thus maintaining the cells at a constant steady state. Once the cell population grows to accommodate the limiting substrate concentration, the growth rate is entirely dependent upon the rate at which fresh media added to the vessel[45]. As evident in the equation below, the doubling time of the cells can be adjusted by controlling the dilution rate (the rate at which fresh media is added to the vessel and continuously removed from it)[54].

$$\text{Doubling time} = \frac{ln2}{\text{dilution rate}} \tag{1}$$

In the experiments used in the study, the dilution rate was maintained at 0.08-0.09 h^{-1}(at a pH of 4.0). This corresponds to an average doubling time of ∼7-8 hours.

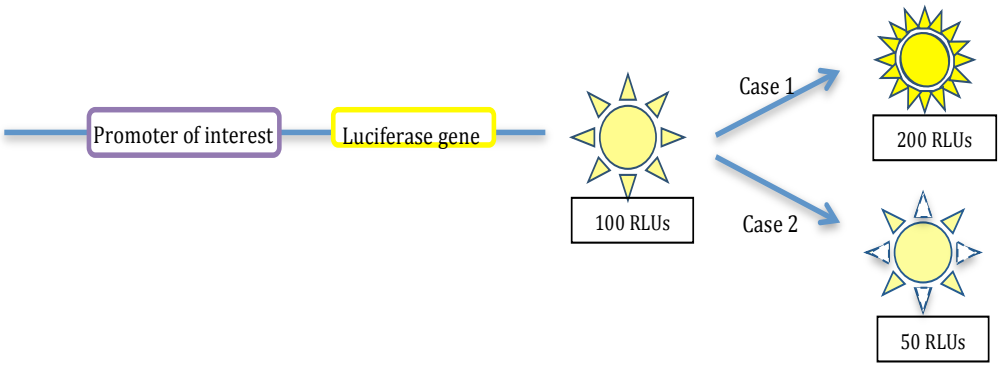

Figure 1.2: Schematic of a single color luciferase reporter. A single color luciferase reporter is insufficient to account for changes in light emission when other factors like cell number and substrate concentration change. A promoter of interest drives expression of a luciferase gene. In case 1, if the light output doubled in a certain period, it could be due to an increase in viable cell number or upregulation of the promoter of interest. In case 2, the light output could have decreased because of decrease in viable cell number, a drop in available oxygen, or downregulation of the promoter of interest.

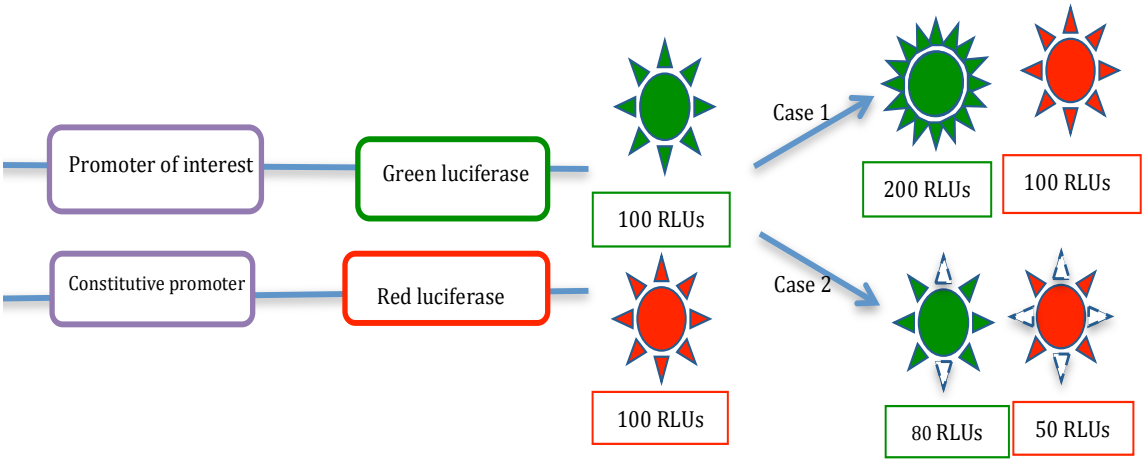

Figure 1.3: Schematic of a dual color luciferase reporter. Dual color light output can monitor two variables at a time, thereby allowing one to normalize gene expression for changes in cell number and substrate levels. In this depiction, a promoter of interest controls the expression of a green-emitting luciferase, while a constitutive promoter controls the expression of a red-emitting luciferase. In case 1, green/red ratio reveals that the promoter of interest has been upregulated. Similarly, in case 2, although it seems that the light output has decreased, the ratio reveals an upregulation in the expression of promoter of interest.

An attractive advantage that this system provides is the control over environmental factors. Variables such as pH, temperature, and nutrient concentration can be adjusted to suit the needs of the experiment thereby making this an indispensible technique to study production of industrially important metabolites[2], pathway analysis[13], and even whole genome analyses[85].

An important implication of maintaining *S. cerevisiae* in an aerobic, nutrient limited continuous culture condition is the expression of sustained, robust oscillations known as the Yeast Respiratory Oscillations (YRO; also known as the Yeast Metabolic Cycle, YMC or Energy Metabolic Oscillation, EMO). Simply put, these biological rhythms metabolically orchestrate the cells through alternating oxidative and reductive states with metabolic intermediates being passed on from one phase to the next[87]. Many cellular parameters such as the dissolved oxygen (DO) concentration, intracellular carbohydrate storage concentration, and oxygen uptake rate vary with the YRO[87]. A typical YRO trend is as shown in Figure 1.4.

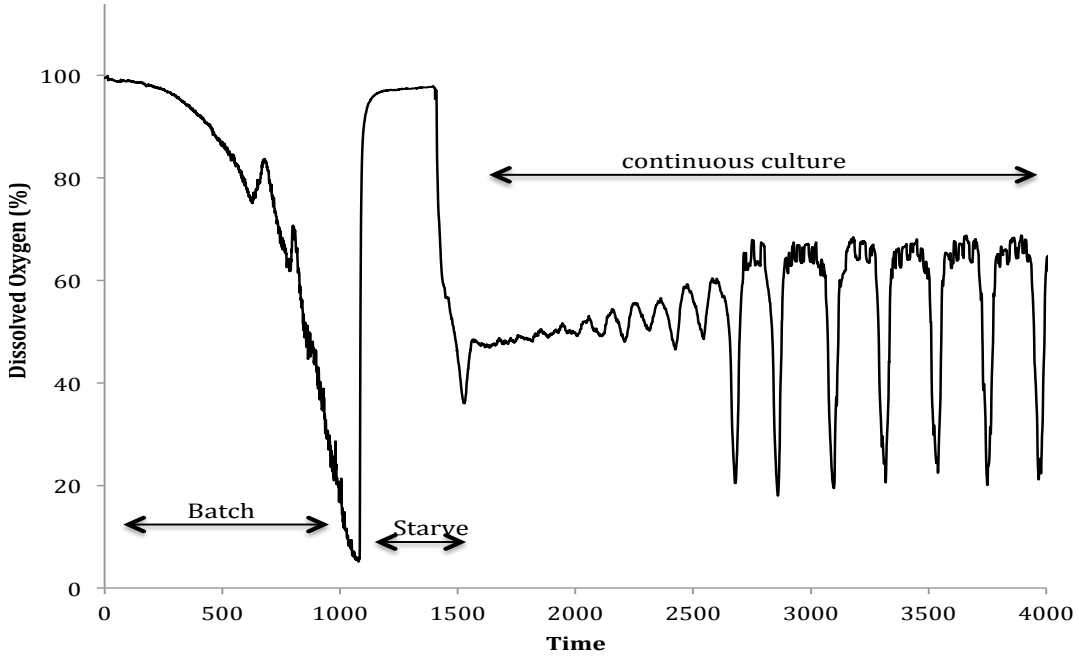

Figure 1.4: Oscillation of DO levels in continuous culture of *S.cerevisiae*. A typical oscillation observed in a culture of *S.cerevisiae* (strain CEN.PK 113-7D). The DO concentration is measured by a DO electrode and is plotted against time to generate the YRO trend. It should be noted that this strain generates oscillations for weeks if maintained in a continuous culture.

Yeast exhibits different periods of oscillations depending on various factors such as dilution rate, nutrient load, strain, and environmental light[38,70,87]. Oscillations are primarily divided into two types: long period (∼4-16 hours)[87], and short period (∼8-60 min)[38]. Experimental parameters in this study are designed to produce long period oscillations; therefore the discussion of the YRO is restricted to them.

Each long period oscillation typically consists of three phases (as indicated in figure 1.5): a highly oxidative phase (Ox phase), during which cells undergo a burst of respiration to consume oxygen as indicated by the dipping dissolved oxygen levels[87,88]. Followed by that, a Reductive, Building phase (R/B phase) occurs in which the mitochondrial respiration is completed and the levels of dissolved oxygen begin to rise[97]. The last and the longest phase is the Reductive, Charging phase (R/C phase) where mitochondrial respiration begins to cease and the cell prepares itself for the following Ox phase[67,88].

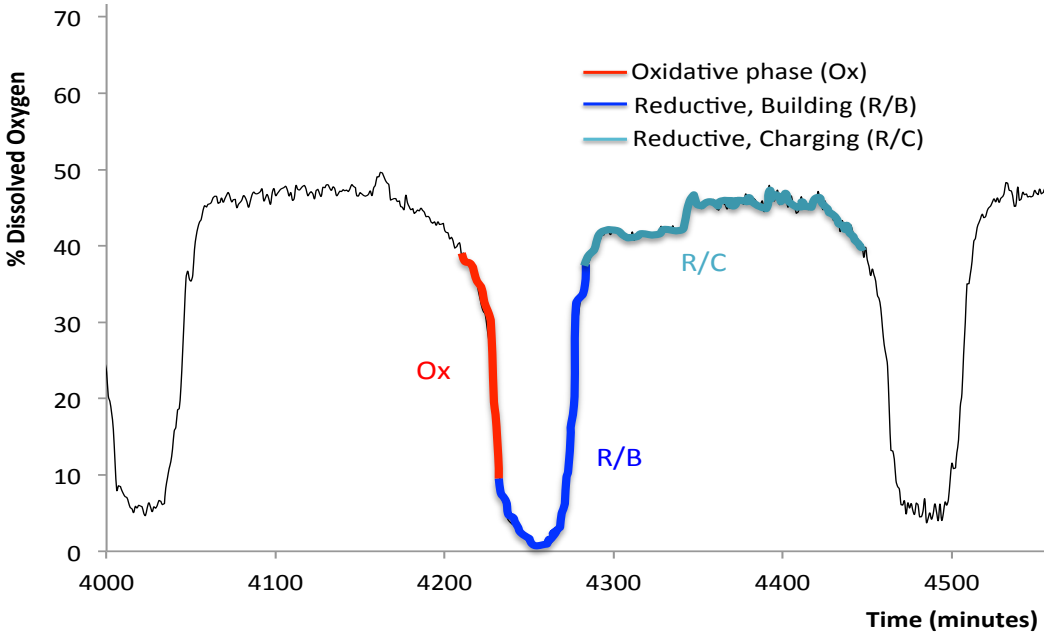

Figure 1.5: Typical phases of the YRO. An oscillation of YRO dissected to show the different phases (Ox, R/B, and R/C phase). As described in the text, metabolic intermediates are passed on from one phase to the next thereby allowing temporal compartmentalization of gene expression.

The metabolic basis of the YRO has been explored in many studies on continuous cultures and cell-free extracts[9,92] and is briefly outlined as follows. In the Ox phase, stored carbohydrate reserves (such as glycogen and trehalose) are broken down to yield glucose. This contributes to fueling a round of cell growth and proliferation in the following phases. The free glucose is channeled through glycolysis and mitochondrial respiration to yield large amounts of ATP and NAD+. This results in a dip in dissolved oxygen levels accompanied by an increase in oxygen uptake rate. Although there is a theoretical increase in ATP, experimental evidence points to higher levels of AMP and ADP than ATP suggestive of its higher rate of breakdown[98]. It is proposed that ATP is used in biomass buildup for budding[10].

The R/B phase, which follows the Ox phase, is characterized by a dip in ATP and NADP levels accompanied by the completion of biomass formation and mitochondrial respiration[47,97]. This repression of mitochondrial respiration induces the synthesis of glycogen and trehalose. A hallmark characteristic of this phase is the rise in dissolved oxygen levels[87]. As discussed in detail at the end of this section, the cell division cycle is strictly initiated in this phase, possibly to prevent oxidative damage[87,97].

In the last phase of an oscillation (R/C phase), glucose is anabolized to form storage carbohydrates and experimental evidence indicates that glycogen is accumulated three times more than trehalose hinting the role of glycogen as the primary source of storage carbohydrate[97]. This phase is also known to produce acetyl-CoA that is used in the following Ox phase as a substrate for respiration[87]. Interestingly, this phase is known to resemble a nutrient starvation state within the cell by upregulating stress response and heat shock proteins. Tu *et al.*, propose that this machinery acts to restore the cell and prepare it for the next oscillation[87].

To understand the molecular basis of YRO, Tu *et al.* (2005) performed a microarray analysis; the study reveals that about 57% of the genome oscillates

with the YRO[87]. Gene expression is separated temporally to coordinate with the various metabolic events that occur within an oscillation[67]. For example, genes that code for enzymes that breakdown glycogen and trehalose such as glycogen phosphorylase (*GPH1*) and neutral trehalase (*NTH1* and *NTH2*) are known to be highly upregulated in the Ox phase[97]. Genes essential for cell division such as mitochondrial proteins, histones, and spindle body components are expressed in the R/B phase[87] and storage carbohydrate synthesis genes are upregulated in the R/C phase[98].

Another chief oscillatory behavior exhibited by yeast is the cell division cycle (described in the next section). It is well known that these two oscillatory behavior coordinate (to a certain extent) yet the underlying reason is not clear[11,72]. Studies by Chen *et al.*,[11] and Tu *et al.*,[87] provide specific clues to the temporal segregation of cell cycle events to specific phases of the YRO. For instance, microarray analyses and DNA labeling assays indicate that the entirety of the cell division is restricted to the reductive phase of the YRO. Many hypotheses have been proposed to explain this rather interesting observation: Some of them propose that this indicates the preference of the cell to shield its nucleic acids from oxidative damage; thereby suggesting that the biological role of YRO is to provide such an environment for replication[11,38,87]. However, another study indicates that a sudden burst of burning through storage carbohydrates pushes the cell through the cell cycle[19]. An alternative hypothesis suggests the separation of cell cycle from the Ox phase is due to the biosynthesis of replicative machinery coupled with the degradation of unprocessed RNA transcripts occurring at the end of Ox phase[79].

Regardless of the underlying explanation, it is now well known that DNA replication begins at early stages of the R/B phase, peaks soon after and diminishes as oxygen levels begin to rise[11]. Furthermore, analyses of bud index (ratio of budded cells to unbudded cells) shows that the highest ratio of budded cells is found in the reductive phase[69]. Therefore, depending on conditions such as pH, dilution

rate, and oxygen levels, when cells are grown in glucose-limited chemostat, they display a remarkable synchrony of the cell cycle with the respiratory oscillation. As outlined in the next section, although asynchrony in cell cycle is observed to be a dominant phenomenon in other culture conditions; cells in the continuous culture spontaneously coordinates with respect to the cell cycle chiefly due to the YRO.

This study demonstrates that a dual color reporter can be used to probe gene regulation during a complex and dynamic process such as the YRO.

1.5 Cell cycle

The cell division cycle (CDC or cell cycle) (as shown in figure 1.5) of *S.cerevisiae* is a highly concerted series of events concerned with the replication and segregation of DNA and the mechanism closely resembles that of more complex eukaryotes[23]. It is divided into four stages: G_1 (Gap 1), S (Synthesis), G_2 (Gap 2), and M (Mitosis/Meiosis). In G_1, the cell grows to build up its size and reserve of nutrients. The cell then passes through a point called 'Start' that deems the cell to be irreversibly committed to one complete round of cell division. It is known that the duration of G_1 phase varies in response to different growth conditions thus indicating that nutrient availability could impact the cell cycle[32,78]. When a cell senses carbon limitation, it cannot pass through 'Start' and hence transits into a quiescent state known as G_0 phase[22]. Another requirement for the cell to pass through G_1 is its size; a cell must attain a critical size for it to pass through G_1. Daughter cells, that spend a lot of time in the G_1 phase, were proposed to do so to build up their cell mass to attain the critical size[31]. However, recent studies indicate that the G_1 delay in daughter cells is size independent indicating that this pause may be an intrinsic property of the daughter cells[41]. Regardless of the reason for the unequal mother-daughter cell division, when in culture (batch or continuous), the population tends towards cell cycle asynchrony.

The S phase commits the cell to a round of genome duplication and is accompanied by the upregulation of certain genes such as cell cyclins and *POL1* (required for the polymerization of DNA). As indicated in figure 1.5, the bud starts to emerge from the mother cell in this phase. In G_2 phase, the bud continues to grow as the cell prepares for the next phase. Chromosome segregation and generation of daughter cells occur in the M phase. Mitosis results in genetically identical diploid daughter cells while certain sporulating conditions direct meiosis to create four haploid daughter cells from diploid parent cells. At the end of the M phase, the bud completely separates from the mother cell. If nutrient conditions are favorable, the mother and daughter cell undergo another round of cell division.

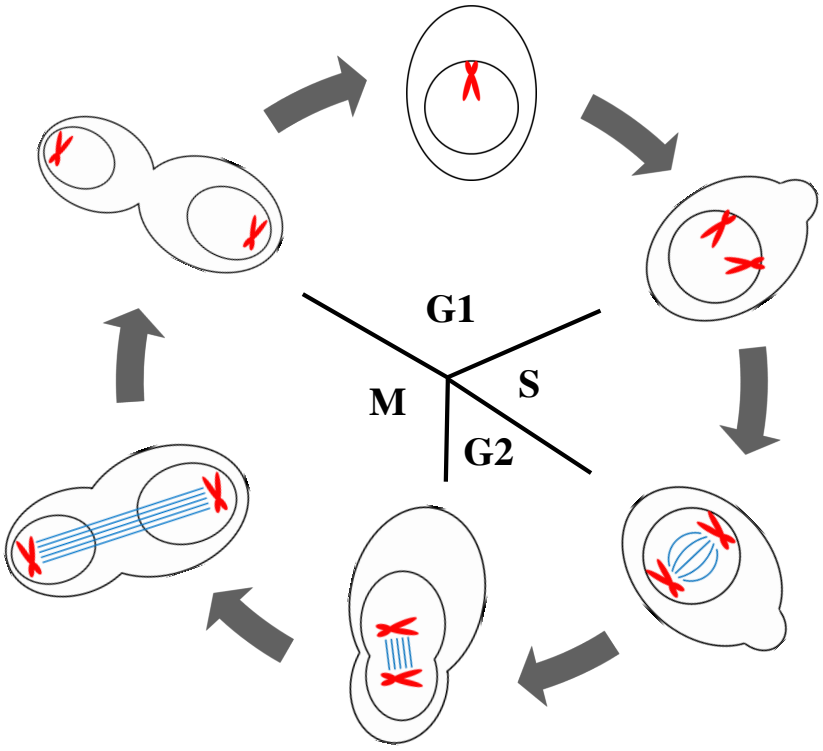

Figure 1.6: Landmarks of the *S.cerevisiae* cell cycle. The cell passes through different checkpoints before committing itself to the next stage of the cell cycle. The doubling time (the time required for a cell to give rise to another cell) in *S.cerevisiae* can be as short as 90 minutes under ideal conditions.

1.6 Significance of the study

As stated earlier, the concept of using a dual color luciferase to monitor complex gene expression patterns has been reported in literature. However, this study will demonstrate the utility of a sensitive, quantitative reporter of transcriptional activity during times of rapid metabolic change in *S.cerevisiae*. Currently, no system exists to distinguish the effect of change in cell number and oxygen level (besides other external variables such as light) on gene expression. The work presented here attempts to illustrate the use of this reporter by testing its efficiency on various promoter combinations and different culture conditions. This study also caters to the YRO research community by providing a tool to further probe this rhythmic process.

In this report, the relative brightness levels of various reporter genes available in the organism of interest are discussed. In addition, well-known promoter systems are tested with the reporter to gauge its efficiency and drawbacks (Chapter II). The reporter was used to investigate the limits of a light sensitive promoter system that was developed in *S.cerevisiae* (Chapter III). All promoters were tested in a variety of conditions (on plates, batch, and continuous cultures) and the effect of YRO on luminescence was also studied extensively. The discussion culminates with general conclusions and future directions in the field of genetic reporters and YRO (Chapter IV).

We believe that this tool will permit our lab (as well as other labs) in investigating rhythmic genetic control in fast growing organisms to optimize protein expression while enabling research in more complex organisms to explore rapid changes in gene expression.

1.7 Obectives of the study

- Find compatible, spectrally resolved pair of luciferases that use the same substrate.
- Construct genetic systems with luciferases under a specific set of well-characterized promoters that will allow us to test the systems effectiveness.
- Use the reporter system to measure gene expression using a light sensitive promoter system developed in *S.cerevisiae*

1.8 Hypotheses

- Dual color reporter will monitor and compensate for substrate loss
- The reporter system will track changes in cell number
- The pair of luciferases chosen for the study will result in minimal spectral cross talk with respect to the filter sets used to distinguish light emission.

Chapter II

DEMONSTRATION OF THE UTILITY OF A DUAL LUCIFERASE REPORTER USING VARIOUS PROMOTER SYSTEMS IN *S.cerevisiae*

2.1 Introduction

Gene expression assays further our understanding of the intricacies of genetic control in complex organisms[37]. Many techniques of varying degrees of efficiency have been established to study gene expression, some of which include: reporter genes, western and northern blots[94], and microarray analyses[55]. Reporter genes have been extensively used to study various biological processes since it reveals transcriptional regulation by producing a protein product that is easy to detect[20]. Although different types of reporter genes exist, for specific applications, luciferases are significantly advantageous over other reporter systems as sensitive, non-invasive detectors of promoter activity.

Since studies of using luciferase reporters to monitor gene regulation emerged, advances in this field have led to the development of a dual color reporter. These dual color reporters have been used for protein complementation assays (PCA)[91], bioluminescent imaging (BLI)[51], and as biosensors[14]. These studies indicate the efficiency of a dual color reporter but most involve luciferases with different reaction chemistries and therefore warrant the use of a second substrate, namely coelenterazine. This investigation details the applicability of a dual color reporter with the same substrate to study gene expression patterns in fast growing organisms, with particular emphasis on monitoring rhythmic gene regulation.

To demonstrate the utility of this tool, we construct vectors that place these luciferases under the control of specific promoters that are then integrated into the genome of *S.cerevisiae*. Having one color of luciferase under the control of a reference gene provides a baseline reference for cell number and substrate avail-

ability, thus compensating for changes in these variables. We hypothesize that this tool is a more accurate reporter of gene expression during conditions of complex metabolic activity, as supported by the data below. Specifically, we show the utility of this tool for analyzing gene expression patterns during the hypoxic mask of the YMC, a phenomenon that has traditionally been inaccessible with a single luciferase reporter.

This chapter begins with a detailed discussion of the use of a dual color reporter to identify gene expression using well-known promoter systems while exploring the advantages and limitations of this system. The following section describes the results of our findings categorized by the objectives outlined in the previous chapter. The chapter concludes with the possibility of extending the use of this reporter system with other not-so-well characterized promoters.

2.2 Results and Discussion

2.2.1 Evaluating the available set of luciferases for *in vivo* brightness and spectral resolution

The initial objective of the study was to evaluate the available range of luciferases that use the same substrate to identify the most suitable pair for experimentation. To ensure that the chosen pair of luciferases was an efficient monitor of gene expression, factors such as their total brightness and spectral resolution were considered. The initial plasmid constructs included luciferases under the control of a constitutive promoter (P_{TEF1} (translational elongation factor) or P_{ACT1} (actin)- luciferase). These constructs were individually transformed into *S. cerevisiae* (strain CEN.PK 113-7D) using the standard lithium acetate method[29]. The transformants were then patched on a yeast peptone dextrose (YPD) plate with 100mM luciferin and imaged with an ultrasensitive CCD camera fitted with filters. The image, shown in figure , was analyzed quantitatively using the ImageJ software.

The results showed that the brightest green and red-emitting luciferases were CBG and CBR respectively (figure 2.1). The analysis also revealed the amount of red signal detected by the green filter and vice versa; this overlap between spectra is known as cross talk. Minimization of this non-specific signal overlap is highly advantageous for sensitive detection of gene expression using the dual color reporter[5]. Therefore, it is essential to choose the pair of luciferases with well-separated emission spectra and also select filters with narrow bandwidth detection.

To corroborate evidence for cross talk between the emission spectra of the luciferases and the transmission spectra of the filters, a sensitive spectral scan was conducted. The candidate luciferases possessed an emission maxima of ∼535 nm and ∼600 nm for CBG and CBR respectively at 25℃ . The transmittance of the filters used in the imager and the Photomultiplier tubes (PMT) was also measured. Then, the plots were overlaid to calculate the cross talk as shown in figure . Based on the data shown below, CBG and CBR seem to be ideal candidate luciferases for the dual color reporter tool in *S. cerevisiae* and were hence used in the study throughout.

2.2.2 Modifications to the CBG luciferase to shorten its half-life

The central idea in the design of the dual color reporter tool in *S. cerevisiae* involves using one color of luciferase to report transcription of the gene of interest and another color to simultaneously monitor transcription of a stably expressed reference gene. In this study, the green emitting luciferase was used to monitor activity of a gene of interest; therefore, a destabilized version of the CBG reporter was developed. We hypothesized that this modification will allow us to explore transient changes in gene expression more accurately.

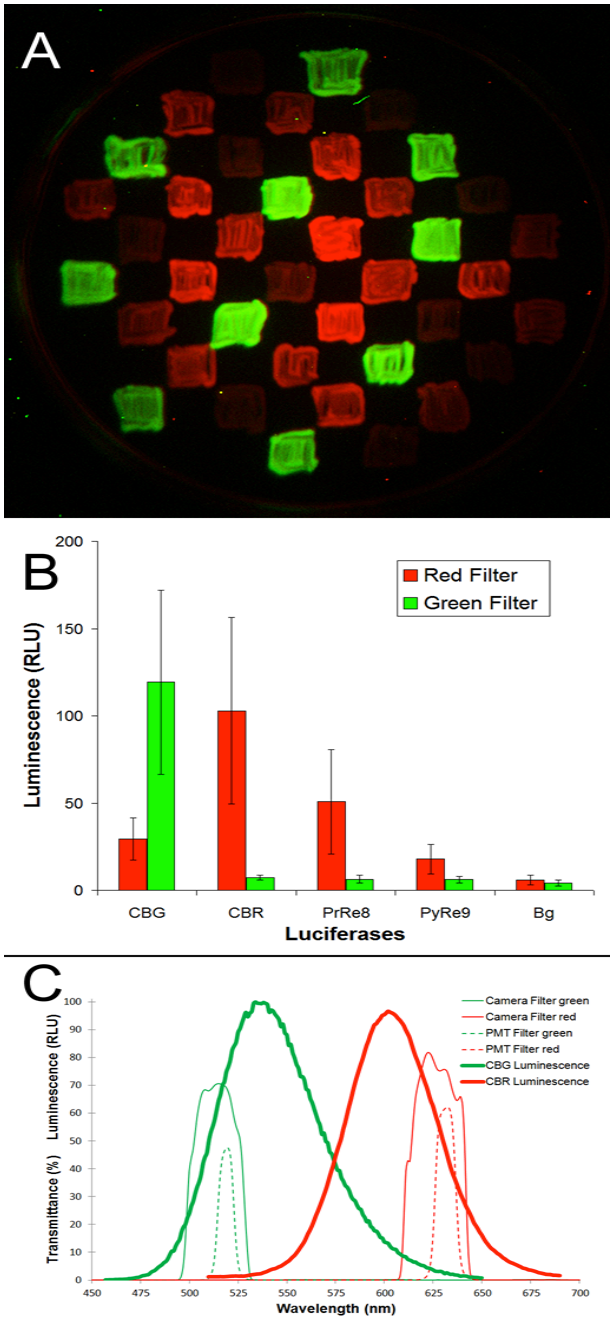

Figure 2.1: Demonstration of CBG99 and CBR as the most suitable pair of luciferases for studying gene expression patterns in *S. cerevisiae*. (**A**) Yeast stably transformed with P_{TEF1} driving various luciferases were patched onto solid media and imaged with an ultrasensitive camera fitted with filters. The identity of each patch (abbreviated "G" for CBG99, "R" for CBR, "8" for PpyRE8, and "9" for PpyRE9), in order left to right grouped by rows: 9G R89 G9RG 8RG89 98RG8 GR98R 8GR98 R8G9 G9R8 G9. (**B**) Each patch was quantified for brightness with the green (green) and red filters (red) using the ImageJ software. Bars are means ± S.D. (**C**) Spectral scans of CBG99 and CBR luciferases were compared with the transmission spectra of the filter sets used in the imager and Photomultiplier tubes (PMT) to identify cross talk between channels.

The half-life of native click beetle luciferases is 3h in mammalian cells. This turnover time is not sufficient for monitoring rapid changes in gene expression[24]; therefore, a PEST protein motif from the *CLN2* gene was added downstream of the coding sequence of the luciferase gene. This genetic modification shortened the turnover time in yeast from 4.75h to 40min, shown in figure 2.2.

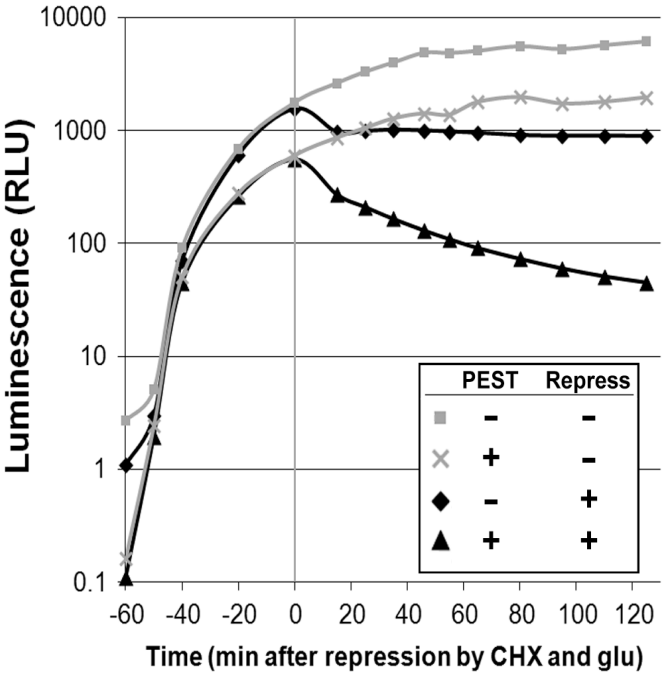

Figure 2.2: Shortening the half-life of CBG in yeast. Half-life of CBG activity in yeast is shortened from ∼5 h to ∼40 min by the addition of a PEST destabilizing sequence to the 3' end of the coding region of the CBG luciferase gene. Luminescence was periodically measured from yeast cultures transformed with galactose-inducible/glucose-repressible reporters with or without a PEST sequence. Luciferase transcription was induced by addition of galactose to the media at time -60 min for all cultures. Cultures with destabilized reporters (Xs and triangles) showed a lower amount of expression compared to cultures with unmodified reporters (squares and diamonds). Transcription and translation of luciferase was repressed at time 0 min by the addition of glucose and cycloheximide in cultures with the destabilized reporter (black triangles) and with the unmodified reporter (black diamonds). Similar cultures were not repressed at time 0 (grey squares and Xs). Luminescence from all repressed cultures dropped after time 0, but cultures with the destabilized luciferase reporter (black triangles) dropped much faster than cultures with the unmodified luciferase reporter (black diamonds); half-lives were calculated as 4.75 h for CBG and 40 min for CBG with PEST. Cultures that were not repressed at time 0 (gray squares and Xs) continued to increase their luminescence over the course of the experiment. Luminescence was graphed on a log scale and time was graphed in negative minutes before repression and positive minutes after repression.

2.2.3 Using the dual color luciferase system to investigate the expression of well-studied promoters

2.2.3.1 Constitutive promoter

As proof-of-principle, preliminary experiments were conducted to explore gene expression using well characterized promoter systems. Initial experiments included placing both of the luciferases under the control of the same constitutive promoter, translational elongation factor, *TEF1*. The promoter region of the *TEF1* gene was chosen because it is a commonly used, constitutively expressed gene with a well-characterized function[62]. The gene product of *TEF1* is responsible for binding aminoacyl t-RNA to the ribosome during translation[73]. We hypothesized that a dual color reporter controlled by the same promoter system would allow us to perform system calibration while letting us explore subtle differences in luciferase compatibility, if any.

Plasmid constructs with individual luciferases under the control of the same constitutive promoter were sequentially integrated into the genome of *S. cerevisiae* and tested in various culture conditions. We predicted that the ratio of green to red would remain stable regardless of perturbations to the individual luminescence levels. The reporter should reveal subtle changes to gene expression in real time more effectively than a single color luciferase alone.

As shown in figure 2.3, yeast when maintained in continuous culture can express sustained, robust oscillations known as the Yeast Respiratory Oscillation (YRO). During this respiratory cycling, levels of Dissolved Oxygen (DO) tend to fluctuate between ∼10-50% of the atmospheric saturation, depending on the conditions[69]. These rapid changes in oxygen levels can affect luminescence output since oxygen is one of the required substrates for the bioluminescence reaction[50]. The deficiency of this reaction is especially apparent during hypoxic mask, a portion of an oscillation in the YRO when the DO levels drop due to respiring cells that rapidly consume oxygen[69,87]. When a single luciferase reporter controlled by

a constitutive promoter is used to monitor gene expression during the YRO, the promoter activity is masked during hypoxia (shown in figure 2.3).

To circumvent this problem, as shown in figure 2.4, a dual reporter was used under the same conditions as described above. Although the luminescence trends follow the same pattern as that of a single color reporter, the green/red ratio reveals the activity of the promoter to be near constant at all times (Figure 2.4; panel B). In a recent study by Robertson *et al.*, a western blot of samples collected over one cycle of the YRO reveals the same data as that of a dual color reporter[69]; the gene expression remains fairly constant through the hypoxic mask. Although the promoter activity can be detected by alternative techniques such as FACS, microarray, and immunoblots, these methods are labor intensive and expensive. A dual color reporter would serve as an inexpensive, efficient, and real time tool to monitor gene expression during active metabolic conditions.

Although the activity of the promoter within hypoxia is revealed through the G/R ratio, the data is not always seamless. Our experiments indicate that the ratio largely depends upon the luminescence output of the individual luciferases. For a smooth and continuous transition, it was observed that the red signal be maintained at ∼3-5 times the brightness of the green signal. The transitionary spikes observed in the ratio at the beginning and end of hypoxia (figure 2.4) are artifacts and are known to arise from the sudden changes in luminescence.

2.2.3.2 Inducible promoter

Another promoter system that was used to explore the utility of the dual color reporter was *GAL1*, a galactokinase coding gene involved in the catabolism of galactose whose expression is stimulated in the presence of galactose and repressed in the presence of glucose[17]. This promoter was chosen because it would allow us to control the expression of luciferase by inducing with galactose. The *GAL1* promoter was fused to CBG and integrated into CEN.PK that already has a constitutive promoter (P_{TEF1}) driving the expression of CBR.

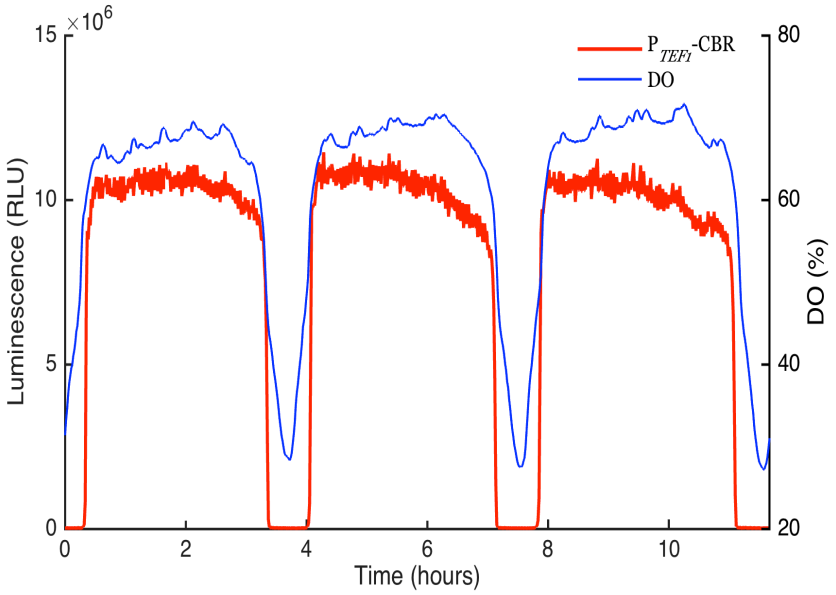

Figure 2.3: The ability of a single luciferase gene to report gene expression is limited due to recurring hypoxia during the YRO. Yeast strain transformed with P_{TEF1} driving CBR is grown in continuous culture to establish oscillation (DO-blue, CBR-red). It is observed that the signal is masked during hypoxia thereby restricting the use of a single color luciferase reporter alone.

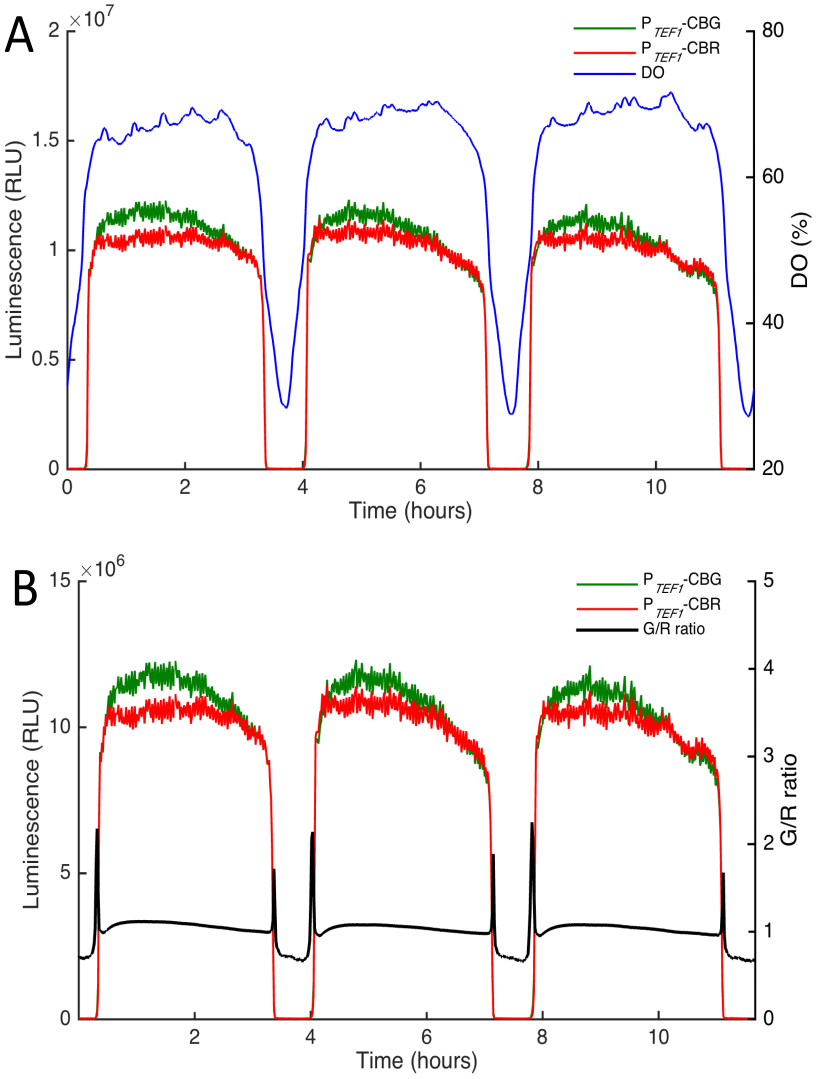

Figure 2.4: A constitutively expressed promoter controlling the expression of both luciferases reveals transcriptional activity during the hypoxic mask. (**A**) Real time luminescence monitoring of a dual luciferase reporter across a ∼11h period. The luminescence output alone (green CBG, red-CBR) is hampered by the recurring dip in dissolved oxygen levels (blue). (**B**) G/R ratio plotted against luminescence output and time reveals the gene expression in hypoxia. It should be noted that the ratio stays fairly constant throughout the entire period except for the transition spikes observed due to sudden changes in oxygen levels.

When tested under different culture conditions with various sugar treatments, this system shows the effectiveness of G/R ratio to predict the gene expression.

As proof of feasibility, yeast stably transformed with the luciferases was patched onto plates containing glycerol as the carbon source. Yeast with only a single reporter was also patched onto the plate as controls. Then, one half of the plate was treated with galactose and the other half was treated with glucose (figure 2.5); the plate was imaged with an ultrasensitive camera ∼7h after sugar treatment. When yeast cells are treated with glucose, they metabolize this sugar extensively and upregulate ∼20% of their genome[93]; *TEF1* being one of them[62]. Therefore, these cells luminesce red. Galactose treated cells, on the other hand, increase the activity of *GAL1* and *TEF1*. *GAL1*, outlined earlier, is required for metabolism of the sugar, galactose while *TEF1* being constitutive, shows stable activity during galactose treatment as supported in other studies by Partow *et al.*, 2010[62]. These patches appear yellow since both luciferases are co-expressed.

When yeast cells containing luciferase constructs as described above are grown in batch cultures and the luminescence output is monitored continuously, the ratio showed constant promoter activity and compensated for changes in variables such as oxygen and cell number. Figure 2.6 helps illustrate the efficiency of this tool. The culture was allowed to grow for 8 hours and then treated with either glucose (panel A) or galactose (panel B) to induce expression of either or both of the luciferases. In the glucose treated sample, the red luminescence peaks while the ratio stays flat indicating that the *GAL1* promoter is not activated. The ratio peaks in the galactose treated sample because the promoter of interest is activated. It should be noted that both of these samples have a constant change in external variables such as cell number and substrate concentration and the G/R ratio compensates for these rapid variations.

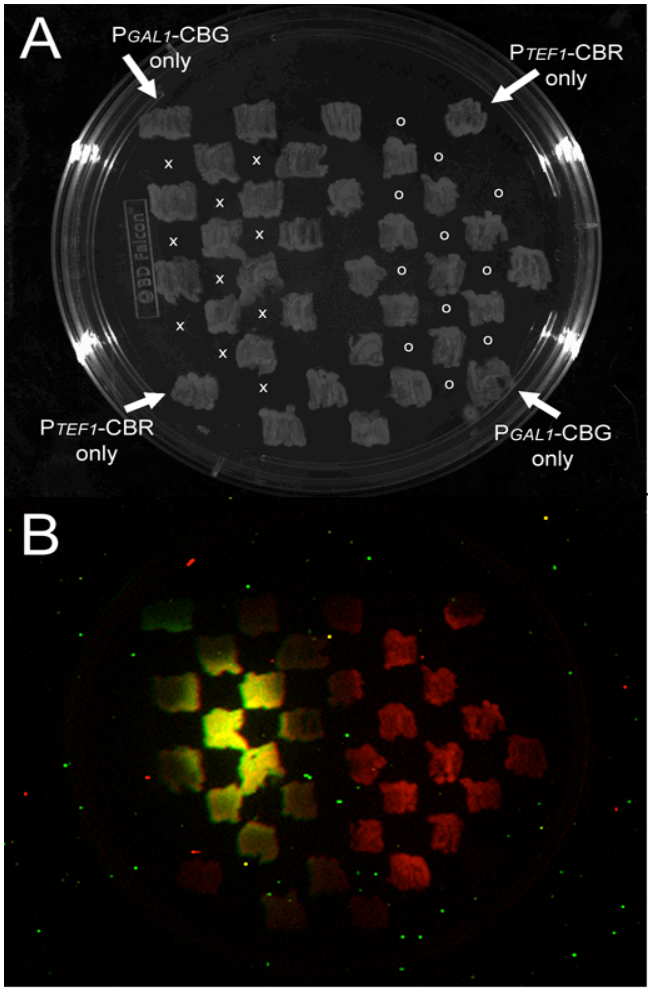

Figure 2.5: Effect of sugar treatment on a dual color luciferase system with galactose inducible CBG99 (P_{GAL1}-CBG) and constitutively expressed CBR (P_{TEF1}-CBR). (**A**) Brightfield image of a patch plate of yeast stably transformed with a dual color reporter. Yeast transformed with single color luciferases were also patched onto the plate as controls. Galactose (4µl) and glucose (2µl) were dotted onto the spaces between patches; indicated with X and O respectively (**B**) Multicolor image of the patch plate after treatment with sugar. The output indicates upregulation of different promoters with regard to the treatment received. Yellow indicates co-expression of red and green luciferases, while red indicates expression of *TEF1* only.

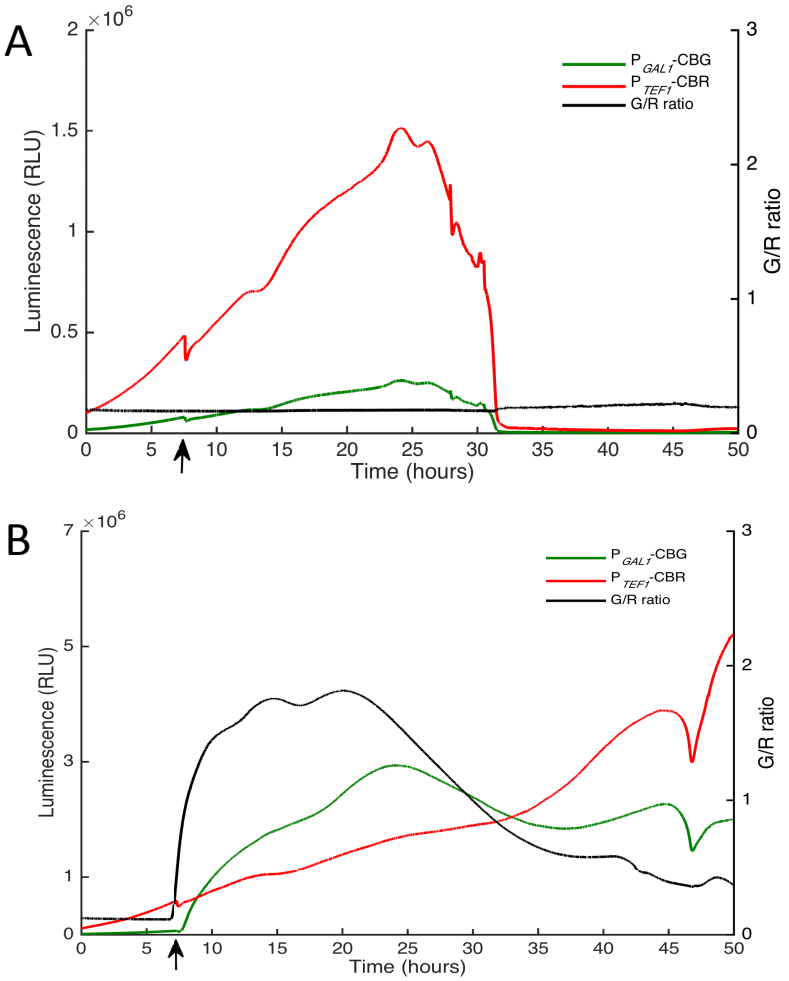

Figure 2.6: Real-time luminescence monitoring of a batch culture of *S. cerevisiae* co-transformed with P_{GAL1}-CBG and P_{TEF1}-CBR. (**A and B**) Luminescence from the yeast culture was monitored before and after treatment with 444µl of 36% glucose and 400µl of 40% galactose (time of addition of sugar indicated by an arrow). Luminescence output from the constitutively expressed luciferase (red trace) and galactose inducible luciferase (green trace) was plotted against time. The G/R ratio (black trace) stays flat (A) during glucose addition indicating that the expression of P_{GAL1}-CBG is not affected while the ratio peaks in panel B; demonstrating the upregulation of the promoter of interest.

The expression of luciferase under the control of P_{GAL1} promoter can be examined by a western blot. An immunoblot of cells collected over the course of a batch culture experiment (before and after induction with galactose) was probed for luciferase. As shown in figure 2.7 (panel B), the blot reveals increasing amounts of the luciferase protein as supported by the luminescence data.

2.2.3.3 Cell-cycle regulated promoter

Recent studies suggest that a portion of the cell population divides synchronously with the YRO indicating a tight coupling between the two phenomena[69,87]. Moreover, microarray analyses have pointed to the peak of expression of cell cycle related genes in the reductive phase, possibly to maintain the cell at a physiologically active state[79]. Out of the genes that are periodically expressed during the YRO, *POL1* is chosen for investigation in this study. According to the online timecourse microarray database, SCEPTRANS (*S.cerevisiae* periodic transcription server), the peak expression of the *POL1* gene coincides with a rise in DO levels[39]. This matches with the experimental evidence that point to its function; it is an essential gene that encodes the catalytic subunit of DNA polymerase and is maximally transcribed at the G_1/S boundary[90].

Earlier studies indicate that the output from a single color luciferase reporter driven by P_{POL1} becomes masked during hypoxia (due to low O_2) (figure 2.8)[69]. A dual color reporter in the same conditions (figure 2.9) shows continuity in *POL1* expression between two continuous oscillations of the YRO. This provides further evidence to the idea of the existence of two subpopulations in antiphase with each other in CDC[44,69]. As explained earlier, the dilution rate of the continuous culture supports a doubling time of ∼8h, average time required for two complete oscillations of the YRO. Other reports involving the use of microarrays or fluorescent reporters support the two-population hypothesis although there seems to be heterogeneity in reporter expression possibly hinting at the complexity of the phenomenon of YRO[44,87].

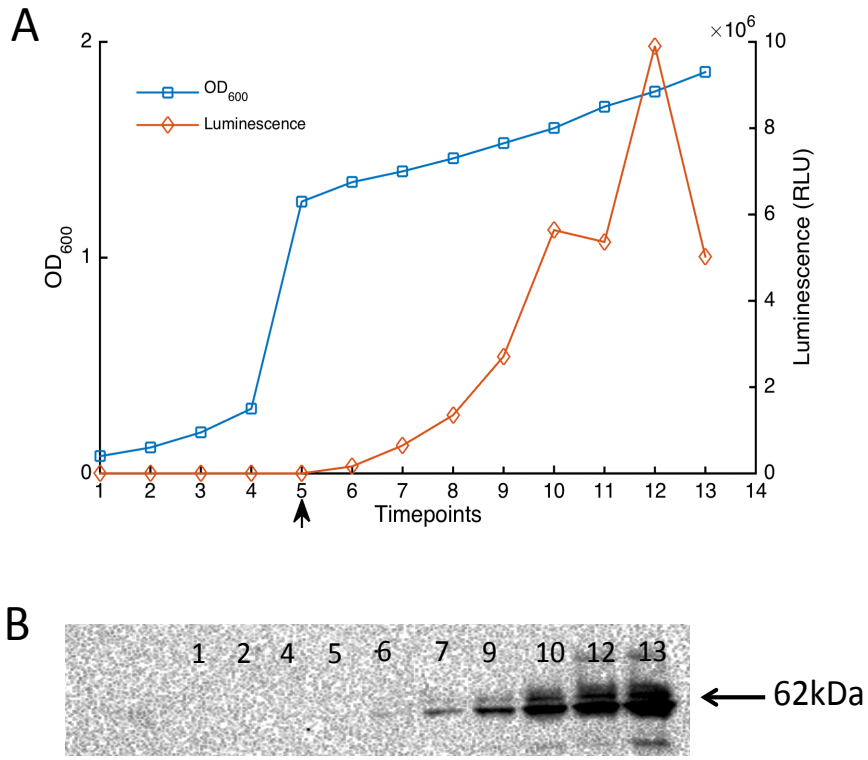

Figure 2.7: Immunoblot reveals that the luciferase protein concentration correlates with the observed luminescence trend. (**A**) Yeast transformed with P_{GAL1}-luc was grown in a batch culture (∼200mls) and the OD_{600} (blue) and luminescence (orange) readings were noted every 3 hours for ∼24 hours. The culture was treated with 4ml of 40% galactose at timepoint 5 (marked by an arrow). About 800µl of sample was collected at indicated timepoints and total protein was extracted. (**B**) An immunoblot of total protein probed with anti-luc antibody shows that the luminescence data corresponds with the protein concentration observed.

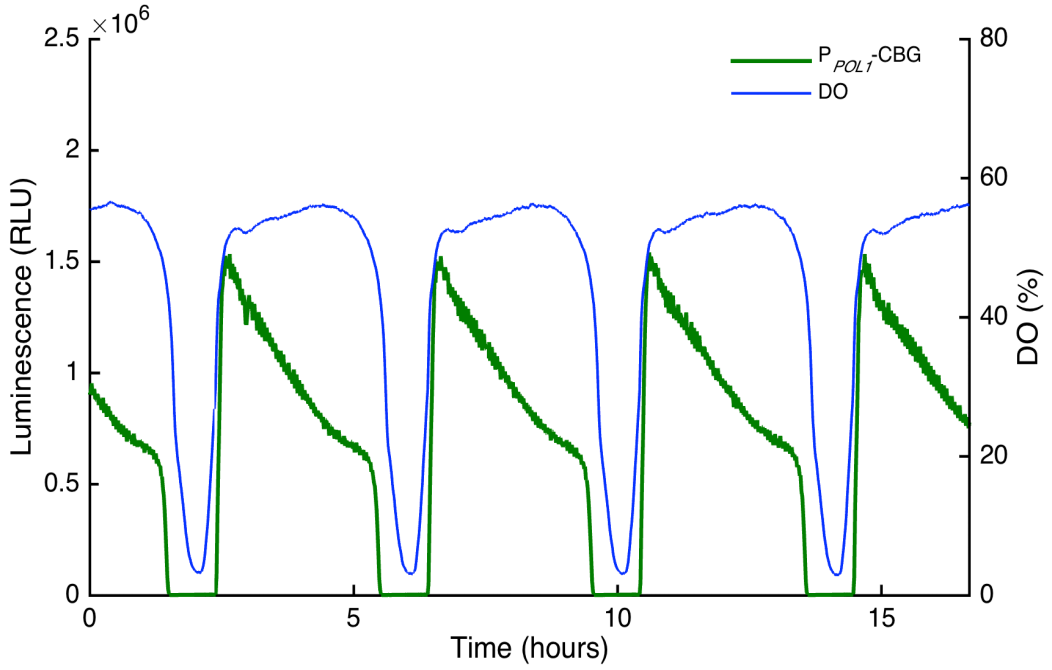

Figure 2.8: Expression of the *POL1* gene in a continuous culture. Real-time activity of P_{POL1} (green trace) monitored across a 30 hour time period of the YRO (Dissolved Oxygen (DO) indicated by a blue trace). During the hypoxic mask, luminescence data is concealed, making it appear as though the expression of *POL1* is discontinuous between oscillations. However, we predict that the G/R ratio will reveal a continuum in expression during hypoxia.

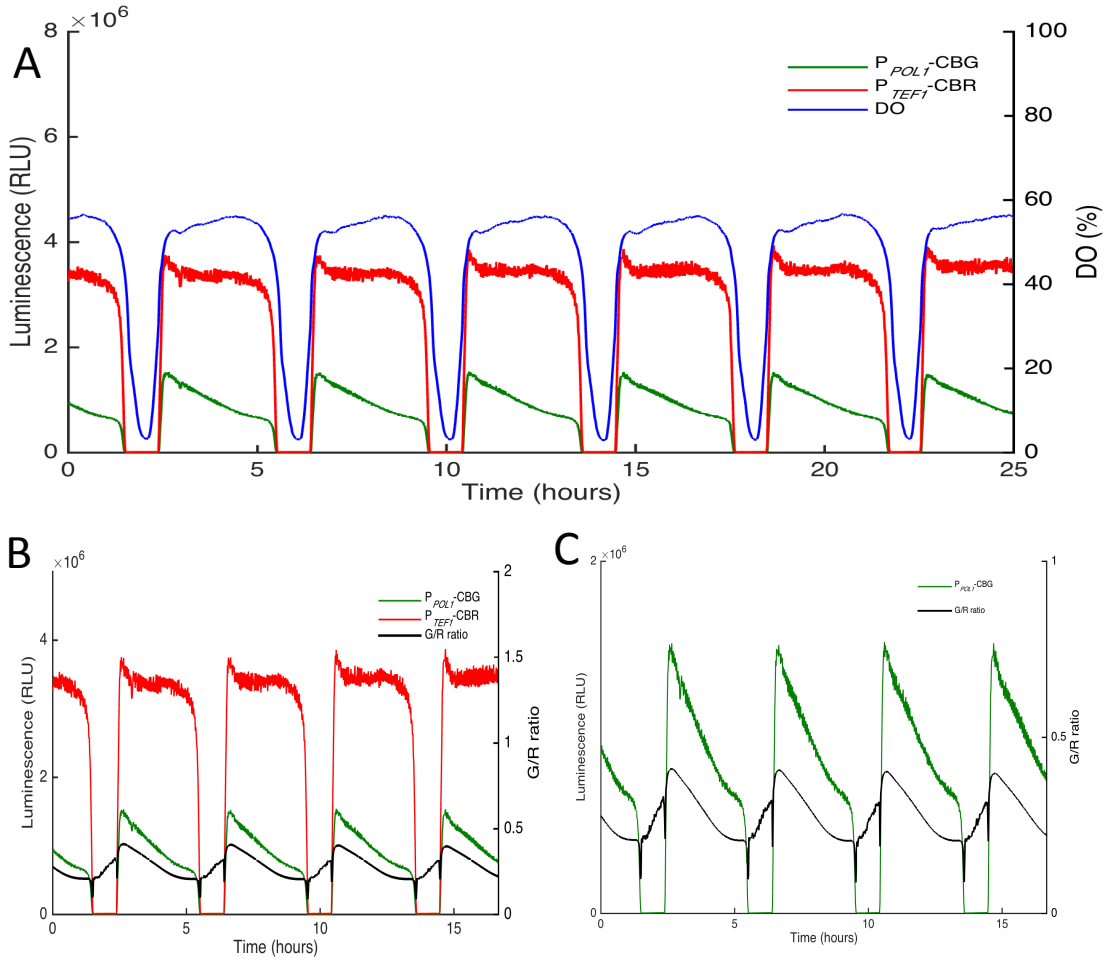

Figure 2.9: A dual color luciferase reporter can be used to study transcriptional activity of genes involved in cell cycle regulation in continuous culture. (**A**) A dual color reporter that monitors P_{POL1} activity with CBG (green trace) and P_{TEF1} activity with CBR (red trace) can be used to observe the expression of genes involved in cell-cycle regulation during hypoxia. Both luciferases succumb to the dip in DO levels (blue) across the oscillation. (**B**) The G/R ratio (black trace) reveals that P_{POL1} activity increases relative to P_{TEF1} activity during the hypoxic mask (**C**) P_{POL1}-CBG(green) and G/R ratio (black) are plotted as a function of time and the continuity in gene activity is clearly observed. Transition spikes (artifacts due to sudden changes in DO levels) are also visible.

2.3 Materials and Methods

2.3.1 Naming conventions

For plasmid names, the letter p is added at the beginning of the backbone name. Any modification to the plasmid (addition of a luciferase gene or an antibiotic resistance marker) is indicated after the backbone name. The letter 'P' followed by the actual promoter name in the subscript indicates the promoter driving the luciferase gene. For instance, the plasmid 'pRS305HPH-P$_{TEF1}$-CBG99PEST' refers to a pRS305 backbone[77] containing a hygromycin resistance cassette and a *TEF1* promoter driving the destabilized CBG99 luciferase gene. In this study, all CBG99 luciferase genes were added to the pRS305 vector backbone and all the CBR luciferase genes were added to the pRS306 vector backbone. Therefore, P$_{TEF1}$-CBG99PEST simply refers to the construct in the pRS305 backbone.

2.3.2 Reporter gene plasmid construction

The CBG99 and CBR plasmid constructs were designed to integrate into the genome of the *S. cerevisiae* strain CEN.PK113-7D (Mat**a**) (provided by Dr. Peter Kötter, University of Frankfurt). The pRS305 and pRS306 vector backbones were used as the template to append additional genetic elements. The construction of the plasmid is described as follows. The SV40 terminator of the pCBG99-basic and pCBR-basic (Promega) was replaced with *ADH1* terminator from pRS315-Luc(A4V) using XbaI and SalI[69]. To move the reporter-terminator combination to the vector backbone, a PCR product containing the coding sequence (cds) of the luciferase gene with the *ADH1* terminator was produced using primers 1,2, and 3 (see Table 2.1) and XmaI and SalI[77].

As detailed below, additional genetic modifications were made to the pRS305-CBG99 plasmid. The first modification included the addition of an antibiotic resistance selectable marker, Hygromycin (*HPH*). To do this, the *HPH* gene was PCR amplified from pYM-24[30] using primers 4 and 5 that added a BamHI site

at the 5' end and used an endogenous NotI site at the 3' end. The amplicon was then moved to the plasmid using BamHI and NotI, thereby yielding pRS305*HPH*-CBG99. The second modification added a unique NheI site just upstream of the stop codon of the CBG99 cds, for purposes of generating C-terminal fusions for later applications. Primers 6 and 7 were used to generate a CBG99 cds with a BglII site at the 5' end and a NheI site and XbaI site at the 3' end (prior to the stop codon). This PCR product replaced the cds on the earlier construct thus yielding pRS305*HPH*-CBG99(NheI).

A PEST degron motif was amplified from the pRS303-P_{POL1}-Luc(A4V)-PEST plasmid using primers 8 and 9[69]. The original PEST motif comes from the C-terminus of the CLN2 gene that codes for the C-terminal end of the gene product. The primers were designed to amplify the PEST motif along with the *ADH1* terminator; the amplicon was then moved to pRS305HPH-CBG99(NheI) with NheI and SalI to yield the final construct pRS305*HPH*-CBG99(NheI)-PEST.

To design plasmids with different promoters (P_{TEF1}, P_{GAL1}, P_{POL1}, P_{ACT1}) driving the CBG99 luciferase, the promoter regions of the P_{ACT1}, P_{POL1}, and P_{GAL1}-Luc(A4V)-PEST were amplified using primers 12-17. The P_{TEF1} promoter was amplified from pYM-N18[30] using primers 10 and 11. These PCR products were moved into the desired vector using XmaI and BglII. A description of the primers used in the construction of the reporter plasmids is shown in Table 2

2.3.3 Transformation of *S. cerevisiae* with plasmid constructs

Once the integrative plasmids were constructed, they were transformed into *S. cerevisiae* (strain: CEN.PK 113-7D Ura-). Plasmids with the pRS305 and pRS306 backbone were linearized with AflII and StuI respectively and transformed with the standard Lithium acetate protocol[29]. Sequential transformation was preferred for ease of selection and efficiency. pRS306 plasmid transformants were selected on CSM-Ura- plate (Per 100ml: 2g Glucose, 0.67g Yeast nitrogen base without

Table 2: Primers used for the construction of a dual color reporter system in *S. cerevisiae*

#	**Name**	**Sequence**
1	CBG99-5(XmaI,BglII)	ttcact**CCCGGG**ttactt**AGATCT**ATGGTGAAGCGTGAGAAAAATG
2	CBR-5(XmaI,BglII)	ttcact**CCCGGG**ttactt**AGATCT**ATGGTAAAGCGTGAGAAAAATG
3	CBG or CBR-3(SalI)	atgatt**GTCGAC**GGATCTATATTACCC
4	*HPH*-5(BamHI)	ttcact**GGATCC**GACATGGAGGCCCAGAATAC
5	*HPH*-3	TACGACTCACTATAGGGAGACC
6	CBG99cds-5(BglII)	TTAACA**AGATCT**ATGGTGAAGCG
7	CBG99cds-3(NheI, XbaI)	atgatt**TCTAGA**gatgat**GCTAGC**ACCGCCGGCCTTCTCCAACAA
8	PEST-5(NheI)	ttcact**GCTAGC**GCATCCAACTTGAACATTTCG
9	ADH1term-3(SalI)	atgatt**GTCGAC**GGATCTATATTACCCTG
10	P_{TEF1}-5(XmaI)	ttcact**CCCGGG**GAGCTCATAGCTTCAAAATGTT
11	P_{TEF1}-3(BglII)	atgatt**AGATCT**AAAACTTAGATTAGATTGCTATGC
12	P_{GAL1}-5(XmaI)	ttcact**CCCGGG**GGCATTACCACCATATACATATC
13	P_{GAL1}-3(BglII)	atgatt**AGATCT**TATAGTTTTTTCTCCTTGACGTTA
14	P_{POL1}-5(XmaI)	ttcact**CCCGGG**TGCATTTTTCTTAAAGAAATATAAC
15	P_{POL1}-3(BglII)	atgatt**AGATCT**TTTCCACTGTTTATTATATGCCT
16	P_{ACT1}-5 (XmaI)	ttcact**CCCGGG**TAAGTAAATAAGACACACGCGAG
17	P_{ACT1}-3 (BglII)	atgatt**AGATCT**TTGTTAATTCAGTAAATTTTCGATC

amino acids, 0.77mg complete supplement mixture drop out (-URA), 2% agar) while pRS305 plasmid transformants were selected on (4µl/ml) hygromycin supplemeted YPD plates.

2.3.4 Batch culture cultivation and monitoring luminescence

2.3.4.1 Culture conditions The setup consisted of a 15ml conical glass test tube, one 4 inch 18-gauge needle (septum penetration needle, Popper & Sons, inc., New Hyde Park, NY), a 5 inch glass capillary tube (VWR 53432-921), a 1.5 inch 21-gauge syringe needle (Becton Dickson 305167), and a size-0 rubber stopper that fits the conical tube (see fig)

2.3.4.2 Media conditions A 4ml overnight culture of the desired strain was inoculated in YPG (Yeast extract (1%), Peptone (2%), 50% Glycerol (240µl)). The following day, a 1:50 dilution was inoculated in YPG (10 ml YEP (Yeast extract (1%), Peptone (2%)) including 600µl of 50% glycerol, 200µl from the overnight culture, and 10µl of luciferin). To prevent excessive foaming, 1.6µl of antifoam A (Sigma-Aldrich) was added to the culture.

2.3.4.3 Recording luminescence The 1:50 dilution initiated on the day of the experiment was incubated in a shaker for about 4 hours (until the OD was 0.6). The culture (∼8ml) was then transferred to a conical test tube where filtered air was sparged through a capillary tube from an aquarium pump. The constant infusion of air ensured two things: proper mixing and relatively constant aeration of the culture. The sparged culture tube was then placed in a light tight box with photomultiplier tubes (PMTs) fitted with appropriate filters monitoring luminescence (put a figure of the setup in the blackbox). The output (RLUs) was continuously plotted as a function of time. For sugar injection, the 1.5-inch needle through the rubber stopper was used as a port.

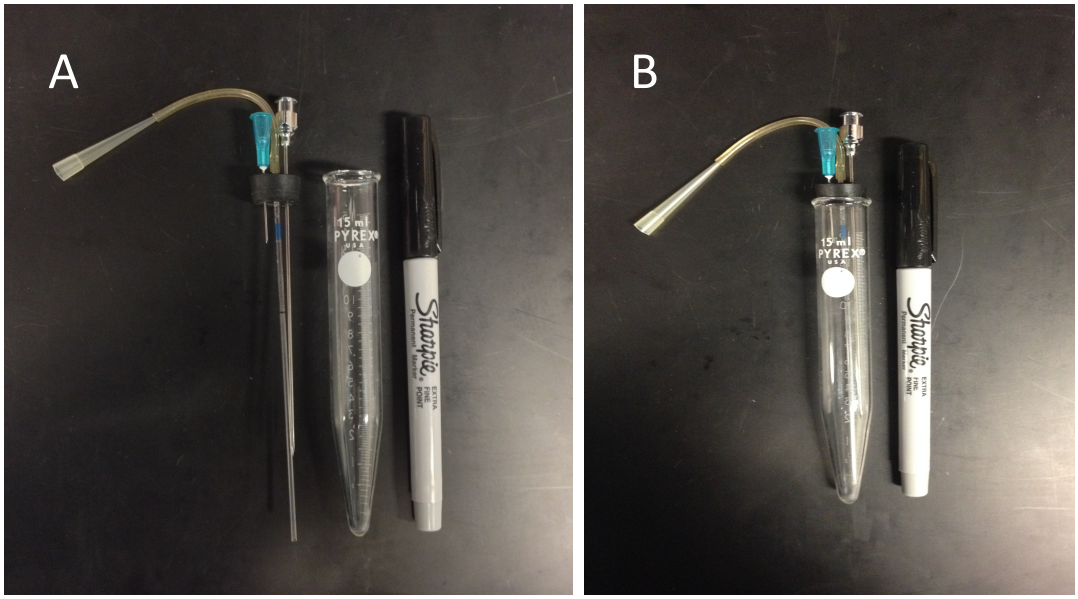

Figure 2.10: Components of the mini batch fermentor used to monitor luminescence. (**A**) The stopper head consists of (from right to left): a long syringe needle (for addition of chemicals), a glass capillary long enough to reach the bottom of the conical tube (for aeration), and a short needle (for vent). All of these components are fit inside a 15ml glass conical tube (Sharpie shown for scale). The tubing (Tygon) that runs from the glass capillary is hooked up to a pump that supplies air to the culture. (**B**) A completely assembled miniature batch fermentor vessel.

2.3.5 Continuous culture cultivation and recording luminescence

2.3.5.1 Culture conditions Continuous culture experiments were conducted in a 3 L BioFlo 115 benchtop fermentor (New Brunswick). The setup, briefly described below, is as indicated in the study by Robertson *et al.*, 2008[69]. The features of the bioreactor included a headplate that consisted of ports for continuous media supply and removal, filtered air supply, NaOH supply (for pH adjustment), and probes to monitor the DO, temperature, and pH levels. In this study, unless otherwise specified, the fermentor was operated at an agitation rate of 550 rpm, an aeration rate of 0.9 L/min, pH of 4.0, and a temperature of 30℃. A schematic of the layout of the fermentor is shown in (put a figure of the setup of a fermentor).

2.3.5.2 Media conditions About 20 ml of an overnight culture of the desired strain grown at 30℃ was used to inoculate ∼850 ml of bioreactor media. The media composition (per liter) is defined as follows: 5g Ammonium sulfate, 2g Potassium phosphate monobasic, 0.5g Magnesium sulfate heptahydrate, 1g Bacto Yeast extract, 10g glucose, 0.5ml of 0.25M CaCl2, 0.5ml of 70% sulfuric acid, 0.5ml metal stock and desired amount of antifoam A. The metal stock solution consisted of 40g/L Ferrous sulfate heptahydrate, 20g/L Zinc sulfate heptahydrate, 10g/L Cupric sulfate pentahydrate, 2g/L Manganese chloride tetrahydrate, 20ml/L 75% sulfuric acid.

2.3.5.3 Monitoring luminescence To initiate oscillations, conditions as defined in the study by Tu *et al.,*[87] were maintained. In brief, cells were allowed to grow in a batch culture until glucose was consumed, indicated by the return of DO levels to ∼95%-100% after which they were starved for ∼ 4hours. Continuous culture was then established by supplying media at a dilution rate of 0.08-0.09/h. To monitor luminescence, (100µM) of D-luciferin (potassium salt, GoldBio LUCK-100) was directly injected into the fermentor during the hypoxic phase of an oscillation. Following this, the culture was continuously supplied with

luciferin stock (rate = 0.532 ml/h) using a standard Harvard Apparatus syringe pump. Using a high speed peristaltic pump, media was continuously drawn from the fermentor in a tube (Nalgene) and wrapped around an opaque conical in a light tight box where the luminescence was recorded using the Hamamatsu PMTs (HC135-01) fitted with appropriate filters (Edmund optics: 65711 and 64699). The culture was then returned to the fermentor.

2.3.6 Protein extraction and immunoblot

Protein extraction was carried out as described by Robertson *et al.*,[69]. A dense overnight culture of pRS303-kan-P$_{GAL1}$-Luc-PEST stably transformed in CEN.PK 113-7D was setup on YPG (composition described above). The following day, a 1:50 dilution of the overnight culture was established in 200ml of YPG (with 200µl of luciferin) and incubated in a shaker at 30℃. About 800µl of sample was then collected every hour for the next 5 hours. Approximately 18 hours later, the culture was treated with 4ml of 40% galactose solution, following which samples were collected every 30 minutes.

The collected samples were monitored for optical density using a spectrophotometer (Spectronic Genesys 5) and luminescence using a luminometer (Berthold FB 12). Subsequently, cells were pelleted in Fischer Scientific Marathon 16km at 2000rpm for 5min at 4℃, washed with 500µl of TMG (For a 50ml solution: 500µl of 1M Tris-Cl (pH8.0), 100µl of 0.5M $MgCl_2$, 10ml 50% glycerol, 2ml of 5M NaCl, and 5µl of 1M DTT)), and resuspended in 500µl of chilled TMG + proteinase inhibitor (1 tablet of EDTA-free proteinase inhibitor cocktail (Roche) per 7.5ml of TMG).

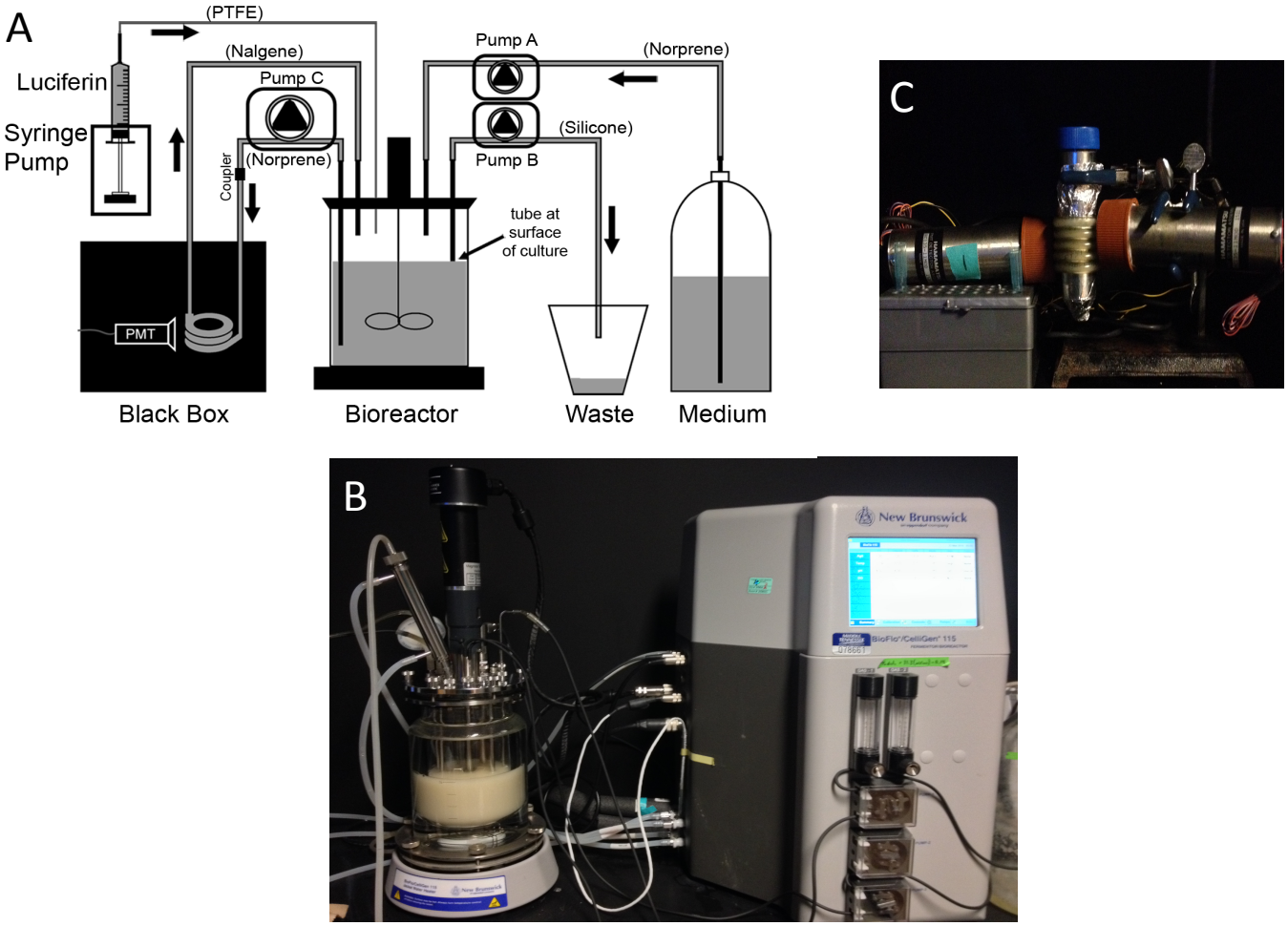

Figure 2.11: A schematic of the setup used to continuously culture yeast and monitor luminescence. (**A**) Pumps A and B are used to feed and remove culture from the bioreactor respectively. Pump C circulates ∼20ml of culture through another tube that is placed in a light tight box where two photomultiplier tubes (PMT) record luminescence simultaneously. Luciferin is slowly injected into the fermentor at the rate of 0.532ml/h. Arrowheads indicate direction of flow of the culture. (Figure used with permission from Robertson *et al.*,[68]). (**B**) shows a fully assembled fermentor with the controller. The water jacket controls the temperature at 30°C. (**C**) Setup of the 'blackbox' for real-time monitoring of luminescence. PMTs (fitted with appropriate filters) are placed on either side of an opaque conical tube wrapped with a Nalgene tube through which the culture is continuously moving.

For protein extraction, the treated time point samples (∼300µl) were mixed with an equal volume of glass beads (Sigma G8772) in 1.5ml snap-cap tubes and lysed simultaneously in the Bullet Blender storm 24 (Next Advance) for 30 sec. The samples were subjected to bead beating about 10 times with 2 min of ice between lysis. Lysis was confirmed by microscopic observation. The bottom of the tubes were then pierced with a needle and inserted into a new microcentrifuge tube and centrifuged at 10000 rpm for 20s at 4℃ to separate the lysate from the glass beads. The collected lysate was centrifuged again at top speed for 30 min to remove the insoluble proteins. The supernatant was aliquoted into a new tube for immunoblot analysis.

Protein content in the lysates was quantified by Pierce BCA assay kit (Thermo Scientific). Protein amounts of 15µg from each timepoint sample was mixed with 10X Laemmli sample buffer, boiled at 95℃ for 5 min, and loaded onto each well of a 7.5% acrylamide gel. Protein ladder (PageRuler Plus) (5µl) was loaded on lanes at either ends of the gel. Proteins from the gel were transferred to a nitrocellulose membrane (Thermo Scientific) using Trans-Blot Turbo (Bio-Rad). The membrane was then blocked with 20ml of 5% milk in 1X TBST (For a 100 ml solution: 0.12g Tris, 0.88g NaCl, and 100l of Tween-20) for 1 hour at room temperature. Primary antibody (1:2000; Sigma L0159, polyclonal rabbit anti-luciferase) was also diluted in the blocking solution and the membrane was incubated in the antibody solution overnight at 4℃. The following day, the membrane was washed 5 times in 1X TBST for 10 minutes each, then incubated in secondary antibody (1:20000; goat anti-rabbit) for 1 hour at room temperature with gentle rocking. Following the treatment with the secondary antibody, the membrane was washed again in the same procedure as described above. The membrane was then incubated in HRP substrate (SuperSignal West Pico, Thermo Scientific) for 7 minutes and imaged using a chemiluminescence detector (ChemiDoc MP, BioRad).

2.3.7 Spectral scan

Dense overnight cultures of yeast transformed with either pRS306-P$_{ACT1}$-CBR or pRS305HPH-P$_{ACT1}$-CBG99PEST were used to obtain the emission spectra. For luminescence measurements, QuantaMaster QM-7/SE (Photon Technology International, Birmingham NJ) spectrofluorometer was used. The measurements were recorded as running averages (± 10 nm) at 25℃ and reported in Relative Luminescence Units (RLU).

2.3.8 Half-life test (unpublished results, J.B. Robertson)

Overnight cultures of SEY 6210 (MATα leu2 ura3 his3 trp1 lys2 suc2)[71] containing either pRS315-P$_{GAL1}$-CBG99 or pRS315-P$_{GAL1}$-CBG99-PEST were grown on supplemented minimal media lacking leucine containing raffinose and luciferin (Composition per liter: 6.5g Difco yeast nitrogen base without amino acids, 20g raffinose, 15.6mg uracil, 15.6mg tryptophan, 15.6mg adenine, 23.6 mg lysine, 15.6mg histidine, and 50µM luciferin). Throughout the duration of the experiment, the culture was grown at 28℃ with constant agitation. At OD_{600} 0.8, the culture was divided into 5ml aliquots and added to scintillation vials. At time minus 60 minutes, 250µl of 40% galactose was added to induce the *GAL1* promoter. Luminescence was measured (Zylux Femtomaster FB12) at an interval of 20 minutes until time 0 when the culture was treated with a mixture of 400µl of 25% glucose and 125µg/ml cycloheximide. Post repression, bioluminescence was measured every two hours. Luminescence measurements were carried out in six vials, two of which were controls. One control did not receive any galactose (uninduced) while the other control was not treated with glucose/cycloheximide (not repressed).

2.4 Conclusion

This chapter highlights the effectiveness of a dual luciferase reporter in monitoring gene expression in various culture conditions. We conclude by summarizing our

findings while raising a few questions about the CDC-YRO relationship. The recurring hypoxic mask during YRO restricts the use of a single luciferase reporter, however, the design of using a luciferase as a normalizing control for gene expression while another luciferase monitors the activity of a promoter of interest has allowed us to detect genetic events in yeast. The ease of continuous, real-time measurement of transcriptional activity is illustrated with the use of constitutive, inducible, and CDC-regulated promoter systems. Our experiments with the *POL1* promoter prompt several questions: What factors dictate the cells to divide in two distinct populations during the YRO? What do the non-dividing cells in each oscillation do to resist the signals, if any, that contribute to this alternate division? Is the whole phenomenon restrained by the conditions maintained i.e., do external factors force the cells to behave this way? Further studies are required to answer this complicated phenomenon in question. We expect this tool to be one step toward opening up new avenues for investigating other fascinating phenomenon in populations of metabolically cycling yeast.

Chapter III

USING A PHOTOSENSITIVE PROMOTER SYSTEM TO TEST GENE REGULATION DURING YRO

3.1 Introduction

The products of gene expression are pivotal in dictating the growth and development of the cell. Researchers now have the advantage of studying physiological/chemical processes that occur within the cell by controlling the expression patterns of specific genes. Bakers yeast, *Saccharomyces cerevisiae*, is an attractive model for understanding gene expression profiles because of its genetic tractability and the strong genetic homology that it shares with more complex organisms[4].

To monitor gene expression, fusions of well-characterized yeast promoters with native or foreign genes are used. Many inducible and non-inducible promoter systems have been identified for this purpose. The non-inducible promoters include P_{TEF1}, P_{GPD}, P_{CYC1}, and P_{ADH1}; all of which are constitutively expressed but at different magnitudes owing to their varying strengths[3,12]. To exercise more control over gene expression, a complementary promoter system that can be conditionally induced with small molecules have been characterized. These include well-known promoters such as P_{GAL1}, P_{CUP1}, and P_{MET25}. P_{GAL1} can be induced with the sugar galactose to increase its expression ∼1000-fold over a span of four hours[33], the expression of P_{CUP1} is rapidly induced in the presence of copper (30 min for maximal expression)[42], whereas P_{MET25} is induced in the absence of methionine[35]. Other systems that are genetically engineered to respond to orthogonal molecules such as hormones or antibiotics have also been employed to identify transcriptional control, examples of which include the TET-ON/TET-OFF system[21,33] and Gal-ER-VP16[48].

As promising as the prospect of using inducible promoters are, they suffer from certain drawbacks. The response to induction with chemicals can be graded,

discontinuous, or even over-expressed. Moreover, the expression of these promoters cannot be completely turned "off" even in the absence of these chemicals, since they tend to linger in the media and become difficult to remove after addition[12]. Perhaps the most important detriment of using small molecules for induction is the reversibility of the reaction, i.e., media manipulation can have adverse effects on the output of a reaction.

Several groups have reported the use of light-responsive DNA elements to control protein expression using various approaches. One technique exploits split transcription factors that dimerize in the presence of light, as in yeast two-hybrid systems[34,74]. Another technique uses plant phytochromes in genetically encoded plasmid constructs in bacteria[61], and mammalian cells. This system has certain advantageous features: (i) This does not require altering media components, and hence can be fully mechanized, (ii) The reaction is completely reversible, and (iii) The expression levels are entirely dose-dependent and cause minimal damage to the cells.

This chapter focuses on the CRY2 (cryptochrome 2)/CIB1 system, derived from *A. thaliana*, which is stimulated by blue light and rapidly shut off in the absence of light. This photoreceptor system, originally designed by Hughes *et al.*, 2012[27] was placed under the influence of a *GAL1* promoter and could only be used in specific strains of yeast, as detailed below.

Plasmid constructs containing the CRY2/CIB1 domain, have been shown to work well in a Gal4/Gal80 deletion strain, however, to expand its utility in other strains, the DNA binding domain of the E.coli LexA protein[7] was fused to CRY2 while the Gal4 activation domain of the Herpes Simplex Virus, VP16 was fused to CIB1. Taken together, this system employs a split transcription factor where the activator and binding domains for LexA are protein fusions with CRY2 and CIB1 that rapidly dimerize in the presence of blue light. To further increase the efficiency, 8 operator regions of LexA (driving LacZ) from pSH18-34 were used. The

modifications described above resulted in an efficient blue-light mediated response while minimizing background protein expression levels[27].

Here, we discuss the use of a light mediated protein interaction in the context of the Yeast Respiratory Oscillation (YRO), a rhythmic process observed under certain conditions of continuous culture, to assess the advantages and drawbacks of using a photosensitive system to investigate protein expression during hypoxia. Furthermore, we predict that using a normalizing luciferase with a light-dependent promoter driving another luciferase will allow us to envision a pattern of productivity in gene expression during the YRO that would otherwise be hampered by using a single color luciferase since light itself affects luciferin stability and the YRO[70].

3.2 Results and discussion

3.2.1 Light-dependent transcriptional activation

To optimize experimental parameters, initial tests involving the lexA-CRY2PHR and VP16-CIB1 constructs were conducted. A single color luciferase reporter was sufficient to estimate the intensity of light needed and length of time required to induce and turn off the promoter system. Therefore, preliminary experiments only included the destabilized CBG luciferase reporter in conjunction with the CRY2 and CIB1 elements. Investigations revealed that different light intensities for varying lengths of time induced the promoter correspondingly, although longer durations of light exposure significantly altered the YRO, as shown in previous studies by Robertson *et al.*, 2013[70].

Our investigation showed that a light pulse with an intensity of 90 µEinstein/m^2/s for 1h was sufficient to activate the promoter without hampering the YRO. The peak in promoter expression occurred ∼5min after the lights were turned off and the promoter system returned to basal expression levels after about 2h in the dark (figure 3.1). It was observed that longer periods of light treatment induced

stronger levels of expression (figure3.1, panelA and B), while increasing light intensities (180 and 300 µE/m^2/s) also proportionally increased expression levels.

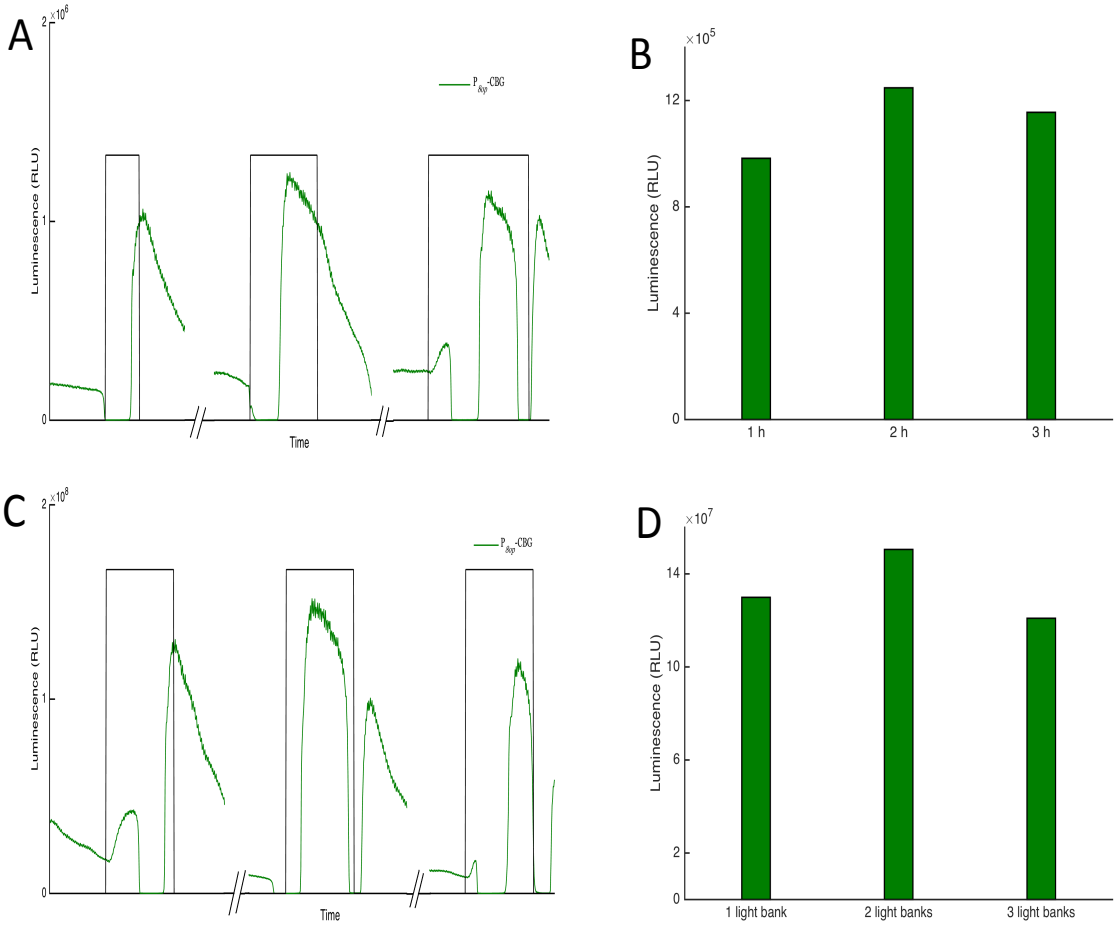

Figure 3.1: Optimization of experimental parameters for light-dependent transcriptional activation. (**A and C**) The luminescence output from a single color luciferase reporter (P_{8op}-CBG; green trace) constructed in a strain containing light sensitive elements was tested for induction with pulses of light for 1,2, and 3h (A) or with different intensities of light (90, 180, and 300 µE/m^2/s) (C). It was observed that the luminescence output increases proportionally for the first two treatments while it was affected in the last light pulse treatment since longer/more intense pulses of light interfere with luciferin stability. (**B and D**) A bar graph shows the effect of light treatment period (B) or varying intensities of light (D).

3.2.2 Phase-dependent luciferase expression

We then investigated whether light-dependent gene expression was influenced by the phase of an oscillation of the YRO. To test this, YRO was established in a continuous culture of *S. cerevisiae* stably transformed with all the light sensitive promoter elements and both of the luciferases, where the light-sensitive promoter controlled the green luciferase and the red luciferase was controlled by the constitutive P_{TEF1}. The culture was then treated with light at different phases of the oscillation (fig 3.2). Each pulse of light was followed by a period of darkness lasting at least 4h; this time was to let the cells recover from any oxidative damage that they might have accumulated during the light treatment[57,70,89] also, more importantly, to allow the luciferase expression to return to baseline before another pulse of induction with light. It appeared that the maximal expression was present in the hypoxic mask but the exact phase for peak protein production was determined in another experiment, described below[87].

The plant phytochromes used in the study for the construction of the light sensitive promoter seemed to respond maximally to light treatment during the hypoxic mask. It should be noted that hypoxia is composed of the Ox phase and the beginning of the R/B phase. Earlier studies by Tu *et al.*, indicate that the Ox and R/B phase upregulate gene clusters associated with ribosomal proteins, RNA processing enzymes, initiation factors, as well as proteins required for the DNA replication and cell division machinery[87]. The process of manufacturing proteins is a highly energy-draining process for the cell[66] and is hence assembled only in abundance of ATP within the cell[87]. The reason, outlined above, could be one of the underlying reasons for explaining the observed pattern in gene expression.

Although it is generally assumed that the CRY2-CIB1 interaction in *S. cerevisiae* in the presence of light is independent of any other *A. thaliana* protein[81], we wanted to be sure that the observed transcriptional upregulation was entirely devoid of the influence of the phytochrome origin. This led us to several questions:

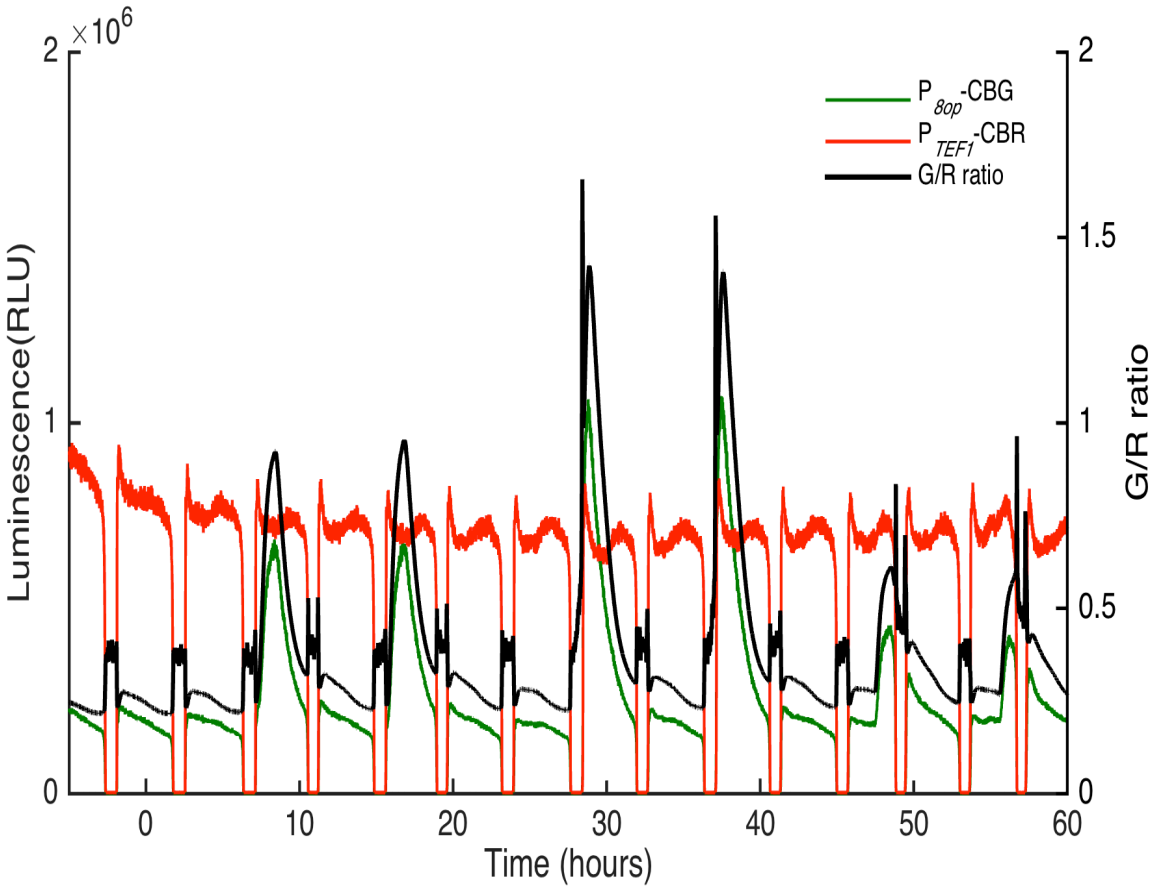

Figure 3.2: A dual luciferase reporter (P_{8op}-CBG, P_{TEF1}-CBR) was tested for induction of gene expression with visible light. The culture was treated with a 1h light pulse (90 µE/m^2/s) at three different phases of the YRO (the treatments were repeated once more for testing reproducibility). Each pulse of light treatment was followed by a period of darkness to allow luminescence to attain baseline. It appeared from the G/R ratio data (black trace) that the gene expression was highest soon after the hypoxic mask, but this hypothesis could not be confirmed in this trial.

(i) Could the observed pattern in gene expression be attributed to the origin of the photosensitive genetic elements? (ii) Do we only observe this pattern because the plant phytochromes are more responsive to light in a particular phase? To exclude this possibility, the normalizing red luciferase was placed under the control of an *ADH1* promoter. This particular promoter was chosen since the CRY2 and CIB1 plasmids (obtained from Hughes *et al.*,) were driven by *ADH1* promoters themselves[27].

A strain transformed with the light dependent genetic elements, P_{8op}-CBG (destabilized), and P_{ADH1}-CBR was constructed. By treating the oscillating culture of the above-mentioned strain with light at several different phases, we generated a phase-dependent productivity curve. The result, as summarized in fig 3.3, was in agreement with our previous experiment where a peak in gene expression lay within the hypoxic mask and gradually declined through the rest of the oscillation.

3.2.3 Dose-dependent luciferase expression

Once the phase for maximal gene expression was established, we attempted to identify dose dependent response of the photosensitive system. The culture was treated with light pulses at different intensities, 90, 180, and 300 µE/m^2/s, at the same phase for 1h (figure 3.4, panel A). As predicted, the brightest light elicited maximal response although the individual luminescence outputs were themselves affected by light. Figure 3.4, panel B shows the increase in G/R ratio with a proportional increase in light intensity. The G/R ratio is a useful indicator of gene activity in this case since luminescence data, if taken alone, can be misleading.

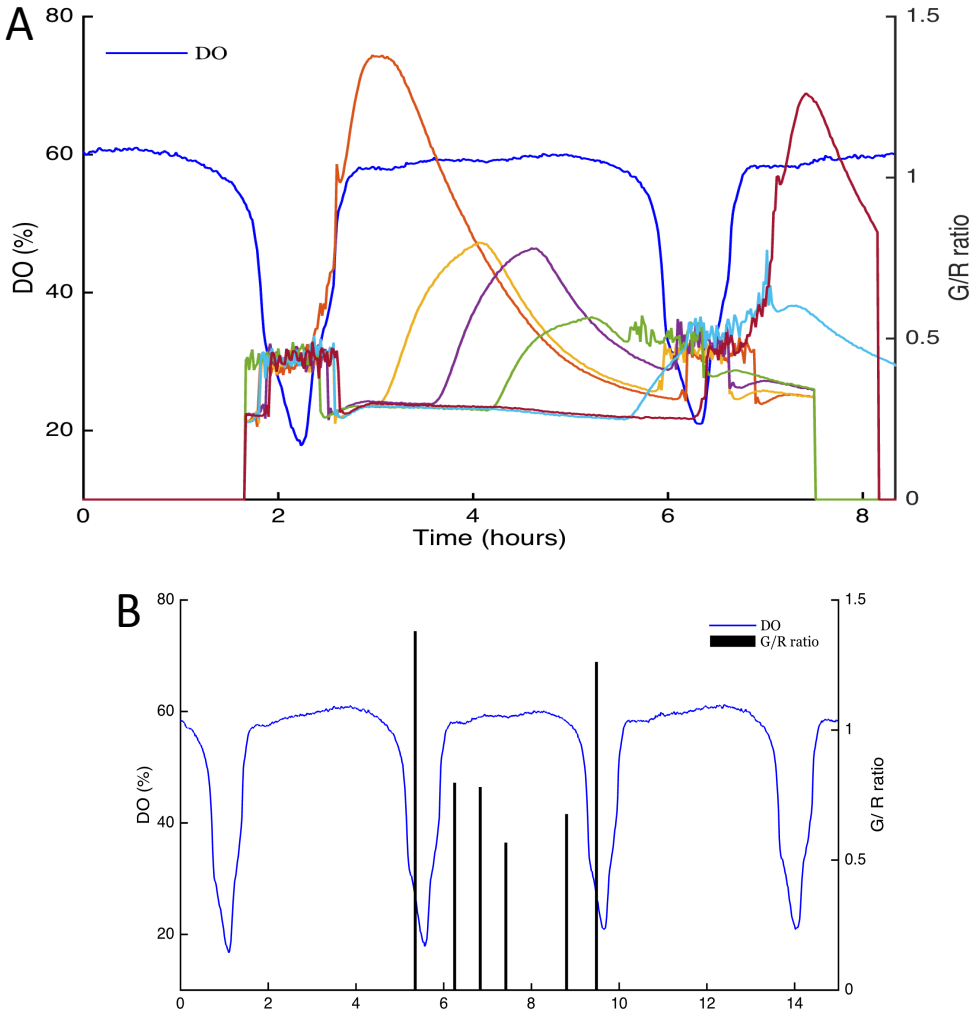

Figure 3.3: Phase-dependent productivity response curve using a photosensitive system. (**A**) A dual luciferase light sensitive reporter was treated with visible light for 1h (90 µE/m^2/s) at different phases of the oscillation (different colors correspond to different phases). Pooled data shows that maximal protein expression was observed during the hypoxic mask (DO-blue) and the response slowly declined through the rest of the oscillation. (**B**) A bar graph of the above data better indicates the peak of protein expression. Phase points 0°, 60°, 120°, 180°, 240°, and 300° were tested in this experiment.

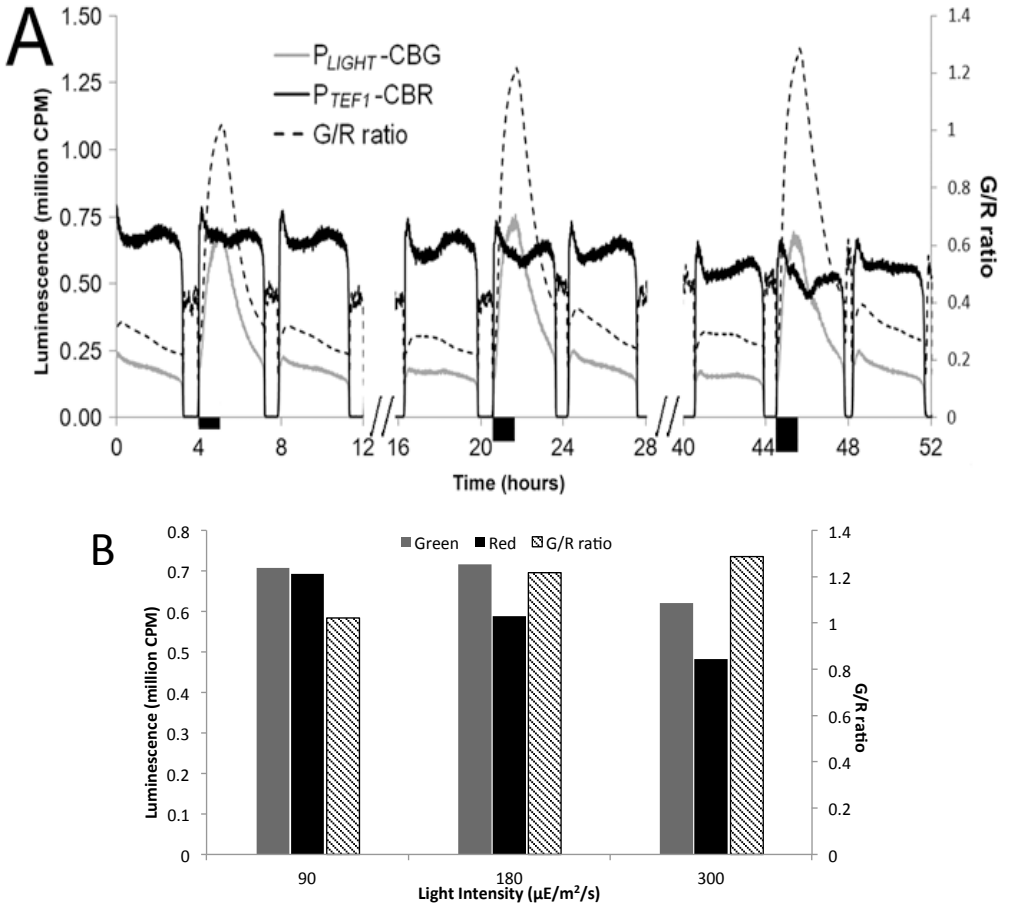

Figure 3.4: Induction of CBG expression with various intensities of visible light. (**A**) A dual color luciferase reporter with CBR driven by a $P_{TEF}1$ promoter and CBG driven by a light inducible promoter was constructed in S.cerevisiae. Oscillations were induced in the culture and luminescence (CBR-black trace; CBG-gray trace) was monitored continuously. Then, a 1-hour white light pulse (black rectangle) with increasing intensities was administered to induce luciferase expression. The G/R ratio (dotted line) reveals increasing expression levels of luciferase. (**B**) Effect of visible light with varying intensities (90, 180, 300 µE/m^2/s) on luminescence. The luminescence data (CBG- gray; CBR- black), if taken alone, indicates that light affects luciferase levels. However, the G/R ratio (pattern) shows an increase in protein levels with increasing light intensities.

3.3 Materials and Methods

3.3.1 Reporter gene plasmid construction

In this study, a light sensitive CEN.PK 113-7D strain was constructed using the pRMH122-LexA-CRY2PHR and pRMH124-VP16-CIB1 plasmids [obtained from Hughes *et al.*,[27]]. Additional modifications were made to the above-mentioned plasmids to allow them to integrate into the genome of CEN.PK.

To construct pRS304-NAT-LexA-CRY2PHR, an antibiotic resistance marker, Nourseothricin (NAT) from pYM-N9 was moved to pRS304 using SacI and BamHI (this brought with it an AscI site at the 5 end); then, the LexA-CRY2PHR region was PCR amplified from pRMH122 using primers 1 and 2 (table 3) that added an AscI and ApaI restriction site at the ends. The amplicon was then added to pRS304-NAT plasmid to produce the desired construct. To modify the pRMH124-VP16-CIB1 plasmid, the region containing VP16-CIB1 was PCR amplified using the primers 3 and 4 (table 3). The PCR product was then cloned into pRS303(d)-Kan using NotI and SpeI to generate pRS303(d)-Kan-VP16-CIB1 plasmid.

The CBG and CBR genes were constructed in pRS305-HPH and pRS306, as described in Chapter II. Further modifications were made to the plasmid containing CBG to include the 8 operator (8op) region of pSH18-34 (driving lacZ). Primers 5 and 6 (table 3) were used to amplify the 700 bp 8op region and then added upstream of the CBG coding sequence using XmaI and BglII to construct pRS305-HPH-P_{8op}-CBG99-PEST.

To construct a plasmid with *ADH1* promoter driving the CBR luciferase, the promoter region was amplified from pRS303(d)-Kan-VP16-CIB1 or pRS304-NAT-LexA-CRY2PHR using primers 7 and 8 (table 3).

3.3.2 Light treatment during continuous culture

The strain of CEN.PK 113-7D Ura- (Peter Kötter, U. of Frankfurt) stably transformed with the plasmid constructs (mentioned above) was cultivated in continu-

ous culture conditions as described previously (chapter II). The culture was then treated with one, two, or three banks of light using the 65-W compact CWF floodlights (Lithonia lighting), placed around the vessels water jacket. The intensity of the delivered light pulse was measured using the LI-COR quantum radiometer/photometer (LI-250A). To control the time period of light delivery, the light banks were set on a timer (Traceable Lab Controller, Fischer Scientific). If two light banks were required, they were placed ∼180° apart from each other. For three light banks, they were set at ∼120° to each other around the water jacket of the fermentor.

3.3.3 Phase-dependent productivity assay

S. cerevisiae transformed with the CRY2-CIB1 elements, P_{8op}-CBG, and P_{ADH1}-CBR are grown in continuous culture till oscillations establish. The period was then calculated and divided into 6 equal phases. Phase 0° was defined as the time when the DO was about 15min before the trough. The oscillating culture was then treated with a light pulse of 90 µE/m^2/s intensity for 1h. The phase shift was calculated by extrapolating the previous oscillations period to predict when the next phase would occur. Phase points 0°, 60°, 120°, 180°, 240°, and 300° were tested. Each light pulse was followed by an oscillation in darkness.

Table 3: Primers used for the construction of a light sensitive reporter system in *S.cerevisiae*

#	**Name**	**Sequence**
1	LexACRY2PHR-5(AscI)	atcata**GGCGCGCC**ATCTATTGAAGTAATAATAG
2	LexACRY2PHR-3(ApaI)	atgctt**GGGCCC**GGTTATTACTGAGTAGTATTTAT
3	VP16-CIB1-5(NotI)	atcata**GCGGCCGC**CATCTATTGAAGTAATAATAGG
4	VP16-CIB1-3(SpeI)	atgctt**ACTAGT**AGGGGAATTTCGACCG
5	8op-5(XmaI)	ttatat**CCCGGG**ACAGGTTATCAGCAACAACAC
6	8op-3(BglII)	acttcc**AGATCT**TATAGTTTTTTCTCCTTGACG
7	P_{ADH1}-5(XmaI)	catcat**CCCGGG**AAGTAATAATAGGCGCATGC
8	P_{ADH1}-3(BglII)	ctacat**AGATCT**GGAGTTGATTGTATGCTTGG

3.4 Conclusion

Light-mediated control of temporal and dose dependent transcription using plant phytochromes to study a rhythmic phenomenon such as the YRO is described in this chapter. Using a photosensitive system from plants in a heterologous system such as *S. cerevisiae* has allowed us to precisely control gene expression to study transcriptional patterns during the YRO, especially in hypoxia. This is especially important since light is otherwise known to hamper cell growth, respiration, and the YRO[70,89,96]. However, given these limitations, the benefit of terminating the stimulating signal by simply returning the culture to darkness was a great advantage compared to other methods of promoter activation (where the inducer would remain in the culture after addition, and would be difficult to remove).

Interesting observations from this study has prompted several questions. First, since the gene expression pattern in the presence of light is very distinct, could this lead us to determine productivity in populations of metabolically cycling yeast? If there does exist a specific format in productivity in protein expression, what is the underlying reason? As outlined earlier, yeast are generally more efficient at producing majority of their proteins during hypoxia and thus, are generally more productive in this phase than the others[66,87]. Other reasons for phase dependent producitivity, if any, still remain to be elucidated.

Second, it is well known that the cell division cycle (CDC) in yeast coordinate with the YRO[11,72]. Specifically, the entirety of the CDC is restricted to the R/B phase[87,97]. Could the CDC have a possible role to play in the productivity of protein expression? If the CDC and YRO were uncoupled, would that result in a different productivity pattern? Further studies are required to understand this intricately orchestrated process.

Chapter IV

GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

The investigation, described above, involved the exploration of the utility of a sensitive, robust monitor of transcriptional activity, especially during times of rapid metabolic turnover in *S. cerevisiae*. This work also provided interesting insights on genetic regulation during YRO, a rhythmic oscillatory phenomenon observed in continuous cultures of yeast. Researchers can also use this tool to expand their understanding of other genetic phenomena that lie beyond the scope of what is outlined in this study. We envision this biological tool to provide valuable insights into genetic control in simple organisms such as *S. cerevisiae* so as to expand it to studying more complex metazoans.

4.1 Summary

Luciferases, proteins obtained from bioluminescent organisms such as fireflies and click beetles, can be used as reporter systems in a wide variety of organisms[16]. The reporter confers to an organism the ability to bioluminesce when transcriptional activity is detected. The idea was then extended to using two separate luciferases; one luciferase to monitor the activity of a gene of interest while the other simultaneously reports the activity of a constitutively expressed reference gene. We hypothesized that the output (in the form of ratio) from the two luciferases would normalize gene expression data during times of rapid metabolic activity, as observed in continuous culture conditions.

One of the repercussions of maintaining yeast in glucose-limited continuous cultures is the exhibition of sustained, robust oscillations known as the Yeast Respiratory Oscillation (YRO). In each oscillation, the cell is driven through alternating oxidative and reductive states while biochemical pathway metabolites

are passed on from one phase to another. The other form of oscillatory behavior observed in yeast is the cell division cycle (CDC); it is now well known that the CDC and YRO strategically coordinate to maintain uniformity in cellular behavior. However, the underlying reason for this coordination still remains to be elucidated. Although the YRO was first reported more than four decades ago[40], many intricate details underpinning this fascinating phenomenon still remain to be understood.

We find the YRO very interesting to investigate because of the remarkable orchestration of cellular events, all of which are temporally compartmentalized, very reminiscent of a circadian cycle. Majority of the study, summarized below, deals with the exploration of genetic events in YRO with the dual luciferase tool.

Luciferases (green and red) obtained from click beetle (*Pyrophorus plagiophthalamus*) were chosen as the most suitable pair for this study. The effectiveness of a single color luciferase to monitor the YRO is limited because of the recurring hypoxia during which the luminescence signal is masked since oxygen is one of the substrates for the reaction. The G/R ratio is very useful during these conditions due to the presence of a normalizing luciferase that compensates for changes in oxygen concentration.

Various promoter systems of interest (constitutive, chemically inducible, and cell-cycle regulated) were tested to study the efficacy of this tool in monitoring gene activity in continuous culture. Our data with the cell cycle regulated promoter indicate continuity in *POL1* expression from one oscillation to the next, possibly supporting the theory of the two-population hypothesis. The hypothesis claims that only ∼50% of the population undergoes cell division at any given oscillation.

The study was then extended to using a photosensitive system from plants to control transcription of a luciferase gene. This system was highly advantageous since it allowed us to regulate transcription using visible light. Data from these investigations revealed the existence of a phase-dependent productivity response

within each oscillation. Further studies are required to evaluate the underlying reason. Regardless of the reason, this pattern of productivity is an interesting observation that could be exploited in industries under specific conditions[80].

4.2 Future directions

We envision the use of light inducible promoter to play a major role in carrying this study forward. Studying the effect of resetting the YRO and/or CDC by placing genes involved in sugar metabolism, oxidative stress resistance, or cell cycle progression under the control of a light sensitive promoter could provide insights into the genetic regulation underlying the YRO.

Questions with larger implications to the field of YRO such as: What factors (metabolic or otherwise) entrain this process?, Could the CDC provide clues to the maintenance of this oscillatory behavior?, and What are the evolutionary origins of the YRO? still remain to be answered. However, this study is an initial step toward understanding this elaborate, rhythmic process.

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