SHEDDING LIGHT ON THE YEAST RESPIRATORY OSCILLATION: USING LUCIFERASE AND VISIBLE LIGHT TO INVESTIGATE BIOLOGICAL RHYTHMS IN YEAST

By

James Brian Robertson

Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Biological Sciences

December, 2009

Nashville, Tennessee

Approved:

Professor Carl H. Johnson

Professor Douglas G. McMahon

Professor Antonis Rokas

Professor Katherine L. Friedman

Professor Kathy Gould

To my wonderful daughters, Abigail and Mareyn

and

To my loving wife, Jessica

ACKNOWLEDGEMENTS

I am forever grateful to my professor and mentor Dr. Carl H. Johnson for accepting me as a student into his lab and for providing me with the encouragement and resources needed to pursue a project outside of the central focus of his lab. I often felt out of place being the only investigator researching yeast rhythms in a lab with many accomplished cyanobacterial and mammalian circadian biologists; however, Carl always showed interest in my project and my ideas. I am also thankful for the support of Dr. Mark Woelfle and the Johnson lab members for their valuable feedback regarding my project, experiments, and presentations.

Because Carl's lab was not particularly equipped to investigate yeast when I joined the lab, my work would not have progressed as it did without the help, expertise, and resources of several other people. Therefore, I would especially like to thank Dr. Kathy Friedman and her lab members for teaching me all that I know about growing and manipulating yeast. In many ways, I also feel like a member of Kathy's lab because she and her students were always willing to share their time, equipment, reagents, and expertise with me. Kathy was always willing to read my drafts and provide critical feedback on my writing and experiments; and for that, I will always be grateful. I would also like to thank Dr. Rick Haselton and Dr. Steve Baskauf for their generous contribution of lab space and equipment when I needed it. I believe that I spent more time conducting experiments in Kathy's, Rick's and Steve's labs than I did in Carl's lab.

The efficiency at which my research progressed can largely be attributed to a fortunate collaboration with two people that began about the time I was looking to apply my luciferase reporter system to oscillating yeast. Therefore, I am extremely grateful to Dr. Erik Boczko for letting me use his bioreactor and equipment, and for the valuable insight he brought to modeling yeast growth and behavior. Without Erik, I would still be

trying to generate yeast respiratory oscillations in test tubes with an array of homemade equipment. Erik's selfless contributions allowed me to utilize techniques developed by leading experts in the field of YRO research and to produce work that could be compared and integrated with their methods and findings. I am also thankful to Dr. Chris Stowers, Erik's former grad student, for teaching me everything I know about bioreactors. The conversations Chris and I had while we waited patiently between time points from the bioreactor helped shape my ideas and goals for the future, in addition to paving an enduring friendship.

My committee has been greatly supportive of my work, so I would like to thank the three members of my committee whom I have not already mentioned. I appreciate Dr. Doug McMahon's chairing my committee and reminding me of the reason I went back to school, namely to develop a career where I can study and research things that interest me. Thanks also to Dr. Kathy Gould, not only for initiating our collaboration with Erik and Chris, but also for providing helpful suggestions regarding the yeast cell cycle and for working side-by-side with me on some of my experiments. And I was extremely fortunate that Dr. Antonis Rokas's joined our department (and my committee) when my ideas regarding yeast rhythm evolution and light responsiveness were in their infancy. His insights into fungal evolution and relationships, as well as experimental forethought and design have been indispensable for my work and future plans. So thanks again to all of my committee members. You have each uniquely impacted my scientific carrier.

Finally, I must thank my family, both immediate and extended, for supporting me morally (and financially) through these four additional years as a graduate student. It was a tremendous undertaking for me, at 31 years of age and with two young daughters, to leave the security of my job as a decently-paid research assistant to go back to school with a class, many of whom were 10 years younger than me and fresh out of college. I received encouragement from many members of my family who told me that it was

never too late to go back to school. This message was especially meaningful from my grandparents, Basil and Pauline Snider who both had also gone back to school later in life. I would like to thank my parents for letting me pursue my dreams. Watching me grow up, you must have known I was bound to be a scientist. Thank you for helping me find my way here. And I will never be able to express in words how thankful I am for my wife Jessica's support and the sacrifices she has made so that I could pursue my school work, regardless of how many overnight experiments I had to run. And finally, I would like to thank my daughters Abigail and Mareyn for providing me the motivation and reasons for taking this next step in my education. I love to see their fascination with science and their interest in the natural world from simple backyard exploration of insects, worms, and plants to visits to the lab to help mix media and look through microscopes. Thank you everyone.

TABLE OF CONTENTS

Page

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

°	degrees (angle, or 1/360 of a period)
°C	degrees Celsius (temperature)
μ	growth rate
$\mu E/m^2/s$	micro Einsteins per meter squared per second (unit of light intensity)
βGal	beta galactosidase
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BCECF	2'-7'-bis(carboxyethyl)-5(6)-carboxyfluorescein
BRET	bioluminescence resonance energy transfer
cAMP	cyclic adenosine monophosphate
CAT	chloramphenicol acetyltransferase
CCD	charge-coupled device
CDC	cell division cycle
CHX	cycloheximide
cpm	counts per minute
D	dilution rate
DNA	deoxyribonucleic acid
DO	dissolved oxygen
DPA	diphenylamine
DTT	dithiothreitol
EBD	estrogen binding domain
ECFP	enhanced cyan fluorescent protein
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein

EMO	energy metabolism oscillation
EYFP	enhanced yellow fluorescent protein
FACS	fluorescence-activated cell sorting
FITC	fluorescein isothiocyanate
G0 or G_0	quiescent phase or exit from cell cycle
G1 or G_1	growth or gap phase 1 of cell cycle
G2 or G_2	growth or gap phase 2 of cell cycle
G3P	glyceraldehyde 3-phosphate
GFP	green fluorescent protein
glu	glucose
h	hour
H^+	hydron or proton
HRP	horseradish peroxidase
IgG	immunoglobulin G
in.	inches
Kan	kanamycin resistance gene
kDa	kiloDaltons
LED	light emitting diode
Luc	luciferase
m	meters
M	mitosis phase of cell cycle
M	molar
min	minutes
mRNA	messenger ribonucleic acid
N	normal
n	number of replicates

NAD(P)H	reduced nicotinamide adenine dinucleotide with/without phosphorylation
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
OD_{600}	optical density at a wavelength of 600 nanometers
ORF	open reading frame
PBST	phosphate buffered saline with Tween-20
PEST	proline, glutamine, serine, threonine-rich protein destabilization domain
PMT	photomultiplier tube
PRC	phase response curve
r.p.m.	revolutions per minute
RLU	relative light units
S	DNA synthesis phase of cell cycle
S.D.	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec or s	seconds
SOEing	splicing by overhang extension
TMG	buffer comprising tris, magnesium, and glycerol
USA$	American dollars
UV	ultraviolet light
V	volts
VDC	volts of direct current
W	watts
YMC	yeast metabolic cycle
YPD	media comprising yeast extract, peptone, and dextrose
YRO	yeast respiratory oscillation

CHAPTER I

INTRODUCTION

Focus

Under a narrow range of continuous culture conditions, the budding yeast *Saccharomyces cerevisiae* strain CEN.PK exhibits a robust respiratory oscillation with a period around 3 to 5 hours. The work presented in this dissertation focuses on this rhythmic phenomenon and the question, “What is the relationship between the cell division cycle and this respiratory oscillation?” This dissertation constitutes a series of steps within a much larger quest to fully understand the nature of biological rhythms in yeast. Although not specifically addressed here, other questions that motivate this research are, “Is the respiratory oscillation evidence of an endogenous biological clock?” and “Does this biological rhythm in yeast exist in nature, i.e. outside of the tightly controlled laboratory conditions of continuous culture?” To pursue answers to these and other related questions, a number of techniques and investigations involving the production and perception of light are used. In fact, every chapter of this dissertation revolves around the use of light for exploring the biology of yeast in some way; from developing bioluminescent yeast that report gene activity, to studying effects that visible light has on yeast respiration and growth, to developing a low cost fluorescent excitation light source for use in microscopy. Important concepts for understanding this material and their contexts are addressed in the following sections of this introduction.

The Cell Division Cycle

The cell division cycle (a.k.a. the cell cycle) is the process of replication and division whereby one cell makes a duplicate of itself through a highly coordinated series of events. The cycle is divided into four phases and is described here in terms of the budding yeast *Saccharomyces cerevisiae*. G1 is a growth phase during which the cell grows in size and builds up stores of carbohydrates. Once a critical size is obtained and the surrounding environment is deemed suitable for cell division, the cell passes a critical point in G1 called Start where the cell irreversibly commits to one round of cell division. The passage of Start is accompanied by the upregulation of certain cell cycle genes like the G1 cyclins (*CLN1, CLN2, CLN3*) as well as other genes (like *POL1*) needed for the next phase of the cell cycle, S phase. During S phase, all of the chromosomes are duplicated in a very tightly controlled process. During S phase, *S. cerevisiae* forms a small bud on its periphery, which continues to get larger through the rest of the cell cycle. After DNA synthesis in S phase, the cell enters a second growth (or gap) phase called G2, in which the cell and bud continue growing and making preparations for mitosis. M phase, or mitosis, is the portion of the cell cycle in which the newly synthesized genetic material moves to the growing bud and is concluded by separation of the bud (the daughter cell) from the mother cell. After mitosis is complete, both mother and daughter cells reenter G1, the only phase of the cell cycle in which a bud is not evident. If conditions are favorable, the mother and daughter cells can begin the cell division cycle again. However there are reasons why a yeast cell may postpone cell division when in G1.

For haploid yeast cells, the presence of mating pheromone from the opposite mating type (e.g. α-factor to MAT**a** cells) arrests progression through G1 prior to Start. This cell cycle arrest ensures that the two nuclei that fuse during mating each contain

only one copy of each chromosome and that a proper diploid cell arises [1]. Mating pheromone can therefore be used in a laboratory setting to artificially arrest yeast cells in G1 and then later release them from this block by removing the pheromone. This procedure results in a synchronized cell cycle for the majority of the population and allows researchers to collect/study many cells in similar phases of the CDC. Unequal sizes of mothers and daughters after cell division, however, cause synchronized cells to have different G1 durations and therefore lose synchrony after one to three cycles [2].

Another reason yeast may postpone cell division and instead "choose" to continue living in G1 is that the nutritional conditions of the environment are poor. Yeast must harvest high-energy molecules (e.g. sugars) from their environment as well as other nutritional requirements that they cannot produce themselves (e.g. organic nitrogen, sulfur, potassium). When these nutrients become low, yeast sense their depletion and respond by slowing their growth rate and extending the time the cells spend in G1 [3]. Longer G1 intervals slow the rate at which the culture as a whole divides. Eventually the cells arrest in G1 altogether until the limiting nutrients are returned. When cells completely arrest in G1 due to depleted nutrients (usually carbon), the cells are referred to as quiescent or G0 denoting that they have exited from the CDC [4]. Reintroduction of nutrients (carbon) returns the quiescent cells to G1 and the CDC resumes. The methods that yeast use to sense nutritional conditions of their surroundings and relay these conditions to mechanisms that regulate growth are largely unknown and are likely multifaceted, but one cell cycle related protein believed to play a role in glucose regulation of the cell cycle is Cln3p, a G1 cyclin that regulates the expression of other G1 cyclins (e.g. Cln1p and Cln2p) which in turn (along with Cdc28p) regulate progression through Start [5].

A third reason cells may delay progression through G1 and forego commitment to cell division is insufficient cell size. There is a critical size that cells must attain during

G1 before being allowed to pass Start, however this critical cell size is somewhat variable, dependent on nutritional composition of the media [6] as well as overall growth rate of the culture [7]. As a result, newly budded daughter cells, which are smaller than their mothers, generally spend longer in G1 attaining the critical mass than their mothers [2]. This unequal mother-daughter growth rate results in yeast cultures dividing asynchronously.

Biological Rhythms and Clocks

Biological clocks (the most well-known being the circadian clock) are biological pathways that measure time, allowing organisms to synchronize and ultimately optimize their behavior and physiology with respect to the rhythmic changes in the environment. In addition to providing advantages of anticipating changes to the external environment, biological timing systems also permit synchrony and coordination of metabolic processes and gene regulation within the organism that provide optimum energy usage and temporal separation of mutually unfavorable reactions or reaction conditions [8, 9]. Scientists have found circadian clocks in cyanobacteria and in nearly all eukaryotes investigated, strengthening the claim that for an organism, the capability of measuring time on a day-length scale is an advantage [10]. But despite the circadian clock's virtual ubiquity, differences in genes and mechanisms for prokaryotes, plants, animals, and fungi point to no clear clock progenitor.

Interestingly for one of the most genetically well-characterized eukaryotes, the budding yeast *Saccharomyces cerevisiae*, researchers have yet to find evidence for a circadian clock. Despite this apparent clock deficiency, *S. cerevisiae* does display some rhythmic behavior and has historically been pivotal for understanding two different biological rhythms: the glycolytic oscillator with a period ranging from 1 to 70 minutes

[11] and the cell division cycle with a period ultimately dependent on strain, nutrition, and other factors [12]. Recently, attention has been given to another yeast biological rhythm with a period ranging from 40 minutes to 5 hours (or more) manifested under certain continuous growing condition in bioreactors (a.k.a. chemostats or fermentors) [13-18]. This specific biological rhythm that manifests during continuous culture is the primary focus of this dissertation and is referred to as the Yeast Respiratory Oscillation, or YRO, in this work. The YRO is described in detail in a later section; but briefly, it displays several robust outputs under continuous culture including oscillating levels of dissolved oxygen, redox state, and concentrations of various metabolites [13-18]. Under some conditions, the respiratory oscillation manifests spontaneously and hampers industrial use of continuous culture by reducing the efficiency of bioreactor outputs [19]. The specific nature of this ultradian oscillation (an oscillation with a period much shorter than a day) and the mechanisms that permit the culture to exhibit respiratory (and sometimes cell cycle) synchrony remain to be clearly identified and are important questions to industrial users of *S. cerevisiae*, researchers who use *S. cerevisiae* as a model organism, and investigators of biological rhythms, alike.

Some have suggested that the YRO comprises a biological clock allowing cells to "tell time" but these arguments are based on the oscillations generated in the artificial continuous culture conditions of bioreactors and have yet to be shown to exist in nature [13, 16, 20]. That is not to say that the YRO is not a biological rhythm or oscillation. Oscillations in biological systems (and other systems comprised of networks) are common and often form spontaneously when three conditions are met: 1) input, energy, or a motivational force that is available to drive a change, 2) feedback to control the rate of change or the amount of change, and 3) a delay in the onset of the feedback control such that some regulated limit or "desired level" of a variable is overshot before the feedback system can affect the level of the variable [21]. These parameters account for

the oscillatory behavior of a wide variety of systems, both biological and non-biological, e.g. predator-prey balance, factory assembly lines, stock markets, temperature regulation of buildings, and biochemical pathways within cells such as glycolysis. In fact, many biological pathways with feedback regulation exhibit some degree of oscillatory behavior. Such oscillations often reduce the efficiency of the system/pathway making the rhythmic activity of the system undesirable; therefore in such situations, natural selection has sought to dampen these rhythms, often by reducing the amount of "delay" before feedback [22]. In fact, factors that affect any of the three conditions for oscillation affect the rhythm itself (e.g. period, shape, amplitude, phase, etc.). In some cases naturally occurring oscillations have been reinforced by natural selection, and have presumably formed the basis for biological (e.g. circadian) clocks where the resulting rhythms could serve as a signal for properly timing other biological processes and behaviors, allowing anticipation of environmental rhythmic phenomena [22]. Determining whether the YRO is one such oscillation that has been reinforced by natural selection to serve as a "clock" is beyond the scope of this work; however, the material presented here will hopefully shed additional light on the debate and offer counterpoints to consider regarding this question. As mentioned above, biological pathways with delayed feedback regulation are notorious for oscillating, and energy metabolism pathways for the production and allocation of ATP within cells are rife with such regulation.

Energy Metabolism in Yeast

When *S. cerevisiae* grows in the presence of excess glucose, as would be the case with bursting fruit in the wild, the cells rapidly divide and ferment glucose to ethanol even in the presence of oxygen [23]. This phenomenon of fermentation in the presence

of oxygen is called the Crabtree effect [24] and is found in only certain species of yeast, *S. cerevisiae* being one [25]. Although some respiration takes place during this rapid fermentation, *S. cerevisiae* demonstrates a strong preference for energetically less favorable fermentation until glucose levels become very low, after which they undergo a genetically widespread metabolic retooling and then grow slowly, oxidatively metabolizing the ethanol as a carbon source [23, 26]. Carbon source transition is called a diauxic shift and the one between sugar (glucose) and ethanol is accompanied by a general environmental stress response involving hundreds of genes as well as a slowing in cell division rate [4, 23, 27]. It is believed that about the time fleshy fruits appeared on earth, yeast evolved this Crabtree strategy to protect their food sources from other microbial competitors for which ethanol was toxic and could not serve as a nutrition source [26]. After exhausting their supply of ethanol and other non-fermentable carbon sources (e.g. acetate, glycerol), yeast undergo another massive genetic and metabolic transition. They enter a state of protective dormancy called quiescence (G0) in which they arrest as unbudded cells, reduce transcription rate, inhibit protein degradation, constrain protein synthesis to ~0.3% of that in log phase, and await a new sugar supply [4].

The Crabtree effect occurs as a long-term or short-term phenomenon and is sometimes called respiro-fermentative metabolism because both respiration and fermentation occur at the same time (Fig. 1.1). The long-term Crabtree effect that describes the fully adaptive, aerobic fermentation of rapidly growing yeast in the presence of abundant glucose is thought to be the result of *S. cerevisiae's* limited respiratory capability when glucose levels exceed some threshold. The short-term Crabtree effect occurs as a fermentative burst when excess glucose is added to a non-fermenting yeast culture, and is thought to result from an overflow response at the level of pyruvate due to temporary saturation of respiratory capability (Fig. 1.1) [25]. Under

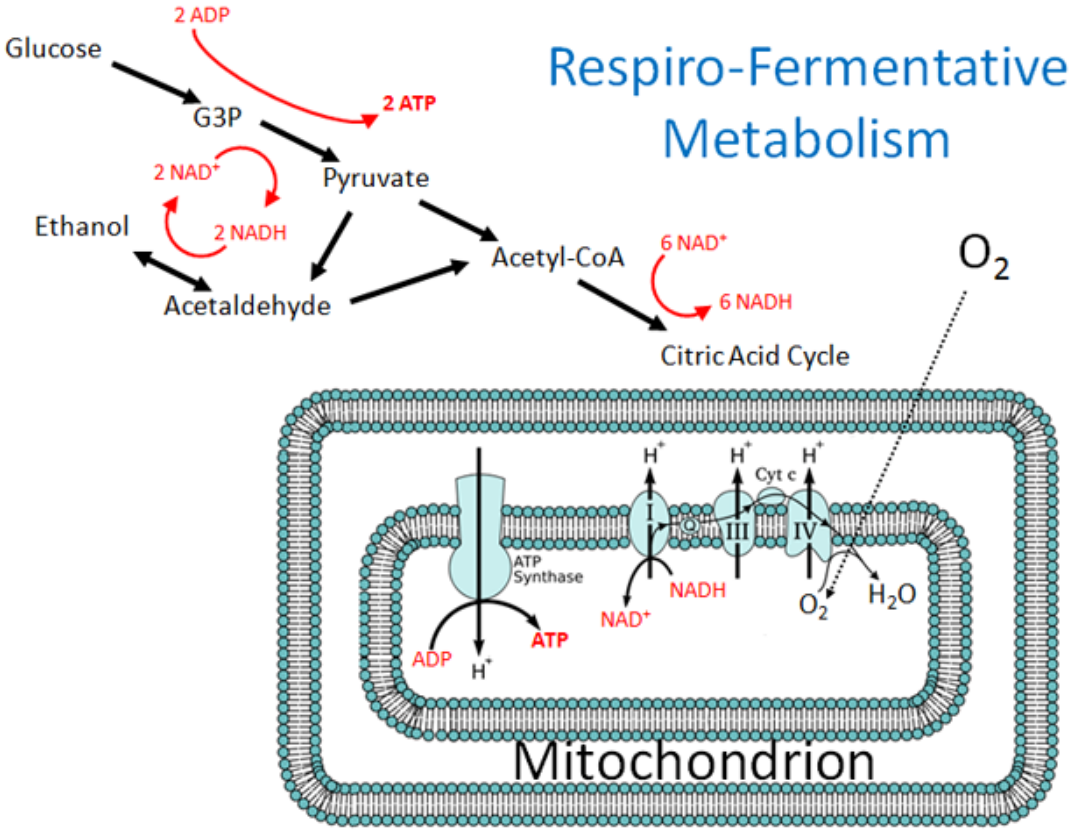

Figure 1.1. A schematic depicting simplified respiro-fermentative metabolism in *S. cerevisiae* where pathways for fermentation and respiration operate simultaneously. Glucose goes through glycolysis and is broken down into pyruvate which is both metabolized into acetaldehyde for fermentation and into Acetyl-CoA entering the citric acid cycle for respiration. The high energy state of the NADH produced from these reactions is both captured for ATP production by electron transport in mitochondria, as well as used to power the conversion of acetaldehyde into ethanol during fermentation. Ethanol can then (or later) be converted back to acetaldehyde and then to actyl-CoA where it can be respired.

lower oxygen concentrations of limited glucose, an opposite phenomenon from the Crabtree effect, called the Pasteur effect [28], is seen where fermentation is suppressed and respiration is favored due to the greater affinity of the enzyme which shuttles pyruvate to respiration (pyruvate dehydrogenase) for pyruvate than the enzyme that shuttles pyruvate to fermentation (pyruvate decarboxylase) [25]. So the pathway(s) of energy metabolism yeast use at any one time largely depend(s) on the levels of nutrients and metabolites (e.g. sugar, nitrogen, oxygen, ethanol, etc.) in the environment.

The ability to sense levels of metabolites and nutrients in the environment is of vital importance for regulating energy metabolism at the level of transcription as well as at the level of enzyme activity. Catabolite repression is one method in which gene expression is regulated, whereby transcriptional activators or repressors bind metabolites or nutritional molecules (e.g. glucose) and either upregulate or downregulate transcription of relevant genes (e.g. those for sugar uptake, respiration, or gluconeogenesis) accordingly. A more rapid response to metabolic cues can come from catabolite inactivation, where the presence of a molecule (e.g. glucose, ATP, cAMP, etc.) affects the activity of certain key enzymes of related pathways, such as by affecting the enzyme's phosphorylation status [25]. Both catabolite repression and inactivation are examples of feedback regulation which often include delays in their responses, hence providing the framework for oscillations of metabolite concentrations or pathway utilization to develop. As mentioned earlier, when such oscillations are detrimental for growth, viability, or effective pathway regulation, natural selection has often sought steps to minimize these rhythms. When yeast grow in the wild on limited substrates, the availability of glucose and other nutrients constantly changes as the yeast consumes the nutrients. Such constantly changing variables (such as the diminishing motive force for change) can work to destabilize or dampen oscillations [21], thus in conditions where nutrient concentrations are constantly decreasing, it stands to reason that fewer

measures may have been needed by evolving yeast to counter detrimental oscillatory consequences from delayed feedback regulation under these natural conditions. However, what if yeast are grown in an unnatural situation in which the supply of nutrients to the culture is maintained at a constant level? Under such unnatural conditions, the measures that yeast may have developed to dampen metabolic oscillations could prove inadequate to quench them.

Bioreactors and Continuous Culture

A bioreactor (sometimes called a fermentor or chemostat) is a continuous culture apparatus that maintains a microorganism culture in a near steady-state level of exponential growth in which one component of the media is the growth-limiting factor (Fig. 1.2A) [23, 29]. Within the reactor's vessel, a specified volume of aerated media sustains yeast growth in much the same way batch growth occurs, but unlike batch growth, the growth environment (including pH, temperature, nutrition, biomass, and metabolic byproducts) is kept relatively constant by continually monitoring and adjusting variables like pH and temperature in addition to constantly introducing fresh media at a steady rate while removing culture (i.e. media, cells, and byproducts) from the vessel at the same rate. As a result of these conditions, an inoculated culture grows to a concentration that the limiting component of the media allows and from that time onward, the growth rate is determined by the rate at which fresh media is supplied [29]. That is, growth rate (μ) equals the dilution rate (D) and thereby, both are equal to the natural log of 2 divided by generation time (i.e. divided by the average cell division time) [30].

$$\mu = D = \frac{ln2}{\text{generation time}} \qquad \text{Equation 1}$$

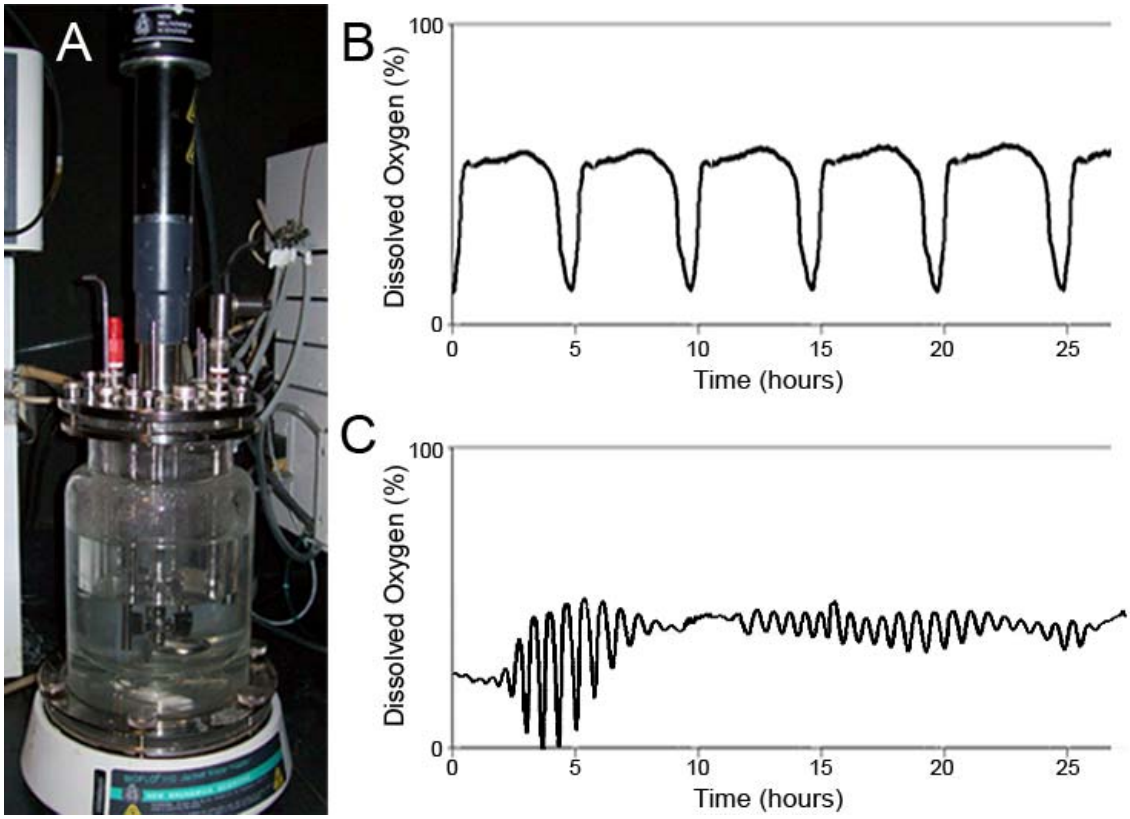

Figure 1.2. The bioreactor is a continuous culture vessel in which yeast can exhibit respiratory oscillations. **A.** shows a bioreactor (New Brunswick Bioflo 110) which is capable of controlling temperature, pH, agitation, air flow, and dilution rate of continuous culture. **B.** is an example of the longer period YRO characteristic of the strain CEN.PK grown in continuous culture. **C.** is an example of the shorter period YRO that forms for the strain IFO0233 grown in continuous culture. The YRO in B. has stable amplitude and has a period of about 5 hours. The YRO in C. has a stable period of about 40 min but its amplitude and midpoint are not stable in this example.

As a result, the average cell division cycle time can be calculated for continuous culture by rearranging the terms in equation 1, and can ultimately be controlled by the user by controlling the dilution rate (the rate at which media is supplied to and removed from the continuous culture).

$$\text{average cell division time} = \frac{ln2}{\mathrm{D}} \qquad \text{Equation 2}$$

Under traditional bioreactor operation (i.e. for non-oscillating conditions or strains of yeast) a steady-state is achieved where metabolite levels remain virtually constant and gene expression involved in metabolism is stabilized [23]. This stability has proven useful in the field of genomics and proteomics where researchers need to control environmental growth conditions and growing states of cells [29]. The importance for continuously fed bioreactors in the industrial sector is also growing. As transgenic technology improves to provide new medicines, fuel sources, and biopolymers, systems that allow rapid, uninterrupted production of these products are of great commercial importance. These are reasons it is necessary to fully understand microorganism behavior in bioreactors, especially when non-steady-state situations arise such as the YRO and cell division oscillations demonstrated in laboratory and industrial bioreactors.

The Yeast Respiratory Oscillation

Under a range of specific conditions of glucose-limited, aerobic continuous culture in bioreactors, spontaneous perturbations of the steady-state can lead to oscillations in various metabolite concentrations in the media that are sometimes accompanied by (and possibly reinforced by) subpopulations of synchronously dividing cells [31]. The most often and most easily observed oscillating metabolite in the continuous culture is the dissolved oxygen concentration, which reflects the culture

alternating between respiro-fermentative metabolism and respiration (Fig. 1.2B, C) [16, 18, 32]. We call this phenomenon the yeast respiratory oscillation (YRO), but it also goes by other names including the yeast metabolic cycle (YMC) [16] and the energy metabolism oscillation (EMO) [18].

Researchers have investigated the YRO for decades (e.g. see [33, 34]) however controversy remains regarding specifics of the timing mechanism involved, synchronizing cues that permit population stability, and the importance of cell division synchrony to the YRO. Some of the contention stems from different oscillatory periods research groups observe in their bioreactors [35-37]. Those that use a specific yeast strain and culture conditions outlined by Satroutinov, *et al*. [38] report short DO (dissolved oxygen) oscillations between 40 and 50 minutes (Fig. 1.2C) [13-15, 32, 38]; while others using different strains and conditions report longer period DO oscillations on the order of 4 to 5 hours (Fig. 1.2B) (or longer for very slow dilution rates) [16-18, 39]. Some have claimed that the longer period ultradian rhythm is merely a reflection of a cell division oscillation [35] and is fundamentally different from the shorter period rhythm, but others defend the longer rhythm as a more robust and significant occurrence than the 40-minute rhythm [36]. Regardless of their perspective, some members in both camps agree on two things. One is that the oscillation consists of yeast synchronously alternating between respiro-fermentative (reductive) and respiratory (oxidative) states. The other is that the oscillation is a legitimate, biological phenomenon that points to a yeast ultradian clock; i.e. an endogenous oscillator that allows a yeast cell to temporally separate sensitive DNA replication from reactive oxygen species produced during respiration, thus having adaptive significance [15, 16, 40]. Researchers speculate that such a clock, with modest temperature compensation ability might be a yeast's attempt to emulate benefits from a circadian clock while retaining capability for rapid cell division [14, 16]. However, it remains unclear whether the YRO is the result of an endogenous

oscillator (an ultradian clock) that ticks in all yeast or whether the phenomenon is simply an artifact or harmonic that propagates from feedback regulation that is stabilized by the conditions of continuous culture. Aside from one study where yeast cultures were grown in batch on the slowly digestible carbohydrate trehalose (which was digested at a relatively steady rate into glucose molecules), the YRO has not been demonstrated outside of continuous culture conditions [41]. An aerobic environment where glucose is limiting but supplied at a slow, steady rate seems to be required for the YRO to manifest.

A generalized model describing the YRO is as follows. Glucose that is constantly introduced into the continuous culture is immediately metabolized by the highly dense culture of slowly growing yeast [16]. This glucose feeds into the respiratory and other pathways to provide ATP to the cells, however the percentage of glucose that goes to respiration depends on two things: 1) the capability of respiration to occur (which depends on availability of oxygen, suitable NAD^+/NADH ratio, sufficient respiratory machinery like cytochromes, etc.), and 2) the ATP levels and energy demands of the cells. At times when lower levels of respiration can adequately satisfy the cell's ATP demands, some of the consumed glucose is shuttled to storage carbohydrates like trehalose and glycogen as well as to the fermentative pathway (hence respiro-fermentative metabolism) [18]. If respiration is inhibited for some reason, more of the glucose is sent to the fermentative pathway. On the other hand, when ATP demands increase, more glucose is directed to respiration than glucose-storage or fermentation pathways. And, if ATP requirements become greater than both respiration and fermentation can sustain from the media-supplied glucose, then the cells mobilize their storage carbohydrates to increase levels of intracellular glucose [18]. Therefore, the YRO is an oscillation where cells spend most of their time storing glucose in an ATP satisfied state but a recurring demand on ATP (or inadequate glucose level) requires the cells to spend their carbohydrate stores increasing intracellular glucose and respiration

rates. This results in the observed dissolved oxygen oscillation along with oscillating levels of a whole host of other metabolic and redox factors (e.g. NADH vs. NAD^+, storage carbohydrates, intracellular glucose, extracellular ethanol, and acetate). [16, 18]

The cell division cycle oscillates along with the YRO [13-18]. But it remains unclear whether the CDC plays a part in the YRO's stability/existence, or whether the CDC synchrony observed during the YRO is merely an output of the oscillating energy metabolism (or an unidentified "clock"). One hypothesis is that the CDC falls into lockstep with the YRO because slowly growing cells in G1 delay progression through Start due to the low glucose levels in the environment and within the cell, and the synchronous release of glucose from carbohydrate stores (during a particular phase of the YRO) satisfies Start-related nutritional requirements allowing cells to simultaneously progress through Start after the intracellular glucose surge. As a result of a large population of cells synchronously progressing through S, G2, and M phases of cell division, CDC-related metabolic influences (e.g. energy demands, pathway preferences, byproduct formation, etc) could then in turn feed back on the regulation of the YRO, thus allowing the cell division rate (determined by the dilution rate of the culture) to influence the period and stability of the YRO.

Because the CDC, energy metabolism, and responses to environmental and nutritional cues are highly regulated at the transcriptional level, it is not surprising that more than half of the yeast genome has been found to have periodic expression with the YRO [16]. For some genes, this periodic coordination supports the hypothesis that the YRO is the result of an ultradian clock (an endogenous oscillator), similar to the circadian clock but operating on a more rapid timeframe [15, 16]. However, it is also possible that the rhythmic expression of more than half of the genome could simply be due to gene regulation that accompanies two things: 1) a synchronized cell division cycle under these conditions, and 2) environmental/nutritional sensing of an oscillating

environment dependent on an hourglass-like metabolic timer of glucose storage and usage. It would therefore be the coincidence of an oscillating environment that manifests under the artificial growth conditions of continuous culture that results in rhythmic gene expression rather than a predictive, endogenous clock controlling these genes.

Reporters of Temporal Changes in Gene Expression

Measuring regular changes within organisms over time is necessary for understanding their biological rhythms. Prior to advances in molecular biology, circadian biologists were limited to observing behavioral, morphological, or physiological rhythms [40]. Later, advances in biochemistry allowed scientists to examine cellular extracts and characterize rhythmic occurrences of certain substances. It was not until genetically encodable reporters of temporal changes were developed that genes for circadian systems were found in simple single-celled organisms like cyanobacteria [42]. Observing rhythms in living organisms that lack behavioral and morphological complexity requires molecular tools capable of revealing intracellular changes as they happen. It has been through the use of *in vivo* genetic reporting systems that we can now witness an organism's rhythmic gene activity and characterize biological oscillations on a molecular scale.

The bioluminescent reaction catalyzed by the enzyme luciferase has become a useful genetic reporting system for monitoring promoter activity in circadian studies of mammals, insects, plants, cyanobacteria, and filamentous fungi. The eukaryotic luciferase from fireflies emits light when the 62-kDa protein catalyses the oxidation of a bioluminescent substrate "luciferin" (in the presence of O_2, ATP, and Mg^{+2}) into oxyluciferin (and ADP and CO_2) [43]. The light emission is therefore an immediate and

measurable indication of the enzyme's activity that can be transgenically coupled to an organism's endogenous regulation of transcription for a specified gene. The relatively short half-life of luciferase (~2-4 hours, depending on the host organism) allows its expression to dynamically reflect transcription on a faster time scale than longer-lived reporters like βGal, CAT, or GFP [43, 44]. Additionally, luciferase does not need excitation from an external light source as do GFP and other fluorescent reporters. Therefore, issues of photobleaching, autofluorescence, phototoxicity, and biological responses to light can be avoided with a luciferase reporter.

Molecular tools for characterizing subcellular spatial expression and constraints of yeast proteins have revolutionized our understanding of *where* proteins reside within yeast, for example using spatial reporters like GFP-fusion proteins [45]. Similarly, quantum leaps in circadian biology of other eukaryotes have been made using luciferase reporters and similar protein fusions to characterize *when* genes and proteins are expressed [46]. Therefore a valuable opportunity exists for using luciferase to characterize the timing aspects of gene expression in yeast or to visualize gene expression in situations where GPF is inadequate. Consequently, I developed luciferase reporters for yeast that provide real-time gene expression data during the YRO.

Significance of these Studies

The work presented here is important on two levels: 1) from a broad perspective, these investigations pursue questions that concern the larger fields of cell cycle regulation, microbial communication and environmental sensing, chronobiology, and industrial uses for *S. cerevisiae*, and 2) more narrowly, these investigations target the yeast and YRO research communities by providing additional tools to study rhythmic gene expression of yeast, as well as attempting to resolve differences between the two

types of reported YROs (i.e. the longer period YRO ranging from 3 to 5+ hours and the shorter period YRO of about 40-50 min).

For this work, luciferase reporters of promoter activity were developed for *S. cerevisiae* to investigate real-time rhythmic activity of gene expression during the YRO and the cell division cycle (Chapter II). From the luminescent reporter data of these studies, cell-cycle landmarks were determined for the longer period YRO and a relationship between the phase resetting capability of the YRO and CDC was shown (Chapter II). A model describing the interaction between the YRO and CDC was proposed (Chapter II) and then later tested (Chapter III). The effects of metabolic signals (e.g. ethanol, acetaldehyde, and oxygen) on the YRO were tested and all showed similar phase resetting characteristics (Chapters II and IV) implicating those metabolites in possible signaling pathways that regulate and maintain the YRO. The large amplitude and ~3.5 to ~5 hour period characteristic of the longer period YRO was shown to be strengthened by the rhythmic participation of synchronized cell division (chapter III) and could be disrupted by either blocking the CDC (chapter III) or by exposing the oscillating culture to visible light (chapter IV). Light was further shown to interfere with yeast respiration and growth rates, and caused *S. cerevisiae* to upregulate the production of pigments, most likely carotenoids to protect the cells from the damaging effects of visible light. And finally, diminishing respiration during the YRO by exposing the culture to light, caused the period of the longer period YRO to shorten from 3.5+ hours to as little as 70 min, which puts the oscillation on a similar timeframe with that of the shorter period YRO reported by others. This observation might show how these two YROs may be related.

Until the YRO is demonstrated in “the wild” or on an individual cellular scale outside of continuous culture, the phenomenon of oscillating respiratory phases will remain an academic curiosity with limited application to the understanding of yeast as

they exist in nature. Such evidence remains the primary obstacle for demonstrating that *S. cerevisiae* has an endogenous timekeeping system like other organisms with circadian clocks. However this deficiency does not diminish the importance of researching the YRO. Independent of its possible existence in nature, the YRO can continue to be used as a tool to investigate other, physiologically relevant questions regarding cell cycle regulation, communication, environmental sensing, metabolic control, and cellular coordination. Additionally, the YRO can be potentially exploited by industry, by taking advantage of the synchrony of gene expression that accompanies the cell cycle to harvest products from continuous culture in phase with their rhythmic production, thereby increasing yields of natural or heterologous yeast products [47].

CHAPTER II

REAL-TIME LUMINESCENCE MONITORING OF CELL-CYCLE AND RESPIRATORY OSCILLATIONS IN YEAST[1]

Introduction

Measuring regular changes within organisms over time is essential for understanding rhythmic biological phenomena and the design principles of biological circuits [48]. Historically, such measurements were limited to observing behavioral, morphological, or physiological parameters [40]. Later biochemical advances allowed scientists to assay cellular extracts for the rhythmic occurrence of specific molecules. All of these methods are labor intensive, relatively low-throughput, and/or require the destruction of the organism (e.g., to make a cell extract). Even modern assay techniques such as microarrays require the destruction of biological samples. However, the use of *in vivo* genetic reporting systems allows us to witness an organism's rhythmic gene activity in real time and to characterize biological oscillations at a molecular level. For example, the field of circadian rhythms (period ~24 h) was revolutionized by the application of luminescence reporting of clock-controlled promoter activities as a non-invasive real-time assay [42, 49-51]. These luminescence reporting applications have extended to high-throughput screening of circadian mutants [52, 53] and traps for clock-controlled promoters and enhancers [54, 55].

[1] The work presented in Chapter II was published as Robertson, J.B., C.C. Stowers, E.M. Boczko, and C.H. Johnson (2008) *Proc. Natl. Acad. Sci. U. S. A.* 105:17988-93.

For circadian applications in eukaryotic cells from filamentous fungi to plants to animals, the luminescence reporter of choice has been firefly luciferase [49-51, 56-58]. The luciferase gene can be coupled to an endogenous promoter and used as a genetically encodable, non-invasive reporter of the promoter's activity. Firefly luciferase (Luc) is a 62-kDa protein that catalyses the oxidation of the bioluminescent substrate "luciferin" in the presence of O_2, ATP, and Mg^{+2}; the energy released by this reaction produces an electronically excited state, which then emits a photon [49]. Light emission is therefore an immediate and measurable indicator of luciferase activity. The relatively short half-life of luciferase (~2-4 h) allows its expression to dynamically reflect transcriptional activity on a faster time scale than longer-lived reporters such as GFP, βGal, or CAT, [43, 44, 49]. Additionally, luciferase does not require excitation from an external light source as do fluorescent reporters, thereby avoiding complications such as photobleaching, phototoxicity, and endogenous responses to light. Most importantly for applications to yeast, luciferase reporters circumvent the problematic autofluorescence that stems from complex media components, dense cultures, or intense excitation needed to detect low concentrations of fluorescent reporters.

Molecular tools for characterizing subcellular spatial expression and localization of yeast proteins have revolutionized investigations of *where* proteins reside within yeast, e.g., by using GFP-fusion proteins [45]. However, even though luciferase has often been the reporter of choice for temporal events such as circadian rhythms, it has not yet been applied as a real-time, non-invasive reporter in yeast. Rhythmic phenomena in yeast include the cell division cycle (CDC) and the yeast respiratory oscillation (YRO). The former phenomenon is intrinsic to all cells, but detection of the YRO has been limited to several strains of *Saccharomyces cerevisiae* growing under specific conditions of continuous culture. While the phenomenon has been known for decades [33], laboratories are only now beginning to elucidate the mechanisms

underlying its stability and maintenance of population synchrony [13, 16, 59]. A coincidence between the CDC and the YRO has been described [16, 34] but the relationship between these two rhythmic phenomena is unclear [35, 36].

In this chapter, we modify firefly luciferase to function as a rapid, continuous, real-time reporter of promoter activity for cell-cycle regulated genes in populations of yeast and use this reporter as a gauge of gene activity during the CDC and in undisturbed, dense cultures that are exhibiting the YRO. This reporter provides an inexpensive, dependable read-out of a synchronized yeast population's phase in the CDC, and thus can serve for some experiments as an alternative to the labor-intensive and/or costly methods of microscopy, FACS, microarray, or blotting. Moreover, we use this novel yeast reporter to probe the interdependency of the CDC to the YRO and show that there is a direct correlation between premature induction of cell division and phase resetting in the YRO under the conditions we tested.

Results and Discussion

The half-life of native firefly luciferase in mammalian cells ranges from 1.4-4.4 h [58, 60]. While this rate of turnover is adequate for measuring circadian oscillations that occur over a ~24 h time course, cycles in yeast (e.g. the CDC and YRO) occur more rapidly, on the order of 40 min to 12 h or more depending on conditions [38, 39]. A reporter with a 1-4 h turnover would not be adequate for accurately reporting oscillations occurring over time spans of 0.5-6 h [49]. Therefore, for measuring more rapid changes in promoter activity, we developed a luciferase reporter with a shorter half-life by modifying the coding region of firefly luciferase to include the destabilizing PEST sequence from the *CLN2* gene of *S. cerevisiae* [44]. This modification shortened the half-life of luciferase's activity in yeast from ~3 h to 35 min (Fig. 2.1). This destabilized

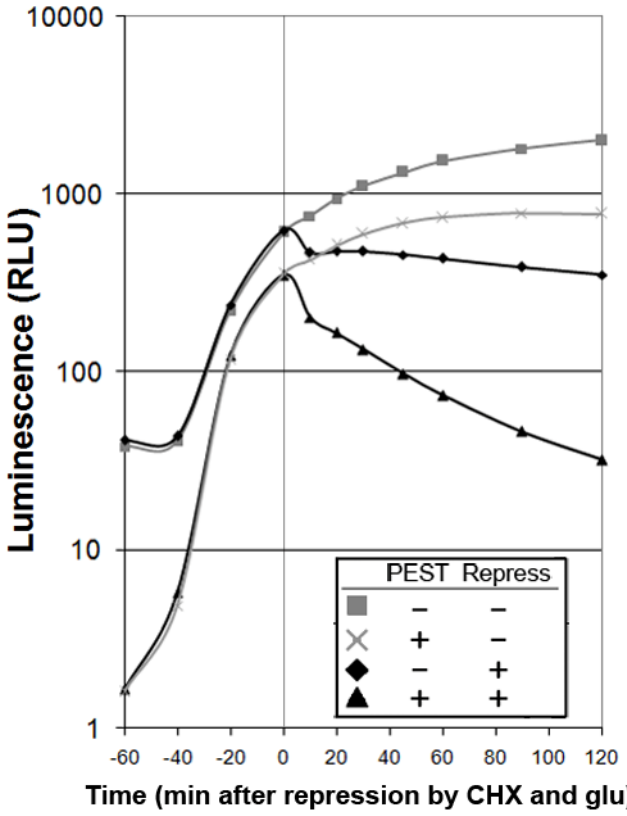

Figure 2.1. Half-life of luciferase activity in yeast is shortened from 3 h to 35 min by the addition of a PEST destabilizing sequence to the 3' end of the coding region of the firefly luciferase gene (Luc). Luminescence was continually measured from yeast cultures transformed with galactose-inducible/glucose-repressible reporters with or without a PEST sequence (P_{GAL1}-LucPEST or P_{GAL1}-Luc respectfully). 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, n=4) and with the unmodified reporter (black diamonds, n=4). Plotted points are averages. S.D. error bars are not shown because they were not large enough to be seen beyond the symbols. 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 3 h for Luc and 35 min for LucPEST. Cultures that were not repressed at time 0 (gray squares and Xs) continued to increase their luminescent output 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.

Luc also showed a lower level of background expression and a level of induction over background that was 13 times greater than the unmodified Luc, providing a greater dynamic range than the unmodified version.

Luciferase can be used as an accurate reporter of the yeast CDC by expressing the destabilized Luc under the control of a cell cycle regulated promoter. *POL1* encodes the largest subunit of DNA polymerase α [61] and is transcribed during the late G1 to early S phase of the cell cycle [62]. The reporter gene was fused to the *POL1* promoter (P_{POL1}-LucPEST) and integrated into the genome of the *MAT***a**, *bar1* strain LHY3865. These yeast, released from cell-cycle arrest with alpha factor, showed at least five noticeable oscillations of luminescence with a period of about 70 min (Fig. 2.2A). The luminescence signal increased with time as the cell density of the culture increased, but the amplitude of the oscillation dampened. When the luminescence data were detrended by subtraction of the luminescence trace from a third order polynomial trendline, the ongoing oscillation became more apparent (Fig. 2.2B). A similar result is obtained when luminescence data from a separate, asynchronously growing culture of the same yeast were used to detrend the data from the synchronized culture (Fig. 2.3). This luminescence oscillation of the synchronized culture coincided with the period of the microscopically determined population budding percentage (bud index) that was taken for 3 h; the luminescence rhythm phase-leads the rhythm of bud index by about 10 minutes (Fig. 2.2B). These data indicate that the luminescence rhythm (reflecting P_{POL1} activity) is a good reporter of CDC phase and period.

The YRO is characterized by a robust oscillation of dissolved oxygen (DO) concentration [13, 16, 18, 20, 34, 38, 39, 63] as well as some degree of synchronous cell division [13, 16, 34, 39]. DO can be monitored continuously and automatically with an electrode, but DO electrodes can only be used in relatively large volumes of liquid cultures. Luminescence reporting technology can potentially allow intracellular O_2 levels

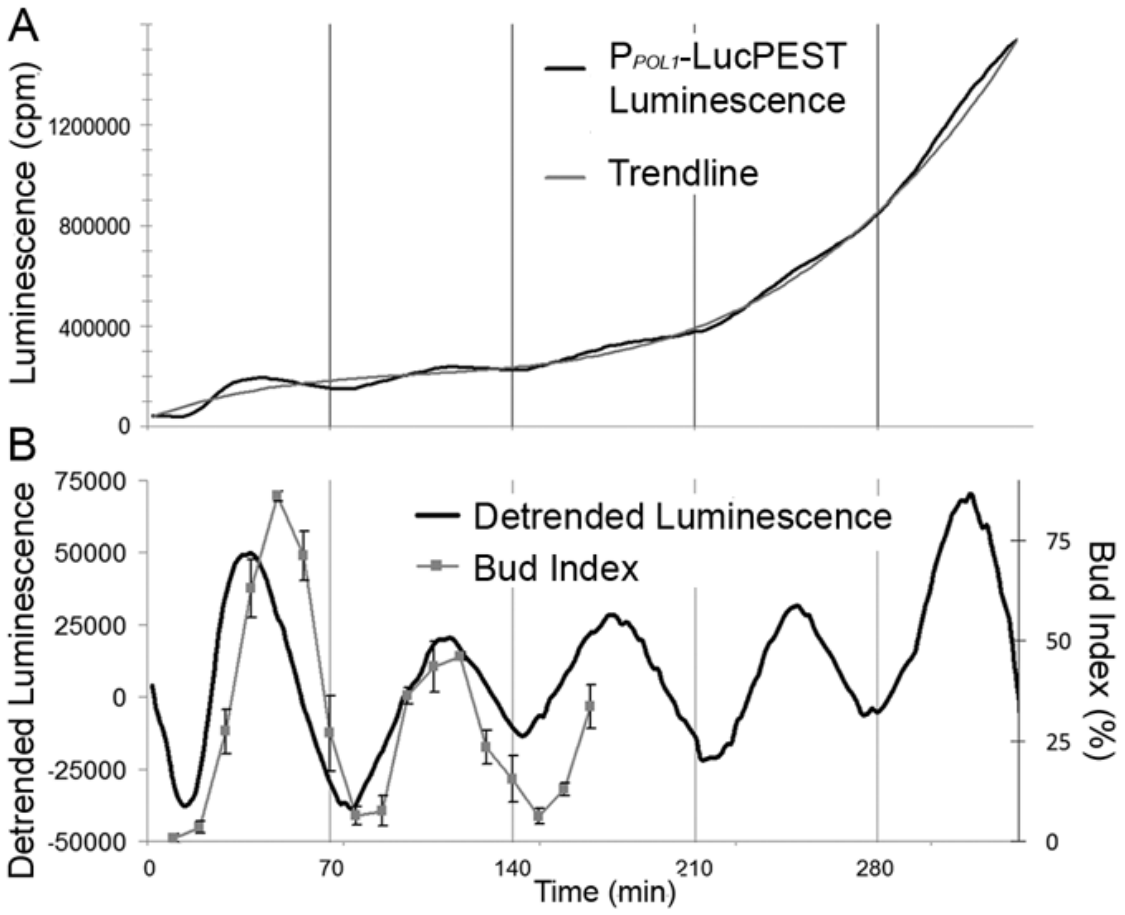

Figure 2.2. Luminescence from P_{POL1}-LucPEST oscillates with the CDC. **A.** Luminescence from a yeast culture transformed with P_{POL1}-LucPEST was recorded over time. The culture was arrested with α-factor for 3 h and released (A, black line). Overall luminescence from the culture increased over time as cell density increased roughly following a trend described by a 3rd order polynomial (A, gray line). The influence of growth was detrended from the data by subtracting the polynomial from the luminescence of the synchronized culture (B, black line). The difference was then compared to the oscillation of cell division calculated by microscopically scoring bud index (B, gray line) (n= 3-5 samples of >100 cells ea. per timepoint, from the same synchrony experiment, +/-S.D.).

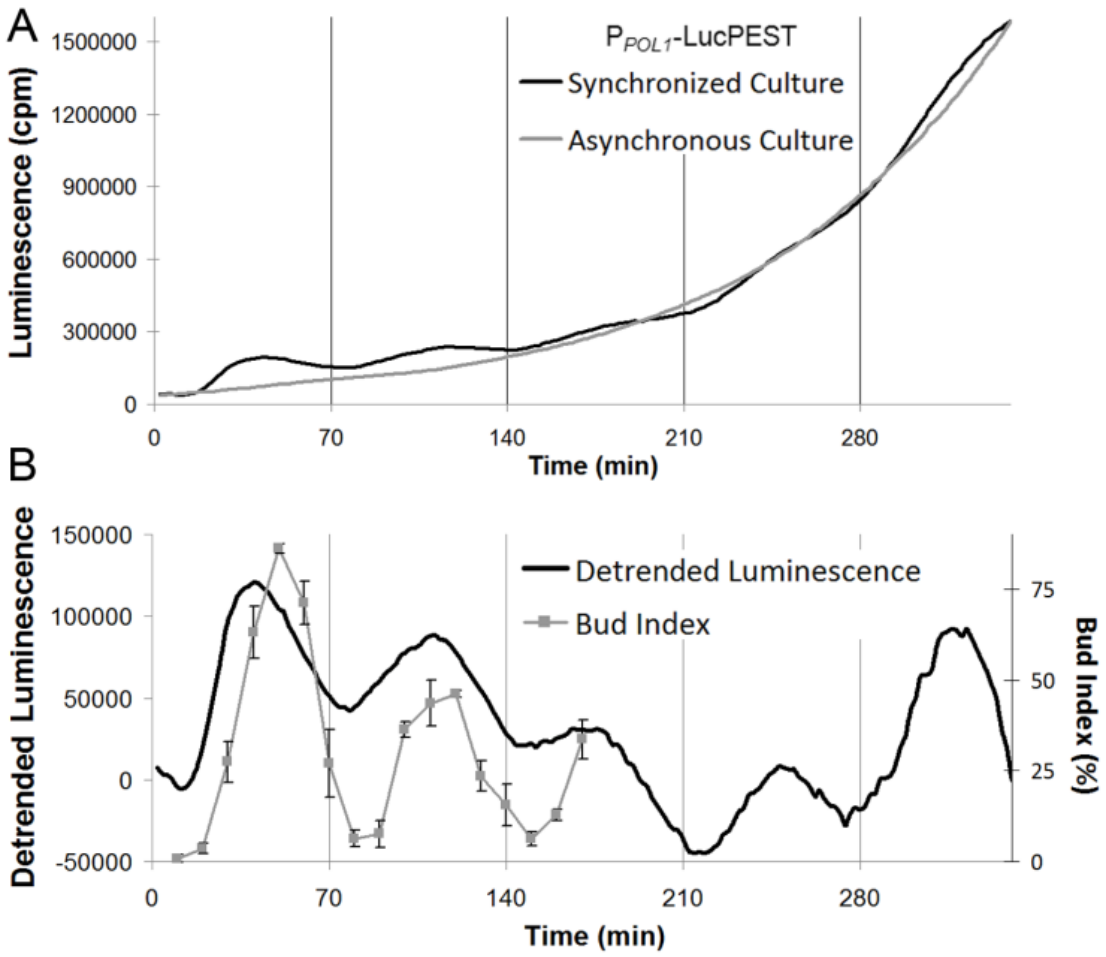

Figure 2.3. Plot of detrended luminescence from Fig. 2.2 using data of a separate, asynchronously growing culture of the same yeast strain. **A.** Luminescence from yeast cultures transformed with P_{POL1}-LucPEST was recorded over time. One culture was arrested with α-factor for 3 h and released (black line) while another culture of similar volume and density was allowed to grow asynchronously (gray line). Overall luminescence from both cultures increased over time as the culture density increased. However, only the synchronized culture showed an oscillation of luminescence. **B.** The influence of growth on luminescence signal was removed by data detrending via the subtraction of luminescence of the asynchronous culture from that of the synchronized culture in panel A. This detrended line was then compared to the oscillation of cell division calculated by microscopically scoring bud index, i.e. the bud percentage in the population (gray points and line).

to be monitored under a wider range of culture conditions. Luciferase is an oxygen-dependent enzyme that maintains a relatively stable light output when [O_2] remains above 5% (~25% atmospheric saturation), declines gradually as [O_2] falls below 5%, and plummets at [O_2] below ~2% [64, 65]. Because DO oscillates between ~2-10% (~10-50% atmospheric saturation) over the course of the YRO, the medium is periodically depleted of one of luciferase's cofactors. The interval of relative hypoxia inhibits luciferase activity, thereby "masking" promoter activity information. To develop a luciferase reporter for the O_2 decrease during the YRO, we (i) used unmodified Luc for its greater stability, and (ii) fused Luc to the constitutively expressed [66] promoter for actin (P_{ACT1}-Luc). Fig. 2.4 shows that the luminescence from yeast transformed with P_{ACT1}-Luc closely matches that of the DO trace. An immunoblot of yeast cells collected over one cycle of the YRO reveals a relatively constant amount of an antiluciferase-reacting protein. These immunoblot results confirm that P_{ACT1} is driving relatively constant expression of Luc and the correlation of dim luminescence with low DO levels supports the conclusion that the luminescence signal disappears at low O_2 levels. Therefore, the P_{ACT1}-Luc reporter could be used as a genetically encodable, *in vivo* monitor for low O_2 levels in situations where a DO probe is not practical–e.g., in colonies or very small culture vessels.

Can luciferase reporters be used to study gene expression during the YRO? Under some conditions, yeast cells divide in synchrony during the YRO (see below and also [16, 39]), and mRNA levels of various genes oscillate in or out of phase with the DO oscillation, as monitored by microarrays [13, 16] or northern blots [18]. Microarray/blot methods are informative, but they are expensive, labor intensive, and are limited by the frequency of sampling over the YRO. We tested the P_{POL1}-LucPEST luminescent reporter in continuous, oscillating cultures of strain CEN.PK to determine whether it could report promoter activity and cell cycle position continuously in real time without the

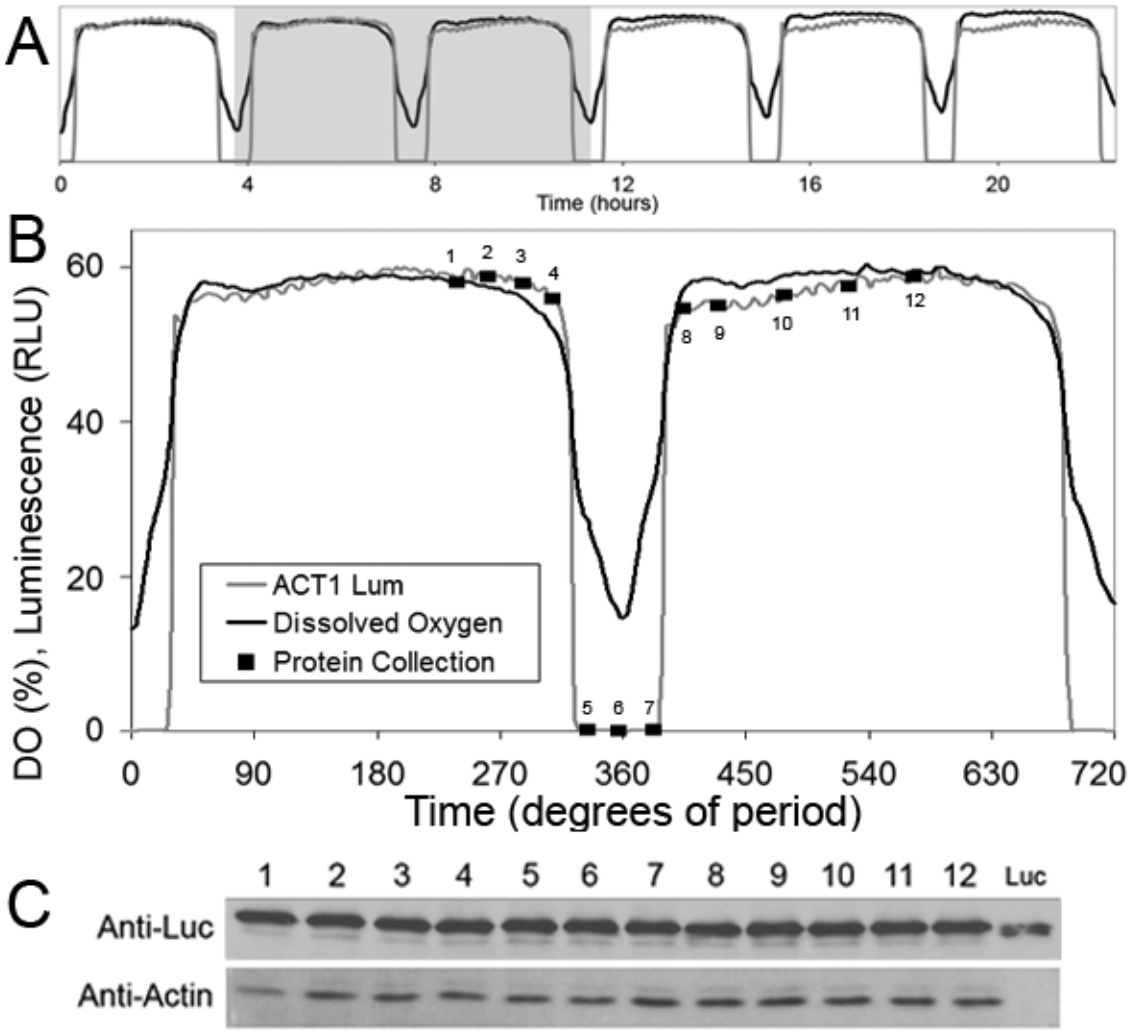

Figure 2.4. The P_{ACT1}-Luc reporter can be used to monitor intervals of low oxygen tension during the YRO. **A.** Six cycles of the YRO showing DO (black trace) and luminescence from P_{ACT1}-Luc (gray trace). The two cycles highlighted in the gray box are shown in panel B. **B.** Total protein was prepared from cell samples taken at various phases of the YRO (timepoints 1-12). DO is plotted as % atmospheric saturation. (360° = 3.75 h in this experiment.) **C.** Reporter protein concentration shows stable expression over the YRO. Immunoblots of total protein using anti-Luc and anti-actin (loading control) antibodies show that Luc protein concentration is stable across the oscillation when driven by the actin promoter, even during the hypoxic mask. The lane marked Luc is a control of 20 ng of purified luciferase. The absence of luminescence signal during the hypoxic mask therefore is likely due to low O_2 levels rather than a change in reporter protein concentration.

need for sampling. Fig. 2.5A shows 7 consecutive cycles of the YRO over 35 h. In each cycle, luminescence peaks soon after DO rises and then gradually decays. Samples were taken from one cycle of the YRO and assayed for bud index. The luminescence signal from P_{POL1}-LucPEST followed the budding percentage except for recurring times of culture hypoxia (Fig. 2.5B). The luminescence traces can be roughly corrected for the intervals of hypoxia (Fig. 2.6)–with this correction, the bud index rhythm clearly phase-lags that of P_{POL1}-LucPEST luminescence. Therefore, as long as the luminescence data are corrected for the hypoxic masking, the data are very similar to those shown for P_{POL1}-LucPEST in yeast cells that are synchronously dividing under conditions in which O_2 is not limiting (Fig. 2.2).

Different cell-cycle promoters can be used in conjunction with LucPEST to identify different CDC phases during the YRO. *CLN2* is a gene that is maximally expressed in late G1 phase just prior to transition to S [67] while *PIR1* is a gene whose transcription peaks during the M to G1 transition [66]. Therefore, expression of these two genes occupies opposite ends of the G1 phase. When the promoters for these genes are fused to LucPEST (P_{CLN2}-LucPEST and P_{PIR1}-LucPEST) and integrated into CEN.PK, they produce two distinctly different expression patterns in continuous, oscillating cultures, revealing characteristic landmarks of the cell division cycle within the YRO (Fig. 2.7). In a separate analysis of the YRO using data drawn from analyzing population budding percentages and cell concentrations, we find that the YRO of CEN.PK yeast can be optimally modeled as two populations of cells that synchronously divide 180° out of phase with one another, one CDC occurring every 2 YROs (Chris Stowers and Erik Boczko, unpublished results). Our data with luminescence reporters (and bud index, Fig. 2.4) are consistent with the “two antiphase populations" model. Fig. 2.7 illustrates how a model in which each population’s short S/G2/M phase may be

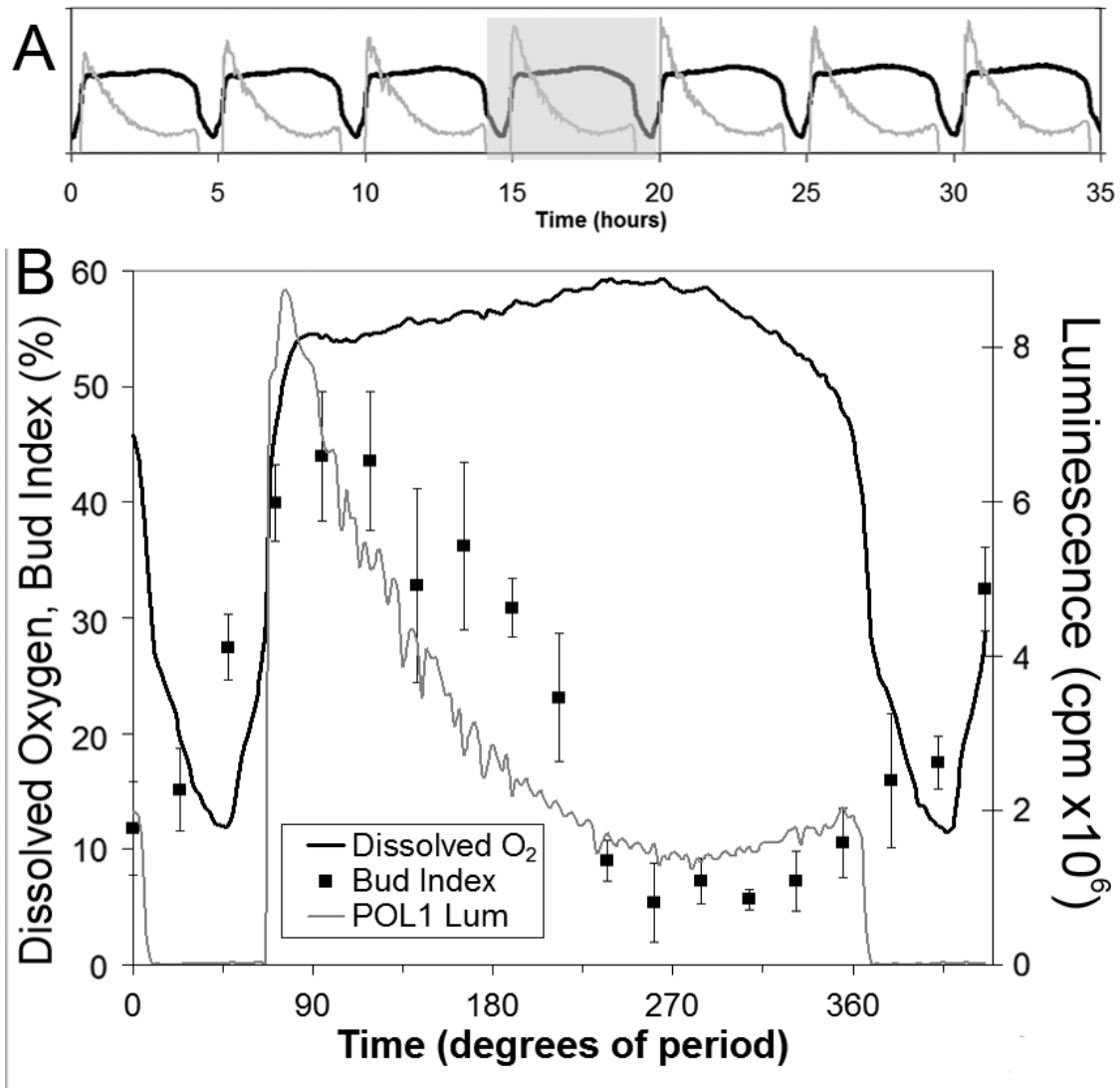

Figure 2.5. Luminescent P_{POL1}-LucPEST reporter in yeast undergoing respiratory oscillations in continuous culture. **A.** The reporter shows real-time *POL1* promoter activity (RLU) over 35 h (A, gray line) of the YRO. Oscillating DO concentration (A, black line) measured continuously with a DO electrode tracks the YRO. **B.** Samples were removed from the oscillating culture at different phases during the gray highlighted section of panel A and cells were scored for bud index to show that luminescence from the P_{POL1}-LucPEST reporter corresponds with a rhythm of cell division in the population (n=3-9 samples/timepoint, ± S.D.). Luminescence (gray) and DO levels (black) are also shown for comparison (100% DO = media saturated by atmospheric O_2). Hypoxia masks the luminescence signal when DO concentration drops below ~40% saturation. (360° = 5 h in this experiment.)

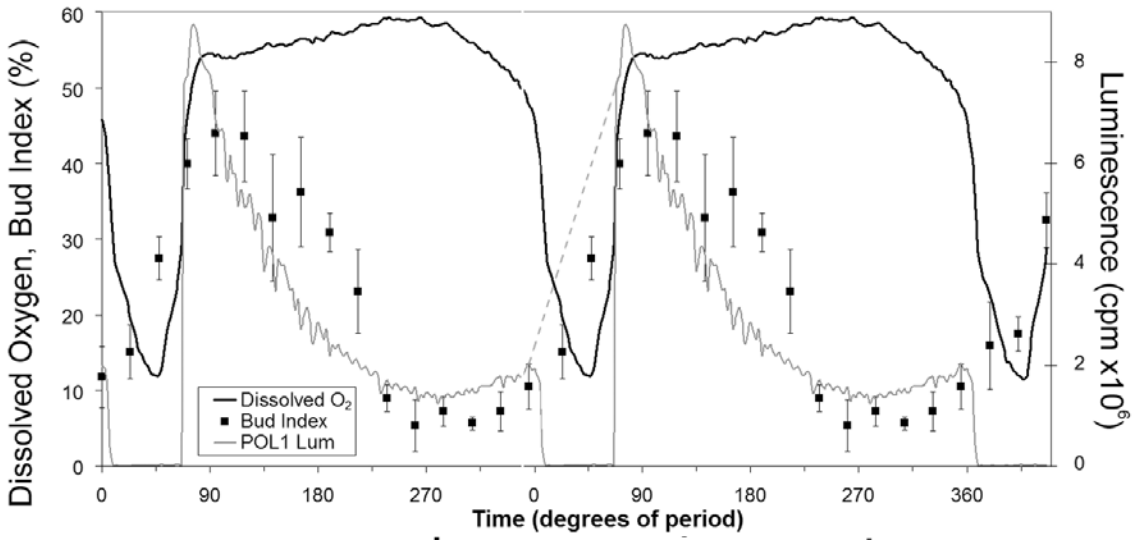

Figure 2.6. The data of Fig. 2.5 are double plotted here to underscore the transitions from cycle to cycle including an extrapolation of luminescence during the hypoxic mask (extrapolation shown as dashed line). Dissolved oxygen concentration is graphed in percent of atmospheric saturation and time is double-plotted in degrees of period (360° = 5 h). Bud index data collected over one cycle are double-plotted.

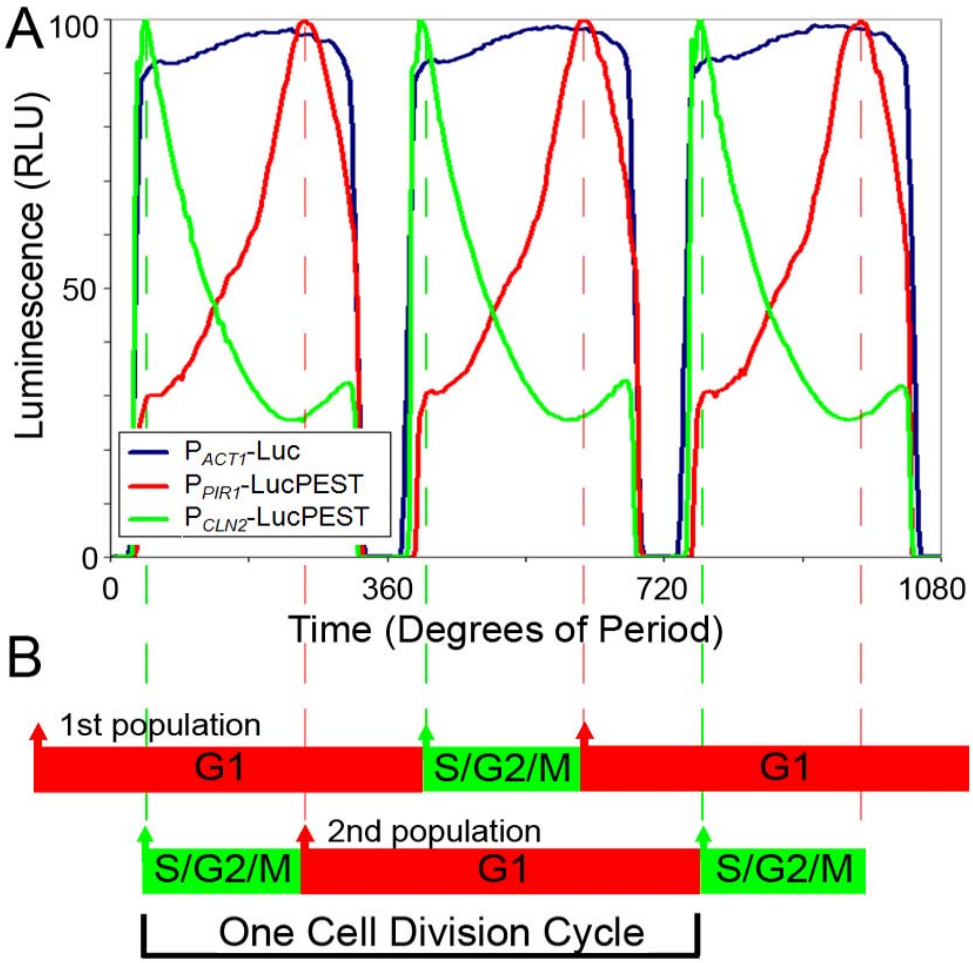

Figure 2.7. Luminescent reporters driven from 3 different promoters in continuous cultures show distinctly different patterns of expression over the YRO. **A.** Peak luminescence from three separate cultures, each expressing a Luc reporter for a different promoter (each trace was normalized to 100 RLU for comparison). Three YRO cycles (which comprise 1.5 cell divisions for a single cell) are graphed. The peak of P_{CLN2}-LucPEST luminescence (green) corresponds with exit from G1 and entrance into S phase of the CDC. The peak of P_{PIR1}-LucPEST luminescence (red) corresponds with exit from M phase and entrance into G1. Luminescence from P_{ACT1}-Luc (blue) indicates intervals of relative hypoxia. (360° = 3.75 h in this experiment.) **B.** Model of how two populations of cells occupy the culture and how their positions in the CDC are diagramed relative to the time axis of A and to each other (see Fig. 2.8 for a deconvolution of the two populations). Transitions between G1 and S/G2/M were determined from the peaks of the luminescence profile from A (vertical dashed red and green lines). The time of one predicted cell division is indicated by the black bracket.

roughly centered in the middle of the other population's long G1 phase and that the hypoxic mask occurs during a time when both populations are in G1 phase (see Fig. 2.8 for correction of the hypoxic mask and deconvolution of the two populations). These two reporters and the cell cycle transitions they delineate demonstrate that for each population, G1 occupies about 70% of the cell cycle and S/G2/M occupies about 30% during the YRO.

Acetaldehyde and ethanol may serve as synchronizing agents for oscillating respiration in continuous cultures because they are rhythmically released from yeast cells and they can strongly reset the phase of the YRO [59, 63]. We wanted to know whether these phase altering substances also alter the timing of cell division associated with the YRO. Phase response curves (PRCs) to injections of 1mM acetaldehyde or 1 mM ethanol into oscillating cultures of CEN.PK/P_{POL1}-LucPEST were measured with regard to the DO oscillation (Fig. 2.9). Fig. 2.10A shows that both stimuli induce strong Type 0 resetting [63, 68]. Independent of the phase of injection, either acetaldehyde or ethanol produces an immediate, but temporary, depletion of O_2 in the culture as the metabolite is consumed (Figs. 2.10B, 2.9), whereas the resuming DO rhythm displays phase-dependent shifts (Figs. 2.10A, 2.10D, 2.9). For example, injection at phases between 0°~45° and 270°~405° have small effects on phase; injections between 45°~135° cause increasingly larger phase delays; injections between 180°~270° cause progressively smaller phase advances (Figs. 2.10A, 2.9).

To ascertain CDC activity during the phase resetting experiment, luminescence from P_{POL1}-LucPEST was recorded following perturbations with acetaldehyde or ethanol. The data for acetaldehyde are summarized in Fig. 2.10B by showing the reporter's initial response to acetaldehyde over a full cycle of 360° (data for ethanol are similar but not shown). The normalized luminescence traces for each of the acetaldehyde injections following the 0° injection were aligned with the peak of the 0° injection for comparison.

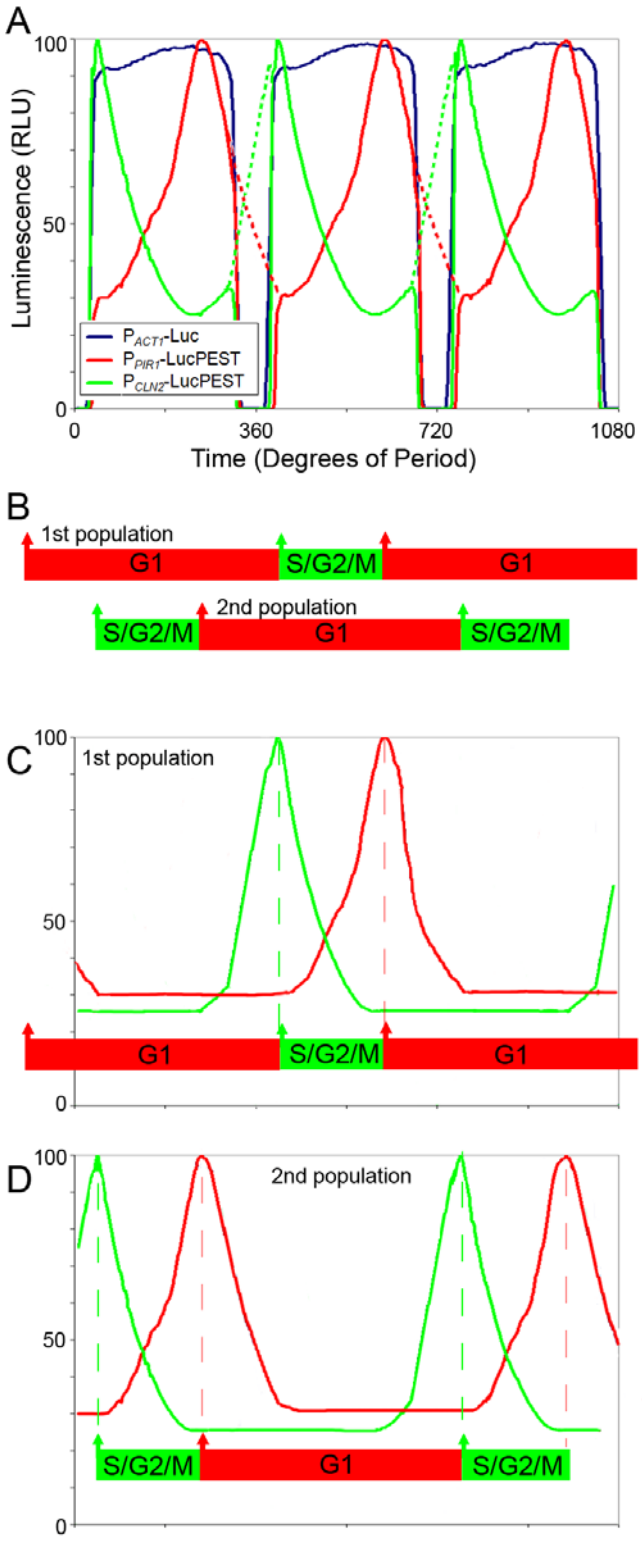
A
100
50
0
Luminescence (RLU)
P_{ACT1}-Luc
P_{PIR1}-LucPEST
P_{CLN2}-LucPEST
0
360
720
1080
Time (Degrees of Period)
B
1st population
G1
S/G2/M
G1
2nd population
S/G2/M
G1
S/G2/M
C
100
50
0
1st population
G1
S/G2/M
G1
D
100
50
0
2nd population
S/G2/M
G1
S/G2/M

Figure 2.8.

Figure 2.8. Data of Fig. 2.7 showing extrapolation of promoter activity during the hypoxic mask and deconvolution of the two hypothetical populations responsible for the luminescent signals. **A.** Peak luminescence from three separate cultures, each containing a luciferase reporter for a different promoter, was normalized to 100 RLU for comparison. Three cycles of the YRO (that comprise 1.5 cell divisions per cell) are graphed. The peak of P_{CLN2}-LucPEST luminescence (green) corresponds with exit from G1 and entrance into S phase of the CDC. The peak of P_{PIR1}-LucPEST luminescence (red) corresponds with exit from M phase and entrance into G1. Luminescence from P_{ACT1}-Luc (blue) indicates intervals of relative hypoxia. Dotted red and green lines connect the respective traces at the boundaries of the hypoxic masks to show likely promoter trends during these times. **B.** We hypothesize that two populations of cells occupy the culture and their positions in the CDC are diagramed relative to the time axis of panel A and to each other (360° = 3.75 h). The contribution of these two predicted populations to the overall luminescence signals of the P_{CLN2}-LucPEST and P_{PIR1}-LucPEST reporters are illustrated in panels C and D. **C.** The first population in the culture completes cell division at approximately phase 620° of the YRO (approximately 720° of the YRO is required for one full cell division cycle to be completed). Transitions between G1 and S/G2/M were determined from the peaks of the luminescence profile from A (vertical dashed red and green lines). **D.** The second population in the culture undergoes cell division out of phase with the first population. In this population, the CDC also takes 720° of the YRO to complete.

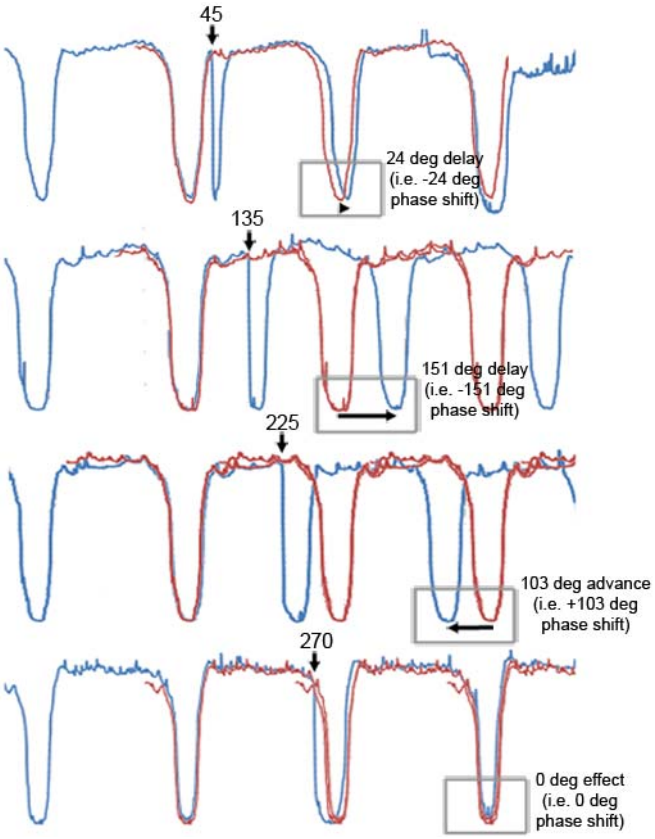

Figure 2.9. Examples of data used to generate the phase response curve (PRC) for acetaldehyde in Fig 2.10A (data for ethanol are comparable but not shown). Four representative DO traces (blue) for phase resetting experiments are depicted where 1 mM acetaldehyde was injected at the phase points indicated by vertical arrows (at phases 45°, 135°, 225°, and 270° of the period). Phase point "0°" is defined as the time when DO begins to rise from hypoxia. The red traces duplicate the cycles prior to injection in order to project the DO trace in the absence of an injection. The phase shift that resulted for each case was measured during the first cycle after injection (gray boxes) and the intensity and direction of the effect is shown by horizontal arrows.

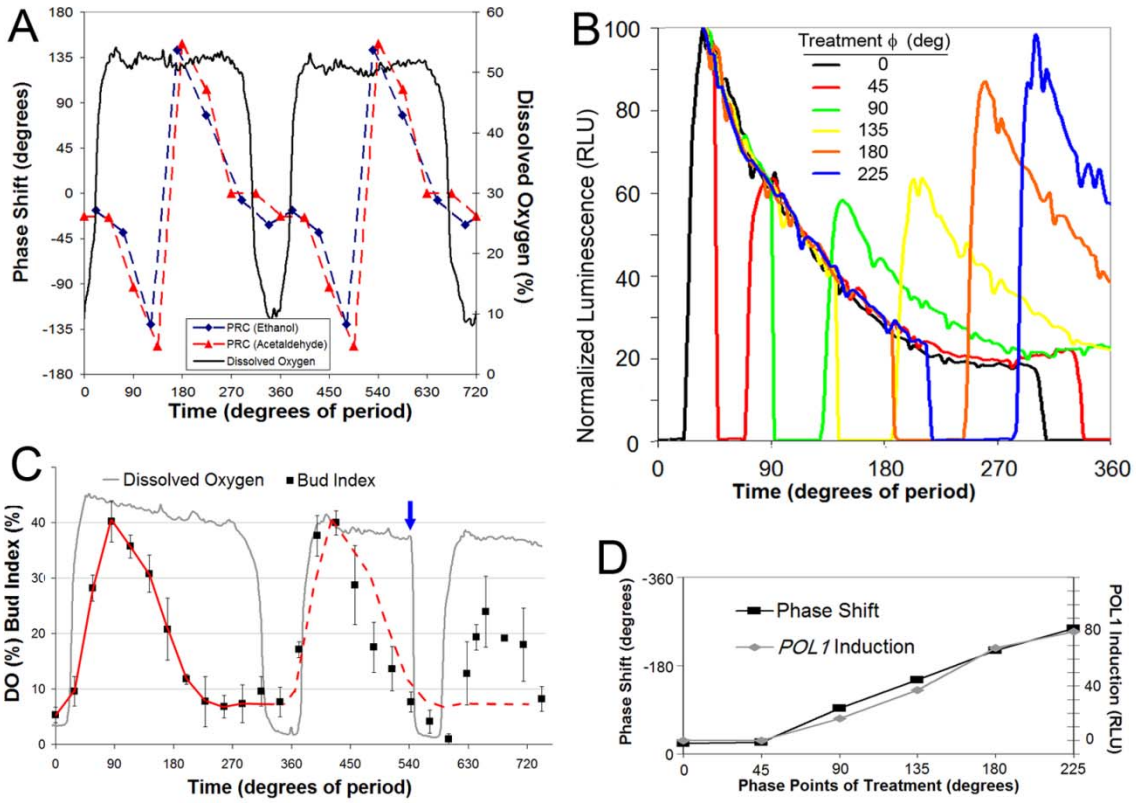

Figure 2.10. Phase responses of the YRO to 1 mM acetaldehyde or 1 mM ethanol. **A.** shows a double-plotted phase-response curve (PRC) to ethanol (blue) or acetaldehyde (red) relative to a DO oscillation. Phase shifts are plotted in degrees of period with advance shifts plotted as positive values and delay shifts as negative values (see Fig. 2.9 for raw data that illustrate the method of calculation). (360° = 4.0 h for the acetaldehyde PRC experiment and 3.75 h for the ethanol PRC experiment.) **B.** Luminescence responses of the P_{POL1}-LucPEST reporter to acetaldehyde treatments at the indicated phase points. The peak luminescence for each oscillation during which an acetaldehyde injection was made was normalized to 100 RLU and aligned with the peak of the 0° treatment. Treatments at phases 0° or 45° showed virtually no difference in luminescence aside from a brief hypoxic mask. Treatments made after phase 45° showed progressively increasing levels of P_{POL1}-LucPEST luminescence rebounding from the hypoxic mask as compared to the controls without treatment. **C.** Bud index in response to 1mM acetaldehyde injection made at phase 540° of the YRO (blue arrow). Bud index is the mean of 3-6 samples at each time point of >100 cells/sample (± S.D.). The red line is the bud index trend from 0-360°; the dotted red line projects this trend onto the next period. **D.** shows a strong correlation between the amount of rebounding *POL1* induction after treatment and the magnitude of phase shift caused by the treatment. For comparison to luminescence, the phase shift in panel D is plotted on a monotonic 0° to -360° scale rather than the -180° to +180° scale of panel A.

The initial trend and shape of all of the luminescent P_{POL1}-LucPEST traces are virtually the same prior to injection, denoted in Fig. 2.10B by the group of multicolored lines following the same decay pattern after their peak. For traces of injections after 0°, a noticeable hypoxic mask occurs soon after injection followed by a return of the signal. Interestingly, when the signal returns from the hypoxic mask of the early treatments at 0° or 45° (black and red traces), the luminescence signal returns to the same level that it would have attained if no injection had occurred, indicated by the red trace rejoining the trend of the black and multicolored traces; these are the same phases at which practically no resetting of the YRO occurs (Fig. 2.10A,D). However, as injections occur progressively later in the cycle (e.g. at 90° (green), 135° (yellow), etc.) the luminescence signal rebounds to a significantly higher level than in the no-injection controls.

These results implied that some cells enter premature replication as a result of acetaldehyde/ethanol injections. If this were true, the percentage of budded cells in the culture should increase shortly after an injection. We confirmed this novel prediction by examining samples across two cycles of the YRO in which acetaldehyde was injected at phase 540° (the first cycle in Fig. 2.10C (0-360°) serves as an unperturbed control). Following the injection, ~25% of the culture prematurely buds (Fig. 2.10C), as predicted by the LucPEST data of Fig. 2.10B. The data in Fig. 2.10A-C suggest a clear correlation between (i) phases at which the acetaldehyde/ethanol stimulate new rounds of the CDC and (ii) phase shifts of the DO oscillation (Fig. 2.10D).

Some researchers have concluded that the YRO may be the result of an endogenous self-sustained ultradian oscillator that functions analogously to the longer period circadian oscillators that regulate many cellular events including cell division [13, 16, 20]. That conclusion is based at least in part on yeast strains and conditions that exhibit YROs that cycle with periods as short as 40 min, and may reflect different underlying mechanisms than the ones investigated in this study. With our strains and

conditions, the YRO has a period of ~4-5 h (as reported by other investigators [16, 18, 39]. Under the latter conditions, the data support an alternative hypothesis, namely that there are two populations of dividing cells in antiphase whose CDC rate and G1 duration are constrained by the dilution rate of the continuous culture environment [3] so that the CDC and the YRO appear to be linked [34]. We hypothesize that one population signals to the other its position in the cell cycle through non-fermentable metabolites like acetaldehyde and ethanol or possibly the hypoxia that results from their consumption. As a result, the YRO is the product of a balanced population structure and environmental signaling under our continuous culture conditions.

Responses to phase-shifting substances/signals disclose important clues to the mechanism of the oscillation itself. For example, do these substances synchronize cells in the population, thereby sustaining a robust oscillation in the presence of noise? Acetaldehyde and ethanol are potential YRO synchronizing signals [59, 63] and their resetting efficacy is phase-dependent (Fig. 2.10). Moreover, this phase shifting is correlated with premature induction of cell division during the oscillation, thereby initiating new cell division and thus resetting the CDC's "timer." Very late in the oscillation (e.g. 270°-315°), introduction of the potential signal has little effect on the phase of the YRO and this correlates with the phase in the natural state of the system at which cells are secreting the greatest amount of non-fermentable carbon compounds into the medium [16, 18]. We hypothesize that during the early phases of the YRO (e.g. 0°-45°, immediately after the secretion of carbon compounds), cells that could have responded to the endogenous signal have done so, and have committed to cell division, entering a fermentation-preferring state (Fig. 2.11). Therefore the culture is deprived of "signal sensitive" cells that are capable of responding to new signal. Only the few straggling cells that narrowly missed the commitment to cell division and have since matured in the intervening time are potentially responsive to an injection of acetaldehyde

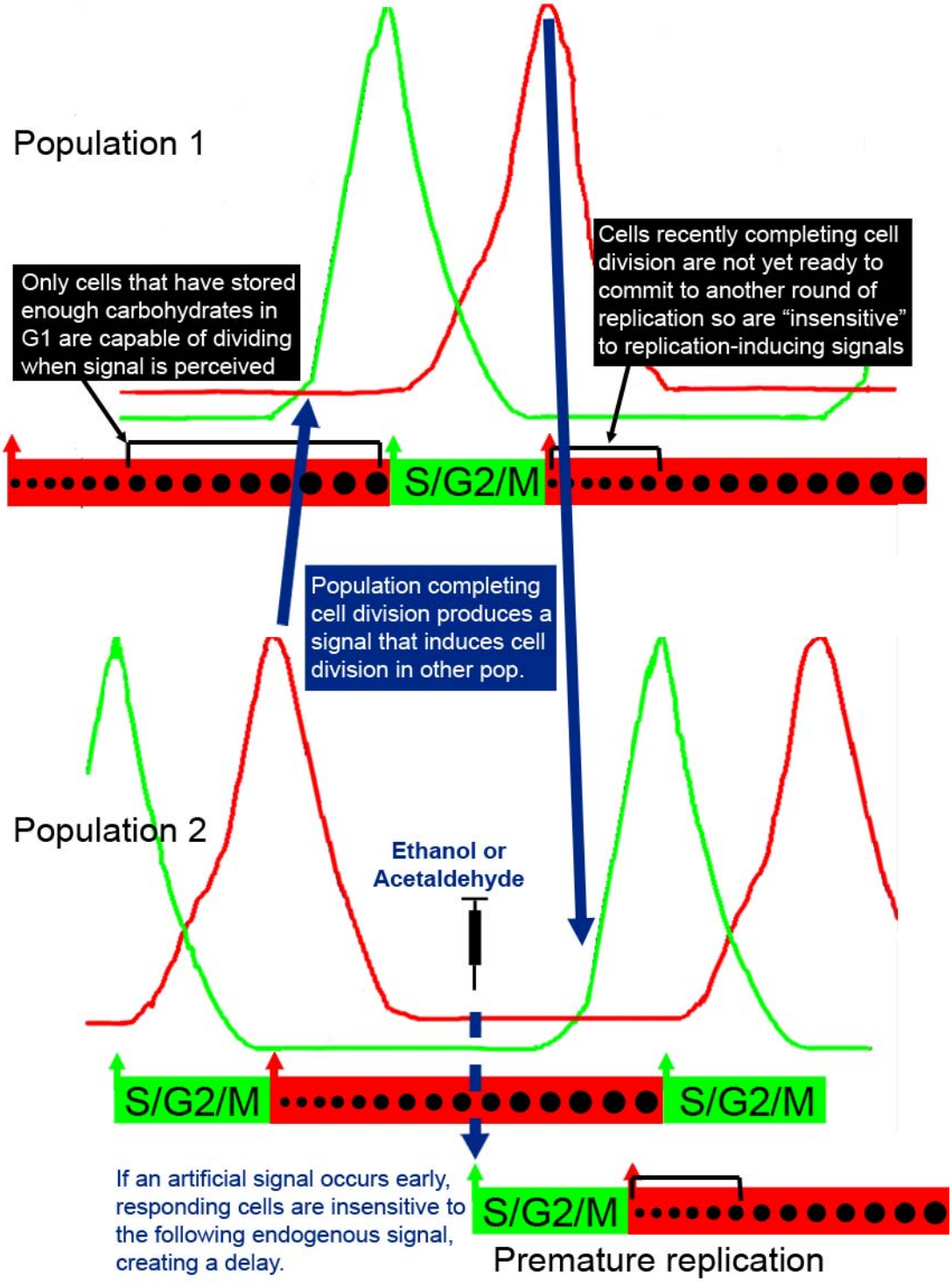
Population 1
Only cells that have stored enough carbohydrates in G1 are capable of dividing when signal is perceived
Cells recently completing cell division are not yet ready to commit to another round of replication so are "insensitive" to replication-inducing signals
S/G2/M
Population completing cell division produces a signal that induces cell division in other pop.
Population 2
Ethanol or Acetaldehyde
S/G2/M
S/G2/M
If an artificial signal occurs early, responding cells are insensitive to the following endogenous signal, creating a delay.
S/G2/M
Premature replication

Figure 2.11

Figure 2.11. The two-population signaling model of the YRO illustrates the underlying cell-division/phase resetting relationship hypothesized to be responsible for the data of Fig. 2.10. Population 1 is shown in the top half of the figure and population 2 is shown in the bottom half. Green and red signal traces for both populations indicate luminescence levels from P_{CLN2}-LucPEST and P_{PIR1}-LucPEST representing onset of S and G1 phases respectively. Cell cycle phase for each population is shown by red (G1) and green (S/G2/M) horizontal bars. Black circles of increasing size in G1 portions of the bars represent cells maturing over time (e.g. by gaining mass, storing carbohydrates, etc). G1 cells of population 1 mature over time and reach a replication-capable state (indicated by the black bracket on the left); however the cells delay replication due to the absence of signals in the medium that allow the passage through the G1/S checkpoint or "gate." Meanwhile cells in population 2 progress through cell replication (S/G2/M) in a fermentation-preferring state, thereby gradually producing non-fermentable carbon compounds (e.g. ethanol) that peak near the end of M phase. These compounds (blue arrow pointing up) serve directly or indirectly as an extracellular signal to open the G1/S "gate" for the waiting cells of population 1. This signal(s) permits the cells of population 1 to commit to cell replication. As population 1 completes replication, its cells also produce a signal (blue arrow pointing down). This second signal has no effect on the G1 cells of its own population (indicated by the short, black bracket on the right) however the second signal opens the gate for cells of population 2, allowing them to begin replication. Underneath population 2's cell cycle bar is an alternative CDC bar that shows how population 2's replication cycle is altered if a premature signal is administered at the point of the dashed blue arrow. An artificial signal (such as the experimental injection of acetaldehyde or ethanol) prematurely administered during the G1 phase of either population can elicit the cell-replication response from only those few cells that have matured in the population up to that point. Since the number of mature cells continues to increase the longer the population remains in G1, greater numbers of cells begin replication as a result of the premature signal the later in G1 the acetaldehyde/ethanol is administered. Because the luminescence signal of P_{POL1}-LucPEST reflects the initiation of replication, progressively later injections of acetaldehyde/ethanol cause concomitantly larger luminescence emissions (Fig. 2.10B).

or ethanol. If left unperturbed, increasing numbers of cells become capable of committing to cell division due to acquisition of required storage carbohydrates, and concomitantly become responsive to signals such as acetaldehyde or ethanol (and/or currently unidentified signals?). Premature injection of ethanol or acetaldehyde into the culture later in the YRO causes drastically larger phase delays because it interrupts the accrual of replication-competent cells, spurs early cell division, and thus desensitizes that group to the next endogenous signal that occurs. Missing this endogenous signal causes a delay. Afterwards, the prematurely induced population produces its own endogenous signal and continues the cycle from that phase.

The principles and mechanisms upon which the YRO depend are likely to have biological relevance in nature, e.g., in colonies or dense films of yeast on substrates such as grapes. With the aid of luminescent reporters of gene regulation and metabolism such as the ones described here, future investigations will hopefully reveal evidence of cell-cell signaling and environmentally regulated cell division synchrony. We show here that the use of luminescence reporters for real-time measurements of promoter activity allow non-invasive measurement of yeast CDC events in batch and continuous cultures. This reporter system also uncovered a linkage in our culture conditions between resetting the phase of the YRO and the induction of new rounds of cell division. The precision and ease of continuous monitoring of CDC-regulated promoter activity is just one example of the utility of this system. The reporting system presented here does have limitations; for example, the necessity for O_2 restricts Luc's ability to report under hypoxic conditions. However, we take advantage of this apparent limitation to develop a reporter that accurately monitors the episodes of hypoxia that recur during the YRO. Additionally, the 35 min half-life of LucPEST restricts its usefulness for investigating shorter-period (e.g., 40 min) metabolic oscillations such as those described from the strain IFO 0233 [13, 20, 38, 63]. Nevertheless, further refinements of Luc (e.g. codon

optimization and/or additional degradation signals) may enable the reporting of shorter-period oscillations. We envision the application of luminescent reporters in yeast to the assay of rapidly changing gene expression, *in vivo* oxygen monitoring, and further investigation of yeast rhythms.

Materials and Methods

Naming Conventions

For purposes of naming, plasmid designations begin with a lowercase "p" followed by relevant labels. When a luminescent reporter is mentioned in the text, it is labeled simply with a capital "P" (promoter) followed by the gene name for the promoter driving the version of the luciferase gene used. For example "pRS315-Kan-P_{POL1}-Luc(A4V)PEST" refers to a plasmid made from the pRS315 backbone and contains a kanamycin resistance cassette and the modified luminescent reporter driven by the *POL1* promoter. However in the text, a yeast strain transformed with this plasmid would be said to have the P_{POL1}-LucPEST reporter.

Bioluminescent Reporter Construction

The plasmid pGL3-*ADH1*term was generated by replacing the SV40 late terminator of pGL3-Basic (Promega) with the *S. cerevisiae ADH1* terminator from pFA6a-GFP(S65T)-HIS3MX [69]. The *ADH1* terminator was PCR amplified from pFA6a-GFP(S65T)-HIS3MX using the forward primer TTCATCTCTAGAGGCGCGCCACTTCTAAAT that contained an *Xba*I site overhang and the reverse primer GGACGAGGCAAGCTAAAC that annealed immediately downstream from the terminator and a *Bgl*II site. The PCR product was digested with *Xba*I and *Bgl*II

and ligated into pGL3-Basic which had been digested with *Xba*I and *BamH*I to remove its SV40 late terminator.

The PEST sequence from *CLN2* of *S. cerevisiae* (3' terminal 534 nucleotides) was amplified by PCR from genomic DNA of strain SEY6210 [70] using the forward primer GAATAAGCTTGCATCCAACTTGAACATT that contained a *Hind*III site overhang and the reverse primer GAAGTTCTAGACTATATTACTTGGGTATTGCC that contained an *Xba*I site overhang. These sites were used to clone the PEST PCR product into pFA6a-GFP(S65T)-HIS3MX for maintenance (designated pFA6a-*CLN2*PEST).

Gene SOEing [71, 72] was used to fuse the PEST sequence from pFA6a-CLN2PEST to the 3' end of the luciferase ORF of pGL3-*ADH1*term just prior to the stop codon. Gene SOEing was performed as described by Wurch et al., 1998 [72]. Specifically, the luciferase component of the fusion was generated from pGL3-*ADH1*term using the forward primer GCTAGCCCGGGCTCGAGATC and the reverse primer AATGTTCAAGTTGGATGCCACGGCGATCTTTCC. The forward primer annealed upstream from the luciferase start codon and included an *Xho*I site. The reverse primer included an overhang with homology to the 5' end of the PEST sequence. The PEST portion of the fusion was generated from pFA6a-*CLN2*PEST using the forward primer GGAAAGATCGCCGTGGCATCCAACTTGAACATTTCG and the same reverse primer that was used to amplify the PEST sequence from the genomic source mentioned above. The forward primer contained an overhang with homology to the 3' end of luciferase, and the reverse primer contained an *Xba*I site overhang. The two PCR products were agarose gel-purified (Qiagen), mixed in equimolar (10 nM) concentrations, and were fused using 10 rounds of PCR without primers. The Luc-PEST fusion was amplified by a final PCR reaction involving flanking primers (i.e. the *Xho*I-containing forward primer of the previous luciferase-generating PCR and the *Xba*I-containing reverse primer of the previous PEST-generating PCR) and cloned into pGL3-

*ADH1*term using *Xho*I and *Xba*I, replacing the original luciferase ORF to create the plasmid pGL3-PEST-*ADH*1term.

The region of DNA containing the luciferase-PEST fusion and the *ADH1* terminator was amplified by PCR from pGL3-PEST-*ADH*1term using the forward primer AAGTAACTGCAGATGGAAGACGTCAAAAACATAAAGAAAGGCCCG and the reverse primer GACGATAGTCATGCCCGGG. The forward primer annealed at the beginning of the start codon of luciferase and included a mismatch that mutated the fourth amino acid from adenine to valine to introduce an *Aat*II site. The forward primer also included a *Pst*I overhang. The reverse primer annealed downstream of *ADH1*term and a *Sal*I site. This PCR product was cloned into the yeast shuttle vector pRS315 [73] using *Pst*I and *Sal*I to create the versatile destabilized-luciferase expression vector pRS315-Luc(A4V)PEST for *S. cerevisiae*. A similar luciferase expression vector (pRS315-Luc(A4V)) without the PEST sequence was constructed in the same way by using pGL3-*ADH1*term as the PCR template instead of pGL3-PEST-*ADH*1term.

Promoters of interest (*POL1*, *CLN2*, *ACT1*, *GAL1*, *PIR1*) were amplified by PCR using yeast genomic template from strain SEY 6210 and primer pairs that targeted nearly all of the intergenic region upstream of the gene of interest. The forward primers contained an overhang that included either the *Pst*I or *Xma*I restriction site. The reverse primers contained an overhang that included an *Aat*II site and the first 4 amino acids of luciferase. Specific primers used to amplify the promoters are listed in Table 2.1. The resulting PCR products (promoters) were individually cloned into pRS315-Luc(A4V)PEST and/or pRS315-Luc(A4V) to make complete luciferase reporter constructs that could be maintained in yeast. The “promoter-Luc(PEST)-terminator” portion of the constructs was also moved to other pRS vectors (e.g. pRS314, pRS303, pRS306, pRS424) when different selection markers were needed or when the luficerase

Table 2.1: Primers used for amplifying promoter regions for different genes

Promoter	Forward Primer (*Pst*I or *Xma*I)	Reverse Primer (*Aat*II)
POL1	AAGTAACTGCAGTGCATTTTTCTTAAAGAAATATAAC	ATGATTGACGTCTTCCATTTTCCACTGTTTATTATATGCCT
CLN2	TTCACTCCCGGGATTCGCCTCTATGCTCCTTC	ATGATTGACGTCTTCCATTGTCTGTCGTTAAATTTAATGAATG
ACT1	TTCACTCCCGGGTAAGTAAATAAGACACACGCGAG	ATGATTGACGTCTTCCATTGTTAATTCAGTAAATTTTCGATC
GAL1	AAGTAACTGCAGGGCATTACCACCATATACATATCC	ATGATTGACGTCTTCCATTATAGTTTTTTCTCCTTGACGTTA
PIR1	TTCACTCCCGGGATATGCCAAATTTAGAAAGCC	ATGATTGACGTCTTCCATTTTCTAGAATATACTATTAGGGGAG
GPH1	AAGTAACTGCAGGTCATAAAAAGTAACTTACAATGC	ATGATTGACGTCTTCCATTGTTCAAAATTATTATAAGTTGA

reporter needed to be stably integrated into the host's genome or over-expressed on 2-micron plasmids [73]. These rearrangements were made with *Pst*I or *Xma*I and *Sal*I.

The yeast integration vectors pRS303 and pRS306 were modified for selection on G418 antibiotic by introducing the kanamycin resistance gene from pFA6a-KanMX6 [69]. This was done in a three-step process. First, the Kan resistance gene was cloned from pFA6a-KanMX6 into pRS315-P_{GPH1}-Luc(A4V)PEST using *Not*I and *BamH*I, creating pRS315-Kan -P_{GPH1}-Luc(A4V)PEST. This plasmid served as a backbone for the introduction of several promoters of interest by swapping out the *GPH1* promoter with the one of interest (e.g. *ACT1*, *PIR1*, *CLN2*, etc) using *Xma*I and *Aat*II. Then the entire region containing the Kan resistance gene, promoter of interest, destabilized luciferase, and the *ADH1* terminator was cloned into either pRS303 or pRS306 using *Not*I and *Sal*I or *EcoR*I and *Sal*I. This pRS315-Kan intermediate was necessary due to an inconvenient *Aat*II site in pRS303 and pRS306 that prevented the direct introduction of promoters of interest into them. This was later overcome by removing the native *Aat*II site from pRS303 and pRS306 by digesting the plasmids with AatII, blunting the digestion with Klenow fragment, and ligating the blunt ends back together. These modified pRS integration vectors were designated pRS303d and pRS306d for the purposes of this investigation.

Luciferase Reporter Stability Assay

Cultures of strain SEY 6210 (*MAT***α** *leu2 ura3 his3 trpl lys2 suc2*) [70] containing either the pRS315-P_{GAL1}-Luc(A4V) or pRS315-P_{GAL1}-Luc(A4V)PEST plasmids/reporters were inoculated in 30 ml of supplemented minimal media lacking leucine and containing raffinose and luciferin [6.5 g/L Difco yeast nitrogen base w/o amino acids, 20 g/L raffinose, 15.6 mg/L uracil, 15.6 mg/L tryptophan, 15.6 mg/L histidine, 15.6 mg/L adenine, 23.6 mg/L lysine, and 50 μM beetle luciferin (potassium salt; Promega E1602)].

They were grown at 28°C with constant agitation for the duration of this experiment. When cultures reached OD_{600} 0.8, they were subdivided into 5 ml aliquots in scintillation vials. At time minus 60 minutes, luciferase biosynthesis (under the control of the *GAL1* promoter) was induced with 0.25 ml of 40% galactose. Luminescence measurements were taken with a Zylux Femtomaster FB12 luminometer every 20 minutes until time 0. At time 0, luciferase biosynthesis was repressed by the addition of 0.4 ml of a 25% glucose and 125 µg/ml cycloheximide mixture. Bioluminescent measurements were taken periodically for two hours after repression. Of the 6 vials for each reporter, two vials were used as controls: one was never induced with galactose and the other was never repressed with glucose/cycloheximide. The remaining 4 replicates were both induced and repressed.

Cell Cycle Synchronization of LHY3865

Strain LHY3865 (*MAT***a** *ura3 leu2 bar1*) (kindly provided by Dr. Linda Breeden, U. of Washington) was stably transformed with pRS303Kan-P_{POL1}-Luc(A4V)PEST by homologous recombination of the entire linearized plasmid (*Nhe*I digestion) into the genomic *HIS3* locus. From a dense overnight culture in YPD, 250 µl was added to 100 ml of YPD (initial OD_{600} = 0.05). The culture was grown at 28°C with agitation for 4.5 h (until OD_{600} = 0.18). 10 ml of this culture was moved to a 50 ml flask and treated with 15 µM α-factor (BioChemika 63591) and incubated for 3 h at 28°C with agitation. The remaining culture that was not treated with α-factor was centrifuged at 2000 rpm for 15 min at room temperature in a Beckman TJ-6 centrifuge. 50 ml of the supernatant was collected and treated with 15 µg/ml of proteinase E (Sigma P-6911) and 50 µM beetle luciferin (potassium salt; Promega E1602) to be used as conditioned media. α-factor treated cells were collected and washed three times with 10 ml of conditioned media. The final washed pellet was resuspended in 10 ml of conditioned media and moved to a

50 ml flask. The synchronized culture was incubated with stirring on a magnetic stir-plate at 28°C in a dark-box. Bioluminescence from the luciferase reporter was monitored continuously by a Hamamatsu HC135-01 photomultiplier positioned 1 inch from the culture. 100 µl samples were collected from the culture and frozen every 10 min for 3 h to determine changes in bud index over time.

Respiratory Oscillations and Monitoring Luminescence

The continuous culture apparatus consisted of a 3L New Brunswick Scientific Bioflow 110 reactor equipped with two Rushton type impellers operated at 550 rpm agitation, 900 ml/min air flow, 30° C, pH 3.4 (by 2N NaOH).

Dense 20 ml starter cultures of stably transformed luminescent CEN.PK113-7D (*MAT***a**) (provided by Dr. Peter Kötter, U. of Frankfurt) were grown in YPD overnight and inoculated into 850 ml of media consisting of 10 g/L anhydrous glucose (Sigma G71528), 5 g/L ammonium sulfate (Sigma A2939), 0.5 g/L magnesium sulfate heptahydrate (Sigma M2773), 1 g/L Yeast Extract (Becton Dickinson 288620), 2 g/L potassium phosphate (Sigma P5379), 0.5 ml/L of 70% v/v sulfuric acid, 0.5 ml/L of antifoam A (Sigma 10794), 0.5 ml/L 250 mM calcium chloride, and 0.5 ml/L mineral solution A. Mineral solution A consisted of 40 g/L $FeSO_4$• 7 H_2O, 20 g/L $ZnSO_4$• 7 H_2O, 10 g/L $CuSO_4$• 5 H_2O, 2 g/L MnCl2 • 4 H_2O, 20 ml/ L 75% sulfuric acid.

Oscillations were obtained as described by Tu, *et al*., 2005 [16]. Briefly, cells were grown in the Bioflow 110 in batch culture until they consumed the limiting media component and DO returned to ~95%-100%. The dense culture was then starved for 4-12 h before beginning continuous culture with the media described above at a dilution rate of 0.08-0.09/h.

Luminescence was monitored as follows. Once oscillations formed, 5 µM beetle luciferin potassium salt (Gold Biotechnology LUCK-300) was injected into the culture

during the hypoxic phase of an oscillation, and then maintained in the culture at this concentration either by adding luciferin to the feeding media or by constantly supplying a steady drip of a chilled, concentrated luciferin stock (0.66 mM) using a Harvard Apparatus syringe pump. The latter method proved to show more stable luminescence for experiments lasting several days as luciferin tended to degrade over time in the acidic media. Luminescence from the Bioflow 110 was constantly observed and recorded by running a closed loop of culture in transparent tubing from the Bioflow 110 into a dark box in front of a Hamamatsu HC135-01 photomultiplier, and then back into the culture vessel with a circuit time of about 30 s (fig. 2.12).

Extract Preparation and Immunoblot

From an oscillating culture of CEN.PK113-7D stably transformed with pRS303dKan-P_{ACT1}-Luc(A4V), 15 ml samples were collected from the Bioflow 110 effluent at either 15 or 30 min intervals across one oscillation (12 samples total). Each collection was immediately centrifuged at 2000 rpm for 5 min at 4°C in a Beckman Coulter Allegra™ X-22R centrifuge to pellet the cells. The supernatant was discarded and the pellet was flash-frozen on a mixture of dry ice and ethanol, and then transferred to -80°C for long-term storage. All samples were simultaneously thawed on ice and washed at 4°C in 4 ml TMG (10 mM Tris-Cl pH 8, 1 mM $MgCl_2$, 10% glycerol, 200 mM NaCl, 0.1 mM DTT). The cells were resuspended in 1 ml of cold TMG + proteinase inhibitor cocktail (1 tablet per 7.5 ml, Complete Mini EDTA-free protease inhibitor cocktail, Roche 04693159001). 300 µl of yeast resuspension from each sample was mixed with an approximately equal volume of glass beads (Sigma G8772) in 1.5 ml screw-cap tubes. The twelve yeast samples were simultaneously lysed on a MP Bio FastPrep-24 by three applications of 4.0 m/s agitation for 30 sec with 5 min on ice between each agitation cycle. Approximately 90% lysis was confirmed by microscopic

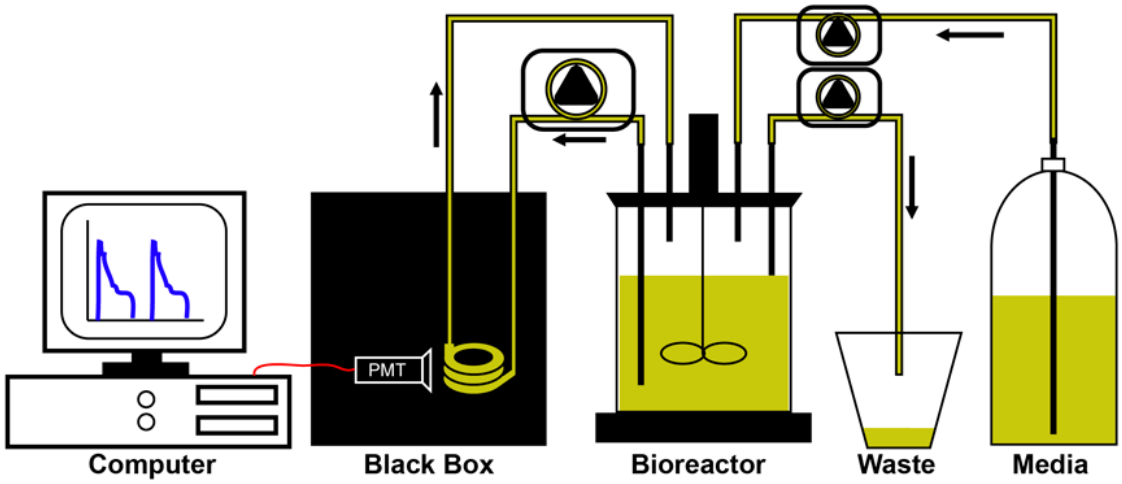

Figure 2.12. Configuration of apparatus for measuring luminescence continuously over the YRO. Arrows show direction of media flow from peristaltic pumps (triangles in circles). Media was gradually fed to the Bioflow 110 (bioreactor) (at ~2L/day) while an equal volume of culture (media + cells) was removed at the same rate. A separate, faster pump continually moved a stream of culture from the reactor and into a black box where it was monitored by a photomultiplier apparatus before returning to the bioreactor. The circuit time from bioreactor to black box and back was ~30 s. Luminescence was continuously recorded by a computer.

inspection. The lysate was separated from the beads by piercing the bottom of each tube with a 26.5 gauge needle, inserting each tube into a clean microcentrifuge tube, and centrifuging them at 10,000 rpm for 20 seconds at 4°C. The lysate was centrifuged at top speed at 4°C for an additional 30 min and the supernatant containing the soluble protein was aliquoted into clean tubes.

The protein concentration from each sample was quantified by Bio-Rad protein assay (BioRad 500-0006). 10 µg of total protein from each sample was mixed with SDS PAGE loading buffer (with DTT), boiled for 10 m, and loaded into the wells of 10% acrylamide gels for SDS-PAGE. A luciferase control of 20 ng of purified firefly luciferase (Sigma L1792) was loaded in one lane of the gel. Protein from gels was transferred to nitrocellulose membranes for immunoblotting. Membranes were blocked in 0.2% I-Block™ (Applied Biosystems T2015) in PBST for 1 h at room temperature, then incubated overnight in primary antibody at 4°C. Membranes were washed 4 times for 15 min in PBST, incubated in secondary antibody for 2 h at room temperature, and then washed again as before. Membranes were treated with HRP substrate (Pierce 32106) for 3.5 min and exposed to X-ray film for various exposure durations. The antibodies and concentrations used are as follows: 1:2000 polyclonal anti-luciferase (Sigma L0159) was detected with 1:4000 donkey anti-rabbit IgG HRP conjugated secondary antibody (Promega W401B). Polyclonal anti-yeast actin at 1:2000 (Santa Cruz 1615) was detected with 1:10,000 donkey anti-goat HRP conjugated secondary antibody (Santa Cruz 2020).

Phase Response Curves

An oscillating culture of CEN.PK113-7D stably transformed with pRS303dKan-P_{POL1}-Luc(A4V)PEST was allowed to establish a stable period and monitored for at least 4 oscillations before injection of acetaldehyde or ethanol. 850 µl of 1M acetaldehyde

(Sigma 00070) or 1M ethanol (~1 mM final concentration) were injected into the Bioflow 110 at phase points 0°, 45°, 90°, 135°, 180°, 225°, 270°, and 315° allowing at least 3 oscillations between injections. Phase 0° was defined as the time when dissolved oxygen started to rise from hypoxia. Phase shifts were determined from measuring the difference between (1) the time of the DO trough in the cycle after the injection (ignoring the immediate hypoxic response from the injection) and (2) the time of the DO trough in the extrapolated control (i.e., where the DO trough was projected to occur in the absence of an injection based on the 3 cycles prior to injection, see Fig. 2.9).

Acknowledgements

We dedicate this paper to the memory of Dr. Robert Klevecz, a scientific pioneer in the study of the YRO. We are grateful to Dr. Kathy Friedman for use of reagents and equipment and to Drs. Robert Klevecz and Caroline Li for helping us to configure our culture apparatus so as to achieve reproducible respiratory oscillations. We thank Dr. Peter Kötter for the CEN.PK113-7D strain and Dr. Linda Breeden for the LHY3865 strain. This research was supported by a grant from the National Institute of General Medical Sciences (NIGMS R01 067152) to CHJ.

CHAPTER III

TESTING A TWO-POPULATION MODEL FOR THE PARTICIPATION OF ALTERNATING CELL DIVISION CYCLES IN THE YEAST RESPIRATORY OSCILLATION

Introduction

The yeast respiratory oscillation (YRO) is a robust biological rhythm that manifests in some strains of *S. cerevisiae* under a narrow range of aerobic, glucose-limited conditions of continuous culture. For the strain CEN.PK, using conditions outlined previously [16, 74], the YRO has a stable period ranging from 3-5 h that largely depends on dilution rate and other conditions (e.g. pH) of the continuous culture [39, 75]. During this oscillation, yeast alternate between phases of respiro-fermentative metabolism and respiration, causing oscillations of intracellular and extracellular levels of metabolites (e.g. O_2, CO_2, ethanol, NADH) as well as transcription of over half of the genome [16, 18]. A correlation between the cell division cycle (CDC) and the yeast respiratory oscillation (YRO) has previously been shown where about 50% of the cells in the culture synchronously progress through the CDC during each cycle of the YRO [16, 74]. However, the importance of this relationship to the persistence of the rhythm and the nature by which it exists are still unknown.

There are three possibilities regarding the relationship between the CDC and the YRO. The first is that the relationship is merely a coincidence. This is highly unlikely for the strain CEN.PK since the association between the CDC and YRO is very reproducible. Moreover, treatments that reset the YRO's phase also result in a wave of

newly dividing cells at the same time [74], supporting the existence of a non-coincidental linkage between the CDC and YRO.

The second possibility regarding this relationship is that it is one of oscillator and output, where one phenomenon is (or is part of) an endogenous oscillator (capable of maintaining a self-sustained, stable oscillation) and the other is merely an output of the oscillator. For example, some researchers have proposed that the YRO could be a part of a metabolic timer, responsible for rhythmic events seen during continuous culture, and synchronous cell division could be a mere output of this oscillator [16, 36, 76]. The reverse example is also possible; where the CDC is the core biological oscillation and the 3 to 5 hour YRO seen in CEN.PK is merely a reflection (or perception) of CDC-related metabolic preferences in continuous culture [35]. Either of these relationships between oscillator and output is possible, but both imply that there is little to no feedback between the two phenomena.

A third (and more likely) possibility is that the relationship between the CDC and YRO is one of interdependency. This scenario is similar to two coupled oscillators where the CDC and YRO are both legitimate biological oscillators; but on their own, each is weak and quickly dampens. However, separate outputs from the rhythmic CDC and YRO could influence the other such that the coupling provides reinforcement which strengthens the overall oscillation of the union. Observations described in chapter II using cell cycle regulated luciferase reporters to track cell division following YRO perturbation are consistent with such an interdependent relationship [74].

In this work, we used firefly luciferase under the control of cell cycle regulated promoters (for various phases of the cell cycle), to demonstrate cell cycle landmarks within the YRO, namely the G1/S and M/G1 boundaries. As a result, cells in G1 could be distinguished from those that were actively dividing [74]. The luminescent CDC reporters also revealed a direct correlation between phase resetting of the YRO by

artificially delivered metabolites (e.g. ethanol and acetaldehyde) and premature induction of new cell division in a YRO-phase-dependent manner. These results demonstrated an interrelationship between the YRO and CDC. The observations from this previous study can most easily be envisioned as two distinct populations of cells (each comprising approximately half of the cells in the continuous culture) dividing 180° out of phase with each other such that the overlapping of these long, alternating CDCs provides the timing mechanism for the oscillations observed, and the YRO (with a period of one half of the CDC period) provides synchronizing signals that keep the CDC in phase (Fig. 2.11)

We took the approach of first modeling the data in a simple way, because there was no need to put forward a complex model if a simple one would do as well. In this chapter, we further investigate the relationship between the CDC and YRO by testing aspects of the alternating two-population model under a more targeted study. We used a luminescent CDC reporter to track a "population" of cells under oscillating continuous culture, and used a conditional knockout of a CDC-dependent gene to observe the effects of the YRO when the CDC is removed from (or added to) the oscillation/culture. The results from these experiments show that for CEN.PK under the conditions tested, the two-alternating population model was too simple to fully explain the complexity of the YRO/CDC relationship but that the long period and large amplitude of CEN.PK's respiratory oscillation is dependent on the CDC, and quickly dampens without it.

Results and Discussion

The YRO Period is pH-dependent

The existence of two "discrete" populations of cells can be tested by looking for examples of YROs where the dilution rate remains the same, but the YRO's period stably changes to something other than roughly half of the period of the average cell

division cycle. In such a way, the “strength” of the separation between (or attractors within) the populations can be tested. The pH of the continuous culture has been reported to alter the YRO period at constant dilution rates in some cases [75] and I therefore tested under our continuous culture conditions whether the period of the YRO was a function of the extracellular pH.

An oscillating culture was established with a dilution rate of ~0.095/h at a pH of 3.4. The average cell division time for this dilution rate was about 7.3 hours (7 h and 18 min) and the YRO had a period of 3.26 hours (3h and15 min), i.e. within 23 min of being half of the cell division time (Fig. 3.1). When the pH of the culture was raised by perfusion of NaOH, the period immediately lengthened, even though the dilution rate and optical density (OD_{600}) of the culture remained unchanged (and therefore the cell cycle time was presumably unaffected). A pH of 4.3 resulted in YRO periods of about 4 hours, and a pH of 5 produced periods close to 5 hours (Fig. 3.1). Increasing the pH to 6 destroyed the oscillation.

These results show that the YRO period does not have to fall close to half of the average cell division time, and that there may be a range of permissible YRO periods that can maintain the oscillation. Environmental conditions aside from nutrition can influence the YRO’s period despite the stabilizing effect that a constant dilution rate has on cell division time, suggesting that the cell division rate alone does not provide the timing mechanism for the YRO. This does not disprove the existence of multiple populations signaling to each other through respiratory metabolites; however it does rule out the existence of two “distinct” (equal) populations of dividing cells where cells in one population (and their daughter cells) always remain with that group of dividing cells. In other words, a model where synchronously dividing groups of cells signal via the YRO to each other could still be valid as long as those cells (and/or their offspring) whose division times did not conform to the rhythm of the YRO-related signals could migrate

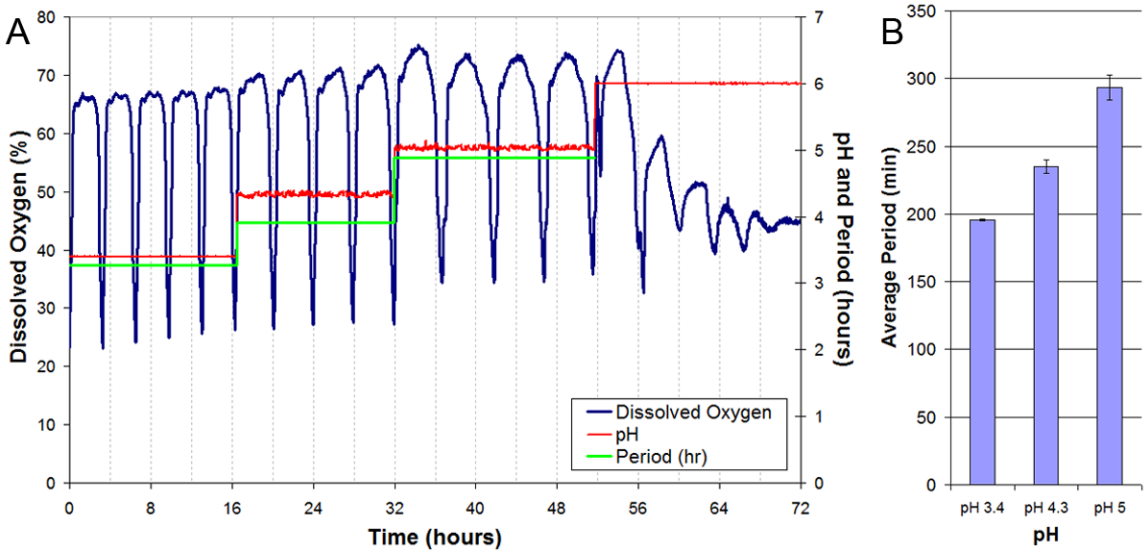

Figure 3.1. The YRO period is affected by pH. **A.** The YRO is shown by the oscillating dissolved oxygen levels (blue) and its period at the given time is shown in hours by the green line. As pH is raised in a stepwise manner (red line), the period of the YRO lengthens in response and remains stable for the duration of the treatment. At pH 6, the YRO destabilizes. **B.** Average periods (in minutes) for oscillations at different pH. Error bars are ± 1 S.D.

from one population to another as needed in order to participate in the next oscillation. The boundaries that separate the populations are therefore rather weak under certain circumstances and cells can move between populations when factors distort the CDC/YRO relationship.

The Population Structure of the YRO is More Complex than Simple Alternating Populations of Dividing Cells

If the YRO is comprised of groups of cells that synchronously divide as a result of a YRO-related signal (that occurs during the DO trough), but cells can move between groups when their cell cycle times do not conform with the timing of the YRO signal, then the population structure of the YRO may be more complicated than alternating populations of dividing cells. We used cell-cycle arrested CEN.PK with a CDC-regulated luminescent reporter to follow what became of cells that entered the YRO at a similar phase by injecting them into an oscillating culture of non-luminescent CEN.PK. Therefore, the luminescent cells become a "reporter" for the behavior of dividing, non-luminescent yeast in the bioreactor. Do such cells wait for a YRO-related signal before synchronously dividing? Do they assimilate into the YRO by dividing every other oscillation of the YRO as would be predicted by the alternating-population model proposed previously? If the CDC occurs once for every two YROs, then injecting a synchronized set of cells into the oscillating continuous culture should result in the injected "tracer-cells" synchronously dividing every other YRO (see Fig. 3.2A for experimental design and prediction). On the other hand, the population structure within the bioreactor may be more complex than modeled, and cell size differences may play a larger role in determining cell cycle duration than previously envisioned. Some larger

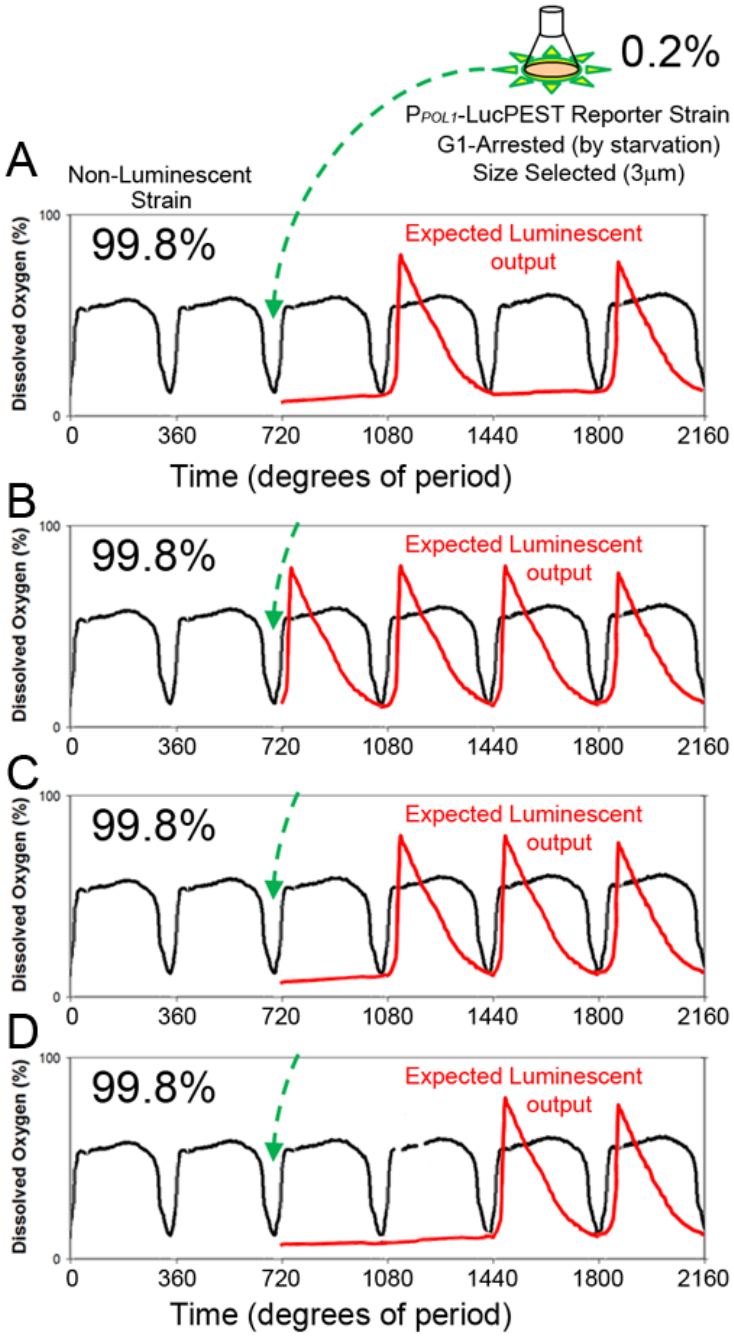
0.2%
PPOL1-LucPEST Reporter Strain
G1-Arrested (by starvation)
Size Selected (3µm)
A
Non-Luminescent Strain
99.8%
Expected Luminescent output
Dissolved Oxygen (%)
100
0
0
360
720
1080
1440
1800
2160
Time (degrees of period)
B
99.8%
Expected Luminescent output
C
99.8%
Expected Luminescent output
D
99.8%
Expected Luminescent output
Time (degrees of period)

Figure 3.2

Figure 3.2. Experimental design and predictions of injecting a luminescent cell cycle reporter strain that has been arrested in G1 into a continuous culture of a non-luminescent strain exhibiting the YRO. A batch culture of CEN.PK113-7D containing P_{POL1}-Luc(A4V)PEST was arrested in G1 by carbon-limited growth for 36 hours (represented in the figure by the flask), and was either used directly or size-selected for 3 µm cells, depending on the experiment. These arrested, luminescent cells (comprising about 0.2% of the number of cells in the continuous culture) were injected at phase point 720° (green dashed line) into a continuous culture of non-luminescent CEN.PK113-7D undergoing the YRO (black oscillating trace). The rationale of this experiment is that the injected, arrested cells should assimilate into that sub-population of the continuous culture population for which their cell cycle state was most similar and begin dividing in time with that oscillating sub-population. **A.** Prediction: if the "alternating population" model is true, and cells within each of the two sub-populations together undergo synchronous cell division every other YRO period, then the cell-cycle-dependent luminescence should stay low for the period in which the cells are injected and should peak every other YRO period following that. The luminescence prediction is shown by the red trace. Alternative outcomes are also possible (B-D). **B.** The predicted result (in red) if the arrested sample is comprised of cells that divide every oscillation of the YRO and begin to divide right after injection. **C.** The predicted result (in red) if the arrested sample is comprised of cells that divide every oscillation of the YRO but wait for the presence of a signal that occurs during the trough of the YRO before beginning cell division. **D.** A similar prediction as C. if the cells delay entry into the cell division cycle for two YRO periods. However, other alternatives are also possible, such as cells delaying entry into the cell cycle for 3 or more YRO periods.

cells may divide every YRO while other smaller cells may require two or more YRO periods to complete cell division. Fig. 3.2B-D shows alternative expectations from luminescent “tracer cells” if some cells in the bioreactor can divide every YRO period rather than every other one.

We grew a batch culture of CEN.PK113-7D containing the destabilized luciferase reporter under the control of the *POL1* promoter which is maximally expressed during late G1 and early S phase of the cell cycle [62, 74]. This culture was grown to stationary phase in similar media to that for which oscillating yeast in continuous culture are grown (at this time, ~95% of the cells were arrested in G1). At the same time, a stable YRO was established in a continuous culture of CEN.PK113-7D (without the luminescent reporter) (Fig 3.3A). A sample of the cell cycle arrested luminescent yeast comprising about 1/600 of the cell density of the non-luminescent oscillating culture was injected into the continuous culture at a phase point shortly after the DO trough (Figs. 3.2 and 3.3C). A low ratio of arrested, luminescent cells (~0.2%) to oscillating, non-luminescent cells (~99.8%) was used so that the injected cells would not perturb the oscillation but would still be bright enough to report the CDC timing of the population into which they assimilated. The experimental design and expected result (according to the alternating population model) is shown in Fig. 3.2A. If the starved, luminescent cells were G1-arrested/prolonged similarly to the carbon-limited G1-arrested/prolonged cells awaiting the YRO-related signal for synchronous progression into the CDC, then the injected cells should serve as tracers for the oscillating CDC population into which they were injected.

After injecting the luminescent cells, a small rise in luminescence was observed due to the background level of luciferase in the injected cells (Fig.3.3C, blue trace). After about 90° (about 1 hour) into the period following the injection, a rapid increase in *POL1* expression was observed, indicating that the arrested cells were passing Start and beginning cell division. However, a noticeable 2-fold jump in *POL1* expression was

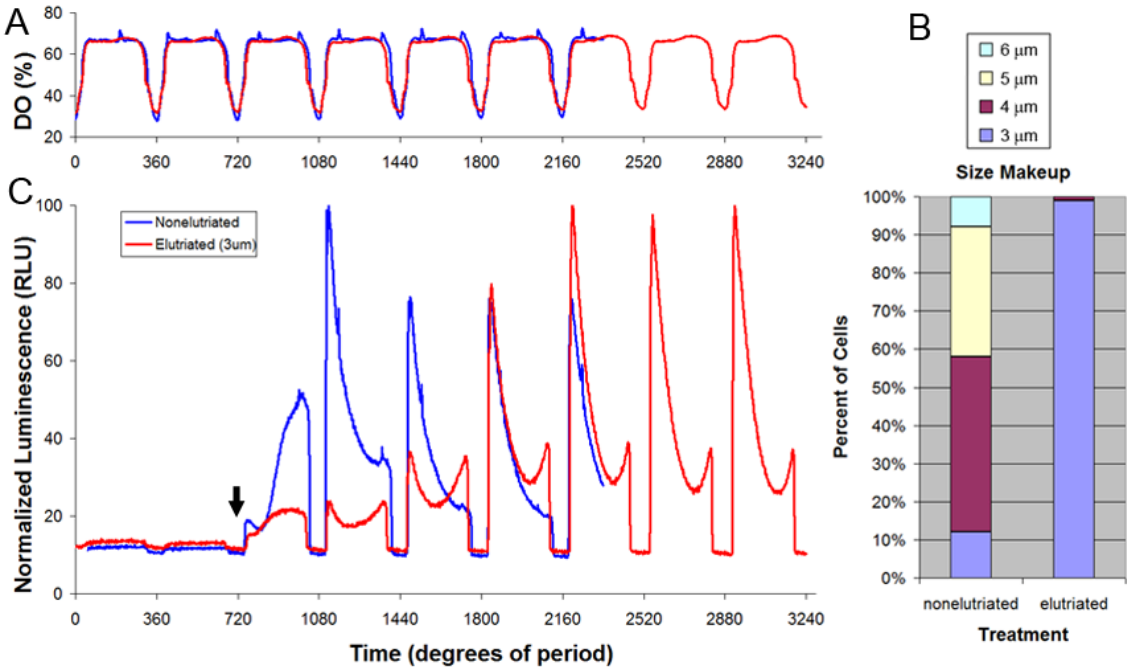

Figure 3.3. Injection of a luminescent cell cycle reporter strain that has been arrested in G1 by starvation into continuous culture of a non-luminescent strain exhibiting the YRO. The results from two experiments are shown. Traces in blue show results for the experiment where injected cells were not size-selected (non-elutriated). Traces in red show results for the experiment where injected cells were size-selected for 3 µm by centrifugal elutriation. **A.** shows the oscillating dissolved oxygen concentrations for the experiments. The x-axis is time in degrees of period where the YRO periods from both experiments were normalized to 360°. The y-axis is DO in percent atmospheric saturation of culture media. **B.** shows the size makeup of the injected cells from each experiment. Samples of 100 cells from each group were microscopically scored for size to the nearest µm. **C.** shows the normalized luminescence from the P_{POL1}-Luc(A4V)PEST reporter for both non-elutriated and elutriated experiments. Cells were injected near 720° at the black arrow. The x-axis is time in degrees of period and aligns with the time scale of panel A. The y-axis is normalized luminescence in relative light units (RLU), where the maximum luminescence for the recorded duration was scaled to 100 RLU. For the non-elutriated experiment (blue), the maximum luminescence occurred at 1122°, and for the elutriated experiment (red) it occurred at 2204°. 360° = 232 min for non-elutriated (blue) experiment, and 360° = 260 min for the 3 µm elutriated (red) experiment.

observed following the first DO trough when the suspected YRO-related cell cycle signal occurred. Interestingly, the CDC-regulated luminescent signal did not skip any of the following respiratory oscillations as was predicted by the alternating population model. Rather, the luminescent signal for the remaining oscillations showed a stable, intermediate amplitude with the characteristic shape of the P_{POL1}-Luc(A4V)PEST reporter.

These results only partially supported the alternating population model in that a portion of the injected cells waited until the YRO trough occurred before beginning to divide (a mix between the alternative predictions shown in Fig 3.2B and C), which provides evidence that there is a YRO related signal that affects cell cycle timing. The remainder of these results did not support the model. It is likely that the substantial increase in *POL1* activity soon after injection (prior to the DO trough) was the result of a small portion (estimated to be ~5% from microscopic observation prior to injection) of the sample that was not arrested, or was due to the largest cells from the injected sample that were ready to undergo cell division prior to arrest and therefore they did not wait for a "signal" to proceed with division. The size makeup of the injected cells ranged from 3 - 6 μm (Fig. 3.3B). It is possible that the size of the arrested cells played a substantial role in determining the G1 duration for oscillating cells. In particular, the fact that no oscillations were skipped (aside from perhaps the first cycle) implies that once cells have attained a size large enough for cell division, they are capable of dividing more rapidly than every other period of the YRO (i.e. faster than the average cell division time for the culture). If so, the average cell division time of roughly 2 YRO periods could be explained by the larger cells dividing every YRO period (having a very short G1) while the smaller cells (e.g. daughters) require multiple (2+) YRO periods to attain the necessary size for division (thus having a very long G1).

To determine the extent to which cell size affected G1 duration during the YRO and whether the initial increase in *POL1* regulated luminescence was the result of non-arrested verses larger arrested cells, a similar experiment as above was performed except that the arrested, luminescent sample was size selected for 3 μm cells (the smallest of the arrested population) by centrifugal elutriation prior to injection into the continuous culture. The arrested population from elutriation was quite uniform in size with 99% of cells being about 3 μm and all were arrested (no buds) (Fig. 3.3B). If the small cell size of the arrested population affected the length of time it took for those cells to reach a cell-division-capable state, then a luminescence trace similar to the one predicted in Fig. 3.2D should have resulted. As seen previously, a small increase in luminescence was observed following injection as a result of background luciferase in the cells (Fig. 3.3C, red trace). Only a very slight increase in *POL1* expression was observed afterward until two YRO periods had passed; after which time, luminescence from *POL1* expression nearly doubled (at phase 1440° in Fig. 3.3C), and then more than doubled again following the next DO trough/signal (at phase 1800°) (similar to the predicted result shown in Fig 3.2D). Therefore overall cell division was obviously delayed as a result of the small cell size (compared to the experiment where small cells were not explicitly selected). Moreover, there was no alternating pattern of luminescence amplitude (high in one YRO, low in the next YRO, high in the next, and so on) that was predicted from the alternating population model, thus supporting the interpretation above that once cells reached a cell-division-capable size, they were able to divide more frequently than every other YRO. The progressive increase of the peak amplitude of *POL1* luminescence in successive YRO periods after cell division began (until equilibrium was reached) also supports this interpretation.

One assumption of the alternating population model is that the poor nutritional quality of the continuous culture medium (or a secreted YRO-related cell division

delaying factor) plays a more influential role in governing G1 duration than cell size. Therefore the assumption is that insufficient nutrition (or the unknown factor) delays larger cells in G1 long enough for smaller cells of the same population to catch up in size before the YRO permits that group of cells to pass Start synchronously. Under such a model, the long G1 durations due to slow growth on limited carbon would make mother-daughter size differences negligible with regard to synchronous division during the YRO. From the evidence shown here, this former assumption was incorrect and cell size influences CDC periods during the YRO. However, if a large enough portion of the culture still divided in phase with the YRO, then (1) whether the dividing cell population is solely composed of the larger cells dividing every YRO, or (2) whether the dividing cell population also includes smaller cells dividing over the course of multiple YROs, the CDC could still provide feedback to influence the YRO's stability and characteristics.

The Cell Division Cycle Influences the YRO's Period and Amplitude

Numerous examples exist from many labs that show when yeast are grown under conditions that do not exhibit the YRO, initially synchronized CDCs rapidly become arrhythmic in the populations due to unequal CDC periods of larger and smaller cells [77-79]. The synchronizing effect of the YRO on the CDC (under continuous culture) has thus far been shown using microscopy, FACS, and cell cycle regulated luminescent reporters [16, 74, 76]. Studying the effect of the inverse relationship, i.e. that of the CDC on the YRO, is more challenging. Cell cycle null mutations are not viable or only permit cells with the mutation to survive/grow in size. Thus the division/growth required to generate the YRO in CDC-null strains are absent from the beginning. Previous studies have made some headway toward this goal by observing the YROs exhibited by strains that have viable mutations that modulate but do not halt the cell cycle [76, 80]. For many of these mutations, the YRO's period was shorter than

wild type by about half; however, it is difficult to determine the extent to which these mutations also affected the initiation of the YRO during startup, or the extent to which they are pleiotrophic. An ideal tool for pursuing this relationship would be a CDC-specific mutation that could be used to inactivate one essential CDC gene while the cells are displaying a stable YRO. The CRE/lox system for conditionally inducing genetic recombination during culture is one such approach.

The *CDC28* gene is essential to the cell cycle because its product is required for a cell to progress pass Start during G1 [81] as well as to progress from G2 to M phase [82]. Deletion of the gene prevents cell division, therefore a null mutation in *CDC28* results in a CDC-arrested strain [83]. Moreover, temperature sensitive (conditional) mutants of *CDC28* have been well characterized. They arrest cell division in G1 or G2 at a restrictive temperature and become large and oddly shaped the longer they remain at the restrictive temperature [81, 84, 85]. We generated a conditional *CDC28*-null strain of CEN.PK in which *CDC28* was flanked by loxP sites (floxed), and CRE recombinase (which mediates recombination between the loxP sites) was introduced under the control of the glucose-repressible/galactose-inducible *GAL1* promoter. When glucose is present in the media, the *GAL1* promoter is repressed and CRE recombinase is not expressed. However, the absence of glucose and the presence of galactose activate the *GAL1* promoter, expressing CRE. The recombinase then removes the *CDC28* gene between the loxP sites leaving a single loxP site in its place. We tested our floxed *CDC28* strain on glucose and galactose media, confirming that our conditional null strain could not grow on solid galactose medium (Fig. 3.4A) and would become elongated and oddly shaped on transition to liquid galactose medium(Fig. 3.4B). An isogenic strain that possessed wild-type *CDC28* in addition to the floxed version grew well on solid galactose medium and retained normal appearance in liquid galactose medium. Some of the cells of the floxed *CDC28* strain however did exhibit the odd morphology when

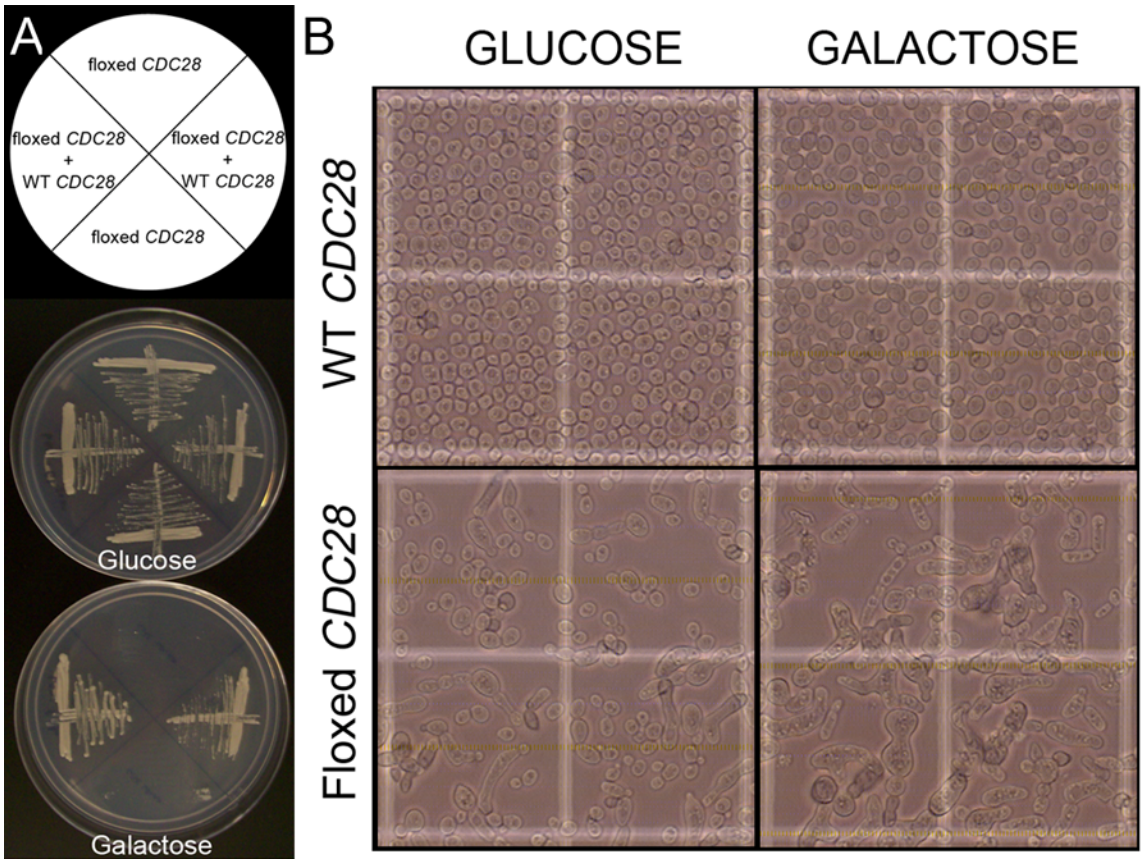

Figure 3.4 Floxed *CDC28* strain of CEN.PK113-7D and a rescued isogenic strain on glucose or galactose media. **A.** The floxed *CDC28* strain with CRE recombinase under the control of the *GAL1* promoter grew well on solid glucose media but did not grow on solid galactose media. Moreover, an isogenic strain with a wild type (i.e. non-floxed) *CDC28* gene in addition to the floxed *CDC28* gene and P_{GAL1}-CRE grew on both glucose and galactose media. **B.** Cellular morphology differences for two of the strains grown to stationary phase in glucose and galactose liquid media. Each panel of B. shows four 0.04 mm^2 sections of a hemocytometer (delineated by white horizontal and vertical lines) viewed under a 100X objective. Yellow horizontal bands across the panels are artifacts of the camera's settings. The strain containing WT *CDC28* had normal cell morphology in glucose and galactose media. However the strain with only floxed *CDC28* had elongated, oddly shaped cells in both glucose and galactose media. The odd morphology was more pronounced in cells grown in galactose, while only some (~35%) of the cells grown in glucose exhibited elongation and odd shape.

grown to stationary phase on glucose medium (Fig 3.4B). This background was likely the result of a leaky *GAL1* promoter that became de-repressed once the glucose in the media had been consumed, thus allowing some level of CRE to be expressed and *CDC28* to be excised from some cells.

Before using the floxed *CDC28* strain in continuous culture, a control was performed using CEN.PK with luciferase under the control of the *GAL1* promoter to determine the extent to which the *GAL1* promoter could be induced by galactose during the YRO as well as whether the galactose treatment would adversely affect the YRO. A stable YRO was established with this luminescent strain and then a 30 min galactose treatment was delivered to the continuous culture by switching the medium from one with glucose to one that contained galactose (Fig 3.5). During the time of the galactose treatment, the DO of the culture increased since the cells were not conditioned to metabolize galactose at that time. This DO response was similar to starving the culture of glucose (data not shown). After the treatment, glucose medium was returned to the culture and the DO dropped, resulting in a slight phase shift of the oscillation, but the YRO forged onward. The galactose-induced luminescence began to increase shortly after galactose treatment, peaking during the hypoxic mask or the early part of the following oscillation and then sharply diminishing afterward. These results show that the *GAL1* promoter could be conditionally controlled during the YRO and that the YRO suffered no significant perturbation from the galactose treatment.

A similar experiment was initiated with the floxed *CDC28* strain; however, the complete consumption of glucose during batch phase prior to continuous culture apparently resulted in leaky expression of the *GAL1* promoter, premature induction of CRE recombinase, and removal of *CDC28* in some of the cells before continuous culture was even begun. As a result, the portion of the culture that was affected was not able to undergo cell division after continuous culture was initiated and the YRO was not able to

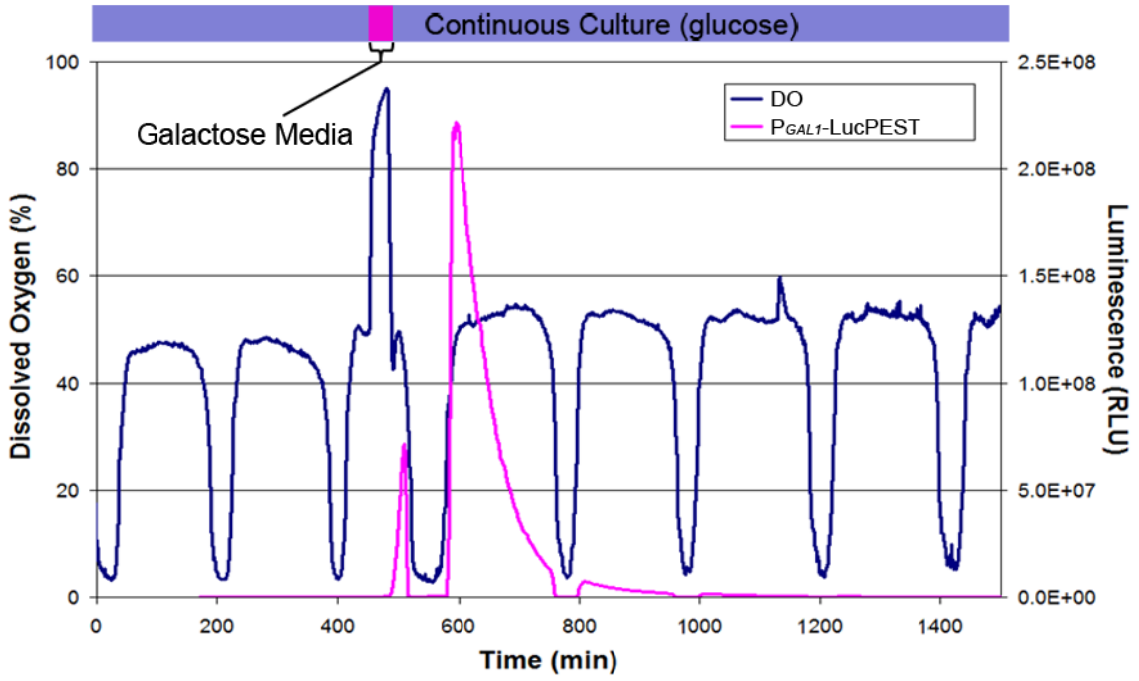

Figure 3.5 Luciferase under the control of the *GAL1* promoter showed maximum expression during the YRO following a temporary switch to galactose medium during continuous culture. This result demonstrated that a galactose treatment could be used during the YRO in continuous culture to manipulate the expression of a gene under the control of the *GAL1* promoter, and that such a treatment had no serious effect on the YRO's stability, period, or amplitude. The x-axis is time in minutes. The y-axis on the left is dissolved oxygen (DO) measured in percent atmospheric saturation of the culture (blue trace). The y-axis on the right is luminescence in relative light units (pink trace). The colored bar across the top shows the media treatment relative to the time scale.

form at the usual time (Fig. 3.6A). After about 24 h of continuous culture, enough of the non-dividing cells were washed out and replaced by newly formed cells (from those unaffected by the leaky promoter) so as to enable short period, low amplitude oscillations. As the culture continued to shift from non-dividing to dividing cells, the short period, low amplitude oscillations gradually transitioned to longer period, larger amplitude oscillations that were more characteristic of CEN.PK's normal YRO. This effect of premature CDC arrest as a result of a leaky *GAL1* promoter turned on by depletion of glucose from the medium was overcome in a subsequent experiment by growing the floxed *CDC28* strain in continuous culture from the beginning (i.e. circumventing the batch phase) (Fig. 3.6B). Under these conditions, the constant supply of glucose minimized premature recombination and the YRO formed soon after the culture reached a saturating density (Fig. 3.6B, ~1250 min).

After a stable YRO had formed in the floxed *CDC28* strain, galactose treatment was accomplished by switching the continuous culture medium from one with glucose to one containing galactose for 40 min (a similar protocol to that of the control in Fig. 3.5). As in the control in Fig. 3.5, the galactose treatment only caused a minor phase shift and had no acute effect on the stability of the YRO (Fig. 3.7, blue). The effects of the galactose treatment were not expected to be immediate since the genetic rearrangement leading to the removal of the *CDC28* gene requires up to 300 min (see Appendix A), and the endogenous level of CDC28p protein (which is reported to be stable and in excess [86]) would continue to permit cell division until it is degraded. About 24 hours after the treatment, the period of the YRO began to shorten and the amplitude began to decrease (Fig. 3.7, at single arrow). The period of the YRO eventually bifurcated into short period, low amplitude rhythms (at double arrow in Fig. 3.7) similar to those seen during the initial phases of YRO formation in Figure. 3.6A that ultimately damp to an undetectable level. The strain also contained an integrated luciferase reporter under the control of the

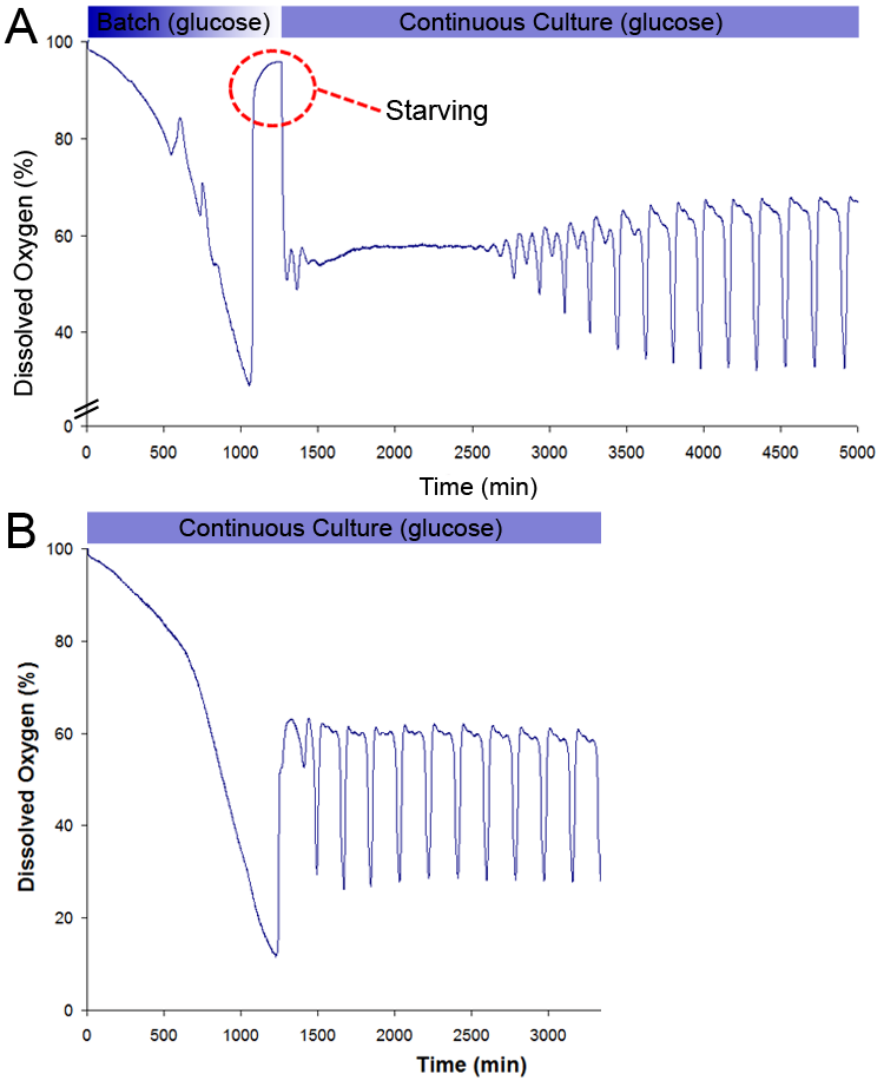

Figure 3.6 Establishment of the YRO for the floxed *CDC28* strain of CEN.PK113-7D. **A.** Under traditional start-up conditions for generating the YRO, where cells are grown in batch prior to continuous culture, the YRO required about 24 hours of continuous culture before oscillations formed. This was likely due to lack of glucose in the media (after it was consumed), causing a leaky *GAL1* promoter to prematurely express CRE recombinase and remove the *CDC28* gene from the growing cells. The lack of cell division prevented the formation of the YRO until enough of the arrested cells had been removed from the culture and replaced by those that could divide. Without sufficient cell division the initial YRO had a very low amplitude and short (80 min) period. **B.** When leaky expression of CRE was minimized in the floxed *CDC28* strain by a continuous supply of glucose (rather than growing in batch), the YRO formed almost immediately after culture saturation was reached and had both a typical period of about 3 h and large amplitude.

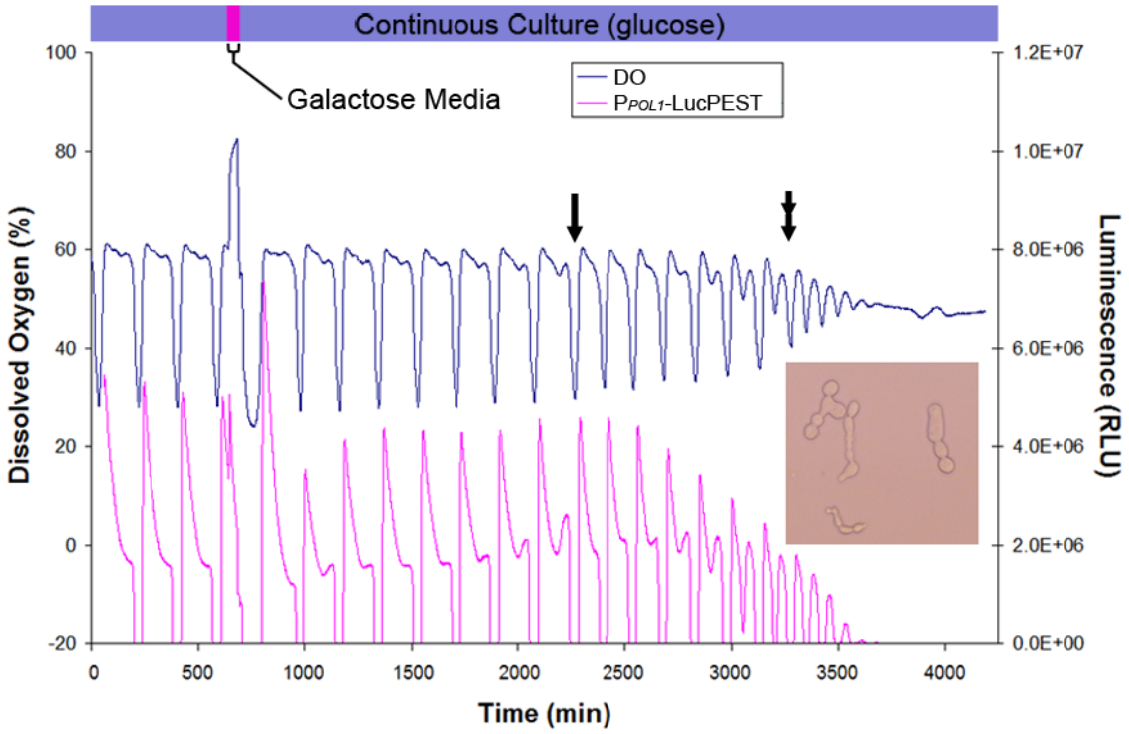

Figure 3.7 Transient galactose treatment during continuous culture induced the removal of *CDC28* and the resulting cell cycle arrest destabilized the YRO. The blue trace shows the oscillating dissolved oxygen concentration during the YRO. The pink trace shows luminescence from the integrated P_{POL1}-luc(A4V)PEST reporter, present in all cells of the culture. The x-axis is time in minutes. The y-axis on the left is dissolved oxygen in percent of atmospheric saturation, and the y-axis on the right is luminescence in relative light units. The colored bar at the top shows the media treatment relative to the time scale. The galactose treatment consisted of switching the continuous culture media source from one with glucose to one containing galactose for 40 min. During the treatment, DO increased because the cells were not conditioned to utilize galactose. Upon returning the culture to glucose, the DO dropped and resulted in a phase shift. About 24 h later, the period and amplitude of the YRO decreased (at single arrow) and eventually bifurcated into a high frequency oscillation (at double arrow) before damping out altogether. The *POL1* CDC-related luminescence showed that disruption of the CDC by *CDC28* deletion caused an unusual amount of cell division late in the oscillations after 2000 min that correlated with the YRO transition to a shorter period oscillation at the single arrow. Afterward, the total amount of luminescence (*POL1* activity) decreased steadily until becoming undetectable at the time when the YRO disappeared. The inset shows a culture sample taken after min 4000. The cell morphology is characteristic of *CDC28* disrupted cells.

CDC-regulated *POL1* promoter (P_{POL1}-Luc(A4V)PEST) used previously in Fig. 3.3. Except for the phases of hypoxia, luminescence reported the expression pattern of *POL1* prior to and following galactose treatment. The amplitude of the *POL1* luminescence rhythm in the culture began to decrease at about the same time that the period effects on the YRO became apparent (Fig. 3.7, pink trace at single arrow) and *POL1* promoter activity was undetectable when oscillations ceased. Microscopic observation of a culture sample taken after oscillations ceased revealed all cells had the CDC-arrested morphology shown previously in Fig. 3.4 (Fig. 3.7, inset). These cells remained metabolically active with regard to respiration, as could be ascertained by the level of DO remaining near the midpoint of the former oscillation (if the culture had stopped respiring, the DO would have increased to the maximum level). We conclude the cells were respiring, but they were not expressing discernable respiratory oscillations.

These experiments demonstrate that when the CDC is impaired, as at the beginning of the continuous culture in Fig. 3.5A and following the galactose treatment of Fig. 3.6, the YRO has a severely dampened amplitude and its period is much shorter. Although a non-cell cycle related effect to the YRO from the loss of *CDC28* cannot be ruled out, these observations may indicate that the YRO cannot operate without the reinforcing influences that cell cycles provide. However, when the arrested cells are replaced with those that can divide (Fig. 3.5A), the period and amplitude are strengthened in turn, demonstrating the importance of the CDC to the maintenance and characteristics of the YRO for CEN.PK.

Summary

We previously proposed a model describing the YRO/CDC relationship comprising two discrete populations of alternating, synchronously dividing cells that are

maintained by signals from rhythmic respiration/fermentation and cell cycle states (Chapter II and [74]). I tested predictions of this model to determine whether the YRO could be so simply maintained. I showed by manipulation of the medium pH that the YRO could achieve periods that differed from half of the average cell division time, ruling out the existence of discrete populations of cells that did not exchange between each other. Secondly, I showed that our previous hypothesis of alternating populations of cells was insufficient to explain data from luminescent "tracer" cells used to label groups of dividing cells in the YRO. Rather, it seems apparent that once cells achieve a sufficiently large size, they can divide more rapidly than once per two YRO periods (approximately the average cell division time for the culture). Such rapidly dividing cells must account for less than half of the culture density to achieve the calculated average cell division time as well as account for the ~50% culture cell division observed in the cultures during each YRO period (Fig. 2.5). Traffic between groups of dividing cells, in addition to a portion of the culture dividing every YRO period, could help explain the stability and uniformity that the YRO exhibits from oscillation to oscillation, and why perturbation to one oscillation does not have an "echo" in later oscillations. Finally, I showed that cell division plays a significant role in YRO maintenance in agreement with our model, and that the CDC is not merely an output of an endogenous oscillator, but rather feeds back upon the respiratory oscillation and affects its stability. As is the case for many biological phenomena, interrelationships among systems are often more complicated than initially believed.

A more complex model is required to explain the YRO and the relationship it has with the CDC. What is the specific population structure regarding cell cycle times within the culture? What is the signal/mechanism by which cells delay their progression through G1 so that they can then simultaneously begin cell division? How is the signal that results in the drop in DO during the YRO related to the cell cycle? Aside from

nutrition and pH, are there other environmental factors that affect the YRO's characteristics? I intend to actively pursue these questions so as to obtain a model that more accurately reflects the relationship between the YRO and CDC.

Materials and Methods

Yeast Strain and Respiratory Oscillations

The yeast strain used for all of these experiments was CEN.PK 113-7D (MAT**a**) kindly provided by Dr. Peter Kötter, University of Frankfurt, Frankfurt, Germany. Unless otherwise stated, respiratory oscillations were obtained as described previously using CEN.PK 113-7D with or without luminescent reporters described previously [74].

Elutriation of G1-Arrested Luminescent Yeast

A colony of CEN.PK113-7D that was stably transformed with the plasmid pRS303-Kan-P_{POL1}-Luc(A4V)PEST [74] was used to inoculate a 100 ml overnight culture in YPD media (1% yeast extract, 2% peptone, 2% dextrose) grown in a 500 ml flask at 28°C with agitation. This overnight culture was used to inoculate 5L of CEN.PK bioreactor media described previously [74] with the following changes: mineral solution A was added and the pH adjusted to 5 with NaOH prior to autoclaving, 50 μM ampicillin was added, and no antifoam was used. The 5L culture was grown at 28°C in an 8L double side arm spinner flask, bubbled with filtered air and magnetically stirred for 36 h. After 36 h, 1L of this culture (OD_{600} = 7.1) was removed and saved for elutriation. The remaining 4L was centrifuged in 250 ml bottles at 1000 r.p.m. for 5 min in a Sorval centrifuge. The supernatant was vacuumed filtered through a 0.2 μm membrane to make cell-free conditioned starvation media.

About 500 ml of the culture was size selected for the smallest cells (~3 μm) by centrifugal elutriation in a Beckman JE-5.0 rotor with a 40 ml chamber [87]. The cell-free conditioned starvation media was used to collect 500 ml (at a concentration of OD_{600} = 0.075) of small cells from the elutriation rotor. These cells were collected on the surface of a 0.2 μm membrane by vacuum filtration and resuspended in 1 ml of cell-free bioreactor media (bioreactor effluent from an oscillating culture of CEN.PK113-7D was collected, centrifuged, and the supernatant filtered through a 0.2 μm syringe filter). 5 μl were used to measure the optical density of the elutriated sample and 5 μl were used for microscopic determination of cell sizes in the sample. The 1 ml elutriated sample of luminescent yeast had an OD_{600} of 37.5 and was directly injected into an 850 ml culture of oscillating (non-bioluminescent) CEN.PK113-7D with an OD_{600} of 26 (the ratio of elutriated, luminescent cells to oscillating, non-luminescent cells was about 1:600). Luminescence was monitored in continuous culture as described previously [74].

Floxed CDC28 Strain Construction

The floxed *CDC28* strain was generated by introducing three constructs into a *ura3* strain of CEN.PK113-7D as described below. A stable *ura3* auxotrophic strain of CEN.PK113-7D was generated by selecting spontaneous *ura3* mutants on YPD media plates containing 1 mg/ml 5-FOA (zymo research). Stability of the mutation(s) was tested by growing cells without the selection pressure for several days and testing the inability to grow on supplemented minimal media plates lacking uracil (6.5 g/L Difco yeast nitrogen base w/o amino acids, 20 g/L glucose, 15.6 mg/L tryptophan, 15.6 mg/L histidine, 15.6 mg/L adenine, 23.6 mg/L lysine, and 23.6 mg/L leucine). One clone that showed a stable *ura3* phenotype was selected and designated CEN.PK113-7D/*ura3*, to which other constructs were added as described below.

The *CDC28* gene, including 300 bp of upstream intergenic DNA (containing the promoter), CDS, and 300 bp of downstream intergenic DNA (containing the terminator) was PCR amplified from genomic DNA (strain S288C) using a 5' primer that added a *Bgl*II site to the 5' end (gataagAGATCTATATATTTACAAGAAAAGCATGGC) and a 3' primer that added an *Xho*I site to the 3' end (ttcttgCTCGAGTTTGTCTTTTTCCTCTTCGCT). This PCR product was used to replace the *KanMX* gene of the loxP-KanMX-loxP cassette of pUG6 [88] at the *Bgl*II and *Xho*I sites. The portion of the pUG6/floxed *CDC28* construct containing the floxed *CDC28* gene was moved to the luminescent reporter plasmid pRS303-Kan-P_{POL1}-Luc(A4V)PEST [74] by digesting both plasmids with *Not*I, agarose gel isolating the fragments of interest, and ligating them together. The resulting construct (pRS303-Kan-P_{POL1}-Luc(A4V)PEST/loxP-*CDC28*-loxP) was linearized by *Nhe*I and integrated into the *HIS3* locus of CEN.PK113-7D/*ura3* by homologous recombination. Transformants were selected on YPD plates containing 200 µg/ml G418.

The native *CDC28* gene was then removed from this strain by replacing the native CDS with the hygromycin resistance cassette (HPH) from pYM24 [89]. This was done by PRC amplifying the HPH cassette from pYM24 using primers that each contained 50 bp overhangs of homology to regions just upstream and downstream of the intergenic regions used to flox *CDC28*. This prevented HPH recombination from targeting the floxed version of *CDC28*. Positive transformants were selected on YPD containing 200 µg/ml hygromycin. The 5' primer used for HPH to *CDC28* recombination was GAATTATCGTTCTCGAGATAGTTTTTATACAATACATATATATATATATAGACATGGAGGCCCAGAATAC, and the 3' primer was GTCAAAGAAGTTCAATTGAGGCCCCAGCATACAATGCGTTATTTCGTTTTCGAGCTCGTTAAAGCCTTCG.

A galactose-inducible CRE recombinase gene was added to the yeast strain as follows. The P_{GAL1}-CRE recombinase gene from pSH47 [88] was moved to the yeast integration vector pRS306 [73] by digesting both plasmids with *Kpn*I and *Sac*I, gel isolating the fragments of interest, and ligating them together. The resulting plasmid was designated pRS306-P_{GAL1}-CRE and was linearized with *Stu*I and integrated into the *ura3* locus of CEN.PK113-7D/*ura3*. Positive transformants were selected on supplemented minimal media lacking uracil. Positive transformants were confirmed by an inability to grow on supplemented minimal media containing galactose instead of glucose, due to galactose inducing CRE which removed the essential floxed *CDC28* gene (Fig. 3.4A)

Galactose Promoter Induction during Continuous Culture

Yeast respiratory oscillations were achieved in continuous culture using either the floxed *CDC28* strain of CEN.PK113-7D/*ura3* described above or the galactose-inducible luciferase reporter strain of CEN.PK113-7D containing pRS303-Kan-P_{GAL1}-Luc(A4V)PEST [74]. After stable yeast respiratory oscillations had formed, the galactose-inducible promoter of either strain was activated by switching the continuous culture medium (at the same infusion rate) to a similar medium that contained 2% galactose instead of 1% glucose, for 30 to 40 min.

CHAPTER IV

LIGHT AFFECTS THE YEAST RESPIRATORY OSCILLATION THROUGH PHOTOSENSITIVE CYTOCHROMES

Introduction

Circadian clocks are biological pathways that measure time on a roughly 24 hour timeframe, allowing organisms to synchronize and ultimately optimize their behavior and physiology with respect to the daily rhythmic changes in the environment. Circadian timing systems provide two major advantages, 1) anticipation of recurring changes to the external environment such as light intensity, temperature, humidity, and food availability; and 2) synchronization and coordination of metabolic processes and gene regulation within the organism that provide optimum energy usage and temporal separation of mutually unfavorable reactions or reaction conditions [8, 9]. Scientists have found circadian clocks spanning organisms from simple prokaryotes like cyanobacteria to complex eukaryotes like humans; but despite the clock's wide prevalence, differences in genes and mechanisms for prokaryotes, plants, animals, and fungi point to no clear clock progenitor suggesting that the clock has evolved independently several times. These two points (the clock's virtual ubiquity and apparent independent origins) strengthen the claim that the capability of measuring time is an advantage worth preserving [10]. Interesting questions therefore arise when organisms like the budding yeast *Saccharomyces cerevisiae* are found that seem to lack circadian clocks. Did these organisms' ancestors once possess clocks? Does a short lifecycle not favor a circadian clock? Does the feast/famine lifestyle of organisms like yeast outweigh the costs of maintaining clocks? Do such organisms possess alternative mechanisms to

achieve some advantages circadian clocks provide without having to have circadian clocks themselves?

One seemingly universal characteristic for all organisms with circadian clocks is the ability to sense light. Although clocks from many organisms can be entrained by a variety of rhythmic environmental cues such as temperature [90], food availability [91], and tides [92], light has been found to be the strongest zeitgeber (time giver) across all kingdoms of life and often heralds changes in other rhythmic phenomena like temperature and humidity. Additionally, the dependency on fixed carbon by primary producers (photosynthetic organisms) is directly tied to the availability of light and the producer's ability to carry out physiological processes in the presence of light. It is not surprising then, that photosynthetic organisms like plants and cyanobacteria have developed circadian clocks to anticipate the arrival of this rhythmic vital energy source. Similarly, light is important to much of the animal world for survival, but as a medium for vision and navigation rather than as a direct energy source. Therefore, it is not surprising that animals have developed (or maintained) circadian clocks to anticipate the sun for reasons including food gathering, predator avoidance, reproduction, and directional orientation. It is less obvious why organisms that are neither photosynthetic nor visual (like fungi) develop or preserve circadian clocks and light detection, but may include advantages that accrue from anticipating changes in temperature and humidity as well as avoiding the damaging properties of solar radiation itself [93].

The ability for an organism to detect or respond to light is dependent on pigmented macromolecules in the organism that receive light energy at specific wavelengths and undergo physical/chemical changes that have biochemical consequences. Photosensitive macromolecules vary as widely in nature as biological uses for light itself, e.g. DNA repair enzymes like photolyases, light harvesting photosynthetic pigments like chlorophylls, and vision-related protein complexes like

rhodopsins. During the evolution of circadian clocks, previously existing photosensitive macromolecules were often co-opted by the clock and provided the evolutionary template for a specialized clock-photoreceptor to develop [94]. As a result, there are evolutionary trends in clock-related photoreceptors, e.g. the photolyase-related family of cryptochrome photoreceptors that are used by both plants and animals, the phytochrome family of photoreceptors that are found in plants and cyanobacteria [94] and the blue light photoreceptor White-Collar-1 found in many fungi [95, 96]. *S. cerevisiae* does possess a photolyase [97]; however, despite the evolutionary trends in photoreceptors that span biological kingdoms, *S. cerevisiae* which apparently lacks a circadian clock, also appears to lack the well-characterized clock-related photoreceptors of other organisms (e.g. cryptochrome, phytochrome, white-collar 1, rhodopsin).

Why does *S. cerevisiae* not have a circadian clock when so many other eukaryotes (including other fungi) do? One answer might be that for an organism with the lifecycle and lifestyle of yeast, maintenance of a circadian clock costs more than the advantages it provides. The advantages of predicting 24 hour environmental cycles are arguably fewer to organisms with lifecycles shorter than a day. Admittedly, this characteristic alone does not preclude the existence of clocks in cyanobacteria that can divide in less than 10 hours yet possess a well characterized circadian clock [98]. However cyanobacteria are photosynthetic and sunlight is part of their nutritional requirement. It is certainly advantageous for an organism to be able to predict the availability and quantity of its food sources (when availability regularly recurs).

Yeast are not photosynthetic, rather their natural habitat provides a lifestyle that is more one of an unpredictable feast or famine. Wild yeast tend to live most of their lives in a dormant/quiescent state (often as a spore) usually in close association with insects like fruit-flies and other diptera, relying on the insects to transport them to the surfaces of fruit where they remain dormant until the fruit bursts or is compromised by

other microbes [99]. The sudden availability of rich, sugary nutrients revives the yeast from their dormancy where they quickly multiply and consume the sugars and starches available [4]. Once the nutrients are depleted (or the yeast are carried away from the fruit by insects), the yeast return to their quiescent and/or sporulated state [4, 100]. Therefore those yeast that live to experience multiple days and nights usually do so in a metabolically quiet form; and those yeast that actively grow and divide usually only live that way for a short time (one or two days). For this type of lifestyle, a circadian clock may be more costly to maintain than it is worth, especially if yeast possess alternative mechanisms that provide similar advantages of a circadian timekeeping system but which are amenable to yeast's short lifecycle and opportunistic fits of growth.

Temporal organization of metabolic reactions within the organism is one advantage provided by a circadian clock, however the circadian clock is not the only way to achieve temporal coordination. Other strategies could include a metabolic "hour-glass" which is reset at the end of each cycle or an ultradian clock, similar to a circadian clock but operating on a timeframe shorter than a day and not exhibiting self-sustained oscillations in a constant environment. Ultradian rhythms of oxygen consumption, metabolite production, and cell division have been studied for decades in *S. cerevisiae* grown under specific continuous culture conditions [33, 34, 38]. Plus, there is evidence that the metabolic coordination these rhythms provide yeast are advantageous for minimizing oxidative damage during DNA replication [16, 76] as well as surviving other stresses [17], at least under the conditions tested. It is unclear whether these oscillations occur in yeast's natural habitat (outside of the continuous culture bioreactors) and also whether these rhythms are due to an ultradian clock verses a metabolic "hour-glass". Nonetheless, they could potentially provide the yeast with a timekeeping capability that is accomplished in other organisms by a circadian clock, lessening the necessity to preserve a circadian timekeeping system during their evolution.

Anticipating external changes related to the day/night cycle is another major advantage provided by a circadian clock. Sunlight can benefit some organisms but there is a trade-off since high-energy rays are detrimental. For some organisms, the circadian clock regulates protection from the sun by gating activity of photosensitive processes (like DNA replication) to the night when high-energy rays are not present [101]. An alternative approach to protection from rhythmic changes in the environment is simply to adapt quickly to external changes as they are perceived rather than anticipated. *S. cerevisiae* alters physiology/metabolism in response to such environmental changes/stresses as temperature, pH, nutrition, and UV-induced DNA damage [27, 102], but if yeast possess a way to rapidly sense and respond to visible light as well, then this could have lessened the necessity to preserve a circadian clock in yeast's evolutionary past.

In this investigation we examined the effects of visible light on the ultradian yeast respiratory oscillation (YRO) as well as the effects on the health/viability of yeast in general. Our goals were to determine 1) if there is evidence of a light-entrainable circadian oscillator in *S. cerevisiae*, 2) if yeast can metabolically respond to visible light, 3) if visible light is in fact harmful to yeast and 4) if yeast can protect themselves from light if it is harmful. We show that visible light (blue and green light in particular) of an intensity less than natural sunlight significantly shortened the YRO's period and amplitude and that no apparent circadian rhythm of this phenomena persisted during constant darkness. Additionally, yeast exposed to light grew more slowly than similar cultures grown in the darkness and they upregulated the production of pigments in response to the light.

Results

Yeast Response to Light in Continuous Culture

Convincing evidence of circadian rhythms in *S. cerevisiae* has yet to be shown, despite the attempts by multiple labs for a number of years. This lack of evidence could simply be due to the fact that *S. cerevisiae* does not possess a clock. Alternatively, the species might possess a circadian clock, but demonstrating it has been hampered by not identifying appropriate outputs as well as complications arising from constantly changing nutritional and metabolic conditions during growth in a confined volume of medium. We considered that one potential output from a circadian clock might be the respiration oscillation seen when certain yeast strains are grown in continuous culture [15, 16, 20, 38]. The steady perfusion of new media into the culture during continuous culture, as well as the constant pH and temperature maintained by the system, would provide a stable environment for growth and hopefully overcome complications from the culture slowing and entering stationary growth as it does in batch or on plates. We established a stable yeast respiratory oscillation in the strain CEN.PK and administered light of increasing intensities from cool white fluorescence bulbs for 12 hour durations, with 12 hours of darkness in between (Fig. 4.1). The YRO had a period of about 250 min in total darkness but white light caused the period of the oscillation to shorten and the amplitude to decrease (Fig. 4.1A,B), becoming more apparent with greater intensities of light (90-300 $\mu E/m^2/s$). The lowest intensity of light applied (90 $\mu E/m^2/s$) had only minor effects on period and amplitude; however, the brightest light applied (300 $\mu E/m^2/s$) caused very short (~85 min) and unstable oscillations. Treating the culture with 300 $\mu E/m^2/s$ of light for longer than 16 hours destroyed the oscillation (Fig. 4.1A), but returning the culture to total darkness did eventually restore the

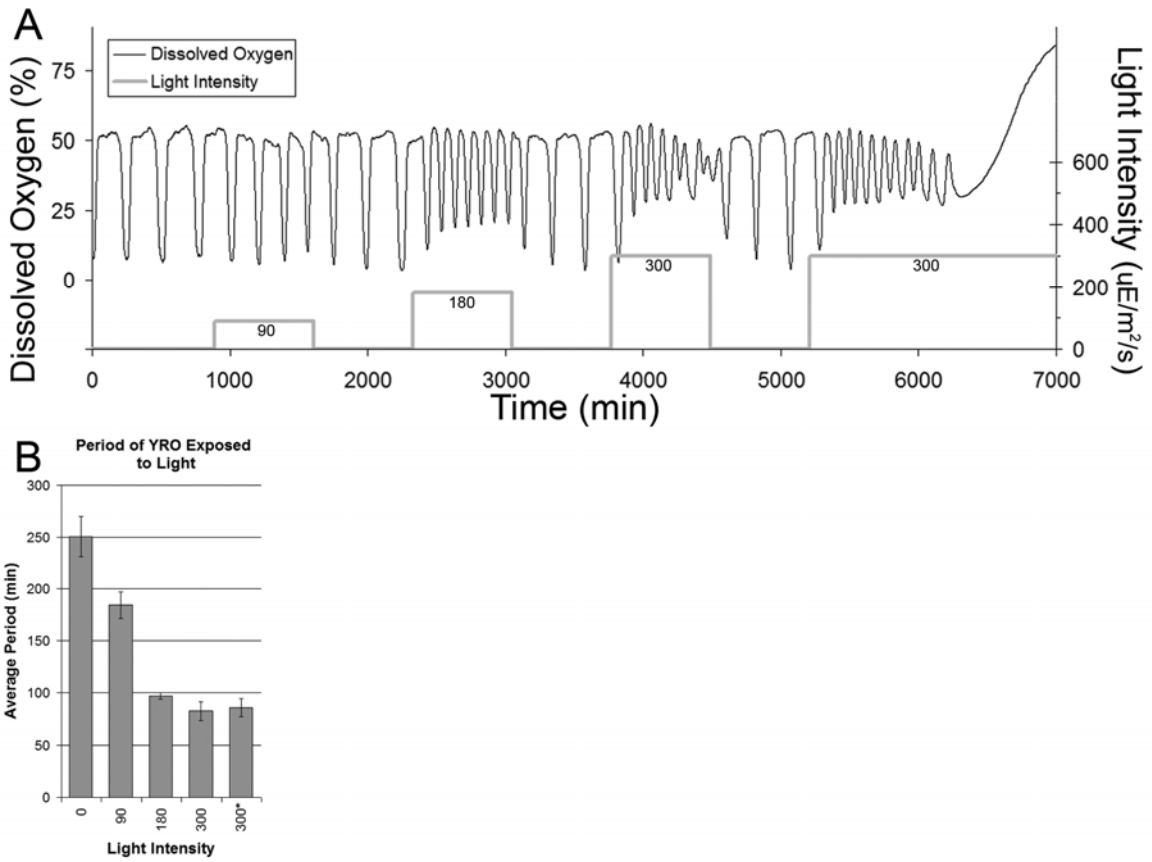

Figure 4.1. The effects of visible light on the yeast respiratory oscillation. **A.** The effect various intensities of light have on the YRO. Oscillations were initiated in a culture grown in the dark until stable oscillations of dissolved oxygen formed (black line, left y-axis). Then 12-hour treatments of light were administered at intensities of 90, 180, and 300 µE/m^2/s (gray line, right y-axis) with 12 hours of darkness in between light treatments. The final 300 µE/m^2/s light treatment was maintained for 35 hours (only 30 hours are shown). Light of increasing intensity had an increasingly obvious effect on period and amplitude, and light treatment at the highest intensity destroyed the oscillation after 18 hours. **B.** The average periods for oscillations occurring during the initial dark phase or during each of the different intensities of light for the YRO shown in A. Each bar represents the average period in minutes of complete oscillations occurring during the treatment, error bars represent ± one standard deviation. The x-axis is arranged by light intensity in µE/m^2/s, and the bar labeled 300* represents the average period for the second, longer 300 µE/m^2/s light treatment.

oscillation about 20 hours later (data not shown). To put these observations into perspective, typical light intensities that are found outside on a sunny day can exceed 2000 μE/m^2/s, while indoor (laboratory) light is often around 10-15 μE/m^2/s. It must be noted that for all applications of light or darkness, the temperature of the culture was maintained at 30°C (± 0.075°C) by a temperature controlled water jacket that surrounded the entire culture and was between the culture and the light sources.

Determining the wavelengths of light that cause a biological response has proved valuable for elucidating the identity of photoreceptors for various biological phenomena in other organisms like plants [103] and filamentous fungi [95], so we tested the effect that red, green, and blue light had on the YRO by placing colored filters in the light path. An oscillating culture with a period of about 210 min was established and filtered light of different colors was administered for 12 hours with 12 hours of darkness in between. The nature of the filters used removed different portions of the spectrum from the light sources so light intensity varied among colors, however the sensitivities to the varying intensities of colored light were also informative. Red light (100 μE/m^2/s) had no effect on the YRO's period and only very minor effects on amplitude, however dim green light (80 μE/m^2/s) and very dim blue light (25 μE/m^2/s) affected the YRO similarly to moderately bright white light (180 μE/m^2/s) (Fig. 4.2A,B).

The three 12-hr treatments of colored light (red, green, blue) were followed by two 12-hr treatments of unfiltered white light of sufficient intensity to cause noticeable effects to the YRO without compromising the oscillation's integrity. After these 5 days of 12 hr light/12 hr darkness, the culture was allowed to free run in constant darkness to see if any circadian patterns of period or amplitude shortening were evident in the YRO. As can be seen in Fig. 4.2A and B, the YRO showed stable periods and amplitudes during this free run similar to those seen prior to light treatment, thus providing no

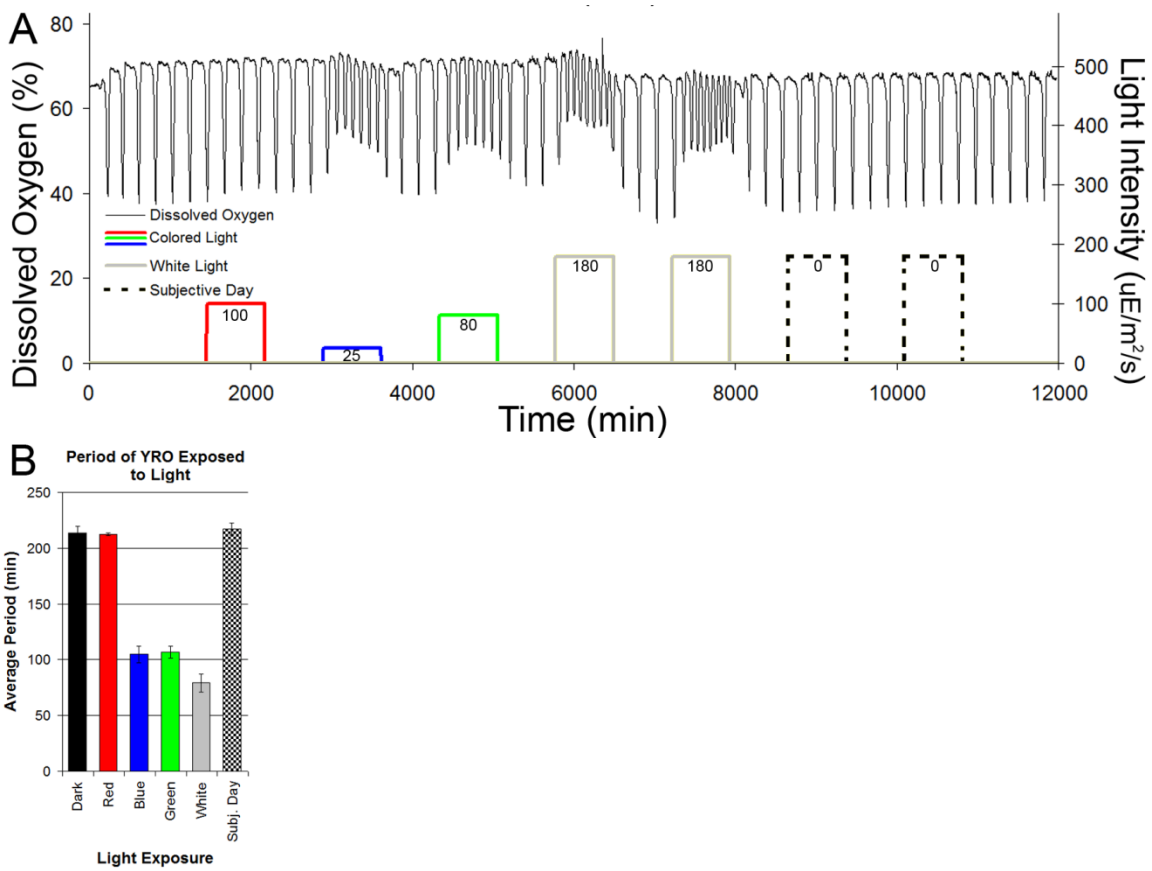

Figure 4.2. The effects of colored light on the yeast respiratory oscillation. **A.** The effects of colored light at different intensities on the YRO. Oscillations were initiated in a culture grown in the dark until stable oscillations of dissolved oxygen formed (black line, left y-axis). Then 12-hour treatments of light of different colors (red, blue, green) were administered (colored lines matching color of light, right y-axis) with 12 hours of darkness in between. After the application of colored light, two 12-hour white light treatments were given. Light intensities of each treatment are shown on the right y-axis as well as indicated by numbers under each of the colored or gray lines showing light treatment. **B.** The average periods for oscillations occurring during the initial dark phase or during each of the different colored light treatments for the YRO shown in A. Each bar represents the average period in minutes of complete oscillations occurring during the treatment, error bars represent ± one standard deviation. The subjective day bar pertains to the portion of the YRO indicated in dashed black lines of A, and represents the times when light would have reoccurred following the 12-hour light/dark cycle maintained for the previous 5 days. These results show that red light has virtually no effect on the period or amplitude of the YRO compared to darkness and that blue light has a very strong effect. At 1/7 the intensity, blue light is capable of reducing the period of the YRO to levels near that of white light. Additionally, there is no evidence of circadian influences on the effects light has on the YRO.

evidence that a circadian clock influences this phenomenon. The changes in the YRO's period and amplitude are direct (and temporary) effects of the light on the organism.

Cytochromes are the Suspected Photosensitive Macromolecules Responsible for Light Effects on the YRO

Sensitivity to blue light is common in fungi [104] due to the photoreceptor White Collar-1, but *S. cerevisiae* lacks the WC-1 gene or any homolog [96]. However, one class of pigmented cellular components that yeast have which are known to absorb heavily in the blue region are the cytochromes which make up part of the electron transport system for ATP synthesis in the mitochondria (for absorption spectra for several cytochromes see [105, 106]). Visible light has been shown to destroy/inactivate some of the cytochromes in mammalian cells, algae, and yeast [107] as well as impair yeast growth, respiration and membrane integrity by affecting the cytochromes [108, 109]. These reports by others point to various cytochromes being candidate photo-transducers for our observed changes in respiration oscillations.

If the shortened period and reduced amplitude of the YRO are due to light's inhibition/destruction of mitochondrial cytochromes, then it is likely that another method of inhibiting or destroying cytochromes (or their effectiveness as an electron transporter) would have a similar effect to light. Sodium azide is a chemical that inhibits respiration by binding and inhibiting cytochrome c [110] and cytochrome oxidase [111] of the electron transport chain. Various concentrations (4-20 μM) of sodium azide were injected into an oscillating culture of CEN.PK at various times in order to determine a working concentration for possible YRO perturbation, however only transient/acute effects to the dissolved oxygen oscillation were observed (data not shown). It is likely that the acidic media of the culture (pH 3.4) converts the sodium azide into azoic acid, a volatile gas, which rapidly leaves the culture through the culture's gaseous effluent. To

compensate for the gaseous loss of azide and the constant dilution of continuous culture, we introduced sodium azide into the oscillating culture at a steady drip (3.4 μmoles/hr) using a syringe pump. Perfusion of azide in 12 hour treatments separated by 12 hours with no azide showed similar effects to the YRO as 12 hour light/dark treatments (Fig. 4.3 A,B). That is, sodium azide shortened the period and reduced the amplitude of the dissolved oxygen oscillation during times of treatment and the oscillation returned to its longer period and amplitude once the chemical's delivery was stopped.

Another way to demonstrate visible light's effect on respiration is to record the changes in dissolved oxygen from a small batch culture as yeast respiration consumes the limited oxygen in the vessel either under conditions of darkness or illumination. Overnight cultures of CEN.PK were grown either in the light (~ 200 $\mu E/m^2/s$) or darkness and then dilutions of these cultures were assayed for respiration rates while either being exposed to light (~ 250 $\mu E/m^2/s$) or darkness. Specifically, aliquots of light-grown or dark-grown yeast were placed into a finite volume (40 ml) of new media in a sealed 50ml flask (magnetically stirred) where the dissolved oxygen in the vessel was recorded over time. Respiration of yeast that had previously been grown in the dark (dark-grown culture) and that were assayed in the dark exhibited a linear consumption rate of oxygen that was directly proportional to the amount of culture added to the assay media (Fig. 4.4A). However, respiration from the dark-grown culture of yeast showed a different trend of oxygen consumption rate once light was applied (Fig. 4.4B). For the experiment showing this result, the dark-grown yeast were allowed to respire in the darkness for 15 min to demonstrate a stable, linear trend of oxygen consumption, then light was applied for the remainder of the measurement. After about 15 min of light treatment (at time = 30 min), oxygen levels began to deviate from that which would be expected from the trend generated from measurements made during the first 15 min of respiration in the

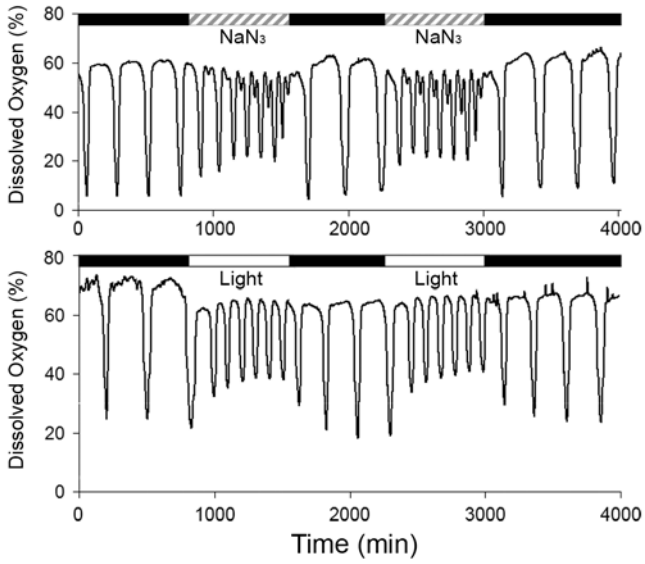

Figure 4.3. Sodium azide causes similar effects as white light on the yeast respiratory oscillation. Separate oscillating 850 ml cultures were given two 12-hour treatments of either sodium azide at 3.4 μmoles/hour (top) or white light at 180 μE/m^2/s (bottom), with 12 hours in between treatments. Both light and azide shorten the period and reduce the amplitude of the YRO and both show a transitional effect after initial administration of each treatment, but their effects on the YRO differ slightly as well. Continual application of sodium azide begins to bifurcate the shorter oscillations later during the treatments, and the culture seems to recover more quickly from cessation of sodium azide treatment than from treatment of light. These results suggest that light causes longer lasting harm to respiration than azide.

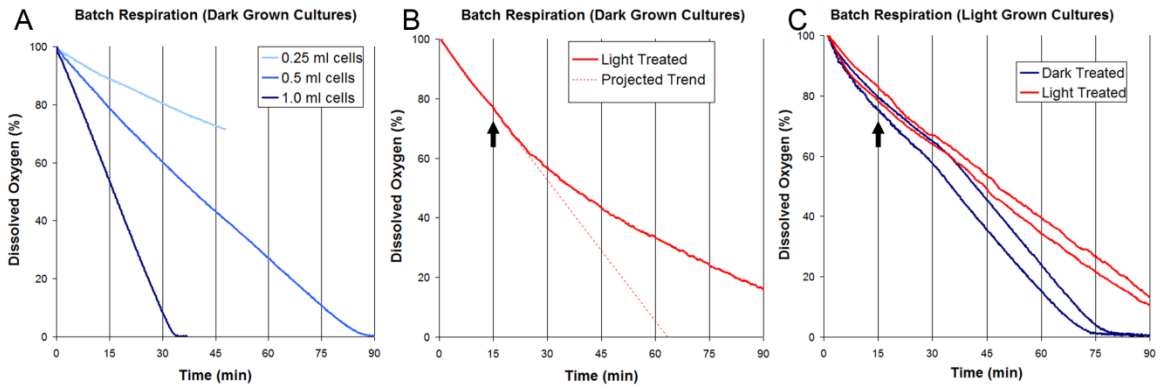

Figure 4.4. Effects of light on respiration measured in batch cultures. **A.** Samples of yeast from a starter culture grown over night in the dark (dark-grown culture) show linear rates of oxygen consumption over time proportional to the number of cells tested. Different amounts of the starter culture (0.25, 0.5, 1.0 ml, light blue, blue, and dark blue lines, respectively) were individually assayed in 40 ml of fresh media. The trial involving 0.25 ml of cells (light blue line) was only measured for 50 min. **B.** Dark-grown yeast subjected to light after the first 15 minutes of dissolved oxygen measurement shows a decreasing rate of oxygen consumption over time (solid red line) compared to the projected rate of oxygen consumption had the culture remained in the dark (dotted red line). A sample of the same starter culture of dark-grown yeast as in A was measured for the first 15 min in the dark to establish a linear trend of respiration. At time = 15 min (arrow), white light (250 $\mu E/m^2/s$) was applied for the remainder of the assay. Light increased the noise of the probe so the solid red line is the smoothed, 11-point moving average of dissolved oxygen measurements. **C.** Samples of a starter culture of yeast grown for 30 hours under 200 $\mu E/m^2/s$ white light (light-grown yeast) show linear rates of oxygen consumption over time when assayed in 250 $\mu E/m^2/s$ of light (red lines) and show increased rates of respiration after 30 min of measurements in the dark (blue lines). Similar colored lines are replicates. For experiments in C. the first 15 min of all samples were measured in the dark. After 15 min (arrow), the samples labeled “light treated” (red lines) were exposed to 250 $\mu E/m^2/s$ white light for the remainder of the assay. For light treated samples light increased the noise of the probe so the solid red lines are the smoothed, 21-point moving average of dissolved oxygen measurements.

dark (Fig. 4.4B). Rather than taking 60 to 70 min to deplete the oxygen in the flask (as would be predicted based on initial respiration rates in the dark), the assayed sample required more than 130 min to deplete the oxygen in the flask under illumination. In the converse experiment where overnight cultures of light-grown yeast were assayed for respiration rates in either the light or the dark, it was the cultures that were assayed in the light that showed the near linear consumption of oxygen, while light-grown cultures that were assayed in the dark increased their respiration rate after about 30 min in the dark (Fig. 4.4C). These results support the hypothesis that light interferes with respiration via photosensitive substances (e.g. cytochromes) that are restored/repaired at a faster rate in the darkness.

Rapid Depletion in Oxygen Tension Can Reset the YRO

The redox state of the culture is believed to play a vital role in the YRO alternating between a respiratory phase and a respiro-fermentative phase [16, 18, 32]. As shown above, damage or impairment to the respiratory cytochromes of the cell resulted in a shortened respiration phase of the YRO (i.e. a shortened period and amplitude) and resulted in the culture more frequently returning to a respiro-fermentative mode of energy metabolism. In addition, I have shown that the introduction of chemicals (metabolites) like ethanol and acetaldehyde have been shown previously to result in differing degrees of YRO phase resetting depending on the phase of the oscillation in which these substances are introduced (Chapter II) [74]. This phase resetting in response to artificial introduction of metabolites prematurely switches the YRO from a respiro-fermentative phase to a respiratory phase, characterized by an immediate drop in dissolved oxygen in the culture. Based on these results (as well as the effects light and azide have on respiration in the YRO), we questioned whether rapidly depriving the culture of oxygen could have a similar phase resetting effect without the addition of

metabolites. Perhaps the oscillating oxygen level in the culture could be an environmental signal that allows the YRO to remain synchronized.

A blend of air and nitrogen was bubbled into the continuous culture (as opposed to only air) to artificially replicate the characteristic dissolved oxygen troughs of the YRO at various phases of the YRO. Simply reproducing these troughs in amplitude and duration by adjusting the extracellular oxygen concentration of the media had no phase resetting effect on the oscillation (Fig. 4.5). That is to say, artificially lowering the dissolved oxygen in the bioreactor to about 20% (the DO concentration normally achieved during each trough of the YRO) for a duration of 20 min (the approximate time that the DO of the culture remains low during each trough of the YRO) had no phase resetting effect on the oscillation at any of the phase points tested. However, extreme deprivation of oxygen in the culture by bubbling 100% nitrogen at 0.9 L/min (in place of air at 0.9 L/min) into the culture for 3 min did result in phase resetting at certain times during the YRO (Fig. 4.6A) similar to those by ethanol that we observed previously (Chapter 2) [74]. In other words, phase shifts similar to those caused by ethanol and acetaldehyde occurred only if the DO of the culture was artificially dropped to near 0% (< 0.5%) for 3 min.

Interestingly, bubbling 100% oxygen into the culture for 30 minutes at different phases had a similar, but smaller, phase resetting effect as removing oxygen from the culture by nitrogen (Fig. 4.6A,B). The fact that both gases (N_2 and O_2) had similar phase resetting properties contradicted expectations regarding the role of oxygen in propagating the YRO, but there is one similarity between the administrations of both gases; namely, both treatments resulted in a sudden depletion of oxygen from the culture (nitrogen at the beginning of the treatment and oxygen at the end of the treatment). We suspect that during the 30 min O_2 treatment, the culture sensed the increased oxygen levels and gradually altered metabolism in response (perhaps either

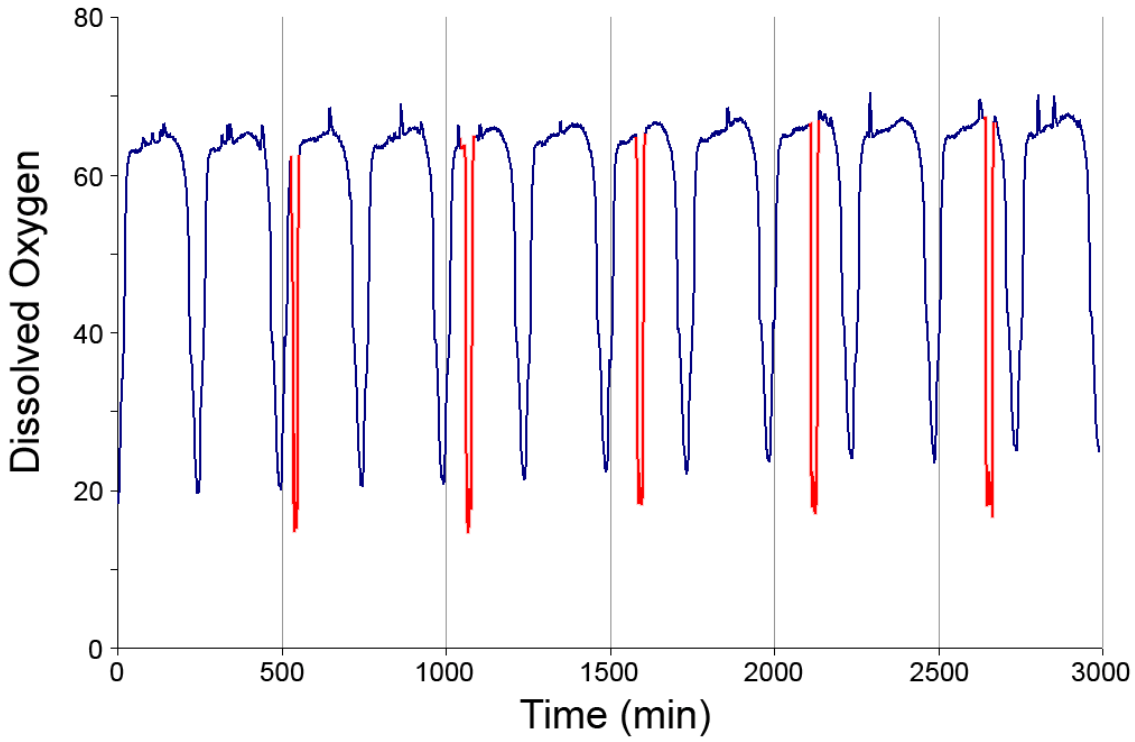

Figure 4.5. Artificially replicated DO troughs are not sufficient to reset phase of YRO. The YRO with a period of about 250 min (blue line) was periodically interrupted with a mixture of air and nitrogen gas in order to artificially drop the dissolved oxygen (DO) in the culture to levels near those experienced during regular DO troughs for 20 min (highlighted in red). The treatments did not affect the phase or period of the YRO as can be seen by the recurring troughs at time intervals of 250 min.

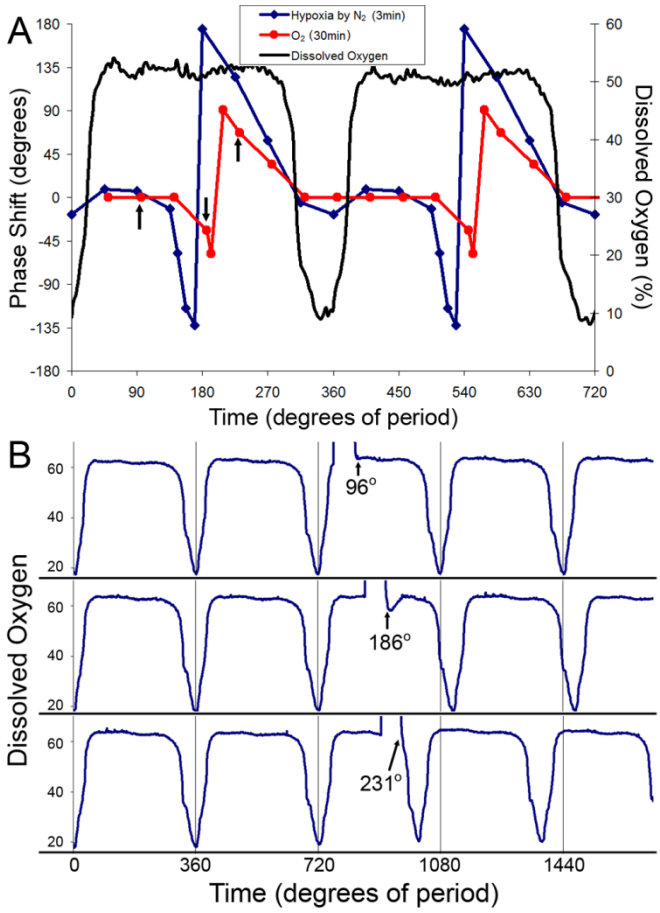

Figure 4.6. Phase responses of the YRO to treatments of hypoxia (by nitrogen gas) and hyperoxia (by oxygen gas). **A.** A double-plotted phase-response curve (PRC) to onset of hypoxia by N_2 (blue) or cessation of hyperoxia by O_2 (red). Phase shifts are plotted in degrees of period with advance shifts plotted as positive values and delay shifts as negative values. A representative dissolved oxygen trace (black) show the YRO for reference. Black arrows show phase points 96°, 186°, and 231° for the O_2 PRC which are shown in more detail in B. 0° was defined as the time when dissolved oxygen started to rise from the YRO trough. 360° = 3.5 h for both PRC experiments. **B.** Representative O_2 treatments used to generate the O_2 PRC in A. At times of treatment, O_2 gas was bubbled in place of air at a rate of 0.9 L/min for 30 min. The phase point for which the PRC was generated was for the time when the culture was switched back to air, shown by black arrows. Treatment at 96° caused no phase shift. Treatment at 186° caused a phase delay. Treatment at 231° caused a phase advance.

by increasing respiration rate, decreasing oxygen uptake rate, or decreasing the cellular levels of respiration machinery). The sudden removal of the O_2 at the end of the 30 min treatment appeared to the yeast as a state of hypoxia based on the rates of respiration or oxygen transport/metabolizing capability to which the cells had become accustomed. The sudden lack of oxygen in this case had a similar effect as removing oxygen by nitrogen displacement; that is, both forced the cells to alter their metabolic strategy, resulting in phase resetting at certain phases of the YRO. These results show that dissolved oxygen levels alone are not the environmental synchronizing signal that maintains the YRO, however intracellular oxygen level could be a downstream signal that plays a role in the redox-dependent switching of the energy strategy during the YRO.

Light Slows Growth and Upregulates Pigment Production in Yeast

Light negatively affects respiration rates and ultimately reduces ATP production, so light should have a negative effect on growth rates as well. Effects like these were reported 30 years ago [108] and experiments in our lab confirmed this effect (Fig 4.7A). Additionally we found that yeast cultures that were grown in the light turned pinkish-orange after about 24 hours while their dark-grown counterparts remained white (Fig 4.7B, and Fig. 4.8). These light-induced pigmented yeast appeared similar to *ade2* strains of yeast, so we first thought the light had caused an *ade2* mutation in our cultures. However pink yeast inoculated into fresh media and grown overnight in the dark turned white, indicating that the coloration was not a permanent change/mutation. Moreover, these yeast also grew well on supplemented minimal media lacking adenine (and remained pink in the light) so the coloration was not the result of an *ade2* mutation.

Some yeast like *Rodotorula mucilaginosa* and *R. glutinis* are naturally pink due to high levels of carotenoids which protect them from oxidative stress [112, 113]. Light

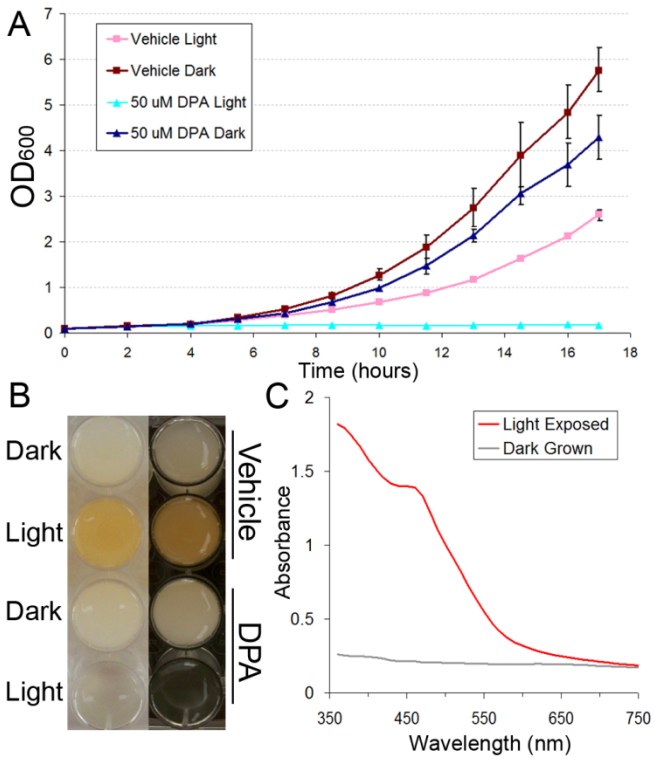

Figure 4.7. Light causes yeast to grow more slowly and to produce pigments. **A.** shows growth curves for yeast cultures grown in 250 μE/m^2/s light (pink and light blue lines) or darkness (dark red and dark blue lines) with 50 μM diphenylamine (light blue and dark blue lines) or 0.05% methanol vehicle (pink and dark red lines). Data points are averages, n=3. Error bars are 1 standard deviation. Cultures grown in the dark grew more rapidly than their light-grown counterparts, and DPA treated cultures only grew in the dark. **B.** Yeast from the experiment in A. grown in the light without DPA produced a pink-to-orange pigment after 24-30 h growth while dark-grown cultures remain white. The three 10 ml cultures from each treatment were pooled, pelleted, and resuspended in 3 ml water for photographing. The column of wells on the left and right are the same, only their backgrounds differ (for contrast). **C.** The absorption spectrum for the chloroform fraction extracted from light-grown and dark-grown yeast. There is a strong shoulder at 450 nm characteristic of C_{40} carotenoids. [113, 114]

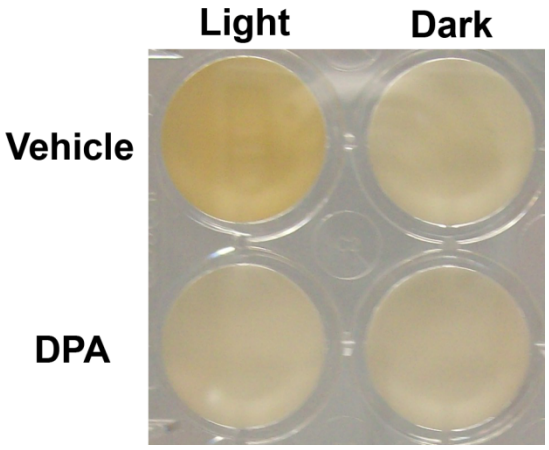

Figure 4.8 Yeast inoculated at a high enough density to overcome growth impairment by light remain white in the presence of DPA, having a similar color to yeast grown in the dark. Yeast grown in the light without DPA turn a pinkish-orange color.

produces reactive oxygen species when it is absorbed by certain pigments like porphyrins [115] (such as those present in heme, cytochromes, and chlorophyll [116]), and carotenoids have been shown to protect against the damaging effects these reactive oxygen species produce [113, 117]. To investigate whether the pink pigment in the light-exposed yeast was a carotenoid, we grew yeast in the presence of diphenylamine (DPA), a carotenoid synthesis inhibitor [118]. We predicted if the pink pigment in these yeast cultures was a carotenoid and that the carotenoid was protecting the yeast from direct photodamage or indirect light-induced oxidative damage, then treating a light-grown culture with DPA would inhibit the pigment's production and make the yeast more sensitive to light's detrimental effects. We subdivided a freshly inoculated culture (supplemented minimal media) into four treatment groups with three replicates each. Two of the treatments received 50 μM DPA and the other two received only the vehicle (methanol). One of each treatment (DPA and vehicle) was grown in the dark, and the other was grown in 250 $\mu E/m^2/s$ light (Fig. 4.7A,B). The cultures grown in the dark grew faster than their light-grown counterparts, and DPA only had a minor inhibitory effect on growth in the dark. However, light-grown cultures that contained 50 μM DPA failed to grow at all during the course of the growth study.

The pigment(s) from the yeast were extracted with methanol:chloroform (50:50) and phase separation with 1% NaCl showed the pigment(s) moved with the chloroform phase (as would be expected with hydrophobic pigments like carotenoids). Spectral analysis of this chloroform extract showed a strong absorption peak/shoulder around 450 nm (Fig. 4.7C) characteristic of many C_{40} carotenoids [113, 114] (e.g. β-carotene and torulorhodin). The high absorption levels below 400 nm could be due to other, lighter polyenes (e.g. carotenoid precursors or degradation products). It was later found that DPA treated cultures could grow to some degree in the light if initially inoculated with an amount of yeast large enough to overcome the growth inhibition from light. Such

DPA treated cultures that grew in the light did not turn pink, but rather remained white like dark-grown cultures (Fig. 4.8), supporting the idea that the pinkish-orange pigment in the light treated cultures (without DPA) is a substance that DPA prevents from forming, namely a carotentoid.

Pigment Production is not Limited to the CEN.PK Strain of Yeast

To determine whether other strains of yeast could also turn pinkish-orange when exposed to light (similarly to the CEN.PK strain), a variety of strains were grown on solid supplemented minimal media under light and dark conditions. The strains that were tested consisted of those we had readily available from a variety of previous studies, and in no way constituted an exhaustive search for photo-induced pigment producing strains. These experiments were done first on a small set of differing strains to determine the prevalence of photo-induced pigment production. The strains tested included CEN.PK113-7D (from Peter Kötter, Frankfurt Germany), IFO0233 (from Robert Klevecz, Institute of the City of Hope Medical Center), SEY6210 [70], YKF201 [119], SK1 (from Angelika Amon, MIT), S288C [120], the yeast two-hybrid strain AH109 (from Clontech), the deletion collection background strain BY4741 [121], and *S. paradoxus* strain YPS138 [122]. S288C, AH109, and BY4741 did not grow well under the conditions used to test growth in either the light or dark (after repeated attempts), so assessment for their viability and ability to change color in the light could not be made. Alternative growth conditions that remain conducive to this assay need to be found before light-dependent phenotypes can be evaluated for these strains. All of the remaining strains, however, showed impaired growth in the light, except SEY6210 (Fig. 4.9). SEY6210 seemed to grow just as well in the light as in the dark and may have had a very faint pink tint to the cells that grew in the light. The color change for CEN.PK113-7D and IFO0233 grown in the light was particularly noticeable, on the other hand (Fig. 4.9 A,B). Interestingly, both

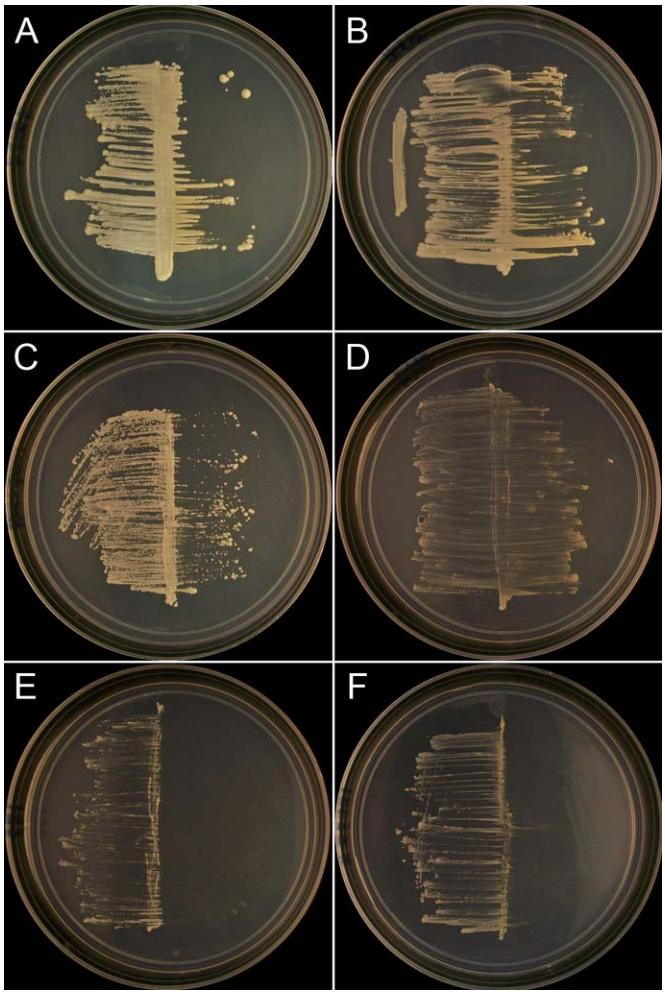

Figure 4.9. Other strains of yeast besides CEN.PK also showed growth inhibition under white light, however only one other strain tested clearly showed the ability to turn pink/orange under the light. Yeast that were spread equally on both left and right sides of plates were covered on the left side with opaque tape and grown at room temperature under 200 μE/m^2/s white light from a cool white fluorescent bulb. A glass dish containing 1 in of distilled water was between the light source and plates to absorb heat from the bulb. Plates contain *S. cerevisiae* strains except where noted: **A.** CEN.PK113-7D. **B.** IFO0233. **C.** *S. paradoxus*. **D.** SEY6210. **E.** SK1. **F.** YKF201. Yeast grew better in the dark (left side) than in the light (right side), except for SEY6210 (D) which grew equally well in both light and dark. Besides CEN.PK (A), the only other strain to clearly turn pink/orange under the light was IFO0233 (B), another yeast strain that exhibits the YRO in continuous culture.

of these strains that noticeably changed color in the light are strains that exhibit the YRO in continuous culture. Whether this is a meaningful association or whether it is a coincidence remains to be seen.

Discussion

We have shown that *S. cerevisiae* (strain CEN.PK) can perceive light (primarily blue and green light) in a physiologically relevant way and that this light can affect yeast's ability to respire in as little as 15 min, presumably by damaging the heme-containing cytochromes that participate in electron transport for oxidative phosphorylation. Ultimately this effect on respiration has consequences to the yeast respiratory oscillation when this strain is grown in continuous culture. Although the overall percent of time that the culture spent in the respiratory phase of the YRO was similar between light-treatment and dark-treatment (~7%), the times when the culture was exposed to light resulted in the YRO having shorter duration respiratory phases that recurred more frequently. This effect was similar to that caused by treating the oscillating culture with sodium azide, an inhibitor of the electron transport chain, which supports the hypothesis that the period and amplitude effects on the YRO are caused by light interfering with the various cytochromes' abilities to transport electrons to oxygen (the terminal electron acceptor) during respiration. With a reduced capacity to respire, the cells' energy demand would require increased fermentation, altering the NAD^+/NADH proportions as well as perhaps requiring the cells to draw on their storage carbohydrates more frequently. Storage carbohydrate levels and NAD^+/NADH ratios are believed to play major roles in regulating the YRO's switch between oxidative and reductive phases of the YRO [18]. A similar effect was seen in a temporary fashion when cells were suddenly deprived of O_2. Rather than the persisting effect of shorter periods and

amplitudes that continually inhibiting cytochromes caused, inhibiting respiration in an acute manner (by dropping O_2 levels), resulted in a temporary effect, namely a phase shift at certain times of the oscillation. One explanation for the phase dependency of this effect is that in early phases of the YRO when O_2 depletion didn't have an effect, the redox state of the cells (e.g. the NAD^+/NADH ratio) already favored fermentative/reductive metabolism so no change between oxidative and reductive phases of the YRO could be made by removing oxygen [16]. In addition, the storage carbohydrate levels are generally low during the early phases of the YRO [18].

The finding that light affects the YRO is relevant for more reasons than simply to warn YRO investigators to control for this variable. The ease at which light can be added to or removed from continuous culture provides researchers another way to manipulate the YRO for research purposes. There remains a debate as to why some yeast produce YROs with short (40 min) periods while others (like the ones studied here) have longer periods (3-5 h) [35-37]. Some argue that these are two separate phenomena with different underlying mechanisms, but perhaps the differences primarily lie in the strains' intrinsic respiratory rates. We have shown that light is capable of reducing the longer period YRO to something closer to that of the shorter period YRO, and hopefully through such techniques the two YROs can eventually be reconciled. These findings also warn of problems that may come from using fluorescent reporters to study the YRO, especially those that are excited by blue light like GFP. Although the issue of fluorescence excitation was not specifically addressed in this investigation, it would be naïve to think that the blue wavelength excitation used to view live yeast does not impact their physiology.

Finally, we showed that light inhibited growth in various strains of yeast, and that treating the CEN.PK and IFO0233 strains with light caused those yeast to upregulate the production of pigments which are most likely carotenoids. It is quite interesting that both

of the strains that show the light-induced pigmentation effect can also manifest the YRO. It could be that qualities related to the formation of the YRO are also involved in the pigmentation under light, or that these phenotypes have coevolved. With only two strains, coincidence cannot be ruled out. We need to test other YRO-competent strains for pigment production under white light.

The adaptive significance of the pigmentation (and/or respiratory) effect as a response to light has yet to be shown, but the absorption spectrum of the pigment (absorbing strongly in the UV to blue range) suggests that the pigments may protect the yeast from damaging rays present in or associated with visible light. Experiments testing the ability of light-exposed yeast to survive UVB-irradiation compared to yeast grown in the dark do not show a significant fitness advantage for expressing the pigment, but relative fitness will be more closely investigated in a later study. The primary achievement of this work was to show that yeast could perceive light in a manner relevant to the YRO and provide a glimpse into mechanisms by which this may occur.

Materials and Methods

Illumination during Continuous Culture

The YRO was established with the CEN.PK113-7D strain of *Saccharomyces cerevisiae* in continuous culture in a Bioflo 110 (New Brunswick) as described previously [74]. White light of 90, 180, or 300 μEinsteins/m^2/s was applied to the Bioflo 110 culture vessel by placing 1, 2, or 3 compact cool white fluorescent lamps around the vessel's water jacket. When two lamps were used, the lamps were placed on opposite sides of the vessel. When 3 lamps were used, two were placed on opposite sides and the third was placed in between the other two (forming a C shape around the vessel). For colored light treatment, a single layer of Roscolux color filter (#74 for blue, #89 for green,

#19 for red; Rosco Laboratories, Stamford, CT) was wrapped around the water jacket of the Bioflo 110 and three compact cool white fluorescent lamps were used as described above. For the red light treatment, in addition to the three compact cool white fluorescent lamps, a 60 watt incandescent lamp was positioned 6 inches from the filtered vessel. Light intensity from these arrangements was measured in the Bioflo 110 with the headplate and culture removed. Light intensity was measured using a Li-Cor quantum radiometer/photometer (LI-185B) and was the average of 8 measurements taken at 45° increments around the water jacked from the vessel's interior.

Batch Respiration Measurements

Eight 10-ml cultures of CEN.PK113-7D were grown for 24 h in 50 ml flasks shaking at 28°C. Supplemented minimal media consisted of 6.5 g/L Difco yeast nitrogen base w/o amino acids, 20 g/L glucose, 23.6 mg/L leucine, 15.6 mg/L uracil, 15.6 mg/L tryptophan, 15.6 mg/L histidine, 15.6 mg/L adenine, and 23.6 mg/L lysine. Four of the cultures were wrapped in aluminum foil (grown in the dark) and four of the cultures were unwrapped and exposed to 200 $\mu E/m^2/s$ of white light from a compact cool white fluorescent lamp (positioned overhead about 4 in. away). After overnight growth, light-grown cultures were pooled (40 ml total), gently centrifuged, and resuspended in 4 ml of fresh media, on ice. The same was done for dark-grown cultures. Batch respiration measurements were made at room temperature by adding 0.25 – 1.0 ml of the concentrated starter cultures described above to 40 ml of fresh supplemented minimal media in 50 ml flasks. The mouth of the measurement flask was sealed with a #0 rubber stopper surrounding the dissolved oxygen probe (Vernier #DO-BTA). The cultures were gently stirred magnetically during measurements. Prior to measurements, the dissolved oxygen probe was calibrated to 0% DO with 2M Na_2SO_3 and to 100% DO in gently stirred media without yeast (stirred at the same rate as DO measurements). DO

measurements were recorded over time with Logger Pro3 software (Vernier). Light from a compact cool white fluorescent lamp was applied to cultures during DO measurements for designated experiments after the first 15 min of DO recording. A 75 cm^2 cell culture flask (Corning #25111-75) filled with room temperature distilled water was placed between the light source and the culture as an infrared screen. Additionally, the DO probe (except for the tip) was covered with aluminum foil to reduce the noise that light added to the probe's measurements.

Phase Response Curves

An 850 ml oscillating culture of CEN.PK113-7D was allowed to establish a stable period as described previously [74], sparged with 0.9 L/min air. At various phase points described below the gas supply to the Bioflow 110 was switched from air to either 0.9 L/min of 100% O_2 (for 30 min treatments) or 100% N_2 (for 3 min treatments). Following gas treatment, the air supply was returned to normal (0.9 L/min air). Phase 0° was defined as the time when dissolved oxygen started to rise from hypoxia. Phase shifts were determined from measuring the difference between (1) the time of the DO trough in the cycle after the gas treatment and (2) the time that the DO was predicted to reach the trough without a gas treatment extrapolated from the previous oscillation's period. Phase response curves were normalized for 360° of period and were double-plotted for the beginning of N_2 treatment (at 0°, 45°, 90°, 135°, 146°, 157.5°, 169°, 180°, 225°, 270°, and 315°) and the cessation of O_2 treatment (at 51°, 96°, 141°, 186°, 192°, 208.5°, 231°, 276°, 321°, and 366°).

Growth Curves

An overnight culture of CEN.PK113-7D was grown in supplemented minimal media (described above), diluted 1/100 in 150 ml of fresh supplemented minimal media,

and aliquoted into twelve 50-ml flasks (10 ml culture into each). Each 10 ml culture either received 5 µl of methanol (vehicle) or 5 µl of 100 mM diphenylamine dissolved in methanol (50 µM final concentration DPA). The mouths of all flasks were covered with aluminum foil with 4 needle holes punched in each for air. Three of the flasks with vehicle and 3 flasks with DPA were covered completely with foil. All flasks were grown at 28°C with moderate agitation under 250 $\mu E/m^2/s$ white light from compact cool white fluorescent lamps positioned about 4 in. above the cultures (but cultures in foil were shielded from the light). A 9 in. fan circulated air across all flasks to help maintain a uniform temperature. Samples (100 µl) were taken from each flask periodically and analyzed in a Eppendorf BioPhotometer for optical density at 600 nm.

Pigment Analysis

After 36 hours of growth, the three light-grown cultures treated with vehicle (from the growth curve experiment described above) were pooled, gently centrifuged, washed once with 50 ml of distilled water, and resuspended in 3 ml of distilled water. The same was done for the other cultures from the growth curve experiment and these concentrated cultures were photographed in a 24-well dish. Pigments were extracted from light-grown and dark-grown vehicle-treated cultures as follows. Light-grown and dark-grown cells were diluted as needed to give pellets of equal weight, ~ 100 mg (i.e. some cells from the dark-grown culture were discarded since these cultures grew denser). The pellets were mixed with an equal weight of glass beads (sigma #G8772) and resuspended in 500 µl of lysis buffer (10 mM Tris pH8, 1 mM EDTA, 100 mM NaCl, 1% SDS, 2% Triton X-100). Cells were chilled on ice then lysed by vortex for 10 min at 4°C. Lysates were separated from the beads by punching a hole in the bottom of both centrifuge tubes with a syringe needle (18 gauge), placing each tube (containing the hole and lysate) into a separate tube, and collecting the lysate by centrifuging the stack

for 10 sec at 1000 rpm. The lysate was centrifuged for 5 min at 13,000 rpm and the supernatant was discarded. The pellets were each resuspended in 1 ml of cold methanol/chloroform (50:50) with aggressive pipetting and vortexing. Tubes were centrifuged at 13,000 rpm for 20 min at 4°C. Supernatant from each tube (containing pigments) was moved to a new tube and an aqueous solution of 1% NaCl was added with vortexing until phase separation occurred (pigments followed the chloroform layer). Phases were separated by centrifuging at 13,000 rpm for 10 min at 4°C. The aqueous phase from each tube was discarded and the chloroform phase was concentrated to ~ 300 µl by speedvac. Absorption spectrum of the concentrated chloroform phase was measured from 350 nm to 800 nm by a Shimadzu UV-160 spectrophotometer.

CHAPTER V

LIGHT EMITTING DIODE FLASHLIGHTS AS EFFECTIVE AND INEXPENSIVE LIGHT SOURCES FOR FLUORESCENCE MICROSCOPY[2]

Preface

Firefly luciferase has previously been shown to be a useful tool for monitoring promoter activity in *S. cerevisiae* both in batch cultures and continuous culture (chapter II), however we are also currently working on another *in vivo* luciferase reporter for *S. cerevisiae* that reveals a cell's (or a colony's, or a culture's) intracellular pH. This is of particular importance because one of the factors believed to oscillate in the YRO is intracellular pH [38]. In addition, intracellular and extracellular pH changes have been implicated in cell-cell communication and colony-colony communication in yeast [123], therefore developing methods that reveal these effects in real time agrees with the focus of the work presented in this dissertation.

The prototype bioluminescent pH reporter makes use of a pH-dependent Bioluminescent Resonance Energy Transfer (BRET) between two ends of a luciferase-YFP protein fusion. In a pH dependent manner, differing amounts of light generated from the luciferase reaction are passed to the YFP fluorophore, resulting in a ratiometric color change in the light emitted. One of the early recognized caveats for using microscopy to visualize pH changes in colonies or groups of cells was the necessity of finding yeast (or cells of other types) that strongly expressed the reporter in the microscope's field of view, in addition to having them in the proper focal plane, before

[2] The material in chapter V following the preface is in press by the *Journal of Microscopy* (2009) by Robertson, J.B., Y. Zhang, and C.H. Johnson.

making the pH measurements. Relying on the cells' bioluminescence for indication of strong reporter expression and proper focus was not practical since such measurements require exposure times in excess of 1 min, as well as requiring the investigator to sacrifice luminescence intensity as a result of substrate consumption. The better approach is to use the fluorescent quality of the reporter (YFP) to find strongly expressing cells and the proper focus for measurement. But this approach, too, had drawbacks that needed to be overcome.

Visualizing BRET by microscopy requires a photon-free environment due to the very low light levels emitted by single cells. Stray light from non-sample sources increases noise to the measurement and can hide the BRET signal altogether. Therefore BRET microscopes must be shielded from light by a black box and measurements must be taken in the dark. Using a traditional mercury lamp for excitation of the YFP in our lab was impractical since measurements would require the mercury lamp to be turned on and off repeatedly. Leaving the lamp on but shuttered was not sensible because heat would build up within our box. In addition, stray light would leak from the lamp through the lamp's vent. The solution was to devise a low cost LED light source that could excite YFP through the microscope and that could be instantly turned on and off from outside of the black box. The material that follows describes this LED light source development.

Introduction

Traditionally, broad-spectrum mercury gas discharge lamps have been used as sources for excitation in fluorescence microscopes. The advantage of this type of excitation is that mercury lamps provide bright, broad-spectrum excitation that is filtered to provide specific wavelength bands (although the "broad spectrum" emission of

mercury lamps is not uniformly distributed and is dominated by 7-8 strong peaks between 300 and 900 nm). While these lamps are the standard for microscopic fluorescence applications, they have a number of limitations. First, the wide range of emitted wavelengths includes ultraviolet (UV) light that is highly deleterious to living biological samples. With modern UV-blocking filters most of this excitation is excluded, but there is always a finite leak of UV that can decrease the viability of very sensitive tissues or cells [124]. For example, cell division is sometimes inhibited when irradiating cells with fluorescence excitation derived from mercury discharge lamps [125]. Second, the lifespan of a mercury discharge lamp is usually only 200-300 hours, and the intensity of these lamps decays progressively during this time [124, 126]. Third, gas discharge lamps require at least several minutes to reach an operating equilibrium after being turned on and lamp intensity can fluctuate during use. Therefore, once mercury discharge lamps are turned on, they are usually left on for hours to enable fluorescence measurements as needed without delay [127], thereby shortening lifetime. Fourth, these lamps generate a significant amount of heat and therefore introduce complications when used in a confined space [126]. Finally, mercury discharge lamps can explode, thereby damaging lenses and/or mirrors within the lamp housing [126].

Light emitting diodes (LEDs) show great promise as alternative light sources for fluorescence microscopy [124, 126, 127]. Compared to the broad-spectrum emission of mercury discharge lamps (which then requires filters), LEDs traditionally have been designed to provide illumination at very specific wavelength bands from UV to infrared. However, current "white" LEDs have been designed to emit relatively broad excitation that can be filtered to provide specific wavelength bands as with mercury lamps ("white" LEDs tend to have a preponderance of blue emission, as seen in Figs. 5.1A and 5.2). Moreover, no UV emission is generated as a by-product in LEDs that are not specifically designed for UV emission. When turned on, LEDs can achieve full brightness within

microseconds with a constant intensity thereafter; upon being turned off, they extinguish immediately without a prolonged glow [127]. Previously the intensity of LED emission was rather dim, but new generation LEDs are much brighter and can additionally have lifetimes as long as 50,000 hours (or more). LEDs are relatively cool and therefore can be used in confined spaces. Not surprisingly, several companies now offer LED-based excitation systems for fluorescence microscopy to capitalize upon these advantageous characteristics of LEDs. However, since these commercially available LED fluorescence sources are designed to be capable of excitation at many different wavelength ranges, they are expensive. We have adapted a commercially available white LED flashlight for use as a source for fluorescence excitation. This light source has the advantages of LED light sources as itemized above and is effective for excitation in the range of 440-600 nm, especially when coupled with CCD detection.

Results and Discussion

We have a CCD-coupled microscope in a light-tight box for luminescence and BRET imaging [128] that we wanted to use for fluorescence applications but we needed to keep the box closed throughout imaging measurements; consequently the box could not be opened to change filters, open/close shutters, or focus the microscope. The characteristics of an LED source were attractive for our application, especially the low heat generation and the ability to immediately start and stop fluorescence excitation by simply turning the LED on and off rather than by using a remotely controlled shutter. However, the price of the commercially available LED sources was unappealing. Given that LED flashlights have been used to detect GFP in whole animals [129], we investigated whether a simple commercially available white LED flashlight could serve as an excitation source for fluorescence microscopy, thereby reaping the benefits of LED

excitation while avoiding the high cost of the excitation sources that have been commercially designed for this purpose. We therefore went to a local camping equipment store and purchased the brightest white single-LED flashlight on the shelf; in our case, this was an Inova Bolt 4.6 watt/6 volt flashlight for a price of approximately USA$ 50. The flashlight's emission spectrum was measured with a fluorescence spectrophotometer (Quantamaster QM-7/SE, Photon Technology International, Birmingham NJ, USA) and showed strong emission in the 440-480 nm range (Figs. 5.1A and 5.2), which is a suitable excitation range for commonly used fluorescent probes such as: (i) the fluorescent proteins ECFP, EGFP, EYFP; (ii) the fluorescent pH indicator BCECF; (iii) fluorescein and its derivatives (e.g., FITC and various Alexa Fluors); and (iv) nuclear/nucleic acid probes like YO-PRO-1 and YOYO-1. The spectrum of the Inova Bolt flashlight also exhibited a dimmer emission extending into the red and could therefore be used for longer wavelength excitation in conjunction with a sensitive CCD detector. The manufacturer estimates the lifetime of the LED in this flashlight to be approximately 50,000 hours.

The Inova flashlight is normally powered by two 3 V lithium batteries with an estimated lifetime of 2.5 hours, but as shown in Fig. 5.1B, we replaced the batteries with a dummy battery component that allowed us to connect the battery terminals to an inexpensive power supply operating at 6 V (we used a Tekpower DC Variable Power Supply # HY152A, which cost USA$ 40 from Amazon.com). The illumination of the LED is therefore controllable remotely from outside our light-tight box by turning on and off the power supply. A simple adaptor was also machined on a lathe from aluminum to connect the flashlight to our microscope's port for the fluorescence lamp housing (Fig. 5.1B,C); however, the flashlight could also function effectively as an excitation source when simply held in place by a ring-stand and a clamp. Therefore, the mercury lamp

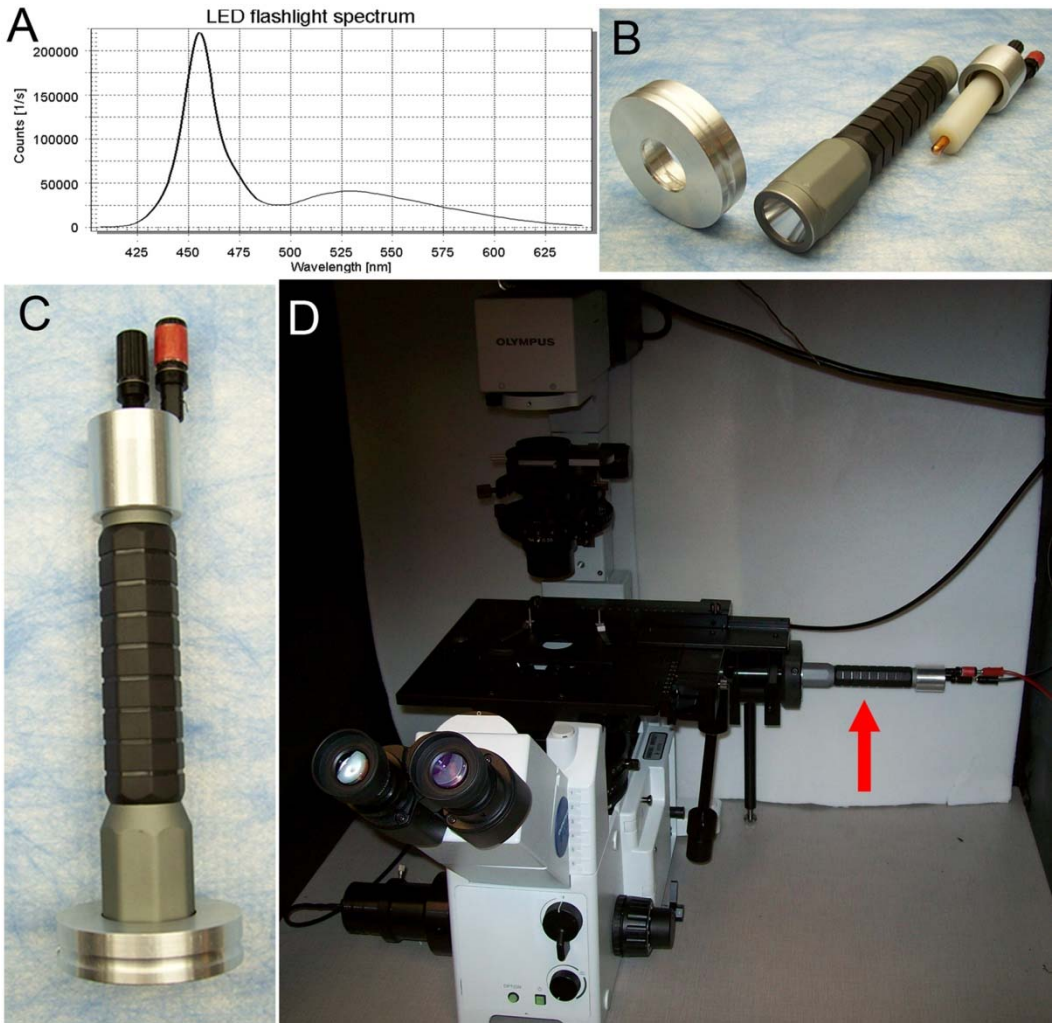

Figure 5.1. The LED flashlight as a tool for fluorescence microscopy. **A.** Emission spectrum from the flashlight from 400 nm to 650 nm. **B.** Disassembled flashlight excitation source showing the microscope adapter ring on the left, the commercial flashlight in the middle, and the 6 VDC dummy battery adapter on the right. **C.** The assembled flashlight excitation source. **D.** The LED flashlight excitation source (red arrow) attached to our Olympus microscope in the light-tight box.

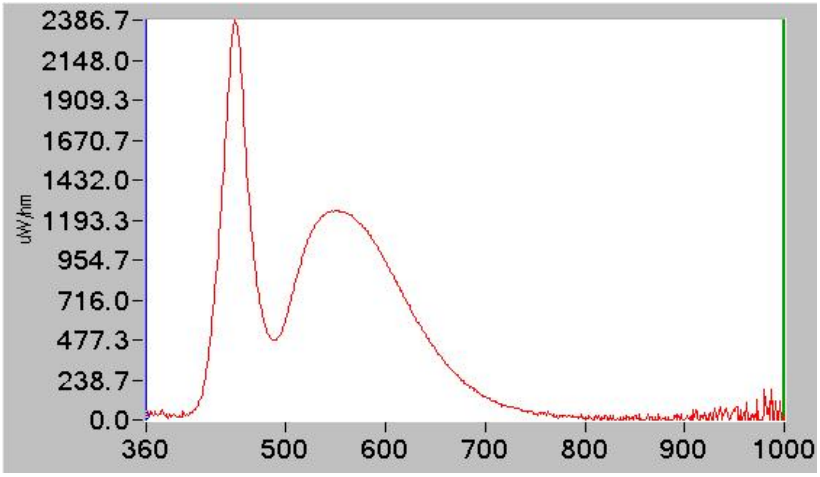

Figure 5.2. The spectrum of the LED in the flashlight as supplied by the manufacturer.

(with its mirror and collector lens) was removed and replaced with the LED flashlight. There is no other difference in the optical train when the LED flashlight is mounted. The reflector within the flashlight acts to focus the beam in the same way as the mirror/lens within a mercury lamp housing. With the mercury lamp housing on the Olympus IX71 inverted microscope replaced with our flashlight, the system appears as in Fig. 5.1D (a color CCD camera [an Olympus DP72] is coupled to the bottom port on the microscope and is therefore out of view).

This white LED flashlight source is less intense than a mercury source, but the fluorescence images shown in Fig. 5.3 were easily visible by eye. For excitation at 470/30 nm ("30" = bandpass width at half peak height), the mercury source was 2X more intense than the LED source and at 500/20 nm, it was 4X more intense (Table 5.1). However, in conjunction with a CCD detector, comparable images were achieved using the LED flashlight source with a camera exposure of about 4-5 times the duration of that required by a 100 Watt mercury lamp. For example, the fluorescence images of Alexa Fluor 488-labeled actin filaments in raccoon uterine cells excited by the 100 W mercury lamp and captured with a 1 second exposure (Fig. 5.3A) are equivalent to those collected by a 5 second exposure with the LED flashlight source (Fig. 5.3B). As another example, we also used our LED source to excite the red autofluorescence of chlorophyll in the stem of a plant seedling (Figs. 5.3C & 2D).

Therefore, for fluorescence microscopy using excitation in the most commonly used range of wavelengths, this flashlight system allows an effective way to reap the benefits of LED excitation. The total cost of this flashlight and power supply (~$ 90 total) was less than the cost of a single replacement lamp for a mercury discharge fluorescence source and has a predicted lifespan of ~300 X longer. This technology provides a way to economically add LED excitation capability to an existing mercury-discharge

Table 5.1. Relative light intensities for LED vs. Mercury Sources. Light intensities from the LED and mercury light sources were measured through the Olympus IX71 microscope at the position of the sample using the excitation filters in the microscope. Light intensity was measured at the position of the sample with a LI-COR 185B Quantum Radiometer/Photometer by attaching the probe to a microscope slide mounted on the microscope. The filter sets used for these measurements were the Chroma YFP filter set #410290 with excitation at half band pass 500/20, and Chroma ZsGreen filter set #42002 with excitation at half band pass 470/30. Light intensity was measured in μEinsteins/m^2/s. Three different microscopic objectives were used (4X, 10X, and 20X), with similar results. Note that the intensity of the LED source compared more favorably with the mercury source for blue excitation (470/30 nm) than for blue-green excitation (500/20 nm), presumably because of the large peak emission of the LED in the 450-475 nm range.

Source	**LED**		**Mercury**		**Ratio (Mercury:LED)**	
Excitation Filter	500/20	470/30	500/20	470/30	500/20	470/30
4x Objective	25	71	115	160	4.6	2.3
10x Objective	28	83	110	155	3.9	1.9
20x Objective	24	72	100	140	4.2	1.9
Average	25.7	75.3	108.3	151.7	4.2	2.0

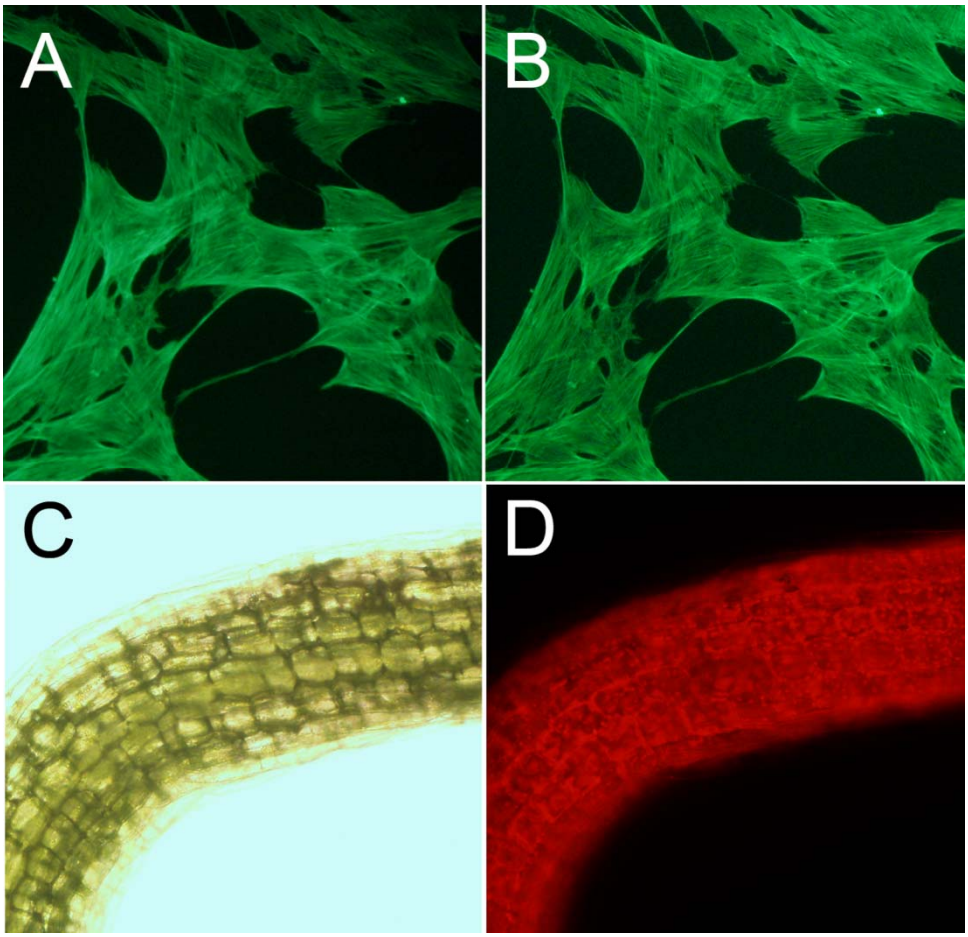

Figure 5.3. Images from the LED-excitation system. **A.** Fluorescence of actin filaments in raccoon uterine cells labeled with phalloidin conjugated to Alexa Fluor 488 excited by the 100 W mercury arc lamp with a 1 s exposure. **B.** The same fluorescent object as in panel A except excited by the LED flashlight with a 5 s exposure. **C.** A bright field image of the stem from an *Arabidopsis* seedling. **D.** The same object as in panel C examined for chlorophyll autofluorescence excited by the LED flashlight source (5 s exposure). For panels A&B, the microscopic objective was 20X, and the fluorescence filters were EX 470/30; EM 520/40 (Chroma ZsGreen filter set #42002); for panels C&D, the microscopic objective was 10X; for panel D, the fluorescence filters were EX 500/20; EM 520LP (Chroma YFP filter set #410290). See Supplementary Material for more information about the spectrum and intensity of the LED source we used as well as information on the fluorescence filter sets.

fluorescence microscope. It may also be an excellent single-source solution for schools with limited resources to set up laboratory exercises involving fluorescence microscopy or research laboratories that wish to economize in the current challenging era of limited research budgets.

Acknowledgements

We thank Jeff Jones and Mark Boyd (Olympus America Inc.) for the loan of the DP72 camera, and Bruce and Roger Williams for machining the adapter and dummy battery. This project was supported by a grant from the NIMH (MH080035).

Supplementary Material

Spectrum of LED light emission

The spectrum from the Inova Bolt 4.6 watt/6 volt flashlight in Fig. 5.1A was measured using a Quantamaster QM-7/SE spectrophotometer with the light source introduced into the black chamber of the device through a 3 mm aperture at a right angle from the detector in order to minimize risk to the sensitive photomultiplier. As a result of unequal reflection, parts of the spectrum in Fig. 5.1A may be underrepresented. Fig 5.2 shows the spectrum form the Inova Bolt provided by the manufacturer.

Filter Sets Used

Two different filter sets were used in this study. Chroma ZsGreen1 #42002 has an excitation filter HQ470/30x, a dichroic Q495LP, and an emission filter HQ520/40m. The spectrum and specifications for this filter set can be seen at Chroma's website: http://www.chroma.com/products/catalog/42000_Series/42002

The other filter set Chroma Yellow GFP LP (10/c Topaz) #41029 has an excitation filter HQ500/20x, a dichroic Q515LP, and an emission filter HQ520LP. The spectrum and specifications for this filter set can be seen at Chroma's website: http://www.chroma.com/products/catalog/41000_Series/41029

CHAPTER VI

GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Impact, Principal Findings, and Achievements

The work presented here involved exploring various aspects of the yeast respiratory oscillation in relationship to the cell division cycle and mechanisms by which the YRO operates/persists. The influences of respiratory capability and CDC synchrony contribute to the stability, amplitude, and period that are characteristic of CEN.PK's YRO. This work has potentially provided clues as to how the longer period YRO of CEN.PK may be similar to (and different from) the shorter period YRO of other strains by the degree to which the CDC can align with the YRO and the degree to which respiration can occur in these strains. Additionally through this work, technical tools were developed that can aid other researchers in questions unrelated to these topics, thus expanding the impact this work has on the global community. Materials developed in this work have already been requested by labs around the world, from Harvard to as far away as Europe and China.

The principal findings and achievements of this work are:

- A destabilized firefly luciferase reporter was developed for monitoring promoter activity for genes of interest *in vivo* and in real-time.
- Cell cycle landmarks were established for the YRO using luciferase reporters.
- A direct correlation was shown for phase resetting of the YRO to non-fermentable metabolites and premature induction of new cell division.

- The methods by which the YRO and CDC communicate and maintain a stable interaction are more complex than two populations that alternate in cell division timing.
- The CDC influences the period, amplitude, and ultimately the stability of the YRO for CEN.PK.
- Visible light, primarily in the blue and green wavelengths, affect the period and amplitude of the YRO in CEN.PK, presumably by damaging the cytochromes needed for respiration.
- Visible light inhibits growth in a number of strains of yeast and causes two in particular (CEN.PK and IFO0233) to turn pinkish-orange, presumably by upregulating the production of carotenoids.
- A white LED flashlight was shown to be a low-cost, effective fluorescent excitation source for use with BRET and bioluminescent microscopy where mercury lamps are problematic.

How Cell Division Synchrony May Be Achieved During the YRO

The cell division cycle oscillates along with the YRO (chapter II) [13-18]. And evidence from chapter III shows that the CDC plays a part in the YRO's stability/existence (at least for the longer period YRO of CEN.PK), rather than the CDC synchrony being merely an output of the oscillating energy metabolism. One way that the CDC might synchronize with the YRO is through a common nutritional molecule (e.g. glucose) that oscillates in the YRO and is required at a certain concentration to pass Start. For instance, slowly growing cells in G1 delay progression through Start due to the low glucose levels in the environment and within the cell, however the synchronous release of glucose from carbohydrate stores (during a particular phase of the YRO near

the trough) satisfies Start-related nutritional requirements allowing the cells that have been held in G1 to simultaneously progress through Start.

There is no direct evidence for an increase of intracellular glucose levels during or near the YRO trough (such as from glucose assays or reporters), however we can infer this increase occurs by observing the response/expression of glucose-regulated genes during the YRO. Because the timing of this event occurs during the hypoxic mask, luciferase reporters unfortunately cannot detect this glucose spike. However, publically available microarray data across three oscillations of the YRO that are online on the *Saccharomyces cerevisiae* Periodic Transcription Server (SCEPTRANS) [130] allows the construction of gene activity profiles for such glucose-regulated genes [16]. Fig. 6.1 shows the microarray expression profile for four genes oscillating during the YRO: *POL1* – a CDC related gene that provides a familiar reference for the YRO shown in chapters II and III, *ADH2* – a gene that is greatly downregulated by the presence of glucose [131], *PMA1* – a gene that is upregulated by the presence of glucose [132], and *CLN3* – a CDC-related gene believed to translate nutritional information into CDC regulation [5] .

As Fig. 6.1 shows, *ADH2* expression sharply decreases at the same time that *PMA1* and *CLN3* sharply increase, followed later by an increase in *POL1* expression showing entry into the cell cycle. The increase of *PMA1* expression is timed exactly with an increase of *CLN3* expression. The collective expression profile of these glucose-regulated genes suggest that a large portion of cells in the YRO experience a simultaneous increase in glucose levels prior to commitment to cell division and that it could be this glucose bolus that permits synchronous cell division. As a result of a large population of cells synchronously progressing through S, G2, and M phases of cell division, CDC-related metabolic influences (e.g. energy demands, pathway preferences, byproduct formation, etc) could then in turn feed back on the regulation of the YRO, thus

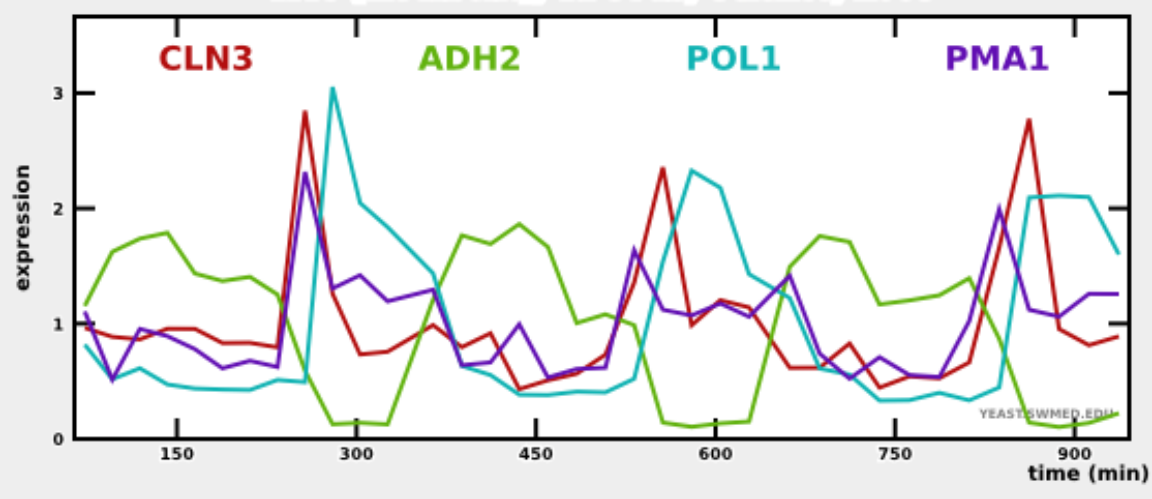

Figure 6.1. Gene expression profiles by microarray for four genes during the YRO. The graph was generated by the SCEPTRANS web tool using data from Tu, et al. [16]. POL1 marks synchronous cell division with a pattern similar to that of the luminescent *POL1* reporter shown in earlier figures. PMA1 is a gene that is upregulated by glucose. ADH2 is a gene that is down regulated by glucose. CLN3 is a gene that is believed to regulate cell cycle entry based on nutritional status of the cell. CLN3 and PMA1 begin to rise at the same time as ADH2 begins to decrease each cycle, suggesting that the cell is exposed to increased levels of glucose at these times. Shortly after, POL1 levels increase showing that cells have synchronously entered S phase.

allowing the cell division rate (determined by the dilution rate of the culture) to influence the period and stability of the YRO.

Future Directions

Determining whether the YRO is a legitimate natural phenomenon rather than an artifact of the continuous culture conditions within the bioreactor depends on demonstrating that it operates in yeast outside of the bioreactor, either on a single-cell level or in populations in a different setting (e.g. colonies on solid media). It is possible that the YRO only occurs in the specialized conditions of the bioreactor. On the other hand, it is possible that the YRO commonly exists on a single-cell level in a wide range of culture conditions; however the bioreactor simply allows individual cells' YROs to align and become measurable in the laboratory. Another idea is that the YRO may exist in a more natural setting when certain requirements (namely high cell density for the generation and dispersal of YRO related signals, low glucose/slow cell division, and the presence of oxygen) are met. Colonies growing on solid media may provide these conditions. The luciferase reporter system developed in this work will be instrumental for these continuing studies.

Pursuing the YRO on a Single-Cell Level

The YRO does not manifest under all conditions of continuous culture. The oscillation requires a specific range of pH, dilution rate, temperature, oxygen, light intensity, culture density, and growth rate in order to persist. One question that remains to be answered is, "Under continuous culture conditions where the YRO is not observable for the culture as a whole, does the YRO still operate in uncoupled individual

cells?" In other words, it is possible that in addition to the CDC, actively dividing cells also undergo their own individual YRO (each with its own period, etc) that is not coupled, transmitted, or communicated to other cells in the population. As a result, cultures of such uncoupled YROs would appear to be arrhythmic. Whether this occurs could be investigated if individual cells were able to be continually monitored for rhythmic metabolism as they grew. What is needed is a microscopically observable continuous culture device on a nano-scale where metabolism can be monitored. We are investigating whether one micro-fluidic device in particular will suit these purposes.

Figure 6.2 shows the basic shape and function of the micro-fluidic device. It consists of a triangular chamber with small "c-shaped" traps in which inoculated yeast settle against a current of media. The traps can be loaded with single cells or groups of cells, and their cell division followed over time by microscopic observation/photography. Once the traps are full, (from either growth or loading) continued cell division results in cells spilling over the sides and out through the effluent. Therefore the device reproduces several features of continuous culture achieved in liter-scale bioreactors. Using the luciferase reporter either as an oxygen sensor or a promoter monitor, the cells can be individually observed for uncoupled (or coupled) rhythmic behavior (Fig. 6.2).

We plan to also use these devices to search for diffusible signals which may be influencing the YRO or allowing cells to communicate their position in the oscillation with other cells in the culture. The nature of the device is such that the direction of the media flow allows cells upstream of the flow to condition the media for cells that are downstream. As a result, the downstream cells would be exposed to potential secreted signals/metabolites to which the upstream cells are not exposed, and there may arise an observable difference (e.g. entrainment) in rhythmic metabolism in the chamber. Another way the presence of diffusible/media-borne signals may be tested with these devices is to feed the cells in the nano-devices with cell-free bioreactor effluent from the

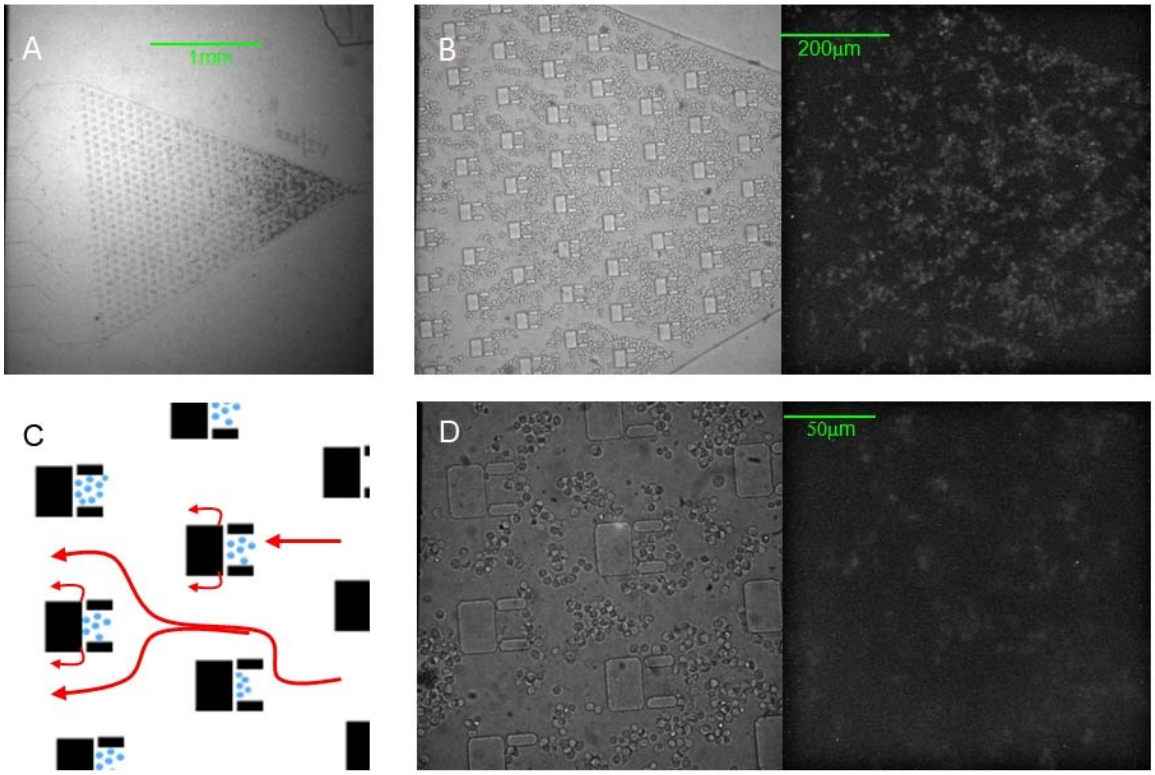

Figure 6.2. A micro-fluidic device for growing small groups of cells in continuous culture on a nano-scale. **A.** shows the entire triangular chamber. Cells are loaded and then later fed from the single channel at the point of the triangle on the right. The darker area toward the right is yeast. The far left of the chamber shows 6 outflow channels. **B.** shows the device at a higher magnification. Left is bright field, Right shows luminescence from P_{GAL1}-Luc yeast in galactose media. The C-shaped traps are visible; however media is not being perfused through the device so the cells do not remain in the traps. **C.** A diagram showing how media flows through the device (red arrows) and keeps cells (blue circles) in the traps (black boxes). **D.** shows an even closer view of the device and cells. Left is bright field and Right is luminescence from P_{GAL1}-Luc yeast in galactose media. Green bars indicate length as shown.

larger (liter-scale) bioreactor in which the YRO is taking place. By continuous microscopic observation of cells exposed to the YRO-conditioned media, we could see whether the CDC (or metabolism) from cells outside of the YRO could be entrained with the media; and if so, we could observe the population structure that results. A preliminary experiment where media from an oscillating bioreactor was washed over a bag of dialysis tubing containing cells with the luminescence CDC reporter P_{POL1}-LucPEST showed that the YRO-conditioned media could entrain synchronous cell division in a culture of cells outside of the bioreactor (Fig. 6.3). The micro-fluidic device will let us observe this synchronization microscopically, which may give additional spatial information, such as gradients of entrainment.

Using the Luciferase Reporter to Observe Cells in Colonies

The YRO is not the only example of cellular communication in yeast. Yeast growing in colonies have been reported to signal to other colonies through diffusible signals (like ammonia) to influence surrounding colonies' growth [123]. It is also possible that the YRO occurs in cells growing in colonies. We want to investigate such signaling among and within colonies using the luciferase reporters developed in this work. GFP and other fluorescent reporters are not very useful for observing colonies of yeast due to the high degree of autofluorescence that arises from densely packed cells and solid media. The luciferase reporter shows promise for revealing spatial gene expression within colonies for a variety of genes (Fig. 6.4). One goal for future work is to develop microscopy techniques and conditions to use these reporters for monitoring temporal gene expression in colonies as well. The most difficult challenges thus far have been obtaining samples that are bright enough to record over a short exposure time, keeping luminescent cells/colonies in focus long enough to photograph them, and

keeping the colony/agar from drying out during time-lapse. The solution thus far has been to observe young/small colonies that are thin and rapidly growing.

A Future for the YRO?

What does the future hold for researching yeast respiratory oscillations? The answer largely depends on whether the YRO occurs in nature. If the YRO is shown to occur under conditions more closely resembling a natural setting (i.e. outside of bioreactors) then increased interest in this field would likely follow. Evidence of a naturally occurring YRO would open the field for questions regarding YRO-based clocks and their adaptive significance, but such evidence is not critical for the YRO to have future importance. Even if the YRO only manifests in continuous culture, the rhythmic gene expression and partially synchronous cell divisions that accompany the YRO may provide useful applications for industrial production of yeast products.

One can envision a scenario in which production or refinement of a desired product from yeast is hampered by the presence of a complicating protein, enzyme, or pathway naturally present in yeast. For example, this complicating factor could be an interfering protease that degrades the product of interest or could be a substance that competes with the product of interest during affinity column purification. If such a complicating factor provides an essential function to growth or health of the yeast, then a deletion strain that lacks the problematic factor would not solve the problem. One potential solution would be to assay for the rhythmic occurrence of the problematic factor during the YRO and if a timeframe exists when the problematic factor is in low abundance, targeting production of the product of interest for that time window could circumvent complications that arise when both product and factor are present at the same time. Pursuing this concept, continued research may allow industry to use the YRO as a tool for enhancing productivity in some circumstances [47].

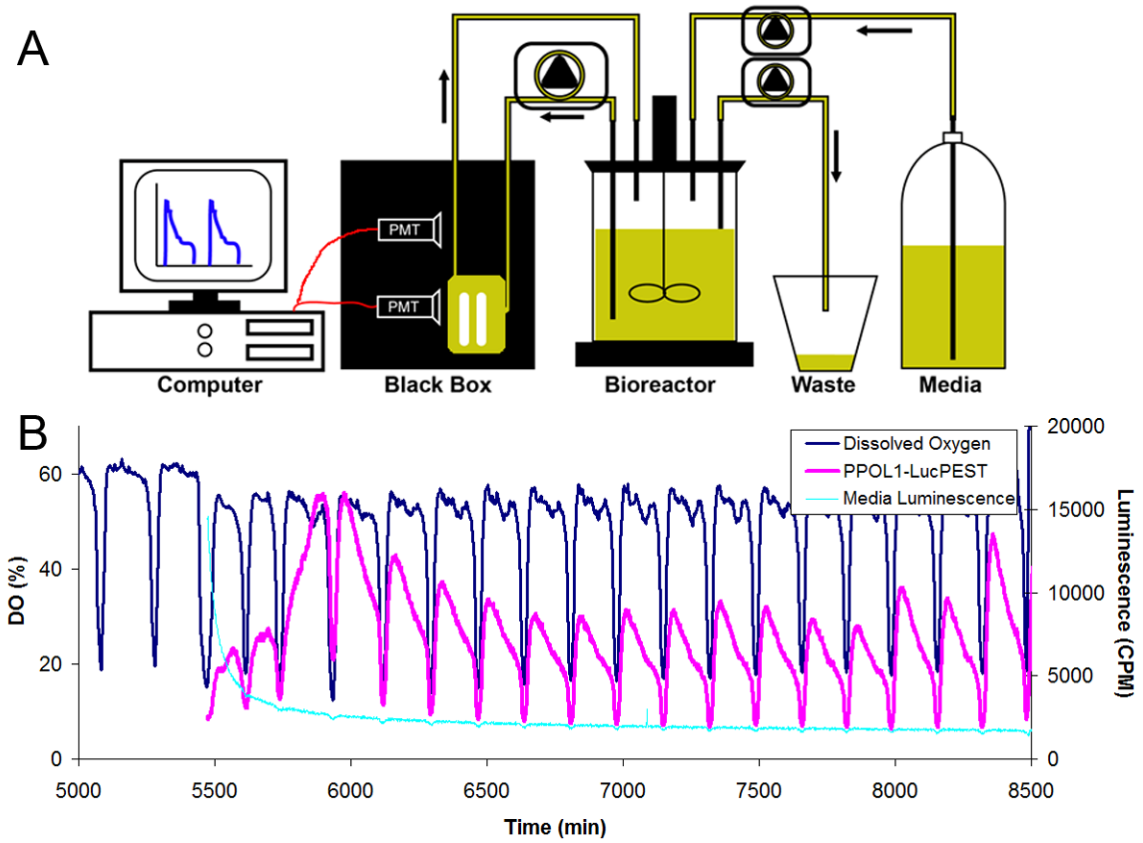

Figure 6.3. Luminescent yeast with the P_{POL1}-Luc(A4V)PEST reporter grown in a dialysis tube outside of the bioreactor show synchronous cell division when exposed to media from non-luminescent yeast expressing the YRO in a bioreactor. **A.** shows the configuration of the apparatus used to monitor luminescence from cells grown in dialysis tubing. Within the blackbox, one PMT (top) monitors light from the non-luminescent culture within the bioreactor exhibiting the YRO. A second PMT (bottom) monitors light from luminescent yeast growing in two dialysis tubes within a 100 ml bottle that has culture from the bioreactor constantly running through it and back to the bioreactor. **B.** The YRO was established using non-luminescent CEN.PK113-7D in a bioreactor (dark blue trace shows oscillating DO levels from the YRO, left y-axis in % atmospheric saturation). The oscillating culture itself is not luminescent (shown by the light blue trace, right y-axis in CPM). However, a culture of yeast containing the cell cycle luciferase reporter P_{POL1}-Luc(A4V)PEST grown in sealed bags of dialysis tubing immersed in culture from the oscillating bioreactor showed synchronized cell division (pink trace, right y-axis) after an initial growth phase for the first 3 oscillations. Redirecting media from the bioreactor to the dialysis chamber caused a perturbation in the oscillation at time 5500 min, but this effect was largely overcome after the third oscillation around time 6000 min. Luminescence shown in pink and light blue traces cannot be directly compared with each other as measures of equal luminescence due to differing sensitivities of the separate photomultipliers used to record each sample simultaneously.

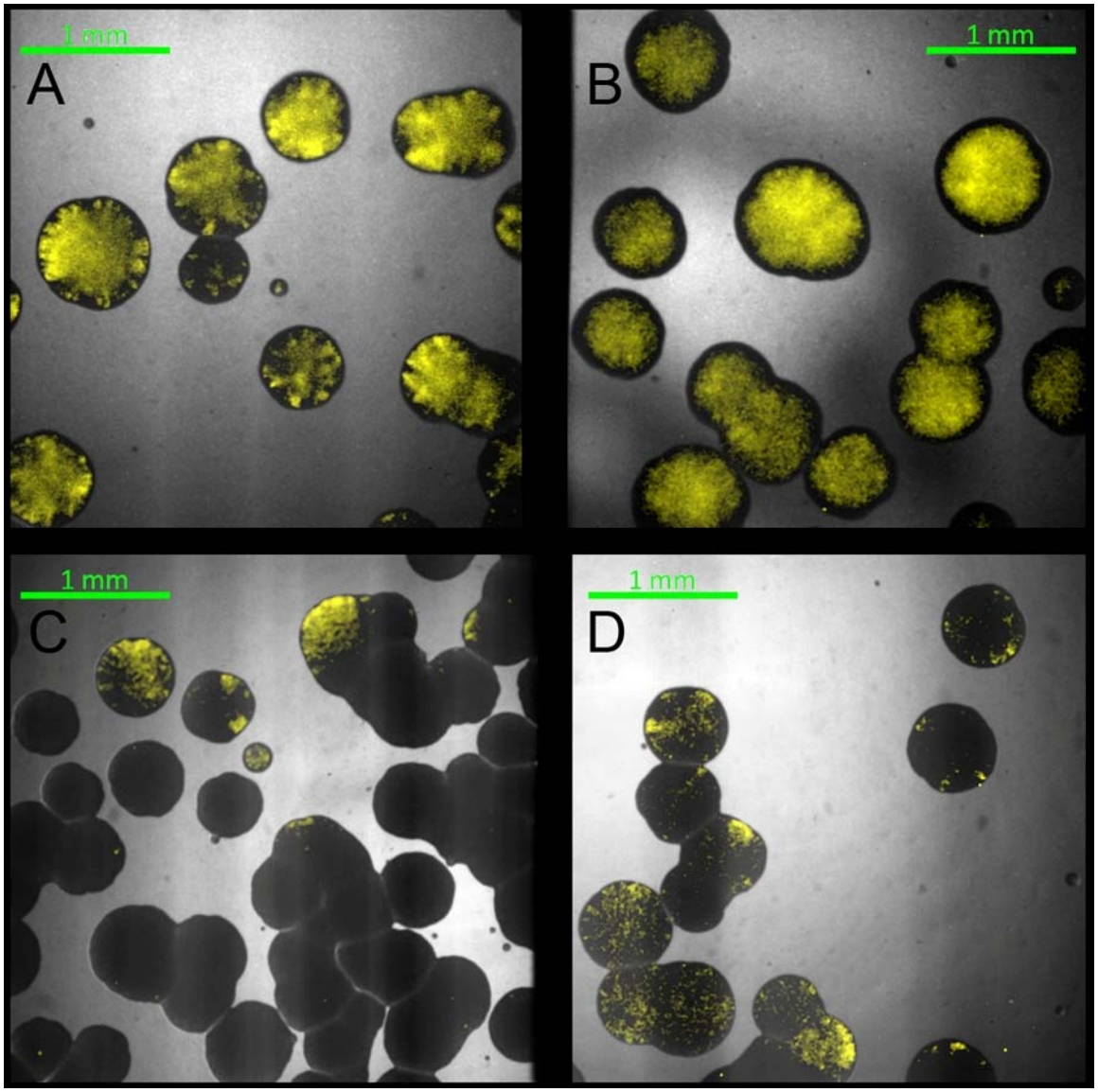

Figure 6.4. Bioluminescent 2-day old colonies of yeast containing different luciferase reporters are visualized at room temperature by an inverted microscope with a 4x objective. Light from bioluminescent yeast was photographed (yellow) and overlaid on the bright field exposure of the same sample to show locations within colonies where promoters for various genes are active. **A.** P_{CYS3}-Luc(A4V) shows areas of active cysteine biosynthesis (exposure time = 1 min). **B.** P_{GPH1}-Luc(A4V) shows areas where glycogen is being mobilized by glycogen phosphorylase (exposure time = 5 min). **C.** and **D.** P_{POL1}-Luc(A4V) show areas of active cell division (exposure times = 5 min). Green scale bars indicate 1 mm. Luciferase reporters do not contain destabilizing PEST sequences and are expressed on high copy number (2 micron) plasmids for purposes of signal detection by microscopy.

APPENDIX A

A LUCIFERASE REPORTER REVEALS THE RATE OF CRE RECOMBINASE INDUCTION AND ACTIVITY IN VIVO

Introduction

The experiment shown here was part of an alternative approach to investigate population structure of the YRO that was abandoned due to complications. However, this experiment is still valuable for providing data regarding the rate at which CRE recombinase functions to remove a segment of DNA flanked by loxP sites. Such information is helpful for interpreting the results of experiments where galactose-inducible CRE recombinase is used to inactivate/remove genes during continuous culture (e.g. Fig 3.7). These results are included in the appendix and not chapter III because this experiment was conducted under different growth conditions than continuous culture and because the assay used a slightly different CRE recombinase construct from the one used in chapter III. Although the experiment reveals a timeframe on which CRE recombinase can be created by the cells and then act to recombine loxP sites in batch culture, the timeframe on which this occurs in continuous culture may be different. Ultimately the strain and the methods developed here can be further modified to conform to the conditions of growth and promoter expression for a variety of systems, e.g. continuous culture, colonies on plates, and in microfluidic devices.

Results

I modified the yeast strain CEN.PK113-7D to include CRE recombinase under the control of the *GAL1* promoter, similar to the construct used in chapter III, with one notable difference. The coding sequence of CRE was fused in frame to the coding sequence of the estrogen biding domain from the human estrogen receptor so that the enzyme would require the presence of estradiol in order to function (Fig. A.1A) [133]. The estrogen binding domain reduces the amount of CRE activity when estradiol is not present [133], and was a feature that was important to the abandoned project.

In addition to CRE recombinase, I modified the strain to include a specialized luciferase reporter (under the control of the *ACT1* promoter) that contained a floxed interruption cassette so that luciferase would only be produced after CRE had removed the interruption cassette. Specifically, the interruption cassette consisted of a floxed kanamycin resistance gene nested within a copy of the intron of *S. cervisiae's ACT1* gene that was then inserted between amino acids 4 and 5 of the luciferase coding sequence. (Fig. A.1A). Within yeast, the transcriptional terminator of the Kan resistance gene arrests transcription of the reporter before the 3' portion of the *ACT1* intron can be transcribed, thus preventing removal of the intron from the pre-mRNA, and preventing transcription of luciferase [133]. However, if CRE recombinase is present, it removes the Kan^r gene from the genomic source by recombination at the loxP sites. The Kan resistance terminator is therefore removed and the intron (containing the remaining single loxP site) can be transcribed in full, along with the remaining 3' portion of the luciferase reporter. The intron containing the remaining loxP site is spliced out by the yeast's native splicing machinery and produces an uninterrupted Luc coding sequence. Once this sequence of events has occurred, luciferase can be translated normally.

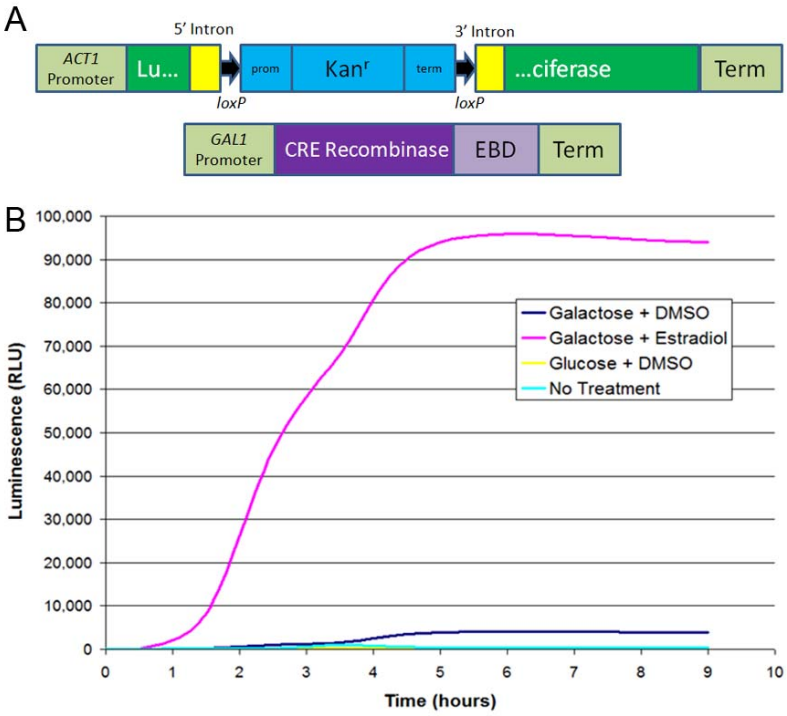

Figure A.1. A luciferase reporter that contains a floxed interruption cassette shows the timeframe that CRE recombinase can act *in vivo*. **A.** shows the features of the two constructs that were integrated into the genome of the yeast strain used for this experiment. An interruption cassette consisting of a kanamycin resistance gene (blue) flanked by loxP sites (black arrows) and nested inside the intron of *ACT1* (yellow) interrupts the coding sequence of luciferase (green) which is under the control of the constitutive *ACT1* promoter. The second construct consists of a fusion of the open reading frames of CRE recombinase (dark purple) and the human estrogen receptor's estrogen binding domain (EBD)(light purple) under the control of the *GAL1* promoter. **B.** shows the luminescence traces for yeast with the constructs from A. treated with either galactose or glucose and either DMSO or estradiol. The only culture that shows a drastic increase in luminescence is the one treated with galactose and estradiol (pink). The increasing luminescence (between 30 min and 5 h) reveals the timeframe of *GAL1* promoter induction, CRE recombinase's transcription and translation, CRE removing the DNA that is flanked by the loxP sites, splicing out of the intron, and transcription and translation of luciferase in the culture.

The light that is produced from the enzyme shows that this sequence (*GAL1* promoter induction, CRE production, recombination, splicing, and translation) has occurred, and the length of time it takes can be measured.

I inoculated a culture of the yeast just described (1:20 dilution from an overnight culture) in yeast extract peptone media supplemented with 2% raffinose and 50 μM beetle luciferin, and grew this culture for 6 h at 28°C with agitation. Prior to the assay, I subdivided the culture into four scintillation vials (1 ml each) and continued to culture the samples at 28°C with agitation in the dark. At time zero, each sample was treated in one of the following ways: 2% galactose + 10 μM estradiol, 2% galactose + 1 μl DMSO (vehicle), 2% glucose + 1 μl DMSO, or no treatment. Luminescent measurements of all cultures were taken periodically for 9 hours. The results show that for the culture in which CRE was induced and functional (i.e. galactose + estradiol) luciferase activity began to increase in as little as 30 min but was not fully expressed until 5 hours later (Fig. A.1B, pink trace). As expected, the presence of estradiol made a difference in whether luciferase was expressed or not (Fig. A.1B, pink vs. dark blue traces).

Discussion

This experiment was a preliminary experiment to show that the combination of constructs within the yeast strain functioned and could serve as a temporal reporter of CRE activity. As a preliminary experiment for a project that was not pursued, there are admittedly a number of controls that were not performed, including the rest of the combinations of galactose, glucose, estradiol, and vehicle. Additionally, replicates of each culture were not measured so variability among samples could not be addressed in this experiment, but the experiment was repeated with different clones of the transformed yeast strain and all showed similar trends. It should be noted that the

>90,000 RLU difference in luminescence seen between the galactose + estradiol sample and the other samples is a large difference for this assay and would not likely be the result of sample variability.

One should be cautious when using the timeframe of CRE activity that this experiment reveals to infer the timing of CRE activity from other experiments if the growth conditions or CRE constructs differ from those used here. Even though the luciferase reporter was under the control of the actin promoter (which should be constitutively expressed), it is possible that the growth status or culture density influenced the luciferase activity and/or the CRE activity. The assay was performed when the culture was predicted to be in late log phase or early stationary phase so that the change in luminescence signal over time would be more likely to reflect CRE activity rather than culture growth. However, a growth influence on the luminescence signal can not be ruled out. Additionally, the timeframe reported by this experiment also included the time needed for the yeast to remove the intron within the reporter, a step that is not needed for actual CRE recombinase activity, just for the processing of the reporter for this assay.

Because the conditions for which galactose-induced CRE used in chapter III differed significantly from those used here to test CRE's timing, only very broad conclusions can be made about how quickly CRE can appear and act within continuous culture in a glucose medium (e.g. in chapter III). A more curtailed experiment can be designed to answer such a question using a strain similar to the one described here that did not include the estrogen binding domain. By following a protocol similar to the one used in Fig. 3.5 that showed the timing and extent of *GAL1* promoter induction of luciferase directly, I could use the luminescence peak that follows a galactose treatment to measure the time it takes for CRE recombinase to be expressed and do its job during the yeast metabolic oscillation.

Materials and Methods

Yeast Strain Used

The *ura3* strain of *Saccharomyces cerevisiae* CEN.PK113-7D described in chapter III was used for this experiment.

Construction of pRS306-P_{GAL1}-CREEBD

A DNA sequence containing the *GAL1* promoter, CRE recombinase fused to the estragen binding domain, and the *CYC1* terminator was PCR amplified from pSH62-EBD [133] using the 5' primer aaagctggagctctagtacgg that added a *Sac*I overhang and the 3' primer acagatgcggccgcaaattaaagc that added a *Not*I overhang. The PCR product was cloned into pRS306 using *Sac*I and *NotI*.

Construction of the CRE responsive luciferase reporter pRS303(d)-P_{ACT1}-Luc(Int/Kan)

The portion of pRS303(d)-Kan-P_{ACT1}-Luc [74] containing the promoter, luciferase, and terminator was moved to pRS303(d) [74] with *Xma*I and *Sal*I in order to eliminate the kanamycin resistance gene from the construct. The *ACT1* Intron/kanMX floxed interruption cassette was PRC amplified from pRKO [133] using the 5' primer aagtaagacgtcgtatgttctagcgcttgcac and the 3' primer atgattgacgtcctaaacatataatatagcaacaaaaagaatg, each of which added an *Aat*II overhang to the ends of the product. The PCR product (the interruption cassette) was cloned into the coding region of pRS303(d)- P_{ACT1}-Luc using *Aat*II.

Luminescence Monitoring

Luminescence measurements were taken with a Zylux Femtomaster FB12 luminometer at times 0, 0.5, 1.5, 2.5, 3.5, 5, and 9 hours.

APPENDIX B

YEAST IN A PROTOTYPE MINATURE BIOREACTOR CAN EXHIBIT RESPIRATORY OSCILLATIONS

Before I had access to a large (3L) bioreactor in which to study the yeast respiratory oscillation, I designed a functional prototype of a miniature bioreactor with a working volume of 3-10 ml. This work was abandoned after we began collaborating with Erik Boczko and developed methods for detecting yeast luciferase expression in his large bioreactor (Bioflo 110, New Brunswick), however applications for the economical homemade miniature version may exist. Therefore, I present work relating to its development and capability here.

The requirements of a bioreactor for continuous culture are fairly simple: a stable temperature and pH, a steady flow of air, sufficient mixing, and a constant infusion of new media at the same rate at which culture is removed. The ability to sterilize the bioreactor is also important. Aside from the ability to control pH, these features were reproduced in a device consisting of a glass 15 ml test tube for the reactor vessel, a 5-inch glass capillary micropipette to bubble air into the bottom of the vessel and provide agitation, two 4-inch stainless steel syringe needles (18 gauge) to deliver and remove media, one 1.5-inch syringe needle (21 gauge) as a gas vent, and a size-0 rubber stopper as a head plate (Fig. B.1). The device was housed in an incubator for temperature control, however a custom-made water jacket was fashioned for the device for additional temperature stability and the capability for rapid temperature modification (Fig. B.2). Air was supplied to the device by an aquarium air pump and fresh media was supplied by a 60ml syringe pump. A relatively constant volume of culture was maintained in the vessel by setting the tip of the outflow syringe needle to the surface of

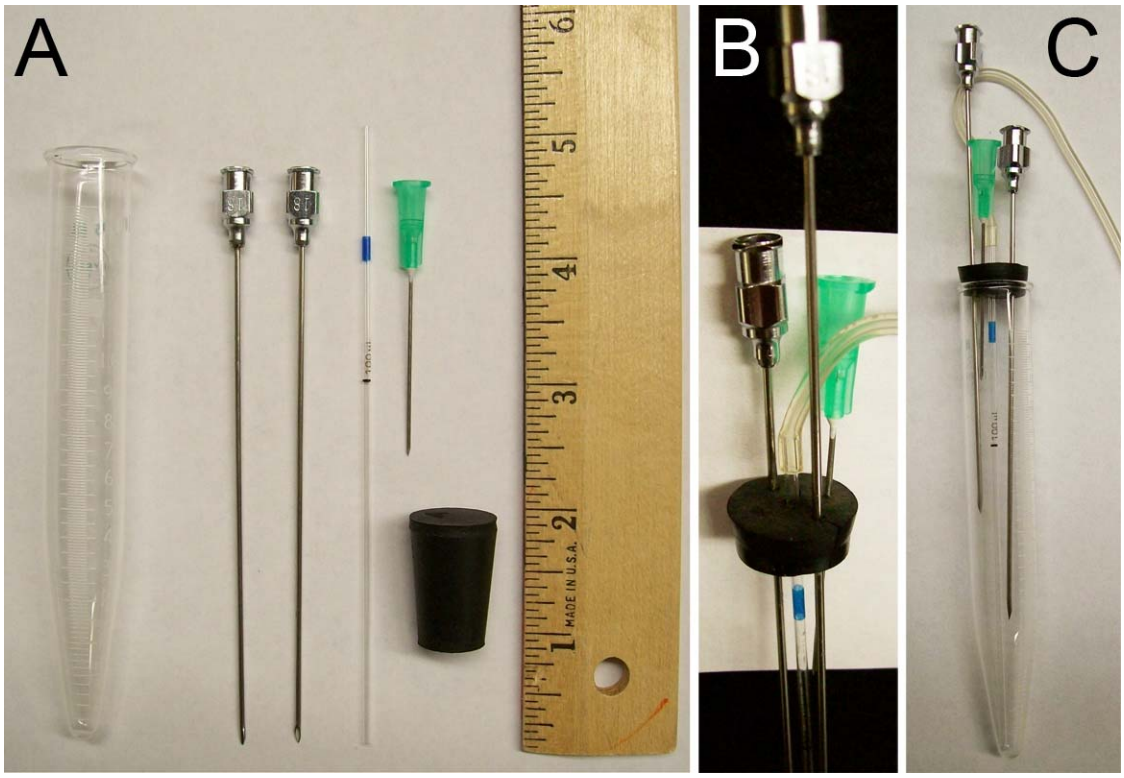

Figure B.1. Simple components are used to build a miniature bioreactor. **A.** shows the components needed (from left to right): A 15ml conical glass test tube, two long syringe needles, a glass capillary tube long enough to reach the bottom of the test tube, a short syringe needle, and a rubber stopper that fits the test tube. The ruler is shown for scale and only applies to panel A. **B.** shows how the components fit through the rubber stopper (head plate). To make pushing the components through the stopper easier, the rubber stopper was cut transversely to make a thinner head plate. **C.** shows the assembled miniature bioreactor. The flexible tubing that is seen in B and C is Tygon tubing (inner diameter = 1/32 in, outer diameter = 3/32 in) connected to the capillary that supplies air to the culture.

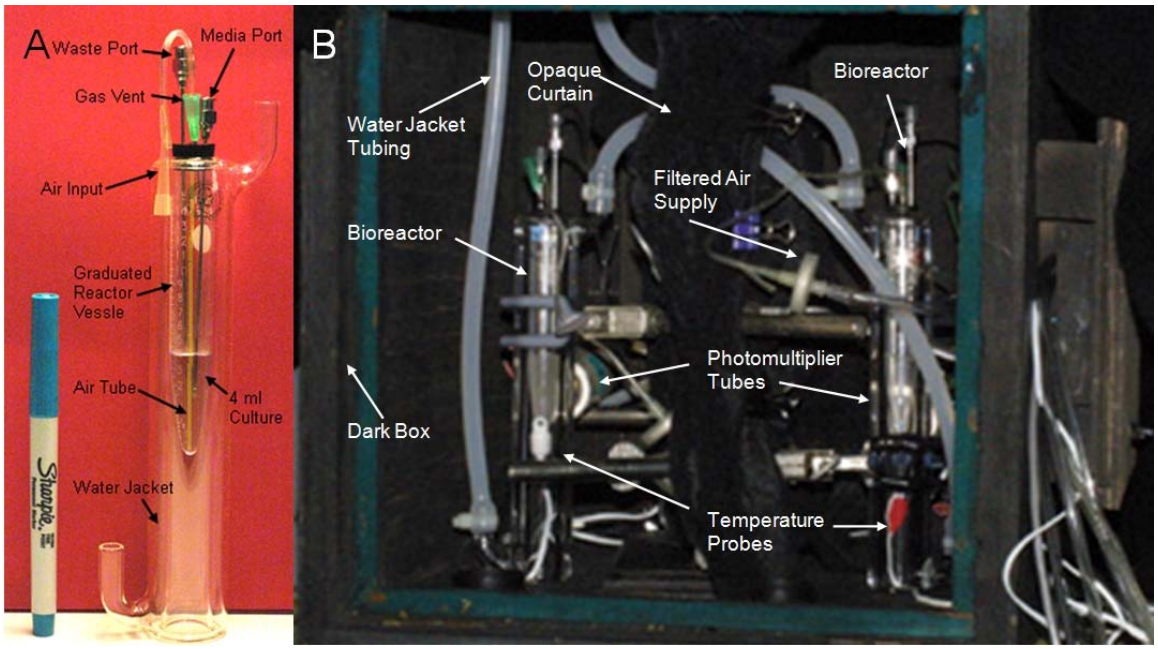

Figure B.2. Bioluminescence is measured from an assembled miniature bioreactor (A) in a black box with its supporting components (B). A 4-ml culture is injected into the reactor vessel through the gas vent needle and the syringe is removed, leaving the needle. The tip of the 4-inch needle that serves as the media port is positioned below the surface of the culture. The tip of the needle that serves as the waste port is positioned at the surface of the culture to remove any portion of the culture that exceeds that level. The air tube (highlighted in yellow for visualization) is a glass capillary tube that supplies humidified air and agitation to the culture. Within a dark box (B), an opaque curtain separates two identical bioreactors. Temperature controlled water flows through the glass water jacket that surrounds each reactor vessel and a temperature probe touching the culture vessel measures culture temperature. Each reactor has a photomultiplyer tube positioned perpendicular to the culture to measure bioluminescence. The dark box and its contents are housed within an incubator.

the culture and running an outflow peristaltic pump at a faster rate than the rate at which the syringe pump infused new medium. If pH control was needed, the supplied medium could be buffered to the desired pH, but otherwise, the device was incapable of regulating pH. The output used to demonstrate the YRO was bioluminescence from firefly luciferase driven by the *GPH1* promoter (glycogen phosphorylase) expressed on low copy plasmids (pRS314-P_{GPH1}-Luc(A4V)PEST) [74] by the yeast strain SEY6210 [70] (Fig. B.3), however the luminescence may represent oscillating oxygen levels more than promoter activity.

Materials and Methods

Miniature bioreactor

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

Medium and Culture Conditions

The medium consisted of supplemented minimal media lacking tryptophan and containing 6.5 g/L Difco yeast nitrogen base w/o amino acids, 20 g/L glucose, 15.6 mg/L uracil, 15.6 mg/L histidine, 15.6 mg/L adenine, 23.6 mg/L lysine, 23.6 mg/L leucine, and 50 µM beetle luciferin (potassium salt; Promega E1602). The 4 ml culture in the miniature bioreactor was inoculated with 50 µl of an overnight culture and grown at 30°C overnight with humidified air bubbling into the culture. Continuous culture was initiated the next day at a dilution rate of ~0.085/h.

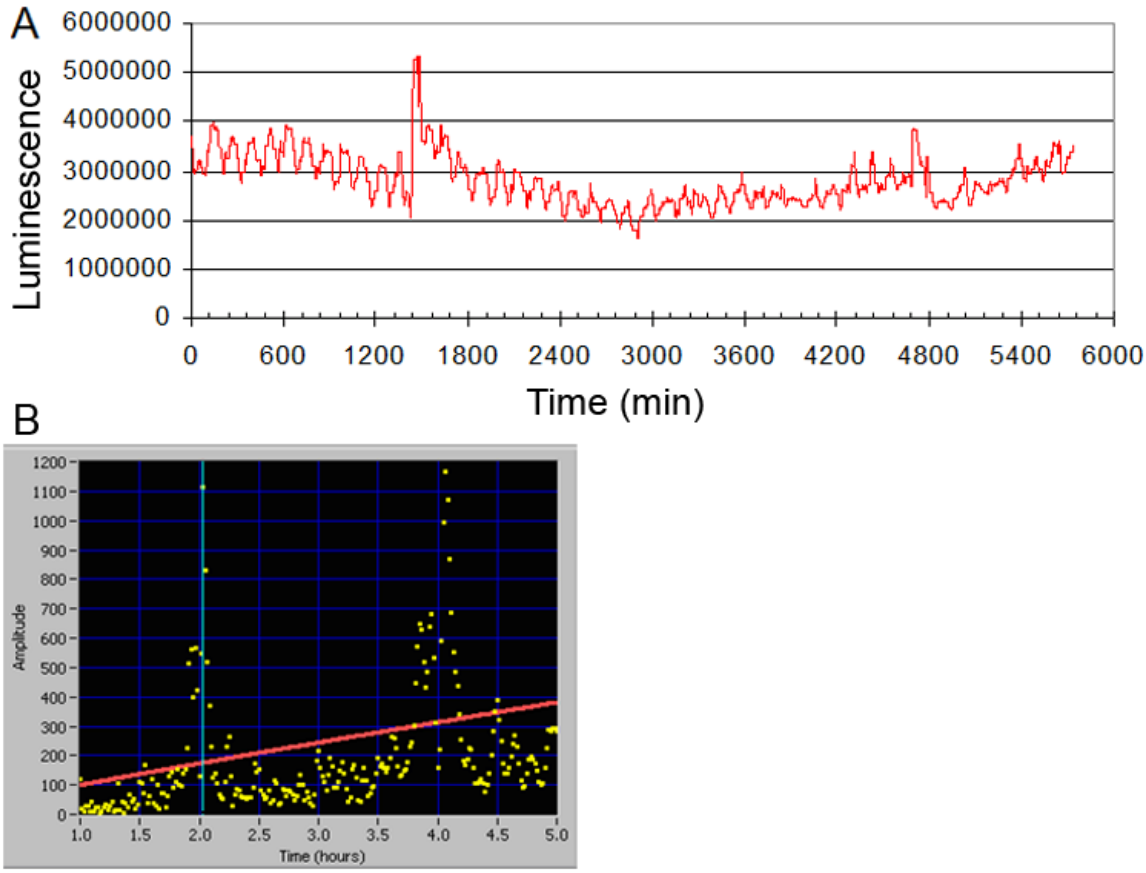

Figure B.3. Rhythmic promoter activity recorded for yeast grown in the miniature bioreactor at a dilution rate of 0.085/h. **A.** shows ninety-six hours of a spontaneous two-hour ultradian rhythm from the yeast strain SEY6210 in continuous culture. The strain was transformed with the destabilized luciferase reporter pRS314-P_{GPH1}-Luc(A4V)PEST which reports transcription of glycogen phosphorylase. Luminescence is in arbitrary units. **B.** shows a chi-square periodogram constructed from baseline subtracted data from panel A and confirms that a two-hour period predominates the rhythm. Peaks that rise above the red diagonal line represent periods with a significance value of 0.001.

REFERENCES

1. Herskowitz, I., (1988) Life cycle of the budding yeast Saccharomyces cerevisiae. Microbiol Rev. 52: 536-53.

2. Brewer, B.J., E. Chlebowicz-Sledziewska, and W.L. Fangman, (1984) Cell cycle phases in the unequal mother/daughter cell cycles of Saccharomyces cerevisiae. Mol Cell Biol. 4: 2529-31.

3. Brauer, M.J., et al., (2008) Coordination of growth rate, cell cycle, stress response, and metabolic activity in yeast. Mol Biol Cell. 19: 352-67.

4. Gray, J.V., et al., (2004) "Sleeping beauty": quiescence in Saccharomyces cerevisiae. Microbiol Mol Biol Rev. 68: 187-206.

5. Newcomb, L.L., J.A. Diderich, M.G. Slattery, and W. Heideman, (2003) Glucose regulation of Saccharomyces cerevisiae cell cycle genes. Eukaryot Cell. 2: 143-9.

6. Flick, K., D. Chapman-Shimshoni, D. Stuart, M. Guaderrama, and C. Wittenberg, (1998) Regulation of cell size by glucose is exerted via repression of the CLN1 promoter. Mol Cell Biol. 18: 2492-501.

7. Johnston, G.C., C.W. Ehrhardt, A. Lorincz, and B.L. Carter, (1979) Regulation of cell size in the yeast Saccharomyces cerevisiae. J Bacteriol. 137: 1-5.

8. Rapp, P.E., (1987) Why are so many biological systems periodic? Prog Neurobiol. 29: 261-73.

9. Sharma, V.K., (2003) Adaptive significance of circadian clocks. Chronobiol Int. 20: 901-19.

10. DeCoursey, P.J., *The behavioral ecology and evolution of biological timing systems*, in *Chronobiology: Biological Timekeeping*, J.C. Dunlap, J.J. Loros, and P.J. DeCoursey, Editors. 2004, Sinauer Assoc. Inc.: Sunderland, MA USA. p. 27-66.

11. Edmunds, L.N., *The glycolytic oscillator*, in *Cellular and Molecular Bases of Biological Clocks: Models and Mechanisms for Circadian Timekeeping*. 1988, Springer-Verlag: New York. p. 7-8.

12. Sherman, F., *Yeast Genetics*, in *The Encyclopedia of Molecular Biology and Molecular Medicine*, R.A. Meyers, Editor. 1997, VCH: Weinheim, Germany. p. 302-325.

13. Klevecz, R.R., J. Bolen, G. Forrest, and D.B. Murray, (2004) A genomewide oscillation in transcription gates DNA replication and cell cycle. Proc Natl Acad Sci U S A. 101: 1200-5.

14. Lloyd, D. and D.B. Murray, (2005) Ultradian metronome: timekeeper for orchestration of cellular coherence. Trends Biochem Sci. 30: 373-7.

15. Lloyd, D., K.M. Lemar, L.E. Salgado, T.M. Gould, and D.B. Murray, (2003) Respiratory oscillations in yeast: mitochondrial reactive oxygen species, apoptosis and time; a hypothesis. FEMS Yeast Res. 3: 333-9.

16. Tu, B.P., A. Kudlicki, M. Rowicka, and S.L. McKnight, (2005) Logic of the yeast metabolic cycle: temporal compartmentalization of cellular processes. Science. 310: 1152-8.

17. Wang, J., et al., (2000) Cellular stress responses oscillate in synchronization with the ultradian oscillation of energy metabolism in the yeast Saccharomyces cerevisiae. FEMS Microbiol Lett. 189: 9-13.

18. Xu, Z. and K. Tsurugi, (2006) A potential mechanism of energy-metabolism oscillation in an aerobic chemostat culture of the yeast Saccharomyces cerevisiae. FEBS J. 273: 1696-709.

19. Moller, H. and S.B. Jorgensen, (1997) Continuous cultivation start-up control--an experimental investigation. J Biotechnol. 53: 55-66.

20. Murray, D.B., S. Roller, H. Kuriyama, and D. Lloyd, (2001) Clock control of ultradian respiratory oscillation found during yeast continuous culture. J Bacteriol. 183: 7253-9.

21. DeCoursey, P.J., *Molecular Biology of Circadian Pacemaker Systems*, in *Chronobiology*, J.C. Dunlap, J.J. Loros, and P.J. DeCoursey, Editors. 2004, Sinauer Associates, Inc.: Sunderland, MA. p. 213-253.

22. Menaker, M., *Biological Clocks at the End of the 20th Century*, in *Biological Rhythms*, V. Kumar, Editor. 2002, Springer: New York. p. 1-4.

23. Brauer, M.J., A.J. Saldanha, K. Dolinski, and D. Botstein, (2005) Homeostatic adjustment and metabolic remodeling in glucose-limited yeast cultures. Mol Biol Cell. 16: 2503-17.

24. De Deken, R.H., (1966) The Crabtree effect: a regulatory system in yeast. J Gen Microbiol. 44: 149-56.

25. Walker, G.M., *Yeast Metabolism*, in *Yeast Physiology and Biotechnology*. 1998, John Wiley and Sons: Chicherter, England. p. 203-264.

26. Thomson, J.M., et al., (2005) Resurrecting ancestral alcohol dehydrogenases from yeast. Nat Genet. 37: 630-5.

27. Gasch, A.P., et al., (2000) Genomic expression programs in the response of yeast cells to environmental changes. Mol Biol Cell. 11: 4241-57.

28. Lagunas, R., (1979) Energetic irrelevance of aerobiosis for S. cerevisiae growing on sugars. Mol Cell Biochem. 27: 139-46.

29. Hoskisson, P.A. and G. Hobbs, (2005) Continuous culture--making a comeback? Microbiology. 151: 3153-9.

30. Gilbert, P., (1985) The theory and relevance of continuous culture. J Antimicrob Chemother. 15 Suppl A: 1-6.

31. Zamamiri, A.Q., G. Birol, and M.A. Hjortso, (2001) Multiple stable states and hysteresis in continuous, oscillating cultures of budding yeast. Biotechnol Bioeng. 75: 305-12.

32. Murray, D.B., F.A. Engelen, M. Keulers, H. Kuriyama, and D. Lloyd, (1998) NO+, but not NO., inhibits respiratory oscillations in ethanol-grown chemostat cultures of Saccharomyces cerevisiae. FEBS Lett. 431: 297-9.

33. Von Meyenburg, H.K., (1969) Energetics of the budding cycle of Saccharomyces cerevisiae during glucose limited aerobic growth. Arch Mikrobiol. 66: 289-303.

34. Porro, D., E. Martegani, B.M. Ranzi, and L. Alberghina, (1988) Oscillations in continuous cultures of budding yeast: A segregated parameter analysis. Biotechnol Bioeng. 32: 411-7.

35. Lloyd, D. (2006) *The ultradian clock: not to be confused with the cell cycle*. Mol Cell Biol 7, Online Correspondence.

36. Tu, B.P., A. Kudlicki, M. Rowicka, and S.L. McKnight (2006) *Let the data speak*. Mol Cell Biol 7, Online Correspondence.

37. Murray, D.B. (2006) *The respiratory oscillation in yeast phase definitions and periodicity*. Mol Cell Biol 7, Online Correspondence.

38. Satroutinov, A.D., H. Kuriyama, and H. Kobayashi, (1992) Oscillatory metabolism of Saccharomyces cerevisiae in continuous culture. FEMS Microbiol Let. 98: 261-268.

39. Beuse, M., R. Bartling, A. Kopmann, H. Diekmann, and M. Thoma, (1998) Effect of the dilution rate on the mode of oscillation in continuous cultures of Saccharomyces cerevisiae. J Biotechnol. 61: 15-31.

40. DeCoursey, P.J., *Overview of biological timing from unicells to humans*, in *Chronobiology: Biological Timekeeping*, J.C. Dunlap, J.J. Loros, and P.J. DeCoursey, Editors. 2004, Sinauer Assoc. Inc: Sunderland, MA USA. p. 3-26.

41. Jules, M., J. Francois, and J.L. Parrou, (2005) Autonomous oscillations in Saccharomyces cerevisiae during batch cultures on trehalose. FEBS J. 272: 1490-500.

42. Kondo, T., et al., (1993) Circadian rhythms in prokaryotes: luciferase as a reporter of circadian gene expression in cyanobacteria. Proc Natl Acad Sci U S A. 90: 5672-6.

43. Thompson, J.F., L.S. Hayes, and D.B. Lloyd, (1991) Modulation of firefly luciferase stability and impact on studies of gene regulation. Gene. 103: 171-7.

44. Mateus, C. and S.V. Avery, (2000) Destabilized green fluorescent protein for monitoring dynamic changes in yeast gene expression with flow cytometry. Yeast. 16: 1313-23.

45. Huh, W.K., et al., (2003) Global analysis of protein localization in budding yeast. Nature. 425: 686-91.

46. Yoo, S.H., et al., (2005) A noncanonical E-box enhancer drives mouse Period2 circadian oscillations in vivo. Proc Natl Acad Sci U S A. 102: 2608-13.

47. Stowers, C.C., J.B. Robertson, H. Ban, R.D. Tanner, and E.M. Boczko, (2009) Periodic fermentor yield and enhanced product enrichment from autonomous oscillations. Appl Biochem Biotechnol. 156: 59-75.

48. Alon, U., *An Introduction to Systems Biology - Design Principles of Biological Circuits*, ed. Chapman and Hall. 2006, Boca Raton, FL: CRC.

49. Hastings, J.W. and C.H. Johnson, in *Methods in Enzymology; Biophotonics*, A.G. Mariott and I. Parker, Editors. 2003, Academic Press: San Diego. p. 75-104.

50. Millar, A.J., S.R. Short, N.H. Chua, and S.A. Kay, (1992) A novel circadian phenotype based on firefly luciferase expression in transgenic plants. Plant Cell. 4: 1075-87.

51. Yamazaki, S., et al., (2000) Resetting central and peripheral circadian oscillators in transgenic rats. Science. 288: 682-5.

52. Kondo, T., et al., (1994) Circadian clock mutants of cyanobacteria. Science. 266: 1233-6.

53. Millar, A.J., I.A. Carre, C.A. Strayer, N.H. Chua, and S.A. Kay, (1995) Circadian clock mutants in Arabidopsis identified by luciferase imaging. Science. 267: 1161-3.

54. Liu, Y., et al., (1995) Circadian orchestration of gene expression in cyanobacteria. Genes Dev. 9: 1469-78.

55. Michael, T.P. and C.R. McClung, (2003) Enhancer trapping reveals widespread circadian clock transcriptional control in Arabidopsis. Plant Physiol. 132: 629-39.

56. Brandes, C., et al., (1996) Novel features of drosophila period Transcription revealed by real-time luciferase reporting. Neuron. 16: 687-92.

57. Gooch, V.D., et al., (2008) Fully codon-optimized luciferase uncovers novel temperature characteristics of the Neurospora clock. Eukaryot Cell. 7: 28-37.

58. Izumo, M., T.R. Sato, M. Straume, and C.H. Johnson, (2006) Quantitative analyses of circadian gene expression in mammalian cell cultures. PLoS Comput Biol. 2: e136.

59. Martegani, E., D. Porro, B.M. Ranzi, and L. Alberghina, (1990) Involvement of a cell size control mechanism in the induction and maintenance of oscillations in continuous cultures of budding yeast. Biotechnol Bioeng. 36: 453-9.

60. Asai, M., et al., (2001) Visualization of mPer1 transcription in vitro: NMDA induces a rapid phase shift of mPer1 gene in cultured SCN. Curr Biol. 11: 1524-7.

61. Plevani, P., et al., (1988) The yeast DNA polymerase-primase complex: genes and proteins. Biochim Biophys Acta. 951: 268-73.

62. Verma, R., A. Patapoutian, C.B. Gordon, and J.L. Campbell, (1991) Identification and purification of a factor that binds to the Mlu I cell cycle box of yeast DNA replication genes. Proc Natl Acad Sci U S A. 88: 7155-9.

63. Murray, D.B., R.R. Klevecz, and D. Lloyd, (2003) Generation and maintenance of synchrony in Saccharomyces cerevisiae continuous culture. Exp Cell Res. 287: 10-5.

64. Hastings, J.W., E.W. Mc, and J. Coulombre, (1953) The effect of oxygen upon the immobilization reaction in firefly luminescence. J Cell Physiol. 42: 137-50.

65. Moriyama, E.H., et al., (2008) The influence of hypoxia on bioluminescence in luciferase-transfected gliosarcoma tumor cells in vitro. Photochem Photobiol Sci. 7: 675-80.

66. Spellman, P.T., et al., (1998) Comprehensive identification of cell cycle-regulated genes of the yeast Saccharomyces cerevisiae by microarray hybridization. Mol Biol Cell. 9: 3273-97.

67. Wittenberg, C., K. Sugimoto, and S.I. Reed, (1990) G1-specific cyclins of S. cerevisiae: cell cycle periodicity, regulation by mating pheromone, and association with the p34CDC28 protein kinase. Cell. 62: 225-37.

68. DeCoursey, P.J., *Fundamental Properties of Circadian Rhythms*, in *Chronobiology*, J.C. Dunlap, J.J. Loros, and P.J. DeCoursey, Editors. 2004, Sinauer Associates, Inc.: Sunderland, MA. p. 67-105.

69. Longtine, M.S., et al., (1998) Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast. 14: 953-61.

70. Robinson, J.S., D.J. Klionsky, L.M. Banta, and S.D. Emr, (1988) Protein sorting in Saccharomyces cerevisiae: isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases. Mol Cell Biol. 8: 4936-48.

71. Horton, R.M., H.D. Hunt, S.N. Ho, J.K. Pullen, and L.R. Pease, (1989) Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene. 77: 61-8.

72. Wurch, T., F. Lestienne, and P.J. Pauwels, (1998) A modified overlap extension PCR method to create chimeric genes in the absence of restriction enzymes. Biotechnol Tech. 12: 653-657.

73. Sikorski, R.S. and P. Hieter, (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics. 122: 19-27.

74. Robertson, J.B., C.C. Stowers, E. Boczko, and C.H. Johnson, (2008) Real-time luminescence monitoring of cell-cycle and respiratory oscillations in yeast. Proc Natl Acad Sci U S A. 105: 17988-93.

75. Beuse, M., A. Kopmann, H. Diekmann, and M. Thoma, (1999) Oxygen, pH value, and carbon source induced changes of the mode of oscillation in synchronous continuous culture of Saccharomyces cerevisiae. Biotechnol Bioeng. 63: 410-7.

76. Chen, Z., E.A. Odstrcil, B.P. Tu, and S.L. McKnight, (2007) Restriction of DNA replication to the reductive phase of the metabolic cycle protects genome integrity. Science. 316: 1916-9.

77. Carter, B.L. and M.N. Jagadish, (1978) The relationship between cell size and cell division in the yeast Saccharomyces cerevisiae. Exp Cell Res. 112: 15-24.

78. Hartwell, L.H. and M.W. Unger, (1977) Unequal division in Saccharomyces cerevisiae and its implications for the control of cell division. J Cell Biol. 75: 422-35.

79. Futcher, B., (1999) Cell cycle synchronization. Methods Cell Sci. 21: 79-86.

80. Xu, Z. and K. Tsurugi, (2007) Role of Gts1p in regulation of energy-metabolism oscillation in continuous cultures of the yeast Saccharomyces cerevisiae. Yeast. 24: 161-70.

81. Reed, S.I., (1980) The selection of S. cerevisiae mutants defective in the start event of cell division. Genetics. 95: 561-77.

82. Surana, U., et al., (1991) The role of CDC28 and cyclins during mitosis in the budding yeast S. cerevisiae. Cell. 65: 145-61.

83. Giaever, G., et al., (2002) Functional profiling of the Saccharomyces cerevisiae genome. Nature. 418: 387-91.

84. Hartwell, L.H., R.K. Mortimer, J. Culotti, and M. Culotti, (1973) Genetic Control of the Cell Division Cycle in Yeast: V. Genetic Analysis of cdc Mutants. Genetics. 74: 267-286.

85. Edgington, N.P., M.J. Blacketer, T.A. Bierwagen, and A.M. Myers, (1999) Control of Saccharomyces cerevisiae filamentous growth by cyclin-dependent kinase Cdc28. Mol Cell Biol. 19: 1369-80.

86. Mendenhall, M.D. and A.E. Hodge, (1998) Regulation of Cdc28 cyclin-dependent protein kinase activity during the cell cycle of the yeast Saccharomyces cerevisiae. Microbiol Mol Biol Rev. 62: 1191-243.

87. Johnston, L.H. and A.L. Johnson, (1997) Elutriation of budding yeast. Methods Enzymol. 283: 342-50.

88. Guldener, U., S. Heck, T. Fielder, J. Beinhauer, and J.H. Hegemann, (1996) A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic Acids Res. 24: 2519-24.

89. Janke, C., et al., (2004) A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. Yeast. 21: 947-62.

90. Liu, Y., M. Merrow, J.J. Loros, and J.C. Dunlap, (1998) How temperature changes reset a circadian oscillator. Science. 281: 825-9.

91. Pitts, S., E. Perone, and R. Silver, (2003) Food-entrained circadian rhythms are sustained in arrhythmic Clk/Clk mutant mice. Am J Physiol Regul Integr Comp Physiol. 285: R57-67.

92. Enright, J.T., (1965) Entrainment of a Tidal Rhythm. Science. 147: 864-867.

93. Pittendrigh, C.S., (1993) Temporal organization: reflections of a Darwinian clock-watcher. Annu Rev Physiol. 55: 16-54.

94. Falciatore, A. and C. Bowler, (2005) The evolution and function of blue and red light photoreceptors. Curr Top Dev Biol. 68: 317-50.

95. Linden, H., P. Ballario, and G. Macino, (1997) Blue light regulation in Neurospora crassa. Fungal Genet Biol. 22: 141-50.

96. Salichos, L. and A. Rokas, (2009) The Diversity and Evolution of Circadian Clock Proteins in Fungi. Mycologia. in press.

97. Green, G. and A.M. MacQuillan, (1980) Photorepair of ultraviolet-induced petite mutational damage in Saccharomyces cerevisiae requires the product of the PHR1 gene. J Bacteriol. 144: 826-9.

98. Kondo, T., et al., (1997) Circadian rhythms in rapidly dividing cyanobacteria. Science. 275: 224-7.

99. Spencer, J.F.T., *Yeasts in natural and artificial habitats*. 1997, New York: Springer. 381.

100. Esposito, R.E. and S. Klapholz, *Meiosis and ascospore development*, in *The molecular biology of the yeast Saccharomyces: life cycle and inheritance*, J.N. Strathern, E.W. Jones, and J.R. Broach, Editors. 1981, Cold Spring Harbor Laboratory Press: Cold Spring Harbor, N.Y. p. 211–287.

101. Nikaido, S.S. and C.H. Johnson, (2000) Daily and circadian variation in survival from ultraviolet radiation in Chlamydomonas reinhardtii. Photochem Photobiol. 71: 758-65.

102. Gasch, A.P. and M. Werner-Washburne, (2002) The genomics of yeast responses to environmental stress and starvation. Funct Integr Genomics. 2: 181-92.

103. Devlin, P.F. and S.A. Kay, (2001) Circadian photoperception. Annu Rev Physiol. 63: 677-94.

104. He, Q. and Y. Liu, (2005) Molecular mechanism of light responses in Neurospora: from light-induced transcription to photoadaptation. Genes Dev. 19: 2888-99.

105. Butt, W.D. and D. Keilin, (1962) Absorption spectra and some other properties of cytochrome c and of its compounds with ligands. Proc R Soc Lond B Biol Sci. 156: 429-58.

106. Horie, S., (1964) On the Absorption Spectrum of Cytochrome a-3. J Biochem. 56: 57-66.

107. Epel, B. and W.L. Butler, (1969) Cytochrome a3: destruction by light. Science. 166: 621-2.

108. Woodward, J.R., V.P. Cirillo, and L.N. Edmunds, Jr., (1978) Light effects in yeast: inhibition by visible light of growth and transport in Saccharomyces cerevisiae grown at low temperatures. J Bacteriol. 133: 692-8.

109. Ulaszewski, S., et al., (1979) Light effects in yeast: evidence for participation of cytochromes in photoinhibition of growth and transport in Saccharomyces cerevisiae cultured at low temperatures. J Bacteriol. 138: 523-9.

110. Horecker, B.L. and J.N. Stannard, (1948) The cytochrome c-azide complex. J Biol Chem. 172: 589-97.

111. Stannard, J.N. and B.L. Horecker, (1948) The in vitro inhibition of cytochrome oxidase by azide and cyanide. J Biol Chem. 172: 599-608.

112. Matsunaka, S., S. Morita, and S.F. Conti, (1966) Respiratory system of Rhodotorula glutinis. I. Inhibitor tolerance and cytochrome components. Plant Physiol. 41: 1364-9.

113. Moore, M.M., M.W. Breedveld, and A.P. Autor, (1989) The role of carotenoids in preventing oxidative damage in the pigmented yeast, Rhodotorula mucilaginosa. Arch Biochem Biophys. 270: 419-31.

114. Britton, G., (1995) Structure and properties of carotenoids in relation to function. FASEB J. 9: 1551-8.

115. Maclean, M., S.J. MacGregor, J.G. Anderson, and G. Woolsey, (2009) Inactivation of bacterial pathogens following exposure to light from a 405-nanometer light-emitting diode array. Appl Environ Microbiol. 75: 1932-7.

116. Caughey, W.S., G.A. Smythe, D.H. O'Keeffe, J.E. Maskasky, and M.I. Smith, (1975) Heme A of cytochrome c oxicase. Structure and properties: comparisons with hemes B, C, and S and derivatives. J Biol Chem. 250: 7602-22.

117. Olson, J.A., (1993) Vitamin A and carotenoids as antioxidants in a physiological context. J Nutr Sci Vitaminol (Tokyo). 39 Suppl: S57-65.

118. Jensen, S.L., (1965) Biosynthesis and function of carotenoid pigments in microorganisms. Annu Rev Microbiol. 19: 163-82.

119. Ji, H., M.H. Platts, L.M. Dharamsi, and K.L. Friedman, (2005) Regulation of telomere length by an N-terminal region of the yeast telomerase reverse transcriptase. Mol Cell Biol. 25: 9103-14.

120. Mortimer, R.K. and J.R. Johnston, (1986) Genealogy of principal strains of the yeast genetic stock center. Genetics. 113: 35-43.

121. Winzeler, E.A., et al., (1999) Functional characterization of the S. cerevisiae genome by gene deletion and parallel analysis. Science. 285: 901-6.

122. Liti, G., D.B. Barton, and E.J. Louis, (2006) Sequence diversity, reproductive isolation and species concepts in Saccharomyces. Genetics. 174: 839-50.

123. Palkova, Z. and L. Vachova, (2006) Life within a community: benefit to yeast long-term survival. FEMS Microbiol Rev. 30: 806-24.

124. Hohman, B., (2007) LED light source: major advance in fluorescence microscopy. Biomed Instrum Technol. 41: 461-4.

125. Khodjakov, A. and C.L. Rieder, (2006) Imaging the division process in living tissue culture cells. Methods. 38: 2-16.

126. Martin, G., H.T. Agostini, and L.L. Hansen, (2005) Light emitting diode microscope illumination for green fluorescent protein or fluorescein isothiocyanate epifluorescence. Biotechniques. 38: 204, 206.

127. Albeanu, D.F., E. Soucy, T.F. Sato, M. Meister, and V.N. Murthy, (2008) LED arrays as cost effective and efficient light sources for widefield microscopy. PLoS ONE. 3: e2146.

128. Xu, X., et al., (2007) Imaging protein interactions with bioluminescence resonance energy transfer (BRET) in plant and mammalian cells and tissues. Proc Natl Acad Sci U S A. 104: 10264-9.

129. Yang, M., G. Luiken, E. Baranov, and R.M. Hoffman, (2005) Facile whole-body imaging of internal fluorescent tumors in mice with an LED flashlight. Biotechniques. 39: 170, 172.

130. Kudlicki, A., M. Rowicka, and Z. Otwinowski, (2007) SCEPTRANS: an online tool for analyzing periodic transcription in yeast. Bioinformatics. 23: 1559-61.

131. Walther, K. and H.J. Schuller, (2001) Adr1 and Cat8 synergistically activate the glucose-regulated alcohol dehydrogenase gene ADH2 of the yeast Saccharomyces cerevisiae. Microbiology. 147: 2037-44.

132. Rao, R., D. Drummond-Barbosa, and C.W. Slayman, (1993) Transcriptional regulation by glucose of the yeast PMA1 gene encoding the plasma membrane H(+)-ATPase. Yeast. 9: 1075-84.

133. Cheng, T.H., C.R. Chang, P. Joy, S. Yablok, and M.R. Gartenberg, (2000) Controlling gene expression in yeast by inducible site-specific recombination. Nucleic Acids Res. 28: E108.