Chapter 1

Introduction

1.1 Motivation of thesis

This thesis aims to understand how an organism adapts its metabolism and cellular processes in response to external conditions. I do so by ~~through~~ using the yeast metabolic cycle (YMC) as a framework for biological oscillators. My reasons are twofold: (a) biological oscillators are important for coordination of responses and are present across kingdoms, (b) there are unanswered questions about the mechanistic basis of the YMC and about reconciling evidence from two types of experimental studies. Therefore, I study YMC regulation in isolated cells in different nutrient conditions.

This thesis is divided into six chapters:

1. Chapter 1 discusses the background behind the yeast metabolic cycle and using flavin autofluorescence as a way to monitor the yeast metabolic cycle.
2. Chapter 2 discusses the methods: single-cell microfluidics of yeast cells followed by an automated image analysis pipeline.
3. Chapter 3 discusses the analysis of oscillatory time series. Given the size of the datasets and the challenges of analysing noisy low-resolution time series, this deserves discussion in its own right. This chapter steps through the process of analysis and provides a review and justification of the computational methods at each stage.
4. Chapter 4 presents biological results, using the analysis methods discussed in chapter 3. I show that the metabolic cycle and cell division cycle are autonomous and synchronise in permissive conditions, while perturbations affect the relationship between these two biological oscillators.
5. Chapter 5 discusses using flux balance analysis to answer whether temporal partitioning of biosynthesis under proteome constraints explains the timing of the yeast metabolic cycle.
6. Finally, chapter 6 combines previous understanding of the metabolic cycle, experimental observations, and mathematical models to propose a coarse-grained, phenomenological model of the yeast metabolic cycle. This chapter also suggests further avenues of study.

1.2 Yeast metabolic cycle

1.2.1 Introduction to biological rhythms

Biological basis of biological rhythms

Biological rhythms are repeating physiological or cellular processes. Genetic oscillators, biochemical oscillators, and metabolic oscillators, all linked to a cellular redox cycle, govern biological rhythms (Mellor, 2016). Biological rhythms can occur at different time scales, from seconds (e.g. glycolytic cycle), to ultradian cycles (i.e. cycles that are more frequent than 24 hours), to circadian rhythms (24 hours). Biological rhythms are important in temporally separating physiological processes. This is instrumental in responding to external conditions, including nutrient conditions, growth requirements, or the day-night cycle. Thus, this means that biological rhythms can vary according to conditions.

Such biological rhythms include the circadian rhythm and the cell division cycle. To demonstrate the definition of biological rhythm, I discuss the cell division cycle, which is well-characterised. The cell division cycle in budding yeast is governed by a series of gene regulatory networks that interact in a feedback loop, resulting in oscillatory expression of regulatory proteins, namely, cyclin-CDK complexes that regulate cellular events in a temporal manner (Adler et al., 2022; Orlando et al., 2008; A. W. Murray, 2004). This cycle also includes biochemical and metabolic oscillators, for example, biosynthesis during S phase. As with other biological rhythms, the cell division cycle also includes a system to control it, so that DNA replication occurs once every cell division cycle and so that the cell only divides when necessary. The importance of such control systems are highlighted by disorders when these systems are impaired, such as chromosome aberrations and cancer.

The yeast metabolic cycle is a type of biological rhythm because it has the properties that define a biological rhythm. Namely, it has gene-expression oscillators as evidenced by transcript cycling in its phases, it has biochemical oscillators as evidenced by changes in dissolved oxygen in the chemostat, and it has metabolite oscillations as evidence by changes in the levels of compounds that undergo redox reactions like NADH/NADPH and flavins. I discuss further this evidence in the context of the known progression of the yeast metabolic cycle in section 1.2.2. However, in contrast to the cell division cycle, the control mechanisms of the yeast metabolic cycle are less well characterised — I discuss this further in section 1.2.6.

Theoretical basis of biological rhythms

The theoretical basis of biological rhythms originated in work in the 1960s, which included simple systems of ordinary differential equations to describe negative feedback control circuits (Goodwin, 1965; Griffith, 1968). Experimental observations have then informed the development of models with finer detail. Furthermore, synthetic genetic circuits have also been modelled and developed (Elowitz and Leibler, 2000).

To illustrate a natural biological rhythm, I discuss the cell division cycle. The well-characterised cell division cycle has inspired models with a variety of approaches. Early models are based on a negative feedback loop of key components as identified by experimental studies. For example, Goldbeter (1991) assumed a minimal model of one cyclin, one kinase, and one protease to construct a negative feedback loop with a delay, giving rise to stable oscillations. Such a strategy forms the basis of later models that incorporate more detail, including additional control points of the cell division cycle (Chen et al., 2004), responses to perturbations such as osmotic stress (Adrover et al., 2011), and relationship with other oscillators like the circadian rhythm (Gérard and Goldbeter, 2012; Charvin et al., 2009; Droin et al., 2019). More recent, comprehensive models include Adler et al. (2022) which is based on a system of ordinary differential equations adapted for the modelling to pheromone and osmotic shock responses, and Novak and Tyson (2022), which models the cell division cycle as a series of switches between two stable steady states whose behaviour is regulated by the CDK oscillator.

In contrast, less well-characterised natural biological rhythms have given rise to models with fewer detail and precision. An example is the glycolytic oscillation. The glycolytic oscillation is a type of ultradian biochemical oscillator, characterised by oscillations in NADH levels (and other cofactors) in budding yeast cells at the time scale of 10 seconds (Dodd and Kralj, 2017; Lloyd, 2019; Olsen and Lunding, 2021), when they are in high-glucose conditions. A recent model is a coarse-grained model driven by positive feedback loops as a system of two coupled instability-generating mechanisms (Goldbeter and Yan, 2022).

As biological rhythms are often coupled with each other, forced and coupled oscillators have been modelled. If an oscillator is forced, it has a natural oscillation frequency, but is forced from it due to an external force applied at a regular interval. An example is the circadian clock, which is entrained to the light-dark cycle (Goldbeter and Yan, 2022). Yeast glycolytic oscillations can also be entrained via a periodic input of substrate. Forced oscillators are closely linked to coupled oscillators, in which two oscillators are coupled to each other by certain activation or deactivation events. Two coupled oscillators tend to oscillate at a compromise frequency if the natural frequencies of each are close enough to each other. Otherwise, complex oscillations can occur: the oscillators lock to a rational ratio of frequencies — i.e. one oscillator goes through p periods while the other goes through q periods. In this case, the exact ratio depends on the ratio of the natural frequencies. Furthermore, in certain cases, chaos can occur. There

is a mathematical basis in Arnold tongues (Heltberg et al., 2021). Experimental observations support this. For example, Charvin et al. (2009) showed that externally forcing cell division cycles via glucose pulsing leads to phase-locking of the cell division cycle oscillator only within a range of extrinsic periods.

The yeast metabolic cycle has been modelled as a system of coupled oscillators (Papagiannakis et al., 2017; Özsezen et al., 2019), based on how it is linked to the cell division cycle. I will discuss this in section 1.2.4.

1.2.2 Definition and description of the yeast metabolic cycle

History of evidence for the yeast metabolic cycle

have you defined the metabolic cycle?

Aspects of the YMC have been observed over decades. Nosoh and Takamiya (1962) discovered that synchronised *S. cerevisiae* cultures show oscillatory oxygen consumption. Kaspar von Meyenburg (1969) showed that gas metabolism and energy generation increase upon budding, while Mochan and Pye (1973) described a high-amplitude respiratory oscillation following a substrate shift from glucose to ethanol. Satroutdinov et al. (1992) were the first to describe the metabolic components of a 40-minute YMC for cells in continuous culture. Tu, Kudlicki et al. (2005) first incorporated transcript cycling in the description of the YMC and defined the YMC events, based on a chemostat-based investigation of growth of budding yeast on glucose-starved conditions.

The yeast metabolic cycle is longer in duration and is more robust than a similar biological oscillator, the glycolytic oscillation. The glycolytic oscillation has a period on the scale of 40 seconds (Olsen, Andersen et al., 2009). In contrast, the yeast metabolic cycle has been described, using various definitions, to either exhibit a 40-minute short-phase cycle (Lloyd and D. B. Murray, 2005; C. M. Li and Klevecz, 2006; Lloyd and D. B. Murray, 2007), or a long-phase cycle, which is most commonly described to be 4–5 hours (Tu, Kudlicki et al., 2005; Tu, Mohler et al., 2007), but also ranges between 1.4 hours to 14 hours, depending on the chemostat dilution rate (Beuse et al., 1998; O' Neill et al., 2020).

Glycolytic oscillations are highly damped, but yeast metabolic oscillations are robust, lasting for weeks (Lloyd and D. B. Murray, 2007). Additionally, glycolytic oscillations have been observed in anaerobic conditions (Lloyd, 2019), but yeast metabolic cycles have been observed in aerobic conditions. Moreover, glycolytic oscillations are characterised by fluctuations in NADH fluorescence only, but yeast metabolic cycles consist of fluctuations in NADH fluorescence as well as fluctuations in other compounds like ATP and flavins.

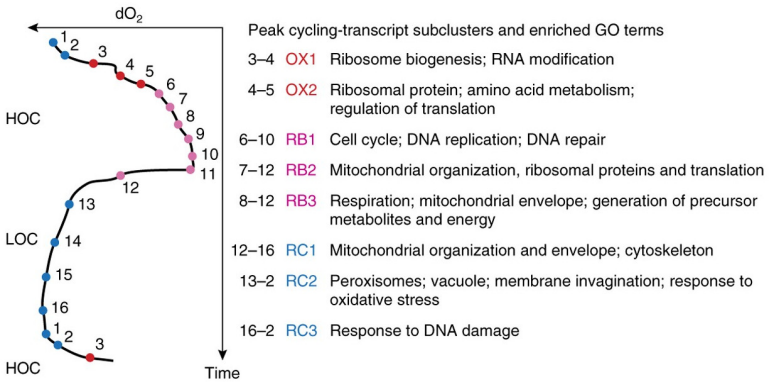

Figure 1.1: Phases of the yeast metabolic cycle, with (left) high- (HOC) and low-oxygen consumption (LOC) phases defined by changes in dissolved oxygen concentration (dO_2) over time in the chemostat and (right) oxidative (OX), reductive-building (RB) and reductive-charging (RC) phases defined by cycling of transcripts. Adapted from Mellor (2016).

Phases of the yeast metabolic cycle

Based on chemostat studies, the YMC can be divided into two major phases: an oxidative, high-oxygen consumption (OX/HOC) phase and a reductive, low-oxygen consumption (RED/LOC) phase (figure 1.1). Many authors (Slavov, Macinskas et al., 2011; D. B. Murray, Haynes et al., 2011; Causton, 2018) use oxygen consumption rates, evidenced by the change of dissolved oxygen concentrations over time, as a basis to refer to the YMC as a two-phase cycle. Though, there are authors (Machné and D. B. Murray, 2012) that base their two-phase model on the clustering of gene expression patterns. Krishna and Laxman (2018) interpret the oxidative phase as a growth state, while the reductive phase is a quiescent state. In contrast to the two-phase model, some authors identify a three-phase model with a reductive-building (RB) phase and a reductive-charging (RC) phase within the reductive phase, especially within the long-phase (4–5 hours) yeast metabolic cycle. This three-phase model is primarily based on cellular events, including clustering of transcript trajectories (Tu, Kudlicki et al., 2005) and of metabolite concentration trajectories (Tu, Mohler et al., 2007).

Single-cell studies (Papagiannakis et al., 2017; Baumgartner et al., 2018) do not discuss phases as the single-cell microfluidic set-up does not allow live monitoring of transcription, and oxygen consumption rate is an emergent property from chemostat cultures. Possibly, the two- or three-phase response results from cellular adaptation to glucose limitation in chemostat cultures, and it is unknown whether these dynamics hold true in glucose-rich conditions, which cannot be created in a chemostat (Slavov and Botstein, 2011).

In the oxidative phase, cells consume oxygen at a high rate as respiration, fermentation, and energy-demanding processes like biosynthesis and gene expression occur. Occurrence of biosynthesis and associated gene expression is confirmed by increased transcripts from genes encoding components of the translation machinery and amino acid biosynthesis (Tu,

Kudlicki et al., 2005). As the oxidative phase transitions to the reductive phase, ethanol and acetate concentrations in the medium peak as respiration finishes (Tu, Kudlicki et al., 2005). 'Redox state' metabolites, including NADH, NADPH, glutathione (Lloyd and D. B. Murray, 2005), and flavins (FMN and FAD) (D. B. Murray, Haynes et al., 2011) become most oxidised in this phase. Here, 70% of metabolite concentrations peak with the combined autofluorescence of NADH and NADPH (D. B. Murray, Beckmann et al., 2007).

fermentation?

I don't follow

In the reductive phase, cells consume oxygen at a low rate. During the reductive-building phase, activities linked to mitochondrial growth occur. There is evidence to suggest that activities linked to cell proliferation — such as initiation of the cell division cycle, DNA replication, and spindle pole activity — are gated to the reductive-building phase for both the short-period and long-period YMC. Such evidence includes budding activity and the pattern of the expression of *YOX1*, which encodes a cell division cycle repressor (Tu, Kudlicki et al., 2005).

Finally, during the reductive-charging phase, non-respiratory metabolism and degradation processes occur to prepare the cell for the oxidative phase. This non-respiratory metabolism includes glycolysis, ethanol and fatty acid metabolism, and nitrogen metabolism. With these metabolic modes, under the regulation of the transcription factors Msn2p and Msn4p (Kuang et al., 2017), acetyl CoA accumulates so ATP can be produced in the oxidative phase (Tu, Kudlicki et al., 2005). After acetyl CoA levels reach a threshold, it promotes histone acetylation and thus induces the oxidative phase. These metabolic pathways also optimise production of NADPH — based on the induction of *GND2* — to buffer against oxidative stress in the oxidative phase. Genes associated with protein degradation, ubiquitinylation, peroxisomes, vacuoles, and the proteosome also peak in the reductive-charging phase.

It has been hypothesised that gating activities linked to cell proliferation to the reductive-building phase creates a temporal separation between oxidative biochemical processes (in OX) and the cell division cycle. This temporal separation may prevent reactive oxygen species generated by oxidative process from damaging DNA. However, measuring DNA content and oxygen consumption in cells grown at different growth rates (Slavov and Botstein, 2011) showed that the S phase of the cell cycle may occur in the oxidative phase if the cells have a slow growth rate. This may be explained by the YMC gating the early and late cell cycle independently, as evidenced by periodic localisation of the anaphase-promoting complex and mitotic exit activator Cdc14 phosphatase in metaphase-arrested cells (Y. Lu and Cross, 2010) and, conversely, the persistence of NAD(P)H cycling upon arresting of the late cell cycle by depletion of Cdc14 (Papagiannakis et al., 2017), all observed in single cells. This evidence indicates that the gating between the YMC and the cell cycle is flexible. Though there is no meaningful consensus as to the function of such flexible gating, such gating can be instrumental in the allocation of metabolites to different temporal phases of both cellular oscillators.

1.2.3 Yeast metabolic cycles under perturbations

Perturbations in growth conditions can affect the length of the metabolic cycle and its relationship with other cellular events. The long-phase cycle may vary from 1.4 to 14 hours (Causton, 2018). The main nutrient perturbations that have been studied are perturbations in carbon sources and in nitrogen sources.

Perturbations in growth conditions

Perturbations in carbon sources are well-documented. Lower glucose concentrations prolong the metabolic cycle, as evidenced by both chemostat studies that assess the effect of changing the dilution rate (Burnetti et al., 2016; O' Neill et al., 2020) and by single-cell studies that assess the effect of glucose concentrations in the limiting region (Papagiannakis et al., 2017). For example, decreasing the dilution rate decreases the growth rate, and in turn increases the duration of the oxidative phase relative to the reductive phases (Slavov and Botstein, 2011), thus prolonging the metabolic cycle. This effect is pronounced in the region of glucose limitation. Increasing the glucose concentration beyond a certain point does not produce an effect. Additionally, several studies (Slavov and Botstein, 2011; O' Neill et al., 2020) show that the duration of the low oxygen consumption phase increases while the duration of the high oxygen consumption phase holds constant if the metabolic cycle duration increases due to these reasons.

Such experimental observations could by explained by models such as K. D. Jones and Kompala (1999), which suggest that as the dilution rate is decreased, metabolic oscillations acquire greater amplitudes and longer periods, but if it is low enough, metabolism becomes entirely oxidative, and metabolic oscillations disappear. However, non-fermentable carbon sources like pyruvate give long-duration metabolic cycles in single cells, comparable to cells under limiting levels of glucose (Papagiannakis et al., 2017).

In addition, bulk depletion or addition of a carbon source can reset the phase of the YMC. Chemostat studies show that an initial starvation phase is needed to generate long-lasting synchronous metabolic cycles (Tu, Kudlicki et al., 2005). On the other hand, adding a bulk carbon source such as acetate, ethanol, or acetaldehyde can reset the phase of the YMC (Kuang et al., 2017; Krishna and Laxman, 2018) or eliminate it (K. D. Jones and Kompala, 1999).

Perturbations in nitrogen sources are less well-studied. Baumgartner et al. (2018), based on single-cell observations, suggest that decreasing nitrogen source concentration prolongs the YMC, as evidenced by longer flavin oscillations when cells are grown on lower concentrations of yeast nitrogen base (YNB) media or on urea, a non-preferred nitrogen source.

In addition, perturbations outside nutrient sources also affect the YMC. For example, externally applied hydrogen peroxide, as a source of oxidative stress, shifts the YMC to the oxidative phase (Amponsah et al., 2021). The oscillation period is insensitive to temperatures from 25 °C to 35 °C and media pH values from 2.9 to 6.0 (Lloyd and D. B. Murray, 2005) — though the period of dissolved-oxygen oscillations decrease as pH decreases to below 2.9 and the oscillations disappear when conditions are too acidic (O' Neill et al., 2020). Additionally, the dissolved-oxygen oscillations are robust to media potassium ion concentrations varying between 1 and 10 mM, but such oscillations disappear when the potassium concentration falls below 1 mM (O' Neill et al., 2020).

Finally, the phase difference between the YMC and cell cycle varies in different conditions (Ewald et al., 2016).

Genetic perturbations

Although the molecular basis of the yeast metabolic cycle is not well-characterised, gene deletions shed light on it. Genes that control the cell division cycle and metabolism have been deleted in studies.

Several deletions have been shown to remove the metabolic oscillations in chemostats: zwf1Δ (Tu, Mohler et al., 2007), gsy2Δ, and gph1Δ (O' Neill et al., 2020). *ZWF1* codes for glucose-6-phosphate dehydrogenase and is thus responsible for entry into the pentose phosphate pathway and subsequently a major source of NADPH generation, so deleting this gene may impair control of cellular redox. However, because of its role, this gene deletion impairs adapting to oxidative and pH stress and also causes methionine auxotrophy, so it may be difficult to draw conclusions from this deletion in particular. Furthermore, other enzyme-catalysed reactions in the cell that generate NADPH exist (Idp2p, Ald6p) and have shown to compensate for the loss of *ZWF1* when cells are grown on lactate plates or on liquid cultures with glucose as the carbon source (Minard and McAlister-Henn, 2005). This therefore raises the question of just how important *ZWF1* is to the yeast metabolic cycle, and to what extent is NADPH generation needed for control of cellular redox. On the other hand, *GSY2* and *GPH1* both have roles in glucose/glycogen mobilisation and storage. The absence of dissolved oxygen cycles in the associated deletions thus suggest that cycling of carbohydrate stores may be needed for the function of the metabolic cycle. However, metabolic oscillations have been observed in high-glucose conditions (Papagiannakis et al., 2017; Baumgartner et al., 2018) in which glycogen synthesis is repressed, therefore suggesting that glycogen cycling may play a more minor role in defining the yeast metabolic cycle and another nutrient cycling phenomenon may be more responsible. In addition, *MSN2* and *MSN4* have been shown to regulate acetyl CoA accumulation in the reductive-charging phase, as evidenced by the lack of YMCs in deletion strains (Kuang et al., 2017). This tells us that genes involved in signalling pathways play an important role in the integrity of the metabolic cycle too.

Additionally, other deletions have been shown to change the frequency or shape of dissolved oxygen cycles. Causton et al. (2015) provide several examples, of which I discuss rim11Δ, swe1Δ, and tsa1Δ tsa2Δ. Rim11p is the yeast homolog of the GSK3β serine/threonine kinase, which regulates metabolism and plays a role in setting the speed of the circadian clock. The *RIM11* deletion has been shown to give shortened periods of dissolved-oxygen metabolic cycles in the chemostat, thus pointing towards a common mechanism for both biological oscillators. Swe1p is a conserved cell division cycle regulator that functions at the G2/M checkpoint and has roles in coupling the cell division cycle with the circadian rhythm. Deleting *SWE1* also resulted in shortened periods of dissolved-oxygen metabolic cycles but with the same rate of DNA replication, suggesting a dysregulation in the coupling between the yeast metabolic cycle and the cell division cycle. Tsa1p and Tsa2p are paralogous cytoplasmic thioredoxin peroxidases that cooperate in the peroxiredoxin-thioredoxin system to eliminate reactive oxygen species and have been shown to be a marker for circadian rhythms. A double deletion of the two associated genes still results in metabolic cycles, but with an additional burst in high oxygen consumption during what would otherwise be the reductive-charging phase, showing that the peroxiredoxin-thioredoxin system is instrumental in the integrity of the yeast metabolic cycle. In addition, Amponsah et al. (2021) show the presence of cycling peroxiredoxin oxidation during the YMC using chemostat-based studies, with a corresponding cycling of hydrogen peroxide. They also confirm that inactivating peroxiredoxins — tsa1Δ tsa2Δ, additionally with inducible degradation of Ahp1, another cytosolic peroxiredoxin — disrupts the metabolic cycle and decouples it from the cell division cycle. Taken together, these deletion studies show that regulators of other biological rhythms and of redox metabolism play a role in the regulation of the YMC.

However, few genetic perturbation studies have been attempted in single-cell studies. The most significant is in Baumgartner et al. (2018), in which by deleting genes (atp5Δ, cyt1Δ) required for respiration, they showed that metabolic cycling does not require respiration.

1.2.4 Modelling the yeast metabolic cycle

Mathematical models have been developed to explain the aspects of the YMC. An early model is K. D. Jones and Kompala (1999) which simulates dynamic competition between three modes of metabolism — fermentation, glucose oxidation, and ethanol oxidation — using differential equations. This model predicts spontaneous generation of oscillations in dissolved oxygen, cell mass, and storage carbohydrates in continuous cultures. This prediction is consistent with chemostat-based studies of the yeast metabolic cycle. Furthermore, the model predicts that, within a window of dilution rate values, if the dilution rate decreases, the

dissolved oxygen oscillations increase in amplitude and period. The increase in period agrees with experimental studies such as O' Neill et al. (2020). However, the model also predicts oscillations in the extracellular concentrations of glucose and ethanol, which conflicts with the steady-state assumption of chemostat studies.

Krishna and Laxman (2018) use a frustrated bistability model to describe a relaxation oscillator that explains how a population of yeast cells switches between quiescent and growth states when faced with a limited amount of metabolic resources. This model assumes that the cells retain hysteresis of their current state and posits that cells of two populations communicate through diffused acetyl-CoA to sustain population-level oscillatory behaviour. Burnetti et al. (2016) also propose that yeast cells committed to the metabolic cycle secrete metabolites that induce other cells to enter the metabolic cycle, provided that they have enough storage carbohydrates. Taken together, the models provide an attractive cell-to-cell signalling explanation for the population-level behaviour observed in the chemostat. However, such an explanation does not explain the presence of metabolic cycling in single-cell conditions in which cells are physically separated and thus signalling between cells cannot occur — though it must be noted that autonomous generation of metabolic cycles and synchrony of metabolic cycles in a population can each arise from mechanisms that are independent of each other.

Based on single-cell experimental observations, Özsezen et al. (2019) use a deterministic Kuramoto model to explain the interaction between one metabolic oscillator and three cell cycle oscillators at different stages. This study builds upon use of the Kuramoto model to model collective oscillatory behaviour in other biological systems. The study uses growth on different carbon source conditions to determine parameters that define the natural frequencies of the cell division cycle oscillators and the strength of the coupling between the four oscillators. Parameter optimisation predicts that the metabolic cycle most strongly influences the START point of the cell division cycle, and more weakly influences the M and S phases, while the three points of the cell division cycle negligibly influence each other. Under perturbations, the model system also exhibits stability but also a shift in oscillation frequency, agreeing with experimental observations, and also predicts the effects of Cdc20 and Cdc14 dynamic depletions. However, a key criticism of this model-based study is that by using the Kuramoto model, it makes simplistic assumptions about the oscillators, which may be unrealistic especially given how little is known about the mechanistic basis of the metabolic oscillator.

Taken together, modelling approaches have been able to predict some aspects of the metabolic cycle. But, most focus on specific aspects to the detriment of other experimental observations, and none sufficiently reconcile observations from both chemostat-based and single-cell studies. Constructing more accurate models is complicated by how little of the mechanistic basis of the yeast metabolic cycle has been elucidated thus far.

1.2.5 Big picture/Hypothesis: a nutrient sensor than entrains the cell division cycle?

From existing evidence, we can create a big picture of the yeast metabolic cycle. The yeast metabolic cycle is a autonomous biological oscillator that operates at a range of frequencies in response to a range of (permissive) growth conditions, as evidenced by how extreme conditions impair the oscillator. Based on chemostat-based studies, such extreme conditions include poor nutrient quality, media being too acidic, and potassium ion concentration being too low. However, there is reason to believe that the metabolic oscillator can function in some conditions previously deemed to be unfavourable. For example, single-cell studies show that yeast cells show metabolic oscillations in high-glucose conditions. Within the permissive growth conditions, different conditions affect the frequency of the metabolic cycle. For example, a low concentration of glucose or nitrogen source results in longer cycles, and bulk addition of certain compounds can reset the phase of the metabolic cycle. These observations support the idea that the metabolic oscillator includes the functionality of a nutrient sensor.

This suggest that

The yeast metabolic cycle then creates windows of opportunities for the cell to commit to START if conditions are favourable, for example, good carbohydrate or lipid stores. Thus, this oscillator acts as a timing mechanism for cellular processes, most importantly the cell division cycle and biosynthetic/redox processes. Though the relationship between the metabolic cycle and the cell division cycle is governed by the mathematical basis of coupled oscillators. Most importantly, there is a small window of frequencies in which both oscillators can be phase-locked, and that other, complicated relationships exist: e.g. multiple metabolic cycles per cell division cycle — in line in the window-of-opportunity idea above.

1.2.6 Disputes and unresolved questions with the yeast metabolic cycle

Chemostat vs single-cell studies

There is a dispute of whether the same conclusions can be drawn from chemostat-based studies and from single-cell based studies. Most studies of the YMC arise from chemostat experiments and any conclusion from a single-cell study is subject to the question of whether it recapitulates the YMC in the chemostat. Reconciling the two types of studies is difficult because the readouts and conditions are different: chemostat studies produce dissolved oxygen and transcript cycling readings, while single-cell experiments cannot report on dissolved oxygen and chiefly report metabolite cycling. This leads to differing definitions of the YMC: some authors (Laxman et al., 2010; Causton, 2018) only use the term metabolic cycle to refer to synchronised cycles of dissolved oxygen concentrations observed in chemostat cultures that must have gone through a starvation phase, while single-cell studies (Baumgartner et

al., 2018; Zylstra and Heinemann, 2022) naturally have to expand that definition to include metabolite cycling and sequences of cellular events that are associated with the chemostat metabolic cycle. For the purposes of my thesis, I adhere to the definition used by single-cell studies.

I argue that there are three caveats to chemostat-based studies: the experimenter cannot assume that the chemostat is in steady-state, the chemostat obscures contributions from sub-populations, and the chemostat imposes glucose starvation. These caveats affect the interpretation of YMC studies.

There is a wide assumption that the chemostat is in steady-state, but it may not be true. A mathematical model shows that levels of solutes change over time (K. D. Jones and Kompala, 1999). In addition, O' Neill et al. (2020) shows a chemostat setup that promotes evaporation of hydrogen sulfide gas, thus shifting the equilibrium of the reduction of bisulfides. This may affect redox metabolism in the cell. In such a case, chemostat observations may not reflect cell-autonomous behaviour. Instead, the oscillations may reflect individual cells' responses to the initial starvation imposed at the start of chemostat-based studies The subsequent response to regularly changing media conditions could explain temporal segregation of physiological processes in phases of the YMC. In other words, the conditions of the chemostat may force the population of cells to behave in a certain way. However, temporal segregation of physiological process has also been reported in single-cell studies (Takhaveev et al., 2023), suggesting that the cycling of solutes in the chemostat could affect some, but not all, temporal aspects of the metabolic cycle.

The chemostat obscures contribution of sub-populations of cells. Burnetti et al. (2016) suggest that sub-populations within the yeast culture that enter the yeast metabolic cycle in a staggered manner can be responsible for chemostat observations, as evidenced by how the yeast cells spend proportionately more time in the reductive phase at lower dilution rates. Contributions from sub-populations of cells are further highlighted by Bagamery et al. (2020), who used a microfluidic platform to show that a group of genetically identical yeast cells divide themselves into two populations. Such a bet-hedging strategy results in some percentage of the population surviving in a glucose-starved or a glucose-rich condition, beneficial for long-term population survival. Taken together, it is possible that phenotypically different sub-populations in the chemostat culture may partially explain the observations in the chemostat so far. In addition, there is the question of whether cells individually generate the metabolic cycle or is a diffusible chemical responsible for synchrony, as proposed by Krishna and Laxman (2018). Bulk culture set-ups, including chemostats, are not able to address questions about cell sub-populations and autonomy of the metabolic cycle. However, single-cell set-ups may fill in such a technical gap.

Finally, the chemostat imposes glucose starvation, and single-cell studies with different carbon sources give a different picture in terms of metabolic requirements. Chemostat studies and related models suggest that glucose starvation and oxidative metabolism are required for oscillations in dissolved oxygen level that define YMCs. NAD(P)H oscillations have been recorded in non-fermentative conditions, such as pyruvate or low-glucose media (Papagiannakis et al., 2017). However, NAD(P)H (Papagiannakis et al., 2017; Özsezen et al., 2019) and flavin (Baumgartner et al., 2018) oscillations still occur in constant high-glucose conditions, and only within a window of periods, in contrast to the 1.4–14 hour range reported for chemostat-based studies. Furthermore, *ATP5* and *CYT1* deletions that impair oxidative respiration do not remove single-cell flavin-based metabolic oscillations (Baumgartner et al., 2018), thus giving additional evidence that oxidative metabolism is not required for the YMC.

Single-cell microfluidic studies are well-positioned to address the limitations of the chemostat, although there have been only few studies. Laxman et al. (2010) was an early attempt at using microfluidics to address the bulk vs single-cell issue by culturing strains with fluorescent gene expression reporters for each phase (OX, RB, RC) of the metabolic cycle by transferring cycling cells from a chemostat to a microfluidic device for real-time imaging of the cells. The study showed that low glucose levels were required for the synchrony of metabolic cycles across cells. This study also shows the presence of quiescent cells, and then proposed that during OX phase, cells decide whether to commit to cell growth or enter a quiescent state, leading to a model of two subpopulations in the culture. However, it lacks quantitative time-series analysis, rather, reporting qualitative interpretations of fluorescent images. The microfluidic device did not truly physically separate each cell individually, thus it was unable to eliminate the possibility of cell-to-cell communication via a diffusible signalling chemical. Papagiannakis et al. (2017) revealed that YMCs are an intrinsic feature of single cells and are autonomous with respect to the cell division cycle, based on measurements of the combined level of NADH and NADPH in single cells in microfluidic devices. Furthermore, by measuring flavin fluorescence in the cell, Baumgartner et al. (2018) demonstrated that YMCs persist in mutants deficient in oxidative phosphorylation, and that the cell division cycle inhibitor rapamycin desynchronises the YMC and the cell division cycle. In sum, these single-cell studies address a small fraction of the knowledge covered by chemostat studies, and further such studies are required.

Molecular and genetic mechanisms

There are unknowns in the molecular mechanism that drives YMCs. Genome-wide transcript cycling has two superclusters that correspond to the oxidative and reductive-building phases (Machné and D. B. Murray, 2012). However, there has been no genome-wide analysis of genes that influence cycling (Mellor, 2016), though some genes seem to have key roles. As we lack proteome analysis, it is unclear how protein levels and post-translation modifications are affected.

Metabolome cycling may play a role in the metabolic cycle and can explain temporal partitioning of biosynthesis, but the evidence so far is indirect as it is based on the cell division cycle. Campbell et al. (2020) showed that lipid biosynthesis in budding yeast is periodic with the cell division cycle and peaks during S phase, as evidenced by an increase in the number of metabolites implicated in lipid metabolism in such phases, based on metabolomics analysis of prototrophic cells with synchronised cell division cycles. Here, precursors are synthesised as they are needed. Ewald et al. (2016) also show that the cell division cycle machinery regulate trehalose mobilisation, showing the coupling between carbohydrate store levels and cellular oscillators. They also showed that lipid metabolism increased during S/G2/M, likely due to the synthesis of new cell membranes during bud growth, as evidenced by pathway enrichment analysis. Based on the coupling between the yeast metabolic cycle and the cell division cycle, it can be inferred that lipid store cycling and perhaps to a lesser extent carbohydrate store cycling are likely instrumental to the yeast metabolic cycle. Though, investigation of how an impairment in lipid utilisation affects the yeast metabolic cycle in single cells is needed to truly prove that such cycles are responsible for the metabolic cycle. Preferably, I need evidence to support metabolome cycles that also confirms that the YMC is independent of the cell division cycle.

1.2.7 Implications of the metabolic cycle

The YMC shares regulatory mechanisms with the cell division cycle and the circadian rhythm, leading to the question of whether the metabolic cycle reflects a fundamental system.

Similar metabolic cycles have been described in other organisms. *E. coli* shows oscillations in NAD(P)H fluorescence coupled to its cell division cycle, as evidenced by time-lapse microscopy of single cells (Z. Zhang et al., 2018). Addition of glucose or hydrogen peroxide to the medium results in global changes in autofluorescence, reflecting a response to nutrient conditions. Metabolic cycles have been observed in mammalian cells. For example, Zhu (2022) describe a 12-hour metabolic cycle in liver cells that includes temporal partitioning of metabolic processes into energy homeostasis, genetic integrity maintenance processes, immune response, and gene expression — linking the processes to the circadian rhythm and the whole cycle to a more general 12-hour mammalian ultradian clock. Importantly, this hepatic metabolic cycle operates independently from the spatial organisation of cells in the

liver, reminiscent of the autonomy of the yeast metabolic cycle. In addition, HeLa cells with synchronised cell division have been shown to exhibit both NAD(P)H and ATP oscillations throughout the cell division cycle (Ahn et al., 2017), but the literature is conflicted about the these oscillations' dynamics across different mammalian cell types (Zylstra and Heinemann, 2022). There is reason to believe that a wide range of organisms exhibit biochemical phenomena similar to the yeast metabolic cycle, as the aims of controlling cell division to match environmental conditions along with the temporal coordination of biosynthesis and cellular redox state with cell division should be fundamental goals that apply to all eukaryotes.

Metabolic oscillations may be the origins of biological timekeeping mechanisms. Lloyd and D. B. Murray (2007) assert that ultradian oscillations form the basis of longer-period biological oscillators like the circadian rhythm or the cell cycle, based on temperature compensation and sensitivity of the period. It is logical for a biological oscillation to have temperature compensation because temperature oscillates with a period of a day on most of the planet. Circadian rhythms can occur in cells of multicellular eukaryotes without transcription (O'Neill et al., 2011), refuting the idea that gene circuits are responsible for such rhythms. Additionally, the eukaryotic cell cycle evolved before cyclin-dependent kinases (Papagiannakis et al., 2017), so metabolic oscillations may have served to regulate the cell cycle before cyclin-dependent kinases evolved. Furthermore, YMCs share mechanisms with the circadian oscillator (Causton et al., 2015; Arata and Takagi, 2019), suggesting a common evolutionary origin. Thus, studying YMCs may shed light on the evolution of biological rhythms.

1.3 Flavins and flavoproteins

1.3.1 Introduction to cellular autofluorescence

Cellular autofluorescence is defined as the intrinsic fluorescence of a cell without fluorescent tags. It is caused by the autofluorescence of compounds that have light emission properties (Maslanka et al., 2018). Such endogenous fluorophores include coenzymes, vitamins, and amino acids with aromatic chemical groups, flavins being one of them. However, autofluorescence pose difficulty in cellular microscopy because their wavelengths can overlap with other fluorophores and therefore it is difficult to draw biochemical conclusions from the signal alone. For example, flavin autofluorescence overlaps with the spectrum of the fluorescent glucose analogue 6-NBDG (Maslanka et al., 2018), and thus interferes with studies that use this analogue to study glucose uptake. Owing to the identity of the compounds that are responsible for autofluorescence, cellular autofluorescence can indicate of the physiology and metabolism of the cell, and it has been used to study the yeast metabolic cycle. Autofluorescence thus offers an easy way to monitor cell physiology without engineering genetic constructs.

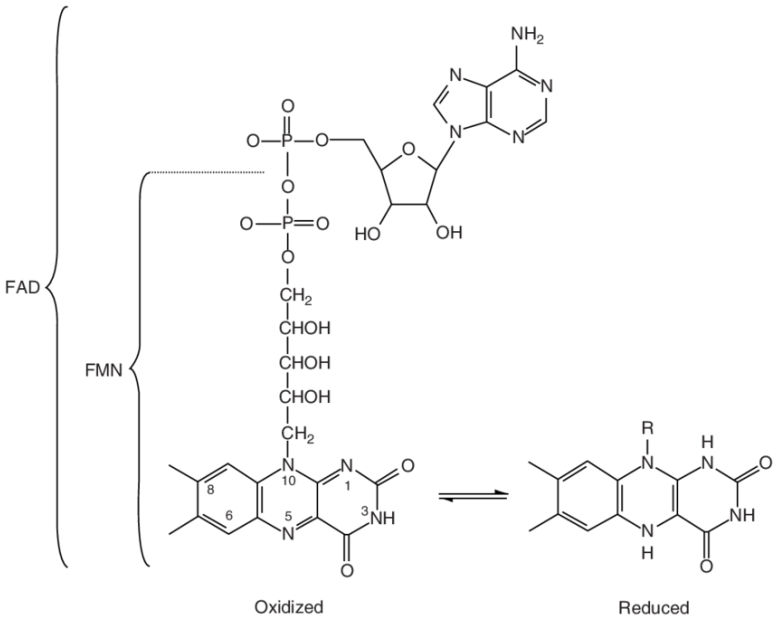

Figure 1.2: Chemical structure of FMN and FAD, with redox states of the aromatic flavin moiety shown. Adapted from Patel (2006).

1.3.2 Biochemical basis of flavins and flavoproteins

Flavins are a group of organic compounds that share an aromatic moiety that allows redox reactions (figure 1.2). Specifically, the flavin moiety can exist in the oxidised, semiquinone, or reduced states. Flavins thus function as electron carriers in the cell. In *Saccharomyces cerevisiae*, flavin is present as FMN and FAD, which function as prosthetic groups in flavin-dependent proteins, or flavoproteins, whose genes account for 1.1% of the genome (Gudipati et al., 2014). FMN and FAD can be covalently bound to these proteins or be free. FAD is a co-enzyme and has major roles in transferring electrons from the TCA cycle to the mitochondrial ETC. Flavins in *Saccharomyces cerevisiae* are derived from riboflavin (figure 1.3). Riboflavin can be synthesised *de novo* from purine biosynthesis and the oxidative pentose phosphate pathway (figure 1.4). Based on the metabolism of flavins, the cell only synthesises new flavin for synthesis of FMN and FAD.

From a technical standpoint, the redox states of flavins reflect the emission and absorption of electromagnetic radiation by the flavin moiety. The redox biochemistry of flavins give rise to fluorescence, so monitoring flavin autofluorescence monitors the redox state of the cell. Flavins, in their oxidised forms (FMN and FAD), have a peak excitation frequency of $\approx$460 nm and a peak emission frequency of $\approx$535 nm (Maslanka et al., 2018; Wagnieres et al., 1998), displayed in figure 1.5. Comparison of *in vivo* autofluorescence in mammalian cells and the fluorescence spectrum of riboflavin in PBS confirms this fluorescence behaviour

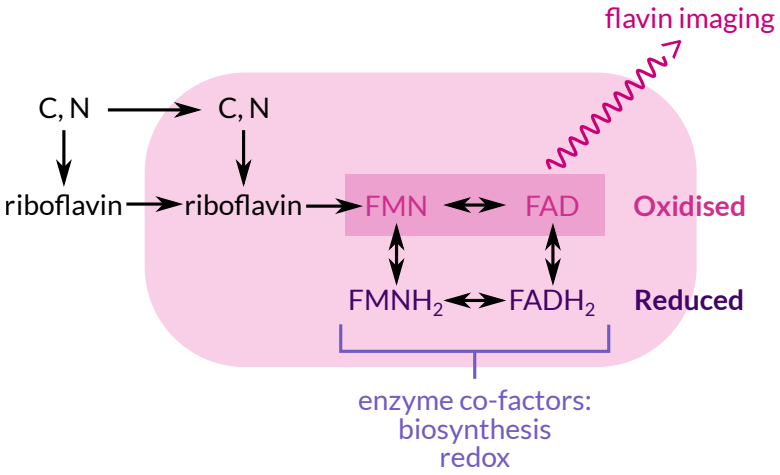

Figure 1.3: Simplified schematic of biosynthesis of flavins and detection of the oxidation states in fluorescence microscopy.

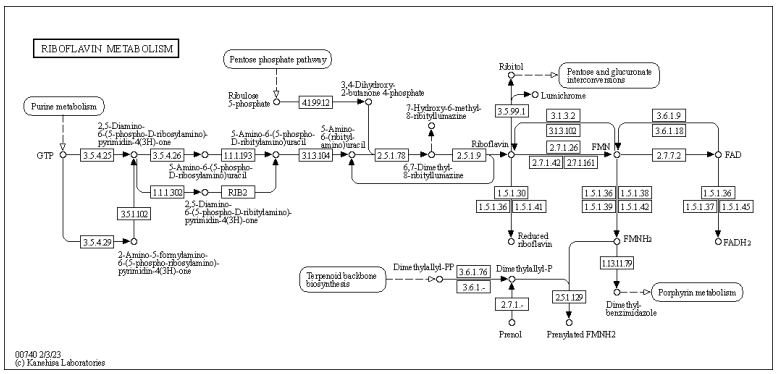

Figure 1.4: Reference pathway for biosynthesis of riboflavin and derivatives, KEGG pathway database (Kanehisa et al., 2023).

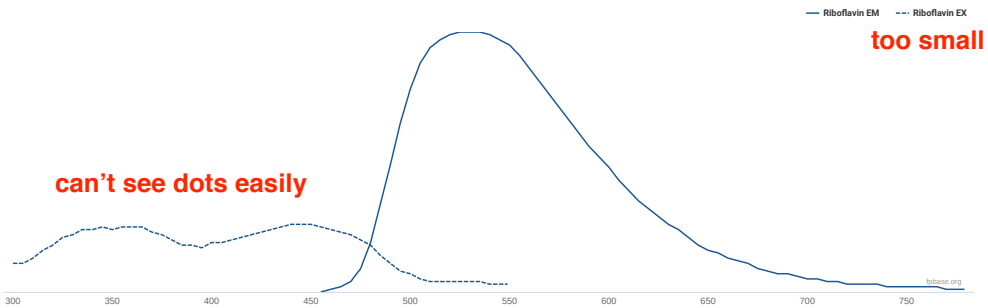

Figure 1.5: Fluorescence spectrum of riboflavin, (dotted line) excitation and (solid line) emission spectra shown, FPbase (Lambert, 2023).

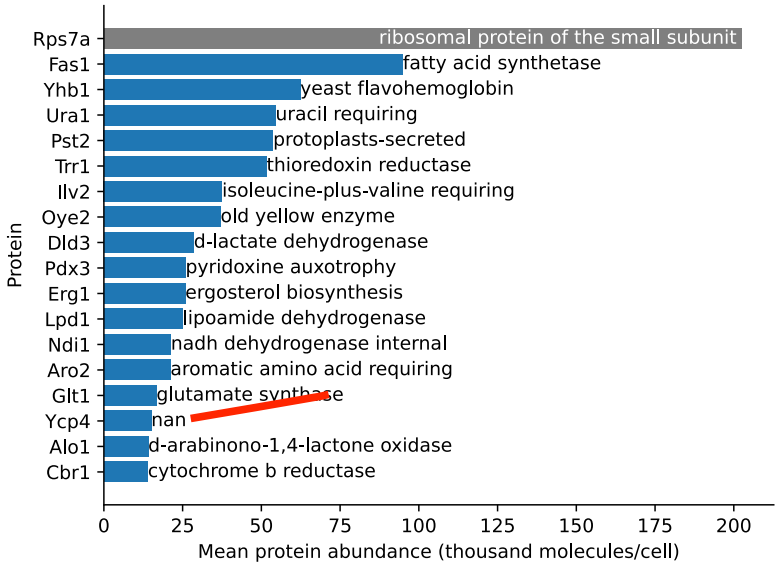

Figure 1.6: Flavoproteins (blue bars) shown by abundance (Ho et al., 2018), with Rps7ap (grey bar) shown as reference. Only the 17 most abundant flavoproteins are shown.

(Aubin, 1979). In contrast, the reduced forms FMNH2 and FADH2 have negligible fluorescence (Masters, 1994). Both chemostat-based (Sasidharan et al., 2012; D. B. Murray, Haynes et al., 2011) and single-cell microfluidic studies (Baumgartner et al., 2018) have monitored flavin autofluorescence to study the YMC.

Descriptions of key flavoproteins and their roles

Gudipati et al. (2014) describe 68 genes that code for 47 flavoproteins in budding yeast (figure 1.6). Of these, 35 require FAD, 15 require FMN, and 3 require both. In budding yeast, most flavins sit in the active site without covalent bonding. The biochemical and enzymatic properties of many flavoproteins are poorly characterised (Koch et al., 2017).

The most abundant flavoproteins catalyse redox reactions (table 1.1). Specifically, these reactions include reduction of reactive chemical species to respond to oxidative stress — though not all enzymes involved in the response to reactive chemical species have flavin co-factors. Additionally, the reactions include biosynthetic reactions. Many of these reactions require NADPH or NADH to donate electrons, suggesting a link between flavins and NAD(P)H in regulating the cellular redox state. In particular, Oye2p catalyses the NADPH redox reaction, thus providing a link between flavins and NAD(P)H. One exception flavoprotein is Ilv2p, which does not catalyse a redox reaction. It has been hypothesised that an ancestral form of Ilv2p catalysed a redox reaction, but the argument is weak because it is inferred from the presence of FAD (Pang et al., 2002). To maintain the cellular redox state, it is thus reasonable to assume that the redox equilibrium of all flavoprotein-catalysed reactions are in the same

Protein	Name	Reaction catalysed	Reference
Fas1 fas1	beta subunit of fatty acid synthetase	acetyl−CoA + malonyl−CoA + NADPH + ATP $\longrightarrow$ palmitate	Singh et al. (2020)
yhb1	nitric oxide oxidoreductase	$2\,NO + 2\,O_2 + NAD(P)H \longrightarrow 2\,NO_3^- + NAD(P)^+ + H^+$	Bonamore and Boffi (2008)
ura1	dihydroorotate dehydrogenase	dihydroorotic acid + fumarate $\longrightarrow$ orotic acid + succinate	Zameitat et al. (2007)
pst2	NAD(P)H-quinone oxidoreductase	$NAD(P)H + H^+$ + quinone $\longrightarrow NAD(P)^+$ + hydroquinone	Koch et al. (2017)
trr1	cytoplasmic thioredoxin reductase	H^+ + NADPH + thioredoxin disulfide $\longrightarrow$ $NADP^+$ + thioredoxin	Machado et al. (1997)
ilv2	acetolactate synthase	2 pyruvate $\longrightarrow$ 2-acetolactate + CO_2	Pang et al. (2002)
oye2	NADPH oxidoreductase	NADPH + H^+ + acceptor $\rightleftharpoons$ $NADP^+$ + reduced acceptor	Odat et al. (2007)
dld3	2-hydroxyglutarate transhydrogenase	D−2-hydroxyglutarate + pyruvate $\longrightarrow$ α-ketoglutarate + lactate	Becker-Kettern et al. (2016)
pdx3	pyridoxine phosphate oxidase	pyridoxamine 5-phosphate + H_2O + O_2 $\longrightarrow$ pyridoxal 5-phosphate + NH_3 + H_2O	Tsuge et al. (1979)
erg1	squalene epoxidase	squalene + H^+ + NADPH + O_2 $\longrightarrow$ 2,3−oxidosqualene + $NADP^+$ + H_2O	Satoh et al. (1993)
lpd1	dihydrolipoamide dehydrogenase	dihydrolipoamide + NAD^+ $\longrightarrow$ lipoamide + $NADH^+ + H^+$	Morrison (2021)

Table 1.1: Roles of the most abundant flavoproteins.

direction at any point of the YMC. Supporting this, Siano and Mutharasan (1989) show that NAD(P)H fluorescence and the fluorescence of lipoamide dehydrogenase, a flavoprotein, indicate simultaneous reduction in response to lowered dissolved oxygen. They further show redox equilibrium in both fluorophores in response to glucose addition.

It is important to rule out the possibility that flavin cycling is merely a function of the cell division cycle to make sure that flavin monitoring monitors the YMC. None of these flavoproteins are strictly cell division cycle proteins, but this does not exclude cycling of flavin autofluorescence linked to the cell division cycle. For example, fatty acid synthesis proteins should cycle along with the cell division cycle as cell synthesises more plasma membrane.

1.3.3 Flavins and flavoproteins in the yeast metabolic cycle

Flavin fluorescence can be used to monitor the metabolic cycle. The biological basis of flavins justifies this use. Flavins are linked to NAD(P)H via nitric oxide oxidoreductase (Yhb1p), as discussed in section 1.3.2, and NAD(P)H cycles have been implicated in bulk-culture (Tu, Kudlicki et al., 2005) and single-cell (Papagiannakis et al., 2017) studies of the YMC, as discussed in section 1.2.2. The timing of flavin cycle peaks — indicating oxidation — coincides with the oxidative state of the YMC, as evidenced by how flavin fluorescence peaks in-phase with oxygen uptake rates in the chemostat (D. B. Murray, Haynes et al., 2011; Sasidharan et al., 2012). Riboflavin has been shown to oscillate and peak in the oxidative state of the YMC, while FAD peaks in the reductive-building phase, as evidenced by metabolic profiling of extracts from chemostat cultures taken at evenly-spaced intervals (Tu, Mohler et al., 2007). Flavoproteins may have roles linked to the YMC. The most abundant is Fas1 (fatty acid synthetase). Because there is evidence that cycles of fatty acid stores are implicated in metabolic cycling in yeast (Campbell et al., 2020), it is likely that fatty acid synthetase is heavily implicated. Following this, the second most abundant is Yhb1, which may play a major role as discussed earlier.

So, for these reasons, I expect flavin autofluorescence to be oscillatory and be a useful read-out of the yeast metabolic cycle. Few studies have characterised how such flavin oscillations respond to changing nutrient conditions or to gene deletions. Thus, filling in this knowledge gap is an avenue for further research.

Nevertheless, there are caveats to using flavin autofluorescence. Riboflavin fluorescence is captured too. Plus, different concentrations of riboflavin influence the autofluorescence signal and influence the physiological state of the cell (Maslanka et al., 2018). The experimenter can eliminate the effects of riboflavin by using riboflavin-free minimal media (Verduyn et al., 1992). Additionally, flavin fluorescence is the aggregate of many flavoprotein components, therefore it cannot be concluded that flavin fluorescence is the readout of one protein in particular — one can only draw conclusions about the overall redox state. Furthermore, the changes in flavin fluorescence can be because of changes in the ‘flavin pool’ — the amount of flavin-derived

moieties in a cell across all their redox states — or due to global changes in intracellular flavin redox state, as a function of intracellular redox state. Most studies assume a constant flavin pool and see oscillations as periodic shifts in redox equilibrium. These caveats are not unique to flavin fluorescence, but are shared limitations with other auto-fluorescing cellular components like NAD(P)H, and the benefits of having a non-invasive method to monitor cellular metabolism outweighs the caveats.