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Glycolytic oscillations and intracellular K⁺ concentration are strongly coupled in the yeast *Saccharomyces cerevisiae*

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Abstract

We measured temporal oscillations of intracellular K⁺ concentration in yeast cells exhibiting glycolytic oscillations using fluorescence spectroscopy and microscopy methods. These oscillations showed the same period as those of glycolytic metabolites (NADH, ATP), indicating a strong coupling between them. We experimentally ruled out that oscillations originate in extra- or intracellular K⁺ fluxes and conclude that these oscillations arise from fluctuations in free and adsorbed states of K⁺ in the cell interior. Oscillations in K⁺ showed a strong dependence on ATP and the organization of the cell cytoskeleton. Our results challenge the widely held view that intracellular K⁺ predominantly exists in a free state. They can, however, be productively understood in terms of Gilbert Ling’s Association-Induction hypothesis.

Keywords: intracellular potassium concentration; PBFI fluorescence; yeast cytosol; glycolytic oscillations; association-induction hypothesis.

Insight, innovation, integration

From a historical perspective there are two opposing views regarding the physical state of intracellular K⁺. The first argues that K⁺ exists as an essentially freely mobile ion, while the second, largely disregarded, proposes that it is mostly in an adsorbed state. Very little effort, however, has been devoted to revisit this problem in the last few decades. Our results, obtained during glycolytic oscillations in yeast, support the idea that the bulk of intracellular
K⁺ exists in an adsorbed state and it is dynamically regulated by central metabolism. These results can be productively understood in terms of the Association-Induction hypothesis.

**Introduction**

That glycolysis in yeast exhibits an oscillatory behavior was discovered over 60 years ago [1] and, since then, the phenomenon has been studied intensely in both intact cells and cell extracts [2, 3]. Remarkably, during these metabolic oscillations it is not just the concentration of glycolytic products and intermediates that oscillate, but also a series of physical-chemical properties such as, for example, mitochondrial membrane potential [4], heat flux [5, 6], conductivity of the medium [7] and intracellular pH [8, 9], among others.

Recent work from our laboratory showed that during glycolytic oscillations there is a tight coupling between intracellular ATP levels and the dynamical state of intracellular water, revealing that the latter parameter is crucial for the emergence of the oscillations [10, 11]. We also found that this coupling requires a functional cytoskeleton [10]. In a subsequent study, we observed that cell volume, heat flux and temperature also oscillate synchronously with the glycolytic oscillations, showing a strong coupling with oscillating metabolites [6]. These results were consistent with a recently proposed thermodynamic formalism in which isentropic thermodynamic systems can display coupled oscillations in all extensive and intensive variables, reminiscent of adiabatic waves [12]. This suggests that glycolytic oscillations may arise as a consequence of the requirement of living cells for a constant low-entropy state while remaining metabolically active. We argued that these results are in line with the Association-Induction hypothesis (AIH) [13, 14] that proposes that the cell interior behaves as a highly structured near-equilibrium system, constraining the emergence and development of glycolytic oscillations and coupled phenomena [6, 15].

The AIH, introduced by G.N. Ling in 1962, developed a rigorous statistical mechanical framework to explain principles of general cell-wide coupling using concepts from colloidal physical chemistry. Succinctly, it proposes that fluctuations in the chemical activity of central metabolites (e.g. ATP, but also others) during active metabolic processes modulate the conformational states of cytoskeletal proteins (e.g., actin) through association and inductive effects. This in turn modulates the binding affinity of intracellular water, ions and metabolites for proteins altering key mechanical properties (e.g. viscoelasticity) of the crowded cell interior and, consequently, the way in which numerous molecular actors perform [13, 14].
the particular case of ATP, Ling demonstrated that its centrality in cellular activity resides in its capacity to modulate the bulk properties of the intracellular environment as an integrated water-protein-solute system. The role of ATP as a biological hydrotrope has been further supported by very recent experimental work by Patel and coworkers [16], although they did not acknowledge the AIH.

In contrast to the view that the cell exists in an energy dependent (dissipative) steady state, the AIH quantitatively approaches the living state as a metastable near-equilibrium system that requires relatively small energy inputs to maintain its highly constrained dynamical properties. Using a mathematical model based on the Yang-Ling isotherm [17, 18], which is entirely based on the principles of the AIH, we were able to successfully simulate the coupling between the oscillations of ATP and intracellular water dynamics observed in our experiments [15]. The isotherm was also shown to be more reliable in describing kinetic aspects of glycolytic enzymes in crowded environments than classical models based on mass action kinetics such as Michaelis-Menten and Monod-Wyman-Changeux [15].

An unexplored aspect of glycolytic oscillations in yeast concerns the behavior of intracellular potassium ion (K\(^+\)). It is well recognized that intracellular K\(^+\) is essential for many processes in both prokaryotic and eukaryotic cells and that a high intracellular concentration of K\(^+\) is a constant in living systems. In yeast, for example, it has been shown that intracellular K\(^+\) is inextricably linked to cell volume, turgor, intracellular pH, electrical membrane potential, enzyme activity and ionic strength [19]. In mammalian cells it is accepted that a high intracellular K\(^+\) concentration is established and maintained by ion pumping mediated by the Na\(^+\)-K\(^+\) ATPase situated on the plasma membrane. In yeast cells, which lack the Na\(^+\)-K\(^+\) ATPase, it is proposed that the high intracellular K\(^+\) concentration (between 200 and 300 mM, depending on the strain and growth conditions) is the result of the action of various potassium transporters that use the proton-motive force created by the plasma membrane H\(^+\) ATPase Pma1 [20]. Both the mammalian and yeast mechanisms require intracellular K\(^+\) to be mostly in a free state to explain the accumulation of this ion in the cell interior. An alternative view, however, is offered by the AIH, where a predominantly adsorbed state of intracellular K\(^+\) [21] is proposed. As briefly discussed above, the AIH postulates that the extent of adsorbed intracellular K\(^+\) is regulated by the concerted action of intracellular ATP levels and cytoskeleton proteins (e.g. actin).
To determine whether intracellular $K^+$ is mostly free or adsorbed, we decided to obtain temporal information of intracellular $K^+$ levels, in parallel to other relevant parameters (such as levels of metabolites, intracellular water relaxation, etc.), during glycolytic oscillations in the yeast *Saccharomyces cerevisiae*. The aim of this study was to test whether the AIH can provide a sound mechanistic basis for glycolytic oscillations and other cell-wide emergent properties in yeast cells, as suggested by previous work from our laboratory.

**Results**

*Temporal behavior of $K^+$ during glycolytic oscillations*

In order to study the temporal behavior of $K^+$ concentration during glycolytic oscillations we used the potassium-sensitive fluorescent indicator PBFI [22, 23]. Upon inducing glycolytic oscillations, as confirmed by the typical temporal behavior of NADH during this process (Figure 1A), the PBFI labelled yeast cells show oscillations in the fluorescent emission of the probe (Fig 1B). The PBFI oscillations are synchronous with oscillations in (endogenous) NADH fluorescence, oscillating in antiphase to (Figure 1C), and with the same frequency of (Figure 1D), the NADH wave.

![Figure 1: NADH and PBFI oscillate with the same frequency and out of phase. (A, B) Oscillations of NADH and PBFI fluorescence; (C) phase plot of PBFI fluorescence versus NADH fluorescence; (D) Power spectra of NADH and PBFI oscillations. BY4743 yeast cells (10% w/v) stained with 40 µM PBFI were suspended in 100 mM potassium phosphate buffer with 20 mM NaCl, pH 6.8. Glycolytic oscillations were induced by addition of 30 mM glucose followed by 5 mM KCN. Temperature was 25 °C.](image-url)
We next endeavored to establish the source of the oscillating $K^+$ ions. The first possibility is that intracellular PBFI oscillations are produced by $K^+$ fluxes from the extracellular milieu and, to examine it, we performed experiments with a suspension of non-electroporated cells containing PBFI exclusively in the external medium (see Material and Methods section for the experimental details). We found that although NADH oscillations are apparent, no oscillations in extracellular $K^+$ were detected (Figure 2). This result shows that $K^+$ oscillations do not involve fluxes of this ion from the extracellular environment, and therefore that PBFI fluorescence oscillations have an intracellular origin. Additional experiments with isogenic yeast strains with mutations of various $K^+$ transporters located at the plasma membrane showed no effect on intracellular $K^+$ oscillations (Table 1, Figure S1), further establishing that $K^+$ oscillations are restricted to the intracellular milieu with no influx of the ion from outside.

**Figure 2:** Fluorescence time series of NADH (top) and PBFI added in the extracellular medium (bottom). BY4743 yeast cells (10% w/v) in 100 mM sodium phosphate buffer with 2 mM KCl, pH 6.8.
Table 1: Glycolytic oscillations in null mutations in sodium or potassium transporters

<table>
<thead>
<tr>
<th>Orf</th>
<th>Protein affected</th>
<th>Ion(s) transported</th>
<th>Location</th>
<th>Oscillation frequency (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YKR050w</td>
<td>Trk2p</td>
<td>K⁺</td>
<td>Plasma membrane</td>
<td>0.025 ± 0.002</td>
</tr>
<tr>
<td>YJL129c</td>
<td>Trk1p</td>
<td>K⁺</td>
<td>Plasma membrane</td>
<td>0.026 ± 0.001</td>
</tr>
<tr>
<td>YNL321w</td>
<td>Vnx1p</td>
<td>Na⁺/H⁺ and K⁺/H⁺</td>
<td>Vacuolar membrane</td>
<td>0.027 ± 0.003</td>
</tr>
<tr>
<td>YLR138w</td>
<td>Nha1p</td>
<td>Na⁺/H⁺</td>
<td>Plasma membrane</td>
<td>0.026 ± 0.002</td>
</tr>
<tr>
<td>YJL093c</td>
<td>Tok1p</td>
<td>Na⁺</td>
<td>Plasma membrane</td>
<td>0.025 ± 0.002</td>
</tr>
<tr>
<td>YBR296c</td>
<td>Pho89p</td>
<td>Na⁺/H₂PO₄⁻</td>
<td>Plasma membrane</td>
<td>0.026 ± 0.001</td>
</tr>
</tbody>
</table>

Intracellular distribution of PBFI

In order to understand how PBFI is distributed in the cell (and therefore where intracellular K⁺ is being measured), we compared oscillating PBFI stained and unstained cells using multiphoton excitation fluorescence microscopy. The results are shown in Figure 3 and show that the PBFI fluorescence signal is quite evenly distributed throughout the cell cytosol and mostly excluded from the vacuole. Since it has been reported that vacuoles in yeast have a higher K⁺ concentration than the rest of the cell [24], we decided to examine whether K⁺ flux between the vacuole and the cytosol is the cause -or contributes to- the observed cytosolic PBFI oscillations. A strain lacking the vacular cation membrane transporter Vnx1p (proposed to transport K⁺) exhibited normal K⁺ oscillations (see Table 1), supporting the notion that there is a cause other than intracellular K⁺ fluxes to K⁺ oscillations.

Figure 3: The PBFI probe distributes evenly in the cell cytoplasm. Two photon excitation fluorescence microscopy images of BY4743 wild type cells supplemented with 30 mM glucose and 5 mM KCN. The cells to the left are unstained while the cells to the right are stained with 10 µM PBFI.
*Estimation of intracellular K⁺ concentration*

Upon inducing glycolytic oscillations PBFI fluorescence fluctuates around a mean value with a (total) amplitude corresponding to about 60% of the average signal (Figure S2), indicating that PBFI interacts with a variable pool of free intracellular K⁺ that shows substantial changes upon induction of glycolytic oscillations. Once the oscillations emerge the PBFI signal oscillates with the same frequency as NADH and endures approximately up to 2000 seconds, as also shown in Figure 1. Using two independent strategies (described in Materials and Methods), we estimated the K⁺ concentration as detected by the probe. Taking into account that these calculations are strongly dependent on the response of the probe in buffers and in the cellular interior we conclude that during oscillations the relative K⁺ concentration oscillates within a range below the dissociation constant of PBFI for K⁺ in the intracellular environment (19.5 mM), much lower than the reported intracellular K⁺ concentration in yeast (between 200-300 mM, [20]).

*K⁺ oscillations are coupled with intracellular ATP levels*

To further explore the mechanism of intracellular K⁺ oscillations we measured NADH, ATP and PBFI (see Materials and Methods for experimental details) after inducing glycolytic oscillations. The results in Figure 4 (A, B and C, respectively) show that the frequencies of all oscillations -NADH, ATP and K⁺ - are the same (~0.026 ± 0.001 s⁻¹), suggesting a strong mechanistic coupling among them. Particularly, we found that the temporal oscillations of PBFI fluorescence are in phase with ATP (Figure 4D). Addition of iodoacetate, which is an inhibitor of glycolysis, causes a correlated disappearance of the three oscillations (Figure 4 A, B and C). Remarkably, while the addition of iodoacetate during the oscillatory regime is followed by an expected drop in the intracellular concentration of ATP, the PBFI labelled cells show a reproducible increase in fluorescence emission. This result suggests active participation of ATP in the regulation of free K⁺ levels in the cell interior.
Figure 4: Iodoacetate (IAA), a strong inhibitor of glycolysis, blocks the oscillations and induces an increase in intracellular $K^+$ and a decrease in intracellular ATP. Oscillations were induced in a 10% (w/v) suspension of BY4743 wild type cells by addition of 30 mM glucose and 5 mM KCN as indicated by the arrows. At time $t=1000$ s 20 mM iodoacetate was added. (A-C) Time series of NADH, PBFI and intracellular ATP. The graph in D shows the phase plot of PBFI fluorescence versus intracellular ATP concentration immediately before the addition of IAA.

Experiments with Latrunculin B and cytoskeleton impaired mutants

In a previous study we determined that the addition of Latrunculin B, which binds to actin monomers and prevents polymerization compromising the integrity of the actin network, obliterates glycolytic oscillations. Specifically, presence of Latrunculin B annihilates the oscillatory coupling between NADH and the dynamical state of intracellular water [10]. We decided therefore to check whether Latrunculin B also affected $K^+$ oscillations. As shown in Figure 5, both intracellular $K^+$ and NADH oscillations are inhibited by it.

To further explore the involvement of the actin cytoskeleton, we also studied the BY4743 isogenic null mutant Arp1pΔ, which lacks the actin-related protein of the dynactin complex. Although ARP1 is a non-essential gene, the Arp1pΔ mutant displays a decreased growth rate
in rich media, is temperature sensitive and, most importantly, has abnormal spindle morphology and nuclear migration. We have previously shown that this mutant strain is unable to display glycolytic oscillations [10]. Figure S3 shows the stationary fluorescence of PBFI in this mutant compared to the corresponding data in the oscillating wild type strain. We note that the fluorescence of PBFI in the Arp1pΔ mutant is always higher than the mean PBFI fluorescence in the oscillating wild type strain, suggesting that in the mutant the concentration of free intracellular K⁺ is higher than that in the wild type.

**Figure 5:** Latrunculin B blocks glycolytic oscillations and increases the intracellular concentration of K⁺. The violet and green graphs show the time series of NADH (top) and PBFI fluorescence (bottom) in cells incubated with Latrunculin B. BY4743 wild type yeast cells were suspended at 10% (w/v) in 100 mM potassium phosphate buffer, pH 6.8, and incubated with 300 μM Latrunculin B for 3.5 h. The suspension was then placed in the spectrofluorometer and at time 180-240 s glucose and KCN were added to final concentrations of 30 mM and 5 mM, respectively. The gray graphs show the corresponding NADH and PBFI time series in a control experiment where cells were incubated with 1% DMSO.
Discussion

From a historical perspective there are two opposed views regarding the physical state of intracellular K\(^+\). The first argues that in the cell interior K\(^+\) exists as an essentially freely mobile ion (a basis of the current standard model of the cell), while the second, largely disregarded, proposes that intracellular K\(^+\) exists mostly in an adsorbed state [14]. Although relatively unknown, the latter view is supported by an abundance of experimental evidence from different laboratories [25-27]. Very little effort has been devoted to revisit this question in the last few decades. Several results from our investigation are, however, difficult to reconcile with the standard view. The first is the detection of coupled K\(^+\) oscillations during glycolytic oscillations in yeast. Specifically, if we consider the intracellular K\(^+\) concentration reported for these cells (from 200 to 300 mM [20]) and assume that most of this intracellular K\(^+\) exists in a free state, we would expect saturation of the PBFI fluorescence signal, since this concentration range exceeds by more than 10 times the measured dissociation constant (K\(_d\)) for PBFI in the cell interior. A more plausible interpretation of this result is, however, that a very large fraction of K\(^+\) (> 90%; considering a total of 200 mM intracellular K\(^+\), we detect less than 20 mM) exists in an adsorbed state in the intracellular environment, i.e. invisible to PBFI. This conclusion is supported by: i) the low intracellular K\(^+\) values (below 20 mM) calculated upon induction of glycolytic oscillations and ii) the observation that intracellular K\(^+\) oscillations operate without intervening K\(^+\) fluxes from the extracellular medium or the (intracellular) vacuole (Figures 1 - 3, Table 1). If K\(^+\) oscillations required fluxes from other compartments (extra- or intracellular) they would show a strong dependence on one or another K\(^+\) membrane transporter (Table 1 and Figure S1); they are, however, completely insensitive to their absence.

Second, and in connection with the identification of potential molecular actors regulating the observed K\(^+\) oscillations, we found: i) an apparent positive correlation between ATP and PBFI oscillations, ii) K\(^+\) oscillations coming to a halt when ATP is depleted by blocking the metabolic pathway using iodoacetate (see Figure 4) with a concomitant rise in the (free) intracellular K\(^+\) concentration and iii) lack of oscillations when the integrity of the cytoskeleton is compromised either by addition of Latrunculin B (Figure 5) or in the Arp1p\(\Delta\) mutant, which lacks the actin-related protein of the dynactin complex (Figure S3). This set of results supports the idea that ATP and the fibrillar cytoskeleton proteins play a linked role in
the origin of K⁺ oscillations, as we previously observed for other cellular properties like water dipolar relaxation, volume, heat flow and temperature [10, 11, 15].

Our observations can be comprehensively explained (unified) in the context of the AIH, which asserts that fluctuations in the levels of key metabolites (e.g., ATP) alter the conformational states of cytoskeletal proteins (e.g. actin). This is caused by association and inductive effects which cooperatively modulate the binding affinity of monovalent ions (K⁺) and intracellular water for intracellular proteins [13, 14, 28]. The AIH proposes that the adsorption of K⁺ onto β- and γ-carboxylic groups of the fibrillar cytoskeleton proteins and the polarized state of water in the intracellular environment are highly favoured when ATP levels are high, and are reverted by decreasing intracellular levels of this metabolite [13, 14]. Thus, the oscillatory behavior of ATP levels during glycolytic oscillations results in a synchronic adsorption-desorption of K⁺ ions, explaining the emergence of K⁺ oscillations. The AIH also provides insight into why the presence of an impaired cytoskeleton (the effect of Latrunculin B and the lack of oscillations in the Arp1p mutant) affects the emergence of the glycolytic oscillations since in this model the polymerization-depolymerization dynamics of actin is a crucial factor affecting the state of intracellular water (and hence oscillations in water relaxation) [13, 14]).

As previously noted, the notion of an adsorbed state of intracellular K⁺ also provides a consistent explanation of the documented regulatory effect of K⁺ on cytosolic enzymes, among them key glycolytic enzymes such as phosphofructokinase and pyruvate kinase [29]. It has been suggested that in muscle cells the availability of free intracellular K⁺ can be controlled by metabolic activity, particularly by a transient and reversible liberation of intracellular K⁺ from an adsorbed state [29]. According to the AIH, the free K⁺ concentration in normal resting cells is only a fraction of that in the external medium (< 2.5 mM) and is thus at a range far lower than what is needed for activating most of the K⁺-sensitive enzymes. This activation occurs at a concentration of free K⁺ in the 10 to 30 mM range, which in turn is far below the >200 mM values reported for intracellular K⁺ in these cells. K⁺ release from adsorbed sites therefore would be essential if the concentration is to be raised to a level sufficient for enzyme activation. This idea seems easily applicable to what happens in the yeast cell during glycolytic oscillations as discussed above.

Finally, and to further test the applicability of the AIH to the ensemble of observations during glycolytic oscillations, we conducted an experiment with the cardiac glycoside Ouabain
which in mammalian cells has been described as an inhibitor of the Na\(^+\)/K\(^+\) ATPase, and has no effect on other P-type ATPases such as Pma1 in S. cerevisiae [30]. The experimental results in yeast displaying glycolytic oscillations are presented in Figure 6 and show that Ouabain causes a correlated halt in the oscillations of NADH, ATP, PBFI and ACDAN GP (Figure 6 A-D respectively). Comparing with the oscillatory process observed in the control (Figure S4; before glucose is exhausted) we can observe: i) a clear increase in the extent of intracellular water dipolar relaxation (Figure 6 D), i.e. the GP values measured are outside the critical GP range where glycolytic oscillations can occur (see [10]), ii) a progressive rise of free K\(^+\) levels and iii) a similar decrease in average ATP levels.

**Figure 6:** Ouabain blocks glycolytic oscillations and modulates the intracellular concentration of K\(^+\) and ATP, also decreasing the extent of polarized intracellular water. Oscillations were induced in a 10\% (w/v) suspension of BY4743 wild type cells by addition of 30 mM glucose and 5 mM KCN as indicated by the arrows. At time t=1000 s 0.1 mM Ouabain (in DMSO) was added. A-D Time series of NADH (A), PBFI (B), intracellular ATP (C) and intracellular water dipolar relaxation (D).
These results during the oscillatory regime are quite puzzling in terms of the standard model of the cell since yeast cells lack an Na\(^+\)/K\(^+\) ATPase. According to the AIH, however, the cardiac glycoside acts as a cardinal adsorbent on the cytoskeleton antagonistically to ATP, resulting in desorption of adsorbed K\(^+\) in the intracellular medium and depolarizing intracellular water [14].

**Concluding remarks**

We studied the behavior of intracellular K\(^+\) during the well characterized glycolytic oscillations in the yeast *Saccharomyces cerevisiae*. This process, which starts when just glycolysis kicks in by the addition of glucose and inhibition of respiration and lasts as long as glucose is available, has a robust period (and a phase with respect to other oscillating variables) that assist in establishing correlations between glycolytic metabolites (ATP) and emergent cellular physicochemical parameters (as described in this paper and previous contributions from our group [6, 10, 11, 15]). Our results show evidence that the bulk of intracellular K\(^+\) exists in an adsorbed state (and thus invisible to PBFI), probably onto the actin cytoskeleton, which is dynamically affected by central metabolism. These findings are satisfactorily unified within the framework of the AIH, which is based on well established concepts from colloidal physical chemistry and up to now remains unrefuted. It is difficult, therefore, to understand why the hypothesis of association-induction has been given so little attention. All the results explored in this system so far (this paper and [6, 10, 11, 15]) are congruous with the yeast cell interior behaving as a highly structured and near equilibrium system, where glycolytic oscillations may arise as a consequence of the requirement of living cells for a near constant low-entropy state while remaining metabolically active.

**Materials and Methods**

PBFI tetraammonium salt was obtained from ThermoFischer Scientific (Waltham, MA). The aptamer switch probe used to measure intracellular ATP concentration was obtained from VBC Biotech (Vienna). 6-acetyl-2-dimethylaminonaphthalene (ACDAN) was purchased from Santa Cruz Biotechnology Inc. (Dallas, TX). All other chemicals were purchased from Sigma Aldrich (Munich, Germany). All measurements were performed in triplicate and the results were shown to be reproducible in independent measurements.

*Cell growth*
Cells of the yeast *Saccharomyces cerevisiae* diploid strain BY4743 wild type and isogenic mutants from the Euroscarf collection were grown and harvested as described previously [31]. The starved cells were suspended to a density of 10% (w/v) in 100 mM potassium phosphate buffer, pH 6.8, with or without 20 mM NaCl or in 100 mM sodium phosphate buffer, pH 6.8, and starved for 3 h at room temperature before use.

**PBFI labelling of cells**

PBFI was loaded into yeast cells by electroporation using a gene pulser transfection apparatus equipped with a capacitor extender, both from Bio-Rad Laboratories (Hercules, CA) as described previously [32] in a 1 M sorbitol solution containing 40 µM or 0 µM of the dye. The cells were then washed twice and finally suspended to a density of 10% (w/v) in the same growth buffer. The experiments performed to detect extracellular K⁺ oscillations (Figure 2) were also performed by incubating the cells with PBFI (without electroporation) in a 100 mM NaCl phosphate buffer supplemented with 2mM KCl, pH 6.8. The cell density in this experiment was also 10% w/v. The ratio intracellular vs extracellular volume in this sample is 1:32.8 (see “Estimations of intracellular K⁺ concentration” section below) such that a 2 mM solution of KCl in a final volume of 1 ml corresponds to 2 µmol of the ion K⁺. Assuming a K⁺ concentration of 200 mM inside the cell in a volume of 29.6 µl (the 1:32.8 volume ratio) one expects to have a total of about 5 µmol of K⁺. A flux of 5 µmol from the inside to the outside of the cell will cause a change from 2 to 7 mM in the extracellular space, which the PBFI would easily detect (see Figure S5).

**Time resolved measurement of NADH, PBFI and ATP**

Measurements of NADH and PBFI in cuvette were made in a SPEX Fluorolog spectrofluorometer (Edison, NJ) fitted with a temperature-controlled cuvette holder (Quantum Northwest, Liberty Lake, WA, USA). The temperature of the sample was maintained at 25 ºC. NADH was excited at 366 nm, and its fluorescence emission was measured at 450 nm. PBFI was excited at 340 nm, and its fluorescence emission was measured at 505 nm. Temporal measurements of PBFI fluorescence were corrected for background fluorescence by subtracting the fluorescence from cells electroporated without the dye (see Figure S2). The sampling frequency was 1 Hz. Temporal measurements of the emission spectra of PBFI were done in aqueous solutions of KCl or NaCl at a resolution of 1 nm. For all excitations and emissions, a slit corresponding to 4.5 nm was used. Changes in cellular volume can also be a
potential source for changes in PBFI fluorescence. However, in a previous work [6] we demonstrated that volume changes measured by light scattering are only noticeable when the cell wall is removed with Zymolyase®, a condition that it was not used in our experiments.

Fluorescence microscopy images of PBFI were obtained using an inverted multiphoton excitation fluorescence microscope (Zeiss LSM 510 META NLO, Carl Zeiss, Jena, Germany) equipped with a Ti:Sa MaiTai XF-W2S laser (Broadband Mai Tai with 10 W Millennia pump laser with a tuneable excitation range of 710–980 nm, Spectra Physics, Mountain View, CA). The excitation wavelength used was 740 nm. The fluorescence signal was collected through a 40X water objective, NA 1.2, separated from the excitation light with a dichroic beamsplitter (NFT 635 VIS) and detected with a photomultiplier tube (H7422 PMT, Hamamatsu, Denmark) using a 520 ± 17.5 nm bandpass (AHF Analysen Technik AG, Tübingen, Germany). Acquisition of the two-photon excitation fluorescence image was performed by adding 300 μl of PBFI-labelled resting yeast cells at a density of 10% by weight (suspended in 100 mM potassium phosphate buffer, pH 6.8) to an 8-well plastic chamber (Lab-tek Brand Products, Naperville, IL).

Estimations of intracellular K⁺ concentration

NADH and PBFI both absorb light in the near-ultraviolet spectrum (300-400 nm) and both contribute to the emission at 500 nm. In contrast to mammalian cells, yeast contains a large amount of NADH and also has an important autofluorescence signal at 500 nm. Hence the normal method for estimating intracellular K⁺ [22, 23], by taking the ratio of emissions at 500 nm of excitations at 340 nm and 380 nm, cannot be used because the signal to noise ratio decreases dramatically when first subtracting the signal from cells without PBFI from the signal from cells with PBFI at each excitation wavelength and then calculating the ratio of the two resulting signals. Moreover, the calibration curves obtained from the excitations at 340 nm and 380 nm of PBFI strongly depend on the background concentration of sodium (Szmacinski and Lakowicz, 1999). Therefore, we used two independent strategies to estimate K⁺ concentration. First, we determined the volume of the cell cytosol using the estimate provided by Richard et al [3] of a cytosolic volume of 3.7 ml/g protein. Using the Bradford method, we measured the protein content of a 10% (w/v) yeast suspension as 8 μg/ml. Thus, the cytosolic volume in 1 ml of 10% (w/v) yeast suspension corresponds to 29.6 μl, i.e. the intracellular to extracellular volume ratio is 1:32.8. The maximum signal from the oscillations of PBFI in a 10% yeast suspension is about 14000 cps while the minimum is about 8000 cps
(from Figure S2), which should correspond to a signal of about 14000*32.8 =459200 and 262400 cps in 1 ml cytosol. Using the spectra of PBFI in water (Figure S5) these values correspond to between 15 and 2 mM intracellular K\(^+\), respectively. The average PBFI fluorescence intensity during oscillations is about 10000 which again corresponds to 10000 cps x 32.8= 328000 cps in 1 ml cytosol. Using the spectra of PBFI in water this should correspond to about 5 mM intracellular K\(^+\).

An alternative way to estimate the intracellular concentration of K\(^+\) is to use the binding curve of intracellular PBFI constructed by adding various concentrations of KCl to yeast cells incubated for several hours in sodium potassium phosphate in the presence of valinomycin and nigericin [22]. Such a curve is shown in Figure S6B. The dissociation constant of intracellular PBFI is estimated to be 19.5 mM. This value is about 5 times higher than the K\(_d\) in water (Figure S6A). Since the oscillations of PBFI fluorescence (see Figures 1 and S2) are sinusoidal and symmetric, the PBFI signal must be on the linear part of the PBFI binding curve, i.e. the K\(^+\) concentration sensed by the probe must be less than the K\(_d\). This evidences a concentration of free potassium during glycolytic oscillations of less than 20 mM, which is consistent with the estimate of 2-15 mM given above. This can be further substantiated by looking at the power spectrum of the PBFI oscillations (Figure 1D). Here we observe only a single peak at a frequency around 0.025 s\(^{-1}\). If the oscillating concentration of PBFI reached a maximum of higher than 20 mM then at least two peaks would be observed. This is illustrated in Figure S7, which shows power spectra of simulations of oscillations of PBFI where it is assumed that potassium oscillations are sinusoidal in the range from either 2-6 mM, 2-12 mM or 2-22 mM, and that PBFI binds potassium with a K\(_d\) of 19.5 mM. In the latter two power spectra there are two peaks, one at the fundamental frequency of 0.025 s\(^{-1}\) and another second harmonic at 0.05 s\(^{-1}\). Since this second peak is absent in the power spectrum in Figure 1D, we may conclude that potassium concentration oscillates with a maximum of 10 mM or less.

Finally and to check for potential interference by Na\(^+\) on PBFI fluorescence, we performed additional control experiments. Specifically, we observed that at the excitation and emission wavelengths used for measuring PBFI there are no significant changes in its fluorescence by varying the concentrations of Na\(^+\) (Figure S8). This is in stark contrast with the effect of K\(^+\) on PBFI. Also, to explore if possible oscillations in Na\(^+\) may interfere in any way with the oscillations measured for intracellular K\(^+\), we performed experiments incorporating Sodium Green (a probe sensitive to Na\(^+\)) in the intracellular environment. No potentially cofounding
Na$^+$ oscillations were detected in these experiments (Figure S9).

**Measurement of intracellular ATP**

Measurements of intracellular ATP using an aptamer-based nanobiosensor were done essentially as described earlier [28, 33]. The nanosensor consists of an approximately 30 nm polyacrylamide particle containing the aptamer switch probe BlackHole2-GTAGTAAGAACTAAAGTAAAAATTTAAAGTAGCCACGCTT-[CH2-CH2-O]36-TTACTAC-TexasRed and with Alexa Fluor 488 dextran as the reference dye. The sensor is inserted into the cells by electroporation as described previously [33]. The sensor uses the ratio of the Texas Red fluorescence and Alexa Fluor 488 to determine the intracellular ATP concentration and can be calibrated *in vitro* [33]. Here the ATP concentration was estimated from a calibration curve constructed by measuring the fluorescence from the ATP sensor in mixtures of ATP and ADP where the total concentration of ATP plus ADP is 4 mM [34]. Temporal measurements of the fluorescence of the ATP nanosensor were made as the ratio of the emission at 605 with an excitation of 580 nm (Texas Red) over the emission at 520 nm with an excitation at 470 nm (Alexa Fluor 488) in a SPEX Fluorolog spectrofluorometer (Edison, NJ) fitted with a temperature-controlled cuvette holder (Quantum Northwest, Liberty Lake, WA, USA). The sampling frequency was 1 Hz.

**Measurements of glycolytic flux**

The glycolytic flux in wild type yeast and in the mutant Arp1pΔ as well as in the wild type strain following incubations with the inhibitors IAA, Latrunculin B or Ouabain was measured either as the rate of ethanol production using a membrane-inlet mass spectrometric technique [9, 33] or as the initial rate of production of CO$_2$ using membrane-inlet mass spectrometry or a home-made Warburg apparatus [32]. The two ways of measuring the flux gave identical results (no significant difference, > 10 replicates). These results are shown in Figure S10. We note that, apart from IAA, there are no significant differences between the glycolytic fluxes measured in cells treated with latrunculin B or ouabain, untreated cells and in the mutant Arp1pΔ. Thus, treatment with Ouabain or Latrunculin B does not affect the viability of the cells. The result for Latrunculin is in accordance with previous observations [35].

**Induction of oscillations in yeast cells**
Yeast cells at 10% (w/v) in 100 mM potassium phosphate buffer with 20 mM NaCl, pH 6.8, were added to a 1 mL stirred cuvette mounted in the spectrofluorometer. Oscillations were induced by adding first 30 mM glucose and 60 s later 5 mM KCN to the suspension.

**Latrunculin B experiments**

Yeast cells at 10% (w/v) in 100 mM potassium phosphate buffer with 20 mM NaCl, pH 6.8, were incubated with 300 µM Latrunculin B for 3.5 h. PBFI was loaded into the cells as described above and the cells were then placed in the spectrofluorometer for measurements of PBFI and NADH.

**Experiments with ACDAN GP in the presence of Ouabain**

The dynamics of intracellular water were measured by the fluorescence response of 6-acetyl-2-dimethylaminonaphthalene (ACDAN) [10, 11]. Specifically, we measured the generalized polarization (GP) function, defined by the equation:

\[
GP = \frac{I_{440} - I_{490}}{I_{440} + I_{490}},
\]

where \(I_{440}\) is the fluorescence intensity at 440 nm and \(I_{490}\) the intensity at 490 nm for an excitation wavelength at 365 nm [10, 11]. Simultaneous measurements of the fluorescence intensities (\(I_{440}\) and \(I_{490}\)) in cells stained with ACDAN were performed in a QE65000 spectrometer (Ocean Optics, Dunedin, FL) fitted with the same temperature-controlled cuvette holder mentioned above. The temperature of the sample was maintained at 25 °C. Light was supplied by a CoolLED pE4000 illumination system (CoolLED, Andover, UK). The optical fiber from the illumination system was mounted perpendicular to the emission beam. Before measurements cells were incubated in 100 mM potassium phosphate buffer, pH 6.8 with 5 µM ACDAN for 30-60 min at 30 °C, the cells were washed twice in the same buffer and finally resuspended in 100 mM potassium phosphate buffer and placed in the spectrofluorometer. Ouabain was added at time \(t=1000\) s. The negative controls corresponding to the Ouabain experiments presented in Figure 6 are included in the supplementary section as Figure S4.
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This paper is dedicated to the memory of Gilbert N. Ling, who passed away on November 10th 2019 at the age of 99.
References


[18] G. Karreman, Cooperative specific adsorption of ions at charged sites in an electric field, Bulletin of Mathematical Biophysics 27 (1965) 91-104.


Supplementary Material

Glycolytic oscillations and intracellular K\(^+\) concentration are strongly coupled in the yeast *Saccharomyces cerevisiae*

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Figure S1. Power spectra of oscillations of NADH in the BY4743 wild type and in three mutant strains defective in Na$^+$ and H$^+/K^+$ transporters (listed in Table 1).
Figure S2. Oscillations in fluorescence of (A) NADH and (B) PBFI after addition of glucose and KCN (added at the indicated arrows). The data in C and D represent the raw data of fluorescence at 505 nm in cells electroporated with 0 and 40 μM PBFI tetraacetate, respectively. The data in B are obtained by subtracting the fluorescence in C from the fluorescence in D.
Figure S3. PBFI Fluorescence in BY4743 wild type strain (blue) and in Arp1pΔ mutant strain (green).
Figure S4. Negative control experiment to that in Figure 6 in the main text. Measurements of NADH (A) and PBFI (B) fluorescence, intracellular ATP (C) and ACDAN GP (D). Oscillations in glycolysis were induced by addition of glucose and KCN at the indicated arrows. At t=1000 s an equal volume of DMSO instead of Ouabain was added to the yeast suspension.
Figure S5. Spectra of PBFI in KCl solutions in 0.1 M Tris buffer, pH 7.0.
Figure S6. PBFI binding curves in (A) 0.1 mM Tris buffer, pH 7.0, or (B) cytosolic PBFI. The latter was constructed for a 10 % (w/v) suspension of BY4743 cells, into which PBFI was inserted using electroporation. The cells were incubated for three hours in a 100 mM sodium phosphate, pH 6.8, containing 10 µM nigericin and 20 µM valinomycin before addition of KCl.
Figure S7. Simulated power spectra of PBFI oscillations assuming sinusoidal potassium oscillations between different concentration limits and a $K_d$ of PBFI of 19.5 mM for binding of $K^+$. The oscillation ranges of potassium concentration are (A) 2 to 6 mM, (B) 2 to 12 mM and (C) 2 to 22 mM.
Figure S8. Spectra of PBFi in KCl (left) and NaCl (right)
Figure S9. Time series of NADH (top) and Sodium Green (bottom) fluorescence. BY4743 yeast cells (10% w/v) in 100 mM potassium phosphate buffer with 20 mM NaCl, pH 6.8. Cells were loaded with sodium green (Sodium Green tetraacetate, Thermo Fischer Scientific, Waltham, MA) by electroporation as described for PBFI in the Materials and Methods section. The K_d of Sodium Green for Na^+, determined as for PBFI (Fig. S7B), was approximately 20 mM.
Figure S10. Relative glycolytic flux in wild type (WT), Arp1pΔ mutant and in wild type cells treated for 10 min with 20 mM iodoacetate (IAA), 3.5 h with 300 μM Latrunculin B (Ltr) or 10 min with 100 μM Ouabain (Oua). The experiments were performed on 10% (w/v) yeast cells in 100 mM potassium phosphate buffer, pH 6.8. Temperature 25 °C. The bars indicate the standard error of the mean. The number of replicas was n=3 for each experiment. ** indicates that the rate is significantly different from the wild type rate (p < 0.00001).
Author Statement

Lars F. Olsen and Luis A. Bagatolli: conceptualization, methodology, validation, investigation, funding acquisition; Lars F. Olsen: formal analysis, software; Lars F. Olsen, Luis A. Bagatolli, Roberto P. Stock: writing-original draft, writing - review & editing