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Review

Heterogeneity of glycolytic oscillatory behaviour in individual yeast cells

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ABSTRACT

There are many examples of oscillations in biological systems and one of the most investigated is glycolytic oscillations in yeast. These oscillations have been studied since the 1950s in dense, synchronized populations and in cell-free extracts, but it has for long been unknown whether a high cell density is a requirement for oscillations to be induced, or if individual cells can oscillate also in isolation without synchronization. Here we present an experimental method and a detailed kinetic model for studying glycolytic oscillations in individual, isolated yeast cells and compare them to previously reported studies of single-cell oscillations. The importance of single-cell studies of this phenomenon and relevant future research questions are also discussed.

1. Introduction

Oscillations can be found in a wide variety of organisms in nature, with period times ranging from fractions of a second to years [1]. One example is the circadian rhythm, which occurs with a period time close to 24 h and allows the organism to adapt to the periodic variations in the environment [1]. Another example is the pulsatile secretion of insulin from islets of β-cells, which in turn might be driven by rhythms within single cells, like membrane potential bursting, oscillations in calcium concentration and metabolic oscillations [2,3]. One of the most studied oscillating biological systems is glycolytic oscillations in yeast and these oscillations will be discussed in more detail in this work.

Glycolytic oscillations in yeast can be induced under specific experimental conditions: harvesting cells at the diauxic shift, starvation for glucose and subsequent addition of glucose and cyanide [4]. The oscillations of the majority of the intermediate metabolites have been studied since the 1950s in dense populations of yeast cells and in cell-free extracts, for a review, see [5]. However, to study oscillations in a population of millions of cells with varying cell cycle phase, age and shape, some kind of synchronization of the oscillations is required. And indeed, when two populations which oscillate 180° out of phase are mixed, they rapidly synchronize [6,7]. Acetaldehyde, a metabolite in glycolysis, has been suggested to be the synchronizing agent in dense populations, since it rapidly diffuses through the cell membrane [7,8] and has been shown to have a strong synchronizing effect [9].

The drawback with studies of cells in a synchronized population is that they will only give information about the average behaviour of the population, and possible heterogeneous behaviour on the single cell level will be lost. It is then, for instance, not possible to tell the difference between a situation where 50% of the cells respond with full intensity and the remaining cells do not respond at all, or a situation where all cells respond with 50% of the intensity. Loss of glycolytic oscillations in a cell population can be due to two different mechanisms: (i) individual cells lose their oscillations collectively or (ii) individual cells continue to oscillate but desynchronize. To study and understand the heterogeneity is important, since different cells might respond in different ways to the same stimulus. Heterogeneous cell responses might then also have implications when new medicines are developed, where a dose required to treat a condition in some cells might leave other cells unaffected.

Several attempts have been made to study oscillations in individual cells, both in a population and in isolation [10–13]. Studies of individual cells in a population indicated heterogeneity where some cells oscillated when the population as a whole did not [10]. However, in more recent studies, cells that were isolated from an oscillatory population did not show oscillatory behaviour [12,13]. It has been speculated that single cells in isolation might not be able to show oscillations and that the onset of oscillations is a collective property and not possible at low cell densities [11].

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Many different models have been used to describe yeast glycolysis, from core models that are used to illustrate a principle [14–16], to detailed kinetic models used for qualitative descriptions and predictions [17–20]. The majority of these models were constructed to study either steady-state [20] or dynamic behaviour [17] and most of the models are available via the BIOMODELS and JWS Online databases (http://www.ebi.ac.uk/biomodels-main and http://jjj.biochem.sun.ac.za) [21,22].

Originally, the core models were highly abstracted and used to investigate the mechanism of oscillations [15,16]. More recently, core models have also been used to study higher-level responses, such as synchronization and oscillation frequency dependence on external glucose concentration [14]. In some cases, using core models instead of detailed models can be advantageous, e.g. for gaining fundamental understanding of the core mechanisms of a certain behaviour. Here the complexity of detailed models can cause difficulties in identifying the dominant mechanism responsible for the emergent behaviour. The disadvantage with core models is that there is no direct mechanistic interpretation of the kinetic parameters; the values of the parameters are chosen so that the behaviour of interest emerges. The disadvantage with previous constructed detailed kinetic models is that they are fitted to the data set of interest in order to investigate whether the model can describe the data or to test a hypothesis. Those models are thus not thoroughly validated and they rarely succeed in predicting results of other studies.

For a model to be useful for investigation and prediction of behaviour also in other data sets, the parameters of the models should preferably be determined experimentally under physiological conditions [23]. For detailed kinetic models describing yeast glycolysis, this means that the enzyme kinetics data should be determined experimentally under conditions where the enzymes are active *in vivo*. Such a model could then be used for other types of studies by varying the external parameters, and the model could also be merged with other detailed models, as in a modular modeling approach, in order to describe a larger network of reactions [24].

In recent publications by du Preez *et al.* [25,26], a previously described steady-state model by Teusink *et al.* [20] was adapted to quantitatively describe and predict most published experimental data for glycolytic oscillations in yeast. Despite the fact that several dynamic models exist, the Teusink model was chosen since (i) it was based on experimentally determined *in vitro* enzyme kinetic parameter values and only small changes were necessary to describe a large experimental data set of dynamic conditions (ii) it was the only detailed model that so far had been used to simulate synchronization; all other models that had shown synchronization were core models [14,27]. The Teusink model had also previously been used to describe glycolytic oscillations [28], but in that case local changes to the model were performed and the resulting limit-cycle oscillations did not resemble existing experimental data.

Using a single parameter set for the enzyme rate equations and using external parameter values, such as external metabolite concentrations and biomass concentration close to the experimental conditions, the du Preez model could predict the observed behaviour qualitatively and in some cases also quantitatively [25]. This is an important finding that indicates that detailed mechanistic models can be used to quantitatively describe a dynamic behaviour.

In a recent work [29], we addressed the question if cells in isolation can oscillate, or if the onset of oscillations is a collective property that requires a high cell density. We also investigated whether there is a large heterogeneity among individual cells when there is no active synchronization and whether individual cells display qualitatively different behaviour than the mean behaviour of a population of cells. The experimental setup consists of a microfluidic flow chamber in which individual yeast cells can be positioned with variable cell-to-cell distance and online NADH fluorescence intensities in up to 100 cells can be monitored simultaneously. The du Preez model [25] was adapted to describe single cell oscillations within the microfluidic flow chamber (Gustavsson model) [29], which included accounting for the removal of the secreted products acetaldehyde and ethanol by the flow in the chamber. Within the microfluidic flow chamber, the environment is not well stirred, as is assumed in the previous models [20,25,26]. The model thus had to be extended to explicitly describe the extracellular concentrations of acetaldehyde and ethanol, using the equation for diffusion through a membrane as a model for diffusion through a boundary layer surrounding each cell. The Gustavsson model was used to determine the required conditions for sustained oscillations to be induced and these conditions were then verified experimentally. Finally, the heterogeneous response of the individual cells were categorized and successfully simulated by allowing small variations in the activity of the glucose transporter.

2. Experimental approach for studies of glycolytic oscillations in single cells

To study oscillations in single cells over an extended period of time, the cells need to be kept within the measurement region. Controlling the position of the cells and the cell density is also crucial in order to discern whether a behaviour is due to cell coupling or if the cells can be regarded as isolated. To investigate how the cells respond to changes in the extracellular milieu, fast environmental changes are required, preferably where a chemical can be added, removed or its concentration changed within a few seconds.

To fulfill these requirements, we combine optical tweezers, microfluidics and fluorescence microscopy (for a detailed description of the experimental methods used, see [29]). Optical tweezers is a tool where a highly focused laser beam is used to catch, move and position yeast cells within a microfluidic flow chamber. The cells are inserted in one of four inlet channels of the microfluidic flow chamber and then positioned in arrays with variable cell-to-cell distance on the bottom of the chamber at the junction between the inlet channels, see Fig. 1. To reduce the risk of photodamaging the cells and to enable simultaneous use with e.g. fluorescence imaging, near-infrared laser light is used. Inside the microfluidic flow chamber, the flows are laminar and the only way for solutions from different inlet channels to mix is by diffusion. This property allows completely different environments to be found in close proximity at the junction between the inlet channels. The extracellular environment can thus be controlled both spatially and temporally by adjusting the flow rates in the inlet channels, causing the flows to cover different areas of the junction depending on flow rates. The advantages with our setup include that a complete change of environment can be performed within 2 s and that a solution can be completely removed from the cells; a task that can be difficult to achieve when working with samples in bulk.

Since all cells in an experiment should experience the same environment, it is important that the entire cell array area is covered by the intended solution, without any concentration gradients. To determine suitable flow rates and an optimal cell array area, the flow rates and the concentration distribution within the chamber were simulated using the Navier–Stokes equation in combination with the continuity equation in COMSOL MultiPhysics.

The responses from the cells due to the environmental changes were then followed by time-lapse imaging of the NADH autofluorescence in the individual cells.

3. Results

Using this setup, we showed that it is indeed possible to induce sustained glycolytic oscillations in isolated yeast cells [29]. Since the oscillations were not synchronized, glycolytic oscillations do

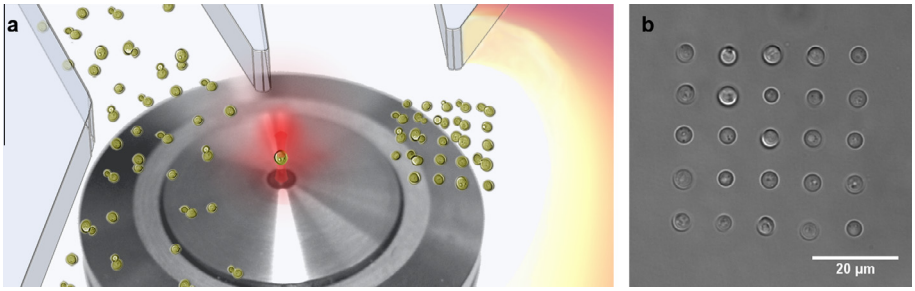

Fig. 1. (a) Schematic image of the experimental method used for studying glycolytic oscillations in isolated yeast cells. Optical tweezers are used to capture yeast cells and position them in arrays with variable cell-to-cell distance within a microfluidic flow chamber. The extracellular environment can then be controlled both spatially and temporally by adjusting the flow rates in the different inlet channels of the chamber, whereby a complete change of environment can be achieved within 2 s [29]. (b) Image showing a typical 5 × 5 cell array with a cell-to-cell distance of 10 µm positioned at the bottom of the microfluidic flow chamber.

not seem to be a collective property that requires a high cell density, but cells can oscillate individually, without any need for synchronization. The response from the individual cells was heterogeneous, where some cells (a) oscillated sustained or (b) damped when only glucose was added, some cells (c) needed both glucose and cyanide to show sustained oscillations and some cells (d) did not show oscillations even when both glucose and cyanide were added, see Fig. 2. These four categories of oscillatory behaviour described the vast majority of the cells in our experimental results and amazingly, by allowing a ±2% variation in the activity of the glucose transporter, all categories were successfully simulated using the Gustavsson model.

It is not known whether it is differences in activity of the glucose transporter that causes the observed heterogeneity; we simply show that comparable heterogeneity can be obtained via small differences in activity of an enzyme of which the expression is known to change during glucose starvation.

4. Perspectives

In summary, our study showed that sustained glycolytic oscillations can be induced in individual isolated yeast cells and thus that a high cell density is not a requirement for oscillations to arise. We have also constructed a detailed kinetic model to describe the oscillations in individual cells.

Our data revealed great heterogeneity in the response from the individual cells and four categories of cell behaviour were identified. By allowing small changes in the activity of the glucose transporter, all four categories of behaviour were successfully simulated.

There can be several possible reasons why previous attempts of studying single-cell oscillations have been inconclusive. In the study by Aon *et al.* in the early 1990s, it was shown that single cells within a dense population continued to oscillate out of phase, also for some time after the macroscopic oscillations had damped out [10]. This study did, however, not address whether a high cell density is required for induction of oscillations in the individual cells, or if oscillations can be induced also in isolated cells. In a later study by de Monte *et al.*, oscillations could not be detected in single cells at low cell densities. It was thus concluded that the oscillations disappear synchronously in the individual cells and in the population and that a high cell density is a requirement for oscillations to be induced [11]. This was explained by a dynamic quorum-sensing mechanism, where the oscillations are a

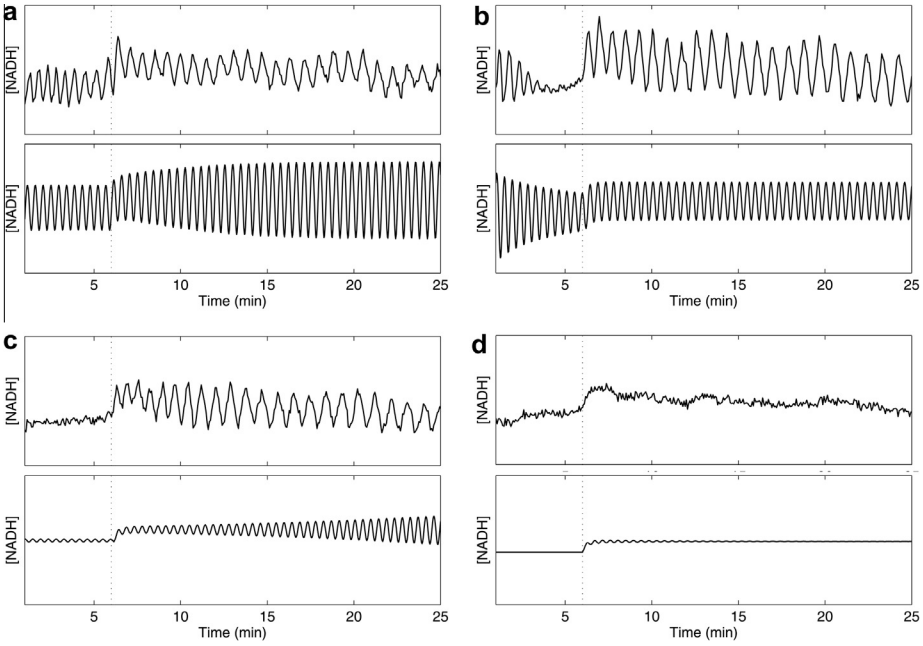

Fig. 2. Graphs showing four different categories of oscillatory behaviour in single cells found experimentally (upper traces) and using model simulations (lower traces). The experimental time series show the measured NADH fluorescence intensities from individual cells from a single experiment, where the cells were exposed to only glucose for the first 6 min, before the flows were changed (dotted lines) and the cells were exposed to both glucose and cyanide. The heterogeneous responses could be divided into categories (a–d) describing the behaviour of the vast majority of all cells and these categories were successfully simulated by allowing the activity of the glucose transporter to vary ±2%. Data reproduced from [29].

collective property of the population. In their experiment, a well-stirred cuvette was used, where cells, glucose and cyanide could be continuously flowed into the cuvette by pumps. The fluorescence was measured from cells in a thin layer near the surface of the cuvette. With this setup, it should be possible to ensure oscillatory conditions for single cells. One reason why single cell oscillations were not detected could be that the detector was not sensitive enough to resolve oscillations from single cells. Another reason might be that the detector measured the average signal from many cells, which can average to a steady-state appearance if a significant distribution of oscillatory phases is present. In a study by Poulsen *et al.* [12], cells were immobilized in isolation on the bottom of a flow chamber, where the flow of chemicals were controlled by pumps. Individual cells were then imaged using epi-fluorescence microscopy and a light-sensitive camera. The experimental setup resembles that used in our studies [29], but despite several attempts, the authors were not able to induce oscillations in isolated cells. It is difficult to address which differences in experimental setup can cause the cells to become non-oscillatory. The dimensions of the flow chamber are larger in the Poulsen-paper compared to the chamber described in this work, and this might have led to more turbulent flows and different flow rates. However, neither turbulent flow nor higher/lower flow rates should in principle hinder oscillations. In a recent publication by Weber *et al.* [30], the desynchronisation with respect to cell density is investigated in the form of the Kuramoto order parameter. This study highlights the importance of understanding the heterogeneous behaviour of individual cells and verifies that single cells can oscillate at low cell densities, without synchronization.

In our study we have answered some questions that for long have been unanswered. However, there are many more questions to address before we fully comprehend the complex role of the reactions of glycolysis in emergent behaviour and there are several questions to be answered by single cell analysis. For example, by studying single cells within a microfluidic flow chamber, the precise conditions where oscillations arise could be investigated and compared to the conditions required for oscillations in a synchronized population. This might further elucidate if the conditions for synchronized oscillations in a population is a subset of the conditions for single cell oscillations and might suggest a new regime of conditions for the study of oscillatory behaviour.

Another open question is the precise role of cyanide in glycolytic oscillations. In most studies, a certain concentration range of cyanide is required to induce oscillations, where cyanide acts by both inhibiting respiration and by binding acetaldehyde [8]. If a flow chamber is used, both anaerobic environment and removal of acetaldehyde can be achieved by blowing an inert gas through the sample, as in the study by Poulsen *et al.*, where argon was used [31]. KOH was also added instead of KCN to adjust pH to facilitate oscillations. In the case of single-cell oscillations, we investigated the need for cyanide by making all solutions hypoxic by bubbling through N_2-gas and letting the flow in the chamber remove excess acetaldehyde [29]. Also when only glucose was added, sustained oscillations were induced. However, the cells showed a stronger tendency to oscillate when KCN was present, indicating that there might be an additional role of cyanide. And indeed, in a recent study, it was shown that cyanide also reacts with other metabolic intermediates, such as pyruvate and dihydroxyacetone phosphate (DHAP) [32]. Further studies are needed to elucidate the precise effect of the intracellular cyanide reactions.

A third interesting and relevant mechanism to investigate is that of synchronization and the role of acetaldehyde as a synchronizing agent. Using the optical tweezers, the cell-to-cell distance can be carefully controlled and the dependence of cell coupling on cell-to-cell distance can be investigated. That the cell density is important has been verified in several different studies in bulk (e.g. [10,11,33]), and recently also in single cell studies [30]. Using microfluidics, the concentration of extracellular acetaldehyde can be controlled and varied in order to see how strongly the individual cells couple to the external acetaldehyde. Since acetaldehyde is involved in the final branch of yeast glycolysis, it can also be of interest to investigate and verify if and how strongly other parts of glycolysis is affected by changes of extracellular acetaldehyde concentrations. One way of studying this would be to measure the concentration of several intermediate metabolites simultaneously, and the development of intracellular metabolic probes (e.g. ATP probes [34]) might give the necessary tools to achieve this. Using microfluidics, also the role of flow rates on oscillatory behaviour and cell coupling can be investigated. We have previously shown that for single cells within a microfluidic flow chamber, the chemicals in the solutions rather than the flow rates determine the oscillatory behaviour, at least in the range of flow rates used in our setup [35]. However, the dependence of cell coupling on flow rate is still to be determined.

Despite intense research, many questions about glycolytic oscillations remain unanswered. Here we have shown how single-cell analysis can provide a useful tool for investigation of glycolytic oscillations in individual cells and discussed how it can be utilized to, for example, gain a better understanding of the mechanism behind cell coupling and synchronization.

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