

A label-free real-time method for measuring glucose uptake kinetics in yeast

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Abstract

*Glucose uptake assays commonly rely on the isotope-labeled sugar, which is associated with radioactive waste and exposure of the experimenter to radiation. Here, we show that the rapid decrease of the cytosolic pH after a glucose pulse to starved *Saccharomyces cerevisiae* cells is dependent on the rate of sugar uptake and can be used to determine the kinetic parameters of sugar transporters. The pH-sensitive GFP variant pHluorin is employed as a genetically encoded biosensor to measure the rate of acidification as a proxy of transport*

velocity in real time. The measurements are performed in the hexose transporter-deficient (*hxt⁰*) strain *EBY.VW4000* that has been previously used to characterize a plethora of sugar transporters from various organisms. Therefore, this method provides an isotope-free, fluorometric approach for kinetic characterization of hexose transporters in a well-established yeast expression system.

Introduction

As the entry point of central carbon metabolism, the transfer of sugars (mainly glucose) across cellular membranes is essential for cell survival in most life forms. Based on this premise, sugar transporters are regarded as promising drug targets to combat serious human diseases, including cancer and diabetes (Schmidl *et al.* 2018), or pathogens such as the malaria causative *Plasmodium falciparum* (Qureshi *et al.* 2020). Therefore, mechanisms and kinetics of sugar transporters are an object of intensive research. Current methods for measuring glucose uptake, mainly relying on isotope labeling, are cost-intensive and necessitate specialized laboratories (Walsh, Smits, Scholte and Smits *et al.* 1994; Walsh, Smits, Scholte and van Dam 1994; Yamamoto *et al.* 2011). Furthermore, these experiments contribute to radioactive waste accumulation, a threat to human and environmental health that requires complex waste management strategies of governments and institutions (Poškas *et al.* 2019).

Isotope-free assays have also been described, employing the non-metabolizable glucose analog 2-deoxyglucose and externally added fluorogenic (Yamamoto *et al.* 2011) or bioluminescent (Valley *et al.* 2016) substrates. By using sugar analogs, the latter methods are not suitable for determining the kinetic parameters of the transporters. Here, we describe an alternative method, based on the intracellular pH change upon glucose uptake. The acidification of the yeast cytosol in response to a glucose pulse to glucose-starved cells is a well-known phenomenon (Orij *et al.* 2009; Ramos *et al.* 1989). During glucose starvation, the cytosolic pH (pH_{cyt}) of yeast cells drops to approximately 6 (Orij, Brul and Smits 2011).

Under these conditions, the lack of glycolysis substrates diminishes ATP production, which leads to lower activities of the plasma membrane ATPase and the vacuolar V-ATPase, both of which consume ATP to translocate protons out of the cytosol (Orij, Brul and Smits 2011). When glucose is provided, it is transported across the plasma membrane and phosphorylated by the hexokinase as the first step of glycolysis. In this step, one molecule of ATP is hydrolyzed, and one proton is released (Fig. 1), causing the observed rapid additional acidification shortly after the glucose pulse (Orij *et al.* 2009; Ramos *et al.* 1989). Subsequently, a recovery of the neutral pH_{cyt} up to pre-starvation conditions ($\text{pH}=7$) (Reifenrath and Boles 2018) occurs, as glucose metabolism yields sufficient ATP to fuel the export of protons by the plasma membrane ATPase.

Since the initial acidification is a direct consequence of glucose uptake, we reasoned that kinetics of both processes should be strongly correlated. For real-time measurements of the fast changes of pH_{cyt} , we use the pH-sensitive green fluorescent protein (GFP) variant, the ratiometric pHluorin, which allows accurate and fast measurements in the pH range from 5.5 to 8 that were derived from emission intensity ratios from two different excitation wavelengths - 390 and 470 nm (Orij *et al.* 2009; Miesenböck, Angelis and Rothman 1998). Importantly, overexpression of pHluorin in *Saccharomyces cerevisiae* cells does not affect the cellular metabolism (Orij *et al.* 2009) and a tuning of pHluorin expression levels can be neglected because of its ratiometric character (Kuhn *et al.* 2007), qualifying it as an ideal biosensor for our approach. Fluorescent dyes like fluorescein (Ramos *et al.* 1989), 2',7'-bis-2-carboxyethyl-5- (and -6)-carboxyfluorescein (BCECF) (Ozkan and Mutharasan 2002) or semi-naphthorhodafluor (SNARF) (Blank *et al.* 1992) are also often used to estimate intracellular pH but exhibit certain deficits like leakage of the dyes (Ozkan and Mutharasan 2002), the compartmentalization in particular cell types (Blank *et al.* 1992), or a loss of pH sensitivity over time (Weiner and Hamm 1989), in addition to considerable costs of these dyes. We developed the pHluorin-based assay in the well-established EBY.VW4000 *hxt*⁰

yeast strain, which lacks all endogenous transporters capable of hexose transport (i.e., Gal2, Hxt1-Hxt17, Agt1) (Boles and Oreb 2018; Wieczorke *et al.* 1999). Therefore, this strain is practically free of residual hexose uptake and has been used to express and characterize heterologous sugar transporters, including human Gluts (Boles and Oreb 2018).

Results

Using pHluorin as a biosensor for time-resolved measurements of glucose uptake

With ratiometric pHluorin, the decrease of pH_{cyt} is represented by a drop in the emission intensities ratio at 512 nm at the two excitation wavelengths 390 and 470 nm ($R_{390/470}$) (Orij *et al.* 2009). For the purposes described in this study, we consider the time-dependent $R_{390/470}$ decrease as an adequate kinetic parameter and its translation to pH_{cyt} is therefore not necessary. Nevertheless, in initial experiments, we generated a calibration curve as described previously (Reifenrath and Boles 2018) for $R_{390/470}$ values in the range from pH 5 to pH 9 (Supplementary Fig. S1). The formula (indicated within the graph, see Supplementary Figure S1) confirmed the correlation between the $R_{390/470}$ and pH_{cyt} as published in previous studies (Orij *et al.* 2009; Reifenrath and Boles 2018) under our experimental conditions and it can therefore be used to estimate the pH range variation in the measurements shown below. We then demonstrated that the rapid changes of $R_{390/470}$ can be measured in a time-resolved manner using the high-performance spectrofluorometer PTI QuantaMasterTM 8000 (HORIBA Scientific). We expressed pHluorin and the endogenous transporter Hxt5 (each from a separate multicopy plasmid) in the *hxt⁰* yeast system EBY.VW4000 to avoid any interferences of the measurement with other hexose transporters. The use of multicopy plasmids for pHluorin expression has been established before (Orij *et al.* 2009; Reifenrath and Boles 2018), but expression levels of both, transporters and pHluorin, might vary under these circumstances (Lee *et al.* 2015). To take into account any putative variances due to expression levels or the physiological conditions of the cells, the average of biological triplicates was

calculated for all experiments. The cells were grown on selective, low fluorescent maltose medium (see Methods), harvested, and starved for 3.5 hours. One minute after starting the fluorescence measurement, the cells were pulsed with 10 mM glucose, leading to the expected cytoplasm acidification. Within approximately 10 seconds, a nearly linear decrease of $R_{390/470}$ was observed (Fig. 2A), followed by a slower recovery phase. Moreover, the acidification rate was dependent on the glucose concentration (Fig. 2B), supporting the idea that it mirrors the velocity of glucose uptake.

To validate the hypothesis that the initial acidification after the glucose pulse is caused by the hexokinase-catalyzed phosphorylation of glucose (Ramos *et al.* 1989) under our experimental conditions, we tested pHluorin-expressing cells of the *Saccharomyces cerevisiae* strain TSY11, which is devoid of all three yeast hexokinases (Subtil and Boles 2012). Indeed, the rapid, intense drop of pH_{cyt} after the glucose pulse is absent in these cells but is recovered when *HXK2*, the predominant yeast hexokinase (Rodríguez *et al.* 2001), is reintroduced on a plasmid (Supplementary Fig. S2). However, we observed a small residual decrease of $R_{390/470}$ for hexokinase devoid cells (*hvk*⁰, TSY11 (Subtil and Boles 2012)), especially when pulsed with the higher concentration of 200 mM glucose. The same was also true for the hexose transporter devoid cells (*hxt*⁰, EBY.VW4000 (Wieczorke *et al.* 1999)) and even for cells that contain neither transporters nor kinases (*hxt*⁰/*hvk*⁰, AFY10 (Farwick *et al.* 2014)) (Supplementary Fig. S3 A). Also, the non-metabolizable sugar lactose induces this residual decrease of $R_{390/470}$ (Supplementary Fig. S3 B), indicating that neither the sugar metabolism nor transport-related processes are the cause of this effect. More likely, the osmotic pressure that increases due to the addition of a sugar drives water out of the cell affecting the pH_{cyt} in a concentration-dependent manner. Overall, this “background” changes of pH_{cyt} were low compared to those caused by glucose transport and - since they were dependent on the sugar concentration – we corrected the slopes measured in the presence of hexose transporters by

subtracting the values measured for each glucose concentration in the absence of a transporter (i.e. the empty vector control) in subsequent analyses.

Suitability of the pHluorin-based method for determination of kinetic constants of glucose transporters

To evaluate the method as a tool for kinetic characterization of transporters, we selected three well-described yeast hexose transporters with glucose affinities differing within two orders of magnitude - Hxt1 ($K_M=100$ mM (Roy *et al.* 2015; Reifenberger, Boles and Ciriacy 1997)), Hxt5 ($K_M=6-10$ mM (Buziol *et al.* 2002; Diderich *et al.* 2001)), and Hxt7 ($K_M=1-2$ mM (Reifenberger, Boles and Ciriacy 1997)). All three transporters were expressed from multicopy plasmids in EBY.VW4000 and the measurements were performed as described above. The slopes of the first time points after the pulse (as exemplified in Fig. 2A) at different glucose concentrations were determined, corrected as described above, and their reciprocal values (to avoid negative values) were plotted against respective glucose concentrations, revealing saturable kinetics (Fig. 3A-C). Fitting to the Michaelis-Menten equation produced the following apparent K_M values for the transporters tested: 6.2 mM (Hxt1, Fig. 3A), 3.3 mM (Hxt5, Fig. 3B), and 1.3 mM (Hxt7, Fig. 3C).

For Hxt7, the determined K_M of 1.3 mM with this method is in agreement with the K_M of 1–2 mM as determined by Reifenberger *et al.* (Reifenberger, Boles, and Ciriacy 1997). However, the discrepancy between previously published data and the K_M values of Hxt5 and (especially) Hxt1 determined by our method prompted us to re-determine the kinetics of these transporters using radiolabeled glucose but under conditions otherwise equal to those of the pHluorin-based assay (Supplementary Fig. S4A, B). The results for Hxt1 ($K_M=111.3$ mM) confirmed those of Reifenberger *et al.* (Reifenberger, Boles and Ciriacy 1997) ($K_M=100$ mM) within the error range of the measurement. For Hxt5, we determined a slightly lower K_M of 3.1 mM compared to the published values of 6.1 mM (Buziol *et al.* 2002) and 10 mM

(Diderich *et al.* 2001)). Strikingly, the K_M values determined for Hxt5 with the pHluorin-based assay were in agreement with our results from the C^{14} -glucose uptake assay, thereby, like the results for Hxt7, validating the new method. However, the measured K_M values for Hxt1 obtained from the two methods still show severe discrepancies. We, therefore, analyzed the correlation of V_0 values measured by the pHluorin-based versus the radiolabeled glucose assay (Fig. 4).

A linear correlation is observed for both Hxt5 ($r = 0.98$) and Hxt1 ($r = 0.96$) up to a threshold at approximately $0.006 R_{390/470} s^{-1}$ and $1.5 \text{ nmol min}^{-1} \text{ mg}_{CDW}^{-1}$ (Fig. 4A, B), which apparently marks the upper limit of the acidification slope. We reasoned that the intrinsically high transport capacity of Hxt1 (Reifenberger, Boles and Ciriacy 1997), in combination with the expression from a strong promoter and a multicopy plasmid, could lead to approaching this threshold already at lower glucose concentration and, consequently, to an underestimation of the K_M value. To test this hypothesis, we expressed Hxt1 on a low-copy plasmid (the CEN.ARS plasmid pUCPY1) and repeated the measurement of its glucose uptake kinetics (Fig. 5). By lowering its expression and, consequently, the number of transporters present at the plasma membrane, the transport capacity is diminished, reflected by lower $R_{390/470} s^{-1}$ values (compare Figures 3A and 5).

This experiment revealed a K_M of 99.1 mM for the tested transporter (Fig. 5), matching both the published data (Reifenberger, Boles and Ciriacy 1997) and those from our radiolabeled glucose uptake assay (Supplementary Fig. S4A) very well. It demonstrates that a very high V_{max} value imposes an upper limit to the pHluorin-based method, likely since other factors than the transport might become rate-limiting and lead to underestimating transporter-saturating glucose concentrations. To test if hexokinase activity is a possible limiting factor, we overexpressed *HXK2* along with *HXT1* and pHluorin. However, despite a 3.6-fold increase in hexokinase activity (Supplementary Fig. S5A), the apparent K_M value of Hxt1 expressed

from a multicopy plasmid was still underestimated (Supplementary Fig. S5B, C). Hence, as the phosphorylation of glucose is ATP dependent, rapid ATP depletion is a likely explanation for our observation. However, we cannot rule out other bottlenecks, e.g., in the downstream glycolytic reactions.

Discussion

The most commonly used method for determining glucose uptake in yeast (Boles and Oreb 2018) requires radiolabeled (^{14}C) sugar and includes the following steps: (1) incubation of the cells with the sugar solution; (2) quenching with an excess of unlabeled sugar; (3) collecting the cells by filtering; (4) washing (twice) the filter; (5) inserting the filter into a scintillation vial and (6) measurement using a scintillation counter. The hands-on time requirement for steps (1)-(5) is considerable (about 2-3 min per sample), adding up to 60-90 min of work necessary for a typical determination of the K_M value for each transporter. Moreover, scintillation counting requires 10 min per sample, and obtaining the raw data for the complete dataset is only possible after several hours (e.g., 5 h for a typical K_M determination/30 samples). Our new method reduces the handling time and raw data collection to about 90 min for the same number of samples. Importantly, signal acquisition can be monitored in real-time, enabling data quality control during the measurement, in contrast to ^{14}C assays that can be evaluated only *a posteriori*. Also, the pHluorin assay substantially reduces the costs for consumables, as no radiochemicals, filters, scintillation liquid, and scintillation vials are necessary. Radiochemicals are not only expensive themselves, but their handling, storage, and disposal requires specialized laboratories and spaces, associated with substantial costs and administrative effort. Most importantly, the pHluorin-based method avoids the exposure of the experimenter to radiation and radioactive waste production.

It should be noted that the hexokinase-dependence cannot be regarded as a particular disadvantage of the pHluorin-based assay; previous work by others has shown that the uptake

of (^{14}C -labelled) phosphorylatable sugars is dependent on intracellular ATP concentration (Walsh, Smits and van Dam 1994) and hexokinase activity (Smits *et al.* 1996), when the assays are performed on the 5 s-timescale, according to the common protocol (Boles and Oreb 2018). In fact, hexose phosphorylation prevents the accumulation of intracellular sugar, which would – due to the breakdown of the concentration gradient across the membrane - cause an underestimation of the transport rates, especially for facilitative transporters, as it has been observed in hexokinase deficient cells (Smits *et al.* 1996). The authors demonstrated that for determination of the “true” initial velocity of sugar uptake, which is not dependent on the events downstream of the transport, the assays must be performed on the 200 ms-timescale using the so-called quench-flow technique (Walsh, Smits and van Dam 1994). Nevertheless, the kinetic constants of sugar uptake measured in wildtype (hexokinase expressing) cells were comparable on both time scales (Smits *et al.* 1996), demonstrating that a fairly good approximation of the transport kinetics can be obtained even on the 5 s-timescale, if the sugar transport does not exceed the capacity of sugar phosphorylation. This is consistent with our observation that the upper limit of the accuracy of the pHluorin assay is determined by the capacity ($V_{\max}$) of the transporter, since a very high uptake rate leads to a saturation of the system and underestimation of the K_M value, as shown here for Hxt1 when expressed from a multicopy plasmid. Based on correlation analyses (Fig. 4), we determined a threshold value of $0.006 R_{390/470} \text{ s}^{-1}$ (corresponding to $1.5 \text{ nmol min}^{-1} \text{ mg}_{\text{CDW}}^{-1}$ with the radiolabeled glucose uptake assay), after which the pHluorin-based assay becomes inaccurate for determination of kinetic constants in EBY.VW4000 cells. By defining the threshold value within the method itself, no *a priori* knowledge about the transport capacity for a particular transporter is necessary. If the upper limit is reached, low copy plasmids (as shown for Hxt1, Fig. 5), a genomic integration, weak or regulatable promoters can be applied to reduce the expression and, thereby, allow for an accurate measurement. However, if the determination of transport rates of, for example, wild-type strains (and not plasmid-expressed transporters in the *hxt₀*

strain) is desired, transport capacities cannot be altered, which restricts the applicability of our method. Also, the prerequisite of using starved cells precludes the possibility to measure transport rates in growing cells. However, in this context it is noteworthy that all available sugar uptake assays require washing of the cells and storing them in a sugar-free solution prior to the measurement, which also likely induces starvation-like changes of cellular physiology.

In starved EBY.VW4000 cells, we have shown for three model transporters that the K_M values (spanning the range from 1 to 100 mM) can be accurately determined with the pHluorin-based method. Whereas the K_M value is an intrinsic property (a kinetic constant) of each transporter, the V_{max} is variable, as it depends on the protein abundance (i.e., the expression level) of the transporter. Nevertheless, in some circumstances, it is desirable to determine the V_{max} , e.g., for comparative purposes. Based on the strong correlation shown in Fig. 4, linear regression (Formula: $[\text{nmol min}^{-1} \text{ mg}_{CDW}^{-1}] = [R_{390/470} \text{ s}^{-1}] / 0.00466$) can be used to interconvert the values, at least under experimental conditions comparable to those applied here (i.e. regarding strain background, hexokinase levels and preculture conditions). For validation of the assay conditions in other laboratories, we recommend including at least one transporter plasmid used in this study as a control. While adapting the expression levels of transporters must be considered due to the above-mentioned reasons, the signal sensitivity of measurements should not be influenced by the expression level of pHluorin. Due to their ratiometric character, pHluorin-based measurements are independent of the biosensor concentration and not affected by artefacts such as bleaching (Kuhn et al. 2007). In previous studies, a super-folder pHluorin was expressed in *Saccharomyces cerevisiae* cells under the control of the repressible promotor *MET25* and pH calibration was performed (as in this study, see Methods) for cells grown under fully inducible conditions (no methionine) and partly repressible conditions (0.1 mM methionine) (Reifenrath and Boles 2018). The sigmoidal-dose response fits of both experiments were in agreement within the error range

(Reifenrath and Boles 2018), further indicating that the quantity of pHluorin molecules is not a relevant factor. In summary, the pHluorin-based method provides a comparably accurate, but simpler, faster, cheaper, and safer alternative to the common ^{14}C -based method for measuring uptake of glucose and other phosphorylatable sugars, which account for the preferred and largest fraction of carbohydrates used by living organisms, including humans and most human pathogens. In combination with the widely used *hxt⁰* background, this approach can be used to characterize all heterologous transporters that can be functionally expressed in yeast.

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Author contributions

S.S. performed the experiments, analyzed the data and drafted the manuscript. C.V.I. helped with the experiments. M.R. suggested using pHluorin as a sensor for glucose uptake. J.C. provided advice and resources. M.O. conceived the kinetic analysis approach and guided the project. All authors edited and approved the manuscript.

Competing interests

The authors declare no competing interests.

Methods

Strains and media

The construction of *S. cerevisiae* strains EBY.VW4000 (*hxt⁰*), TSY11 (*hxx⁰*), and AFY10 (*hxt⁰/hxx⁰*) was reported previously (Wieczorke *et al.* 1999; Subtil and Boles 2012; Farwick *et al.* 2014). Plasmid-free cells were grown in standard YEP-media (1% (w/v) yeast extract, 2% (w/v) peptone) supplemented with 1% (w/v) maltose (for EBY.VW4000 cells) or 2% (v/v)

ethanol (for TSY11 and AFY10 cells) for maintenance and preparation of competent cells. Frozen competent cells were prepared and transformed according to Gietz and Schiestl (Gietz and Schiestl 2007). The transformants were plated on solid, selective synthetic complete (SC) medium (Bruder *et al.* 2016) with 1% (w/v) maltose (SCM) or 2% (v/v) ethanol (SCEtOH), where leucine, uracil or histidine were omitted as required for the selection of the plasmids. Filter-sterilized, low fluorescent, synthetic complete medium (lf-SC) containing 6.9 g/L YNB with ammonium sulfate, without amino acids, without folic acid and without riboflavin (MP Biomedicals), containing 1% (w/v) maltose or 2% (v/v) ethanol and amino acids as stated in Bruder *et al.* (Bruder *et al.* 2016), in which the respective amino acid(s) were omitted, was used for cultivation of cultures for the pHluorin measurements. For subcloning of plasmids, *E. coli* strain DH10B (Gibco BRL, Gaithersburg, MD) was used.

Plasmid construction

For plasmid assembly, the vector backbone p425H7 (for pHluorin), p426H7 (for all applied transporters or *HXX2*), pUCPY1 (to express Hxt1 with lower capacity), and p423H7 (for *HXT1* in the *HXX2* overexpression experiment) were linearized with restriction enzymes BamH1 and EcoR1 (New England Biolabs GmbH). The backbones were transformed together with the corresponding PCR fragment in EBY.VW4000 for homologous recombination, according to Oldenburg *et al.* (Oldenburg *et al.* 1997). All genes applied in this study were under the control of the strong, constitutive, truncated *HXT7*¹⁻⁻³⁹² promoter and the *CYC1* terminator. PCRs were performed with Phusion polymerase (New England Biolabs GmbH) and primers listed in Supplementary Table S1. The plasmids are listed in Supplementary Table S2.

Calibration of pHluorin

EBY.VW4000 cells expressing pHluorin on the multicopy p425H7 plasmid were grown overnight in 15 ml lf-SC -LEU medium containing maltose (1%, w/v) and harvested the next

day in the exponential growth phase at an OD_{600nm} of 0.5 – 1.5. The pellets were resuspended in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , pH 7.4) containing 100 μ g/ml digitonin and incubated 10 min on ice to permeabilize the cell wall as described by Orij *et al.*, 2009. Thereafter, cells were washed twice and resuspended in PBS. pH calibration was performed as described in Reifenrath and Boles, 2018, where the cells were added (to a final $OD_{600nm} = 0.5$) to citric acid/ Na_2HPO_4 buffer solutions, with different pH values ranging from 5.0 to 9.0. The cell/buffer suspensions were transferred into a black polystyrene clear-bottom 96-well microtitre plate (Greiner Bio One, article Nr. 655097) and mounted onto the Clariostar microplate reader (BMG LABTECH GmbH) (set at 30°C) to measure the emission intensities at 512 nm when excited at 390 nm or 470 nm, respectively, to calculate the ratio $R_{390/470}$. Data from biological triplicates was analyzed with the sigmoidal dose-response fit in GraphPad Prism 5.

The pHluorin-based assay and data analysis

To test the response of a sugar-pulse to sugar-starved cells, 15 ml of selective If-SCM (or If-SC-EtOH) medium was inoculated with the transformed cells in biological triplicates, and grown overnight in 100 ml Erlenmeyer-flasks at 30 °C and 180 rpm. On the next day, the cultures were harvested in the exponential growth phase (at an OD_{600nm} between 0.5 and 1.5). Cells were washed twice with double-distilled water and resuspended in the If-SC medium without any carbon source to a final OD_{600nm} of 1. These suspensions were incubated at 30 °C and 180 rpm in Erlenmeyer flasks for 3.5 – 4 hours to induce starvation.

To monitor the effect of a sugar pulse to sugar-starved cells on the intracellular pH, we used a PTI QuantaMasterTM 8000 Spectrofluorometer (Model QM-8075-11-C, Horiba Scientific).

After starvation, 1800 μ l of the cell suspension was transferred into a macro cell quartz-glass cuvette (type 100-QS, layer thickness 10 mm, volume 3.5 ml, Hellma Analytics) together with a magnetic stirring bar (3 mm x 6 mm, Sigma-Aldrich). Cells were kept in a water bath at

30 °C until just before the measurement, and the temperature in the fluorometer was set to 30 °C. The cuvette was mounted into the sample compartment, and the lid, which contained a sample port, was closed. The magnetic stirring function was switched on and adjusted to medium velocity. Integration time was set to 0.5 s, and slit widths were set to 3 nm. The measurement was performed by exciting the samples successively at the two excitation wavelengths 390 nm and 470 nm and recording the emission intensity at 512 nm. The ratio of these intensities ($R_{390/470}$) was calculated and plotted on-line during the measurement, presenting one $R_{390/470}$ value every 2.2 s. After 60 s, 200 μ l of the 10x sugar solution was injected through the port into the cuvette with a Hamilton syringe (500 μ l, Sigma-Aldrich) and the $R_{390/470}$ value was recorded for another 3 min. After a total time of 4 min, the cuvette was removed from the device. For the determination of transport kinetics, this measurement was performed in biological triplicates for the following final glucose concentrations: 5, 10, 20, 50, 100, 200, and 400 mM (for Hxt1, for *HXK2* overexpression experiments, 400 mM was omitted); 1, 1.5, 2, 5, 10, 20, 50, and 100 mM (for Hxt5); and 0.5, 1, 1.5, 2, 5, 10, 20, 50, 100, and 200 mM (for Hxt7).

For each derived data set, the time frame after the glucose pulse, in which $R_{390/470}$ decreased linearly, was identified. In this time frame, the slope ($\Delta R_{390/470}/\Delta t$) was calculated and multiplied by -1 to obtain positive values (for plotting over glucose concentrations for Michaelis-Menten analysis). The same was done with the data sets derived from the empty vector control (pHluorin-expressing EBY.VW4000 cells without any transporter), pulsed with the corresponding glucose concentration, and the average from triplicates was calculated. The corresponding empty vector control average values were subtracted for each glucose concentration from slopes obtained in transporter measurements to correct for the background change of $R_{390/470}$ (see Supplementary Figure S3). The values were plotted against glucose concentrations and analyzed by nonlinear regression using GraphPad Prism 5 to calculate the K_M parameter.

Radiolabeled glucose uptake assay

K_M values for Hxt1 and Hxt5 were also determined by the conventional uptake assay using radiolabeled glucose to compare the results with the K_M values derived from the pHluorin-based assay. Precultures expressing pHluorin and concomitantly either *HXT1* or *HXT5* on a multicopy plasmid were grown for 6 hours in 30 ml If-SCM medium, in which leucine and uracil were omitted, in a 300 ml Erlenmeyer-flask. Pre-grown cells were transferred to pre-warmed If-SCM medium, without leucine or uracil, to a total volume of 200 ml, in 2 L Erlenmeyer-flasks. Despite the larger volume of the culture, cells were grown overnight under the same conditions as applied for the pHluorin-based assay (30 °C, 180 rpm) and harvested on the next day in the early exponential growth phase (at an OD_{600nm} between 0.5 and 1.5). Cells were washed twice with double-distilled water and resuspended in selective If-SC medium without any carbon source to a final $OD_{600nm} = 1$. Cell suspensions were incubated at 30 °C and 180 rpm in 1 l Erlenmeyer flasks for 3.5 – 4 hours to induce starvation. After this time, cells were transferred to 50 ml Falcon tubes, of which the empty weight has been determined before to later calculate the cell fresh and dry weight, and centrifuged (4,000 x g, 5 min, 20 °C). Pellets were washed once in ice-cold 0.1 M potassium phosphate buffer (KH_2PO_4 , pH 6.5, adjusted with KOH). After another centrifugation (4,000 x g, 5 min, 4 °C), the supernatant was discarded; the pellet was weighed and resuspended in 0.1 M potassium phosphate buffer to a wet-weight concentration of 60 mg/ml. 110 μ l aliquots of these cell suspensions were prepared and kept on ice. The volume of the leftover cell suspension was determined, and cells were centrifuged (4,000 x g, 5 min, 4 °C). The pellet was frozen at -80 °C overnight or longer to prepare for freeze-drying. The procedure of the uptake assay, the freeze-drying, and the calculation of data have been described previously in detail by Boles and Oreb (Boles and Oreb 2018). The measured uptake velocity was expressed as $nmol\ C^{14}$ glucose taken up per minute per milligram cell dry weight ($nmol\ min^{-1}\ mg_{CDW}^{-1}$). The cell suspension- radiolabeled glucose mixture was incubated for only 5 s before application to the

membrane filter. Uptake was measured at glucose concentrations 5, 10, 20, 50, 100, 200, and 400 mM (for Hxt1) and 1, 1.5, 2, 5, 10, 20, 50, 100, and 200 mM (for Hxt5).

Calculation of the K_M and the V_{max} was done by nonlinear regression analysis and global curve fitting in Prism 5 (GraphPad Software) with values from technical triplicates.

Preparation of protein crude extracts

To produce crude extracts, EBY.VW4000 cells expressing *HXX2* on the high-copy p426H7 plasmid as well as EBY.VW4000 and TSY11 cells containing an empty p426H7 plasmid as controls were grown overnight in 30 ml SC medium without uracil and with 1% (w/v) maltose (for EBY.VW4000 cells) or 2% (w/v) galactose (for TSY11 cells). Cells were harvested at the exponential phase (at an OD_{600nm} between 2 and 3), transferred in 50 ml falcon tubes, and centrifuged (4,000 x g, 10 min, 20 °C). Pellets were washed twice in double-distilled water and transferred to 2 ml Eppendorf reaction tubes during the second washing step. The pellets were suspended in 1x pellet volume of 50 mM HEPES buffer (pH 7.6, adjusted with KOH), containing 1x Halt Protease Inhibitor Cocktail (ThermoFisher) and 1 mM PMSF (Sigma-Aldrich) to prevent proteolytic degradation. The same volume glass beads (0.25 – 0.5 mm, Roth) was added, and cells were disrupted by vortexing on the vibrax for 10 min at 4 °C. After centrifugation (13,000 x g, 5 min, 4 °C), the supernatant was transferred to a fresh Eppendorf reaction tube and kept on ice. Protein concentration was determined by Bradford assay (Bradford 1976).

Enzyme activity assay

Hexokinase activity was assayed, in technical triplicates, based on the coupled reaction of the hexokinase and the glucose-6-phosphate dehydrogenase (G6P-DH), releasing $NADPH + H^+$. The accumulation of $NADPH + H^+$ can be quantified photometrically as an increase of the absorption at 340 nm (Bergmeyer 1974).

The following components of the assay were pre-mixed in cold 50 mM HEPES buffer (pH 7.6, adjusted with KOH) and stored on ice: Glucose (217 mM), MgCl₂ (8 mM), NADP (1.027 mM), ATP (0.71 mM), and G6P-DH (0.11 Units/reaction). 190 µl of the mix was dispensed in a cuvette (UV-Cuvette micro, Brand) and incubated in the photometer (Ultrospec 2100 pro, Amersham Biosciences), which was pre-warmed to 30 °C, for 2 min. 10 µl of the crude extract was added, and the sample was mixed thoroughly by pipetting up and down. A continuous measurement of the absorption at OD_{340nm} was started and stopped after approximately 10 min, when no increase of OD_{340nm} was observed anymore. Specific activity was calculated from the slope ($\Delta\text{OD}_{340\text{nm}}/\Delta t$) (Bergmeyer 1974), taking into account the determined protein concentration.

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ORIGINAL UNEDITED MANUSCRIPT

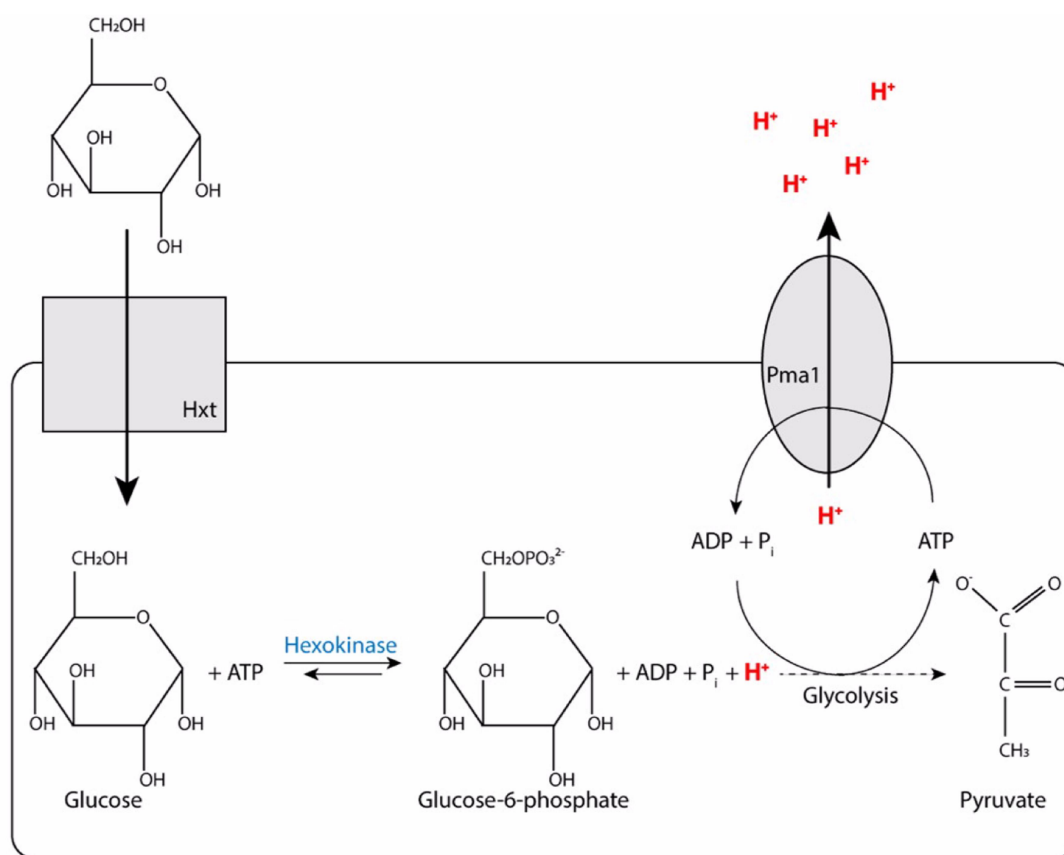


Figure 1. Mechanism of cytosol acidification in yeast cells after a glucose pulse.

Glucose is transported into the cell through a hexose transporter (Hxt) and immediately phosphorylated by the hexokinase. In this process, one molecule ATP per molecule glucose is hydrolyzed, releasing one proton and causing the drop of the pH_{cyt} (see Fig. 2A and Supplementary Fig. S2). With the progress of glycolysis, ATP is regenerated, fueling the plasma membrane ATPase Pma1, which actively transports protons out of the cell and thereby recovers the pH_{cyt} . For simplicity, multiple glycolytic steps between glucose-6-phosphate and pyruvate are indicated by the dashed arrow and the stoichiometry is neglected.

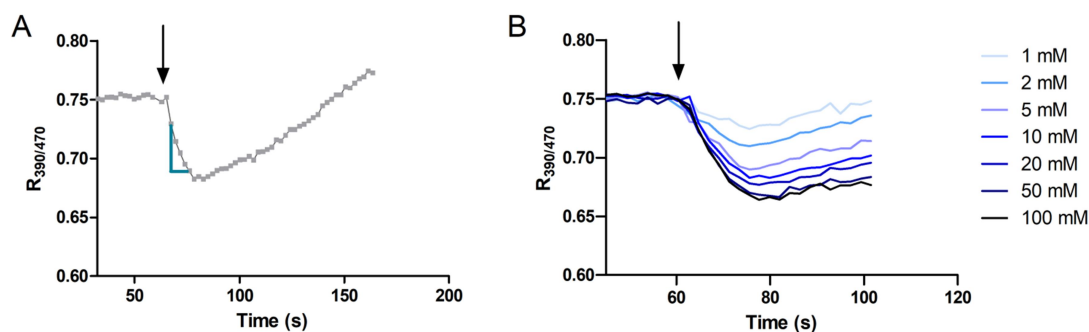


Figure 2. pHluorin-based measurement of acidification rate following a glucose pulse.

The arrow indicates the time point of the glucose addition. (A) A fluorimetric measurement of pH_{cyt} changes after a glucose pulse to glucose-starved yeast cells. The change in the emission ratios (512 nm) at the two excitation wavelengths 390 nm and 470 nm ($R_{390/470}$) of pHluorin serves as a proxy for the pH_{cyt} change. The blue lines indicate the acidification phase in which the slope decreases linearly. Values from this phase were used to calculate the reciprocal $R_{390/470}$ slope as a parameter of velocity. After the acidification phase, the $R_{390/470}$ values rise gradually to the pre-starving state. The average from biological triplicates (EBY.VW4000 cells harboring the Hxt5 transporter, pulsed with 10 mM glucose) is shown exemplarily. Error bars are omitted for clarity. According to the calibration curve shown in Supplementary Figure S1, the pH_{cyt} decrease spans the range from 6.6 to 6.4 in these measurements. (B) The dependence of acidification velocity on glucose concentration. Indicated glucose concentrations were added to glucose-starved *hxt⁰* yeast cells, expressing pHluorin and the transporter of interest (here Hxt5). The acidification velocity increases until reaching saturation.

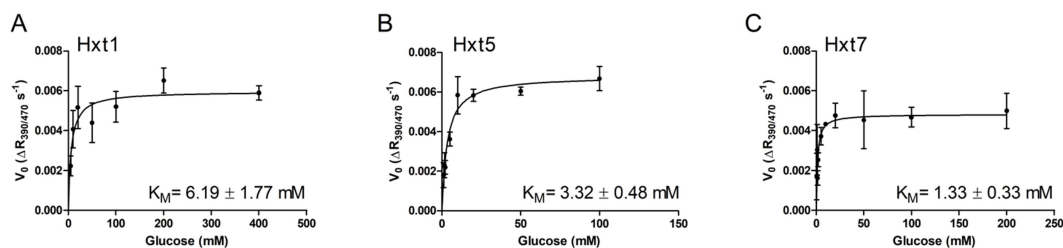


Figure 3. Glucose transport kinetics determined by the pHluorin-based assay.

Michaelis-Menten constants (K_M) of three prominent yeast hexose transporters Hxt1 (A), Hxt5 (B), and Hxt7 (C) were determined in the *hxt⁰* strain background. Reciprocal values of the acidification slopes were plotted against the glucose concentration. Mean values and standard deviations were calculated from biological triplicates. The lines represent a least-square fit to the Michaelis-Menten equation. Determined K_M values are indicated within the graphs.

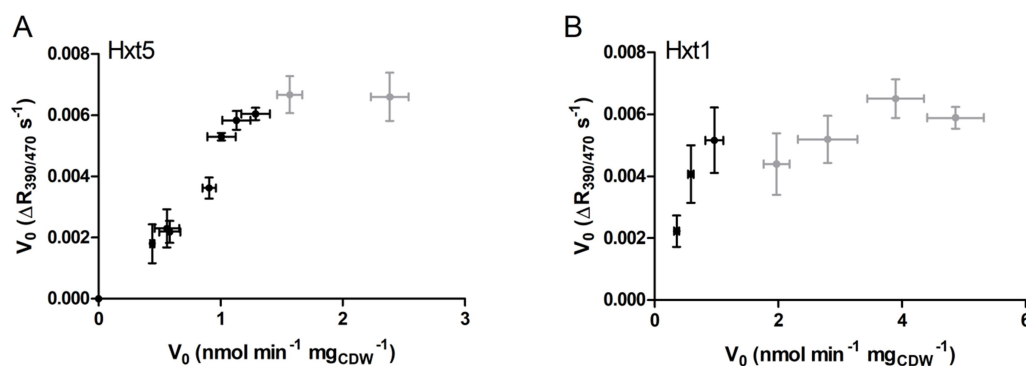


Figure 4. Correlation of initial transport velocity (V_0) values measured with the pHluorin-based and radiolabeled glucose assays.

V_0 values measured by the radiolabeled glucose method are shown on the x-axes and those measured by the pHluorin-based assay on the y-axes, for the medium capacity transporter Hxt5 (A) and the high capacity transporter Hxt1 (B). Error bars (standard deviation) of three replicates are shown. Correlation was calculated only for values below a threshold of 0.006 $R_{390/470} \text{ s}^{-1}$ or 1.5 $\text{nmol min}^{-1} \text{ mg}_{\text{CDW}}^{-1}$ (excluding the data points which are colored grey).

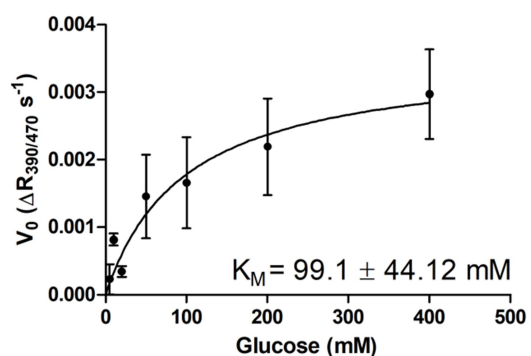


Figure 5. Glucose transport kinetics of the yeast hexose transporter Hxt1.

The Michaelis-Menten constant (K_M) was determined by the pHluorin-based assay with Hxt1 expressed on a low-copy CEN.ARS (pUCPY1) plasmid in the *hxt⁰* strain background, and its value is indicated within the graph. Mean values and standard deviations of two (for 5, 50, and 100 mM) or three (for 10, 20, 200, and 400 mM) biological replicates are shown. The lines represent a least-square fit to the Michaelis-Menten equation.