Published in final edited form as:

Biochem J. 2010 November 12; 432(2): 399–406. doi:10.1042/BJ20100946.

DYNAMIC ANALYSIS OF CYTOSOLIC GLUCOSE AND ATP LEVELS IN YEAST WITH OPTICAL SENSORS

Clara Bermejo[#], Farzad Haerizadeh^{#,1}, Hitomi Takanaga, Diane Chermak, and Wolf B. Frommer^{*}

Carnegie Institution for Science, 260 Panama St., Stanford CA 94305, USA

Abstract

Precise and dynamic measurement of intracellular metabolite levels has been hampered by difficulties in differentiating between adsorbed and imported fractions and the subcellular distribution between cytosol, endomembrane compartments and mitochondria. Here, genetically encoded Förster Resonance Energy Transfer (FRET)-based sensors were deployed for dynamic measurements of free cytosolic glucose and ATP at varying external supply and in glucose transport mutants. Moreover, by using the FRET sensors in a microfluidic platform, we were able to monitor *in vivo* changes in intracellular free glucose in individual yeast cells. We demonstrate the suitability of the FRET sensors for gaining physiological insight by demonstrating that free intracellular glucose and ATP levels are reduced in an *hxt5Δ* hexose transporter mutant compared to wild type and to other *hxtΔ* strains.

Keywords

nutrient starvation; hexose transport; flux; Förster resonance energy transfer; microfluidics

INTRODUCTION

Cellular metabolite levels have predominantly been determined using destructive assays. Such assays can only provide dynamic information when carried out with parallel samples and have limited spatial resolution, i.e. average over cells in a culture or organ. Isotopes have been used to monitor glucose transport activity, but cannot measure glucose uptake in single living cells and interpretations are occluded by metabolism. Thus minimally invasive technologies that can provide real time information of metabolite levels in a given subcellular compartment at varying external supply levels would help to advance our understanding of the mechanisms and control of metabolic fluxes.

For many organisms including yeast, glucose is the preferred source of energy. In yeast, glucose uptake is mediated by a family of 17 hexose transporters Gal2, Hxt1-11 and Hxt13-17 [1]. These transporters are characterized by broad selectivity for various hexoses and different affinities thus covering a wide range of external supply level with Hxt1–Hxt7 being the dominant ones at typical supply levels of 2% glucose in the medium [2,3]. *Saccharomyces cerevisiae* is a facultative fermentor, which, depending on the growth conditions, the type and concentration of sugars and/or oxygen availability, will display

fully respiratory, fermentative or mixed respiratory/fermentative metabolism [4]. During fermentation, i.e. under anaerobic conditions, cytosolic glucose is oxidized to pyruvate generating two ATP per glucose molecule. In yeast, ATP levels have been typically measured using bioluminescence assays in cell extracts or NMR [5–7]. To determine the yield of ATP in the cytosol and in mitochondria under varying conditions and its relation to cytosolic glucose levels *in vivo*, a suitable quantitative analytic method for both molecules would be advantageous.

Genetically encoded Förster resonance energy transfer (FRET) nanosensors provide a unique tool enabling dynamic quantitation of metabolite analysis with subcellular resolution [8,9]. Genetically encoded FRET-based nanosensors have been developed for a variety of sugars and amino acids. The nanosensors are composed of the bacterial periplasmic binding proteins as a recognition element coupled allosterically to a set of two spectral variants of the Green Fluorescent Protein (GFP) as reporter elements [10–13]. Conformational changes induced by ligand-binding to the recognition element are translated into a change in FRET between attached eCFP and eYFP moieties, permitting non-invasive measurements of analyte levels in living cells [11]. To determine analyte levels inside organelles, these genetically encoded nanosensors can be targeted to the respective subcellular compartments such as nuclei or the endoplasmic reticulum [14,15]. Recently a new FRET-based ATP sensor that uses the ϵ subunit of the bacterial F_0F_1 -ATP synthase has been reported [16]. This sensor provides information about the ATP level changes in the cytosol and mitochondria of individual HeLa cells and in response to nutritional changes.

Here we expressed FRET glucose and ATP sensors in yeast and developed a simple fluorimetric assay for measuring ATP level changes, glucose accumulation rates and steady state levels of glucose and ATP in yeast cultures using genetically encoded glucose FRET sensors. Furthermore, we implemented the use of a microfluidic platform to monitor glucose accumulation and elimination in individual yeast cells. Consistent with previous work showing induction of the *HXT5* gene during starvation [17–19], we found that Hxt5p is the dominating transporter for glucose in starved cells. In agreement with a low level of glucose accumulation in starved *hxt5Δ* cells we observed a reduced rate of ATP accumulation in *hxt5Δ* compared to the wild type strain.

EXPERIMENTAL

Yeast strains

Yeast strains used here were isogenic to BY4743 [*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0*] (Open Biosystems, Huntsville, AL). Additional yeast strains are described in Table S1. KY98 was kindly provided by Jos Arents, SILS, Amsterdam.

Plasmid constructs

A new sensor FLIPglu-30μΔ13V with an affinity for glucose of 30 μM was generated by site-directed mutagenesis of D154A in FLIPglu-170nΔ13 (Quickchange, Stratagene; primers: CCGGGCCATCCGGCTGCAGAAGCACGTACC and GGTACGTGCTTCTGCAGCCGGATGGCCCGG). The affinity of the sensor was determined with affinity-purified protein isolated from *E. coli* as described [20]. A set of more robust sensors covering the low nanomolar to high millimolar range of glucose concentrations was constructed on the backbone of FLIPglu-170nΔ13. The eYFP gene in FLIPglu-170nΔ13/pRSETB [21] was exchanged with Venus [22] using the unique *XhoI* and *HindIII* sites. The plasmid was first cleaved with *HindIII*, 5'-overhangs were filled in by treatment with DNA Polymerase I, Large (Klenow) Fragment, the nanosensor cassette was excised using *BamHI*, and inserted into the *BamHI/EcoRV* sites of pENTR1A (Invitrogen).

The nanosensor cassette in FLIPglu-170n Δ 13V/pENTR1A was introduced into pDRf1GW-ura3 [23] using Gateway® LR reactions utilizing pDONR201 as an intermediate vector. Subsequently, different affinities for glucose from 2 μ M, 30 μ M and 600 μ M were generated by site directed mutagenesis [24] of H152A, D154A, F16A using mutagenesis primers (F16A: CTATAAGTACGACGATAACgcgATGTCTGTAGTGCGCAAGGC, H152A/D154A: GCTGAAAGGTGAACCGGGCgctCCGGATGCAGAAGCACGTACC) in FLIPglu-170n Δ 13/pDRf1GW-ura3. All construction steps were verified by sequencing.

The ATP sensors, AT1.03, AT1.03^{YEMK} and AT1.03^{R122K/R126K} [16] were introduced in a pDONR221 intermediate vector using a Gateway® BP reaction and subsequently into pDRf1GW-ura3 using Gateway® LR reaction. Construction steps were verified by DNA sequencing. Yeast cells were transformed with the pDR-AT1.03, pDR-AT1.03^{YEMK} and pDR-AT1.03^{R122K/R126K}, respectively, and grown in selective media for fluorimetric analysis of mVenus versus mscCFP emission levels.

The hexose transporter *HXT5* from pTH[HXT5] [25] was introduced into pDONR221 as an intermediate vector using a Gateway® BP reaction and subsequently into pDRf1GW-ura3 using Gateway® LR reaction for yeast transformation.

Growth conditions and treatments

Yeast cells were grown in YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose) or in synthetic complete medium [26] containing 2% glucose (SC^{glc}), 2% maltose (SC^{mal}) lacking uracil for selection. For starvation, yeast was incubated either for 16 hours in 2% ethanol (SC^{eth}) or for 6 hours in medium lacking a carbon source (SC^{-C}). Cultures were grown at 30°C and harvested in log phase (OD₆₀₀ 0.6–1.0) for all analyses described.

Growth monitoring assay

Yeast growth was monitored by light scattering using a microplate reader (Tecan GENios™ at 595 nm). Selected strains were grown O/N in SC^{glc}. Cultures were divided into two parts, one part was grown in fresh SC^{glc}; the other was glucose-starved in SC^{eth}, both diluted to OD 0.05. After 16 hours in SC^{glc} or SC^{eth}, 5 $\times$ 10⁴ cells were inoculated in a final volume of 100 μ l SC^{glc} in 96-well microplates. Plates were incubated at 30°C with constant shaking; growth was monitored using light scattering measurements in 20 min intervals.

Fluorimetric analysis of FRET sensor responses in yeast cultures

For determination of the *in vivo* response, yeast transformed with the new Venus-versions of the Δ 13 series FLIPglu sensors [21], FLII¹²Pglu-700 μ δ 6 [20,27] or the ATP sensors [16] were grown at 30°C in SC^{glc} to OD₆₀₀ 0.8–0.9 (*CEN.PK2-1C* and *EBY4000* cells were grown in SC^{mal}). Cells were centrifuged (4,000 rpm), resuspended and incubated for glucose starvation either in SC^{-C} for 6h, or SC^{eth} for 16h. Cells were washed in 20 mM MES pH6, OD₆₀₀ was set to 0.5, and 180 μ l were transferred to a 96-well microplate (Greiner PS, F-bottom) for analysis in a microplate reader (Tecan Safire™; ex 428/12 nm; em 485/12, 530/12 nm). Sensor expression in yeast was verified by analyzing the emission spectra of CFP (428 nm) and Venus (500 nm). Typically, 42 wells of the microplate were filled with cultures. A row of six wells was filled with cells from the same culture; the first row was filled with non-transformed wild type cells, three adjacent rows were filled with cultures derived from three wild type independent transformants using the same plasmid. The next three rows were filled with cultures derived from independent transformants of the mutant to be tested. Emission intensities of CFP and Venus at CFP_{ex} were acquired for two cycles (each cycle ~100 sec) before addition of 20 μ l glucose. The plate was ejected from the reader, glucose (or other metabolite) was added using a multichannel pipette, reloaded and immediately read for 8 cycles. Different concentrations of glucose (0–100 mM) were added

to each of the six individual wells in a single row. Emission ratio is defined here as the background-corrected fluorescence intensity at 528 nm divided by the background-corrected intensity at 485nm. All analyses were repeated (with three biological replicates) at least three times independently. Emission ratios were normalized to the average of the two initial ratio values before glucose addition.

Real-time glucose accumulation assays

For real-time glucose accumulation assays, transformed cells were grown in SC^{glc} and glucose-starved in SC^{eth}. Cells were resuspended in 20 mM MES pH 6 to OD 0.5. Ratiometric measurements (Tecan Infinite™ M200; ex 428/12 nm; em 485/12, 530/12 nm) were taken until a stable baseline had been established and time was set to zero. Fluorescence readings were obtained every 20 sec. Glucose was injected into individual wells after 20 sec (duration 0.2 sec) to a final glucose concentration of 50 mM. Subsequently the procedure was repeated for the next well. Experiments were repeated at least three times independently with three biological replicates.

FRET imaging analysis for single yeast cells

For single cell FRET analysis cells transformed with FLII¹²Pglu-700μδ6 or FLIPglu-30μΔ13V were grown in SC^{glc} or SC^{mal} to OD ~0.8. Cells were washed in 20 mM MES pH 6 and starved in SC^{eth}. Cells were trapped in a Y2 microfluidic plate (CellASIC, San Leandro, CA) and subjected to reversible changes of the external sugar supply using ONIX™ Microfluidic Perfusion Platform. Fluorescence intensities in single cells were recorded by FRET imaging as previously described [20]. In short, imaging was performed on an inverted fluorescence microscope (Leica DMIRE2) with a Quant EM digital camera (Photometrics) and 40×/N.A. 1.25–0.75-oil immersion lens (IMM HCxPL Apo CS). Dual emission intensity ratios were simultaneously recorded using a DualView unit with a Dual CFP/YFP-ET filter set (ET470/24m, ET535/30m Chroma) and Slidebook 4.0 software (Intelligent Imaging Innovations, Inc.). Excitation (filter ET430/24× Chroma) was provided by a Lambda LS light source (Sutter Instruments; 100 % lamp output). Images were acquired within the linear detection range of the camera at intervals of 20 s. Exposure time was typically 1000 ms with an EM gain of 3× at 10 MHz. Two genotypes (wild type/mutant) were compared one by one using the two trapping areas in the Y2 microfluidic plate. Cells were perfused with glucose, maltose or galactose. After each pulse, sugar was removed from the cell trapping area by perfusion with buffer (20 mM MES pH 6). Analysis of emission ratios (535/470 nm) was done for > 30 cells in each experiment. The experiments were repeated 3 times independently. Dead volume was corrected in the resulting graphs considering the chamber refresh time reported by the microfluidic plate manufacturer at 3 psi perfusion pressure (information available at www.cellasic.com).

Confocal microscopy

Confocal imaging of yeast cells was performed with a Leica SP5 confocal microscope (Germany) using a 63×/N.A. 1.3 glycerol immersion lens (IMM HCX PL APO, Leica). GFP was excited at 488 nm. Image processing was performed using ImageJ (NIH). To determine the Average Plot Profile, 50 randomly selected cells were analyzed and sizes were normalized to the smallest cell in the analyzed population.

RESULTS AND DISCUSSION

FRET glucose sensors covering the nano- to millimolar range

Due to technical challenges, conventional methods have provided limited information on steady state glucose levels in the cytosol of yeast [28]. To measure free glucose levels of

yeast as well as the rate of accumulation and elimination, we developed a simple quantitative detection system using genetically encoded FRET sensors. We had previously constructed FRET glucose nanosensors with affinities of 170 nM, 2 μ M and 600 μ M. This set of sensors reports glucose levels between 20 nM and 6 mM, but left a gap in the range between 20 μ M to 60 μ M [21]; a range corresponding to the K_m of hexokinase. A new FRET sensor with a K_d for glucose of 28.5 μ M (FLIPglu-30 μ) was constructed by mutation of aspartate-154 to alanine in the MglB recognition element (Fig. S1A). To increase sensor robustness, eYFP was replaced by the pH- and chloride-tolerant Venus, producing FLIPglu-170n Δ 13V, FLIPglu-2 μ Δ 13V, FLIPglu-30 μ Δ 13V and in FLIPglu-600 μ Δ 13V [13]. All four sensors show a comparable ratio change of approximately -1.0 *in vitro* in response to glucose addition ([13], Fig. S1B), and assuming a linear detection range between 10 and 90% saturation, report glucose levels over six orders of magnitude ($\sim$ 20 nM to 6 mM; diagram in Fig. S1C).

Glucose accumulation potential of sugar-starved yeast cells

To measure cytosolic glucose levels, and to test whether yeast has the potential to accumulate glucose when grown in media lacking glucose, the FRET glucose nanosensors were expressed in yeast. To starve cells for glucose, cultures grown in SC^{glc} medium were transferred to SC^{eth} for 16 hours. Cells were then transferred to microtiter plates and the ratio of Venus/eCFP emission peak intensities were recorded in a microplate spectrofluorimeter before and after addition of glucose. Consistent with the potential of the starved cells to accumulate glucose, the cultures showed sustained concentration-dependent decreases in Venus/eCFP ratio indicating accumulation of cytosolic glucose after exposure to extracellular glucose (Fig. 1A). Since all four sensors differ in only one or two amino acids in the glucose binding pockets and show a similar maximal ratio change of -1.0 *in vitro* [21], all four sensors were expected to yield similar maximum ratio changes *in vivo*, provided that the basal glucose concentration is below the sensor saturation and that uptake is not limiting. Consistent with this prediction, the intermediate affinity sensors FLIPglu-2 μ Δ 13V (K_d 2 μ M) and FLIPglu-30 μ Δ 13V (K_d 28 μ M) gave maximal *in vivo* ratio changes of ~ -0.3 (note that *in vitro* and *in vivo* ratios are not comparable directly due to differences in the instrumentation; the maximal *in vitro* ratio change of -1.0 corresponds to -0.3 *in vivo*). In contrast, FLIPglu-170n Δ 13V and FLIPglu-600 μ Δ 13V showed reduced responses (Fig. 1B). The reduced response of FLIPglu-600 μ Δ 13V at high levels of added glucose is consistent with its maximum response being limited by saturation of the cell's uptake systems. The reduced response of FLIPglu-170n Δ 13V suggests that the sensor did not reach its full dynamic range because the sensor concentration exceeded ligand levels, or that before external addition of glucose a fraction of the sensor had bound glucose, e.g. due to low basal glucose levels. If we assume that sensor output is largely unaffected by the *in vivo* conditions, steady state cytosolic glucose levels in cells grown in media containing 2% ethanol are at or below $\sim$ 100 nM (assumed maximal ratio change of 0.3; observed 0.2 suggests partial saturation; Fig. 1B). Intracellular levels could be measured over the full range of the four sensors. The extracellular concentration was 16–46 $\times$ higher relative to intracellular level when calculated for half saturation (corresponding to the K_d of the respective sensor; Fig. S1D). The measured cytosolic steady state levels (at a given external concentration) are a function of the relative rates of uptake and hexokinase-mediated conversion into glucose-6-phosphate. Our data demonstrate that yeast either has the potential to take up glucose before addition of glucose ('ajar strategy'), or the uptake activity was induced within minutes after re-exposure to glucose ('intermediate or induction strategy').

The approach described above required removal of the microplate from the fluorimeter, creating a time lag between glucose addition and the first measurement. To analyze the

response in starved cells immediately after glucose addition, we injected glucose into the wells using a computer-controlled injector and recorded the response instantaneously, or assayed individual cells trapped in a microfluidic device. To increase the signal-to-noise ratio, and thus assay sensitivity, the high sensitivity FRET glucose nanosensor FLII¹²Pglu-700 μ δ 6 (K_d 700 μ M; note positive ratio change in Fig. 1C) was utilized. As expected from the large *in vitro* ratio change of FLII¹²Pglu-700 μ δ 6 [20], the maximal *in vivo* ratio change in the fluorimetric assay was positive and significantly larger (normalized ratio change *in vivo* $\sim +0.65$; Fig. 1C) compared to FLIPglu-600 μ Δ 13V (Fig. 1C). The specificity of the *in vivo* response of FLII¹²Pglu-700 μ δ 6 to glucose and galactose was confirmed in the wild type strain BY4743 using several compounds D-glucose, D-galactose, D-mannose, sucrose, fructose, trehalose, xylose, mannitol, D-maltose. Cytosolic glucose accumulation was detected in wild type cell populations within the first minute after injection and reached saturation within ~ 3 min (Fig. 2A, **squares**). For individual wild type cells trapped in the microfluidic platform, an initial response (increase in cytosolic glucose levels) was observed within <20 sec (Fig. 2B, **black line**). The response was linear over the first few 20 sec acquisition intervals and saturated within <1 min after glucose addition. Accumulation was reversible immediately after removal of glucose, probably caused by a combination of glucose efflux and conversion by hexokinase (Fig. 2B, **black line**), and an example of ratio changes caused by changes in cytosolic glucose is represented in Fig. 2C,D. The almost instantaneous ability of starved yeast cells to accumulate glucose even at low external supply strongly suggests that yeast expresses functional glucose transporters even in the absence of glucose, despite the fact that the production of an ‘unnecessary’ transporter requires energy (‘ajar pathway’).

To further verify that the sensors report actual changes in glucose, trapped cells were challenged with maltose. No response was observed upon addition of maltose to wild type yeast (Fig. 3A). A yeast strain lacking all 18 HXT hexose transporters showed no significant glucose accumulation (Fig. 3B), but responded to maltose, most probably due to a lack of HXT-mediated glucose export leading to net accumulation of glucose derived from maltose metabolism [as described by 29]. A mutant unable to phosphorylate cytosolic glucose (due to deletion of all three hexokinase genes) responded to much lower external glucose levels as expected for a condition in which glucose metabolism is reduced or absent (Fig. 3C,D).

Both the tests of specificity and the finding that the response range of the affinity mutants, which differ in only one or two amino acids, correlate with their K_d for glucose strongly support the hypothesis that the FRET changes are specific for glucose or galactose and thus provide a reliable measure of the change in cytosolic sugar levels. To test whether the response of the sensors is caused by changes in the cytosolic glucose concentration and not changes of the cytosolic pH, we tested the response of BY4743 cell cultures transformed with FLII¹²Pglu-700 μ δ 6 to glucose in media adjusted to different pH (Fig. S2). Single cells were also perfused with 25 mM MES buffer at different pH showed no significant change in the FRET sensor response (Fig. S3).

A high sensitivity FRET sensor for mutant screens

To identify the transporters responsible for the ability of starved yeast cells to accumulate glucose, mutants lacking individual members of the Hxt hexose transporter family (all 10 mutants from the stock collection; *hxt1 Δ* , *hxt2 Δ* , *hxt3 Δ* , *hxt4 Δ* , *hxt5 Δ* , *hxt8 Δ* , *hxt10 Δ* , *hxt12 Δ* , *hxt14 Δ* , *hxt17 Δ*) were analyzed using the FLII¹²Pglu-700 μ δ 6-based fluorimetric assay system. While most *hxt* mutants were unaffected with respect to glucose accumulation, deletion of *HXT5* led to a significant reduction in cytosolic glucose accumulation (Fig. 4A,B). Given the fact that *HXT5* is highly induced, e.g. during the transition from fermentative to glycerol-based respiratory growth [30] and its function as a hexose transporter, the simplest hypothesis is that *HXT5* is the dominant uptake system

under starvation conditions. The reduced capacity for glucose accumulation of the *hxt5Δ* strain was confirmed in real time glucose accumulation assays and single cell analyses (Fig. 2A,B **respectively, triangles and grey line**). A specific effect of ethanol on Hxt5p activity was excluded since a comparable reduction in uptake capacity was observed in *hxt5Δ* starved in medium lacking a carbon source (Fig. 4C,D). Thus Hxt5p appears not only to be induced as shown previously [17], but also to dominate glucose accumulation under various starvation conditions. When the *hxt5Δ* strain was exposed to low amounts of glucose (0.5 mM) before the measurement, no difference to wild type was observed (Fig. 4E,F), suggesting that low amounts of glucose induce other Hxts, which then mask the lack of Hxt5p-mediated glucose uptake activity. It is noteworthy that *hxt5Δ* does not completely lose its ability to accumulate glucose. Also, since the FRET sensors measure the relative rates of uptake and metabolism, the absolute cellular uptake capacity is most probably underestimated by the assay. Since starved *hxt5Δ* cells still show residual transport activity, other Hxts, e.g. those not in the *knock-out* collection or with redundant activities, must contribute during starvation. A few double mutants (*hxt3Δ/hxt5Δ* and *hxt6Δ/hxt7Δ*) were tested but showed no significant differences from the single *hxt5Δ* or wild type, respectively (data not shown). Since none of the expression profiles of the other *HXT* genes clustered with *HXT5* (data not shown), a systematic analysis of all single mutants and all *hxt5Δ/hxtxΔ*-double mutants will be required to identify the other contributors.

Increase of ATP levels after glucose resupply is limited in an *hxt5Δ* strain

To measure the accumulation of cytosolic ATP levels, the FRET ATP sensors AT1.03 (K_d 3.3 mM), AT1.03^{YEMK} (K_d 1.2 mM) and the control sensor AT1.03^{R122K/R126K} developed by the Merckx lab [16] were expressed in yeast BY4743 and the response to addition of glucose to starved cells was measured in parallel with cells expressing the glucose nanosensor FLII¹²Pglu-700μδ6. Yeast cells were grown in SC^{glc} and then transferred to SC^{-C} for 5 hours. Quantification of FRET from different sensors and culture replicates were performed by determining the ratio of Venus/eCFP emission peak intensities before and after addition of glucose in a microplate spectrofluorimeter. Glucose addition lead to a slow and concentration-dependent increase in the Venus/eCFP ratio in yeast cells transformed with AT1.03, AT1.03^{YEMK} after exposure to low levels of glucose (0.1 mM and 0.5 mM), and saturated within 7–10 min after exposure to concentrations greater than 2 mM (2 mM, 5 mM and 10 mM) (Fig. 5A,B). The negative control sensor AT1.03^{R122K/R126K} did not show a significant response after glucose addition (Fig. 5C). Yeast cells lacking *HXT5* were analyzed in parallel. The non-saturating glucose concentration of 0.5 mM resulted in a reduced ratio change in *hxt5Δ* compared to wild type for both ATP sensors AT1.03 and AT1.03^{YEMK} (Fig. 5E,F). Thus not unexpectedly, the reduced ability of *hxt5Δ* to accumulate glucose limits the availability of cytosolic ATP. For comparison, glucose accumulation was measured in parallel after glucose exposure to same external glucose concentrations for both wild type and *hxt5Δ* (Fig. 5D,H). Using the formula $[S] = K_d \times (r - r_{min}) / (r_{max} - r)$ [11], we calculated the glucose cytosolic concentrations after perfusion of the different external concentrations for FLII¹²Pglu-700μδ6 in yeast. The cytosolic glucose concentration in wild type cells was estimated to 228 μM when cells were exposed to 0.5 mM glucose outside the cells whereas *hxt5Δ* cells contained 158 μM under the same conditions. The estimates of the cytosolic glc levels are based on the assumption that r_{min} and r_{max} correspond to the apo and saturated forms of the sensor and that the *in vivo* environment has not affected the K_d of the sensors. While the use of a wide spectrum of sensors with differing affinity suggests that at least the assumption for r_{min} and r_{max} are adequate for the glucose measurements, we did not calculate the cytosolic ATP levels since r_{max} has probably not been reached, thus making an estimate highly unreliable. Independent *in vivo* calibration of the sensors will be necessary before they can be used to reliably quantify cellular ATP levels. It is however obvious that cytosolic ATP levels correlate with increased cytosolic glc levels both in a time-dependent

manner, but that ATP levels lag significantly behind the glucose accumulation. The implementation of ATP sensors in yeast will provide a fast assay for changes in the energy status of yeast cells under varying growth conditions, e.g. oxygen levels. Furthermore the FRET sensors can be targeted to mitochondria, as has successfully been done in mammalian cells to compare cytosolic and mitochondrial ATP levels with striking results (lower mitochondrial ATP levels compared to the cytosol) [16]. Moreover, expressing ATP sensors in yeast mutants would help elucidating the function of putative energy-related genes and the participation of glycolysis and the TCA cycle to overall cellular energy status in different growth conditions.

A specific role of Hxt5p during early growth phase and delayed growth during transition to glucose

The *hxt5Δ* mutant had been reported to grow slightly slower compared to wild type after glucose addition to glucose-depleted cells [17]. Also under the conditions used here for starvation, i.e. transfer from ethanol to glucose medium, *hxt5Δ* showed delayed half-saturation (567 ± 65 min) compared to wild type (405 ± 63 min). Growth curves of microorganisms show a lag phase, controlled by cell cycle checkpoints that determine the energy status; only when sufficient nutrients are available cell division proceeds. *HXT5* transcript and protein levels are low during exponential growth on fermentable carbon sources [17]. Hxt5p protein levels increase during carbon source depletion [17] and decrease after glucose re-addition [31]. To confirm whether Hxt5p localizes to internal membranes, or is present at the plasma membrane under the specific starvation conditions used in this study, Hxt5p-GFP was localized by confocal microscopy (Fig. S4A). Hxt5p-GFP was detected mainly at the plasma membrane. Within 60 minutes after resupply, plasma membrane levels of Hxt5p decreased and intracellular levels increased. Within <5 hours Hxt5p levels dropped to almost undetectable levels (Fig. S4C,D), indicating that Hxt5p is specifically used for priming and is then rapidly endocytosed and a suite of other Hxts takes over [32].

Interestingly, overexpression of *HXT5* in the hexose uptake-deficient yeast strain *EBY4000* [lacking *GAL2* and all the *HXT*s transporters; 33] using a strong *PMA1* promoter fragment in the pDRf1GW-ura3 plasmid [25], lead to a greatly increased transformation efficiency if the cells were incubated for three hours in ethanol, glucose or maltose (Figure S5B,C,D) compared to direct plating after transformation (Figure S5A) on selective media plates containing glucose as the sole C source. No recovery was needed when transformation reactions were plated on maltose plates (Figure S5F), consistent with the presence of maltose transporters in *EBY4000*. These findings suggest the need for the presence of an uptake system for immediate glucose accumulation and limited availability of energy for Hxt5p synthesis during early stages of recovery from starvation or transformation.

The presence of Hxt5p in the absence of external glucose appears counterintuitive, since its production requires energy, which is limiting under starvation conditions, and since the transporter is 'inactive' since no substrate is available. Such an expense is only useful if the transporter provides a competitive advantage, e.g. if it allows the cells to take up glucose immediately after new resources become available. Cells with a preformed metabolic pathway can import glucose immediately as energy source. The earlier checkpoint requirements are fulfilled, the earlier the organism can divide and grow. In its natural habitat yeast experiences cycles of extreme sugar supply and subsequent exhaustion. Yeast cells typically are transferred from carbon-poor environments such as soil to new habitats rich in nutrients such as grapes or fruits. *Saccharomyces* is successful in outpacing other microbes also present on a grape or fruit [34].

In summary, we developed a set of simple assays for dynamic analysis of cytosolic glucose and ATP levels using optical sensors. The use of the sensors allowed exploration of how glucose-starved yeast prepare for future exposure to sugar. FRET sensors allow nonradioactive, simple, cost-effective and rapid analysis of steady state levels using either fluorimetric assays or FRET imaging of cells trapped in microfluidic devices. The sensors are genetically encoded and can thus be targeted to subcellular compartments such as organelles to provide for the first time information on subcellular levels using a minimally invasive approach [15]. Further analysis using this technology is expected to contribute to a better understanding of the signaling pathways that lead to induction of Hxt5p activity. Moreover, the FRET sensors can be implemented in fermentor technology to monitor the physiological state of the culture in real time in industrial settings [35].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbrev

FLIP	fluorescent sensor protein
FRET	Förster resonance energy transfer
glc	glucose
ATP	adenosine triphosphate
Hxt	hexose transporter
EtOH	ethanol

Acknowledgments

We are very grateful to Maarten Merckx for providing the ATP FRET sensors. We would like to especially thank Johan Thevelein for critical reading of the manuscript. This work was made possible by grants to WBF from NIH (NIDDK; 1RO1DK079109).

REFERENCES

1. Kruckeberg AL. The hexose transporter family of *Saccharomyces cerevisiae*. Arch Microbiol 1996;166:283–292. [PubMed: 8929273]
2. Reifemberger E, Freidel K, Ciriacy M. Identification of novel HXT genes in *Saccharomyces cerevisiae* reveals the impact of individual hexose transporters on glycolytic flux. Mol Microbiol 1995;16:157–167. [PubMed: 7651133]
3. Diderich JA, Teusink B, Valkier J, Anjos J, Spencer-Martins I, van Dam K, Walsh MC. Strategies to determine the extent of control exerted by glucose transport on glycolytic flux in the yeast *Saccharomyces bayanus*. Microbiol 1999;145:3447–3454.
4. Rodrigues, F.; Ludovico, P.; Leao, C. Sugar metabolism in yeasts: an overview of aerobic and anaerobic glucose catabolism. Berlin: Springer; 2006.
5. Freimoser FM, Hurlimann HC, Jakob CA, Werner TP, Amrhein N. Systematic screening of polyphosphate (poly P) levels in yeast mutant cells reveals strong interdependence with primary metabolism. Genome Biol 2006;7:R109. [PubMed: 17107617]
6. Guimaraes PMR, Londesborough J. The adenylate energy charge and specific fermentation rate of brewer's yeasts fermenting high- and very high-gravity worts. Yeast 2008;25:47–58. [PubMed: 17944006]
7. Thevelein JM, Beullens M, Honshoven F, Hoebeeck G, Detremmerie K, Griewel B, den Hollander JA, Jans AW. Regulation of the cAMP level in the yeast *Saccharomyces cerevisiae*: the glucose-

induced cAMP signal is not mediated by a transient drop in the intracellular pH. *J. Gen. Microbiol* 1987;133:2197–2205. [PubMed: 2832519]

8. Frommer WB, Davidson MW, Campbell RE. Genetically encoded biosensors based on engineered fluorescent proteins. *Chem. Soc. Rev* 2009;38:2833–2841. [PubMed: 19771330]
9. Okumoto S, Takanaga H, Frommer WB. Quantitative imaging for discovery and assembly of the metabo-regulome. *New Phytol* 2008;180:271–295. [PubMed: 19138219]
10. Fehr M, Frommer WB, Lalonde S. Visualization of maltose uptake in living yeast cells by fluorescent nanosensors. *Proc. Natl. Acad. Sci. USA* 2002;99:9846–9851. [PubMed: 12097642]
11. Fehr M, Lalonde S, Lager I, Wolff MW, Frommer WB. *In vivo* imaging of the dynamics of glucose uptake in the cytosol of COS-7 cells by fluorescent nanosensors. *J. Biol. Chem* 2003;278:19127–19133. [PubMed: 12649277]
12. Lager I, Fehr M, Frommer WB, Lalonde S. Development of a fluorescent nanosensor for ribose. *FEBS Lett* 2003;553:85–89. [PubMed: 14550551]
13. Deuschle K, Okumoto S, Fehr M, Looger LL, Kozhukh L, Frommer WB. Construction and optimization of a family of genetically encoded metabolite sensors by semirational protein engineering. *Protein Sci* 2005;14:2304–2314. [PubMed: 16131659]
14. Fehr M, Ehrhardt DW, Lalonde S, Frommer WB. Live imaging of glucose homeostasis in nuclei of COS-7 cells. *J. Fluoresc* 2004;14:603–609. [PubMed: 15617267]
15. Takanaga H, Frommer WB. Facilitative plasma membrane transporters function during ER transit. *FASEB J.* 2010 in press.
16. Imamura H, Nhat KP, Togawa H, Saito K, Iino R, Kato-Yamada Y, Nagai T, Noji H. Visualization of ATP levels inside single living cells with fluorescence resonance energy transfer-based genetically encoded indicators. *Proc Natl Acad Sci U S A* 2009;106:15651–15656. [PubMed: 19720993]
17. Diderich JA, Schuurmans JM, Van Gaalen MC, Kruckeberg AL, Van Dam K. Functional analysis of the hexose transporter homologue *HXT5* in *Saccharomyces cerevisiae*. *Yeast* 2001;18:1515–1524. [PubMed: 11748728]
18. Verwaal R, Arako M, Kapur R, Verkleij AJ, Verrips CT, Boonstra J. *HXT5* expression is under control of STRE and HAP elements in the *HXT5* promoter. *Yeast* 2004;21:747–757. [PubMed: 15282798]
19. Verwaal R, Paalman JW, Hogenkamp A, Verkleij AJ, Verrips CT, Boonstra J. *HXT5* expression is determined by growth rates in *Saccharomyces cerevisiae*. *Yeast* 2002;19:1029–1038. [PubMed: 12210898]
20. Takanaga H, Chaudhuri B, Frommer WB. GLUT1 and GLUT9 as major contributors to glucose influx in HepG2 cells identified by a high sensitivity intramolecular FRET glucose sensor. *Biochim. Biophys. Acta* 2008;1778:1091–1099. [PubMed: 18177733]
21. Deuschle K, Chaudhuri B, Okumoto S, Lager I, Lalonde S, Frommer WB. Rapid metabolism of glucose detected with FRET glucose nanosensors in epidermal cells and intact roots of Arabidopsis RNA-silencing mutants. *Plant Cell* 2006;18:2314–2325. [PubMed: 16935985]
22. Nagai T, Ibata K, Park ES, Kubota M, Mikoshiba K, Miyawaki A. A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. *Nature Biotech* 2002;20:87–90.
23. Loqué D, Lalonde S, Looger LL, von Wiren N, Frommer WB. A cytosolic trans-activation domain essential for ammonium uptake. *Nature* 2007;446:195–198. [PubMed: 17293878]
24. Kunkel TA, Roberts JD, Zakour RA. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol* 1987;154:367–382. [PubMed: 3323813]
25. Hamacher T, Becker J, Gardonyi M, Hahn-Hagerdal B, Boles E. Characterization of the xylose-transporting properties of yeast hexose transporters and their influence on xylose utilization. *Microbiol* 2002;148:2783–2788.
26. Sherman F. Getting started with yeast. *Methods Enzymol* 1991;194:3–21. [PubMed: 2005794]
27. Loqué D, Lalonde S, Looger LL, von Wirén N, Frommer WB. A cytosolic trans-activation domain essential for ammonium uptake. *Nature* 2007;446:195–198. [PubMed: 17293878]

28. Teusink B, Diderich JA, Westerhoff HV, van Dam K, Walsh MC. Intracellular glucose concentration in derepressed yeast cells consuming glucose is high enough to reduce the glucose transport rate by 50%. *J. Bacteriol* 1998;180:556–562. [PubMed: 9457857]
29. Jansen ML, De Winde JH, Pronk JT. Hxt-carrier-mediated glucose efflux upon exposure of *Saccharomyces cerevisiae* to excess maltose. *Appl. Environ. Microbiol* 2002;68:4259–4265.
30. Roberts GG, Hudson AP. Transcriptome profiling of *Saccharomyces cerevisiae* during a transition from fermentative to glycerol-based respiratory growth reveals extensive metabolic and structural remodeling. *Mol. Genet. Genomics* 2006;276:170–186. [PubMed: 16741729]
31. van Suylekom D, van Donselaar E, Blanchetot C, Do Ngoc LN, Humbel BM, Boonstra J. Degradation of the hexose transporter Hxt5p in *Saccharomyces cerevisiae*. *Biol. Cell* 2007;99:13–23. [PubMed: 16928197]
32. Zaman S, Lippman SI, Schnepfer L, Slonim N, Broach JR. Glucose regulates transcription in yeast through a network of signaling pathways. *Mol Syst Biol* 2009;5:245. [PubMed: 19225458]
33. Wiczorke R, Krampe S, Weierstall T, Freidel K, Hollenberg CP, Boles E. Concurrent knock-out of at least 20 transporter genes is required to block uptake of hexoses in *Saccharomyces cerevisiae*. *FEBS Lett* 1999;464:123–128. [PubMed: 10618490]
34. Fleet GH. Wine yeasts for the future. *FEMS Yeast Research* 2008;8:979–995. [PubMed: 18793201]
35. Kaper T, Lager I, Looger LL, Chermak D, Frommer WB. Fluorescence resonance energy transfer sensors for quantitative monitoring of pentose and disaccharide accumulation in bacteria. *Biotechnol Biofuels* 2008;1:11. [PubMed: 18522753]
36. Rande-Gil F, Herrero P, Sanz P, Prieto JA, Moreno F. Hexokinase PII has a double cytosolic-nuclear localisation in *Saccharomyces cerevisiae*. *FEBS Lett* 1998;425:475–478. [PubMed: 9563516]

Figure 1.

In vivo FRET measurements. (A) Glucose-dose response curves FLIPglu-170nΔ13V in the cytosol of yeast. Cells expressing FLIPglu-170nΔ13V were starved, washed and transferred to microplates. The 528/485 nm ratio was measured fluorimetrically to obtain a baseline, glucose was added and the ratio change was determined (n=3). Normalized emission ratio changes upon addition of glucose are shown. (B) *In vivo* measurements for FLIPglu-170nΔ13V, FLIPglu-2uΔ13V, FLIPglu-30μΔ13V, FLIPglu-600μΔ13V (cf. Fig. 1A). Arrows indicate partial saturation of FLIPglu-170nΔ13V by base glucose levels, or partial saturation of FLIPglu-600μΔ13V due to saturation of uptake systems. (C) Schematic representation of relative responses of FLIPglu-600μΔ13V and FLII¹²Pglu-700μδ6. (D) Relative responses of FLII¹²Pglu-700μδ6 to different sugar analogs at a concentration of 10 mM: glc, glucose; gal, galactose; man, mannose; suc, sucrose; frc, fructose; tre, trehalose; xyl, xylose; mtl, mannitol; mal, maltose; ctrl, control (no sugar added).

Figure 2.

In vivo kinetics of glucose accumulation. **(A)** Real time glucose accumulation in wild type cells (blue) and in the *hxt5Δ* mutant (orange) expressing FLII¹²Pglu-700μδ6. Cells were grown in SC^{glc}, glucose-starved in SC^{eth}, resuspended in MES buffer and perfused with MES buffer (diamonds), or glucose in MES buffer (50 mM, square and triangles). Error bars correspond to SD (n=3). Glucose addition is indicated with a grey bar. **(B)** *In vivo* measurement of FRET responses in wild type and *hxt5Δ* cells expressing FLII¹²Pglu-700μδ6 trapped in a microfluidic platform. Cells were grown in glucose, glucose-starved in SC^{eth}, trapped in microfluidic device, washed with MES buffer and exposed to 4 min square pulses of glucose with increasing concentrations (blue shading). Error bars represent SD (n=30 cells). **(C, D)** Ratiometric images of individual yeast cells trapped in the microfluidic device in false color before (C) and after addition of 100 mM glucose (D).

Figure 3.

Glucose accumulation in single cells in wild type, glucose uptake- and hexokinase-deficient yeast strains. **(A)** FRET responses in a microfluidic chamber (normalized Venus/eCFP emission ratio at 535 nm over 470 nm) for wild type cells expressing FLIPglu-30 μ Δ 13V. **(B)** FRET response of the isogenic mutant lacking all 18 HXTs (*EBY.VW.4000*; [33]). **(C)** FRET responses to glucose measured in a microfluidic chamber (normalized Venus/ eCFP emission ratio) of wild type (*ENY.WA-1A*) cells expressing FLIPglu-30 μ Δ 13V and **(D)** in the isogenic triple hexokinase mutant (*WAY.78-1*) [36].

Figure 4.

Glucose accumulation in wild type and *hxt5Δ* cells. Yeast cells expressing FLII¹²Pglu-700μδ6 were grown in SC^{glc}, glucose-starved, washed and transferred to microplates. Glucose was added after time point #2 (grey bar) at the indicated concentrations 0–100 mM, and time-dependent glucose responses were analyzed in wild type (left panel: A,C,E) and *hxt5Δ* (right panel: B,D,F). (A,B) starved for 16 hrs in ethanol; (C,D) starved for 5 hrs in medium lacking a carbon source; (E,F) grown in the medium containing 0.5 mM glucose. After addition of glucose to the cells, fluorescence intensities in CFP and YFP channels were measured for 8 additional cycles (each cycle ~100 sec). The maximum absolute ratio change in wild type cells was +0.65. Red labeled glc on the top left

of the panels indicates the ligand being measured by the FRET sensor. Error bars represent SD (n=6 transformants).

Figure 5.

Reduced cytosolic glucose accumulation in *hxt5Δ* mutants correlates with reduced cytosolic ATP accumulation. (A–H) Yeast cells expressing either FLII¹²Pglu-700μδ6 (A, E), AT1.03 (B, F), AT1.03^{YEMK} (C, G) or AT1.03^{R122K/R126K} (D, H) were glucose-starved for 5 hours in SC^{-C}, washed and transferred to microplates. Glucose was added after time point #2 at the indicated concentrations of 0–10 mM, and time-dependent glucose responses were analyzed in wild type and *hxt5Δ*. After glucose addition, fluorescence intensities for CFP and YFP channels were measured for 8 additional cycles (each cycle ~100 sec). Red labels for glc and ATP indicate the ligand being measured using FRET sensors. Error bars

represent SD (n=6 transformants). X-axis is broken to indicate interruption of the time course during glucose addition (grey bar).