Video Article

Measurement of Vacuolar and Cytosolic pH *In Vivo* in Yeast Cell Suspensions

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Abstract

Vacuolar and cytosolic pH are highly regulated in yeast cells and occupy a central role in overall pH homeostasis. We describe protocols for ratiometric measurement of pH *in vivo* using pH-sensitive fluorophores localized to the vacuole or cytosol. Vacuolar pH is measured using BCECF, which localizes to the vacuole in yeast when introduced into cells in its acetoxymethyl ester form. Cytosolic pH is measured with a pH-sensitive GFP expressed under control of a yeast promoter, yeast pHluorin. Methods for measurement of fluorescence ratios in yeast cell suspensions in a fluorimeter are described. Through these protocols, single time point measurements of pH under different conditions or in different yeast mutants have been compared and changes in pH over time have been monitored. These methods have also been adapted to a fluorescence plate reader format for high-throughput experiments. Advantages of ratiometric pH measurements over other approaches currently in use, potential experimental problems and solutions, and prospects for future use of these techniques are also described.

Video Link

The video component of this article can be found at https://www.jove.com/video/50261/

Introduction

pH homeostasis is a dynamic and highly regulated process in all organisms [1,2]. Biochemical processes are tightly regulated by pH, and intracellular environments are tuned to narrow pH ranges to allow optimal activity of the resident enzymes. However, intracellular pH homeostasis can be challenged by rapid changes in environmental pH, metabolic shifts, and certain signaling pathways. In addition, intracellular pH can itself serve as an important signal. Finally, many organelles maintain lumenal pH values that are distinct from the surrounding cytosol and essential for organelle-specific functions.

The yeast *Saccharomyces cerevisiae* shares a number of pH homeostasis mechanisms with higher eukaryotes [2]. In the acidic organelles of the endocytic/lysosomal pathway, pH is primarily controlled by the highly conserved vacuolar proton-translocating ATPase (V-ATPase), acting in tandem with many exchangers dependent on the pH gradient. All eukaryotic cells also have proton export mechanisms. In fungi and plants, a second, distinct proton pump at the plasma membrane, Pma1, exports metabolic protons and is believed to be the major determinant of cytosolic pH and plasma membrane potential. The genetic flexibility of *S. cerevisiae* and its commercial importance, have made it a very interesting and important model for studying pH homeostasis [2].

In addition to being the primary drivers of organelle acidification, V-ATPases are highly regulated enzymes and our lab is interested in understanding mechanisms of V-ATPase regulation. Towards this goal, we have been using *in vivo* pH measurements of vacuolar and cytosolic pH: 1) to monitor responses to changing extracellular conditions, such as glucose deprivation and readdition, 2) to examine the effects of mutations that compromise V-ATPase activity, and 3) to explore the coordination of organelle and plasma membrane proton pumps [3-5]. These experiments only became possible through the development of robust ratiometric pH indicators amenable to use in yeast cells. Plant *et al.* first showed that BCECF (2'7'-Bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein), which has been used widely to measure cytosolic pH in mammalian cells, accumulates in the yeast vacuole instead of the cytosol [6]. This difference in BCECF localization has been attributed to the many hydrolytic enzymes in the vacuole, which are likely responsible for cleavage of the acetoxy methyl ester from BCECF-AM (acetoxymethyl ester of BCECF) and vacuolar retention [6]. Ali *et al.* [7] further developed vacuolar pH measurement using BCECF and adapted these measurements to a fluorescence plate reader format. Brett *et al.* introduced yeast pHluorin as a means of measuring cytosolic pH in yeast by expressing a plasmid-borne ratiometric pH-sensitive GFP [8] under control of a yeast-specific promoter [9].

The excitation spectra of both BCECF and yeast pHluorin are sensitive to pH, so they are used as ratiometric pH indicators in which the ratio of fluorescence at two excitation wavelengths, measured at a single emission wavelength, provides a measure of pH [8,10]. These yeast vacuolar and cytosolic pH sensors have been used for both single-cell and population-based measurements. Single-cell measurements [6,11] are performed by fluorescence microscopy and image analysis. Vacuolar or cytosolic fluorescence at the two wavelengths is measured for each cell. The population-based measurements are performed in either a microplate reader with appropriate fluorescence capabilities or in a fluorimeter. We have generally done our measurements in a fluorimeter, because it provides easy access for addition of components such as glucose during

continuous kinetic measurements. Our current lab protocols for measurement of vacuolar and cytosolic pH are listed below; both are also easily adapted to microplate assays.

Protocol

1. Measurement of Vacuolar pH *In Vivo* Using BCECF-AM

1. Grow a 50 ml liquid culture of the yeast strain to be measured in the desired medium overnight. The goal is to have cells in mid-log phase (OD600 (optical density at 600 nm) measurement of approximately 0.8 for the suspension).
2. Pellet the yeast cells by centrifugation. Resuspend the pellet in 0.6 ml of the growth medium and transfer to a microcentrifuge tube that has been weighed previously. Pellet the cells again in a microcentrifuge at 2,000 x g for 60 sec. Remove the supernatant as completely as possible and then weigh the cell pellet. Resuspend the pellet to a final density of 0.5 g/ml (w/v); a culture of this volume will give a cell pellet of approximately 200 mg, and 200 µl of buffer would be added to give a final volume of 400 µl.
3. Add BCECF-AM to the cell suspension at a final concentration of 50 mM from a 12 mM stock prepared in DMSO. Mix well and then incubate the cells at 30 °C for 30 min. on a rocking platform or roller drum.
4. While the cells are incubating, prepare calibration buffers. The calibration buffer contains 50 mM MES (2-(N-morpholino)ethanesulfonic acid), 50 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 50 mM KCl, 50 mM NaCl, 0.2 M ammonium acetate, 10 mM sodium azide, and 10 mM 2-deoxyglucose, and is adjusted to the pH values appropriate for your calibration range with NaOH or HCl. (Caution: Sodium azide is highly toxic and should be handled with care.) For measurement of pH in wild-type cells, we would prepare several calibration mixtures at pH 5.5 to 6.5, but for mutants expected to have a more alkaline vacuolar pH (vma mutants), we would prepare additional, higher pH buffers.
5. Aliquot 2 ml of calibration buffer for each pH into 15 ml conical tubes, and add monensin (15 mM stock) and nigericin (2 mM stock) to give final concentrations of 110 µM monensin and 15 µM nigericin, respectively. Mix well on a vortex mixer. (Caution: Both monensin and nigericin are toxic and should be handled with care.)
6. When the BCECF-AM incubation time is completed, pellet the cell suspension by centrifugation for 30 sec at 2,000 x g in a microcentrifuge. Resuspend the cells is 1 ml of growth medium lacking glucose and centrifuge as above. Repeat this wash step once, and then resuspend the pellet in 200 µl of growth medium without glucose. Place on ice.
7. Add 20 µl of the cell suspension to each pH calibration tube prepared above. Incubate at 30 °C for 30-60 min. on a roller drum.
8. Set the fluorimeter to alternately measure at excitation wavelengths 450 nm and 490 nM, both with an emission wavelength of 535 nm. Set the sample chamber temperature to 30 °C. Perform all measurements with continuous stirring of the mixture in the cuvette.
9. Add 1.96 ml of 1 mM MES (adjusted to pH 5 or 7 depending on the pH of the cells' growth medium and the experimental design). Add 20 µl of cell suspension into the cuvette. Initiate fluorescence measurements. We collect both continuous kinetic and time-point data in different experiments. For continuous kinetic data, we take measurements every 6 sec for 5 min, then add glucose to a final glucose concentration of 50 mM, and continue measurement for 5-10 min. For single time-point measurements, we generally take measurements at 1 and 5 min. after addition of cells to the cuvette, add glucose as in the kinetic measurements, and then take another measurement 5 min. after glucose addition. These additions can be varied, and additional components (inhibitors, etc) can also be added.
10. After the experimental measurements are complete, remove the calibration tubes from the 30 °C incubation and transfer the entire 2 ml volume for each to the fluorimeter cuvette. Measure fluorescence at the same settings (see 1.8) every 5 sec over a total of 30 sec for each sample.
11. Export fluorescence data to Microsoft Excel. (For our fluorimeter, this requires export of data as text in tab-delimited form and import into Excel.) Obtain a calibration curve by calculating the ratio of fluorescence at 490 nm to 450 nm for each calibration mixture. The fluorescence ratio is then plotted vs. pH to get a calibration curve.
12. Convert the experimental data to fluorescence ratio and calculate pH using the standard curve. Vacuolar pH can then plotted against time (kinetics of pH changes) or compared under various conditions (**Figure 1**) or in different mutant strains.

2. Measurement of Cytosolic pH *In Vivo* Using Yeast pHluorin

1. Transform the desired yeast strain with the yeast pHluorin plasmid by standard protocols, and select for transformants on supplemented minimal medium lacking uracil (SC-uracil).
2. Grow a 50 ml liquid culture of transformed cells in SC-uracil to mid-log phase (OD_{600} = 0.8 or lower).
3. Prepare calibration standards as described for vacuolar pH, but buffer to a pH range appropriate for cytosolic pH measurements, generally pH 6-8. Aliquot 2 ml of calibration buffer adjusted to the desired pH into several 15 ml conical tubes. Add monensin and nigericin as described above (1.4) and mix well.
4. Harvest the cells by centrifugation as described above, and resuspend the pellet in 600 µl of the growth medium. Transfer to a weighed microcentrifuge tube, and pellet cells by centrifugation at 5,000 rpm for 30 sec. Resuspend the pellet in 1 ml of growth medium without glucose (SC-uracil, -glucose), pellet cells again and repeat. After the final centrifugation, remove the supernatant as thoroughly as possible and weigh the cell pellet. Resuspend cells to a final density of 0.5 g/ml in SC-uracil with no glucose.
5. Add 20 µl of cell suspension to each tube of calibration buffer and mix well on a vortex mixer. Incubate at 30 °C on a spinning drum rotor for 60 min.
6. Set up the fluorimeter for excitation at wavelengths 405 and 485 nm and an emission wavelength of 508 nm. Proceed with single time point or kinetic measurements and addition of glucose as described above for BCECF measurement.
7. Measure fluorescence of calibration samples, and construct a calibration curve as for BCECF (see 1.10-1.11). A plot of fluorescence ratio vs. pH is linear over the range of most cytosolic pH measurements (pH 6.0-8.0) [9], so experimental fluorescence ratio measurements are easily converted to pH.

Representative Results

Figure 1 presents vacuolar pH data obtained on wild-type yeast cells grown in rich medium (yeast extract-peptone-dextran; YEPD) buffered to pH 5 with 50 mM MES. We often grow the cells in buffered medium because the pH of the medium can change quite dramatically during overnight growth, particularly for minimal medium, and we have found that the pH of the growth medium can affect vacuolar pH responses [3]. However, it is also acceptable for many experiments to grow the cells in unbuffered medium. **Figure 1A** shows a calibration curve for cells incubated with BCECF-AM by the protocol above. Although the relationship between BCECF fluorescence ratio and vacuolar pH is non-linear over a wider pH range [7], it is nearly linear in the relevant range for these experimental measurements, and a linear trendline is shown and used to calculate pH values for the experiment. Vacuolar pH changes resulting from glucose addition to glucose-deprived cells are shown in **Figure 1B**. Vacuolar pH increases during BCECF-AM labeling because cells are glucose deprived, but decreases after glucose readdition, presumably as a result of V-ATPase activation through reassembly [3,4]. A slight increase in vacuolar pH is observed upon addition of 50 mM KCl, three minutes after the glucose addition. In a larger study, we typically run at least three independent experiments (biological replicates) and show the average pH ± S.E. for each condition [3-5].

The kinetics of cytosolic pH change with glucose addition are shown in **Figure 2**. For this experiment, wild-type yeast cells were grown in synthetic complete medium lacking uracil (SC-uracil), buffered to pH 5 with 50 mM MES. This medium is appropriate for maintenance of the plasmid containing yeast phluorin under control of the phosphoglycerate kinase (PGK) promoter, which we have used recently [3,5]; other plasmids may require different select conditions. The calibration curve (**Figure 2A**) shows that the ratiometric response of the pHluorin to pH is linear over a wide range of pH; generally covering any physiologically relevant cytosolic pH. The response of the wild-type cells shown here is very characteristic; there is an immediate decrease in pH followed by an increase to pH 7.2-7.4.

We construct calibration curves for each strain and in every experiment. We use an *in situ* calibration mixture described by Brett *et al.* [9] for both BCECF and pHluorin measurements. This mixture includes cell-permeant ammonium acetate capable of collapsing pH gradients across multiple membranes, sodium azide and deoxyglucose to halt ATP production and inhibit the H+-pumps, and monensin and nigericin, ionophores implicated in collapse of pH gradients in the yeast secretory/vacuolar transport pathways [12] and mitochondrial inner membrane [13], respectively. It is worth noting that we do not subtract a background value for unlabeled cells for the vacuolar or cytosolic pH measurements. Although the cells do have some intrinsic autofluorescence, we have directly compared pH measurements with and without background correction on the calibration and experimental samples and have seen no difference in the final values. Background subtraction will result in steeper calibration curves, and may be desirable under conditions where fluorescence signals are low and signal to noise ratio becomes a problem. For example, in measuring from a Golgi/endosome-localized pHluorin [5], which gives a lower overall signal, we did subtract the background signal from all measurements.

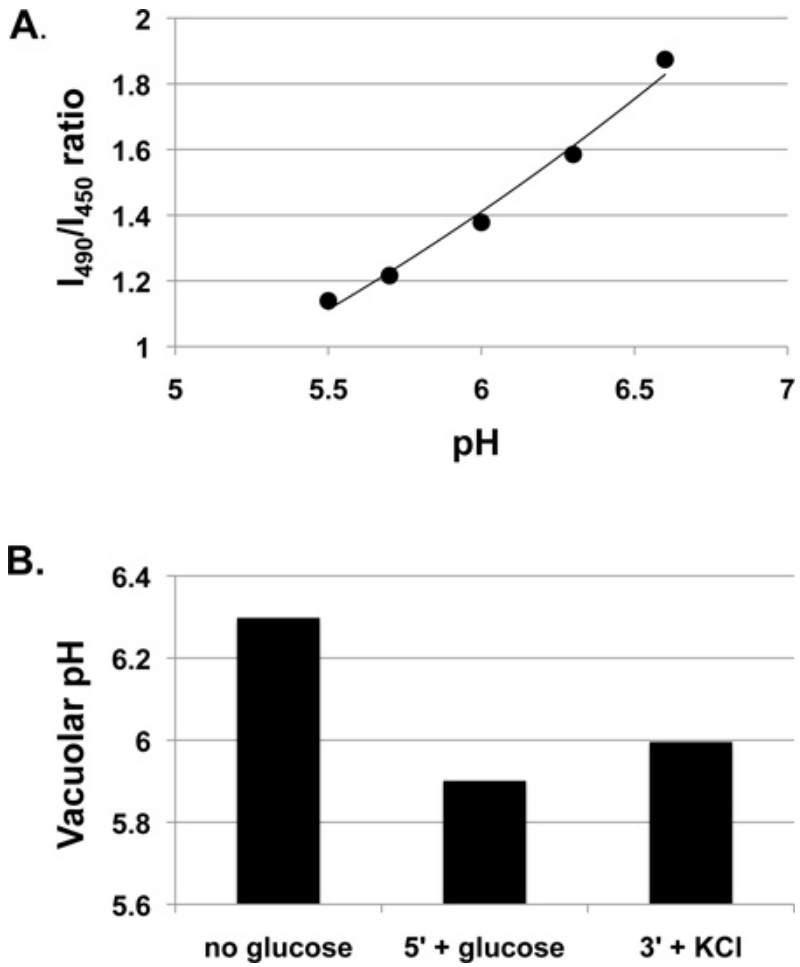

Figure 1. Measurement of vacuolar pH responses in wild-type cells grown in YEPD, pH 5. Wild-type (SF838-5Aα) cells were grown to mid-log phase and then incubated with BCECF-AM as described above. **A.** Calibration curve showing measured ratio of fluorescence intensity from excitation at 490 nm (I490) to intensity from excitation at 450 nm (I450) (both measured at an emission wavelength of 535 nm) at varied pH. A linear trendline is shown. **B.** Vacuolar pH after a brief glucose deprivation was measured in a portion of the same, labeled cell suspension (no glucose). Glucose was then added to a final concentration of 50 mM and fluorescence ratio was measured after 5 min (5' + glucose). 50 mM KCl was then added and fluorescence ratio was measured after 3 min and converted to pH (3'+ KCl).

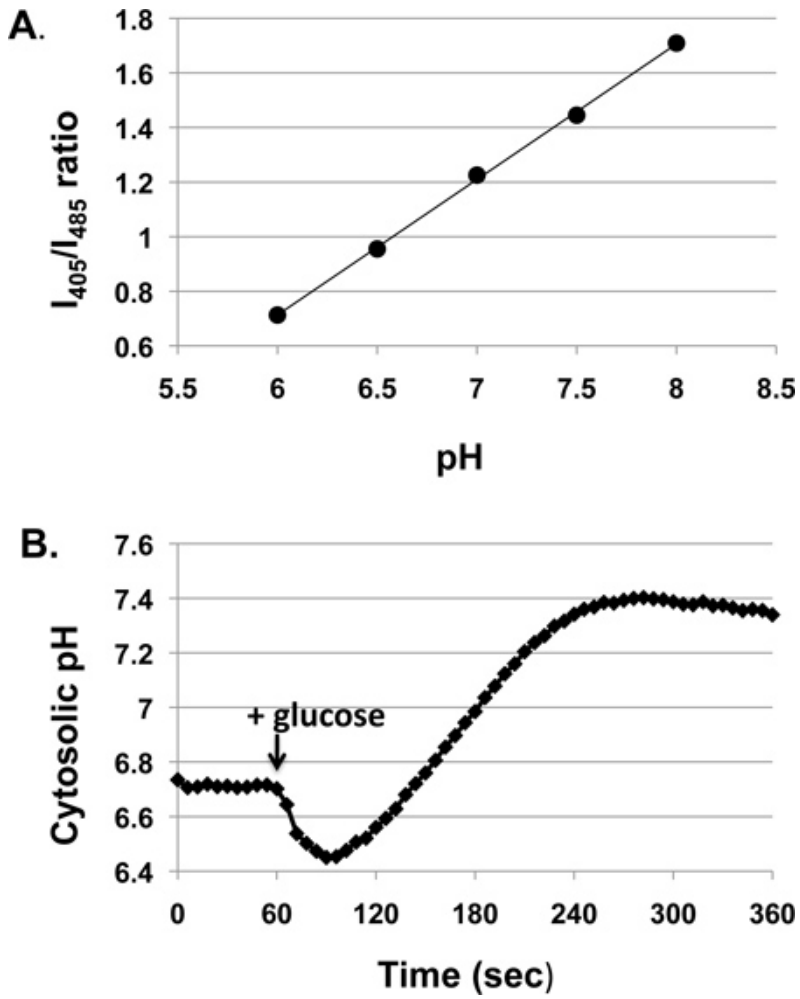

Figure 2. Kinetics of cytosolic pH response to glucose addition in wild-type cells. Wild-type cells were transformed with yeast pHluorin under control of the phosphoglycerate kinase (PGK) promoter and transformants grown as described above. **A.** Calibration curve showing measured ratio of fluorescence intensity at 405 nm (I405) to intensity at 485 nm (I485) vs. pH. **B.** Cytosolic pH was derived from fluorescence intensity measurements taken every 6 sec. over 6 min. Glucose (50 mM final) was added to the cells in the cuvette at the indicated time.

Discussion

We have utilized these protocols to address a number of aspects of pH homeostasis. For example, we have compared cytosolic and pH responses of wild-type and V-ATPase-deficient mutant cells [4,5]. We have also examined the effects of altered growth conditions, particularly extracellular pH, on vacuolar pH response to glucose [3]. Importantly, the responses we observe are both consistent with other methods of quantitative pH measurement and with biochemical data describing altered activities of the proton pumps.

The two most important features of the type of *in vivo* pH measurements described here are localization of the fluorophore and the level of signal; problems with either of these require modification of the method for the specific application or mutant. The vacuolar localization of BCECF is somewhat fortuitous [6], but is preserved in a number of different mutants, including the *vma* mutants [4], which have reduced levels of vacuolar hydrolases. Yeast vacuoles are readily visualized by microscopy under Nomarski optics, and we confirm vacuolar localization of the dye for each strain. pHluorin is more versatile as a pH sensor, particularly given its responsiveness over most of the physiological pH range (**Figure 2A**). The yeast pHluorin that we have used appears to be exclusively cytosolic, presumably because it lacks other targeting information. However, pHluorin has been targeted to the Golgi apparatus by tagging with sequences from the Golgi chloride transporter Gef1, along with an additional insertion of membrane segments from halorhodopsin to give proper topology (pH-Gef1; [14]). Orij *et al.* have successfully targeted pHluorin to the mitochondrial matrix and measured mitochondrial pH changes with metabolism [15]. These results suggest that properly designed fusion proteins may make it possible to monitor pH responses in many organelles. The signal to noise level of vacuolar BCECF is quite high in most of the yeast strains that we have examined. Signal from expressed pHluorin proteins can be manipulated via the promoter driving pHluorin expression. In the original cytosolic pHluorin construct [9], expression was driven by a heat shock element-containing promoter and was not very high. However, later expression constructs from the PGK promoter, *TEF1* promoter, and actin promoter appear to give higher levels of expression [3,15,16]. The Golgi-specific pHluorin pH-Gef1 is expressed from an inducible promoter [14]. Transient induction of expression may be particularly helpful for smaller compartments of the secretory and endocytic pathways, where sustained high level expression could lead to pHluorin mislocalization or even perturbations in compartment pH, manifest through slow growth or mislocalization of other proteins.

In addition to the characteristics of the fluorophores, it is important to recognize that *in vivo* pH measurements are sensitive to the growth and metabolic conditions of the yeast cells themselves. This sensitivity is physiologically relevant and potentially interesting, but can also be a source of variability between measurements. We take care to compare measurements from yeast cells in the same growth phase, usually early to mid logarithmic phase. Because vacuolar pH responses can be sensitive to extracellular pH [3], we monitor pH of the growth medium, particularly for cells grown in unbuffered medium. While it is clear that many different growth media are compatible with ratiometric pH measurement, differences in medium composition can definitely impact pH [17]. In addition, after cells are harvested and prepared for measurement, we have found that prolonged glucose deprivation (hours) not only decreases the initial cytosolic pH and increases the initial vacuolar pH, but also significantly slows responses to glucose. Therefore, we generally initiate experiments within 30 min. or less of beginning the glucose deprivation.

Accumulation of fluorescent lysosomotropic amines, such as quinacrine and acridine orange, has been used extensively to assess compartment acidification in living yeast cells. These methods are rapid and simple, but should be regarded as much more qualitative than ratiometric measurements of pH. In general, methods such as quinacrine uptake rely on membrane permeability of the basic form of the fluorophore, which become entrapped when it is protonated in an acidic compartment and can be visualized under a fluorescence microscope [18]. Accumulation of the dye relies on partitioning according to the pH gradient across the membrane, so uptake into acidic organelles can be weakened by either alkalinization of the organelle or acidification of the cytosol. These methods will remain useful, but should probably be interpreted as providing a relative level of acidification.

The applications of ratiometric fluorescent pH measurements in yeast continue to expand. Brett *et al.* recently measured vacuolar pH in the collection of over 4500 non-essential yeast deletion mutants and uncovered a number of novel mechanisms of pH regulation [17]. BCECF and pHluorin [19,20] have also been adapted to a fluorescence-activated cell sorting format to facilitate screening for new V-ATPase inhibitors. Dechant *et al.* [11] used microfluidics in combination with fluorescence microscopy to monitor V-ATPase assembly and cytosolic pH simultaneously through multiple cycles of glucose deprivation and readdition. These results suggest that ratiometric measurements of pH in yeast are sufficiently robust and versatile to be applied in many types of experiments.

Disclosures

The authors have no conflicts of interest to disclose.

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