

Multimode Metabolic Profiling of Stationary Phase *Saccharomyces Cerevisiae* Exposed to Short-Term Oxidative or Nutrient Stress



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Abstract

Due to the prevalent use of yeast as a model system, the choice of genomic, biochemical, and cell biology tools compatible with these cells is well-developed and wide-ranging. The ability to choose from a variety of assays that can combine optimal workflow productivity with relevant data outcomes can be limited by the need for multiple devices to perform different task and detection methods. This application note demonstrates a flexible and versatile instrument that supports a broad range of yeast studies, combining multiple imaging and detection modes with onboard environmental control and shaking. In this study, multiple assay formats were used to conduct a detailed characterization of oxidative and nutrient stress response in *Saccharomyces cerevisiae*.

Introduction

Yeast is a fungal microbial organism whose cellular processes are well conserved and frequently found similar to higher eukaryotic cells. These attributes, coupled with the tendency of yeast to grow rapidly and their amenability to genetic and molecular biological procedures, make yeast a common model system that continues to reveal new understanding and applications in cellular biology. The *Saccharomyces cerevisiae* yeast vacuole, for example, once thought to play only a terminal role in cellular waste digestion, is now understood to be a dynamic organelle that regulates a variety of cellular processes with a morphology that adapts to diverse extra- and intracellular stimuli (Figure 1). Investigations into the *S. cerevisiae* vacuole have provided data about cellular stress response; protein sorting, synthesis, transport, and trafficking; organelle acidification; ion homeostasis; and autophagy. Importantly, these functions have found equivalencies in mammalian cells and led to many practical applications, including bioengineering yeast-derived vacuoles as a potential drug delivery nanocarrier that could enhance targeted cancer therapy.¹⁻³

Vitality and viability describe two different, yet interdependent metabolic processes that can inform on the overall health of a yeast cell population. One definition of vitality is the fitness of the vacuole to absorb and assimilate nutrients and toxins to maintain the life of the cell, or the work of digestive metabolism. Viability is understood as a state of overall cellular homeostasis, largely maintained by the process of glycolysis that in yeast results in available adenosine triphosphate (ATP) produced either via aerobic respiration or fermentation pathways. While the fermentation process generates approximately 14-fold fewer molecules of ATP compared to aerobic respiration, this pathway can maintain yeast cell viability under oxygen or nutrient starved conditions.^{4,5}

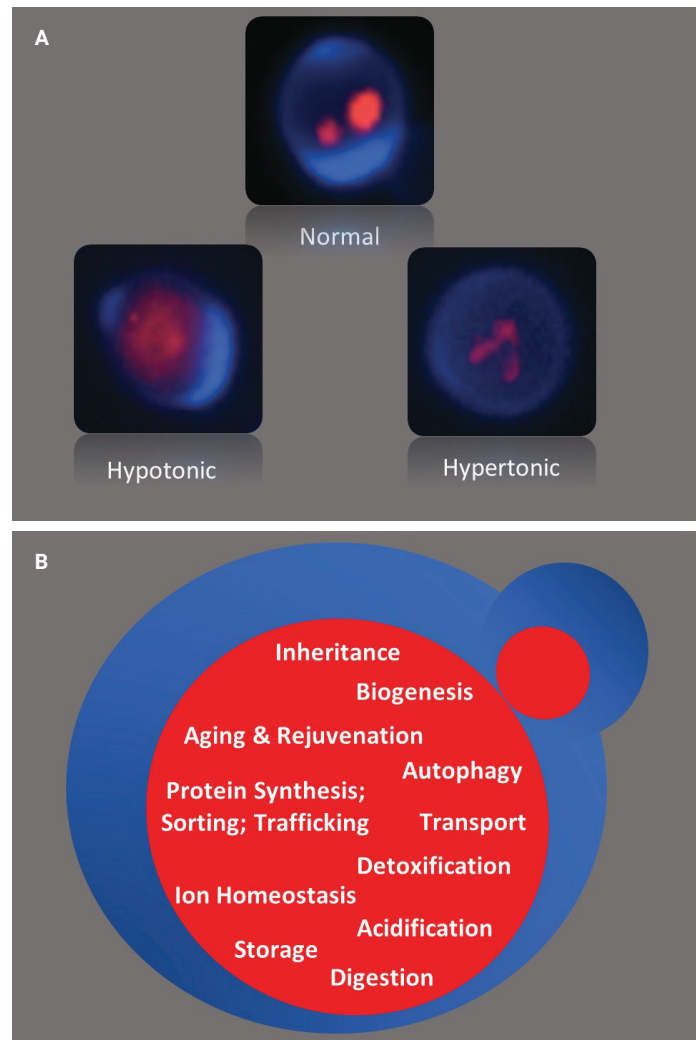


Figure 1. The yeast vacuole (red) is an organelle with a dynamic morphology (A) and diverse functional profile (B) that make it the target of a broad range of research and application areas in cell biology. Zoomed images (A) representative of vacuolar morphology described by Li and Kane 2009¹, taken using a 60x dry objective on the Agilent BioTek Cytation 5 cell imaging multimode reader.

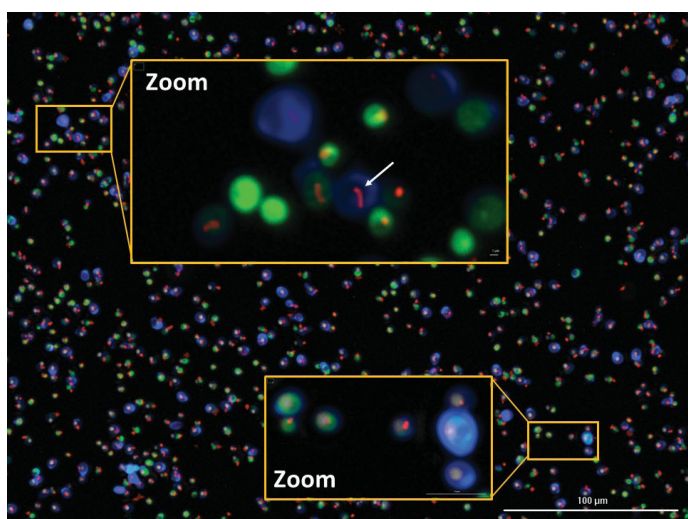


Figure 2. Principle of the staining and imaging assay. ViaVac Red/Green is a dual staining dye that changes from green to red when internalized from the cell cytoplasm to the yeast vacuole. A classic morphology resulting from dye conversion is the formation of cylindrical intravacuolar structures (CIVS), shown by the arrow. Blue fluorescence is cellulose and chitin in the yeast cell wall detected by binding of Calcofluor White (CW). High contrast brightfield imaging (dull white) was used to identify and enumerate total yeast cells that were then characterized for metabolic vitality based on cytoplasmic and vacuolar uptake of the ViaVac reagent. The high contrast channel is deactivated in the top Zoom to provide better visualization of CIVS.

Research into *S. cerevisiae* is often performed using cells in some stage of log phase, typically during exponential growth, when cell reproduction is rapid over a fairly short time frame. Stationary phase yeast, however, have various unique and complex characteristics that distinguish them from yeast cells in other growth phases, most notably a response to signals that result in proliferative arrest and the ability to retain viability in a quiescent state of starvation tolerance.⁶ Some population of stationary phase cells are also naturally senescent, indicating exhaustion or aging that make them vulnerable to acute changes in the environment resulting in increased viability pressure. To interrogate the relationship between ATP and yeast vacuole metabolism in *Saccharomyces cerevisiae* cell growth was monitored to stationary phase at OD600 over 36 hours. Cells were then either exposed to a lethal concentration of the reactive oxygen species hydrogen peroxide (H_2O_2) or to short-term nutrient/glucose starvation in a pH microenvironment buffered above acidic basal preferred by yeast. Vacuolar and glycolytic response to the effect of these treatments on yeast cells has been extensively described and includes triggering a complex network of mechanisms inducing, for example: adaptive stress responses to DNA, lipid, and protein damage; autophagy, apoptosis, and/or necrosis; and conservation of energy production.⁷⁻¹²

Vitality and viability of treated cells were then compared to untreated cells first by an imaging assay that was used to confirm functioning of the vacuole by a dual staining reagent. This membrane permeant dye complexes proteins and nucleic acids in the yeast cytoplasm and fluoresces green. In metabolically active cells the dye becomes internalized to the vacuole producing red fluorescence of intravacuolar structures (Figure 2). The structural patterning depends on the stage of conversion and sequestration of the stain, but one morphology uniquely characteristic of it has been defined as Cylindrical IntraVacuolar Structures (CIVS) indicating terminus of the reagent metabolism.¹³

A luciferase assay that could be performed on the cells directly following the imaging assay was then used to measure relative cell viability as indicated by units of ATP as a comparison to vacuolar metabolic vitality. According to the kit technical bulletin the luminescence assay principle depends on a proprietary thermostable luciferase and buffer reagent that works to extract ATP from microbial cells and generate a luminescent signal proportional to the amount of ATP present that, in turn, is directly proportional to the number of metabolically viable cells in culture.¹⁴

The workflow demonstrated in this study was enabled by the Agilent BioTek Cytation 5 cell imaging multimode reader controlled by Agilent BioTek Gen5 multimode reader and imager software that was used to perform the (1) yeast growth monitoring assay; (2) vitality assay stain mixing, incubation, and 4-channel fluorescence image acquisition and analysis; and (3) luminescence detection and quantitation of viability. As these methods are common in microbiology, a single instrument may prove beneficial compared to multiple pieces of equipment as a means to increase process efficacy and expand assay choices. The instrument features and protocols used by this application are presented by Figure 3.

Materials and methods

Yeast Growth Assay

Stock *Saccharomyces cerevisiae* kept at $\sim 4^{\circ}\text{C}$ was centrifuged and resuspended in 50 mL freshly prepared sterile filtered yeast extract peptone dextrose (YPD). Resuspended 1x bulk stock was dispensed to a Corning #3904 96-well microplate in 200 μL aliquots to wells A1 to D3 and E4 to H6, and in 220 μL aliquots to column 8. Two hundred microliters of YPD (starting pH 6.7) were dispensed to columns 9 to 11, then, starting with column 8, 20 μL were aspirated and dispensed to subsequent columns 9 to 11 to achieve 200 μL of a final 4-point 1:10 volume serial dilution in replicates of eight across columns 8 to 11 (20 μL were aspirated from column 11 at the end of the dilution). Remaining wells on the microplate were left empty. To maintain the same air liquid interface ratio as the aliquots dispensed to the microplate, 28 mL of the resuspended 1x bulk stock was dispensed to a 50 mL Erlenmeyer flask, covered, and placed on an orbital shaker set to 200 rpm and 30°C . In parallel, to monitor growth of the 1x bulk stock to stationary phase, the microplate was loaded and loaded onto an Agilent BioTek Cytation 5 set to a 36-hour kinetic run at 30°C with a 2 minute and 10 second interval of 206 cpm slow orbital shaking for 1 minute 30 seconds followed by a monochromator absorbance read at 600 nm. At the end of 36 hours pH of the bulk stock was determined using an Orion 3 Star benchtop meter (Thermo Fisher Scientific). To determine optimal cell density for the subsequent imaging assay 10 μL of bulk 1x stationary phase cells were diluted in 990 μL of sterile filtered deionized water, followed by three additional 1:10 point dilutions. 100 μL of each dilution was dispensed to a new microplate in quadruplicate and imaged and analyzed using high contrast brightfield (HCBF) and primary masking as described for the imaging assay below. Cell counts per mL were calculated as described elsewhere using the total HCBF cell count for each image.

Treatment and Staining Assay

Two 10 mL aliquots of 1x stationary phase yeast from the bulk growth stock were centrifuged. Supernatant was replaced by 10 mL 3% (w/v) hydrogen peroxide (H_2O_2) in deionized water to induce oxidative stress, or 10 mL phosphate buffered saline (PBS, pH 7.4 using the method described for the yeast stock) to induce nutrient and pH shock. The remaining 1x bulk stock was left untreated in the YPD growth microenvironment (pH 5.5). All three aliquots were returned to 30°C with 200 rpm orbital shaking for one hour. At the end of the hour the H_2O_2 treated aliquot was quenched with 2 mL PBS then all stock aliquots were

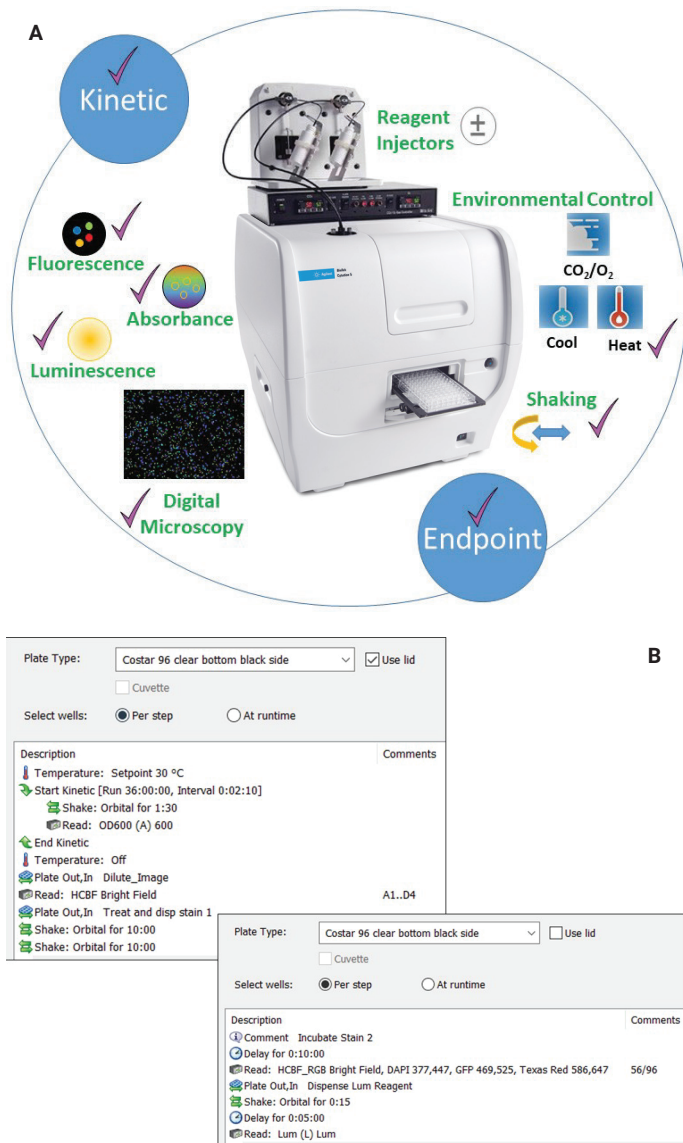


Figure 3. (A) The Agilent BioTek Cytation 5 cell imaging multimode reader has functionality that can enable microbiology workflow tasks typically requiring multiple pieces of equipment to be accomplished using only one instrument. (B) Protocols used in this application integrate Cytation 5 features highlighted with a purple check mark in (A). Options amenable to the procedure are shown with a (±) symbol.

transferred to 25 mL Eppendorf tubes and centrifuged. Supernatant from each aliquot was replaced with 10 mL PBS. Treated cells were diluted 1:1000 in 5 mL final PBS containing 5 μ M of 100x ViaVac Red/Green reagent (Biotium part number 29068), and untreated cells were diluted to a 4 point 1:10 dilution with a starting dilution of 1:100 in 2 mL PBS containing 5 μ M of 100x ViaVac Red/Green reagent (100 μ L ViaVac in 2 mL). Twelve 100 μ L replicates of H₂O₂ treated cells were dispensed to wells A1 to D3; twelve 100 μ L replicates of PBS treated cells were dispensed to wells E4 to H6; and 100 μ L each of a 4-point serial dilution curve of untreated cells were dispensed in quadruplicate to wells A8 to D11 of a 96-well microplate (Corning #3904). One hundred microliters of PBS only were dispensed to column 7, and 100 μ L of PBS containing 5 μ M of staining reagent were dispensed to column 12. Cells were incubated for 20 minutes using slow 205 cpm orbital shaking at ambient on the Agilent BioTek Cytation 5. Calcofluor White (Biotium part number 31062A) was diluted to 50x in sterile deionized water and 2 μ L (25 μ M final in the well) was dispensed to all assay wells except column 7. The plate was incubated for an additional 10 minutes at ambient on the Cytation 5 without shaking before imaging.

Imaging Assay

Stained cells were imaged with a 20x objective (part number 1220517) using a laser autofocus (part number 1225010) reference scan captured in brightfield with a high contrast (HCBF) annulus (part number 1320023) and auto exposure on well A8. Fluorescence imaging was acquired using filter configurations for blue (part number 1225100 and 1225007) to capture Calcofluor White staining of cellulose and chitin in the cell wall; green (part number 1225101 and 1225001) to capture ViaVac cytoplasmic uptake; and red (part numbers 1225102 and 1225002) to capture ViaVac vacuolar uptake. A ten slice z-stack was defined with a step size of 3 μ m to compensate for potential focal shift between channels of live cells during imaging. A final z-projection was created using brightfield (0 μ m offset); blue (+6 μ m); green (+9 μ m); and red (+12 μ m). Preprocessing of the z-projection included inverting HCBF images to a dark background with a 3 μ m rolling ball and fine results. The blue channel was background flattened with a rolling ball size of 8 μ m and fine results with 1 cycle of image smoothing. Auto background flattening was used in the green channel. A background flattening size of 2 μ m with fine results and 1 cycle of smoothing was applied to the red channel.

Image analysis consisted of a primary mask on HCBF objects using a detection threshold value of 8950 and size threshold

range of 1 to 5 μ m. HCBF analysis can result in constrained size of cells, therefore cell membrane staining (CW, blue) was used to determine an average cell size resulting in a secondary mask on all three fluorescent channels defined by expanding the primary mask 2.75 μ m with no thresholding, resulting in lower overall signal intensities but a larger area of total signal recognition. Subpopulation analysis was used to characterize and quantitate cells within one of five categories of metabolic vitality based on a staining profile of untreated cells defined by mean red and green signal cutoff values starting with indeterminate cells (those with low to no green or red signal). Cells per well in 100 μ L was calculated as described elsewhere using the total HCBF cell count for each image.¹⁵

Viability Assay (ATP)

The BacTiter-Glo Microbial Cell Viability Assay (Promega part number G8230) was performed according to the kit insert instructions. Following the imaging assay step above, a pause in the Agilent BioTek Cytation 5 protocol was integrated so that 100 μ L of luminescence reagent could be dispensed into all wells of the plate. The plate was returned to the instrument and shaken for 15 seconds using orbital shaking at 205 cpm, incubated for 5 minutes at room temperature (RT), then luminescence recorded using an excitation plug and emission hole at a gain of 221, integration time of 200 ms, and a read height of 4.50 mm. To reduce potential cross-talk and increase signal detection the clear bottom of the plate was covered with a mat of opaque white tape prior to the reagent dispense step. ATP of treated cells was normalized to untreated cells at 100% ATP as shown by Figure 4.

1. Obtain Relative Luminescence Units (RLU)/cell of untreated cells

$$\text{Average RLU/Average HCBF Cell Count} = \text{UnTx_RLU/cell}$$

$$\text{Example: } 162,500/132 = 1231$$

2. Multiply average HCBF cell count of Tx cells by UnTx_RLU/cell

$$\text{AverageTxCellCount} \times \text{UnTx_RLU/cell} = \text{Expected RLU}$$

$$\text{Example: } 178 \times 1231 = 219,118$$

3. Divide actual RLU by expected RLU and multiply by 100

$$\text{Actual RLU /Expected RLU} \times 100 = \% \text{ Viability}$$

(UnTx cells will equal 100% viability)

$$\text{Example: } 75,770/219,118 = .345795 \times 100 = 34.58\%$$

Figure 4. Equation sequence for normalizing relative luminescence units of ATP to cell count to measure percent cell viability.

Results and discussion

Results at the end of the yeast growth monitoring assay are shown by Figure 5, where compared to wells seeded at lower density, the 1:10 dilution of yeast stock consistently achieved stationary phase sooner (columns 1 to 8) as indicated by a higher mean integral (larger area under the curve) and low integral CV% (not shown). However, the 1:10 dilution had a slightly slower growth rate in log phase, as calculated by the mean maximum velocity metric (Figure 5B). Using HCBF cell count results from 4 dilutions of the 1x stock used to seed wells in column 8 (well A8 represented by the red curve in Figure 5B), an average OD600 of 1.8 in cells maintained in stationary phase for 21 hours was equated with an average cell density of 3.7×10^8 cells/mL (Figure 5C). This was used to inform on the optimal cell density to use for imaging the vitality assay, as due to the high confluence of the 1:10 and 1:100 dilutions of stationary phase stock, a 1:1,000 yeast stock dilution was chosen for staining, plating, and imaging treated cells, and a 4-point 1:10 untreated cell dilution starting with a 1:100 dilution of stationary phase stock was used for staining, plating and imaging untreated cells. For data analysis of vitality and viability, the 1:1,000 dilution of untreated cells was used to compare to treated cells plated at the same dilution. To achieve greater statistical relevance and accuracy it is recommended to define a montage and stitching procedure step when counting cells, thereby capturing multiple images of each well to increase total count area. The data reduction formula used to calculate cells/mL from HCBF cell count corrects for total image area.¹⁵

A number of approaches could be taken for image analysis of the vitality assay. The parameters used for this workflow resulted in 5 distinct categories of cellular response defined by Table 1, with a distribution depicted for a representative well of each treatment by the histogram in Figure 6A, and the final total percent distribution shown by Figure 6B. The two vitality categories identified as LoStainRG and LoStainAll are described by little to no cytoplasmic and vacuolar uptake of the ViaVac reagent, or of the ViaVac and Calcofluor White (CW) respectively, and were defined to indicate an absence of membrane integrity, metabolic fitness, and compromised cell wall integrity as described for the stain mechanism of action.¹³ These cells were unlike the other defined negative cells (Neg) that retained the cytoplasmic dye, signifying membrane integrity, but resulted in no vacuolar uptake, indicating a lack of digestive metabolic competence. Of note in Figure 6B is the response of nutrient/pH stressed cells (PBS) that have elevated HiPos and LoStainRG compared to untreated cells that have significantly fewer of each. Prolonged exposure to the treatment media (no nutrients

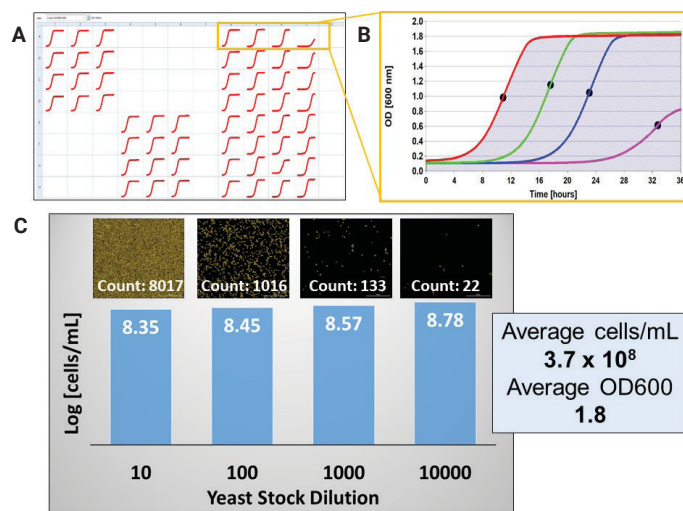


Figure 5. Results of the yeast growth assay. (A) Growth of yeast cells over 36 hours at 30 °C with orbital shaking was monitored using absorbance 600 nm at 2 minute 10 seconds intervals on an Agilent BioTek Cytation 5 cell imaging multimode reader. (B) As illustrated by a representative well at each dilution, time to stationary phase was proportional to dilution of seeded stock as shown by area under the curve (shading), but maximal growth rate (black circles) was slower for cells seeded at higher density. Well A11 (pink) was just entering log phase at a seeded dilution of 1:10,000, and well A8 (red) represents the 1:10 dilution of starting stock. (C) Cell count obtained from HCBF imaging of a serial dilution of the 1x stationary phase stock was converted to cells/mL to inform on cell density for plating the subsequent treatment and imaging assay.

pH 7.4) compared to stationary phase media of untreated cells (nutrient depleted YPD at pH 5.5) may result in a loss of membrane integrity resulting more from pH shock than nutrient deprivation, since both media had low to no nutrients during the treatment phase.

The data suggests that cells may have been in a state of pH stress response resulting in (1) an increased rate of conversion and sequestration of the dye by the vacuole as shown by a higher percent of HiPos (higher conversion and sequestration of the dye) in PBS treated cells versus a higher percent of Pos (lower conversion and sequestration of the dye) in untreated cells, and, in parallel, (2) a weakened membrane potential in PBS treated cells that decreased dye retention 12x in the LoStainRG category compared to untreated cells. One explanation for a greater number of HiPos PBS treated cells may be that enough membrane potential remained to retain sufficient dye to metabolize, but that the concentration of the dye may be low, therefore conversion and sequestration may proceed more rapidly as there is less dye to metabolize. The metabolic profile of cells exposed to either nutrient and alkaline stress or oxidative shock resulted in a loss of vitality 2x and 4x higher than untreated cells respectively as measured by total percent comparison of Neg, LoStainRG, and LoStainAll shown by Figure 6B.

Table 1. Descriptions of vitality response profiles.

Vitality Response	Description	Example
HiPos	Vitality high positive cells were characterized as medium to high sequestration of the dual staining dye from the cytoplasm to the vacuole, resulting in low green cytoplasmic signal and high red intravacuolar signal largely presented as well defined CIVS. GFP <5,800 AND TexasRed ≥1,200	
Pos	Vitality positive cells were characterized as sequestration transitional, resulting in medium to higher green cytoplasmic signal and medium to high red intravacuolar signal respectively. GFP ≥5,800 AND TexasRed ≥1,200	
Neg	Vitality negative cells were characterized as having little to no sequestration of the dual staining dye to the vacuole, resulting in high green cytoplasmic signal and low to no red intravacuolar signal. GFP ≥5,800 AND TexasRed <1,200	
LoStainRG	Cells that retained little to no dual staining dye resulting in lack of green or red signal, but retaining Calcofluor White staining of the cell wall, were considered indeterminate, or vitality negative for purposes of analysis. GFP <5,800 AND TexasRed <1,200 AND DAPI ≥7,150	
LoStainAll	Cells that had little to no staining of any dye but retained cellular object structure as detected by high contrast brightfield imaging (white) were considered vitality negative for purposes of analysis. GFP <5,800 AND TexasRed <1,200 AND DAPI <7,150	

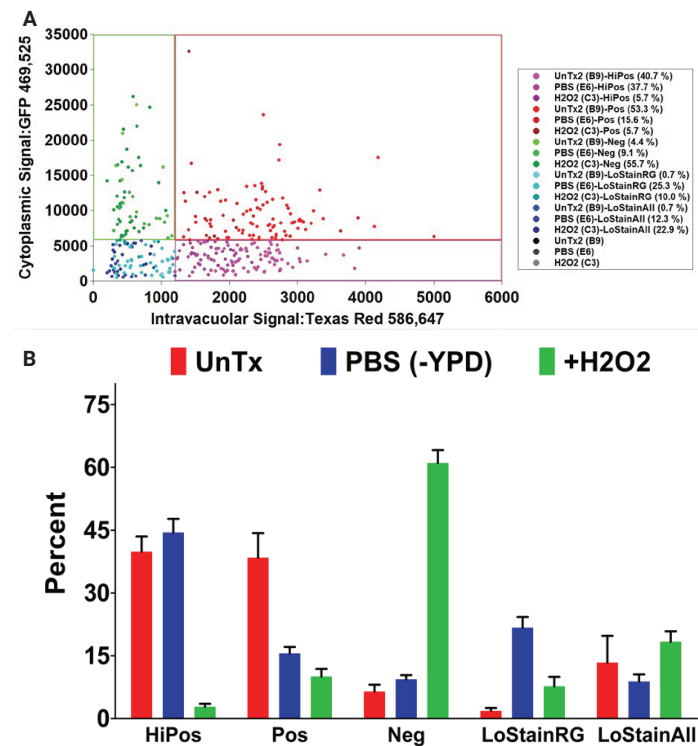


Figure 6. (A) Histogram showing distribution of metabolic response profiles in a representative well from each treatment, and total percent vitality profile distribution for all cells (B).

Figure 7 illustrates the principle of the luminescence assay, where the total yeast count in 100 μ L from a 1:10 dilution series of untreated cells was calculated from the HCBF analysis steps described by the imaging assay method and plotted against corresponding relative luminescence units (RLU) of ATP. Increasing cell counts correspond to higher relative luminescence units of ATP. When comparing results from variable experimental conditions, cell number is shown to be disproportionate to measured viability (Figure 7B). A distinct advantage to acquiring actual cell counts from imaging in parallel to luminescence detection is the ability to normalize ATP to the same scale, correcting for variations in cell number between outcomes of different experimental conditions, thus increasing accuracy. Treated and untreated cells at the dilution shown by the arrow in Figure 7A were normalized as defined previously by Figure 4 resulting in a 6% increase (H_2O_2) and 15% decrease ($-\text{YPD}$) in relative viability for treated cells compared to untreated cells (Figure 7C).

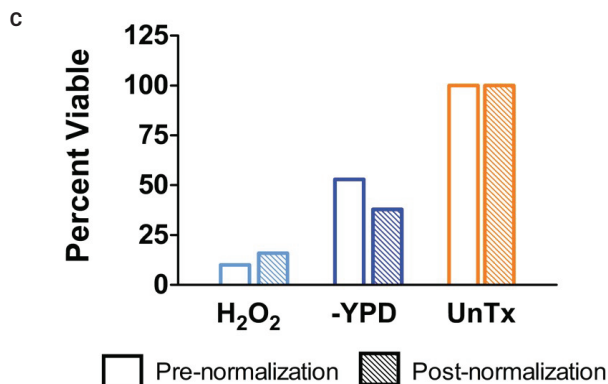
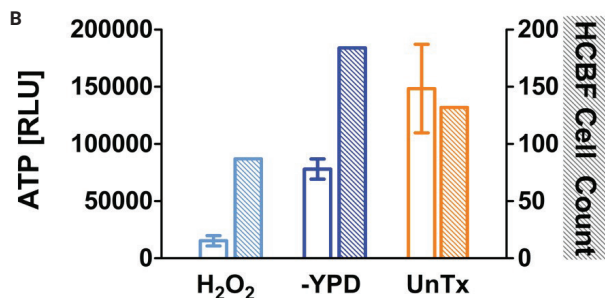
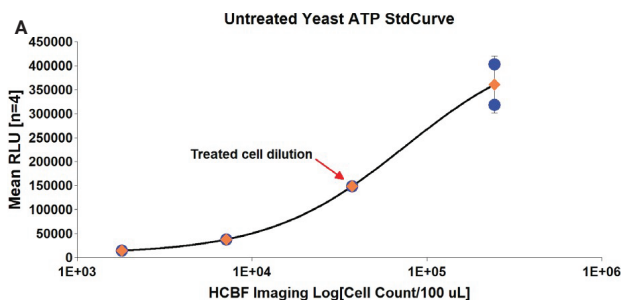


Figure 7. (A) Relative luminescence units (RLU) of ATP is proportional to number of cells as shown by a serial dilution of the untreated stationary phase yeast cells, but does not account for actual cell count differences that occur from variations in cell number due to experimental conditions (shaded, (B)), that can be corrected by normalizing the RLU to cell count (C).

The ViaVac Red/Green dye is reported to have identical structure to the Invitrogen FUN1 Cell Stain that has been described by Millard *et al.* as requiring ATP for uptake and transformation of the dye to intravacuolar structures.¹³ The relationship of percent vitality positive cells and cell viability for treated and untreated cells is shown in Figure 8. Although cells exposed to PBS at 30 °C for one hour still retained 80% normalized vitality, conversion and sequestration of the vitality dye in those cells may have contributed to the 60% loss of normalized viability compared to untreated cells, possibly a result of the stress response as described previously for Figure 6. H₂O₂ is lethal to yeast at certain concentrations, and stationary phase cells have been reported to be more sensitive to H₂O₂ concentrations than cells in log phase.¹⁵ Figure 8 shows that exposure of stationary phase cells to 3% w/v H₂O₂ resulted in a greater than 80% loss of both normalized viability and vitality as expected. Some protection against total cell death from oxidative shock may have been afforded by the high cell density of the treated cells.¹⁵ or by a progressive lack of potency of the H₂O₂ over the one hour treatment time at 30 °C (10 °C above normal room temperature), as warmer temperatures can increase the rate of H₂O₂ decomposition to water and oxygen.¹⁶

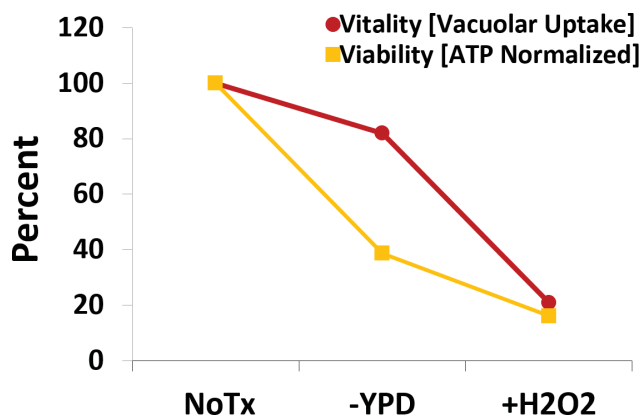


Figure 8. Cellular vitality and viability compared between treated and untreated stationary phase *S. cerevisiae*. Normal yeast digestive and respiratory metabolism is compromised more by exposure to short-term oxidative shock than by alkaline nutrient deprived stress.

Conclusion

S. cerevisiae is a robust yeast strain that retains digestive vitality and respiratory viability after 21 hrs in stationary phase in nutrient depleted media at a pH of 5.5. When these cells are exposed to 90 minutes of nutrient deprivation in an alkaline microenvironment of pH 7.4, although some vacuolar metabolism is retained, data suggests it is at the expense of unrecoverable ATP depletion therefore decreasing cell viability 60% compared to untreated cells. When oxidative stress of stationary phase cells was provoked by one hour of 3% H₂O₂ treatment digestive vitality and respiratory viability were decidedly compromised, but in a population of 3.7×10^8 cells/mL 21% vitality and 16% viability was retained following treatment.

The combination of imaging and detection available on the Agilent BioTek Cytation 5 cell imaging multimode reader enabled the ability to use actual cell counts obtained from HCBF imaging to augment absorbance and luminescence assays performed on the same instrument, in addition to providing comparative quantitation of metabolic vitality using fluorescence imaging. In particular, high contrast brightfield (HCBF) imaging provided a label-free automated calculation of actual yeast cell counts that (1) reduced the need for a cell counting stain and eliminated extraneous manual cell counting steps, (2) provided cell density values used for cell seeding, treating and detection, (3) increased available data points for analysis from kinetic growth monitoring, and (4) enabled normalization of relative luminescence units of ATP to increase statistical accuracy of results between samples with variable cell densities and experimental inducements. This instrument may help consolidate other typical microbiology workflows while increasing assay choices over a broad range of imaging and detection modalities.

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Printed in the USA, May 31, 2022
5994-3401EN