Traffic

Published by John Wiley & Sons Ltd
doi:10.1111/tra.12391

Toolbox

A Toolbox for Rapid Quantitative Assessment of Chronological Lifespan and Survival in *Saccharomyces cerevisiae*

Sarah R. Chadwick[1], Athanasios D. Pananos[2], Sonja E. Di Gregorio[3], Anna E. Park[1], Parnian Etedali-Zadeh[1], Martin L. Duennwald[1,3] and Patrick Lajoie[1]*

[1]Department of Anatomy and Cell Biology, The University of Western Ontario, London N6A 5C1, Canada
[2]Department of Applied Mathematics, University of Waterloo, Waterloo N2L 3G1, Canada
[3]Department of Pathology and Laboratory Medicine, The University of Western Ontario, London N6A 5C1, Canada
*Corresponding author: Patrick Lajoie, plajoie3@uwo.ca

Abstract

Saccharomyces cerevisiae is a well-established model organism to study the mechanisms of longevity. One of the two aging paradigms studied in yeast is termed chronological lifespan (CLS). CLS is defined by the amount of time non-dividing yeast cells can survive at stationary phase. Here, we propose new approaches that allow rapid and efficient quantification of survival rates in aging yeast cultures using either a fluorescent cell counter or microplate imaging. We have generated a software called ANALYSR (**An**alytical **Al**gorithm for **Y**east **S**urvival **R**ates) that allows automated and highly reproducible analysis of cell survival in aging yeast cultures using fluorescent data. To demonstrate the efficiency of our new experimental tools, we tested the previously characterized ability of caloric restriction to extend lifespan. Interestingly, we found that this process is independent of the expression of three central yeast heat shock proteins (Hsp26, Hsp42, Hsp104). Finally, our new assay is easily adaptable to other types of toxicity studies. Here, we assessed the toxicity of various concentrations of acetic acid, a known contributor of yeast chronological aging. These assays provide researchers with cost-effective, low- and high-content assays that can serve as an efficient complement to the time-consuming colony forming unit assay usually used in CLS studies.

Keywords caloric restriction, cell viability, chronological lifespan, computer-assisted analysis, fluorescence, heat shock proteins, yeast

Received 21 December 2015, revised and accepted for publication 1 March 2016, uncorrected manuscript published online 4 March 2016, published online 1 April 2016

The budding yeast *Saccharomyces cerevisiae* has been used successfully as a model organism to study the basis of genetic regulation of cellular homeostasis (1). The availability of yeast deletion and overexpression collections provides scientists with an unmatched model system to study gene regulation and cellular signaling. Among the key cell features studied in yeast are the mechanisms regulating longevity (1–9). Yeast is a powerful and well-established model to study aging, including basic aspects that are directly relevant to human aging. Yet how can aging studies in yeast help us understand what is happening in humans? Martin et al. proposed that highly conserved genes and mechanisms should be referred as 'public mechanisms' of aging as opposed to 'private mechanisms' which are restricted to distinct organisms (10). Public mechanisms include basic, highly conserved pathways initially discovered in yeast, such as caloric restriction (11,12) and the Ras/PKA pathway (13). Further, the role of sirtuins (14), which regulates aging in mammals, was first discovered in yeast.

Yeast allow the study of two distinct paradigms of aging: chronological and replicative lifespan (5,15,16) (Figure 1).

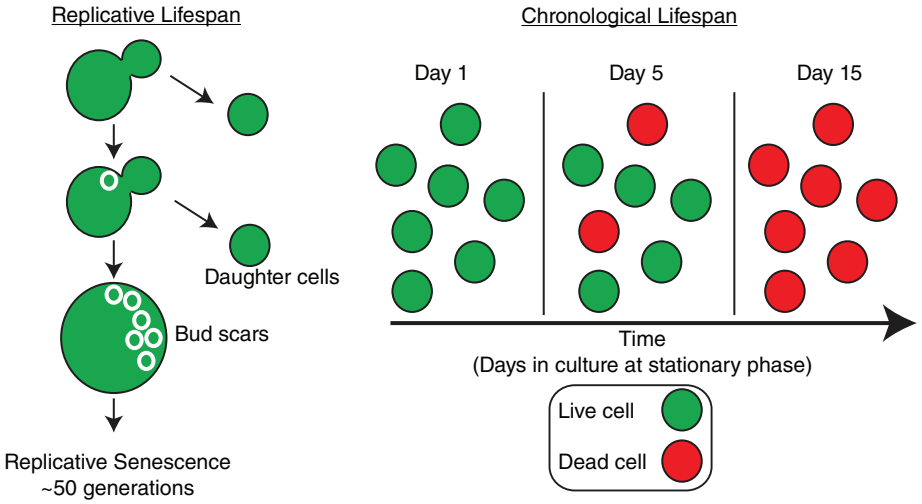


Figure 1: Models of yeast aging. Aging in yeast can be studied using two different paradigms: replicative or chronological lifespan (CLS). Replicative lifespan is defined as the number of times a mother cells can produce daughter cells before entering replicative senescence. CLS is the amount of time a yeast cell can survive in a non-dividing state at stationary phase. CLS has been developed to model aging of post-mitotic cells in higher eukaryotes, such as neurons.

Replicative lifespan is defined by the number of daughter cells a mother cell can produce before entering senescence (17). Chronological lifespan (CLS) is defined as the amount of time non-dividing cells can survive in the absence of nutrients. CLS experiments are typically performed by culturing cells for extensive periods of time at stationary phase. During the course of chronological aging, yeast cells undergo distinct growth phases: the mid-log phase (comprised of a mixture of dividing cells and quiescent cells), diauxic shift (metabolic shift transitioning into stationary phase) and stationary phase. Each of these growth phases is characterized by distinct metabolic activities and gene expression profiles (18), which parallel central aspects of aging mammalian cells, such as increased respiratory activity, arrested cell cycle and the accumulation of damaged proteins (19). This model was developed to serve as a genetically and biochemically traceable model for aging of non-dividing cells in metazoans, such as neurons (20).

CLS is traditionally assessed by aging cells cultures for several days in liquid media to allow cells to enter stationary phase. Every few days, an aliquot is removed from each culture, diluted, and plated on rich media containing plates. The proportion of viable cells within the culture is determined based on the number of colonies growing on the plate (termed colony forming units or CFUs) (21). While this method is well established, it requires a large quantity of agar plates, is time consuming, and is not suitable for high-content analysis. In order to circumvent these problems, scientists have established variations of this assay where the outgrowth of aging culture is performed in liquid media (22). The use of an incubator/shaker/plate reader system allows one to measure the optical density of the cultures over time and generate growth curves (23). From these growth curves, one can use computer algorithms such as the yeast outgrowth data analysis (YODA) (24) or GATHODE (Growth Analysis Tool for High-throughput Optical Density Experiments) and CATHODE (Chronological life span Analysis Tool for High-throughput Optical Density Experiments) (25) programs to calculate the fraction of viable cells in the aging culture. This method, while less quantitative than the CFU methods, allows for high-content screening of yeast libraries, and requires fewer consumables and less time commitment. In addition, a competition survival-based assay has been developed wherein different strains are tagged with fluorescent proteins and cultured simultaneously to assess CLS using flow cytometry analysis of outgrown co-cultures (26). However, all these methods rely on the ability of the cells to re-enter the cell cycle and therefore only indirectly assess viability of aged cells.

It is possible to use a more direct method to measure cell death in aging culture by labeling cells with viability dyes [such as membrane-impermanent propidium iodide (PI)] that only stain dead cells. Dead cells can then be visualized using standard fluorescent microscopy. Again, the drawback of these methods is the significant amount of time it takes to acquire and quantify fluorescent images. Such fluorescently labeled cultures can also be assessed using flow cytometry (27). While this has proven to be efficient, it does not accommodate high-content experiments

unless the facility is equipped with a flow cytometer paired with a high-content sampler capable of accommodating multiwell plates.

Here we describe methods to quantitatively monitor cell viability in aging cultures using fluorescent labeling of dead cells. We developed protocols optimized for quantification of cell viability in 96-well plates using a fluorescent cell counter, a simple ultra violet (UV) trans-illuminator, or a fluorescent plate reader. We designed a simple computer program to enable rapid and efficient quantitation of fluorescent data that can be employed to assess a multitude of genetics or pharmalogical perturbations. As a proof of principle, we used these methods to assess the effects of caloric restriction on CLS and found robust extension of lifespan, which is independent of the expression of three heat shock proteins (Hsp26, 42 and 104). Thus, our study provides researchers with a toolbox of reliable fluorescent assays for cost-efficient and large-scale analysis of CLS in yeast.

Results and discussion

Quantitative assessment of yeast CLS using a fluorescent cell counter

Yeast cell viability can be determined by labeling dead cells with fluorescent dyes such as PI (28,29). These fluorescent nucleic acid stains are known to be membrane-impermeant and therefore are only internalized by cells with compromised plasma membrane, a hallmark of yeast cell death (30). Upon incubation with PI, cells can be analyzed using either fluorescent microscopy or flow cytometry. In contrast, we employed a newly commercially available fluorescent cell counter (Countess II FL) to collect fluorescent images of PI-labeled yeast cultures. In this method, cells are labeled with PI and 10 μL of the culture is loaded onto a counting chamber. All images are collected using the same exposure time (Figure 2A). A negative control (unstained cells) and a positive control (consisting of boiled cells) are included. Both bright field and fluorescent images are acquired. Interestingly, we found that PI staining in aging cultures is much more efficient when performed in PBS rather than in the standard yeast culture media (Figure 2B). We therefore suggest that samples be spun down and resuspended in PBS prior to staining. Although the counter can generate percent viability data, we obtained more consistent results by manually analyzing the raw images in ImageJ. Survival was calculated by measuring the area covered by PI-labeled cells in the red channel divided by the area covered by all cells in the bright field image.

As proof of principal, this method was used to assess the effects of caloric restriction on yeast chronological aging. In yeast, caloric restriction can be achieved by reducing the concentration of glucose in the growth media and is known to extend lifespan (11,31–33). Thus, yeast cells (W303a strain) were cultured in synthetic complete media for several days and viability was measured at different time points during the aging process with PI (Figure 3A). Cells cultured in media containing 0.1% glucose (as opposed to the standard 2%) were used to perform caloric restriction. We found that caloric restriction significantly increased lifespan, demonstrated by both percent survival and survival integral data (the area under the survival curve) (Figure 3B,C). Importantly, the maximal lifespan of W303a strain (~15 days) using this study was similar to previously published data using either regrowth assay or flow cytometry (7,25,27). Previous studies have shown, however, that PI staining is not suitable for extreme caloric restriction experiments using water instead of growth media (34). Thus, researchers should be cautious in the choice of methodology employed according to their type of study, and confirmation using other methods is suitable. While this simple method is suitable to quantify survival at stationary phase, it does not offer the ability to perform high-throughput analysis. Therefore, we next sought to develop an assay that would allow detection of a large number of fluorescent samples simultaneously without the need of expensive automated instrumentation such as a flow cytometer.

Assessing CLS using fluorescence in multiwell plates

PI has an excitation peak at 350 nm, which makes it suitable for imaging using UV light. Thus, we tested if PI fluorescence could be detected in 96-well plates imaged with a UV transilluminator. Interestingly, we found that dead (boiled) cells stained with PI gave a significantly increased signal over unstained cells in a concentration-dependent manner (Figure 4A). We quantified the mean fluorescent signal in each well using the ImageJ software. Data were expressed in percent survival by normalizing to the signal of boiled cells (0% survival) and subtracting background

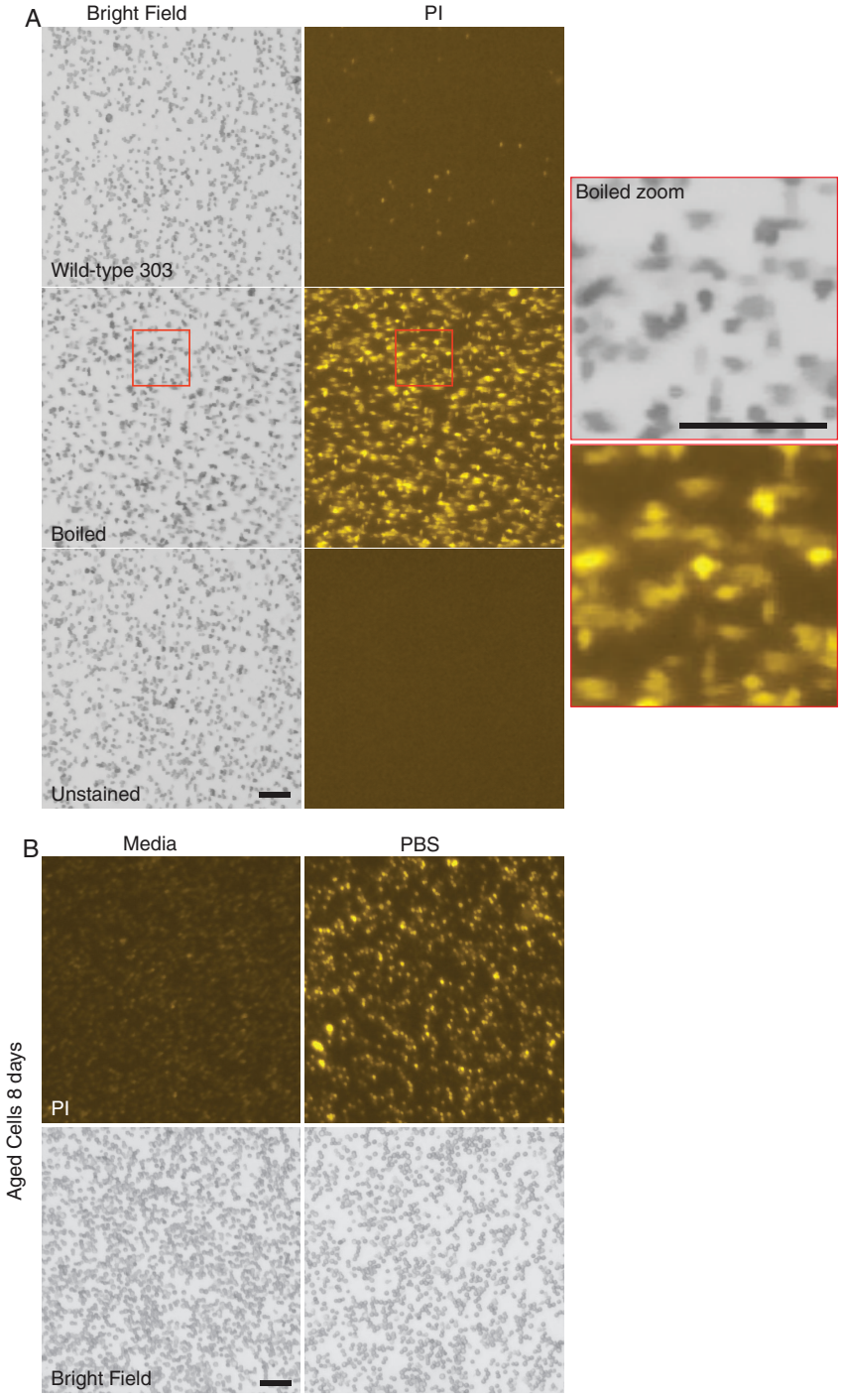


Figure 2: Propidium iodide staining and imaging. A) Cells aged from overnight cultures were stained with propidium iodide and imaged with the Countess II Cell Counter. Bright field images depict all cells in the sample, RFP images depict only fluorescently labeled cells. Boiled cells were used to depict 100% death; unstained cells represent 0% death. B) Cells aged for 8 days were stained with propidium iodide, either in original media or after being resuspended in PBS, and imaged with the Countess II Cell Counter. Bars: 50 μm.

signal from the unstained sample. In order to account for differences in cell population density among replicates, the same plate was also scanned using a plate reader to measure the absorbance at 595 nm (to measure cell density) and all samples were normalized to cell numbers. Several samples per condition were assessed in order to determine the variability within the experiment. Importantly, our assay was sensitive enough to detect as low as 10% cell death in a yeast culture (Figure 4B). This was determined by mixing increasing proportions of dead (boiled) cells stained with PI with live unstained cells prior to imaging. Next, we used this assay to measure survival at stationary phase. We

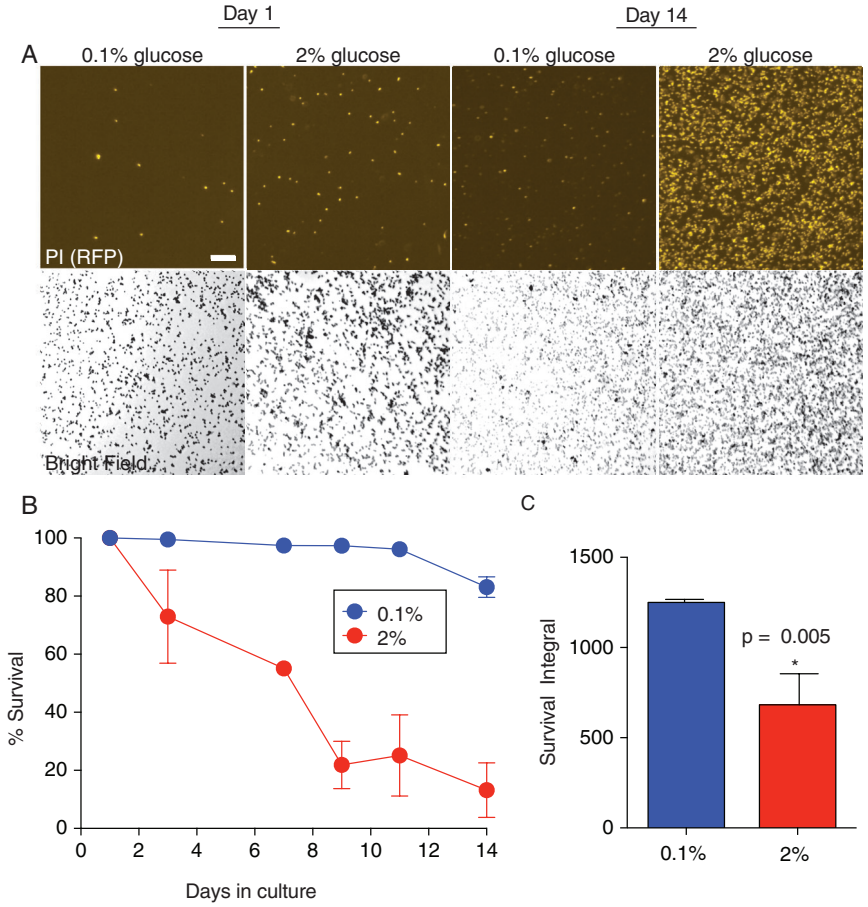


Figure 3: Effects of caloric restriction on CLS using the cell counter. Cells were aged in either standard synthetic media (2% glucose) or caloric restricted synthetic media (0.1% glucose) for the indicated time points before being stained with propidium iodide to measure percent death. Boiled cells were used as a positive control. Cells were imaged using the Countess II Cell Counter and analyzed with ImageJ. A) Side-by-side comparison of cell counter images obtained from days 1 and 14, from cells aged in standard (2% glucose) media and caloric restricted (0.1% glucose) media. Bright field images depict all cells in the sample, RFP images depict only stained (dead) cells. Bar: 50 μm. B) Using the ANALYSR computer program, normalized survival rates were generated based on data from cell counter images; data were normalized by absorbance and by positive and negative controls, with day 1 set at 100% survival. Survival integrals were also generated for mean normalized survival rates (± SD, $n = 6$) (C) and compared using an unpaired *t*-test.

subjected W303a cells to chronological aging. As expected, the intensity of PI staining increased significantly during chronological aging (Figure 4C), indicating that this method can be used to quantitatively assess CLS. Importantly, our data produced a maximal lifespan of ~15 days and reflected the known maximal lifespan of W303a cells (Figure 4D) (7,25,27).

Next, we used our multiwell assay to compare survival at stationary phase in standard (2% glucose) and caloric restricted (0.1% glucose) media, to recapitulate the lifespan extension described before and reproduced using our cell counter assay. Indeed, cells grown in caloric restricted media showed very little increase in PI staining intensity during chronological aging compared with those grown in standard media (Figure 5A). Importantly, we found that our multiwell plate assay yields similar survival results as the ones obtained with the cell counter (Figure 5B,C) or by using a semi-quantitative CLS spotting assay (Figure 5D), highlighting the accuracy of this approach.

The fluorescent CLS assay described in this study allows rapid quantification of yeast survival during the aging process with the ability to assess viability of a large

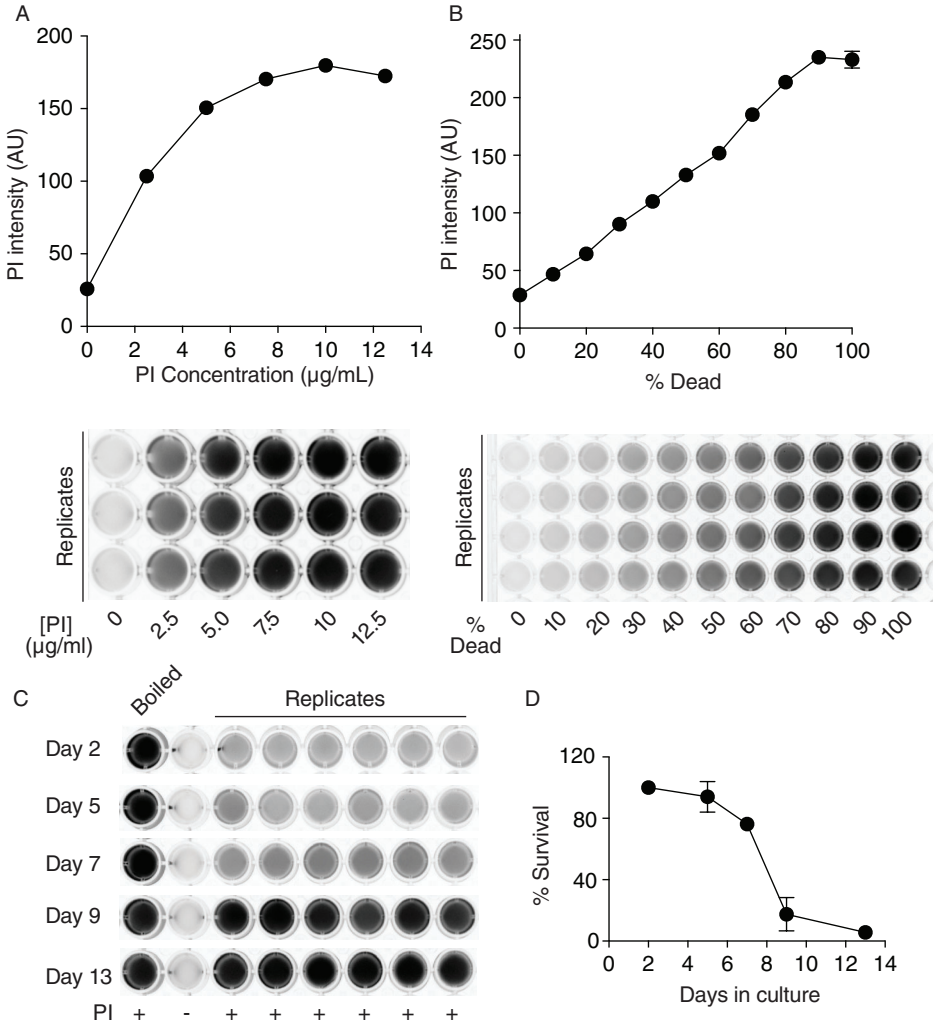


Figure 4: A fluorescent multiwell plate assay for CLS. Aged wild-type W303a cells were stained with propidium iodide and imaged with the UV transilluminator. A) Boiled cells were stained with different concentrations of PI and imaged to find the optimal PI concentration ($n = 3$). B) Boiled, stained cells were mixed with different concentrations of unstained cells to produce different dilutions of PI-labeled dead cells. Four replicates of each dilution of stained cells were imaged. C) Wild-type cells were aged in synthetic media. At various time points, an aliquot was removed from each culture, stained and imaged. One positive control, one negative control and six replicates were analyzed at each time point. D) Using the ANALYSR computer program, mean normalized survival rates (± SD, $n = 6$) were generated based on fluorescent data; data were normalized by absorbance and by positive and negative controls, with day 1 set at 100% survival.

number of samples. This method also does not require any expensive equipment and can be performed using a simple UV gel box. In addition, it is significantly less labor-intensive when compared with the traditional CFU method. It also allows direct measurement of cell viability, as opposed to relying on the ability of cells to re-enter the cell cycle. Our method showed to be suitable for not only studying environmental or genetic perturbations that affect CLS but also effects of toxic conditions. Indeed, we detected a concentration-dependent increase in toxicity of acetic acid (Figure 6), a chemical released by aging cells that is known to contribute to CLS (35). Wild-type yeast cells (W303a) were cultured in complete media for 4 days before being treated with acetic acid, which has previously been shown to be toxic to yeast (35). Cells were exposed to acetic acid ranging from 0 to 500 mM for 200 min

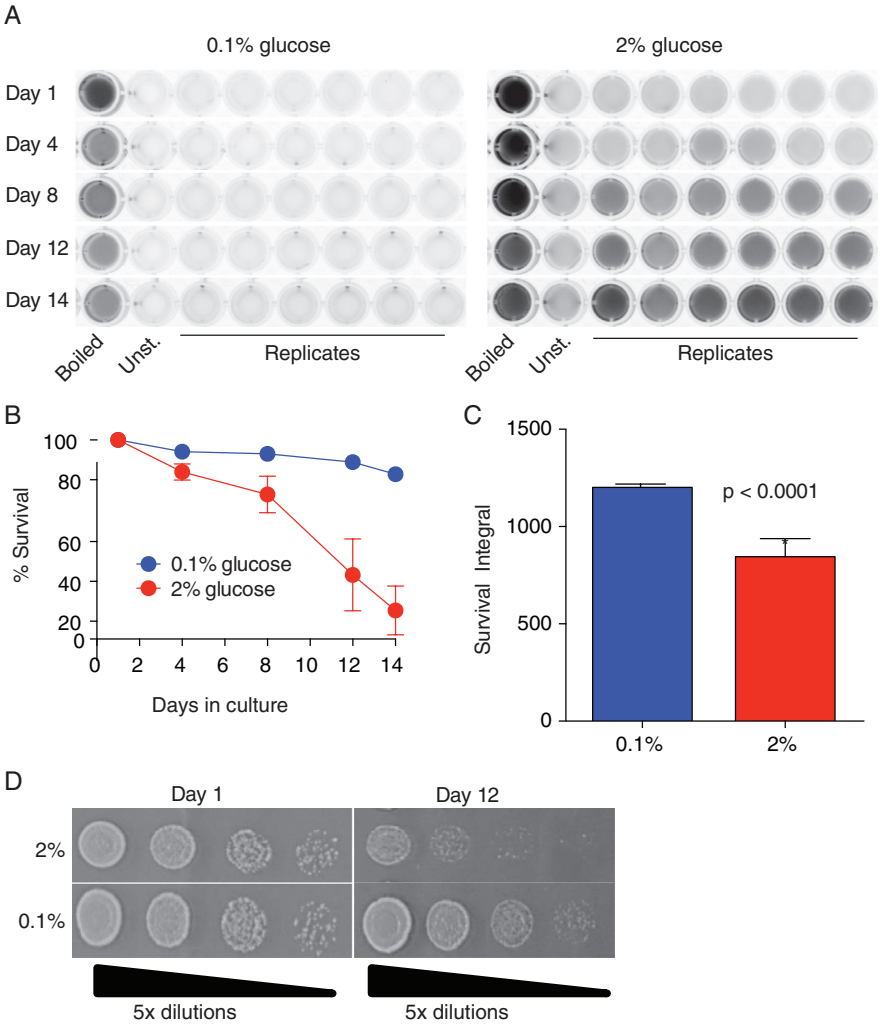


Figure 5: Quantification of lifespan extension by caloric restriction using the PI multiwell plate assay. Cells were aged in either standard synthetic media (2% glucose) or caloric restricted synthetic media (0.1% glucose) for the indicated time points before being stained with propidium iodide to measure percent death. A) 96-well plates were imaged with the transilluminator. One positive control, one negative control and six replicates were analyzed at each time point. B) Using the ANALYSR computer program, mean normalized survival rates (±SD, $n = 10$) were generated based 96-well plate images; data were normalized by absorbance and by positive and negative controls, with day 1 set at 100% survival. C) Survival integrals were calculated for each replicate and compared using an unpaired Student's *t*-test (±SD, $n = 10$). D) Semi-quantitative CLS spot assay for wild-type W303a cells aged in either 2 or 0.1% glucose.

before being stained with PI and imaged (Figure 6A). Quantitation showed that a distinct, linear decline in cell viability could be observed with increasing acetic acid concentration (Figure 6B). These data demonstrate that the methods described here are not limited to assessing CLS but can be used to determine cell viability under a number of different conditions that induce cell death.

It is important to note that our method using a UV transilluminator only allows the detection of one fluorescent dye/protein at the time. Thus, this method is not suitable to determine viability of cells expressing fluorescent proteins. In this case, a fluorescent plate reader can be used to detect specific fluorescent signal using appropriate filter sets. We used the SYTOX Green viability dye in combination with a fluorescent plate reader and this method also highlighted the increased lifespan of caloric restricted cells (Figure 7).

In addition, outgrowth assays, fluorescent microscopy, fluorescent cell counter or flow cytometry could be used. Therefore, our methods could be expanded for screening of yeast deletion collections or chemical libraries. Several studies have shown that yeast cells can be aged in multiwell plates, and our assay would be compatible with such protocols. However, generation of large-scale fluorescent data and subsequent analysis requires assistance of imaging software for rapid quantification. Thus, we generated freely available software that calculates survival rates in yeast cultures from fluorescent images.

Computer program for rapid quantification of CLS using fluorescent data

The first step of data analysis is to determine the fluorescent intensity within the various wells of a plate. This can be done using any available imaging software, such as IMAGEJ.

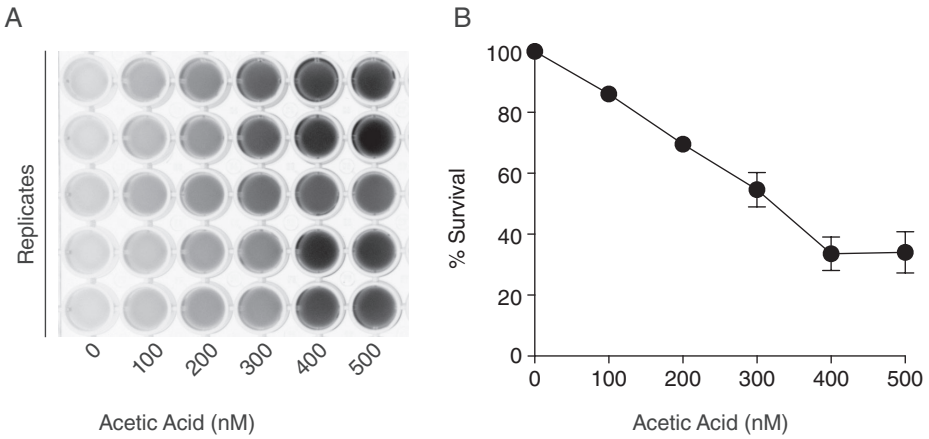


Figure 6: Acetic acid treatment induces cell death in yeast. WT cells were aged for 4 days before being treated with various concentrations of acetic acid for 200 min. A) Cells were stained with propidium iodide and imaged with the UV transilluminator. Five replicates were analyzed for each concentration. B) Mean normalized survival rates (±SD, $n = 5$) based on data from UV transilluminator images using ANALYSR; data were normalized with absorbance and positive and negative controls, with untreated cells values set at 100% survival.

The well to be analyzed is selected and the mean fluorescent intensity from that well is recorded. These data can be saved in a Microsoft Excel format and used to calculate survival rates.

In order to perform such analysis, we designed a new open-source software named ANALYSR (Analytical Algorithm for Yeast Survival Rates). This program was designed specifically for the purpose of analyzing data from fluorescent viability assays. The software runs in Python and accepts .xlsx files as input for the fluorescent data and optical density/absorbance (if required) for these assays over a number of days. Using these data, ANALYSR calculates the percent survival in each replicate of a given condition relative to the positive and negative controls. Then, the data are normalized to the Day 1 of the CLS experiment (set at 100% survival). Users also have the option of normalizing each sample to its optical density/absorbance data. ANALYSR produces two Excel files: one with the raw data and one with the normalized percent survival data (Figure 8A). It also produces two graphs with standard error bars, one showing the trend in percent survival over time in each replicate separately, and the other showing this trend as the average of all replicates (Figure 8B,C). If a sample has a higher degree of fluorescence than the positive (boiled) sample, the percent survival is set at 0%; no negative percentages are calculated.

The software also allows users to measure the area under the curve (survival integral) for each replicate using the trapezoid rule, allowing statistical comparison of survival rates between different conditions.

The ANALYSR software was written in the Python programming language and the source code and instructions are freely available (Appendix S1, Supporting Information). The program can be run using Enthought Canopy, a freely available Python analysis environment that can be downloaded here: https://www.enthought.com/products/canopy/.

Application: Hsp26, 42 and 104 are dispensable for lifespan extension by CR

To provide evidence that the methods presented are effective, we performed further experiments and assessed the impact of genetic mutations on CLS. The deletion of members of the hsp31 minifamily has been previously shown to severely reduce CLS (36). Whether other Hsp proteins are required for CLS is unclear. Thus, we used yeast cells carrying deletions of three heat shock proteins required for heat shock tolerance (*hsp104*Δ, *hsp42*Δ, *hsp26*Δ) (37). First we tested the sensitivity of these cells to heat shock using both qualitative methods (spot assay) (37) and our newly developed methods. Wild-type and triple Hsp deletion mutant cells were either left untreated, or exposed to a tolerance

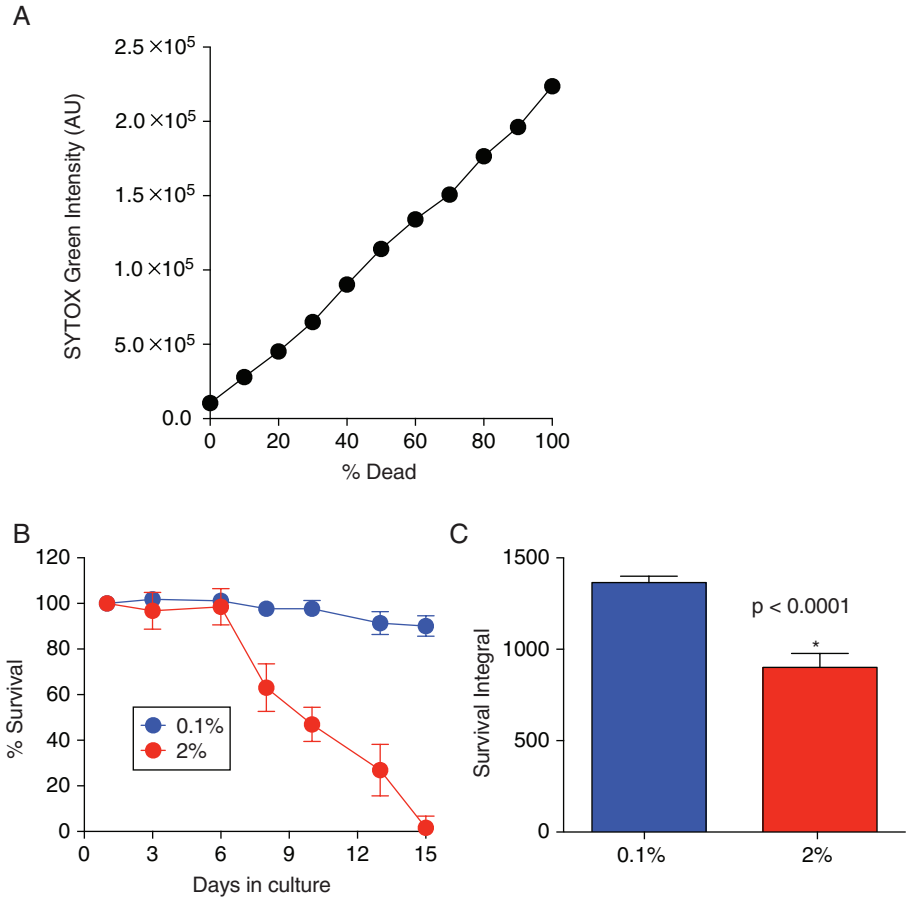
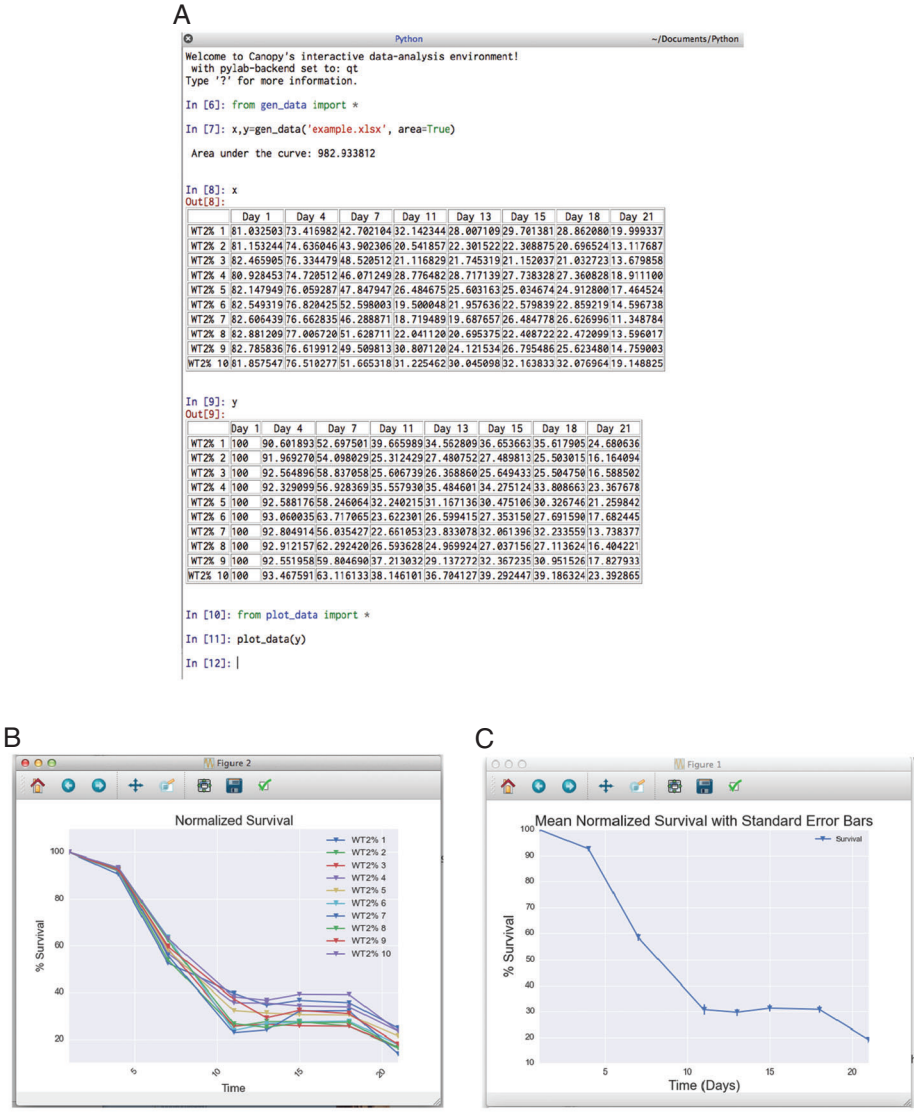


Figure 7: Quantification of lifespan extension by caloric restriction using a fluorescent plate reader. A) Boiled, stained cells were mixed with different concentrations of unstained cells to produce different dilutions of SYTOX Green-labeled dead cells. A total of 4 replicates of each dilution of stained cells were imaged using a fluorescent plate reader. B) Cells were aged in either standard synthetic media (2% glucose) or caloric restricted synthetic media (0.1% glucose) for the indicated time points before being stained with SYTOX Green to measure percent death. 96-well plates were imaged a fluorescent plate reader. Using the ANALYSR computer program, mean normalized survival rates (±SD, $n = 10$) were generated based on 96-well plate images; data were normalized by absorbance and by positive and negative controls, with day 1 set at 100% survival. C) Survival integrals were calculated for each replicate and compared using an unpaired Student's *t*-test (±SD, $n = 10$).

inducing heat shock at 37°C for 30 min followed by either 10- or 20-min incubation at a lethal temperature (50°C) (37). While heat shock did impair growth of the triple deletion mutant during the spot assay (Figure 9A), we surprisingly found that it did not actually result in PI uptake by the cells, indicating that the plasma membrane was not compromised. PI staining revealed that, upon heat shock, the deletion mutant experienced modest cell death similar to what is observed in wild-type cells (Figure 9B). These data suggested that the heat shock proteins are not necessary for cell survival during heat shock, and are perhaps only required for later re-entering the cell cycle after heat shock. However, we did not observe PI uptake even 18 h following heat shock (Figure 9C) suggesting that these cells may die from mechanisms that do not involved a compromised plasma membrane. At the same time, heat shock at higher temperature resulted in PI-positive cells even in wild-type, indicating that 50°C may represent a milder stress that can induce cell death via a different mechanism. Evidence in the literature shows that yeast cells can undergo apoptosis, which is not necessarily associated with increased plasma membrane permeability (38–40).



	Day 1	Day 4	Day 7	Day 11	Day 13	Day 15	Day 18	Day 21
WT2% 1	81.032503	73.415982	42.702104	32.142344	28.007109	29.701381	28.862080	19.999337
WT2% 2	81.153244	74.635046	43.902306	20.541857	22.301522	22.308875	20.696524	13.117687
WT2% 3	82.465905	76.334479	48.520512	21.116829	21.745319	21.152037	21.032723	13.679858
WT2% 4	80.928453	74.720512	46.071249	28.776482	28.717139	27.738328	27.360828	18.911100
WT2% 5	82.147949	76.059287	47.847947	26.484675	25.603163	25.034674	24.912800	17.464524
WT2% 6	82.549319	76.820425	52.598003	19.500048	21.957636	22.579839	22.859219	14.596738
WT2% 7	82.606439	76.662835	46.288871	18.719489	19.687657	26.484778	26.626996	11.348784
WT2% 8	82.881209	77.006720	51.628711	22.041120	20.695375	22.408722	22.472099	13.596017
WT2% 9	82.785836	76.619912	49.509813	30.807120	24.121534	26.795486	25.623480	14.759003
WT2% 10	81.857547	76.510277	51.665318	31.225462	30.045098	32.163833	32.076964	19.148825

	Day 1	Day 4	Day 7	Day 11	Day 13	Day 15	Day 18	Day 21
WT2% 1	100	90.601893	52.697501	39.655989	34.562809	36.653663	35.617905	24.680636
WT2% 2	100	91.969270	54.098029	25.312429	27.480752	27.489813	25.503015	16.164094
WT2% 3	100	92.564896	58.837058	25.606739	26.368860	25.649433	25.504750	16.588502
WT2% 4	100	92.329099	56.928369	35.557930	35.484601	34.275124	33.808663	23.367678
WT2% 5	100	92.588176	58.246064	32.240215	31.167136	30.475106	30.326746	21.259842
WT2% 6	100	93.060035	63.717065	23.622301	26.599415	27.353150	27.691590	17.682445
WT2% 7	100	92.804914	56.035427	22.661053	23.833078	32.061396	32.233559	13.738377
WT2% 8	100	92.912157	62.292420	26.593628	24.969924	27.037156	27.113624	16.404221
WT2% 9	100	92.551958	59.804690	37.213032	29.137272	32.367235	30.951526	17.827933
WT2% 10	100	93.467591	63.116133	38.146101	36.704127	39.292447	39.186324	23.392865

Figure 8: **ANALYSR.** A) A screenshot depicting the user interface and output from the python-based program designed for this research. Output includes percent survival data normalized only to positive and negative controls, and the same data with day 1 set to 100% survival. B) One chart produced by the program, showing the normalized survival of each replicate in one condition. C) Mean normalized survival chart produced by the program, including standard error bars.

Indeed, we observed increased DNA fragmentation in the triple deletion mutant compared with wild-type following heat shock indicating that these cells may be undergoing apoptosis (Figure 9D). It is possible that heat shock induces apoptotic cell death, which can results in secondary necrosis (41) once the plasma membrane is compromised and explained the PI staining observed at higher temperatures (Figure 9C). Thus, while the PI viability assay can provide additional information that cannot be obtained using spotting assays, it should also be used with care, as it might not always allow efficient detection of non-viable cells that do not have compromised plasma membrane.

Next, we assessed survival of the triple mutant at stationary phase. Interestingly, we found that deletions of these specific Hsp proteins did not significantly impair CLS (Figure 10A). Importantly, incubation in low glucose-containing media was still able to increase

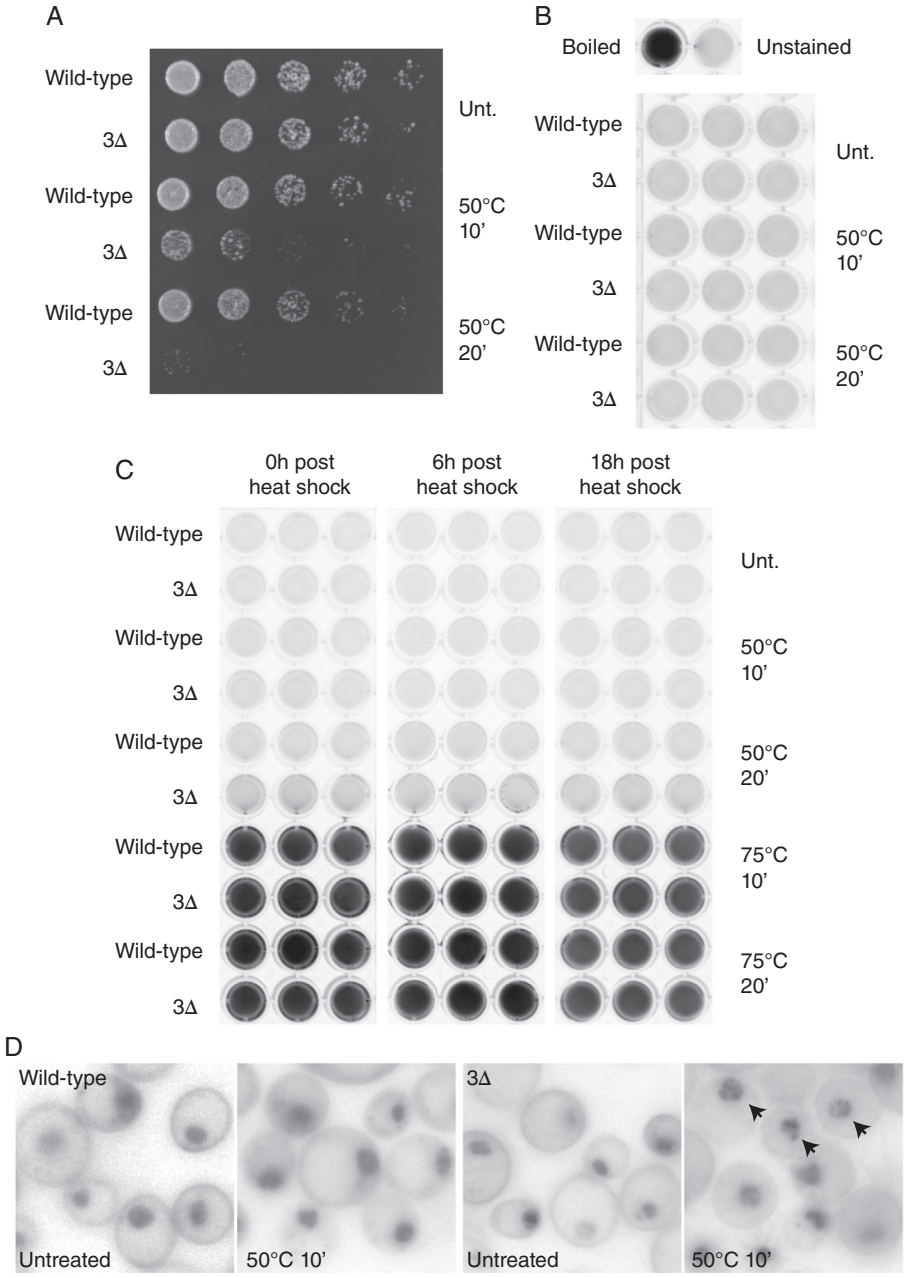


Figure 9: Comparison of regrowth assay versus direct viability staining. A) Wild-type and *hsp104*Δ, *hsp42*Δ, *hsp26*Δ (*3*Δ) cells were left either untreated, or treated at tolerance-inducing temperature (37°C) for 30 min, followed by lethal heat shock at 50°C for either 10 or 20 min. Cells were then spotted on YPD plates. B) Wild-type and *hsp104*Δ, *hsp42*Δ, *hsp26*Δ (*3*Δ) subjected to 50°C heat shock for 10 or 20 min were stained with propidium iodide (triplicates) and imaged with the UV transilluminator. C) Wild-type and *hsp104*Δ, *hsp42*Δ, *hsp26*Δ (*3*Δ) subjected to 50 or 75°C heat shock for 10 or 20 min were stained with propidium iodide (triplicates) and imaged with the UV transilluminator. D) Wild-type and *hsp104*Δ, *hsp42*Δ, *hsp26*Δ (*3*Δ) cells were left either untreated, or treated at tolerance-inducing temperature (37°C) for 30 min, followed by lethal heat shock at 50°C for 10 min. Cells were then labeled with DAPI and imaged using a wide-field fluorescent microscope. Inverted black and white images are shown for clarity. *3*Δ cells show increased DNA fragmentation (arrowheads) upon heat shock indicating that cells undergo apoptosis. Bar: 5 μm.

lifespan in the triple mutant using our assay and was confirmed using a semi-quantitative CLS (Figure 10A–D). These results indicate that, while these three Hsp proteins are known to be upregulated during stationary phase (42), they are not required for extension of lifespan by caloric restriction. We then concluded that their function must be required for tolerance of others type of stresses (such as heat shock) as previously described (43) and highlighted in Figure 9. Our results also indicate that the requirement for Hsp104 is different between CLS and replicative lifespan, because the protein is highly involved in preventing transfer of misfolded protein aggregates from mother to daughter cells (44–46).

Conclusions

The methods described here have proven to be an effective way to assess CLS in yeast. Our new methods successfully confirmed the effects of caloric restriction on CLS determined by other methods. Using a 96-well plates and

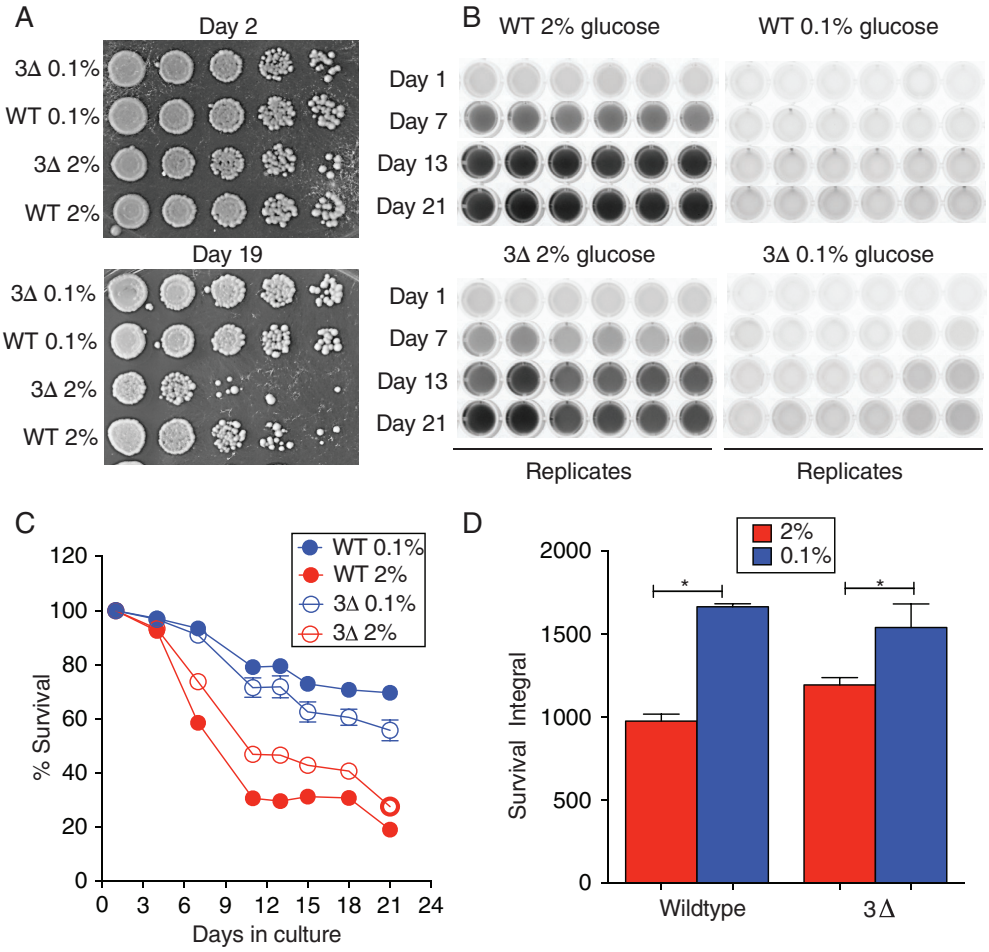


Figure 10: Hsp26, 42 and 104 are dispensable for lifespan extension by caloric restriction. A) Wild-type and *hsp104*Δ, *hsp42*Δ, *hsp26*Δ (*3*Δ) cells were aged for 21 days in either standard (2% glucose) or caloric restricted (0.1% glucose) synthetic media and spotted on YPD plates. B) Chronologically aged wild type and *hsp104*Δ, *hsp42*Δ, *hsp26*Δ (*3*Δ) cells stained with PI in 96 well plates and imaged using the UV transilluminator. One positive control, one negative control and ten replicates were analyzed at each time point. C) Normalized survival rates (±SD, $n = 10$) were generated based on data from UV transilluminator images; data were normalized by absorbance and by positive and negative controls, with day 1 set at 100% survival. D) Survival integrals were generated for each condition and compared to cells in standard media (2% glucose) using 1-way ANOVA and Tukey's test ($p < 0.05$) (±SD, $n = 10$).

readily available technology (such as UV transilluminators), we were able to acquire large amounts of quantitative data relatively quickly, using far fewer resources than the traditional CFU method. This proved to be a significant advantage over traditional CFU assays, which require a high number of agar plates and a large time commitment. Another feature of our assay is the direct quantitation of cell viability, which offers a complementary measure of viability to the regrowth assays. Thus, we have provided researchers with a new toolbox of reliable fluorescent assays that allow rapid, cost-efficient and large-scale analysis of CLS in yeast.

These methods, while effective, also have limitations. It is not always possible, for example, to quantify cell viability of strains expressing fluorescent proteins (i.e. GFP), as the UV transilluminator cannot excite at specific wavelengths. In such cases, a fluorescent plate reader must be used to differentiate the fluorescent signals. The use of multiple assays in measuring CLS and cell viability is important, as results may vary between and within methods. It is therefore important to first assess the sensitivity of the plate reader or translluminator and find the ideal concentration of nucleic acid dye before performing viability assays.

Table 1: Yeast strains used in this study

Strains	Genotype	Description	References
W303a	*MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	Wild-type	(47)
AGC15	*MATα, leu2-3,112 trp1-1 ura3-1 ade2-1 his3-11,15 lys2D can1-100 hsp26::LEU2 hsp104::KanMX, hsp42::HygB*	*hsp26Δ/hsp42Δ/hsp104Δ*	(37)

Although these methods (and the accompanying ANALYSR software) are designed to measure viability during CLS experiments, they could be easily repurposed to suit other needs. They could also be utilized for measuring viability in other conditions, such as treatment with drugs or heat shock, as we demonstrated.

In summary, our methods provide the opportunity for researchers to acquire high quality, high-throughput viability data. The availability of different yeast mutant collections (non-essential gene deletions, temperature sensitive alleles, and overexpression libraries) offers the possibility to assess the roles of specific genes in the aging process on a large scale. Our assays could allow for large amounts of reliable quantitative data to be produced and offer a complementary approach to research currently being performed using traditional CLS methods. Finally, the use of the ANALYSR software is not limited to yeast and can be used to quantify any kind of viability data independently of the organism employed.

Material and Methods

Cell culture and CLS assay

Strains used in this study are listed in Table 1. For every experiment, W303a *S. cerevisiae* cells or derivatives were thawed from frozen stocks and grown on YPD (yeast extract peptone dextrose) media for 2 days at 30°C before being transferred to liquid cultures. A total of 5 mL of liquid cultures were grown to saturation overnight before being separated into replicates (100 µL of overnight cultures in 5 mL of liquid media) for CLS experiments. All experiments were carried out using synthetic complete media (SC). Replicates were incubated in polystyrene snap cap tubes at 30°C in a rotating drum over the course of the aging process.

Cell viability staining

A total of 200 µL of each cell culture were added to 1.5 mL tubes, plus two extra samples to serve as positive and negative controls. The positive control was boiled for 15 min at 100°C. All samples were pelleted for 2.5 min. All samples except the negative control were resuspended in 200 µL of phosphate-buffered saline (PBS) containing a 1 of 200 dilution of a PI stock solution (1 mg/mL in H_2O). The negative control was resuspended in 200 µL of PBS alone. The samples were incubated at room temperature for 10 min. Imaging was then performed using either the Countess II FL cell counter (Life Technologies), a Gel Doc system (Biorad) or a Victor2 plate reader (Perkin Elmer). The optical density (OD_{600}) of each sample was determined with either plate reader, or by measuring the absorbance of diluted samples in cuvettes. Survival rates and integrals were calculated using the ANALYSR program described in this manuscript (see Appendix S1 for extended instructions).

Semi-quantitative CLS assay

At various intervals during the chronological aging process, an aliquot of the aging cultures was removed and cells and serial 5× dilutions of OD_{600} 0.2 were spotted onto YPD agar plates. Plates were then incubated at 30°C for 2–3 days and images using the BioRad GelDoc system.

Fluorescent cell counter

For imaging, 10 µL of each stained sample was loaded onto a reusable hemacytometer and inserted into a Countess II FL Automated Cell Counter. Bright field and RFP/GFP (depending on stain used) images were obtained and manually quantified, as the cell counter's ability to distinguish small budding yeast cells was determined to be unreliable with the current software. To quantify, the positive control images for a given condition were opened first in IMAGEJ (http://imagej.nih.gov/ij/). The threshold was set for each image type, bright field and fluorescent light, ensuring all cells were covered, and 'Analyze Particles' was selected. The percent of the total area covered by the particles (cells) in the sample was recorded. From there, all other samples within that condition were analyzed using the same method, ensuring the threshold set matched that of the positive control. The resulting data were then analyzed using the ANALYSR program.

Imaging with UV transilluminator and fluorescent plate reader

For imaging, 200 µL of each PI-stained sample was loaded into a well in a 96-well plate. The plate was loaded into a BioRad GelDoc and imaged at 300 nm. Exposure time differed between plates, but the use of a positive control (boiled sample) allowed for normalization. The images were analyzed in IMAGEJ by selecting the sample within each well and

measuring the mean grey value for each one. The data were then analyzed via ANALYSR. Alternatively, cells were stained with SYTOX Green (1/200 of a 5 mM stock in DMSO, Life Technologies) and plates were imaged with a fluorescent plate reader (Victor2, Perkin Elmer) equipped with a combination of 485 nm excitation filter and 535 nm emission filter for SYTOX Green imaging.

Fluorescent microscopy

Upon heat shock, cells were stained with 2.5 μg/mL DAPI for 30 min in growth media, then washed and resuspended in PBS and imaged in LABTEK (Nunc Inc.) imaging chambers using a Zeiss Axiovert A1 wide-field fluorescent microscope equipped with a 365 nm excitation/420 emission long pass filter for DAPI and a 63× 1.4 numerical aperture oil objective and a AxioCam ICm1 R1 CCD camera (Carl Zeiss inc.). Images we analyzed using IMAGEJ.

Acknowledgments

This study was supported by an Operating grant from the Canadian Institutes for Health Research (CIHR) to MLD and PL. PL is the recipient of a Discovery grant from the Natural Sciences and Engineering Research Council of Canada (NSERC) and a Strategic Research Award from The University of Western Ontario. We thank Chris Brandl for useful comments during the preparation of the manuscript.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Implementation of the scripts, the algorithms employed, and structuring of data to be used by the ANALYSR program.

References

1. Kaeberlein M, Burtner CR, Kennedy BK. Recent developments in yeast aging. PLoS Genet 2007;3:e84.
2. Kaeberlein M, McVey M, Guarente L. Using yeast to discover the fountain of youth. Sci Aging Knowledge Environ 2001;2001:pe1.
3. Sutphin GL, Olsen BA, Kennedy BK, Kaeberlein M. Genome-wide analysis of yeast aging. Subcell Biochem 2012;57:251–289.
4. Jo MC, Liu W, Gu L, Dang W, Qin L. High-throughput analysis of yeast replicative aging using a microfluidic system. Proc Natl Acad Sci USA 2015;112:9364–9369.
5. Longo VD, Shadel GS, Kaeberlein M, Kennedy B. Replicative and chronological aging in *Saccharomyces cerevisiae*. Cell Metab 2012;16:18–31.
6. Burtner CR, Murakami CJ, Olsen B, Kennedy BK, Kaeberlein M. A genomic analysis of chronological longevity factors in budding yeast. Cell Cycle 2011;10:1385–1396.
7. Murakami C, Kaeberlein M. Quantifying yeast chronological life span by outgrowth of aged cells. J Vis Exp 2009 6;(27). pii: 1156.
8. Mirisola MG, Braun RJ, Petranovic D. Approaches to study yeast cell aging and death. FEMS Yeast Res 2014;14:109–118.
9. Burtner CR, Murakami CJ, Kaeberlein M. A genomic approach to yeast chronological aging. Methods Mol Biol 2009;548:101–114.
10. Martin GM, Austad SN, Johnson TE. Genetic analysis of ageing: role of oxidative damage and environmental stresses. Nat Genet 1996;13:25–34.
11. Kaeberlein M, Hu D, Kerr EO, Tsuchiya M, Westman EA, Dang N, Fields S, Kennedy BK. Increased life span due to calorie restriction in respiratory-deficient yeast. PLoS Genet 2005;1:e69.
12. Jiang JC, Jaruga E, Repnevskaya MV, Jazwinski SM. An intervention resembling caloric restriction prolongs life span and retards aging in yeast. FASEB J 2000;14:2135–2137.
13. Longo VD, Liou LL, Valentine JS, Gralla EB. Mitochondrial superoxide decreases yeast survival in stationary phase. Arch Biochem Biophys 1999;365:131–142.
14. Kennedy BK, Austriaco NR, Zhang J, Guarente L. Mutation in the silencing gene SIR4 can delay aging in S. cerevisiae. Cell 1995;80:485–496.
15. Longo VD, Gralla EB, Valentine JS. Superoxide dismutase activity is essential for stationary phase survival in Saccharomyces cerevisiae. Mitochondrial production of toxic oxygen species in vivo. J Biol Chem 1996;271:12275–12280.
16. Piper PW. Long-lived yeast as a model for ageing research. Yeast 2006;23:215–226.
17. Mortimer RK, Johnston JR. Life span of individual yeast cells. Nature 1959;183:1751–1752.
18. Goldberg AA, Bourque SD, Kyryakov P, Gregg C, Boukh-Viner T, Beach A, Burstein MT, Machkalyan G, Richard V, Rampersad S, Cyr D, Milijevic S, Titorenko VI. Effect of calorie restriction on the metabolic history of chronologically aging yeast. Exp Gerontol 2009;44:555–571.
19. Tissenbaum HA, Guarente L. Model organisms as a guide to mammalian aging. Dev Cell 2002;2:9–19.
20. MacLean M, Harris N, Piper PW. Chronological lifespan of stationary phase yeast cells; a model for investigating the factors that might influence the ageing of postmitotic tissues in higher organisms. Yeast 2001;18:499–509.
21. Hu J, Wei M, Mirisola MG, Longo VD. Assessing chronological aging in *Saccharomyces cerevisiae*. Methods Mol Biol 2013;965:463–472.
22. Powers RW, Kaeberlein M, Caldwell SD, Kennedy BK, Fields S. Extension of chronological life span in yeast by decreased TOR pathway signaling. Genes Dev 2006;20:174–184.
23. Murakami CJ, Wall V, Basisty N, Kaeberlein M. Composition and acidification of the culture medium influences chronological aging similarly in vineyard and laboratory yeast. PLoS One 2011;6:e24530.
24. Olsen B, Murakami CJ, Kaeberlein M. YODA: software to facilitate high-throughput analysis of chronological life span, growth rate, and survival in budding yeast. BMC Bioinformat 2010;11:141.
25. Jung PP, Christian N, Kay DP, Skupin A, Linster CL. Protocols and programs for high-throughput growth and aging phenotyping in yeast. PLoS One 2015;10:e0119807.

26. Garay E, Campos SE, González dela Cruz J, Gaspar AP, Jinich A, Deluna A. High-resolution profiling of stationary-phase survival reveals yeast longevity factors and their genetic interactions. PLoS Genet 2014;10:e1004168.
27. Ocampo A, Barrientos A. Quick and reliable assessment of chronological life span in yeast cell populations by flow cytometry. Mech Ageing Dev 2011;132:315–323.
28. Wallen CA, Higashikubo R, Roti Roti JL. Comparison of the cell kill measured by the Hoechst-propidium iodide flow cytometric assay and the colony formation assay. Cell Tissue Kinet 1983;16:357–365.
29. Krishan A. Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. J Cell Biol 1975;66:188–193.
30. Corliss DA, White WE. Fluorescence of yeast vitally stained with ethidium bromide and propidium iodide. J Histochem Cytochem 1981;29:45–48.
31. Murakami CJ, Burtner CR, Kennedy BK, Kaeberlein M. A method for high-throughput quantitative analysis of yeast chronological life span. J Gerontol A Biol Sci Med Sci 2008;63:113–121.
32. Tsuchiya M, Dang N, Kerr EO, Hu D, Steffen KK, Oakes JA, Kennedy BK, Kaeberlein M. Sirtuin-independent effects of nicotinamide on lifespan extension from calorie restriction in yeast. Aging Cell 2006;5:505–514.
33. Kaeberlein M, Andalis AA, Fink GR, Guarente L. High osmolarity extends life span in *Saccharomyces cerevisiae* by a mechanism related to calorie restriction. Mol Cell Biol 2002;22:8056–8066.
34. Pereira C, Saraiva L. Interference of aging media on the assessment of yeast chronological life span by propidium iodide staining. Folia Microbiol (Praha) 2013;58:81–84.
35. Burtner CR, Murakami CJ, Kennedy BK, Kaeberlein M. A molecular mechanism of chronological aging in yeast. Cell Cycle 2009;8:1256–1270.
36. Miller-Fleming L, Antas P, Pais TF, Smalley JL, Giorgini F, Outeiro TF. Yeast DJ-1 superfamily members are required for diauxic-shift reprogramming and cell survival in stationary phase. Proc Natl Acad Sci USA 2014;111:7012–7017.
37. Cashikar AG, Duennwald M, Lindquist SL. A chaperone pathway in protein disaggregation. Hsp26 alters the nature of protein aggregates to facilitate reactivation by Hsp104. J Biol Chem 2005;280:23869–23875.
38. Madeo F, Fröhlich E, Ligr M, Grey M, Sigrist SJ, Wolf DH, Fröhlich KU. Oxygen stress: a regulator of apoptosis in yeast. J Cell Biol 1999;145:757–767.
39. Aerts AM, Zabrocki P, Govaert G, Mathys J, Carmona-Gutierrez D, Madeo F, Winderickx J, Cammue BP, Thevissen K. Mitochondrial dysfunction leads to reduced chronological lifespan and increased apoptosis in yeast. FEBS Lett 2009;583:113–117.
40. Herker E, Jungwirth H, Lehmann KA, Maldener C, Fröhlich KU, Wissing S, Büttner S, Fehr M, Sigrist S, Madeo F. Chronological aging leads to apoptosis in yeast. J Cell Biol 2004;164:501–507.
41. Carmona-Gutierrez D, Eisenberg T, Büttner S, Meisinger C, Kroemer G, Madeo F. Apoptosis in yeast: triggers, pathways, subroutines. Cell Death Differ 2010;17:763–773.
42. Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz G, Botstein D, Brown PO. Genomic expression programs in the response of yeast cells to environmental changes. Mol Biol Cell 2000;11:4241–4257.
43. Sanchez Y, Taulien J, Borkovich KA, Lindquist S. Hsp104 is required for tolerance to many forms of stress. EMBO J 1992;11:2357–2364.
44. Saarikangas J, Barral Y. Protein aggregates are associated with replicative aging without compromising protein quality control. Elife 2015:4 pii: e06197.
45. Hill SM, Hao X, Liu B, Nyström T. Life-span extension by a metacaspase in the yeast *Saccharomyces cerevisiae*. Science 2014;344:1389–1392.
46. Andersson V, Hanzén S, Liu B, Molin M, Nyström T. Enhancing protein disaggregation restores proteasome activity in aged cells. Aging (Albany NY) 2013;5:802–812.
47. Thomas BJ, Rothstein R. Elevated recombination rates in transcriptionally active DNA. Cell 1989;56:619–630.