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An Optimized LIVE/DEAD Assay Coupled with Flow Cytometry for Quantifying Post-Stress Survival in Yeast Cells

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Abstract

Quantifying survival is a common and critical task in yeast research. LIVE/DEAD stains based on plasma membrane integrity provide a rapid and high-throughput assay for yeast survival when coupled with flow cytometry. However, variations in staining buffer, dye concentration, incubation time, and flow cytometry settings can impact data quality and reproducibility. This protocol presents a standardized LIVE/DEAD assay for post-stress survival quantification in yeast using flow cytometry. After treating *Candida glabrata*, an opportunistic yeast pathogen, with different doses of hydrogen peroxide, the post-stress samples were stained with a two-component LIVE/DEAD stain consisting of SYTO 9 and propidium iodide (PI). Flow cytometry was used to distinguish live, damaged, and dead cell populations and quantify their percentages in each sample. Survival estimates based on the percent live statistic were compared to the Colony Forming Unit (CFU) result on the same sample. The two methods yielded consistent results for the mock- and lethal dose (1 M H_2O_2)-treated samples. At the sublethal dose of 100 mM H_2O_2, SYTO 9/PI estimated a higher survival rate than CFU, reflecting a key difference between the two, where the protocol presented here evaluates cell survival immediately after the stress, while CFU quantifies the percent of cells able to recover and reproduce. Hence, this protocol measures viability at an earlier stage of the cell-death process. In summary, the protocol described here provides a fast and scalable alternative to CFU for post-stress survival quantification in yeast. Its results provide complementary information to CFU by evaluating survival at an earlier stage and distinguishing between dead and damaged cells.

Introduction

The budding yeast subphylum contains many important biological and biotechnological models. A common and critical task in yeast research is quantifying the effect of genetic or environmental perturbations, the latter including

stress or drug treatments. In industrial fermentation, for example, producers must monitor the viability of yeast cultures to ensure the efficiency and quality of the fermentation process[1,2]. When studying pathogenic yeast species, measuring their survival after stress or antifungal treatment is critical for understanding the genetic and mechanistic basis for infection-related traits such as antifungal resistance. Measuring survival on catheters and surfaces is also an important task in clinical settings[3]. These diverse scenarios all call for quantitative approaches that can rapidly and accurately measure the outcome of drug and stress treatments.

Existing methods for achieving the above goal fall into three main categories based on what they measure. The first category measures *clonogenicity*, or the ability of individual yeast cells to form a single colony following the stress. The representative method in this category is the Colony Forming Unit (CFU) assay. The second category measures *vitality*, which relies on detecting enzymatic activities in live cells. Examples include chemical probes such as the FUN-1 dye[4]. The third category of methods measure plasma membrane integrity - an irreversible loss of plasma membrane integrity is considered the "point of no return" for cell death[5]. Examples of this category include fluorescent dyes such as propidium iodide (PI).

This work presents an optimized LIVE/DEAD assay coupled with flow cytometry to achieve fast and scalable quantification of yeast survival after stress treatment. This membrane permeability-based assay is faster than CFU, taking 15-30 min of staining compared with 24-48 h of incubation for colonies to form. Also, samples stained with a LIVE/DEAD stain can be readily assayed using flow cytometry in addition to microscopy, which measures tens of thousands of cells in a few seconds and can easily process 96-well samples for high-throughput assays. As a result, this assay is rapid, quantitative, and scalable. In this protocol, a two-component LIVE/DEAD stain consisting of SYTO 9 and PI is used to achieve enhanced resolving power. SYTO 9 labels all cells, live or dead, while PI only enters cells with compromised plasma membranes[6,7]. Therefore, live cells only accumulate SYTO 9 while dead cells accumulate both SYTO 9 and PI. Because PI has a higher affinity than SYTO 9 for nucleic acid, it competitively excludes the latter in the cell[8]. Also, the two dyes form a Förster Resonance Energy Transfer (FRET) pair, where SYTO 9 emission is absorbed by PI as its excitation. As a result, dead cells exhibit muted green and strong red fluorescence. In contrast, live cells display bright green fluorescence[8]. This difference enables better differentiation between live and dead cells in the presence of variations in fluorescence intensity within each group.

Since an initial report of its application in yeast in 2004, the use of SYTO 9/PI in yeast studies has been spotty and largely limited to qualitative evaluations using microscopy[7,9,10,11,12,13,14,15]. A major limitation to its adoption as a quantitative assay for yeast survival is the lack of systematic characterization and comparison with widely used methods such as CFU. This protocol describes a standardized assay including the staining buffer, dye concentration, staining time, and flow cytometry settings, which were found to minimize artifacts and generate reproducible results when applied to *C. glabrata* and two other yeast species[16]. Because SYTO 9/PI staining reveals an intermediate "damaged" population of cells that differ from both the live and dead cells in their staining pattern, it can complement CFU by revealing more nuanced information

on the effects of stress treatment on yeast cells, potentially enabling new applications.

Protocol

The details of the reagents and the equipment used in this study are listed in the **Table of Materials**.

1. Application of hydrogen peroxide stress

1. At least a day before the experiment, innoculate an overnight culture of *C. glabrata* from a single colony on a freshly-streaked plate into 3 mL of Synthetic Complete (SC) media (1.7 g/L Yeast Nitrogen Base, complete amino acid mix, 2% w/v glucose, water), and culture it by placing the glass tube in a roller drum and grow at 30 °C. Alternatively, an orbital shaker can be used by placing the glass tube at a slanted angle and shaking the culture at 200 rpm.
    1. Measure the optical density (OD_{600}) of the overnight culture next morning and dilute it with fresh SC media to OD_{600} ~0.2 in a total volume of 10 mL. Let it grow for two doublings, which takes about 4 h, to reach the mid-log (OD_{600} ~1) phase.

        EXAMPLE: If an overnight culture has an OD_{600} = 10, add 200 µL of this culture to 9.8 mL SC media.
2. Within 30 min before the stress treatment, prepare the hydrogen peroxide stress media. Serially dilute hydrogen peroxide from the 30% stock (9.798 M) into Synthetic Complete (SC) media to the desired concentration. Include a mock treatment (SC with no hydrogen peroxide added) and a lethal dose (1 M is suitable for *C. glabrata*).

    EXAMPLE: To make a 100 mM hydrogen peroxide stress media, add 10.3 µL of the 9.798 M hydrogen peroxide stock to 1 mL SC. Gently pipette to mix. To make a 10 mM hydrogen peroxide stress media, dilute 500 µL of the 100 mM hydrogen peroxide stress media into 4.5 mL of SC.
3. Measure the OD_{600} of the mid-log culture and standardize it to, e.g., OD_{600} = 1, by adding SC media.

    EXAMPLE: The mid-log culture is at OD_{600} = 1.2. Add 0.2 mL SC media per 1 mL of culture.
4. Transfer 600 µL mid-log culture per testing condition into a 96 deep-well plate. Pellet cells by centrifugation at 3,000 x *g* for 5 min at room temperature. Carefully remove the supernatant by aspiration without disturbing the cell pellet.
5. Add 600 µL of mock or hydrogen peroxide stress media prepared in step 1.2 to each well and gently pipette to resuspend the cells.
6. Incubate the plate for 120 min at 30 °C with shaking at 300 rpm.

    NOTE: Incubation time depends on the biological question of interest.

2. Application of the SYTO 9/PI stain

1. Prior to the experiment, prepare at least 10 mL sterile 0.85% saline buffer.

    NOTE: We found 0.85% saline buffer to produce minimal staining artifacts, i.e., unstained and dead-like cells in mock-treated samples, compared with deionized water or growth media[16].
2. Prepare a working stock solution of PI at 0.2 mM using sterile, deionized water.

    NOTE: This working stock of PI can be stored for up to 6 months at 4 °C.

3. Immediately before the experiment, prepare a working SYTO 9 stock solution at 33.4 µM with sterile, deionized water.

   NOTE: Prepare the SYTO 9 working stock fresh for each experiment. Do not store and reuse.

4. Gently pipette to mix the cells in each well from the treatment plate. Measure OD_{600} of each treatment condition and calculate the amount of buffer needed to resuspend the cells to an OD_{600} of 1.

   NOTE: Multi-channel pipettes can be used for efficient and uniform mixing. Avoid overmixing the samples.

5. Collect yeast cells after the mock or stress treatment by centrifugation at 3,000 x *g* for 5 min. Remove supernatant carefully through aspiration. Resuspend cells in sterile 0.85% saline buffer to OD_{600} = 1 (calculated in step 2.4).

   NOTE: Use a multi-channel pipette to resuspend the cells. Handle all post-treatment cultures with care and avoid overmixing.

6. Reserve 50 µL of each sample to serve as the unstained and single dye staining controls.

   NOTE: Minimal autofluorescence was found for wild-type *C. glabrata* in both channels for SYTO 9 and PI. However, always check unstained samples for autofluorescence and perform proper background subtraction or use low autofluorescence growth media.

7. Aliquot 16 µL of post-treatment sample resuspended in 0.85% saline buffer to a 50 µL PCR tube. Add 2 µL of the 0.2 mM working stock of PI to the cell suspension. Then add 2 µL of the 33.4 µM working stock of SYTO 9 to the same tube to a final volume of 20 µL.

   1. Final concentrations of PI and SYTO 9 will be 20 µM and 3.34 µM, respectively. Gently pipette to mix and incubate for 30 min in the dark (a black box can be used) at room temperature.

3. Setting up and calibrating the flow cytometer

NOTE: This should be done on each new instrument and also on new applications of the SYTO 9/PI staining, including for a different species/strain, new stressor, or growth protocol, etc. The settings below were developed on an Attune NxT flow cytometer. Refer to the user manual of the instrument in use for specific instructions.

1. Choose proper fluorescence channels/filters for the flow cytometer: a 530 nm ± 30 nm bandpass filter (BL1) and a 600 nm long pass filter (> 600 nm, BL3) were used to collect signals in the green (SYTO 9) and red (PI and SYTO 9) channels. Both were excited with a 488 nm blue laser.

2. Set the flow rate to 200 µL/min and stop when at least 30,000 events have been collected.

   NOTE: The number of events per sample can be adjusted with the goal of achieving robust estimates of the percentages. Samples with too few events (cells) should be excluded. The next step is to adjust voltages on the FSC and SSC channels during the initial setup.

3. Dilute 16 µL mock-treated, unstained sample into 200 µL sterile 0.85% saline buffer. Gently pipette to mix immediately before flow cytometry. Run this sample and adjust the voltages of the FSC and SSC channels so that the population is at the center of the FSC *vs*. SSC density plot.

   NOTE: A voltage of 345 mV and 399 mV was used for the FSC and SSC, respectively. The next steps are to adjust voltages on the collection (BL1 and BL3) channels during the initial setup.

4. Mix 24 µL mock-treated sample with 24 µL of 1000 mM hydrogen peroxide-treated or heat-killed sample.

5. Divide the mixed live and dead cell sample into three tubes of 16 µL each. Stain the three tubes with (1) 2 µL SYTO 9 and 2 µL water for 30 min; (2) 2 µL PI and 2 µL water for 30 min; (3) 2 µL SYTO 9 and 2 µL PI for 30 min. Follow the staining protocol from the previous section.

    1. After the incubation, add 200 µL of sterile 0.85% saline buffer to each tube. They are now ready for flow cytometry.

6. Run tube (1) to adjust the voltage of the green fluorescence (BL1) channel; then run tube (2) to adjust the voltage of the red fluorescence (BL3). The goal is to have the mode of each channel's intensity distribution be ~10^4-10^5 so as to utilize the full dynamic range of the instrument.

    NOTE: Please follow the instructions of your specific flow cytometer when adjusting voltages. Some flow cytometers have fixed voltages, and this step is omitted.

7. Run tube (3) to check if live and dead cell populations are well separated. Adjust BL1 or BL3 channel voltages if needed.

    NOTE: A voltage of 200 mV was used for both the BL1 and BL3 channels on the Attune NxT.

8. Run the mock-treated, unstained sample under the established voltage settings to ensure that the sample has minimal autofluorescence in the channels of interest (<10^2).

4. Running samples on the flow cytometer

1. Apply the flow cytometer settings established above and run the unstained sample first to ensure that autofluorescence signals are below 10^2 in both BL1 and BL3 channels.

2. Add 200 µL 0.85% saline buffer to each stained sample and run it on the flow cytometer.

    NOTE: If multiple samples are assayed, stagger the staining step such that each sample is stained for 30 min before being run on the flow cytometer.

3. Export flow cytometry output as .fcs files (FCS 3.0 or 3.1 standard).

5. Data analysis in R

NOTE: A sample analysis script is provided as **Supplementary File 1**. Example gating strategies are provided in the Representative Results section.

1. Install and load the required packages: (1) flowCore (2) ggplot2 (3) ggcyto.

2. Import .fcs files into the current R environment.

3. Adjust file names and specify treatment conditions.

4. Make a 2D density plot for all events on FSC.H against SSC.H. Use a polygon gate to exclude non-cell events. Name this gate "cells".

    NOTE: On certain flow cytometers, the Area parameter is recommended over Height. Check instrument-specific recommendations.

5. For the "cells" gated population, make a 2D density plot on FSC.H against FSC.W. Typically, two clusters are visible with similar ranges of FSC.H but different FSC.W. The cluster with a lower mean FSC.W level contains the single cells ("singlets"). Draw a polygon gate to select them and name the gate "singlets".

6. For the "singlets" gated population, make a 2D density plot on BL1.H against BL3.H. Draw polygon gates for

live, damaged, and dead populations based on live and dead cell controls. An example is provided in the Representative Results section.

7. Determine survival using the percentages of each population and export the statistics.

Representative Results

The SYTO 9/PI stain distinguishes live yeast cells from dead cells based on their fluorescence signals in the green (500 nm-560 nm) and red (>600 nm) channels. Live cells, which only accumulate SYTO 9, fluoresce strongly in the green channel, with minimal red signals. Dead cells accumulate both SYTO 9 and PI and are expected to fluoresce strongly in the red channel, with lower green fluorescence signals compared to live cells due to competitive exclusion of SYTO 9 by PI and FRET.

The above SYTO 9/PI staining protocol was applied to oxidatively stressed *C. glabrata* cells. Mid-log phase *C. glabrata* cells were treated with 0 mM, 100 mM, and 1,000 mM H_2O_2 for 2 h and stained with SYTO 9 and PI. The staining patterns were visually examined by laser scanning confocal microscopy, shown as an overlay of the two fluorescent channels on top of the brightfield (**Figure 1A**, 488 nm excitation, 510-560 nm emission for SYTO 9 and 590-700 nm emission for PI). Mock-treated live cells are green. In the 100 mM H_2O_2-treated sample, cells show an enhanced green signal, with some cells showing both green and red fluorescence, making them appear yellow in the overlay. It has been shown in bacteria that SYTO 9 signals intensify in stressed cells, likely due to increased permeability as a result of plasma membrane damage[17]. The same phenomenon was confirmed in oxidatively stressed *C. glabrata* samples in our own work[16]. Lastly, dead cells appear bright yellow, indicating both SYTO 9 and PI are accumulating in the cell.

Next, the stained samples were subjected to flow cytometry using the settings specified above. The distribution of the BL1 (green) and BL3 (red) channel signals is shown as 2D density plots (**Figure 1B**). The results reveal an increase in the mean green signal at the 100 mM H_2O_2-treated sample compared to the mock, while the 1,000 mM H_2O_2-killed sample exhibits a significant shift towards stronger red fluorescence. To estimate the survival rate, the flow cytometry data were first gated for single cell events based on the forward and side scatters (FSC and SSC, **Figure 2A**,**B**). Then, two polygon gates were used to identify the live and damaged cell events (**Figure 2C**). Ungated events were classified as dead cells. SYTO 9/PI staining patterns are consistent across biological replicates, although the intensity distributions vary, likely due to dye concentration and staining efficiency variations (**Figure 3A**). We quantified the % live and damaged cell events as described above and compared the results to CFU for the same samples (**Figure 3B**). Both CFU and SYTO 9/PI reveal a significant difference between the two H_2O_2-treated samples from the mock (Tukey's HSD $P < 0.05$ for all comparisons). SYTO 9/PI-based % live estimates exhibit larger variance across replicates than CFU. Another difference between CFU and SYTO 9/PI % live estimates is that the latter is a lot higher for the 100 mM H_2O_2-treated samples than the CFU (> 50% *vs*. ~10%). This can be attributed to the two methods measuring different quantities and at different stages - the SYTO 9/PI assay described in this protocol quantifies the fraction of cells with compromised plasma membrane immediately after the treatment, while the CFU counts the number of cells able to recover and form single colonies. It has been shown that cells not accumulating PI after H_2O_2 treatment can undergo secondary necrosis and become PI-positive later[18]. Thus, it is important to understand this difference when applying and interpreting the results of the assay described in this protocol and also other LIVE/DEAD assays based on the same principle.

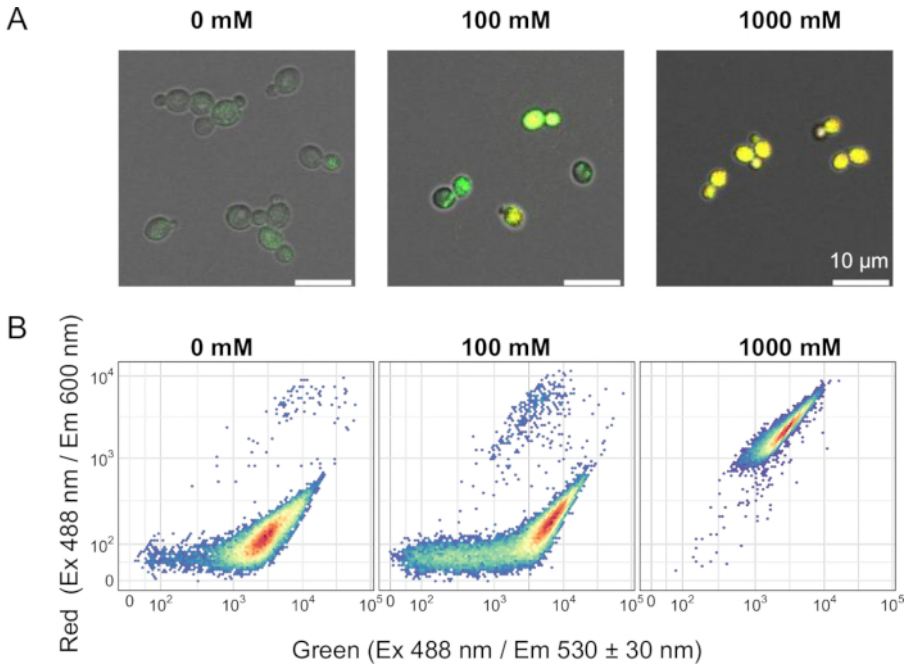

Figure 1: SYTO 9/PI staining distinguishes *C. glabrata* cells treated with different doses of H_2O_2. (**A**) Overlay of fluorescent images of SYTO 9/PI PI-stained *C. glabrata* cells on top of the bright field images. SYTO 9 was imaged with 488 nm excitation and 510-560 nm emission; PI was imaged with 488 nm excitation and 590-700 nm emission on a Laser Scanning Confocal Microscope. Scale bars: 10 µm. (**B**) Flow cytometry results for the same samples as in (**A**). 2D density plots show the distribution of green and red signals for >10,000 cell events. The color gradient corresponds to normalized cell counts in each 2D bin, with red indicating a high density and blue indicating a low density. Please click here to view a larger version of this figure.

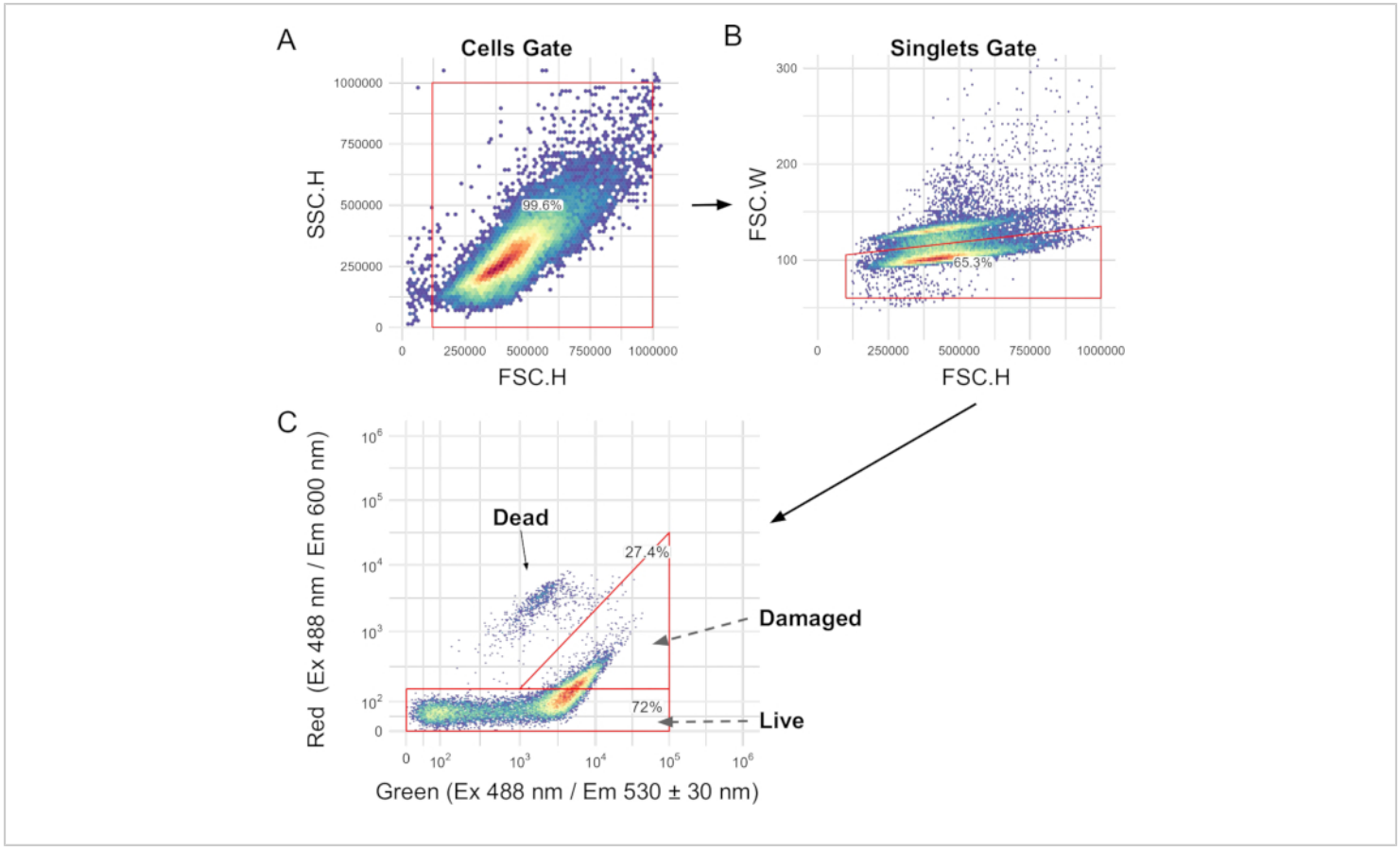

Figure 2: Gating strategies for analyzing flow cytometry results. (**A**) FSC.H and SSC.H parameters were used for excluding non-cell events with a rectangular gate. (**B**) FSC.H and FSC.W were used to distinguish single-cell events (singlets) from aggregates, which have the same FSC.H but higher FSC.W. (**C**) Polygon gates were drawn to identify live, damaged, and dead cells. The percent of events was shown for the first two groups. 2D density plots have the same meaning as in **Figure 1**. Please click here to view a larger version of this figure.

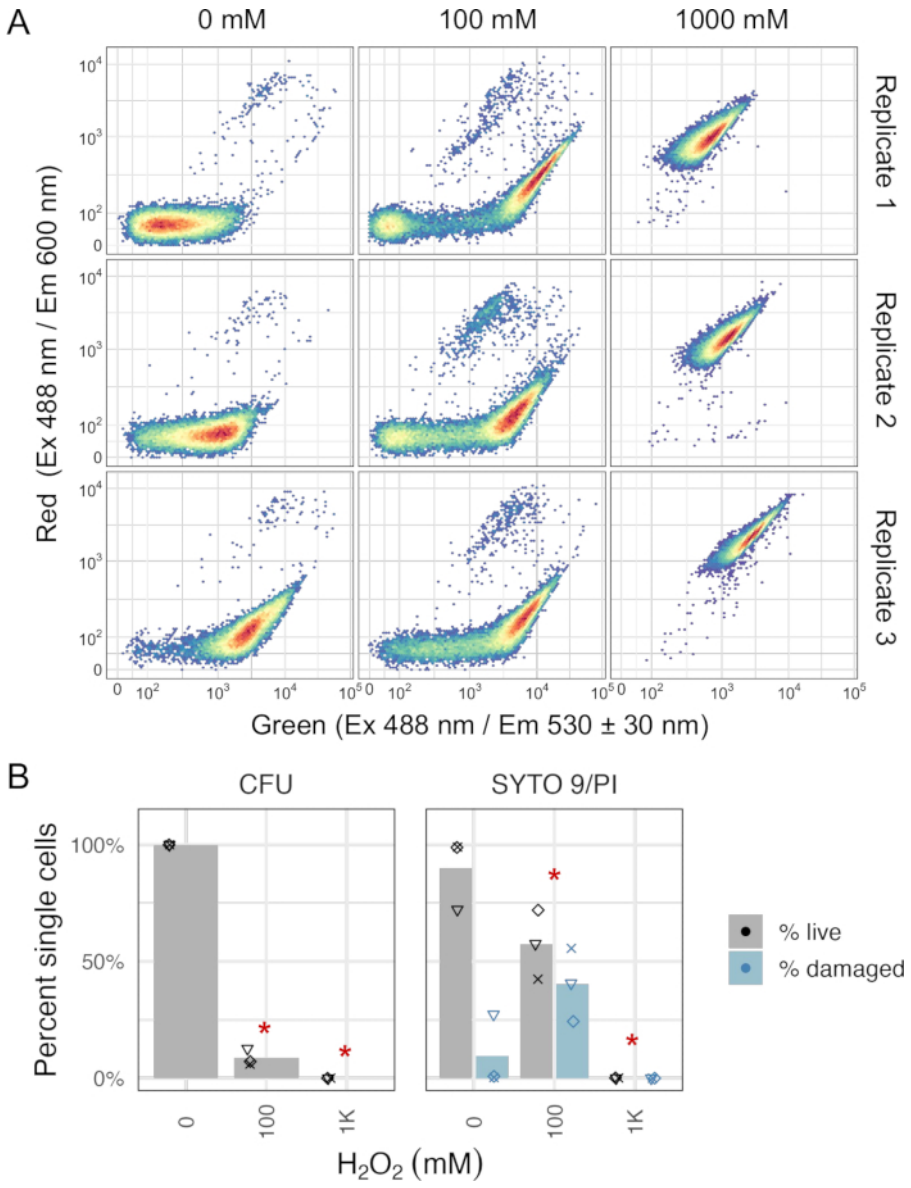

Figure 3: Sample-to-sample variations in SYTO 9/PI staining and comparison to CFU. (**A**) *C. glabrata* cells were treated with the indicated dose of H_2O_2 for 2 h and stained with SYTO 9/PI. Flow cytometry data are shown for three biological replicates in the form of 2D density plots as described in **Figure 1**. (**B**) Comparison of % live quantified by CFU and SYTO 9/PI for the same samples shown in (**A**). Asterisks denote significant differences between an H_2O_2-treated samples and the mock (Tukey's HSD test, $P < 0.05$), % damaged values are shown for information only and not included in the statistical comparisons. Please click here to view a larger version of this figure.

Supplementary File 1: An example sample analysis script. Please click here to download this file.

Discussion

This protocol describes a LIVE/DEAD assay using two fluorescent dyes, SYTO 9 and PI, and using flow cytometry to quantify post-stress survival in yeast. While these two stains have been applied to assess survival in various yeast species in the literature and are available as a commercial kit, neither the manufacturer's manual nor the literature provides sufficient details on the staining protocol or flow cytometry settings[7]. Here, we present a standardized protocol resulting from systematic characterization of the assay, including an optimal staining buffer, dye concentrations, staining time, and flow cytometry settings. Additional experiments described in a separate study apply this assay to diverse yeast species and for other types of stresses, such as antifungal treatments[16]. Thus, the protocol described here may be more generally useful upon further validation.

There are several critical steps in the presented protocol. These include careful pipetting when working with small volumes to ensure consistent dye concentrations, properly setting up the flow cytometer to best distinguish signals from the background (between 10^3 and 10^6 for positive controls and $<10^2$ for negative controls on both fluorescent channels). Once a set of voltage values on different channels was determined for a particular strain and treatment, they should be kept constant to allow for the same gates to be used across samples and for the quantitative estimates to be consistent. To achieve a high signal-to-noise ratio, it is important to follow the incubation time suggested. Short incubation can lead to underestimates of SYTO 9 signals, while both fluorescence signals start to decline after 45 min[16]. Lastly, we found that 0.85% saline buffer minimizes staining artifacts while sterile water or growth media can create either artificial "dead-like" cells or unstained cells[16].

SYTO 9/PI staining measures loss of plasma membrane integrity as an indicator for cell death. CFU, by contrast, quantifies clonogenicity, i.e., the ability of a cell to survive and form a single colony[5]. When SYTO 9/PI staining is applied immediately after the treatment as described in this protocol, the two methods also differ in when they evaluate the survival state of the cells. These reasons can explain the difference in the survival (% live) estimates by the two methods for the 100 mM H_2O_2-treated *C. glabrata* cells (**Figure 3B**). It is therefore important to note that the assay presented here is not a simple replacement of CFU. Instead, one must understand their differences when applying and interpreting the results of the SYTO 9/PI assay.

Notwithstanding their differences, if the goal of an experimenter is to rank or compare the severity of different genotypes or treatment conditions, SYTO 9/PI is a suitable alternative, as its results are consistent in ranking with CFU while being faster and more scalable. Besides, the Nomenclature Committee on Cell Death (NCCD) recommends the usage of a combination of two or more assays to properly identify observed cell death phenotypes[19].

One potential application of this assay is to obtain kinetic information on the cell death process by staining a subculture of the sample at varying times after the treatment. This has been used to reveal distinct features of cell death caused by different stressors[18]. Another use of this assay is to isolate mutants based on survival using Fluorescence Activated Cell Sorting (FACS) in place of flow cytometry. Therefore, this standardized SYTO 9/PI assay adds a valuable tool to the yeast research toolbox.

Several limitations are recognized in the presented method. First, this protocol used fixed polygon gates to measure the percentage of live, damaged, and dead cells. One limitation

of this approach is that the gates need to be adjusted for each new species and stressor. Given the large number of parameters obtainable from flow cytometry, including FSC, SSC, and multiple fluorescence channels, a model-based approach could be developed to automatically identify and quantify these three populations. Furthermore, a distribution of survival scores may be calculated for single cells based on the flow cytometry measurements, circumventing the need for arbitrary grouping and revealing detailed information and heterogeneity of the populations. A second limitation is the higher variance across biological replicates compared with CFU (**Figure 2**), resulting in lower statistical significance at the same sample size. This higher variance can be attributed to variations in staining (dye concentration in small volumes, staining time/condition variability), in flow cytometry (instrument noise), coupled with the use of fixed gates, which don't account for the above. Future efforts will focus on reducing staining efficiency variation and coming up with a flow cytometry quantification that is less dependent or independent of the measurement scales.

What is the best measure for quantifying survival? The widely used CFU assay is simple, robust, and well adopted by the field, but it is labor-intensive and requires days to obtain results. Variations of the plating-based assays have been developed, including recording colony sizes over time with imaging and calculating viability from the "growth curve"[20]. However, these methods are not yet widely adopted due to the need for special equipment and optimization efforts. We believe SYTO 9/PI can be used as an alternative to CFU under certain scenarios to accelerate the experiment and make it more scalable, while it provides complementary information when used alongside CFU assays.

Disclosures

The authors have no conflict of interest.

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