FINAL SCRIPT: APPROVED FOR FILMING

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

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes**

3. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length
Number of Steps: 21
Number of Shots: 42

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

1.1. **Jessica Miller:** We study the phosphate starvation–induced Pho regulon and its role in the evolutionary, genetic, and biochemical differences among related yeast species, focusing on the evolution of stress-responsive genetic networks. The need for frequent, high-throughput quantification of fungal post-stress survival led us to develop this protocol.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

What technologies are currently used to advance research in your field?

1.2. **Bin He:** Colony Form Unit (CFU) assay is the de facto method for quantifying survival in yeast and other single-cellular microbial species. Alternative methods exist but are poorly adopted due to a lack of systematic characterization and comparison with CFU.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

What significant findings have you established in your field?

1.3. **Hanxi Tang:** We established a standardized protocol for quantifying post-oxidative stress survival in the opportunistic pathogen *C. glabrata*. This protocol allows for scalable distinction of live, damaged, and dead cell populations in as little as 30 minutes post-treatment.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.5*

What research gap are you addressing with your protocol?

1.4. **Hanxi Tang:** Even though flow cytometry based fungal survival assays have been applied in the field, we found wide variations in the specific protocols and quantifications. There's a lack of systematic optimization and standardized protocol for these live/dead assays in the field.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

How will your findings advance research in your field?

1.5. **Jessica Miller:** We developed a standardized, scalable method for quantifying survival in *C. glabrata*. Beyond this, the protocol can be used to assess survival and cell damage in yeast mutants, adapted for other yeast species, and applied to diverse stress conditions.

1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: Figure 3*

Videographer: Obtain headshots for all authors available at the filming location.

Testimonial Questions (OPTIONAL):

How do you think publishing with JoVE will enhance the visibility and impact of your research?

1.6. **Jessica Miller, Graduate Student:**

1.6.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Can you share a specific success story or benefit you've experienced—or expect to experience—after using or publishing with JoVE? (This could include increased collaborations, citations, funding opportunities, streamlined lab procedures, reduced training time, cost savings in the lab, or improved lab productivity.)

1.7. **Jessica Miller, Graduate Student:**

1.7.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Protocol

2. Application of the SYTO 9/PI Stain to Quantify Plasma Membrane Integrity

Demonstrator: Jessica Miller

2.1. To begin, prepare at least 10 milliliters of sterile 0.85 percent saline buffer **[1]**. Using sterile, deionized water, prepare a 0.2 millimolar working stock solution of propidium iodide and a 33.4 micromolar working stock solution of SYTO 9 *(Sigh-toe nine)* **[2]**.

2.1.1. WIDE: Talent with 10 milliliters of sterile 0.85 percent saline buffer.

2.1.2. Talent pipetting sterile, deionized water in a labelled microcentrifuge tube.

2.2. Gently mix the *Candida glabrata* cells in each well of the treatment plate **[1]**. Measure the optical density at 600 nanometers for each treatment condition **[2]**. Then, calculate the volume of buffer required to resuspend the cells to an optical density at 600 nanometers of 1 **[3]**.

2.2.1. Talent using a multi-channel pipette to gently mix cells in a 96-well treatment plate.

2.2.2. Talent placing the plate in a spectrophotometer and measuring OD600.

2.2.3. Talent calculating buffer volume based on OD600 values.

2.3. Then, centrifuge the yeast cells after mock or stress treatment at 3,000 *g* for 5 minutes **[1]**. Carefully aspirate and discard the supernatant **[2]**. Resuspend the pellet in sterile saline buffer to an optical density of 1 using the previously calculated value **[3-TXT]**.

2.3.1. Talent placing the samples into a centrifuge and starting the spin.

2.3.2. Talent aspirating the supernatant from each tube with a pipette, leaving the pellet intact.

2.3.3. Talent using a multi-channel pipette to resuspend the cell pellets in the correct volume of sterile 0.85 percent saline buffer. **TXT: Reserve 50 µL from each sample for unstained & single-stain controls**

2.4. Next, aliquot 16 microliters of the post-treatment sample, resuspended in 0.85 percent saline buffer, into a 50-microliter PCR tube **[1]**. Add 2 microliters working stock of the propidium iodide and SYTO 9 to reach a final volume of 20 microliters **[3]**. Gently pipette to mix the contents of the tube **[3]** and incubate the stained sample for 30 minutes in the dark at room temperature **[4]**.

2.4.1. Talent pipetting post-treatment sample into a PCR tube.

2.4.2. Talent adding PI and SYTO 9 working stock to the tube.

2.4.3. Talent gently pipetting to mix the contents of the polymerase chain reaction tube.

2.4.4. Talent placing the tube in a black box and closing the lid to begin incubation.

3. Setting up and Calibrating the Flow Cytometer

3.1. On the flow cytometer, select a 530-nanometer bandpass filter for the green channel and a 600-nanometer long-pass filter for the red channel **[1-TXT]**.

3.1.1. WIDE: Show the flow cytometer interface with BL1 configured for 530 nanometer ± 30 nanometer bandpass and BL3 set for a 600 nanometer long pass filter. **TXT: Use 488 nm blue laser for excitation**

3.2. Set the flow cytometer flow rate to 200 microliters per minute **[1]**. Then, run the sample and stop the acquisition after collecting a minimum of 30,000 events **[2]**.

3.2.1. SCREEN: 3.2.1.-SCREEN-1920x1080.mkv; 00:07-00:11

3.2.2. SCREEN: 3.2.2.-SCREEN-1920x1080.mkv: 00:10-00:20

3.3. Now, dilute 16 microliters of the mock-treated, unstained sample into 200 microliters of sterile 0.85 percent saline buffer **[1]**. Immediately before flow cytometry, gently pipette the sample to mix it **[2]**. Then, run the sample and adjust the voltages of the forward scatter and side scatter channels so that the population appears centered in the forward scatter versus side scatter density plot **[3]**.

3.3.1. Talent pipetting 16 microliters of mock-treated, unstained sample into 200 microliters of sterile saline buffer in a clean tube.

3.3.2. Talent gently pipetting to mix the diluted sample just before placing it into the cytometer.

3.3.3. SCREEN: 3.3.3-SCREEN-1920x1080.mkv: 00:17-00:34, 01:16-01:28, 02:00-02:07

3.4. Next, mix 24 microliters of mock-treated, stained sample with 24 microliters of either 1,000 millimolar hydrogen peroxide-treated or heat-killed stained sample **[1]**.

3.4.1. Talent pipetting equal volumes of live and dead stained samples into a microcentrifuge tube and mixing them.

3.5. Divide the mixed live and dead sample into three separate tubes of 16 microliters each **[1]**. Stain the tubes with 2 microliters each of SYTO 9, propidium iodide, and a mix of SYTO 9 and propidium iodide **[2-TXT]**.

3.5.1. Talent pipetting the sample into 3 tubes.

3.5.2. Talent pipetting SYTO9, PI to the tubes. **TXT: Incubate for 30 min in the dark at RT**

3.6. After incubation, add 200 microliters of sterile 0.85 percent saline buffer to each tube to prepare them for flow cytometry **[1]**.

3.6.1. Talent pipetting 200 microliters of sterile saline buffer into each of the three stained tubes.

3.7. Run tube one, containing SYTO 9-stained cells, to adjust the voltage of the green fluorescence channel **[1]**. Then run tube two, containing propidium iodide-stained cells, to adjust the voltage of the red fluorescence channel **[2]**. Set each channel's intensity distribution mode to between 10^4 and 10^5 to utilize the full dynamic range of the instrument **[3]**.

3.7.1. SCREEN: 3.7.1-SCREEN-1920x1080.MKV: 00:13-00:38.

3.7.2. SCREEN: 3.7.2-SCREEN-1920x1080.MKV: 00:20-00:34, 00:50-01:00

3.7.3. SCREEN: 3.7.3-SCREEN-1920x1080.MKV: 00:17-00:40

3.8. Run tube three, containing cells stained with both SYTO 9 and propidium iodide, to check whether live and dead cell populations are well separated **[1]**. Adjust the voltages on the BL1 or BL3 channels if separation is unclear **[2]**.

3.8.1. SCREEN: 3.8.1-SCREEN-1920x1080.MKV: 00:06-00:08, 00:21-00:35

3.8.2. SCREEN: 3.8.2-SCREEN-1920x1080.MKV: 00:15-00:30

3.9. Then, run the mock-treated, unstained sample using the established voltage settings to confirm minimal autofluorescence in both fluorescence channels, with intensity values remaining below 10^2 **[1]**.

3.9.1. SCREEN: 3.9.1-SCREEN-1920x1080.MKV: 00:22-00:34

4. Running Samples on the Flow Cytometer

4.1. Apply the established flow cytometer settings and run the unstained sample first to confirm that autofluorescence remains below 10^2 in both the BL1 and BL3 channels **[1]**.

4.1.1. SCREEN: 4.1.1.-SCREEN-1920x1080.MKV: 00:05-00:06, 00:23-00:30

4.2. Run the experimental samples on the flow cytometer using the same staining and voltage settings **[1]**. Export the flow cytometry output files in .fcs *(F-C-S)* format using either the FCS 3.0 or 3.1 standard **[2]**.

4.2.1. Talent placing stained experimental samples into the flow cytometer one by one for acquisition.

4.2.2. SCREEN: 4.4.2.SCREEN.MKV: 00:03-00:15

~~4.3. In R, install and load the required packages: **flowCore** *(Flow-Core)*, **ggplot2** *(G-G-Plot-Two)*, and **ggcyto** *(G-G-Cyto)* **[1]**.~~ NOTE: Not filmed

~~4.3.1. SCREEN: Show the R console with the commands for installing and loading the flowCore, ggplot2, and ggcyto packages.~~

~~4.4. Import the .fcs files into the current R environment using an appropriate import function from the **flowCore** package **[1]**. Adjust the file names and assign treatment conditions accordingly for downstream analysis **[2]**.~~

~~4.4.1. SCREEN: Display the R console loading .fcs files.~~

~~4.4.2. SCREEN: Show R code that renames sample files and adds treatment conditions in a data frame or flowSet object.~~

4.5. Generate a two-dimensional density plot of all events using FSC.H *(F-S-C-H)* against SSC.H *(S-S-C-H)* **[1]**. Draw a polygon gate to exclude non-cell events and name the gate "cells" **[2]**.

4.5.1. SCREEN: LIVEDEAD-Assay-Analysis-in-Attune-Software.mp4: 00:00-00:16

4.5.2. SCREEN: LIVEDEAD-Assay-Analysis-in-Attune-Software.mp4: 00:17-00:37

4.6. For the population gated as "cells", create a two-dimensional density plot using FSC.H against FSC.W **[1]**. Identify the cluster with a lower mean FSC.W as the single-cell population and draw a polygon gate around it, naming it "singlets" **[2]**.

4.6.1. SCREEN: LIVEDEAD-Assay-Analysis-in-Attune-Software.mp4: 00:58-01:02, 01:15-01:19

4.6.2. SCREEN: LIVEDEAD-Assay-Analysis-in-Attune-Software.mp4: 01:22-01:32, 02:06-02:11

4.7. For the gated "singlets" population, create a two-dimensional density plot using BL1.H against BL3.H **[1]**. Based on control samples of live and dead cells, draw polygon gates

to define live, damaged, and dead cell populations **[2]**.

4.7.1. SCREEN: LIVEDEAD-Assay-Analysis-in-Attune-Software.mp4: 02:17-02:39

4.7.2. SCREEN: LIVEDEAD-Assay-Analysis-in-Attune-Software.mp4: 03:33-03:46, 04:39-04:49

4.8. Calculate the percentages of live, damaged, and dead cells based on the gated populations **[1]**. Export these statistics to a data table or file for downstream analysis **[2]**.

4.8.1. SCREEN: LIVEDEAD-Assay-Analysis-in-Attune-Software.mp4: 05:07-05:19

4.8.2. SCREEN: LIVEDEAD-Assay-Analysis-in-Attune-Software.mp4: 06:45-06:57

Results

5. Results

5.1. Confocal microscopy showed uniformly green, intact plasma membranes in mock-treated *Candida glabrata* cells **[1],** while 100 millimolar hydrogen peroxide induced stronger green fluorescence with some yellow cells **[2],** and 1,000 millimolar treatment resulted in predominantly yellow cells, indicating complete membrane compromise and dye uptake **[3].**

5.1.1. LAB MEDIA: Figure 1A. *Video editor: Highlight the green-colored cells in the 0 millimolar panel.*

5.1.2. LAB MEDIA: Figure 1A. *Video editor: Highlight the mixture of green and yellow cells in the 100 millimolar panel.*

5.1.3. LAB MEDIA: Figure 1A. *Video editor: Highlight the bright yellow cells in the 1,000 millimolar panel.*

5.2. Flow cytometry analysis showed that treatment with 100 millimolar hydrogen peroxide caused a noticeable increase in green fluorescence intensity compared to untreated cells **[1].** In contrast**,** treatment with 1,000 millimolar shifted the population strongly towards red fluorescence **[2].**

5.2.1. LAB MEDIA: Figure 1B. *Video editor: Highlight the denser and right-shifted population in the green axis of the 100 millimolar panel.*

5.2.2. LAB MEDIA: Figure 1B. *Video editor: Highlight the upward-shifted population in the red axis of the 1,000 millimolar panel.*

5.3. Three populations—live, damaged, and dead—were separated based on green and red fluorescence intensity, with live cells showing strong green and low red signals **[1],** damaged cells showing elevated green and red signals **[2],** and dead cells showing dominant red signals **[3].**

5.3.1. LAB MEDIA: Figure 2C. *Video editor: Highlight the cluster at the lower-right labeled "Live".*

5.3.2. LAB MEDIA: Figure 2C. *Video editor: Highlight the intermediate area labeled "Damaged".*

5.3.3. LAB MEDIA: Figure 2C. *Video editor: Highlight the upper-left region labeled "Dead".*

5.4. Flow cytometry across three replicates showed consistent SYTO 9 and Propidium iodide patterns at each hydrogen peroxide concentration, with only minor variations in fluorescence intensity **[1].**

5.4.1. LAB MEDIA: Figure 3A.

5.5. Colony-forming assays showed a sharp drop in viability at 100 millimolar hydrogen peroxide **[1]** and near-zero survival at 1,000 millimolar **[2],** whereas the SYTO 9 and propidium iodide assay overestimated viability at 100 millimolar by detecting membrane-intact but non-culturable cells **[3].**

5.5.1. LAB MEDIA: Figure 3B *(left panel: CFU). Video editor: Highlight the dramatic drop in gray bars between 0 and 100 millimolar.*

5.5.2. LAB MEDIA: Figure 3B *(left panel: CFU). Video editor: Highlight the near-zero bar at 1,000 millimolar.*

5.5.3. LAB MEDIA: Figure 3B (right panel: SYTO 9/PI). *Video editor: Highlight the tall gray bar labeled "% live" at 100 millimolar.*

1. **Candida glabrata**
   Pronunciation link: https://www.howtopronounce.com/candida-glabrata (How To Pronounce)
   IPA (American): /kænˈdɪdə gləˈbreɪtə/
   Phonetic: kan-DIH-duh gluh-BRAY-tuh
2. **propidium iodide**
   Pronunciation link: https://www.howtopronounce.com/propidium-iodide
   IPA: /prəˈpɪdiəm ˌaɪəˈdaɪd/
   Phonetic: pruh-PID-ee-um eye-uh-dide
3. **SYTO** (as in "SYTO 9")
   Pronunciation link: https://www.howtopronounce.com/syto
   IPA: /ˈsaɪtoʊ/
   Phonetic: sigh-TOE
4. **fluorescence**
   Pronunciation link: https://dictionary.cambridge.org/pronunciation/english/fluorescence (Cambridge Dictionary)
   IPA: /flɔːˈresəns/
   Phonetic: fluh-RESS-ens
5. **optical**
   (Standard English dictionaries will have it, e.g. Merriam-Webster)
   IPA: /ˈɑptɪkəl/
   Phonetic: OP-ti-kul
6. **centrifuge**
   (Standard English dictionaries—Merriam-Webster)
   IPA: /ˈsɛntrəˌfjuːdʒ/
   Phonetic: SEN-truh-fyoohj
7. **microcentrifuge**
   (compound of micro + centrifuge)
   IPA: /ˌmaɪkroʊˈsɛntrəˌfjuːdʒ/
   Phonetic: MY-kroh-SEN-truh-fyoohj
8. **incubate**
   IPA: /ˈɪnkyubeɪt/ (or /ˈɪnkjuˌbeɪt/)
   Phonetic: IN-kyoo-bayt
9. **compensation** (in flow cytometry context)
   (term for correcting spectral overlap) (Wikipedia)
   IPA: /ˌkɑmpənˈseɪʃən/
   Phonetic: kom-puhn-SAY-shuhn
10. **cytometer**
    Pronunciation link: https://www.howtopronounce.com/flow-cytometer (How To Pronounce)
    IPA: /saɪˈtɑmɪtər/
    Phonetic: sigh-TAH-muhter

11. **spectrophotometer**
    (common lab instrument name)
    IPA: /ˌspɛktroʊˈfɑtəmɪtər/
    Phonetic: spek-troh-FAH-toh-mihter
12. **microliter**
    IPA: /ˈmaɪkrəˌliːtər/
    Phonetic: MY-kruh-LEE-ter