An Optimized LIVE/DEAD Assay Using Flow Cytometry to Quantify Post-Stress and Antifungal-Treatment Survival in Diverse Yeasts

Hanxi Tang[1^], Jinye Liang[1*], Bin Z. He[1]

[1] Department of Biology, the University of Iowa, Iowa City, IA, 52241, USA

^ Current address: Department of Chemical and Systems Biology, Stanford University, Stanford, CA, 94305, USA

* Current address: Molecular and Computational Biology Section, Department of Biological Sciences, University of Southern California, Los Angeles, CA 90089, USA

Abstract

Quantifying post-stress survival in yeasts is crucial for biological, biomedical, and industrial research. Traditional methods like Colony Forming Unit (CFU) assays are labor-intensive and time-consuming. In this study, we systematically characterize a two-dye (SYTO 9 / Propidium Iodide) LIVE/DEAD assay coupled with flow cytometry to rapidly and scalably quantify post-stress survival in diverse yeast species. By optimizing staining buffer, dye concentrations and staining time, we minimized artifacts and improved reproducibility. Notably, we identify an "Intermediate" population containing damaged cells with enhanced SYTO 9 uptake but little propidium iodide (PI) accumulation under sublethal stress, providing finer gradations of cellular damage compared to CFU or PI staining alone. We demonstrate the assay's applicability across *Candida glabrata*, *Saccharomyces cerevisiae*, and *Candida albicans* after hydrogen peroxide treatment and in *C. glabrata* after Amphotericin B exposure. While CFU is more sensitive at lower stress levels, the SYTO 9/PI staining effectively distinguishes sublethal from lethal doses, offering a valuable alternative for rapid, high-throughput survival quantification.

Introduction

The budding yeast subphylum (Saccharomycotina) contains many important biological and biotechnological models as well as important human fungal pathogens, including *Candida albicans* [1], *Candida glabrata* [2], and the newly emerged *Candida auris* [3]. A common and critical task in the studies of this group, including both biology and biomedical research [4] as well as in environmental monitoring [5] and the food industry [6], is to quantify survival after stress or drug-treatment. The most widely-used and accepted method is the Colony Forming Unit (CFU) assay, which is relatively easy to perform and robust, but can be labor-intensive when assaying large numbers of samples and time-consuming due to the need to wait for single colonies to form. Therefore, there is a need for alternative methods that are fast and scalable for quantifying post-stress survival in this group.

There are two common measures used to quantify post-stress or post-drug treatment outcomes. The first one is represented by CFU, which measures viability, or the ability of cells to survive and reproduce to generate single colonies. The second is survival, or the lack thereof, i.e., cell death. Viability and survival (or cell death) measure related but distinct quantities: cells that are alive after the stress treatment could either die later or stay in a non-proliferative state, such as senescence [7], such that they do not contribute to the growth quantified by CFU. Conversely, CFU can be influenced by the additional stress introduced when performing dilutions and plating, and also the media and culturing conditions, which may lead to underestimates of the number of viable cells. Adding to the complication is the lack of a universal definition, hence methods to quantify cell death [8,9]. In this study, we follow the Nomenclature Committee on Cell Death and define cell death as the irreversible loss of plasma membrane integrity [9,10]. In yeast, a common way of monitoring plasma membrane integrity

49 relies on the differential ability of certain dye molecules to penetrate intact *vs.* compromised
50 plasma membranes, leading to a number of LIVE/DEAD assay reagents, most of which
51 fluoresces upon binding nucleic acids [11].

52 Currently available assays to quantify post-stress outcomes present many limitations.
53 CFU is robust and easy to perform, and its results are generally comparable between labs and
54 studies. However, it is time consuming and labor intensive when assessing multiple samples. In a
55 hypothetical experiment involving four samples with three replicates, to achieve ~30-500
56 individual colonies per plate, three 10x dilutions of each sample are needed, resulting in nine
57 plates per sample [12]. For *S. cerevisiae*, typically 48 hours of incubation is required for
58 individual colonies to grow to a sufficient size for reliable counting [13]. This time can be even
59 longer for other species. While modified CFU protocols have enabled higher throughput and
60 automated counting, they require special setup or optimization, which limited their wide
61 adoption [14–16]. As a result, manual counting of CFU is still a routine task performed in many
62 microbiology labs.

63 Fluorescence dye-based methods are fast – mostly requiring 10-30 minutes of incubation.
64 Coupled with flow cytometry, they provide fast, scalable and single-cell level information on
65 survival. However, their application is significantly limited by a lack of standardized protocols
66 and no systematic comparison with CFU results. In the literature, live/dead staining are nearly
67 always used with microscopy to provide a qualitative representation of the survival proportion
68 rather than yielding quantitative information [17]. In the few studies where quantification was
69 performed, method details were scarce and varied [18,19].

70 Our goal in this study is to systematically characterize and optimize a fluorescence dye-
71 based LIVE/DEAD assay for quantifying post-stress outcome in yeasts. We chose a two-dye kit

72 composed of SYTO 9 and propidium iodide (PI). SYTO 9 labels all cells, live or dead, while PI
73 is excluded from live cells with intact plasma membranes, hence differentiating between them.
74 Moreover, the excitation and emission spectra of the two dyes make them an efficient pair for
75 Förster Resonance Energy Transfer (FRET, Fig. 1A). FRET plus competitive exclusion of SYTO
76 9 by PI, whose affinity for DNA is higher [20], lead to a strong red fluorescence and muted green
77 emissions in dead cells compared with live cells, hence better separating the two populations
78 than using PI alone (Fig. 1B). This combo was initially developed for quantifying bacterial
79 survival in environmental samples, known as BacLight [21]. Later it was applied to the baker's
80 yeast *S. cerevisiae* [19] and marketed as "FungaLight". Despite its potential as a fast and scalable
81 alternative for survival quantification, its use in the yeast literature is spotty and limited to mostly
82 qualitative assessment. For example, when FungaLight was used to quantify the effect of
83 antifungals on *C. albicans* survival, results were presented as microscopy images and were not
84 quantified [22–29]. In the few studies where samples were assayed with flow cytometry, critical
85 information such as the staining procedure, flow cytometry settings and gating strategies were
86 often missing, making it difficult to compare or reproduce their results [18,19,30]. We believe
87 the lack of systematic characterization of the method and standardization of the protocol is a
88 major factor limiting the use of STYO 9/PI in quantitative assessment of post-stress survival.
89 In this study, we systematically optimized the SYTO 9/PI LIVE/DEAD stain for use with
90 flow cytometry to quantify post-stress survival in diverse yeast species. We found distinct
91 staining patterns in cells that were damaged from those that were dead. We applied the optimized
92 protocol to quantify the survival of several yeast species after stress or drug treatments, showing
93 its broad applicability.

RESULTS

SYTO 9/PI staining for H_2O_2-treated *C. glabrata* cells show complex patterns and high sample-to-sample variability

We applied SYTO 9/PI staining on *C. glabrata* cells treated with different doses of H_2O_2 from 0 mM (mock) to a lethal dose of 1 M for 2 hours. We followed the manufacturer's protocol for FungaLight and recorded the fluorescence signals in the green (530±30 nm) and red (600 nm long pass, or LP) channels with a blue laser (488 nm) as the excitation (Materials and Methods). At the highest H_2O_2 dose, all cells exhibited low green and high red fluorescence as expected for cells with a compromised plasma membrane (Fig. 1C). In mock-treated samples, the majority of the cells exhibited higher green than red signals, consistent with the expected patterns for live cells. However, we observed a subpopulation (red polygon gate) that exhibited "dead-like cell" features: they had lower green and high red signals, with the red fluorescence intensity comparable with or exceeding that of the dead cells (1 M H_2O_2-treated). The fraction of this subpopulation varied between 3.5%-13.2% across three biological replicates. This was unexpected given that the cells were taken from an exponentially growing population. Indeed, when we measured the CFU of the mock-treated sample and compared it to the number of cells plated (estimated by flow cytometry), we obtained a nearly 1:1 ratio (Fig. S1).

Compared with the mock, we observed an increase in the "dead-like cell" frequency in the 10 mM H_2O_2-treated samples, varying from 10% to 50% across replicates. There was also a subset of cells with very low red and green signals (orange arrowhead), which we hypothesize are unstained cells. At the severe 100 mM H_2O_2 dose, rather than a further increase in the fraction of "dead-like cell" population, we observed an increase in the population of cells with high-red and high-green signals (magenta arrowhead). Overall, the patterns we observed for

117 H_2O_2-treated *C. glabrata* cells were more complex than what was shown in the FungaLight
118 manual for a mixture of live and dead cells (Fig. 1B). There were also large sample-to-sample
119 variations that prevented the assay from being reliably used to quantify post-stress survival
120 outcomes. In the sections that follow, we systematically tested different parts of the staining
121 protocol to improve the reproducibility and resolve some of the unexpected patterns.

122 **Using 0.85% saline as the staining buffer minimizes the dead-like and unstained cell**
123 **populations**

124 The FungaLight manual recommended washing and resuspending the cells in "appropriate
125 buffer" and didn't discuss the potential effects of the buffer on the staining pattern. In our initial
126 test, we washed and resuspended cells in room temperature deionized water. To test if staining
127 buffer choice affects staining results, we compared four buffers, i.e., deionized water, Phosphate
128 Saline Buffer (PBS), 0.85% saline solution and Synthetic Defined (SD) media, the last being the
129 base media in our experiments. The same mock- or H_2O_2-treated cultures were split four-ways,
130 washed, resuspended and stained in the respective buffer, and the staining results were compared
131 (Fig. 2A). We found that deionized water resulted in a large amount of "dead-like" staining
132 events (~60%, Fig. 2B top). SD media resulted in the lowest amount of "dead-like" cells but also
133 produced far more unstained cells than the other three buffers (Fig. 2B, lower). By comparison,
134 1xPBS and 0.85% saline solution both provided a low amount of "dead-like" and unstained cells
135 (Fig. 2B), supporting their use as the buffer for FungaLight staining. In our test, 0.85% saline
136 performed slightly better than PBS. Hence, we use 0.85% saline for all subsequent experiments.

137 **Optimizing dye concentrations, staining time, and dye ratios**

138 We then investigated the concentration and staining time for each dye individually as potential
139 factors that could influence the result. The manual recommended using SYTO 9 and PI at 1000x

140 dilutions from the 3.34 mM and 20 mM stock solutions, respectively. For both dyes, we tested
141 250x, 500x, 1000x and 2000x dilutions. For SYTO 9, we found that at 500x and 250x dilutions,
142 more cells in the mock-treated sample fell within the "high-red and high-green" population seen
143 in the 100 mM H_2O_2-treated sample (19% and 70%, respectively, Fig. S2A). Conversely, a
144 2000x dilution resulted in >90% events with a green fluorescence less than 10^2, suggesting they
145 were not stained. We therefore conclude that 3.34 uM (1000x dilution) is optimal for SYTO 9.
146 For PI, we found minimal variation at the four dilutions tested for the mock-treated sample (Fig.
147 S2A).

148 Fluorescence dyes take time to penetrate cells and bind to their target molecules, after
149 which their signal could decay over time due to instability and photobleaching [31]. The manual
150 recommended incubating the sample with the dyes for 15-30 minutes at 37°C and protected from
151 light. We investigated changes in the SYTO 9 and PI's signal intensity as a function of time
152 when incubated at room temperature in the dark. Using exponential phase *C. glabrata* cells, we
153 found that SYTO 9 signals plateaued at 15 min and didn't change significantly up until 55 min
154 post-staining, suggesting that an incubation time between 15-50 minutes can be used for SYTO 9
155 (Fig. S2B). Because PI is not permeable to live cells, we used heat-killed *C. glabrata* cells and
156 found that the PI signal intensity reached a plateau within 5 minutes and began to decrease after
157 35 minutes (Fig. S2C). Together, we found the recommended 15-30 minutes of staining in the
158 dark to be optimal for the combination of the two dyes.

159 We also tested different PI-to-SYTO 9 ratios, reasoning that the relative concentration of
160 PI to SYTO 9 could affect the competitive exclusion and FRET between the two. However, we
161 didn't find significant differences in the staining pattern between 2:1, 1:1 and 1:2 ratios (1000x
162 dilutions used as the base level) (Fig. S2D).

163 In summary, our systematic tests suggest that PI and SYTO 9 should be used at a 1000x
164 dilution as the manual recommends; samples should be incubated with both dyes in the dark for
165 15 to 30 minutes at room temperature for both dyes to reach their stable signal intensity and
166 assayed within 45 minutes.

167 **Sublethal dose of oxidative stress results in enhanced SYTO 9 staining but not PI**

168 In our initial test of SYTO 9/Pi staining on H_2O_2-treated *C. glabrata* cells, we observed an
169 unexpected population of cells with a high-green and high-red fluorescence (Fig. 1C, magenta
170 arrowhead). This population persisted after we optimized the protocol, suggesting it is not a
171 staining artifact. We noted that cells in this population showed stronger fluorescence than live
172 cells in both channels, and they are distinct from dead cells, which have enhanced red and mute
173 green fluorescence due to FRET and competitive exclusion of SYTO 9 by PI. Because SYTO 9
174 fluorescence is stronger than PI at the experimental conditions [20], and its emission bleeds into
175 the red channel (Fig. 1A), we hypothesize the high-green and high-red fluorescence population
176 contain cells with a higher level of SYTO 9 than live cells but little PI. Furthermore, because this
177 population is more prominent in the 100 mM H_2O_2-treated sample, we hypothesize that severe,
178 sublethal doses of H_2O_2 resulted in a population of oxidatively damaged cells that are more
179 permeable to SYTO 9 but not yet permeable to PI. This hypothesis is consistent with the finding
180 that in some bacterial species dead cells accumulate more SYTO 9 than live cells [31].

181 To test the hypothesis that the high-green and high-red signals are due to an increased
182 SYTO 9 level, we performed single-dye staining for *C. glabrata* cells treated with increasing
183 doses of H_2O_2 and quantified their emission patterns in both the green and red channels (Fig.
184 3A). Consistent with our hypothesis, SYTO 9-stained cells showed increased fluorescence when
185 the concentration of hydrogen peroxide increased (Fig. 3A). In the mock sample, only ~1% of

186 the cells had a red fluorescence level greater than 10^2; this proportion jumped to ~25% and ~50%
187 in the 100 mM and 1 M H_2O_2 treated samples (Fig. 3B, Holm-Bonferroni corrected Student's t-
188 test $P < 0.05$ when compared with the mock). By comparison, cells stained with PI alone only
189 showed a significant increase in the proportion of "PI-positive" cells at the lethal 1 M H_2O_2 dose
190 (Fig. 3B, Holm-Bonferroni corrected Student's t-test $P < 0.5$ compared to the mock). We also
191 calculated the median fluorescence intensity (MFI) for all cell events in the SYTO 9 stained
192 samples, confirming that the MFI increased significantly in both green and red channels at the
193 100 mM and 1 M H_2O_2 doses (Fig. 3C). We conclude that at the sublethal dose of 100 mM H_2O_2,
194 ~25% of the *C. glabrata* cells became more permeable to SYTO 9 but not yet to PI. This likely
195 leads to the "Intermediate" high-green and high-red subpopulation in the SYTO 9/PI staining
196 (Fig. 1C). At the lethal 1 M dose, however, cells become more permeable to both SYTO 9 and
197 PI. Competitive exclusion of SYTO 9 by PI and FRET between the two result in enhanced red
198 and reduced green fluorescence relative to live and the "Intermediate" population.

199 **SYTO 9/PI is less sensitive than CFU at medium levels of H_2O_2 but can better distinguish**
200 **sublethal from lethal H_2O_2 doses**

201 Having optimized the staining protocol for SYTO 9/PI (referred to as FungaLight from hereon),
202 we next compared its result to two widely cited methods for quantifying post-stress or drug
203 treatment outcomes, i.e., CFU and PI staining. The former is generally accepted as the standard
204 method for viability while the latter is frequently used to quantify live/death based on plasma
205 membrane permeability [32].

206 To compare these methods, we treated *C. glabrata* cells with 0, 10, 100, and 1000 mM of
207 H_2O_2 for 2 hours. The same post-treatment sample was used for CFU as well as PI or FungaLight
208 staining in separate vials. The percent viable/live cells were quantified and compared. For PI, we

209 gated for live cells only; for FungaLight, we additionally gated for the "Intermediate"
210 population, which we hypothesize contains oxidatively damaged cells that are more permeable to
211 SYTO 9 but not yet permeable to PI (Fig. 4A).

212 PI-staining detected a significant drop in the percent-live cells only at the lethal 1 M
213 H_2O_2 dose (Fig. 4B, middle, Tukey's range test $P < 0.05$). By contrast, CFU results revealed a
214 significant decrease in viability at the 100 mM and 1M H_2O_2 doses compared with the mock and
215 10 mM H_2O_2-treated samples (Fig. 4B). While a drop in CFU in the 10 mM H_2O_2 treated sample
216 was observed compared with the mock, the difference was not significant by Tukey's range test
217 (aka Tukey's HSD) at the 0.05 level. The difference in CFU between the 100 mM and 1 M
218 H_2O_2-treated samples was also insignificant (Fig. 4B).

219 Similar to CFU, FungaLight percent-live estimates also revealed a significant decrease in
220 survival in the 100 mM and 1 M H_2O_2-treated samples compared with the mock and 10 mM
221 H_2O_2-treated ones. However, FungaLight estimates for the 100 mM H_2O_2-treated sample were
222 ~50%, much higher than its CFU and the difference between this condition and the 1 M H_2O_2
223 treatment was significant (Fig. 4B, right). It is worth noting that the two methods measure
224 different quantities and therefore their values are not directly comparable – CFU quantifies the
225 ability of a cell to recover from the oxidative stress and reproduce to form a colony 48-72 hours
226 post treatment, while FungaLight measures the plasma membrane permeability immediately after
227 the treatment. Nonetheless, the goal of our study is to compare these two methods at a practical
228 level for quantifying post-stress outcomes.

229 Since FungaLight revealed a significant difference between the 100 mM and 1M H_2O_2-
230 treated samples, we asked if it is able to resolve different levels of damage caused by a gradient
231 of sublethal doses of oxidative stress. To test this, we treated *C. glabrata* cells with a linear

232 gradient of H_2O_2 from 100 mM to 1 M, then subjected the treated samples to both CFU and
233 FungaLight staining. For the latter, 2D density plots showed distinct fluorescence patterns
234 between the mock, 100 mM and 250 mM H_2O_2-treated samples; above 250 mM, all events
235 appeared in the "dead" population (Fig. 4C). Quantitatively, we found that CFU was able to
236 detect a significant difference between all non-zero doses of H_2O_2 vs the mock sample as well as
237 a significant difference between the 100 mM dose against all higher doses by Tukey's range test
238 at a 0.05 level (Fig. 4D top, only significant tests were labeled below the plot). FungaLight
239 detected a significant difference between doses at 250 mM or above vs either the mock or 100
240 mM H_2O_2-treated sample, but not between the 100 mM H_2O_2-treate and the mock sample (Fig.
241 4D bottom). In general, we observed a higher sample-to-sample variation in the FungaLight
242 results than CFU, limiting its power to distinguish post-stress survival especially at a lower dose.
243 However, we noticed that the percent of "Intermediate" population was significantly different
244 between the 250 mM H_2O_2 dose and all other doses. This extra dimension offers additional
245 information and provides more gradation in assessing the outcomes of oxidative stress.
246 In summary, we found that PI-staining only detects changes when a lethal H_2O_2 dose of 1
247 M was used. Notably, PI estimated percent-live values for 100 mM H_2O_2-treated samples were
248 close to 100% even though CFU shows <10% of the cells were viable following the treatment.
249 Compared with CFU, FungaLight (SYTO 9/PI) can similarly differentiate the sublethal dose of
250 100 mM from the mock and mild 10 mM dose. While it exhibited a higher sample-to-sample
251 variance, limiting its power in detecting differences at a lower dose of H_2O_2, it revealed more
252 information through changes in the percent "Intermediate" population at 100 mM and 250 mM
253 H_2O_2 doses. These observations suggest that FungaLight can be a useful alternative to CFU for
254 quantifying post stress survival especially at higher, sublethal doses.

255 **FungaLight can be used to quantify post-stress or drug treatment survival across diverse**
256 **yeast species**
257 To determine if the optimized FungaLight assay works in other yeast species, we applied it to
258 H_2O_2-treated *S. cerevisiae* and *C. albicans* cells. We chose these two species as they are both the
259 most important and frequently studied yeasts and also at different evolutionary distance from *C.*
260 *glabrata*: the baker's yeast *S. cerevisiae* diverged from *C. glabrata* ~80 million years ago post
261 the Whole Genome Duplication (WGD) event while *C. albicans* diverged from both *S.*
262 *cerevisiae* and *C. glabrata* approximately 240 million years ago prior to the WGD (Shen et al
263 2018). We used different concentrations of H_2O_2 for the two species based on our preliminary
264 experiments with the goal of selecting a serial dilution series of the oxidant to achieve
265 comparable CFU-based viability as compared to our results for *C. glabrata*. Despite the literature
266 reporting a higher H_2O_2 resistance in *C. albicans* compared to *S. cerevisiae* [33], we found
267 similar resistance levels in the two species, both lower than that of *C. glabrata*. We chose 0.6
268 and 6 mM in place of 10 and 100 mM as did in *C. glabrata*. We kept the 1 M as the lethal dose.
269 We applied the same FungaLight protocol to both species after 2 hours of H_2O_2
270 treatment. The fluorescence patterns in the red and green channels are very similar to what we
271 found in *C. glabrata*. However, the absolute intensities shifted between species. We manually
272 adjusted the live and intermediate cell gates for each of the two species to capture the same
273 population based on visual inspections (Fig. 5A, Methods). We also performed CFU assays on
274 the same samples as we did FungaLight staining with, then compared CFU with FungaLight
275 percent live and percent intermediate values (Fig. 5B, C). In both species, CFU estimates were
276 significantly different between the mock and all three H_2O_2-treated samples as well as between
277 different doses of H_2O_2 except for the 6 mM vs 1 M comparison (Fig. 5B, C, top). FungaLight

278 percent live values were much higher in the 0.6 and 6 mM H_2O_2-treated groups than CFU in both
279 species. In *S. cerevisiae*, significant differences were detected in all pairwise comparisons for the
280 percent live values except for the mock vs 0.6 mM pair (Fig. 5B, bottom). In *C. albicans*, while
281 the general trend was similar, a higher sample variance resulted in non-significant differences for
282 most of the percent live value comparisons except for those involving the 1 M H_2O_2-treated
283 group (Fig. 5C, bottom). Percent intermediate values were the highest in the 6 mM group, which
284 were significantly higher than the rest in *S. cerevisiae*, but not in *C. albicans* (Fig. 5B, C,
285 bottom). These results showed that the FungaLight assay can be used to quantify post-oxidative
286 stress survival in diverse yeast species; its results are consistent in ranks with CFU. Similar to
287 our observation in *C. glabrata*, FungaLight can have a higher sample-to-sample variance, but it
288 is useful in distinguishing between medium to lethal doses while CFU is more sensitive towards
289 lower doses. Also, we note that while the same protocol is applicable across multiple species, the
290 percent live and intermediate gate boundaries need to be adjusted for each species based on their
291 mock-treated and dead cell controls.

292 To determine if FungaLight can be used to quantify post-treatment outcomes beyond the
293 oxidative stress, we applied the same protocol to *C. glabrata* cells treated with Amphotericin B
294 (AmpB), a frontline antifungal used to treat systemic candidiasis [34]. We observed a similar
295 "live → intermediate → dead" shift in the FungaLight staining pattern with increasing doses of
296 AmpB from 0 to 1 ng/mL, showing the applicability of FungaLight in this application (Fig. 6A).
297 Viability estimates based on CFU showed a roughly linear decrease with an increasing AmpB
298 dose (Fig. 6B). Significant differences were determined by Tukey's range test and shown at the
299 bottom of Fig. 6B. FungaLight estimates showed a larger sample variance. Percent live events
300 showed significant differences only between the two or three highest doses when compared with

301 the mock or the 0.04 ng/mL dose (Fig. 6C). Percent intermediate values were markedly higher in
302 the 0.08 and 0.1 ng/mL AmpB-treated groups: there were significant differences between these
303 two groups and the 0.04 ng/mL or the 1 ng/mL-treated groups (Fig. 6C). Finally, we observed a
304 strong linear correlation between the percent live values and CFU in the AmpB-treated *C.*
305 *glabrata* cells (Fig. 6D, Pearson's r = 0.94, Spearman's ρ = 0.98).

306 In summary, we successfully applied the FungaLight assay to quantify post-oxidative
307 stress survival in three yeast species, as well as post-antifungal survival in one of them, *C.*
308 *glabrata*, demonstrating the broad applicability of the assay and our optimized protocol.

309 **Discussion**

310 Quantifying post-stress and drug treatment outcomes is a routine yet critical task in studies of
311 diverse yeast species for basic, medical and industrial purposes. In this study we systematically
312 characterized the two-dye LIVE/DEAD assay: SYTO 9/PI, aka FungaLight. Through rigorous
313 testing of buffer conditions, we found that 0.85% saline buffer minimizes staining artifacts such
314 as unstained cells and "dead-like cell" populations, which were present in DI water and growth
315 media-based staining buffer applications. We also determined the optimal dye concentrations and
316 staining incubation time. Interestingly, we discovered that at sublethal doses of oxidative stress,
317 an "Intermediate" cell population exhibited distinct fluorescence patterns than both the live and
318 dead cell populations. Evidence supported our hypothesis that this population includes damaged
319 cells with increased SYTO 9 uptake but minimal PI accumulation. This additional information
320 enhanced the resolving power of FungaLight especially between medium to high doses of stress.
321 Lastly, we applied the optimized FungaLight assay to two other yeasts and after an antifungal
322 treatment, demonstrating its broad utility as a complementary method to traditional CFU assays.

323 Dye-based methods like FungaLight when coupled with flow cytometry are fast and
324 scalable. Compared with CFU, which requires multiple dilutions and a lengthy incubation period
325 for single colonies to emerge, a typical FungaLight assay can be done within 2 hours post stress
326 or drug treatment. Using the autosampler attachment, we can conveniently examine a large
327 number of samples in a 96-well format, further enhancing the scalability of the assay. Another
328 advantage of FungaLight is that it reveals finer gradations in the damage to the cells caused by
329 the stress or drug. Compared with CFU and single dye staining like propidium iodide (PI), which
330 classifies cells into either live or dead, FungaLight staining reveals a transitional population of
331 cells under sublethal stress levels, enhancing our ability to distinguish the outcomes at sublethal
332 doses from those at lethal levels.

333 We also recognized several limitations of FungaLight in our characterization. The most
334 important limitation is the need to manually assign the gates when analyzing the flow cytometry
335 data for each new species and treatment condition. This is because the fluorescence signal
336 intensity is influenced not only by the cell's plasma membrane integrity, but also by staining and
337 flow cytometry conditions as well as species-specific cell size and membrane permeability traits.
338 The first group of variables, such as dye concentrations, flow cytometer voltage settings, can
339 introduce sample-to-sample variations as seen in our experiments (Fig. 4-6), which reduces the
340 statistical power for detecting differences between treatments and can make it difficult to
341 compare results between different labs. The second group of species-specific variables mean that
342 gate boundaries need to be calibrated for each new species and treatment, potentially restricting
343 its wide adoption (Fig. 5, 6).

344 One possible source of sample-to-sample variance in FungaLight staining is variations in
345 cell size distributions as a result of the stress treatment. It has been shown that H_2O_2 can cause

346 cell size changes in a dose-dependent manner in cyanobacteria [35]. This, in turn, could affect
347 the fluorescence intensity by the two dyes in FungaLight. We compared the distributions of the
348 forward scatter height (FSC.H), which is correlated with cell size, among samples treated with
349 different H_2O_2 doses. In all three species, we observed an interesting pattern where the low dose
350 treatment group (10 mM for *C. glabrata* and 0.6 mM for *S. cerevisiae* and *C. albicans*) exhibited
351 a right shift, indicating cell size expansion, while at medium to high doses, including the lethal 1
352 M dose, there was a slight leftward shift, indicating cell size contraction (Fig. S3). Normalizing
353 the fluorescence signal by cell size (Materials and Methods), however, had only small effects on
354 the variance and didn't affect the statistical test results (Fig. S4).

355 Despite its limitations, our results showed that FungaLight is a valuable tool for yeast
356 research and complements the traditional CFU. It is worth emphasizing, however, that the two
357 assays measure fundamentally different traits: CFU quantifies viability while FungaLight like
358 other dye-based methods measure plasma membrane integrity. Therefore, there can be genuine
359 differences between the two measures for the same sample, such as when a subpopulation of
360 cells enter senescence, which will not be classified as dead by FungaLight but also don't
361 contribute to CFU. Such differences are not a bug but a feature that should be appreciated and
362 exploited. That being said, we imagine that in most practical applications, researchers want a
363 method to quantify the effects of a stress or drug but do not explicitly distinguish viability from
364 survival. In such cases, FungaLight can serve as a useful alternative to CFU to either achieve the
365 results faster and / or to examine a large number of samples in parallel. We found the two
366 methods generate broadly consistent results while differing in their resolving power at different
367 parts of the stress level gradient. As shown for H_2O_2 and Amphotericin B-treated *C. glabrata*
368 cells, CFU is better at differentiating mock and low dose treatments but lacks the ability to

369 distinguish between sub-lethal vs lethal doses; FungaLight, by contrast, is more powerful in
370 resolving differences between the sublethal and lethal doses, yet it doesn't distinguish mock and
371 low dose treatments (Figs. 4-6). Knowing these properties will help the researcher choose the
372 proper method or combine their power.

373 In the future, we would like to improve the FungaLight assay by devising novel analysis
374 methods that capture the essence of the observed fluorescence pattern, namely a gradual shift
375 from the low-green, low-red "live" state to the medium-green, high-red "dead" state, through the
376 "intermediate" high-green, high-red "damaged" state. A scale-free approach that doesn't require
377 manual gate drawing and thus not dependent on the absolute fluorescence scales will simplify the
378 assay analysis while also making the results more comparable across studies. In summary, we
379 believe our optimized FungaLight assay provides a rapid and scalable approach for quantifying
380 yeast survival after stress and drug treatments. It can be used as an alternative to CFU when the
381 researchers do not need to distinguish between viability and survival, or it can be used together
382 with CFU to provide in-depth information for the post-stress states of the population.
383
384

385 ## Materials and Methods

386 ### Strains

387 Yeast species and strains used in this study are: *C. glabrata* BG99, derived from a clinical
388 isolate, BG2 [36]; *S. cerevisiae* K699 (ATCC #200903); *C. albicans* SN152, derived from a
389 clinical isolate, SC5314 [37]. All strains used are listed in Table 1.

390 ***Table 1. Yeast strains used in this study***

Species name	ID	Genotype	Source
C. glabrata	yH181	[BG2] his3Δ(1 + 631)	[36]
S. cerevisiae	yH154	[W303] MATa ade2-1 trp1-1 can 1–100 leu2-3,112 his3-11,15ura3 GAL+	ATCC #200903
C. albicans	yH714	[SC5314] SN152, HIS3 and LEU2	[37]

391

392 ### Media and yeast culture

393 Yeast cultures were grown in Synthetic Complete (SC) media, made with Yeast Nitrogen Base
394 without amino acids (Sigma Y0626), 2% glucose, and amino acid mix. For SC agar plates, 2.5%
395 agar was added to the SC liquid media before autoclaving. SC agar plates were stored at 5 °C
396 and used within two weeks. For hydrogen peroxide stress, a 30% (w/w, Sigma H1009) stock
397 solution was added to the SC media to the indicated concentration and used within 30 minutes.

398 ### Hydrogen peroxide treatment

399 For all three species of yeast, overnight culture was diluted in the morning in SC and grown to
400 the mid-log phase (OD_{600} = 1). For each treatment, 600 µL of cells were transferred to a well in a
401 96 deep-well plate (Thermo Fisher 278606). The plate was centrifuged at 3,000g for 5 min and
402 the media was removed by aspiration. Mock (SC) or hydrogen peroxide stress media was added

403 to each well to reach an OD_{600} of 1. The plate was incubated for 120 min at 30 °C with shaking
404 at 300 rpm.

405 **Colony Forming Units (CFU) assay**

406 Yeast cells were collected by centrifugation at 3,000g for 5 minutes. After removing the media,
407 cells were resuspended in 0.85% saline buffer and adjusted to an OD_{600} of 0.2. Serial dilutions
408 were made to aim at 50-500 single colonies per plate (typically 100-1000 fold for H_2O_2-treated
409 cells). 50 µL of the properly diluted sample was spread onto SC plates with sterilized glass
410 beads. Plates were incubated at 30 °C for 48 hours. Colonies were tallied manually. Three
411 biological replicates were used per treatment group.

412 **SYTO 9/PI, aka FungaLight staining**

413 Dye preparation: LIVE/DEAD FungaLight Yeast Viability Kit for flow cytometry was obtained
414 from Thermo Fisher (Cat# L34952). The kit contains two components, which we used either
415 individually or in combination as indicated in the text. SYTO 9 (component A) was diluted 1:100
416 to a working concentration of 3.34×10^{-2} mM with deionized water immediately before staining.
417 PI (component B) was diluted 1:100 to a working concentration of 20×10^{-2} mM with deionized
418 water and stored for less than 6 months at 5 °C.

419 After mock or stress treatment, yeast cells were collected by centrifugation at 3,000 g for
420 5 minutes. The treatment media was removed by aspiration. Cells were resuspended in 0.85%
421 saline buffer to an OD_{600} of 1 (except when testing different staining buffers, in which case
422 another solution was used for resuspension).

423 To stain the sample, the working stock of SYTO 9 (3.34×10^{-2} mM) and PI (20×10^{-2}
424 mM) were added at a 1:10 ratio unless stated otherwise. The final concentrations were 3.34 µM
425 for SYTO 9 and 20 µM for PI. At least three biological replicates were performed per group.

426 To determine the time evolution of fluorescence signals for each dye, after either dye is
427 added to the sample, fluorescence was measured every 5 min from 0 min at the time of adding
428 the dye to 55 min after in the single dye time course. All incubations were performed in the dark
429 at room temperature (~25 °C) until being measured on the flow cytometer.

430 **Flow Cytometry**

431 Samples were stained with one or both dyes of FungaLight. To quantify with flow cytometry, 20
432 µL of the stained sample was diluted into 200 µl of 0.85% saline buffer and directly measured on
433 an Attune NxT instrument fitted with an autosampler (Thermo Fisher Cat#A24858). A voltage of
434 345 mV and 399 mV were used for the FSC and SSC channels.

435 For SYTO 9 staining alone, samples were excited at 488 nm and emission was collected
436 using a 530+/-30 nm bandpass filter at 230 mV. For PI-staining alone, samples were excited by a
437 488 nm blue laser and emission was collected using a 600 nm longpass filter (>600 nm) at 200
438 mV. For PI and SYTO 9 co-staining, samples were excited at 488 nm, and emissions were
439 collected in the BL1 channel using a 530+/-30 nm filter at 230 mV and in the BL3 channel using
440 a 600 nm longpass filter (>600 nm) at 200 mV. Samples were run at 200 µl / min speed and at
441 least 30,000 total events were collected per sample. At least three biological replicates were
442 measured per treatment group.

443 **Flow cytometry data analysis**

444 FCS (v3.1) files were exported from the Attune NxT software and analyzed in R using flowCore
445 (2.12.0), openCyto (2.16.1), flowWorkspace (4.12.2), ggcyto (1.32.0) and tidyverse (2.0.0)
446 packages in R. We used a standard gating strategy to gate for single cells: 1) Non-cell events
447 were removed by gating on FSC.H and SSC.H (Fig. S5A); 2) single cell events were identified
448 based on FSC.H and FSC.W (Fig. S5B). In the main text results, gated single-cell events were

449 then plotted on the FungaLight Green (BL1-H) and FungaLight Red (BL3-H) axes. Live,
450 intermediate and dead cell gates were specified for each species and treatment condition. The
451 gate boundaries used are listed in Table 2. Percent live and intermediate gate events were
452 calculated by dividing the number of events in each gate by the total number of single cell
453 events. In Fig. S3 and Fig. S4 where we examined the effect of normalizing by cell size, the
454 green channel fluorescence was divided by FSC.H^1.2 and the red channel fluorescence by
455 FSC.H^0.8, then multiplied by a constant such that the resulting values have the same median as
456 before the normalization. These normalized values became generally independent of FSC.H as a
457 proxy for cell size. The same procedure as above was followed to calculate the percent live and
458 intermediate events, except instead of the FungaLight Green and FungaLight Red fluorescence,
459 the normalized values were used.

460 ***Table 2. Flow cytometry gate boundaries***

Fig.	Species	Stress	Gate	Boundary coordinates (green, red)
1C	*C. glabrata*	H_2O_2	dead-like	$(10, 10^{2.1})$, $(10^{2.4}, 10^{2.1})$, $(10^{4.2}, 10^{4.2})$, $(10, 10^{4.2})$
2A	*C. glabrata*	H_2O_2	dead-like	$(10, 10^{2.1})$, $(10^{3}, 10^{2.1})$, $(10^{4.5}, 10^{4.2})$, $(10, 10^{4.2})$
2A	*C. glabrata*	H_2O_2	unstained	$(0, 0)$, $(10^{2}, 0)$, $(10^{2}, 10^{2.1})$, $(0, 10^{2.1})$
3A	*C. glabrata*	H_2O_2	PI-positive	$(0, 10^{2.2})$, $(10^{2.5}, 10^{2.2})$, $(10^{2.5}, 10^{5})$, $(0, 10^{5})$
3A	*C. glabrata*	H_2O_2	SYTO 9, high red and green	$(10^{3}, 10^{2.2})$, $(10^{5.5}, 10^{2.2})$, $(10^{5.5}, 10^{4.5})$
4	*C. glabrata*	H_2O_2	Live	$(0, 10^{2.2})$, $(10^{4}, 10^{2.2})$, $(10^{4}, 0)$, $(0, 0)$
4	*C. glabrata*	H_2O_2	Intermediate	$(10^{3}, 10^{2.2})$, $(10^{5.5}, 10^{2.2})$, $(10^{5.5}, 10^{4.5})$
5	*S. cerevisiae*	H_2O_2	Live	$(0, 10^{2.3})$, $(10^{4}, 10^{2.3})$, $(10^{4}, 0)$, $(0, 0)$
5	*S. cerevisiae*	H_2O_2	Intermediate	$(10^{3}, 10^{2.3})$, $(10^{5.5}, 10^{2.3})$, $(10^{5.5}, 10^{4.5})$
5	*C. albicans*	H_2O_2	Live	$(0, 10^{2.7})$, $(10^{4.2}, 10^{2.7})$, $(10^{4.2}, 0)$, $(0, 0)$

5	*C. albicans*	H_2O_2	Intermediate	(10^3, $10^{2.7}$), ($10^{5.5}$, $10^{2.7}$), ($10^{5.5}$, $10^{4.5}$)
6	*C. glabrata*	AmpB	Live	(0, $10^{2.5}$), ($10^{3.5}$, $10^{2.5}$), ($10^{3.5}$, 0), (0, 0)
6	*C. glabrata*	AmpB	Live	($10^{3.5}$, 0), ($10^{3.5}$, $10^{2.5}$), ($10^{5.5}$, $10^{4.5}$), ($10^{5.5}$, 0)
S4	*C. glabrata*	H_2O_2	Live	(0, $10^{2.2}$), (10^4, $10^{2.2}$), (10^4, 0), (0, 0)

Statistical analyses and data plotting

Figure 3B: Significant differences in the percent SYTO 9-intermediate gate events between the H_2O_2-treated samples and the mock were determined by a linear model, i.e., %intermediate ~ [H_2O_2]. H_2O_2 doses were treated as a categorical variable, with mock as the reference (intercept). The effect of each of the three non-zero doses was estimated by the linear model. Statistical significance was determined using a Student's t-test. The two-sided *p*-values were corrected for multiple testing using the Holm-Bonferroni procedure and a test was considered significant when the corrected *p*-value was lower than 0.05. The same procedure was applied to PI-positive events. Figure 4, 5, 6, S4: Pairwise comparisons of either percent live or percent intermediate events were performed using Tukey's range test. A *p*-value of smaller than 0.05 was considered significant and shown as a filled circle in the figures.

Acknowledgement

We would like to thank past and current members of the Gene Regulatory Lab for reading and giving feedback on the manuscript. We also want to thank Drs. Jan Fassler and Ana Llopart for reading an early draft of the paper and giving valuable feedback.

Data Availability

All raw data, including CFU assay and FungaLight staining results, are available at https://github.com/binhe-lab/E041-yeast-viability-assay/releases/tag/preprint. At the time of publication, this repository will be archived with Zenodo to generate a DOI.

Funding

This work is supported by NIH R35GM137831 and a startup fund from the University of Iowa to BZH. HT received summer support from the University of Iowa Provost Office's Investment in Strategic Priorities fund. JL received summer support from the Carol B. and Robert G. Lynch Department of Biology Graduate Fund at the University of Iowa.

Figure Legends

Fig. 1 SYTO 9/PI (FungaLight) LIVE/DEAD stain, properties and application to H_2O_2-treated *C. glabrata* cells. (A) Excitation and emission spectra for SYTO 9 and Propidium Iodide (PI) when bound to DNA. Dashed lines and filled curves show the excitation and emission spectra, respectively. Both are normalized to the 488 nm laser. The plot is generated using FluoroFinder's Spectra Viewer. (B) 2D density plot showing the dead (black) and live (gray) populations, reproduced from the FungaLight manual with permission. 1D density graphs shown next to the x- and y-axes. **(C)** 2D density plots for *C. glabrata* treated with different doses of H_2O_2 and stained with FungaLight. The red polygon gates identify dead and dead-like cells, with percentages on the top. A magenta arrowhead points to a subpopulation distinct from either live or dead cells as shown in B. Three biological replicates are shown as rows.

Fig. 2 Effect of the staining buffer choice on the dead-like and unstained populations in mock-treated and FungaLight stained samples. (A) 2D density plots showing the FungaLight staining patterns of *C. glabrata* cells treated with different doses of H_2O_2, then stained in one of the four buffers: deionized water, 1x PBS, 0.85% Saline and Synthetically Defined (SD) media. In the mock-treated samples, the red trapezoid gate identifies the "Dead-like" population while the gray rectangular gate identifies the "Unstained" population. Their frequencies are further

504 analyzed below. (B) Frequencies of the dead-like and unstained populations in the mock-treated
505 samples (n = 3, biological replicates). Bars show the mean and dots show the individual
506 replicates.
507
508 **Fig. 3 *C. glabrata* cells exposed to sublethal doses of H_2O_2 accumulate more SYTO 9 but not**
509 **PI.** (A) 2D density plots showing *C. glabrata* cells treated with various doses of H_2O_2 for 2 hrs
510 and stained with either SYTO 9 or PI individually. An "intermediate" high-green and high-red
511 population is shown with a triangle gate for the SYTO 9 stained samples (top) and a "PI-
512 positive" population shown with a rectangular gate for the PI stained samples (bottom).
513 Percentages of gated events are shown next to the gates. (B) Percentages of the SYTO 9
514 "Intermediate" and PI-positive populations are shown as dots and their means as red lines (n = 3,
515 biological replicates). A significant difference compared to the mock-treated (0 mM) sample was
516 determined by a linear model. A Holm-Bonferroni corrected Student's t-test P-value < 0.05 is
517 indicated by a red box around the x-axis label. (C) Median Fluorescence Intensity (MFI) for all
518 events in samples stained with SYTO 9 are shown for both the green and the red channels.
519 Biological replicates are shown as dots and their means as red lines. Significant differences from
520 the mock-treated sample were determined and indicated the same way as in (B).
521
522 **Fig. 4 Comparing CFU, Propidium Iodide (PI), and FungaLight in the estimates of viability**
523 **or survival.** (A) Gating strategy for PI and FungaLight stained samples (see Methods for gate
524 boundary coordinates). (B) C. glabrata cells were treated with H_2O_2 at various concentrations for
525 2 hours and then plated for CFU or stained with PI alone or with FungaLight. Percent viable (for
526 CFU) or percent live (PI and FungaLight) estimates for three replicates were shown as dots (n =

527 3, biological replicates). Bars are the means of the replicates. Dot shapes distinguish different
528 days of experiments. Tukey's range test (aka Tukey's HSD) was used to compare all pairs of
529 samples. The results are presented below the x-axis: lines originate from the first sample and end
530 with the other sample, with a filled circle at the end indicating a significant difference at 0.05
531 level, or open circle otherwise (n.s. = not significant). (C) Density plots for one replicate
532 showing the FungaLight staining patterns for *C. glabrata* cells treated with H_2O_2 from 0 to 1 M
533 in 100 mM increments. The axes and live cell gates were the same as in A. Intermediate gates
534 were omitted for clarity. (D) CFU and FungaLight survival estimates for the same concentration
535 series of H_2O_2 in *C. glabrata* (n = 3, performed on three different days indicated by the shape of
536 the dots). Bars represent the mean. For FungaLight, the gray bars and dots indicate the percent of
537 events in the live gate and the light blue bars and dots show the percent of events in the
538 "Intermediate" gate (see A). Tukey's range test was applied to (i) CFU, (ii) FungaLight % live
539 events and (iii) % intermediate events separately. Only significant results at a 0.05 level were
540 indicated by lines and filled circles due to space constraints.
541
542 **Fig. 5 FungaLight quantifies post-oxidative stress survival in diverse yeast species.** (A)
543 Representative 2D density plot for *S. cerevisiae* and *C. albicans* cells treated with different doses
544 of H_2O_2. The live (black) and intermediate (blue) cell gates were shown (see Methods for gate
545 boundary coordinates). (B) Percent viable (for CFU) or percent live (FungaLight) estimates for
546 *S. cerevisiae* cells treated with 0 to 1 M H_2O_2 (2 hours) were shown as dots (n = 3, biological
547 replicates). Bars represent the means. Dots with the same shape represent the same day of
548 experiment across both methods. Tukey's range test (aka Tukey's HSD) was used to compare all
549 pairs of samples. Test results were presented below the x-axis in the same way as in Fig. 4. (C)

550 Same as B for *C. albicans* samples.

551

552 **Fig. 6 FungaLight quantifies survival after treatment with the antifungal Amphotericin B**

553 **(AmpB).** (A) Representative 2D density plot for *C. glabrata* cells treated with different doses of

554 AmpB. The live (black) and intermediate (blue) cell gates were shown (see Methods for gate

555 boundary coordinates). (B) Percent viable estimates for *C. glabrata* cells treated with 0 - 1

556 ng/mL AmpB shown as dots (n = 4, biological replicates). Bars represent the means. Dots with

557 the same shape represent the same day of experiment. Tukey's range test (aka Tukey's HSD)

558 was used to compare all pairs of samples. Test results were presented below the x-axis in the

559 same way as in Fig. 4. (C) Same as B for FungaLight percent live and percent intermediate gated

560 events. Only significant results at a 0.05 level were shown for the % intermediate events. (D)

561 Scatter plot shows the relationship between the FungaLight % live and CFU % viable estimates.

562 Dots are colored by AmpB dose. The dash line indicates y = x. Pearson's correlation coefficient

563 and Spearman's rank correlation coefficient are shown inside the plot.

564

565 **Literature Cited**

566 1. Lopes JP, Lionakis MS. Pathogenesis and virulence of Candida albicans. Virulence. 13: 89–
567 121. doi:10.1080/21505594.2021.2019950

568 2. Hazen KC. New and emerging yeast pathogens. Clin Microbiol Rev. 1995;8: 462–478.

569 3. Welsh RM, Bentz ML, Shams A, Houston H, Lyons A, Rose LJ, et al. Survival,
570 Persistence, and Isolation of the Emerging Multidrug-Resistant Pathogenic Yeast Candida
571 auris on a Plastic Health Care Surface. J Clin Microbiol. 2017;55: 2996–3005.
572 doi:10.1128/jcm.00921-17

573 4. Quinto-Alemany D, Canerina-Amaro A, Hernández-Abad LG, Machín F, Romesberg FE,
574 Gil-Lamaignere C. Yeasts Acquire Resistance Secondary to Antifungal Drug Treatment by
575 Adaptive Mutagenesis. PLoS ONE. 2012;7: e42279. doi:10.1371/journal.pone.0042279

576 5. Novak Babič M, Zalar P, Ženko B, Džeroski S, Gunde-Cimerman N. Yeasts and yeast-like
577 fungi in tap water and groundwater, and their transmission to household appliances. Fungal
578 Ecol. 2016;20: 30–39. doi:10.1016/j.funeco.2015.10.001

579 6. Lourens-Hattingh A, Viljoen BC. Growth and survival of a probiotic yeast in dairy
580 products. Food Res Int. 2001;34: 791–796. doi:10.1016/S0963-9969(01)00085-0

581 7. Blagosklonny MV. Cell senescence: Hypertrophic arrest beyond the restriction point. J Cell
582 Physiol. 2006;209: 592–597. doi:10.1002/jcp.20750

583 8. Davey HM. Life, Death, and In-Between: Meanings and Methods in Microbiology. Appl
584 Environ Microbiol. 2011;77: 5571–5576. doi:10.1128/AEM.00744-11

585 9. Carmona-Gutierrez D, Bauer MA, Zimmermann A, Aguilera A, Austriaco N, Ayscough K,
586 et al. Guidelines and recommendations on yeast cell death nomenclature. Microb Cell.
587 2018;5: 4–31. doi:10.15698/mic2018.01.607

588 10. Wloch-Salamon DM, Bem AE. Types of cell death and methods of their detection in yeast
589 Saccharomyces cerevisiae. J Appl Microbiol. 2013;114: 287–298. doi:10.1111/jam.12024

590 11. Kwolek-Mirek M, Zadrag-Tecza R. Comparison of methods used for assessing the viability
591 and vitality of yeast cells. FEMS Yeast Res. 2014;14: 1068–1079. doi:10.1111/1567-
592 1364.12202

593 12. Sahu SR, Utkalaja BG, Patel SK, Acharya N. Spot Assay and Colony Forming Unit (CFU)
594 Analyses-based sensitivity test for Candida albicans and Saccharomyces cerevisiae. Bio-
595 Protoc. 2023;13: e4872. doi:10.21769/BioProtoc.4872

596 13. Olivares-Marin IK, González-Hernández JC, Regalado-Gonzalez C, Madrigal-Perez LA.
597 Saccharomyces cerevisiae Exponential Growth Kinetics in Batch Culture to Analyze
598 Respiratory and Fermentative Metabolism. J Vis Exp JoVE. 2018; 58192.
599 doi:10.3791/58192

600 14. Khan A ul M, Torelli A, Wolf I, Gretz N. AutoCellSeg: robust automatic colony forming
601 unit (CFU)/cell analysis using adaptive image segmentation and easy-to-use post-editing
602 techniques. Sci Rep. 2018;8: 7302. doi:10.1038/s41598-018-24916-9

603 15. Boukouvalas DT, Prates RA, Lima Leal CR, de Araújo SA. Automatic segmentation
604 method for CFU counting in single plate-serial dilution. Chemom Intell Lab Syst.
605 2019;195: 103889. doi:10.1016/j.chemolab.2019.103889

606 16. Stolze N, Bader C, Henning C, Mastin J, Holmes AE, Sutlief AL. Automated image
607 analysis with ImageJ of yeast colony forming units from cannabis flowers. J Microbiol
608 Methods. 2019;164: 105681. doi:10.1016/j.mimet.2019.105681

609 17. Kwolek-Mirek M, Zadrag-Tecza R. Comparison of methods used for assessing the viability
610 and vitality of yeast cells. FEMS Yeast Res. 2014;14: 1068–1079. doi:10.1111/1567-
611 1364.12202

612 18. Sun S, Baryshnikova A, Brandt N, Gresham D. Genetic interaction profiles of regulatory
613 kinases differ between environmental conditions and cellular states. Mol Syst Biol.
614 2020;16: e9167. doi:10.15252/msb.20199167

615 19. Zhang T, Fang HHP. Quantification of Saccharomyces cerevisiae viability using BacLight.
616 Biotechnol Lett. 2004;26: 989–992. doi:10.1023/b:bile.0000030045.16713.19

617 20. Stocks SM. Mechanism and use of the commercially available viability stain, BacLight.
618 Cytometry A. 2004;61A: 189–195. doi:10.1002/cyto.a.20069

619 21. Boulos L, Prévost M, Barbeau B, Coallier J, Desjardins R. LIVE/DEAD BacLight :
620 application of a new rapid staining method for direct enumeration of viable and total
621 bacteria in drinking water. J Microbiol Methods. 1999;37: 77–86. doi:10.1016/s0167-
622 7012(99)00048-2

623 22. Jin Y, Zhang T, Samaranayake YH, Fang HHP, Yip HK, Samaranayake LP. The use of new
624 probes and stains for improved assessment of cell viability and extracellular polymeric
625 substances in Candida albicans biofilms. Mycopathologia. 2005;159: 353–360.
626 doi:10.1007/s11046-004-6987-7

627 23. Bojsen R, Regenberg B, Folkesson A. Saccharomyces cerevisiae biofilm tolerance towards
628 systemic antifungals depends on growth phase. BMC Microbiol. 2014;14: 305.
629 doi:10.1186/s12866-014-0305-4

630 24. Ryan LK, Freeman KB, Masso-Silva JA, Falkovsky K, Aloyouny A, Markowitz K, et al.
631 Activity of potent and selective host defense peptide mimetics in mouse models of oral
632 candidiasis. Antimicrob Agents Chemother. 2014;58: 3820–3827.
633 doi:10.1128/AAC.02649-13

634 25. Roscetto E, Contursi P, Vollaro A, Fusco S, Notomista E, Catania MR. Antifungal and anti-
635 biofilm activity of the first cryptic antimicrobial peptide from an archaeal protein against
636 Candida spp. clinical isolates. Sci Rep. 2018;8: 17570. doi:10.1038/s41598-018-35530-0

637 26. Chen Y-C, Yang Y, Zhang C, Chen H-Y, Chen F, Wang K-J. A Novel Antimicrobial
638 Peptide Sparamosin26-54 From the Mud Crab Scylla paramamosain Showing Potent
639 Antifungal Activity Against Cryptococcus neoformans. Front Microbiol. 2021;12: 746006.
640 doi:10.3389/fmicb.2021.746006

641 27. Wainschtein P, Jain D, Zheng Z, TOPMed Anthropometry Working Group, NHLBI Trans-
642 Omics for Precision Medicine (TOPMed) Consortium, Cupples LA, et al. Assessing the
643 contribution of rare variants to complex trait heritability from whole-genome sequence data.
644 Nat Genet. 2022;54: 263–273. doi:10.1038/s41588-021-00997-7

645 28. Chudzik B, Koselski M, Czuryło A, Trębacz K, Gagoś M. A new look at the antibiotic
646 amphotericin B effect on Candida albicans plasma membrane permeability and cell
647 viability functions. Eur Biophys J EBJ. 2015;44: 77–90. doi:10.1007/s00249-014-1003-8

29. Sun J, Liu X, Jiang G, Qi Q. Inhibition of Nucleic Acid Biosynthesis Makes Little Difference to Formation of Amphotericin B-Tolerant Persisters in Candida albicans Biofilm. Antimicrob Agents Chemother. 2015;59: 1627–1633. doi:10.1128/aac.03765-14

30. Ponomarova O, Gabrielli N, Sévin DC, Mülleder M, Zirngibl K, Bulyha K, et al. Yeast Creates a Niche for Symbiotic Lactic Acid Bacteria through Nitrogen Overflow. Cell Syst. 2017;5: 345-357.e6. doi:10.1016/j.cels.2017.09.002

31. Stiefel P, Schmidt-Emrich S, Maniura-Weber K, Ren Q. Critical aspects of using bacterial cell viability assays with the fluorophores SYTO9 and propidium iodide. BMC Microbiol. 2015;15: 36. doi:10.1186/s12866-015-0376-x

32. Rego A, Ribeiro A, Côrte-Real M, Chaves SR. Monitoring yeast regulated cell death: trespassing the point of no return to loss of plasma membrane integrity. Apoptosis. 2022;27: 778–786. doi:10.1007/s10495-022-01748-7

33. Nikolaou E, Agrafioti I, Stumpf M, Quinn J, Stansfield I, Brown AJ. Phylogenetic diversity of stress signalling pathways in fungi. BMC Evol Biol. 2009;9: 44. doi:10.1186/1471-2148-9-44

34. Pappas PG, Lionakis MS, Arendrup MC, Ostrosky-Zeichner L, Kullberg BJ. Invasive candidiasis. Nat Rev Dis Primer. 2018;4: 18026. doi:10.1038/nrdp.2018.26

35. Giannuzzi L, Lombardo T, Juárez I, Aguilera A, Blanco G. A Stochastic Characterization of Hydrogen Peroxide-Induced Regulated Cell Death in Microcystis aeruginosa. Front Microbiol. 2021;12: 636157. doi:10.3389/fmicb.2021.636157

36. Cormack BP, Falkow S. Efficient homologous and illegitimate recombination in the opportunistic yeast pathogen Candida glabrata. Genetics. 1999;151: 979–987.

37. Homann OR, Dea J, Noble SM, Johnson AD. A Phenotypic Profile of the Candida albicans Regulatory Network. PLOS Genet. 2009;5: e1000783. doi:10.1371/journal.pgen.1000783

Supporting Information Captions

Fig. S1 Viability of mock-treated mid-log phase *C. glabrata* cells. Ratios between the CFU and the number of cells plated based on flow cytometry in mock-treated (mid-log) *C. glabrata* cells resuspended in the indicated buffers. Dots represent the individual biological replicate and bars represent the mean.

Fig. S2 Testing dye concentrations, incubation time and dye ratio on the staining

680 **pattern.** (A) 2D density plots showing staining patterns of mid-log *C. glabrata* cells at
681 various dilutions of SYTO 9 or PI (stock concentrations: 3.34 mM for SYTO 9, 20 mM
682 for PI). Rectangular gates were used to quantify cell events considered damaged or
683 dead. (B) Median Fluorescence Intensity (MFI) over time for SYTO 9 stained mid-log *C.*
684 *glabrata* cells. Dots show individual biological replicates (n = 4, biological replicates).
685 Thick red bars represent the mean and the error bars represent the 95% confidence
686 intervals. (C) Similar to B for PI-stained, heat-killed *C. glabrata* cells. (D) 2D density
687 plots for *C. glabrata* cells treated with H_2O_2 and stained with various ratios of PI:SYTO 9
688 (right). For all samples, SYTO 9 is applied at a fixed concentration of 3.34 μM; PI is
689 used at 20 μM at 1:1 ratio.
690
691 **Fig. S3 H_2O_2 treatment causes changes in cell size as indicated by FSC (forward**
692 **scattering).** FSC.H (height) distribution of gated single cell events were shown for each
693 of the three species as indicated on the top. Cells were treated for 2 hours at four
694 different H_2O_2 doses (y-axis labels). Three biological replicates were shown for each
695 species and treatment.
696
697 **Fig. S4 Normalizing fluorescence by FSC.H didn't reduce the sample variance**
698 **dramatically..** Fluorescence signals were first normalized by FSC.H (see Methods for
699 details) and then the same gating strategies were applied as for the unnormalized data
700 to calculate the percent live values. The resulting estimates were plotted side-by-side
701 with the non-normalized (Fig. 4B) results for *C. glabrata* treated with different
702 concentrations of H_2O_2. Statistical test results were shown below the graph similarly to

703 Fig. 4B.

704

705 **Fig. S5 Example gating strategy for flow cytometry data.** (A) All events were plotted
706 on FSC.H and SSC.H. A rectangular gate is used to exclude non-cell events. (B) Events
707 within the first gate ("cell") were plotted on FSC.H and FSC.W. Singlets (single cell
708 event as opposed to doublets or multilets) were selected by excluding events with a
709 higher FSC.W given the same FSC.H.

710

711

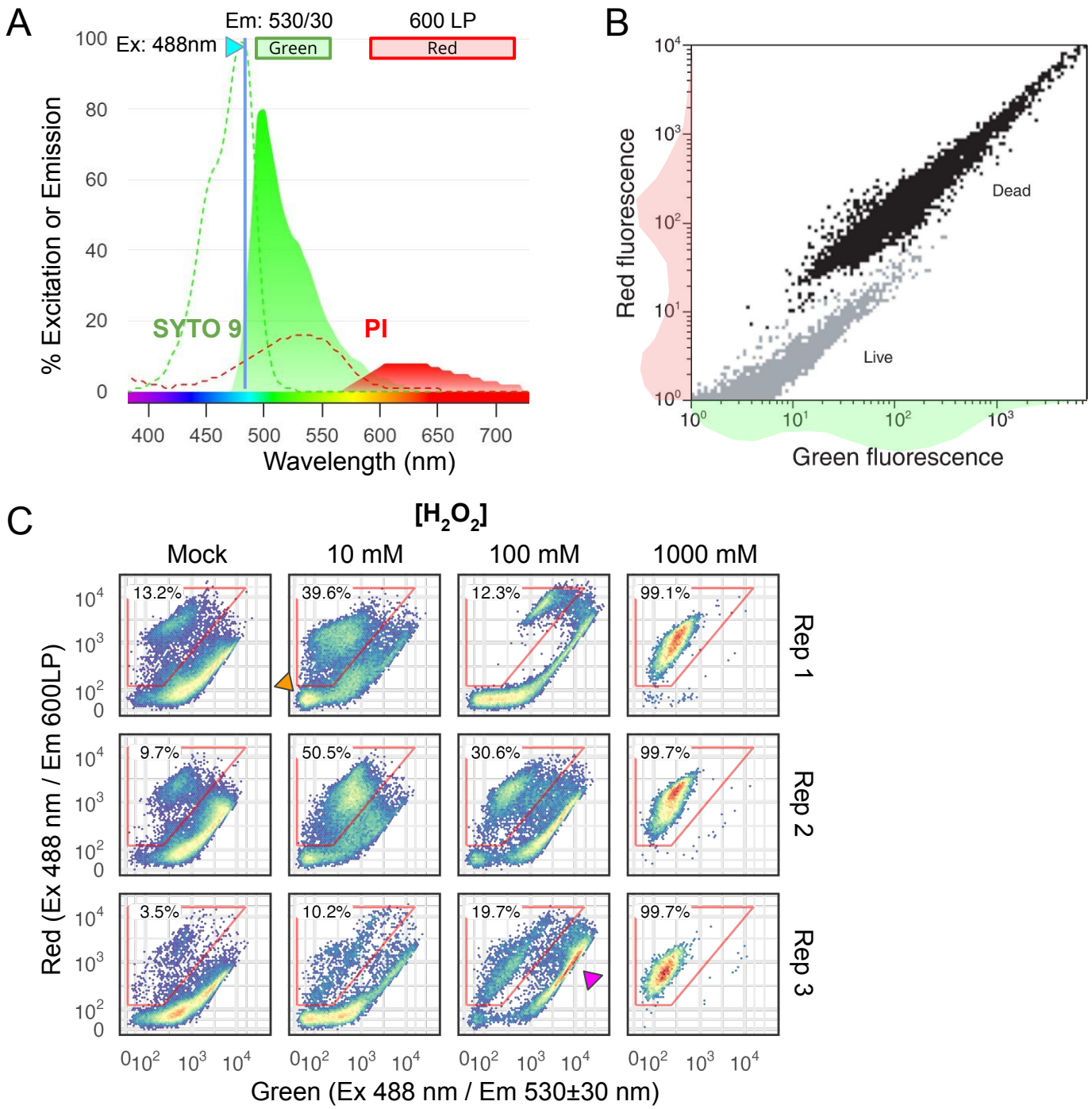

Fig. 1 SYTO 9/PI (FungaLight) LIVE/DEAD stain, properties and application to H_2O_2-treated *C. glabrata* cells. (A) Excitation and emission spectra for SYTO 9 and Propidium Iodide (PI) when bound to DNA. Dashed lines and filled curves show the excitation and emission spectra, respectively. Both are normalized to the 488 nm laser. The plot is generated using FluoroFinder's Spectra Viewer. (B) 2D density plot showing the dead (black) and live (gray) populations, reproduced from the FungaLight manual with permission. 1D density graphs shown next to the x- and y-axes. **(C)** 2D density plots for *C. glabrata* treated with different doses of H_2O_2 and stained with FungaLight. The red polygon gates identify dead and dead-like cells, with percentages on the top. A magenta arrowhead points to a subpopulation distinct from either live or dead cells as shown in B. Three biological replicates are shown as rows.

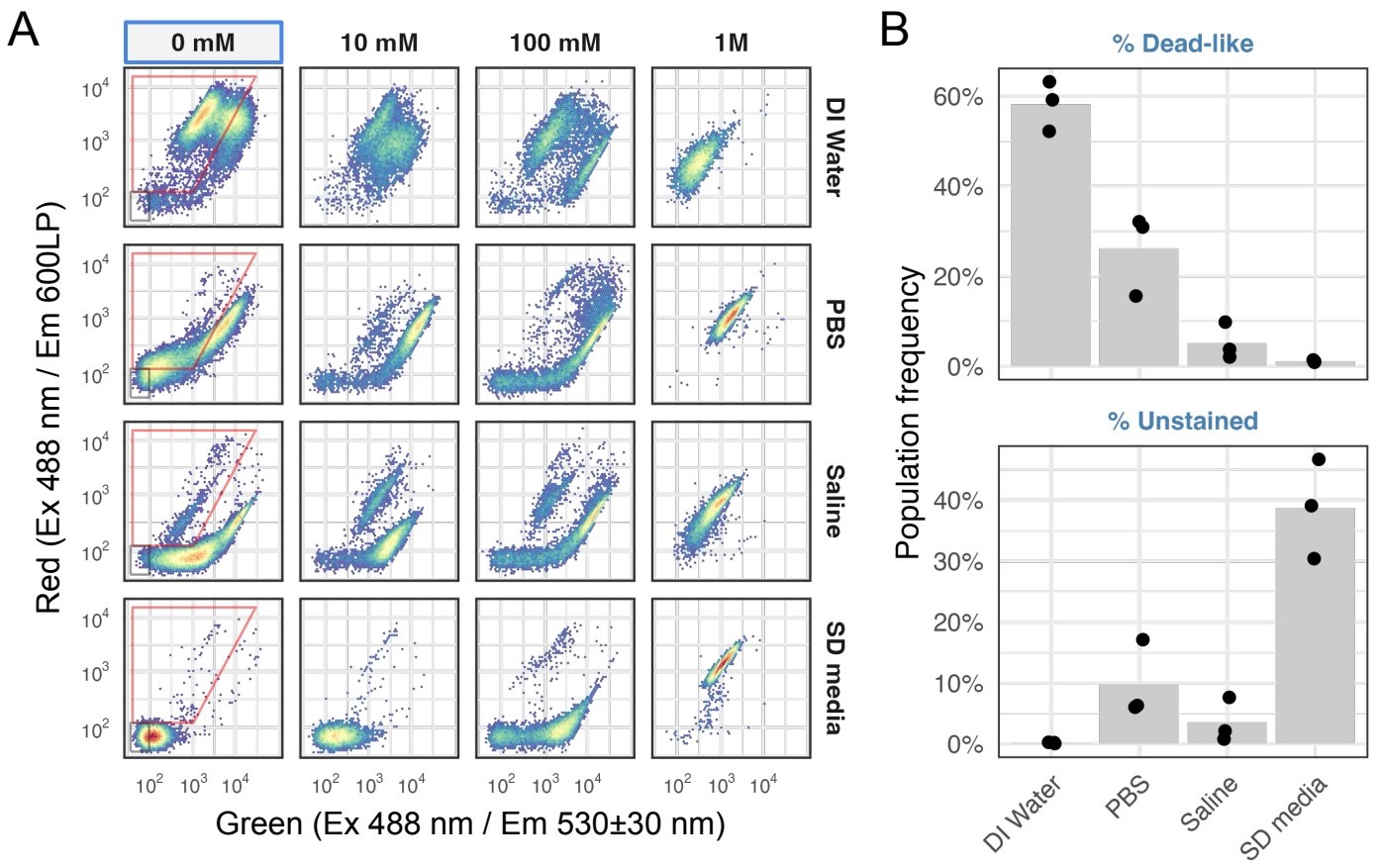

Fig. 2 Effect of the staining buffer choice on the dead-like and unstained populations in mock-treated and FungaLight stained samples. (A) 2D density plots showing the FungaLight staining patterns of *C. glabrata* cells treated with different doses of H_2O_2, then stained in one of the four buffers: deionized water, 1x PBS, 0.85% Saline and Synthetically Defined (SD) media. In the mock-treated samples, the red trapezoid gate identifies the "Dead-like" population while the gray rectangular gate identifies the "Unstained" population. Their frequencies are further analyzed below. (B) Frequencies of the dead-like and unstained populations in the mock-treated samples (n = 3, biological replicates). Bars show the mean and dots show the individual replicates.

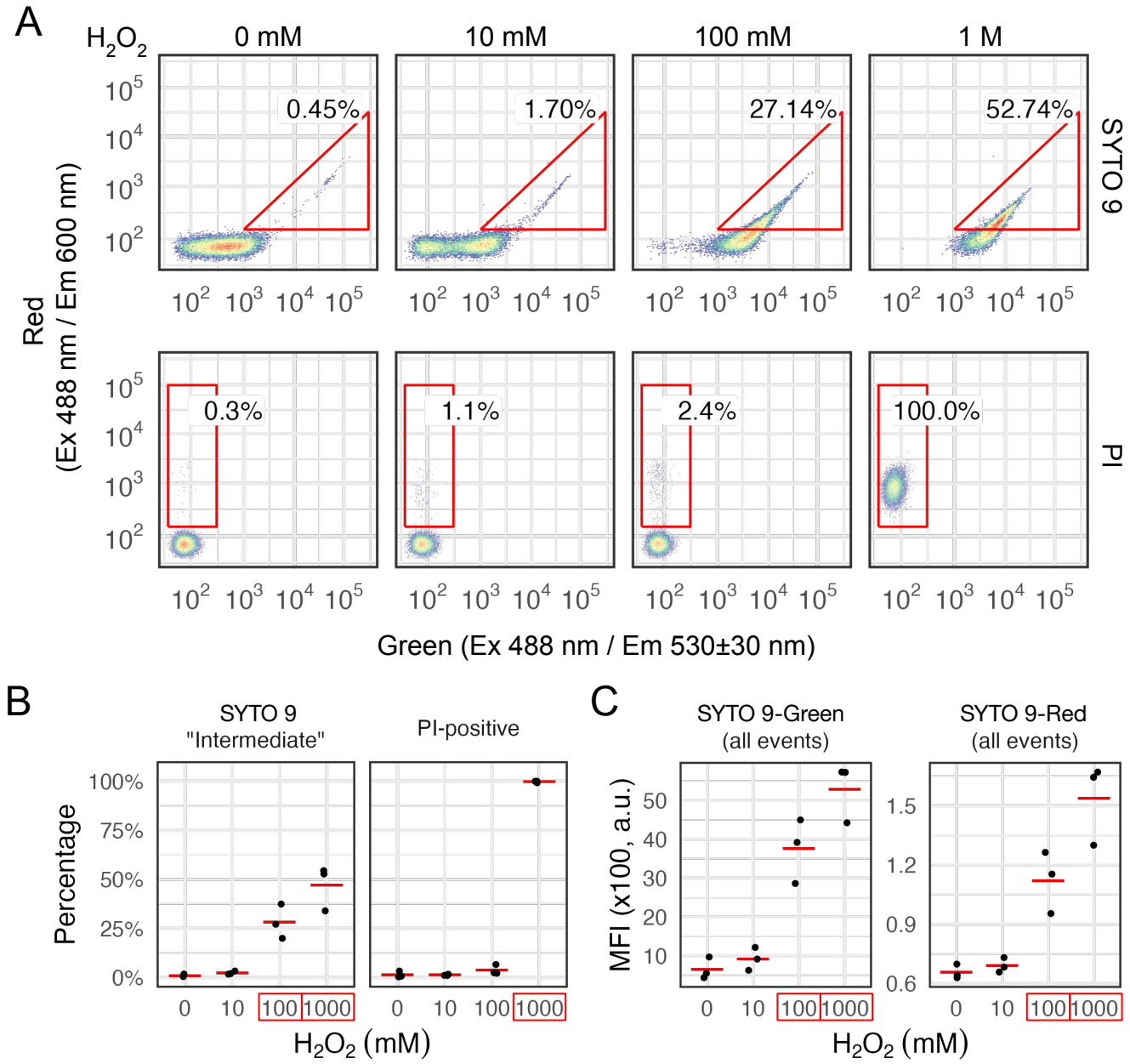

Fig. 3 *C. glabrata* cells exposed to sublethal doses of H_2O_2 accumulate more SYTO 9 but not PI. (A) 2D density plots showing *C. glabrata* cells treated with various doses of H_2O_2 for 2 hrs and stained with either SYTO 9 or PI individually. An "intermediate" high-green and high-red population is shown with a triangle gate for the SYTO 9 stained samples (top) and a "PI-positive" population shown with a rectangular gate for the PI stained samples (bottom). Percentages of gated events are shown next to the gates. (B) Percentages of the SYTO 9 "Intermediate" and PI-positive populations are shown as dots and their means as red lines (n = 3, biological replicates). A significant difference compared to the mock-treated (0 mM) sample was determined by a linear model. A Holm-Bonferroni corrected Student's t-test *P*-value < 0.05 is indicated by a red box around the x-axis label. (C) Median Fluorescence Intensity (MFI) for all events in samples stained with SYTO 9 are shown for both the green and the red channels. Biological replicates are shown as dots and their means as red lines. Significant differences from the mock-treated sample were determined and indicated the same way as in (B).

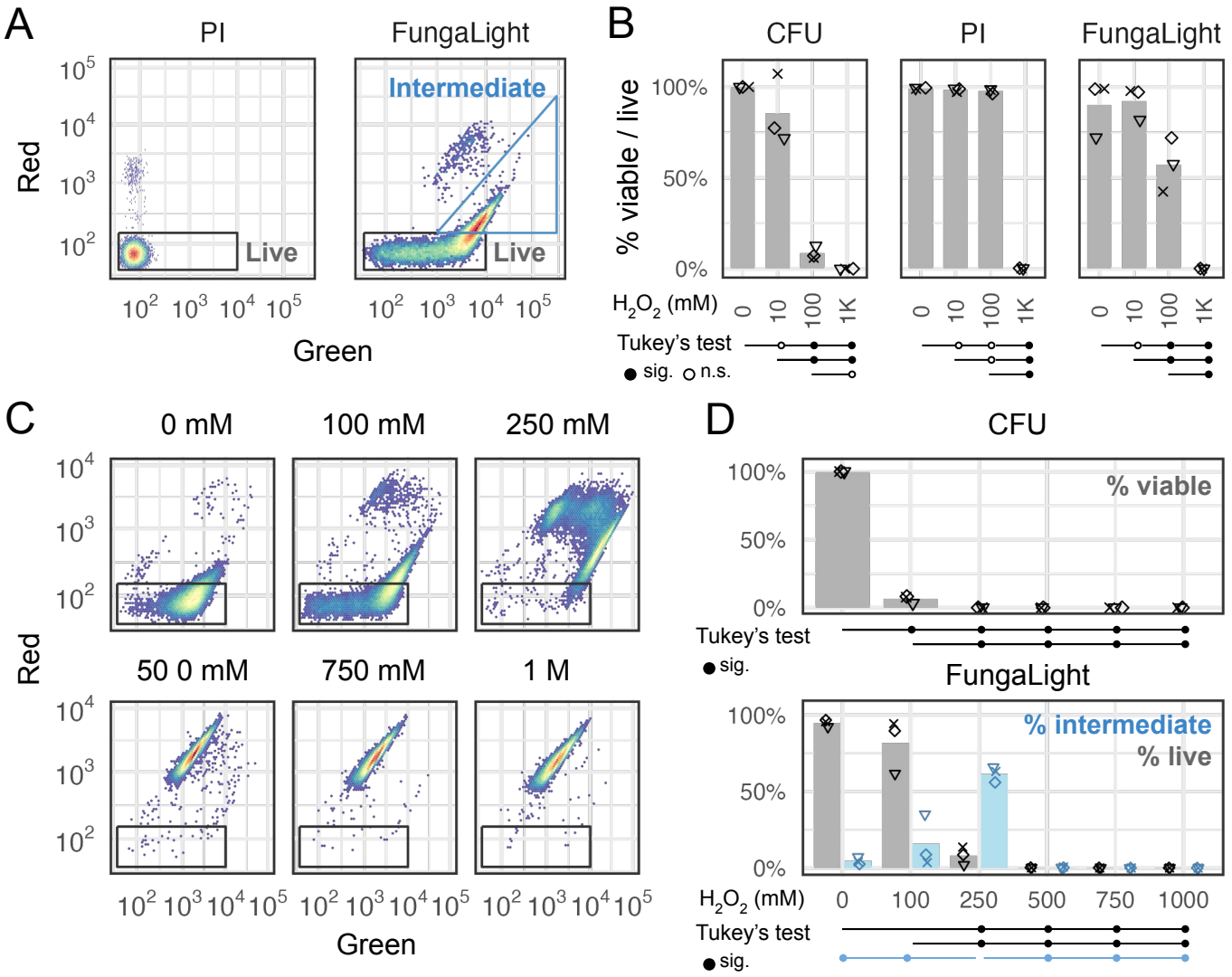

Fig. 4 Comparing CFU, Propidium Iodide (PI), and FungaLight in the estimates of viability or survival. (A) Gating strategy for PI and FungaLight stained samples (see Methods for gate boundary coordinates). (B) *C. glabrata* cells were treated with H_2O_2 at various concentrations for 2 hours and then plated for CFU or stained with PI alone or with FungaLight. Percent viable (for CFU) or percent live (PI and FungaLight) estimates for three replicates were shown as dots (n = 3, biological replicates). Bars are the means of the replicates. Dot shapes distinguish different days of experiments. Tukey's range test (aka Tukey's HSD) was used to compare all pairs of samples. The results are presented below the x-axis: lines originate from the first sample and end with the other sample, with a filled circle at the end indicating a significant difference at 0.05 level, or open circle otherwise (n.s. = not significant). (C) Density plots for one replicate showing the FungaLight staining patterns for *C. glabrata* cells treated with H_2O_2 from 0 to 1 M in 100 mM increments. The axes and live cell gates were the same as in A. Intermediate gates were omitted for clarity. (D) CFU and FungaLight survival estimates for the same concentration series of H_2O_2 in *C. glabrata* (n = 3, performed on three different days indicated by the shape of the dots). Bars represent the mean. For FungaLight, the gray bars and dots indicate the percent of events in the live gate and the light blue bars and dots show the percent of events in the "Intermediate" gate (see A). Tukey's range test was applied to (i) CFU, (ii) FungaLight % live events and (iii) % intermediate events separately. Only significant results at a 0.05 level were indicated by lines and filled circles due to space constraints.

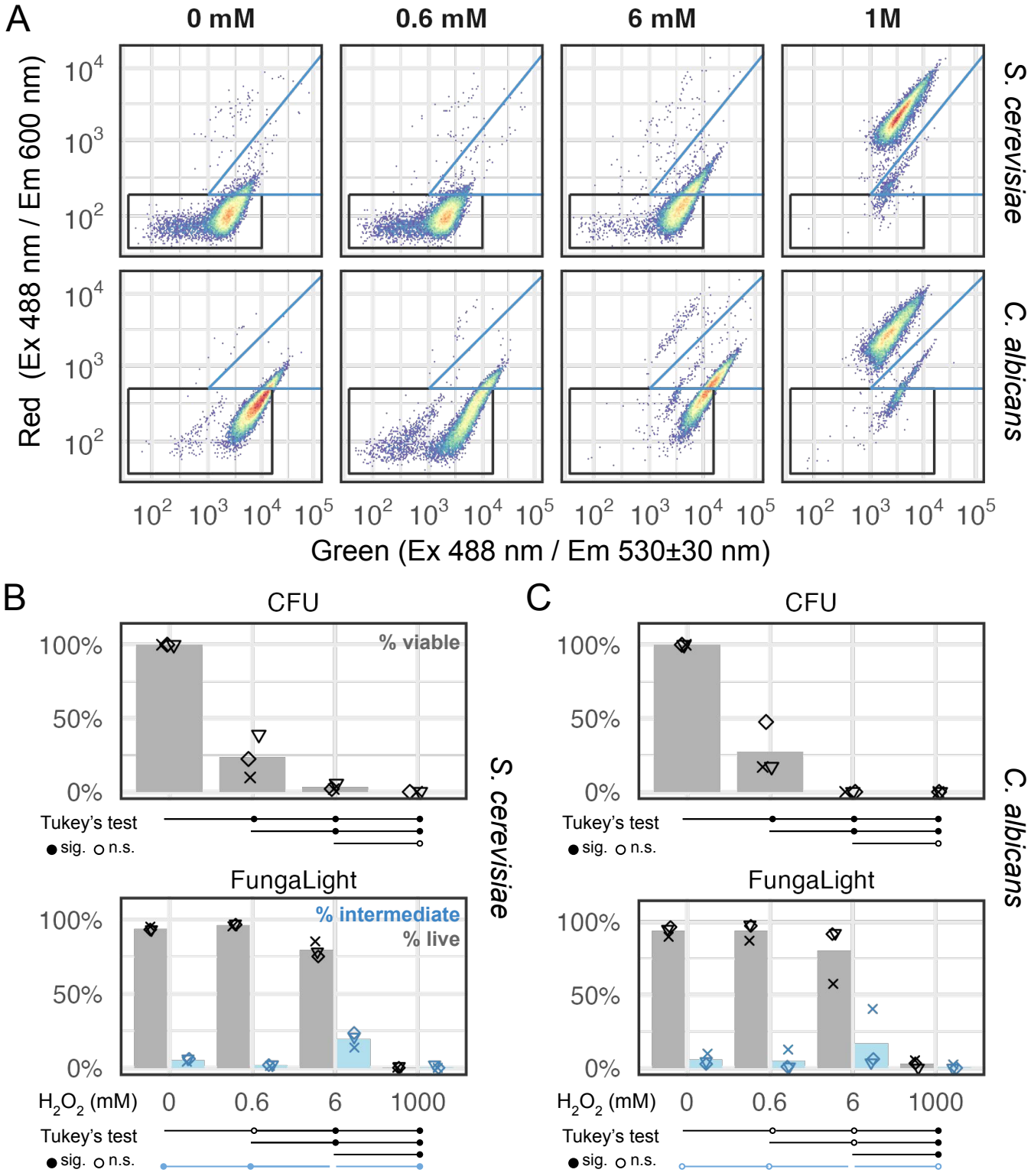

Fig. 5 FungaLight quantifies post-oxidative stress survival in diverse yeast species. (A) Representative 2D density plot for *S. cerevisiae* and *C. albicans* cells treated with different doses of H_2O_2. The live (black) and intermediate (blue) cell gates were shown (see Methods for gate boundary coordinates). (B) Percent viable (for CFU) or percent live (FungaLight) estimates for *S. cerevisiae* cells treated with 0 to 1 M H_2O_2 (2 hours) were shown as dots (n = 3, biological replicates). Bars represent the means. Dots with the same shape represent the same day of experiment across both methods. Tukey's range test (aka Tukey's HSD) was used to compare all pairs of samples. Test results were presented below the x-axis in the same way as in Fig. 4. (C) Same as B for *C. albicans* samples.

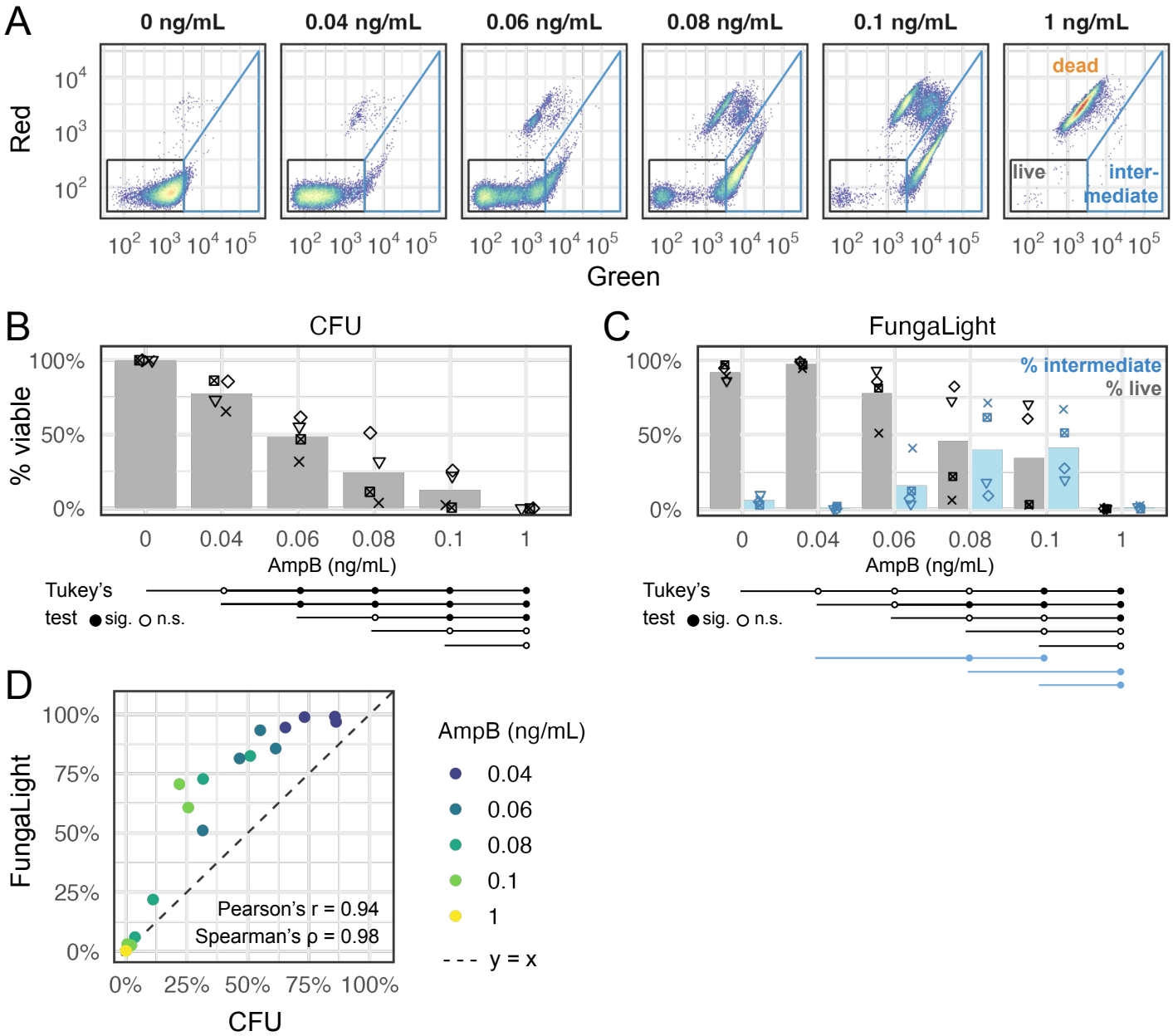

Fig. 6 FungaLight quantifies survival after treatment with the antifungal Amphotericin B (AmpB). (A) Representative 2D density plot for *C. glabrata* cells treated with different doses of AmpB. The live (black) and intermediate (blue) cell gates were shown (see Methods for gate boundary coordinates). (B) Percent viable estimates for *C. glabrata* cells treated with 0 - 1 ng/mL AmpB shown as dots (n = 4, biological replicates). Bars represent the means. Dots with the same shape represent the same day of experiment. Tukey's range test (aka Tukey's HSD) was used to compare all pairs of samples. Test results were presented below the x-axis in the same way as in Fig. 4. (C) Same as B for FungaLight percent live and percent intermediate gated events. Only significant results at a 0.05 level were shown for the % intermediate events. (D) Scatter plot shows the relationship between the FungaLight % live and CFU % viable estimates. Dots are colored by AmpB dose. The dash line indicates y = x. Pearson's correlation coefficient and Spearman's rank correlation coefficient are shown inside the plot.

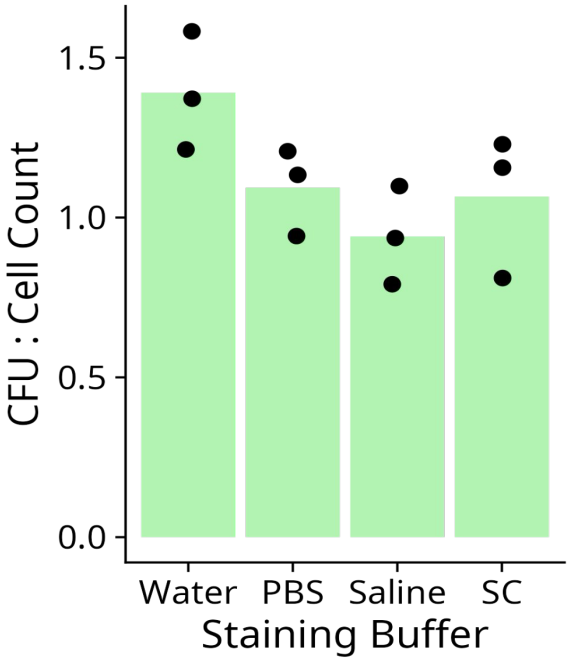

Fig. S1 Viability of mock-treated mid-log phase *C. glabrata* cells. Ratios between the CFU and the number of cells plated based on flow cytometry in mock-treated (mid-log) *C. glabrata* cells resuspended in the indicated buffers. Dots represent the individual biological replicate and bars represent the mean.

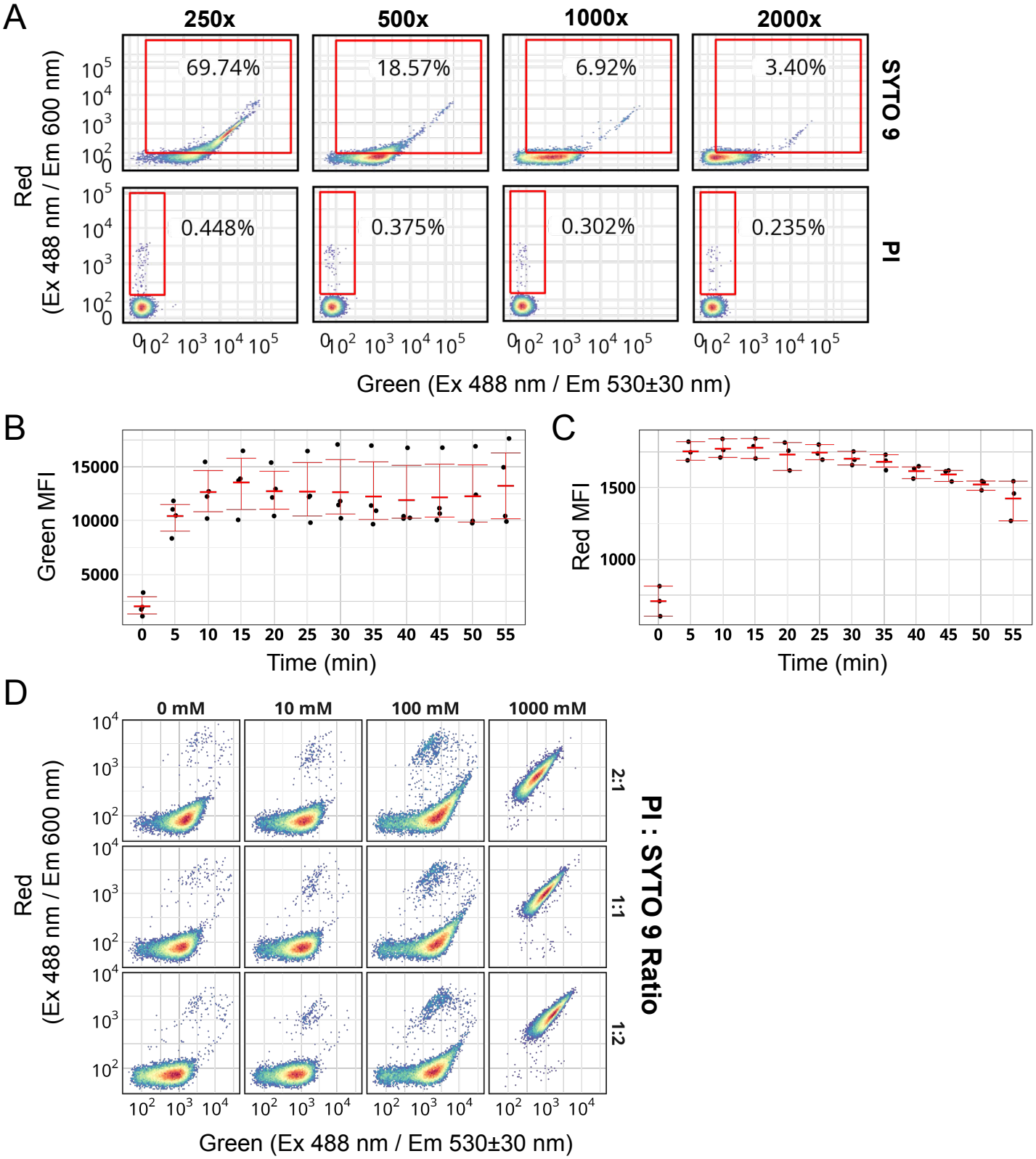

Fig. S2 Testing dye concentrations, incubation time and dye ratio on the staining pattern. (A) 2D density plots showing staining patterns of mid-log *C. glabrata* cells at various dilutions of SYTO 9 or PI (stock concentrations: 3.34 mM for SYTO 9, 20 mM for PI). Rectangular gates were used to quantify cell events considered damaged or dead. (B) Median Fluorescence Intensity (MFI) over time for SYTO9- stained mid-log *C. glabrata* cells. Dots show individual biological replicates (n = 4, biological replicates). Thick red bars represent the mean and the error bars represent the 95% confidence intervals. (C) Similar to B for PI-stained, heat-killed *C. glabrata* cells. (D) 2D density plots for *C. glabrata* cells treated with H_2O_2 and stained with various ratios of PI:SYTO 9 (right). For all samples, SYTO 9 is applied at a fixed concentration of 3.34 μM; PI is used at 20 μM at 1:1 ratio.

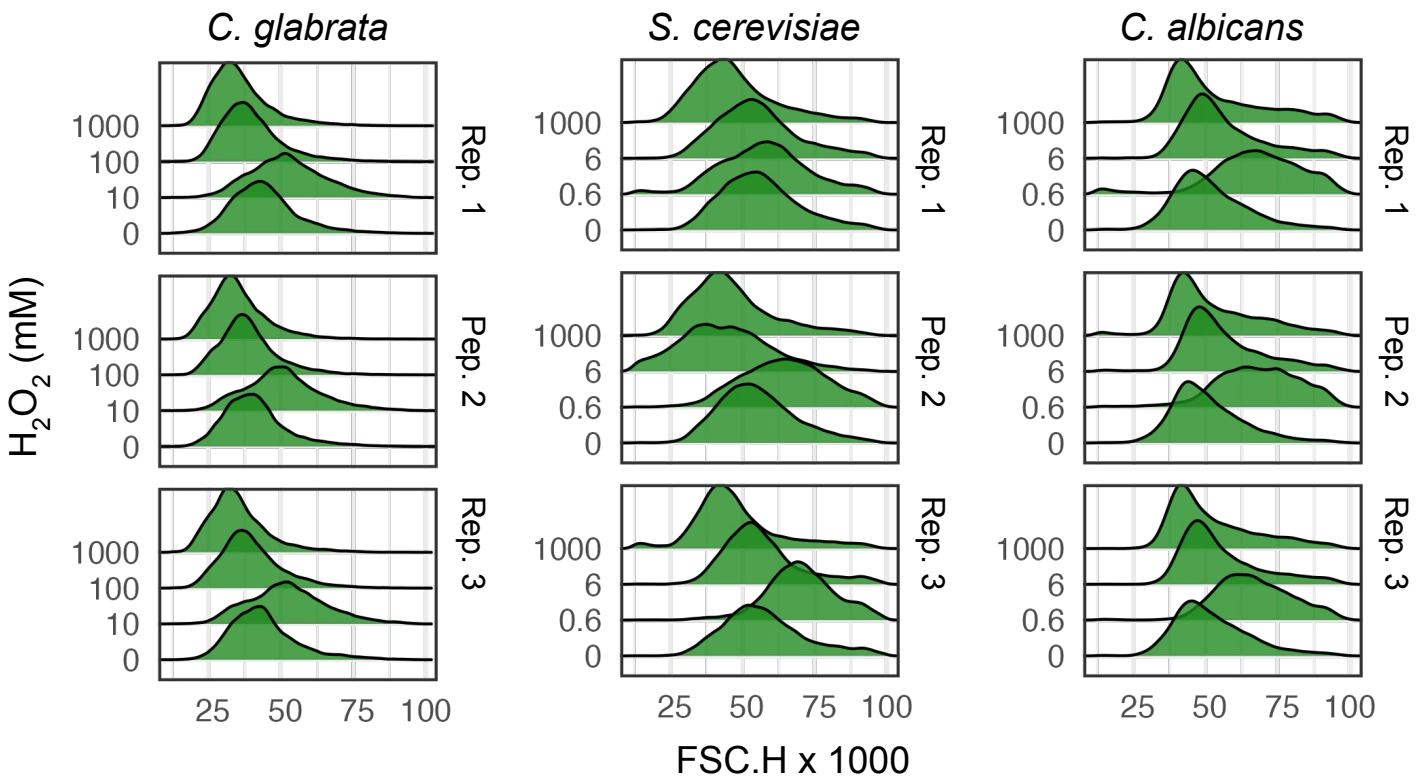

Fig. S3 H_2O_2 treatment causes changes in cell size as indicated by FSC (forward scattering). FSC.H (height) distribution of gated single cell events were shown for each of the three species as indicated on the top. Cells were treated for 2 hours at four different H_2O_2 doses (y-axis labels). Three biological replicates were shown for each species and treatment.

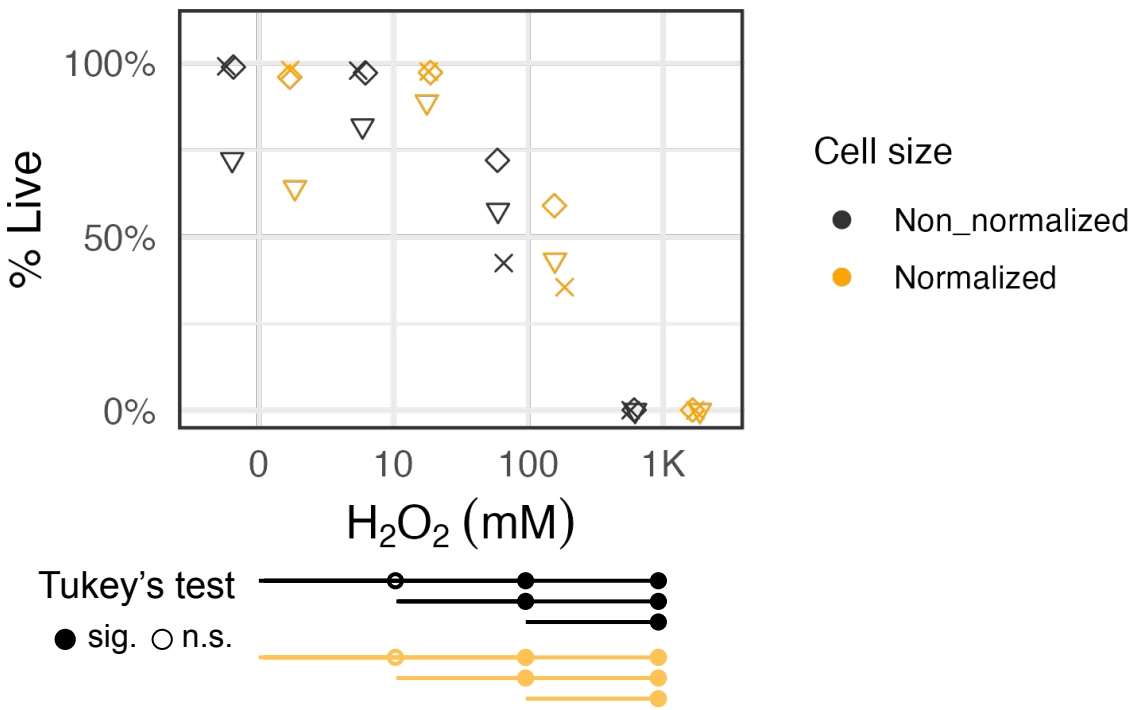

Fig. S4 Normalizing fluorescence by FSC.H didn't reduce the sample variance dramatically.. Fluorescence signals were first normalized by FSC.H (see Methods for details) and then the same gating strategies were applied as for the unnormalized data to calculate the percent live values. The resulting estimates were plotted side-by-side with the non-normalized (Fig. 4B) results for *C. glabrata* treated with different concentrations of H_2O_2. Statistical test results were shown below the graph similarly to Fig. 4B.

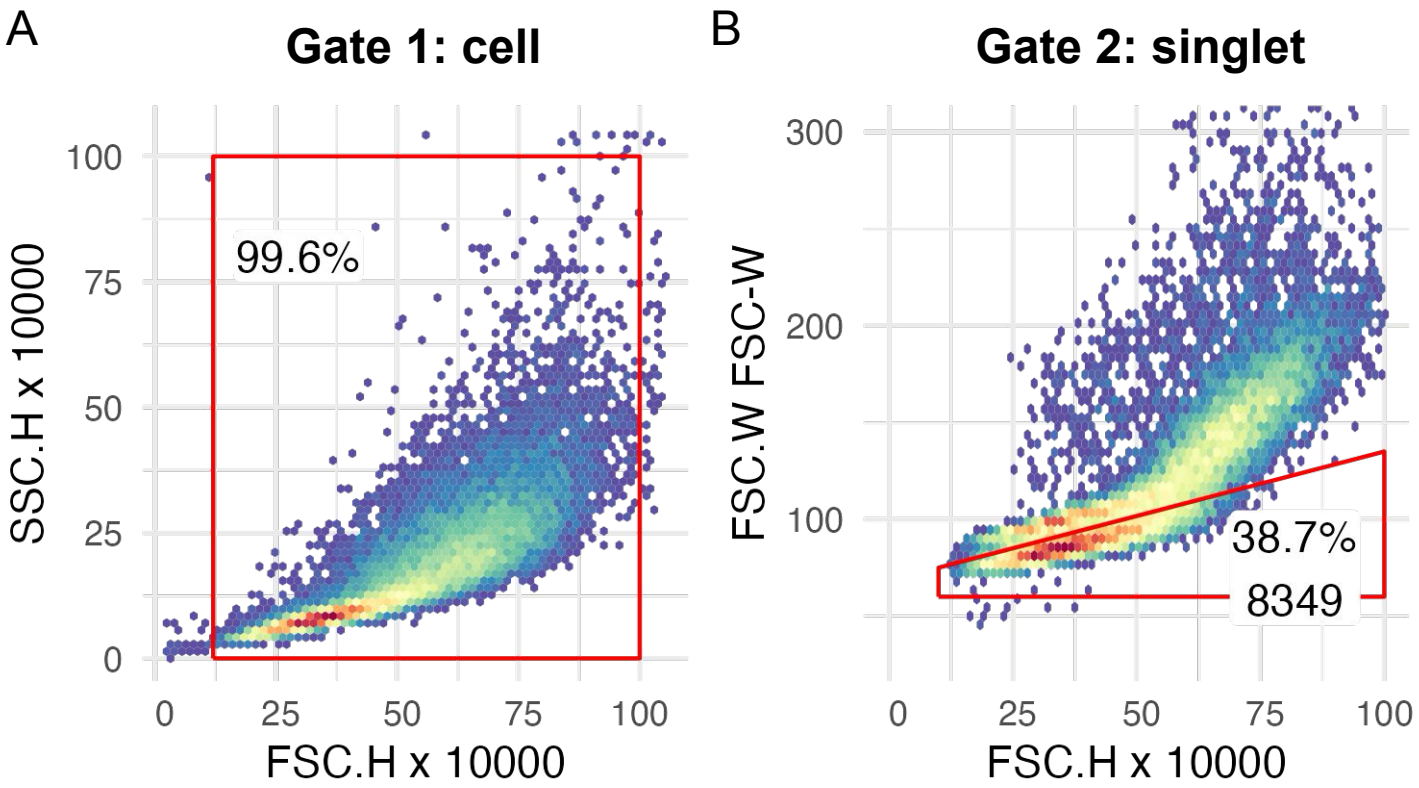

Fig. S5 Example gating strategy for flow cytometry data. (A) All events were plotted on FSC.H and SSC.H. A rectangular gate is used to exclude non-cell events. (B) Events within the first gate ("cell") were plotted on FSC.H and FSC.W. Singlets (single cell event as opposed to doublets or multilets) were selected by excluding events with a higher FSC.W given the same FSC.H.