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Development of the FUN-1 Family of Fluorescent Probes for Vacuole Labeling and Viability Testing of Yeasts

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A new family of fluorescent probes has been developed for assessing the viability and metabolic activity of yeasts. This class of halogenated unsymmetric cyanine dyes is exemplified by the FUN-1 [2-chloro-4-(2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene)-1-phenylquinolinium iodide] stain, a membrane-permeant nucleic acid-binding dye that has been found to give rise to cylindrical intravacuolar structures (CIVS) in *Saccharomyces cerevisiae*. Biochemical processing of the dye by active yeasts yielded CIVS that were markedly red shifted in fluorescence emission and therefore spectrally distinct from the nucleic acid-bound form of the dye. The formation of CIVS occurred under both aerobic and anaerobic conditions and was highly temperature dependent. Treatment of yeasts with the nonmetabolizable glucose analog 2-deoxy-D-glucose reduced cellular ATP levels ~6-fold and completely inhibited CIVS formation. Under aerobic conditions, the formation of CIVS was abrogated by the cytochrome oxidase inhibitors azide and cyanide; however, the H^+ transport uncoupler carbonyl cyanide *m*-chlorophenylhydrazone inhibited CIVS formation under both aerobic and anaerobic conditions. Depletion of cellular thiols, including glutathione, with millimolar concentrations of *N*-ethylmaleimide, iodoacetamide, or allyl alcohol completely inhibited CIVS production. Marked reduction in the formation of CIVS by ethacrynic acid and sulfobromophthalein, inhibitors of glutathione *S*-transferase, suggested that dye processing can involve enzyme-mediated formation of glutathione conjugates. The conversion of FUN-1 by *S. cerevisiae* was studied quantitatively by using several techniques, including fluorometry, flow cytometry, and wide-field and confocal laser scanning fluorescence microscopy.

The widespread use of yeasts to mediate biotransformation of organic materials has promoted the development of numerous techniques for measuring the viability and metabolic activity of these organisms (3, 14–16, 20, 23, 25, 27). There is appreciable variation in the speed, simplicity, sensitivity, and reliability of techniques that are currently in use for this purpose. Although the ability to reproduce ultimately defines cell viability, physiological processes other than those involved in cell division can be of considerable practical importance. A wide range of indicators of physical and metabolic parameters, such as redox potential, membrane permeability, and nutrient utilization, have been used to evaluate the activity of yeast.

Enumeration of viable cells by colony counting is time-consuming and does not reliably report on the metabolic capacity of slow-growing or nondividing cells. Conventional direct-count methods, which typically involve vital staining with indicators such as methylene blue (20) or tetrazolium salts (27), are used to assess the activity of cellular oxidoreductases in yeasts. A technique that has proven to be highly effective in mammalian cells uses the fluorogenic stains calcein acetoxymethyl ester and ethidium homodimer-1 to differentially label the cytosol of live cells and the nuclei of dead cells, respectively. Although this assay is simple to perform, it has been shown to be unreliable in yeasts because of inconsistencies in the permeability or retention of the dyes, as well as the nonspecific surface labeling of the these cells (13). A higher degree of sensitivity may be achieved with a unique new group of indicators that generate differential staining patterns in live and dead yeast cells and do not require the living cells to be in an actively dividing state. The FUN-1 [2-chloro-4-(2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene)-1-phenylquinolinium iodide] stain is representative of this class of indicators, all of which are membrane-permeant, halogenated cyanine compounds that bind nucleic acids and have unexpected and useful properties when used to stain a yeast such as *Saccharomyces cerevisiae* (24).

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MATERIALS AND METHODS

Chemicals and reagents. Allyl alcohol and iodoacetamide were obtained from Aldrich (Milwaukee, Wis.). Sulfobromophthalein, DL-buthionine [*S*,*R*]-sulfoxime (BSO), Calcofluor white M2R, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), 2-deoxy-D-glucose (2-DOG), ethacrynic acid, *N*-ethylmaleimide (NEM), D-glucose (glucose), sodium HEPES (Na-HEPES), sodium cyanide, sodium azide, and other chemicals were obtained from Sigma Chemical Company (St. Louis, Mo.). FUN-1 stain and its analogs were synthesized by Molecular Probes, Inc. (Eugene, Oreg.). Stock solutions of FUN-1 and its analogs contained each compound at 10 mM in dimethyl sulfoxide (DMSO). Stock solutions of allyl alcohol, iodoacetamide, and NEM consisted of 10 mM concentrations of the compounds in E-pure (10 MΩ) water. Potassium cyanide and sodium azide stock solutions contained 100 mM chemicals in 5% glucose and 0.5 mM Na-HEPES in E-pure water. BSO, bromosulfophthalein, and ethacrynic acid stock solutions contained 10 mM compound in 20% glucose and 10 mM Na-HEPES in E-pure water. CCCP and Calcofluor white M2R stock solutions were prepared in DMSO to 100 and 2 mM, respectively. Glucose and 2-DOG were added directly to the buffered solutions as solids.

Yeast cultures. Cultures of *S. cerevisiae* SF838-1Dα and SF838-1Dα vat2Δ were generously provided by Tom Stevens, Institute of Molecular Biology, University of Oregon, Eugene. Lyophilized Fleischmann's baker's yeast was obtained commercially.

Culture conditions. Yeasts were cultured by standard procedures in YTD broth or YTD agar medium containing 1% yeast extract, 2% tryptone, and 2% glucose. Cultures were derived from isolated colonies picked from culture plates grown for 24 to 48 h at 30°C. Overnight broth cultures shaken at 200 to 250 rpm in a 30°C incubator were used for most staining procedures. For anaerobic growth, yeasts were cultured in liquid YTD medium that was purged of oxygen by boiling for 30 min, cooled to 30°C, and incubated in an anaerobic chamber in the presence of an activated BBL GasPak (Baltimore Biological Company, Baltimore, Md.).

Growth studies. Growth studies were conducted at 30°C in 96-well flat-bottom microplates containing broth medium formulated to have low background levels of nucleic acids. The medium consisted of RPMI 1640 (without phenol red; GIBCO) supplemented with 3% tryptone, 5% glucose, and 20 mM Na-HEPES (pH 7.2). This medium was sterilized by filtration through a 0.2-μm-pore-size syringe filter. Absorbance of each well at 410 nm was determined with an MR600 enzyme-linked immunosorbent assay plate reader (Dynatech Laboratories, Inc., Chantilly, Va.). Yeast suspensions were labeled with 5 μM FUN-1 stain for 30 min in the above-specified medium and then subjected to 12 1:2 dilutions in either FUN-1 stain or medium alone. Growth in the wells was indicated by an increase in turbidity after 24 h.

Staining with fluorescent dyes. One milliliter of overnight suspension culture was centrifuged at 10,000 rpm for 3 min in a microcentrifuge and resuspended in staining solution consisting of 20% glucose and 10 mM Na-HEPES (pH 7.2) passed through a 2-μm-pore-size syringe filter. The cell suspension was adjusted to the appropriate density between 5×10^6 and 5×10^8 CFU/ml based on optical density at 410 nm. Dye stocks were prepared in DMSO at a final concentration of 1 to 10 mM. For staining, these stock solutions were added to the yeast cell suspension such that the final DMSO concentration was below 0.2%. The cells were either washed free of dye after staining or allowed to remain in the presence of the dye for observation. Yeasts to be stained under anaerobic conditions were cultivated under anaerobic conditions as described above, pelleted by centrifugation, and resuspended in 20% glucose–10 mM Na-HEPES solution that had been purged of oxygen by boiling. Ten microliters of cell suspension was trapped between a microscope slide and an 18- by 18-mm no. 1.5 coverslip and sealed with paraffin in preparation for microscopy.

Wide-field fluorescence microscopy. All microscopy was carried out with either a Zeiss Axioplan microscope equipped with 20×, 40×, and 100× Neofluar objective lenses or a Nikon Diaphot inverted microscope with a 60×, 1.4-numerical aperture Plan Apo objective lens. Epifluorescence illumination was provided by either a 50- or a 100-W mercury arc lamp. The fluorescence filter sets used were manufactured by Omega Optical (Brattleboro, Vt.). Typical filter sets included the following: for UV, 365-nm excitation, 400-nm dichroic, and 450-nm long-pass emission; for fluorescein, 490-nm excitation, 510-nm dichroic, and 520-nm long-pass emission; for rhodamine, 546-nm excitation, 560-nm dichroic, and 580-nm long-pass emission; and triple-band 4′,6-diamidino-2-phenylindole (DAPI)–fluorescein–Texas Red sets. Both microscopes were fitted with a 35-mm camera back and an electronic light meter and shutter. Photomicrographs were acquired with Ektachrome Daylight 400 ASA color slide film (Kodak, Rochester, N.Y.). Photographic slides were digitized electronically, and composite figures were assembled from the resulting images with Adobe Photoshop software (Adobe Systems Inc., Mountain View, Calif.).

Laser scanning confocal microscopy. FUN-1-stained yeasts were observed with a TCS 4D confocal laser scanning microscope (Leica Inc., Malvern, Pa.) equipped with a 100×, 1.4-numerical aperture objective lens. Stacks of images were taken through the *z* axis of the yeast cells at intervals of 0.5 μm, using the 568-nm line of the Kr/Ar laser (>600-nm emission). Three-dimensional projections of the *z*-axis series were produced with NIH-Image/PPC V1.6 software. Images selected from the three-dimensional projection series were combined to create stereo pairs, which were combined and pseudocolored with Adobe Photoshop software.

Fluorometric analysis of yeast suspensions. Fluorescence spectra were obtained with an Alphascan fluorescence spectrophotometer (Photon Technology International, Inc., South Brunswick, N.J.). Measurement of fluorescence in yeast suspensions was carried out in 1-cm-path-length acrylic cuvettes that were held in a temperature-controlled block and stirred continuously with a magnetic stir bar. Fluorescence microplate assays were carried out in flat-bottom 96-well clear polystyrene plates. Yeast cells in microplates were kept in suspension with an oscillating shaker during all incubations. The fluorescence of cell suspensions in microplates was measured with a Cytofluor 2300 fluorescence microplate reader (PerSeptive Biosystems, Framingham, Mass.). Conversion of FUN-1 stain by yeasts was monitored either by measuring the complete 480-nm excited fluorescence emission spectrum from 500 to 700 nm with the Alphascan fluorometer or by acquiring fluorescence values in a fluorescence microplate reader equipped with a 485-nm excitation filter and an emission filter centered at either 535 or 640 nm. Dye conversion was expressed as a change in the ratio of red (580 to 610 nm) to green (510 to 540 nm) fluorescence (R/G ratio).

Enumeration of cells containing CIVS. Cells were labeled with FUN-1 stain as described above and subsequently labeled with 25 μM Calcofluor white M2R for 10 min. Ten microliters of yeast cell suspension was added to a slide previously coated with poly-L-lysine and sealed as described above. Images were acquired with an integrating color charge-coupled device camera (Optronics Engineering, Goleta, Calif.) and stored digitally as three separate images corresponding to the red, green, and blue outputs of the camera. Cylindrical intravacuolar structures (CIVS) were indicated by the coincidence of a red image of a predetermined minimum intensity that is enclosed by a high-intensity zone in the blue image plane, corresponding to the cell wall chitin labeled with Calcofluor white M2R.

Flow cytometric analysis. Flow cytometry was carried out with a FACS Vantage instrument (Becton Dickinson Immunocytochemistry Systems, San Jose, Calif.) equipped with a Coherent Enterprise laser (Coherent, Santa Clara, Calif.) in which the 488-nm beam was set to a light-regulated output of 100 mW. Fluorescence emission was collected with interference bandpass filters (Omega Optical): 530 ± 30 nm (FL1). The sheath fluid consisted of 0.9% NaCl and sample flow rates through a 70-μm nozzle tip were between 200 and 400 particles/s. Data were stored as list-mode files onto the hard disk of an Apple Macintosh Quadra 650 microcomputer and subsequently analyzed with software supplied by the manufacturer.

Treatment with 2-DOG and cell disruption. *S. cerevisiae* SF838-1Dα was cultured for 22 h at 30°C in 500 ml of YTD medium shaken at 200 rpm. The culture was divided in two equal portions, and one half was immediately refrigerated at 5°C. The remaining culture was pelleted by centrifugation at $500 \times g$ for 10 min, and the cell pellet was resuspended in 200 ml of 30°C filter (0.2-μm pore size)-sterilized 0.5% 2-DOG in 10 mM Na-HEPES, pH 7.4 (2-DOG–HEPES). The cell suspension was shaken for 16 h at 30°C, pelleted by centrifugation, resuspended in 50 ml of 2-DOG–HEPES, and placed on ice for subsequent cell counting. The previously refrigerated yeast culture was warmed slowly to 30°C, pelleted by centrifugation, and resuspended in 30°C 2% glucose in 10 mM Na-HEPES, pH 7.4 (glucose-HEPES). The suspension was shaken at 30°C for 1 h, pelleted by centrifugation, resuspended in 50 ml of glucose-HEPES, and placed on ice for cell counting. Yeast cells in suspension were disrupted with a probe sonic oscillator (Branson Sonifier 250; Branson, Danbury, Conn.). One-milliliter samples were withdrawn from both suspensions immediately prior to and 30 min after sonication and placed on ice. For ATP measurement, 2 ml of sonicated yeast suspension was pelleted at 10,000 rpm in a microcentrifuge, and the supernatant was retained for subsequent measurement of ATP. The initial cell number and the fraction of cells lysed by sonication were quantified microscopically and used to calculate the amount of ATP in 10^8 cells.

ATP measurement. The amount of ATP released from sonicated yeast cells was determined by using a luminometric luciferin-luciferase assay (ATP determination kit; Molecular Probes) in combination with a TD-20/20 luminometer (Turner Designs, Sunnyvale, Calif.) according to a modification of the instructions provided with the kit. In brief, a 1 mM ATP stock solution was diluted serially in nine 10-fold dilutions, and 20 μl of the dilutions was added to 180 μl of reaction buffer containing luciferin and luciferase. Luminescence of the samples was read in 1.5-ml polypropylene microcentrifuge tubes, and a standard curve relating ATP concentration to luminescence was constructed for between 2×10^{-17} and 2×10^{-8} mol of ATP. Twenty microliters of yeast lysate supernatant solution was added to 180 μl of reaction buffer, and luminescence was read as described above. Background luminescence of reaction buffer alone was subtracted from all readings. ATP amount was expressed as picomoles of ATP per 10^8 yeast cells.

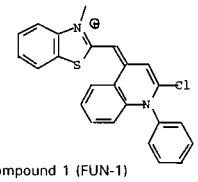
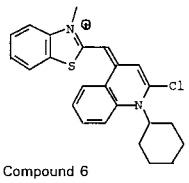
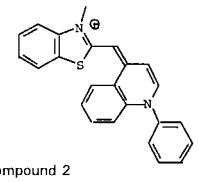
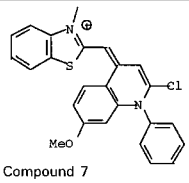
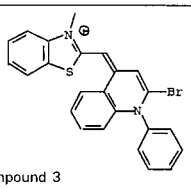
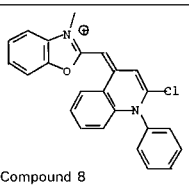
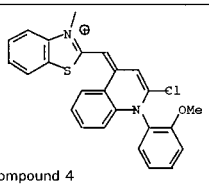
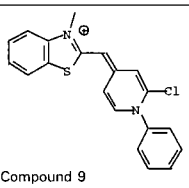
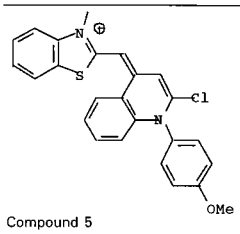
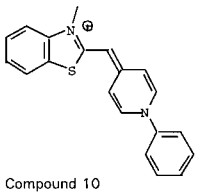
Evaluation of CIVS formation in 2-DOG-treated yeast cells. The ability of yeast cells to produce CIVS from FUN-1 was evaluated by adding 5 μM FUN-1 to a suspension of 10^6 yeast cells per ml of test medium, followed by a 30-min incubation at 30°C. CIVS formation was monitored by direct observation in the fluorescence microscope, using a rhodamine long-pass epifluorescence filter set.

RESULTS

Properties of FUN-1 stain. FUN-1 stain (Table 1, compound 1) is a halogenated unsymmetric cyanine compound with one fixed positive charge. Free FUN-1 stain is virtually nonfluorescent in aqueous solution; however, when complexed with DNA or RNA and excited with light between 470 and 500 nm, FUN-1 stain fluorescence at 530 nm increased as much as 400-fold (24). FUN-1 generally did not stain the nuclei of metabolically active yeast cells in growth medium or glucose-containing staining solutions. The fluorescence of a 5 μM solution of FUN-1 in water was enhanced ~6-fold in the presence of 0.1% bovine serum albumin and ~32-fold in 1% bovine serum albumin with no shift in emission spectrum.

Metabolically active yeast cells sequester FUN-1 stain in their vacuoles. When *S. cerevisiae* cells were stained with FUN-1 dye and observed by differential interference microscopy (DIC) (Fig. 1a), cylindrical refractile bodies could be observed in the vacuolar region of the cells. These same cells observed by epifluorescence microscopy (590-nm excitation and 610-nm emission) contained bright red-orange fluorescent structures (Fig. 1b) that correlated in morphology and position with the refractile bodies observed by DIC. A small number of yeast cells contained no CIVS and were stained uniformly with very bright yellow-green fluorescence. These brightly labeled yeast cells were assumed to be nonviable because heat-killed cells were found to stain with similar fluorescence hue, distribution, and intensity (data not shown). Confocal microscopy of yeast cells stained with FUN-1 stain showed that the CIVS typically reside within the vacuoles (Fig. 2). The CIVS ap-

TABLE 1. Structure-function relationships between FUN-1 stain and related compounds

Chemical Structure	CIVS			Chemical Structure	CIVS		
	Maximum Number	Maximum Length	Fluorescence Emission		Maximum Number	Maximum Length	Fluorescence Emission
 Compound 1 (FUN-1)	>10	>10 μm	red-orange	 Compound 6	>10	>10 μm	red-orange
 Compound 2	None	N/A	N/A	 Compound 7	<5	<3 μm	red-orange
 Compound 3	>10	>10 μm	red-orange	 Compound 8	>10	>10 μm	red-orange
 Compound 4	>10	>10 μm	red-orange	 Compound 9	<5	>10 μm	yellow-orange
 Compound 5	>10	>10 μm	red-orange	 Compound 10	None	N/A	N/A

peared in random orientations, some adjacent to the inner surface of the vacuolar membrane and others projecting through the lumen of the vacuole.

The progression of FUN-1 dye labeling could be observed in the wide-field epifluorescence microscope. Different patterns of cell staining corresponded to the stages of conversion and sequestration of FUN-1 stain by the cells. Figure 3a to f shows separate fields of yeast cells incubated at room temperature for various lengths of time with 5 μM FUN-1 stain and visualized by using a triple-band optical filter set (see Materials and Methods). Within 1 min of FUN-1 dye addition, the live yeast cells were stained uniformly with dim green fluorescence (Fig. 3a). After 3 min had elapsed, regions of more concentrated yellow-green fluorescence began to appear in the cytoplasm. Minute orange spots also began to appear in a few of the cells (Fig. 3b). After about 7 min, minute orange structures could be observed in most of the cells, primarily in the vacuolar region (Fig. 3c). At this early stage, the structures were difficult to resolve and to document by photomicrography or electronic imaging due to their small size, low fluorescence intensity, and rapid saltatory movement. After 60 min (Fig. 3f and 4a and b), the CIVS had reached their maximum size and many cells had lost any trace of green cytoplasmic staining. Many of the CIVS became curved as they conformed to the boundary of the vacuolar membrane.

Compounds that are structurally related to FUN-1 stain also gave rise to fluorescent bodies in the vacuoles of actively growing yeast cells. *S. cerevisiae* stained with compound 7 or 9 (Table 1) produced fluorescent structures that were observed by fluorescence microscopy using a fluorescein long-pass filter set (exitation at 485 nm and emission at >510 nm; Fig. 5a and b, respectively). Brightly fluorescent CIVS were observed in cells in all three of the preparations, but each was unique with respect to its morphology and fluorescence emission. Yeast labeled with FUN-1 stain (Fig. 1) produced numerous long, red-orange fluorescent structures, whereas CIVS in the vacu-

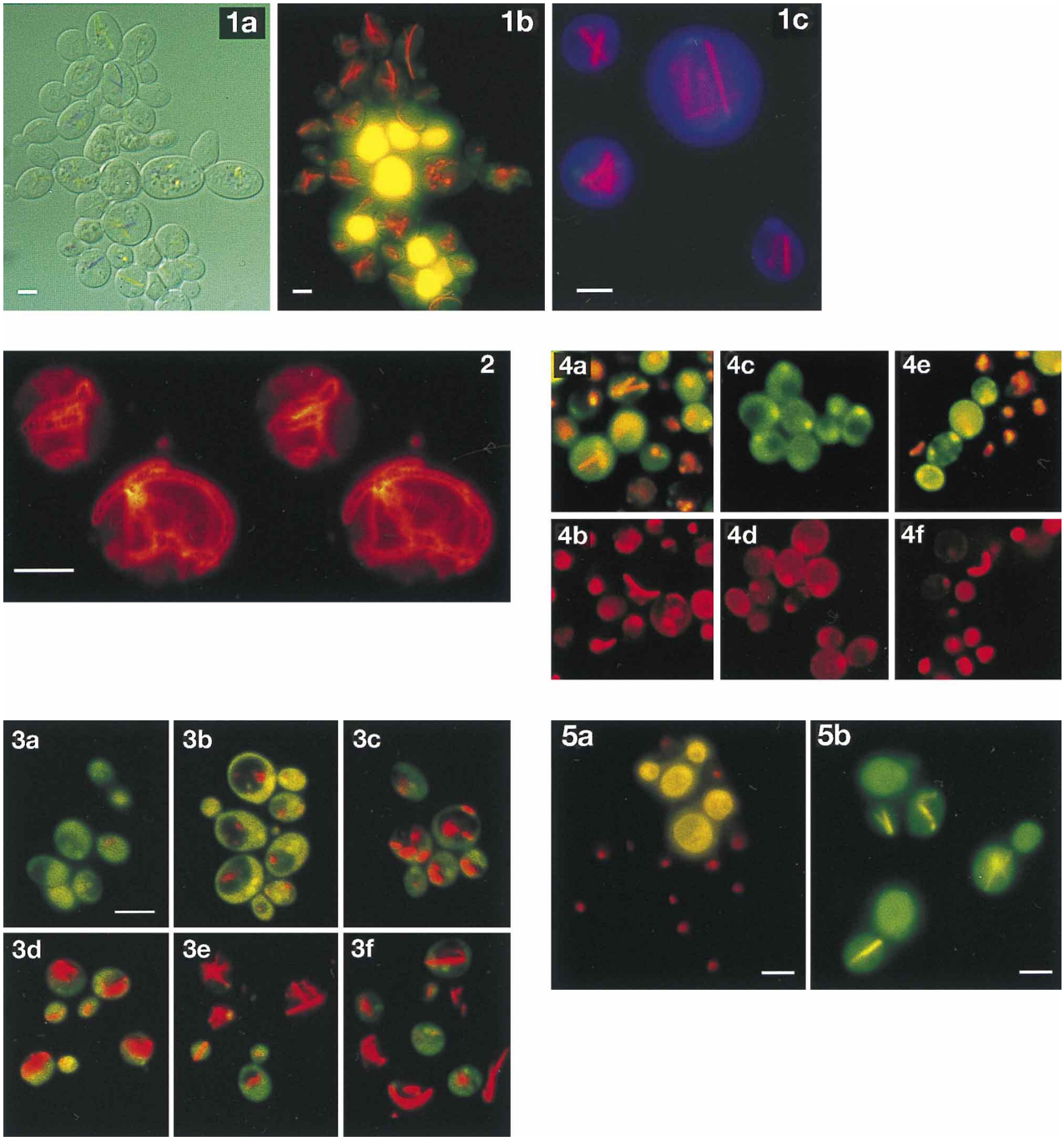


FIG. 1. *S. cerevisiae* SF838-1Dα stained with FUN-1 dye and observed by DIC (a) and epifluorescence microscopy (b and c). In DIC, CIVS appear as yellow or blue rod-shaped inclusions (a), depending on their orientation in the polarized light. The same field of cells observed by epifluorescence microscopy using a fluorescein long-pass filter set (see Materials and Methods) shows bright orange-fluorescent inclusions in green-fluorescent cells (b). A small number of dead yeast cells are stained uniformly with very bright yellow fluorescence and do not contain discrete fluorescent structures. Bars = 5 μm. FUN-1 staining is combined with Calcofluor white M2R, a blue-fluorescent chitin label that can be used to decorate the cell walls of either living or dead cells (c). FUN-1 and Calcofluor staining were observed simultaneously, using a triple-band filter set (see Materials and Methods). Bar = 5 μm.

FIG. 2. Stereo pair of confocal images of two *S. cerevisiae* SF838-1Dα cells labeled with FUN-1 stain for approximately 1 h at 30°C. Only CIVS within the yeast vacuoles are visible in the images. Bar = 2.5 μm.

FIG. 3. Time course of conversion of FUN-1 to CIVS by *S. cerevisiae* SF838-1Dα. The six panels show separate fields of yeast cells stained at room temperature for various lengths of time (1 [a], 3 [b], 7 [c], 20 [d], 30 [e], and 60 [f] min) with 5 μM FUN-1. Photomicrographs were acquired by using a triple-band DAPI-fluorescein-Texas Red filter set (see Materials and Methods). Bar = 5 μm.

FIG. 4. Effect of 2-DOG on CIVS formation. Yeast suspensions were incubated with either 2% glucose (a and b) or 0.5% 2-DOG (c to f) for 16 h, washed by centrifugation, and resuspended in either 2% glucose (a, b, e, and f) or 0.5% 2-DOG (c and d) for 30 min prior to staining with 5 μM FUN-1 stain. Cell staining was observed by using a fluorescein long-pass filter set (a, c, and e) or a Texas Red filter set (b, d, and f).

FIG. 5. Intravacuolar structures in yeast cells labeled with structural analogs of FUN-1 stain. *S. cerevisiae* SF838-1Dα cells were stained with compounds 7 (a) and 9 (b) (Table 1) and observed by epifluorescence microscopy using a fluorescein long-pass filter set (excitation at 485 nm and emission at >510 nm). Small orange spots are associated with the vacuoles of yeast cells that display no other intracellular staining (b), whereas dead cells fluoresce bright yellow. Bars = 5 μm.

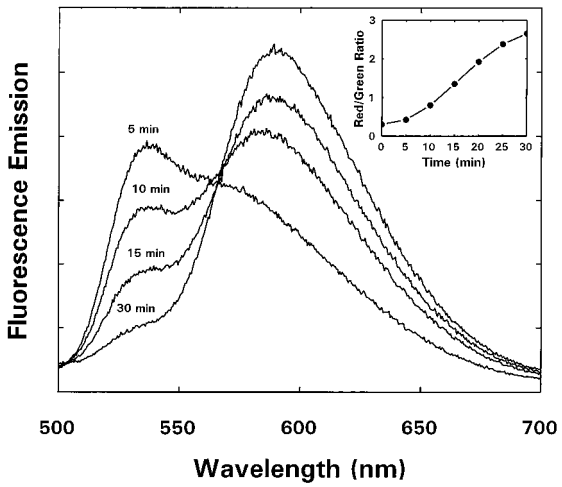


FIG. 6. Conversion of FUN-1 by metabolically active baker's yeast. Fluorescence emission spectra of a suspension of FUN-1-stained yeast cells excited at 480 nm were acquired at 5-min intervals for 30 min. The ratio of red (580 to 610 nm) to green (510 to 540 nm) fluorescence in FUN-1-labeled yeast (inset) increases during the incubation period.

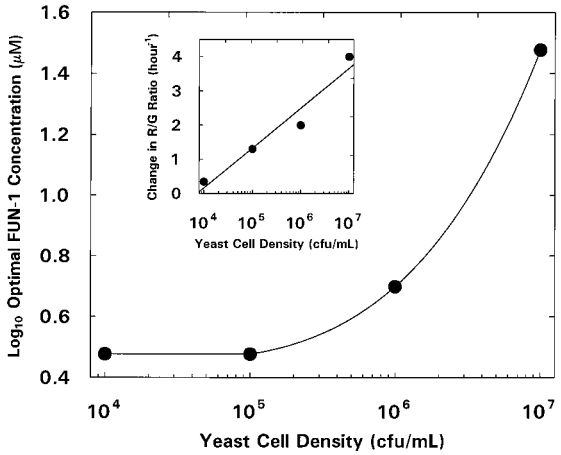


FIG. 7. Effect of yeast cell density and dye concentration on the rate and extent of bioconversion of FUN-1 stain by yeast cells. Each point in the main graph represents the concentration of FUN-1 stain that brought about the most rapid rise in R/G ratio (main plot). The maximum change in R/G ratio that was obtained at each cell density is shown (inset).

oles of yeast cells stained with compound 7 (Fig. 5a) were also red-orange in fluorescence but were only 1 to 2 μm or less in length. Staining with compound 9 (Fig. 5b) resulted in formation of CIVS that were generally fewer in number than those produced from FUN-1 stain; however, the average length of CIVS arising from staining with compound 9 was greater than that of CIVS obtained with FUN-1 stain, and the fluorescence emission was yellow-orange rather than red-orange. As with FUN-1 stain, dead, permeabilized yeast cells were labeled very brightly fluorescent by all three stains, but CIVS were not formed.

The growth of all strains of *S. cerevisiae* was completely arrested by FUN-1 when the cells were incubated continuously with sufficient stain to result in formation of CIVS. Inhibition of yeast growth by FUN-1 stain was also readily reversible. Cells labeled with FUN-1 stain and subsequently washed free of the dye rapidly regained the ability to proliferate normally in broth or solid media (data not shown). The CIVS in these cells did not decrease in size, and the number remained constant until the cells underwent division.

Changes in yeast staining that were observed by fluorescence microscopy over time could also be monitored quantitatively in a fluorometer and fluorescence microplate reader. The primarily green fluorescence emission of a suspension of FUN-1-stained yeast cells that was excited at 480 nm shifted to the red region of the spectrum over time (Fig. 6).

The rate and extent of CIVS formation in *S. cerevisiae* were sensitive to both cell number and dye concentration. Optimal loading was achieved when yeast cells were exposed to limiting levels of the dye, which was then processed by the cells into red-orange fluorescent material. Excessive loading with FUN-1 stain increased the amount of residual green fluorescence, whereas suboptimal loading typically limited both the number and the size of the CIVS. Brief exposure of the yeast cells to a concentrated FUN-1 stain solution (≥50 μM) resulted in maximal staining and structure formation, as did increasing the density of yeast cells to a level high enough for cellular uptake to substantially reduce the extracellular FUN-1 stain concentration. When yeast cells were incubated with FUN-1 stain, centrifuged free of the dye, and subsequently resuspended in fresh glucose solution without the dye, the cells processed most of the retained FUN-1 stain. FUN-1 stain was completely removed from solution by 2×10^7 heat-killed yeast cells/ml but was left virtually unchanged by an equivalent number of live cells. In suspensions of living yeast cells (<5% dead), the optimal FUN-1 stain concentration increased with cell number, and the rate of change in the R/G ratio was related to the concentration of the stain (Fig. 7).

The rate of conversion of FUN-1 stain was highly temperature dependent. Uptake and conversion of FUN-1 stain by yeast cells occurred over a 30°C range in temperature, with an exponential dependency of the initial rate of FUN-1 dye conversion (ΔR/G ratio) on temperature between 10 and 40°C and an abrupt decline at 50°C (Fig. 8).

CIVS are distinct from DAPI-stained intravacuolar polyphosphate bodies. The nucleic acid stain DAPI enters yeast cells, staining nuclei with blue fluorescence when excited with UV light. DAPI binds double-stranded DNA with an

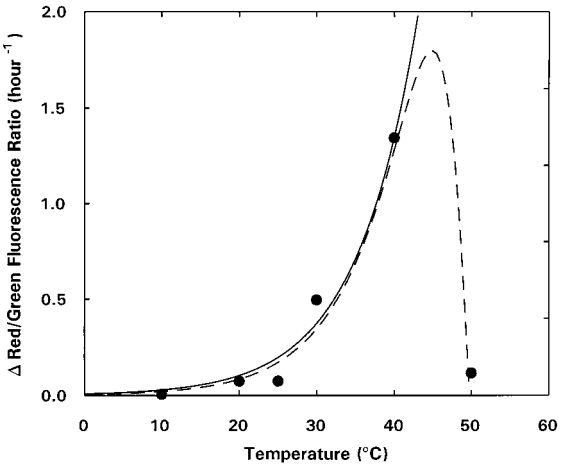


FIG. 8. Temperature dependence of FUN-1 stain conversion. An exponential fit (solid line) of the initial rate of FUN-1 conversion (ΔRed/Green) is shown as a function of temperature (Q_{10} of ~3.6). A best-guess fit of the complete heat-inactivation curve is shown (dashed line).

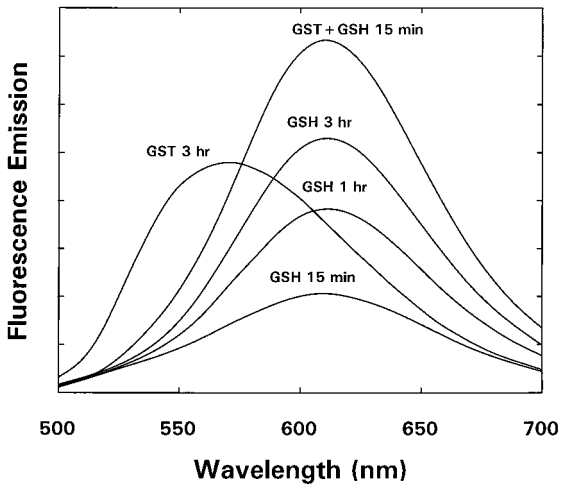


FIG. 9. Comparison of rates of reaction at pH 8.3 of 60 μM GSH with 10 μM FUN-1 stain alone or in the presence of 1 U of GST/ml.

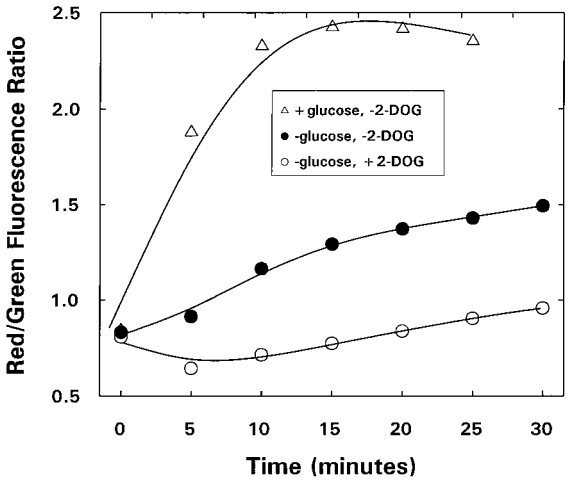


FIG. 10. Rate of change in R/G fluorescence ratio in yeast cells pretreated for 18 h with 2-DOG. Yeast suspensions were incubated in 2% 2-DOG for 18 h, washed free of 2-DOG by centrifugation, and stained with FUN-1 dye with either 2% glucose (+glucose, −2-DOG), without additions (−glucose, −2-DOG), or with 2% 2-DOG (−glucose, +2-DOG). The change in R/G ratio of yeast suspensions was monitored in a fluorometer.

affinity of about 2×10^7 (12). DAPI also facilitates the appearance of spherical, polyphosphate-based, green fluorescent vacuolar bodies (FVB) (28, 32) in some strains of *S. cerevisiae*. Incubation of yeast cells with 10 μM DAPI for 30 min in the presence of 20% glucose resulted in staining of the cell nuclei (365-nm excitation and 450-nm emission) and appearance of green-fluorescent FVB in the vacuoles. When the DAPI-labeled cells were subsequently washed and incubated with 5 μM FUN-1 stain, both green FVB and orange-red CIVS were observed in many of the cells after several minutes. Even though FUN-1 stain is excited optimally in the blue region of the spectrum, fluorescence of the CIVS could be excited with light ranging from the near UV to the green region of the spectrum.

FUN-1 processing involves conjugation with thiol-containing biomolecules. Chemical modifications of the FUN-1 parent compound (Table 1; compare to FUN-1 [compound 1]) involving methoxy group addition to various positions (compounds 4, 5, and 7), Cl-to-Br substitution (compound 3), phenyl-to-cyclohexyl substitution (compound 6), thiazole-to-oxazole substitution (compound 8), or quinoline-to-pyridine substitution (compound 9) did not inhibit CIVS formation. Halogen substituents, such as Cl or Br, were required for CIVS formation (compare compounds 1 [FUN-1] and 2 and also compounds 9 and 10).

FUN-1 stain reacted with reduced glutathione (GSH) in vitro, yielding a red-fluorescent product with a fluorescence emission maximum near 610 nm (Fig. 9). This reaction occurred spontaneously at pH 8.0, and its rate was enhanced threefold under these conditions by addition of glutathione *S*-transferase (GST). FUN-1–GSH conjugates generated in vitro could be separated from free FUN-1 stain by thin-layer chromatography. Furthermore, fluorescent organic solvent-extractable material isolated from crudely fractionated yeast cells containing fluorescent bodies comigrated on thin-layer chromatography with FUN-1–GSH generated in vitro (data not shown). When solutions containing high concentrations (>100 μM) of GSH and FUN-1 stain were trapped between a glass slide and coverslip, rod-shaped structures formed spontaneously within a few minutes. The dimensions of the FUN-1–GSH structures and CIVS were measured and compared by digital image analysis. The diameter of red fluorescent structures that formed from FUN-1–GSH solutions was 0.69 ± 0.05 μm, and the length varied considerably, from 5 to 20 μm, as it did in yeast vacuoles. The average diameter of the intravacuolar structures was 0.62 ± 0.07 μm, while the length varied widely, from 2 to greater than 5 μm.

GSH levels in *S. cerevisiae* have been shown to be reduced to <5% of normal levels after a 1-h incubation with either 1 mM allyl alcohol or 1 mM iodoacetamide and to <20% with 1 mM NEM (7). Incubation of strain SF838-1Dα in 1 mM solutions of any of these compounds for 1 h prior to labeling with 5 μM FUN-1 completely inhibited the formation of CIVS (data not shown). When cells treated with either allyl alcohol or iodoacetamide were transferred to fresh medium without inhibitor, CIVS formation was recovered in more than half of the cells within 3 h. Although no obvious signs of cell death were observed, yeast cells treated with NEM were unable to generate CIVS, even when incubated in staining solution for 24 h after removal of the inhibitor. At 1 mM, the GST inhibitors ethacrynic acid and Sulfobromophthalein inhibited CIVS formation by 50 and 75%, respectively. Pretreatment of yeast cells for 12 h with the GSH synthesis inhibitor BSO (10 mM) reduced GSH levels by 70% but did not affect the formation of CIVS (data not shown).

ATP is required for CIVS formation. CIVS formation could occur in metabolically active yeast cells in water or saline solutions; however, the process was enhanced markedly by addition of glucose to the staining solution (Fig. 10). The dependency of CIVS formation on active oxidative or fermentative metabolism in yeast cells was demonstrated by the inhibitory effect of the nonmetabolizable glucose analog 2-DOG (8, 19) on biochemical conversion of the dye. Replacement of glucose in the medium with 2-DOG inhibited the conversion of FUN-1 stain from a green-fluorescent to a red-fluorescent form (Fig. 10). The initial rate of FUN-1 dye conversion by yeast cells that were incubated with 2-DOG for 18 h and then stained in the presence of 2-DOG alone was 8.6-fold lower than that of yeast cells held in glucose during staining and 2.6-fold lower than that of yeast cells held in medium without glucose or 2-DOG (Fig. 10). Removal of the yeast cells from the 2-DOG solution and addition to fresh staining solution containing glucose resulted in virtually immediate formation of numerous structures throughout the cells, while incubation

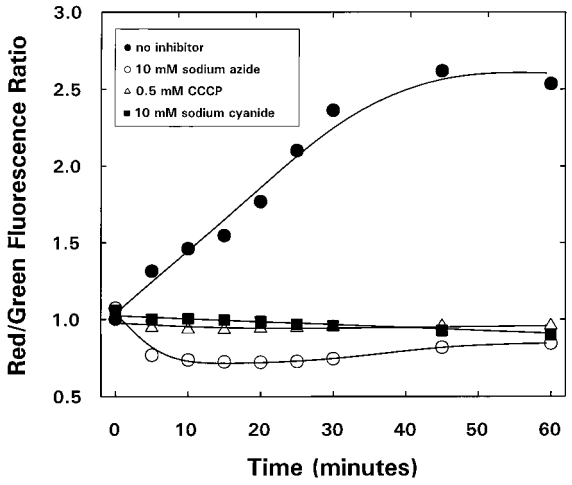


FIG. 11. Inhibition of FUN-1 sequestration by respiration inhibitors. CIVS formation and the accompanying increase in R/G ratio by aerobically grown yeast cells incubated with FUN-1 stain alone or in the presence of 10 mM sodium azide, 10 mM sodium cyanide, or 0.5 mM CCCP are shown.

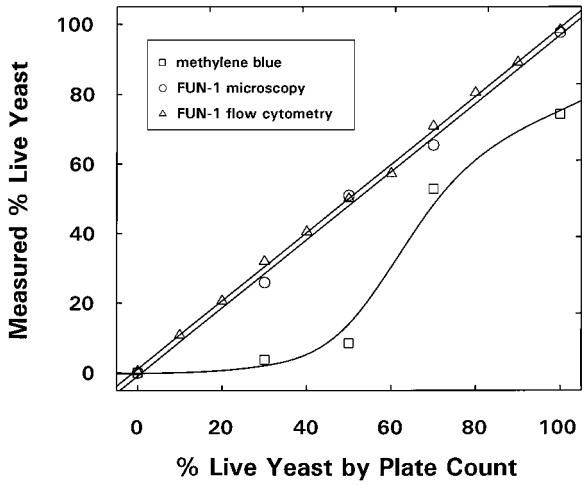


FIG. 12. Comparison of FUN-1 and methylene blue staining for assessing yeast viability. Suspensions of yeast cells with various proportions of live and dead cells were diluted, plated on YTD agar medium, and then incubated at 30°C for 48 h prior to colony counting (CFU/milliliter). Suspensions of yeast cells were also stained with either 5 μM FUN-1 stain or 1% methylene blue. Viability was scored by microscopy for the presence of CIVS in FUN-1-stained yeast cells and for clearing in methylene blue-stained yeast cells. Yeast cells were also labeled with 5 μM FUN-1 stain and subjected to flow cytometric analysis using 488-nm excitation and single-channel, 530-nm emission.

with glucose for 30 min prior to incubation with FUN-1 stain promoted formation of structures only within the vacuole (Fig. 4e and f).

The amount of cellular ATP was measured to determine if 2-DOG inhibition of CIVS formation was correlated with ATP depletion. Cells incubated with 5 μM FUN-1 in the presence of 2% glucose contained 177 pmol of ATP/10^8 cells and exhibited diffuse cytoplasmic green and orange-red fluorescence in addition to CIVS (Fig. 4a and b). Parallel incubation of yeast cells with 0.5% 2-DOG for 16 h in the absence of glucose resulted in an ~5.7-fold reduction in cellular ATP to 31 pmol/10^8 cells. Cells treated with 2-DOG alone were stained with diffuse green and red cytoplasmic fluorescence, and each contained a bright fluorescently labeled nuclear spot (Fig. 4c and d). CIVS production was completely inhibited in these cells. Complete recovery of CIVS formation was brought about by replacement of 2-DOG with 2% glucose 30 min prior to labeling with FUN-1 stain (Fig. 4e and f).

Under aerobic conditions, CIVS formation and the accompanying increase in R/G ratio were completely abrogated by inhibition of mitochondrial cytochrome oxidase with sodium azide or sodium cyanide or by treatment with CCCP to uncouple the H^+ gradient across the mitochondrial inner membrane (Fig. 11). When yeast cells were grown and stained under anaerobic conditions with rigorous exclusion of oxygen, azide and cyanide no longer completely inhibited CIVS formation (data not shown). In contrast, CCCP inhibited CIVS formation completely under both oxidative and fermentative conditions, suggesting a different or additional mode of inhibition by CCCP besides depletion of ATP by mitochondrial uncoupling. A yeast strain devoid of vacuolar H^+-ATPase (SF838-1Dα vat2Δ [32]) produced CIVS equivalent in size and number to those of an otherwise equivalent strain with normal vacuolar H^+-ATPase activity (SF838-1Dα) when both strains were incubated with FUN-1 stain under identical conditions (data not shown).

FUN-1 allows discrimination of live and dead yeast cells. To test the ability of FUN-1 stain to discriminate live from dead yeast cells, suspensions of live and heat-killed yeast cells were mixed in various proportions and analyzed by four methods. To determine the number of CFU/milliliter, samples of the yeast suspension were diluted, plated on YTD agar medium, and incubated at 30°C for 48 h, after which time colonies were counted. Viability determination by staining was scored by the presence of CIVS in FUN-1-stained cells and by clearing in methylene blue-stained cells. When the numbers of viable cells were assessed by colony counts, a closer correlation was observed with CIVS formation in FUN-1 dye-labeled cells than with clearing in methylene blue-stained cells (Fig. 12).

The relative proportions of live and dead yeast cells stained with FUN-1 stain were also quantified by flow cytometry, although not by detection of CIVS. Suspensions containing various proportions of live and heat-killed cells were stained with FUN-1 and analyzed in the flow cytometer, using the 488-nm line of the argon ion laser for excitation while monitoring a single-emission channel (530-nm bandpass filter). Fluorescence microscopy of yeast had shown previously that the heat-killed cells were much more brightly fluorescent in both the green and red regions of the spectrum than were live cells. In the flow cytometer, the fluorescence of live and dead cells fell into two discrete regions that were easily discriminated from one another. When suspensions of live and dead yeast cells were mixed in various proportions and analyzed by flow cytometry, the resulting distributions of fluorescent particles accurately reflected the known numbers of live and dead organisms in the sample (Fig. 12).

DISCUSSION

Members of the FUN-1 family of dyes are nucleic acid stains that are virtually nonfluorescent in solution but can undergo fluorescence enhancement of 10- to 50-fold with protein binding and >400-fold with nucleic acid binding. These stains give rise to the formation of CIVS in the vacuoles of metabolically active yeast cells grown and stained under either oxidative or fermentative conditions.

CIVS derived from FUN-1 stain have been used to specifically identify the vacuolar compartment in *S. cerevisiae* (4), an application that is of particular interest for studies of organelle

inheritance (12). FUN-1 has also been used in conjunction with flow cytometry to measure the susceptibility of *Candida albicans* to antifungal compounds (30) and also has been observed to give rise to CIVS in *Aspergillus nidulans* (5).

CIVS are distinctly different from the polyphosphate bodies sometimes observed in the lumen of vacuoles in yeast cells stained with DAPI. Indeed, the two types of structures, CIVS and FVB (1), could be generated in the same vacuole and observed simultaneously because of their spectrally distinct fluorescence emission.

Adenine-deficient mutants of *S. cerevisiae* (*ade1* and *ade2* strains) accumulate an intracellular red pigment derived from phosphoribosylaminoimidazole and its carboxylate form, respectively (11, 26). Red-pigmented structures, which might be confused with the CIVS generated by FUN-1 dye-stained yeasts or give rise to CIVS, are found in *ade1* and *ade2* mutants but not in yeast strains without these mutations. CIVS are produced both in *ade1* mutants and in normal yeast cells (data not shown) and are therefore not related to this mutation.

ATP is required for the formation of CIVS by *S. cerevisiae*. The relationship between CIVS formation and ATP level was demonstrated by the inhibitory effect of the nonmetabolizable glucose analog 2-DOG on yeast processing of FUN-1 stain. The inhibition of CIVS formation paralleled a 5.7-fold drop in cytoplasmic ATP concentration in 2-DOG-treated yeast cells. Relief from inhibition of glucose catabolism, by removal of 2-DOG and readdition of glucose to the medium, rapidly reversed the inhibitory effect. The respiration inhibitors azide and cyanide completely eliminated FUN-1 conversion to CIVS under aerobic conditions, where mitochondrial ATP production is of primary importance, and were much less effective in yeast cells grown fermentatively and stained under anaerobic conditions.

The mitochondrial uncoupler CCCP also inhibited CIVS formation. CCCP acts by permeabilizing the inner mitochondrial membrane to protons, thereby dissipating the transmembrane proton gradient that is exploited by the mitochondrial ATPase to generate ATP. It has been shown, however, that in addition to uncoupling mitochondria, CCCP can dissipate the H^+ gradient across the yeast vacuolar membrane (2). Since the intravacuolar environment of wild-type *S. cerevisiae* is typically about pH 6.0 (22, 31), the proton gradient between the vacuole and the cytosol, which is about pH 7.0 (6, 10), is ~10-fold. If a pH gradient of this magnitude were required for the transport of dye molecules into the vacuole, or if low vacuolar pH were necessary for their aggregation into CIVS, then hypothetically CCCP could inhibit CIVS formation under fermentative conditions by dissipating the H^+ gradient. Equivalent CIVS formation either in a yeast strain with normal vacuolar H^+-ATPase activity (SF838-1Dα) or in a mutant that does not express the vacuolar membrane proton pump (SF838-1Dα vat2Δ) strongly suggests, however, that neither ΔpH nor vacuolar membrane potential (Δψ) is important for transport of FUN-1 stain into the yeast vacuole. Since vacuolar pH in the H^+-ATPase-lacking mutant is nearly neutral, low vacuolar pH is unlikely to be required for molecular association of FUN-1 stain into CIVS. Taken together, these results show that ATP, generated through either oxidative phosphorylation or glycolysis, is required for one or more steps leading to the vacuolar sequestration of FUN-1 stain and its analogs and that the ATP required for CIVS formation is not related to the functioning of the vacuolar H^+-ATPase; i.e., an intact vacuolar proton gradient is not required for CIVS to form.

The morphological and spectral characteristics of CIVS formed by yeasts can differ markedly when the cells are exposed to FUN-1 analogs with different chemical substituents; however, processing of FUN-1 and related dyes requires that the dye contain a halogen substituent such as Cl or Br (Table 1). Since the halogen moieties are easily displaced by nucleophiles, they may allow the halogenated stains to react with free sulfhydryl groups of peptides and proteins. CIVS formation requires FUN-1 stain to be transported from the cytoplasm of the yeast cell to the vacuole, a process that can be facilitated by generation of a conjugate.

The removal of FUN-1 from the yeast cytosol involves biochemical modification of the dye and sequestration of the resulting adducts in the vacuole. FUN-1–GSH adducts with spectral properties identical to those of CIVS formed in live yeast cells could be generated in vitro. Although the formation of FUN-1–GSH adducts was accelerated by GST in vitro, the reaction did not require GST or ATP. CIVS-like structures could be generated in the absence of enzymes or cells. The inhibition of CIVS formation brought about by ATP depletion in live yeast cells is therefore unlikely to be attributable solely to inhibition of adduct formation and is more likely due to the inability of the cells to transport the FUN-1 stain adducts into the vacuole.

In many eukaryotic cells, detoxification and solubilization of xenobiotic compounds and their metabolites occur through formation of covalent adducts with small biomolecules such as GSH (21). Common strains of *S. cerevisiae* typically express a variety of GST isozymes (18, 21) and maintain millimolar levels of cytosolic GSH (9). Elimination of xenobiotic compounds involves activation, conjugation, and transport from the cytosol. Conjugation with GSH directs compounds for export from the cytosol by at least two transport systems. In *S. cerevisiae*, an uncoupler-insensitive Δψ-dependent uniporter acts on certain negatively charged conjugates, whereas an uncoupler-insensitive Mg-ATP-dependent pump transports other GSH conjugates (17, 29, 32). Transport of GSH adducts by either system is independent of the ΔpH between the vacuole and the cytosol, which is consistent with the observation that CIVS formation by a yeast mutant lacking vacuolar H^+-ATPase activity was indistinguishable from that of a FUN-1-stained yeast strain with a normal intravacuolar pH. Monochlorobimane-GSH and dinitrophenol-GSH conjugates have been shown to be transported into the vacuoles of certain *S. cerevisiae* strains by the yeast cadmium factor protein (YCF1), the uncoupler-insensitive pathway that is responsible for cadmium resistance, which resembles the human multidrug resistance associated protein both structurally and functionally. It is probable that FUN-1–GSH conjugates are also transported into the yeast vacuole by the YCF1 system. It remains unclear how CIVS formation is inhibited by the uncoupler CCCP under anaerobic conditions, since under these conditions the uncoupler should affect neither Mg-ATP levels nor YCF1 function. CCCP may act on a cellular process upstream from transport of FUN-1–GSH conjugates into the vacuole.

Conversion of the FUN-1 stain by yeast cells was found to be highly temperature dependent, with an apparent Q_{10} of 3.6, nearly double the Q_{10} of 2 normally associated with single-enzyme-mediated processes. This observation is consistent with the idea that CIVS formation is a multistep process involving one or more enzyme-mediated conversions in addition to transport of biochemically conjugated dye into the vacuolar compartment. Under static conditions, FUN-1 staining of yeast with intact plasma membranes is much less than that of permeabilized cells in which FUN-1 stain labels accessible proteins and nucleic acids. Dead, permeabilized yeast cells are easily detected by single-color fluorometry or flow cytometry. More precise analytical procedures such as epifluorescence microscopy or ratiometric fluorometry are required to deter-

mine if the less brightly fluorescent yeast cells were metabolically competent.

The transport of FUN-1 stain and its analogs from the cytosol to the vacuole does not occur in dead yeast cells. The kinetics of FUN-1 stain conversion and transport can be quantified either by measurement of the relative contributions of red and green fluorescence after exposure of the yeast to the dye or by visualization of CIVS in the yeast vacuoles. Cell viability determined by fluorometry is reflected either by the difference between the starting and ending R/G ratios or by the rate of change in the ratio. A single ratio cannot be interpreted alone because a large initial fluorescence ratio may be indicative of dead, permeabilized yeast cells. Proteins and nucleic acids in dead, permeabilized cells stain brightly and have a broad fluorescence emission spectrum, which contains appreciable signals in both the green and red regions of the spectrum. Free thiols of proteins and peptides may also react spontaneously with FUN-1 stain, generating red-fluorescent adducts in permeabilized cells. Counting of yeast cells containing CIVS by fluorescence microscopy allows the number of living, culturable organisms under normal growth conditions to be determined. Fluorescence labeling with FUN-1 stain provides spectral and morphological information not available with other single-dye fluorescence-based methods for viability determination in yeasts and fungi. The FUN-1 family of stains may also be useful tools for probing the function of multidrug resistance transporters and naturally occurring resistance factors in yeasts.

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