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Assessment of mitochondrial membrane potential in yeast cell populations by flow cytometry

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In yeast the use of rhodamine 123 (Rh123) has been restricted to the evaluation of mitochondrial respiratory function including the discrimination between respiratory-competent and -deficient cells. This study describes the optimization and validation of a low-concentration Rh123 staining protocol for the flow-cytometric assessment of mitochondrial membrane potential ($\Delta\Psi$m) changes in whole yeast cells. The optimized protocol was validated by the use of compounds that specifically affect mitochondrial energetics. Epifluorescence microscopy was used to monitor Rh123 distribution within the cell. Incubation of yeast cell suspensions with Rh123 (50 nM, 10 min) gave minimal non-specific binding and cytotoxicity of the dye. The ratio (R) between the green fluorescence and forward scatter (both measured as log values) was used to measure $\Delta\Psi$m with only little dependence on cell 'volume' and mitochondrial concentration. Cells treated with mitochondrial membrane de- or hyper-polarizing agents displayed a decrease and an increase of R values respectively, indicating that changes of the Rh123 distribution in cells indicate variations in the $\Delta\Psi$m. Live and dead cells also displayed significantly different R values. The method described here allows assessment of $\Delta\Psi$m changes in whole yeast cells in response to a given drug. Moreover, the relationship between drug effects and disorders of mitochondrial energetics might be addressed.

Keywords: mitochondrial functionality, flow cytometry, rhodamine 123, whole yeast cells

INTRODUCTION

In the eukaryotic cell ATP is provided mainly through mitochondrial oxidative phosphorylation. Such a mechanism is achieved by the respiratory electron-transport chain and by the ATP synthase complex, both localized at the mitochondrial inner membrane. In mitochondria, the energy released by the transfer of electrons is used to pump out H^+. Therefore, H^+ electrochemical potential (protonmotive force; Δp) is formed by a proton gradient (ΔpH) and an electrical gradient ($\Delta\Psi$m). To produce ATP, the ATP synthase complex mediates the H^+ flow back to the matrix and the energy is conserved in the phosphorylation of ADP to ATP. In fact, the Δp is one of the most important physiological parameters that reflects energy status and mitochondrial membrane transport. Direct assessment of Δp is not possible; thus it is necessary to evaluate separately the mitochondrial electrical potential ($\Delta\Psi$m) and the proton chemical gradient (ΔpH) (Chen, 1989).

In recent years, many probes for monitoring $\Delta\Psi$m have been developed and described with increasing emphasis on membrane-permeable lipophilic cations. Mitochondria are the only organelles known to have a significant membrane potential, with a negative charge inside. Hence lipophilic compounds with delocalized positive charge may be accumulated by mitochondria to a much greater extent than by other organelles (Grinius *et al.*, 1970). These compounds can freely enter the cell by simple diffusion and although plasma membrane potential contributes to some accumulation into the cytoplasm, the dye concentration there would be much

Abbreviations: EFM, epifluorescence microscopy; FCM, flow cytometric microscopy; FS, forward scatter; R, ratio; Rh123, rhodamine 123; SS, side scatter.

lower than that in the mitochondria. In fact, the Nernst equation predicts a $\Delta\Psi p$ of -60 mV and a $\Delta\Psi m$ of -180 mV with accumulation ratios of 10 and 1000 in the cytoplasm and mitochondria, respectively. Therefore, the probe concentration inside mitochondria will be 10000-fold higher than that in the extracellular medium (Chen, 1988).

Among the different fluorescent dyes available, rhodamine 123 (Rh123) is the one most frequently used for the assessment of $\Delta\Psi m$ in mammalian cells (Emaus *et al*., 1986; Johnson *et al*., 1981; Goldstein & Korczac, 1981; O'Connor *et al*., 1988; Petit *et al*., 1990; Juan *et al*., 1994). Rh123 stains mitochondria directly, without passage through endocytotic vesicles and lysosomes (Chen *et al*., 1981), and distributes electrophoretically into the mitochondrial matrix in response to $\Delta\Psi m$ (Emaus *et al*., 1986). In yeast the use of that probe has been restricted to the evaluation of the mitochondrial (respiratory) function (Skowronek *et al*., 1990; Lloyd *et al*., 1996; Lloyd, 1999) and the detection of efflux pumps (Prudêncio *et al*., 2000; Kolaczkowski *et al*., 1996). The success of this cationic dye in probing $\Delta\Psi m$ in mammalian living cells is closely related to its lower toxicity than other fluorescent cations. However, depending on the concentration and the cell type, it can also be cytotoxic. So far Rh123 has been used at concentrations up to 10 μg ml^{-1} (Emaus *et al*., 1986; Johnson *et al*., 1981; Goldstein & Korczac, 1981; O'Connor *et al*., 1988; Petit *et al*., 1990; Juan *et al*., 1994; Lloyd *et al*., 1996).

The aim of the present work was to optimize a Rh123 staining protocol to assess qualitative changes of $\Delta\Psi m$ in whole yeast cells by flow cytometry. Flow cytometry provides a sophisticated technique for research on heterogeneous microbial populations to determine physiological characteristics on a single-cell basis.

METHODS

Micro-organisms and culture conditions. *Zygosaccharomyces bailii* ISA 1307, originally isolated from a continuous production plant of sparkling wine (Wium *et al*., 1990), was used. *Saccharomyces cerevisiae* IGC 4072 was originally isolated from a sample of Fremivin, an industrial wine yeast distributed by Rapidase. *Saccharomyces cerevisiae* J-1-3 (*MAT α ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 aac1*::*LEU2 aac2*::*HIS3 aac3*::*URA3*) was kindly provided by Professor Jordan Kolarov (Department of Biochemistry, Faculty of Sciences, Comenius University, Bratislava, Slovakia). All strains were maintained on slants of YEPD medium containing yeast extract (0·5 %, w/v), peptone (1 %, w/v), glucose (2 %, w/v) and agar (2 %, w/v). Cells were grown in 250 ml flasks containing 100 ml synthetic medium (van Uden, 1967) with vitamins, oligoelements and 2 % (w/v) glucose (MGV). The cells were grown to early and late exponential phase in a water bath, with magnetic stirring (250 r.p.m.), at 26 °C.

Fluorochrome solutions. Concentrated stock solutions of Rh123 (500 μM; Molecular Probes) were prepared in 100 % ethanol and stored at −20 °C. Working solutions (25 μM) were prepared by diluting the stock solutions in 100 % ethanol and kept on ice in the dark to minimize degradation.

Preparation of yeast suspensions, flow cytometric (FCM) and epifluorescence microscopy (EFM) analysis. In control assays, cell suspensions were prepared from cultures at early and late exponential phase. Cells were harvested, centrifuged (1600 ***g*** for 4 min at 4 °C) and washed twice with ice-cold distilled water, then resuspended (about 1×10^6 cells ml^{-1}) in sterile commercial water (PARACÉLSIA), pH 6·0. It is generally accepted that this cell concentration allows an adequate data acquisition by flow cytometry. Control suspensions of killed cells were prepared by boiling the cell suspensions, prepared as above, for 10 min.

The Rh123 staining protocol was developed as described in Results. FCM analysis was performed on a EPICS XL-MCL (Beckman-Coulter) flow cytometer, equipped with an argon-ion laser emitting a 488 nm beam at 15 mW. The green fluorescence was collected through a 488 nm blocking filter, a 550 nm long-pass dichroic and a 525 nm band-pass. Twenty thousand cells per sample were analysed. An acquisition protocol was defined to measure forward scatter (FS), side scatter (SS) and green fluorescence (FL1) on a four decades logarithmic scale. Green fluorescence was gated in a scattergram of log SS × log FS in order to include the subpopulation with the highest frequency and homogeneity in the fluorescence measurements. The data were analysed with the Multigraph software included in the system II acquisition software for the EPICS XL/XL-MCL version 1.0. The ratios between green fluorescence and FS were performed offline with Multiparameter Data Analysis Software, MULTIPLUS AV (Phoenix Flow Systems).

The kinetics of Rh123 uptake by the yeast cells was followed by flow cytometry. For this, green fluorescence acquisition over time of an unstained yeast cell suspension, was started and paused after 1 min. As quickly as possible, Rh123 was added to the cell suspension; the acquisition continued again and was followed for 30 min.

The EFM analyses were performed on a Leitz Laborlux S epifluorescence microscope equipped with a 50 W mercury lamp and a filter set (excitation filter BP 450–490, a beam splitter FT510 and an emission filter LP520). Samples of stained cell suspensions (20 μl) were placed between a slide and a cover slip after mixing with an equal volume of antifading reagent (Vectashield Mounting Medium for fluorescence H-1000; Vector Laboratories). The digital images were acquired with a 3CCD Colour Video Camera (SONY, DXC-9100P), a frame grabber (IMAGRAPH, IMASCAN/Chroma-P) and software for image archival and management (HOSPITRANS, Fotoscope version 1.0). In the EFM analysis the concentration of Rh123 used was 500 nM instead of 50 nM, due to the lower sensitivity of EFM compared with FCM. However, the dye to cell concentration ratio was the same as that used in FCM analysis.

Cytotoxicity assays of Rh123. Cytotoxicity of Rh123 was determined by estimating its effect on cell viability assessed by the plate count method. The c.f.u. were counted after 2 d incubation at 26 °C on YEPD agar plates.

Modification of mitochondrial membrane potential. To induce a decrease in the mitochondrial membrane potential ($\Delta\Psi m$), cells were treated for 10 min at room temperature prior to the Rh123 staining, with one of the following chemical agents (all from Sigma): the uncoupler of oxidative phosphorylation carbonyl cyanide *p*-(tri-fluoromethoxy)-phenyl-hydrazone (FCCP; 5, 10 and 15 μM), the electron-transport inhibitor sodium azide (NaN_3; 20 mM) or the potassium ionophore, valinomycin (8 μM). A decrease of $\Delta\Psi m$

was also achieved by cell starvation. For this purpose, cells from an overnight culture, prepared as described above, were resuspended in culture medium without glucose and incubated for 48 h in a water bath, with magnetic stirring (250 r.p.m.), at 26 °C.

Incubation of the cells with 2-deoxy-D-glucose (2-DOG; Sigma) was also carried out. This compound is a glucose analogue that competitively inhibits glucose transport and depletes cells of ATP. Cells were also incubated with 2-DOG (100 mM) at room temperature, for 10 min before Rh123 staining.

The ΔΨm was also evaluated in the presence of other agents that disrupt mitochondrial metabolism, namely oligomycin (0·4 μg ml^{-1}), an F_0-ATP-synthase inhibitor, and nigericin (5 μM), a dissipater of ΔpH (both from Sigma). These agents were added 10 min before Rh123 staining.

Stimulation of ΔΨm was achieved by incubation at room temperature with 100 or 150 mM glucose, or 5 mM succinate, 10 min before Rh123 staining. Incubation with glucose was carried out at pH 6·0 and incubation with succinate at pH 3·0. Both substrates were from Merck.

Reproducibility of the results. All experiments were repeated at least three times. The data reported are from one representative experiment.

RESULTS

The aim of the present work was to optimize a Rh123 staining protocol for the assessment of mitochondrial membrane potential (ΔΨm) in yeast. This protocol was designed to ensure that the total fluorescence level obtained reflected a relative measure of that functional parameter. It was taken into account that the flow cytometer measures, in each individual cell, the total amount of fluorescence and not the actual concentration of the dye. Consequently, cells with the same fluorochrome concentration but different relative volumes will show different levels of fluorescence and could be misinterpreted as cells displaying different ΔΨm values. Therefore, it was necessary to correlate the data on fluorescence intensity from individual cells with the relative cell volume estimated from the forward light scatter (Shapiro, 1994; Seligmann & Gallin, 1986). In addition, we attempted to i) identify other factors that could lead to different levels of fluorescence independent of changes in ΔΨm and ii) select the minimum concentration of the dye to minimize both non-specific binding and cytotoxicity.

Normalized Rh123 fluorescence

The analysis of biparametric histograms, plotting log SS against log FS, of cell suspensions of *Zygosaccharomyces bailii* or *Saccharomyces cerevisiae* revealed a marked heterogeneity, in both relative complexity and size. In the scattergram of a *Z. bailii* cell suspension (Fig. 1a) several subpopulations can be visualized. The subpopulation at the top right consists mainly of cell aggregates, since it disappeared after a brief ultrasonication of the cell suspension (data not shown). The subpopulation with the lowest scatter most probably corresponds to young cells that have been recently separated from their mother cells. The most numerous subpopulation is present in the middle of the scattergram. The monoparametric histogram of the total fluorescence of this subpopulation has a great coefficient of variation (Fig. 1b), and three different gated regions (A, B and C) can be defined in the corresponding biparametric histogram (Fig. 1a). Previous results on DNA distributions (Howlett & Avery, 1999; Fortuna *et al*., 2000) showed that these regions are related to the different phases of the cell cycle; the A, B

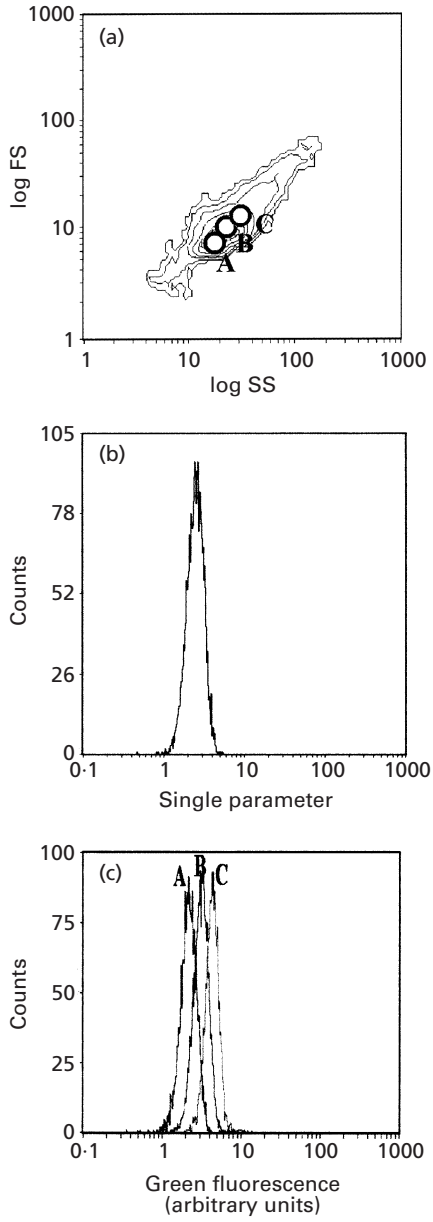

Fig. 1. (a) Scattergram (contour plot) for *Z. bailii* after Rh123 staining. (b) Green fluorescence histogram of *Z. bailii* after Rh123 staining of non-gated population. (c) Overlay of green fluorescence histograms of the same cell suspension of gated regions A, B and C in (a).

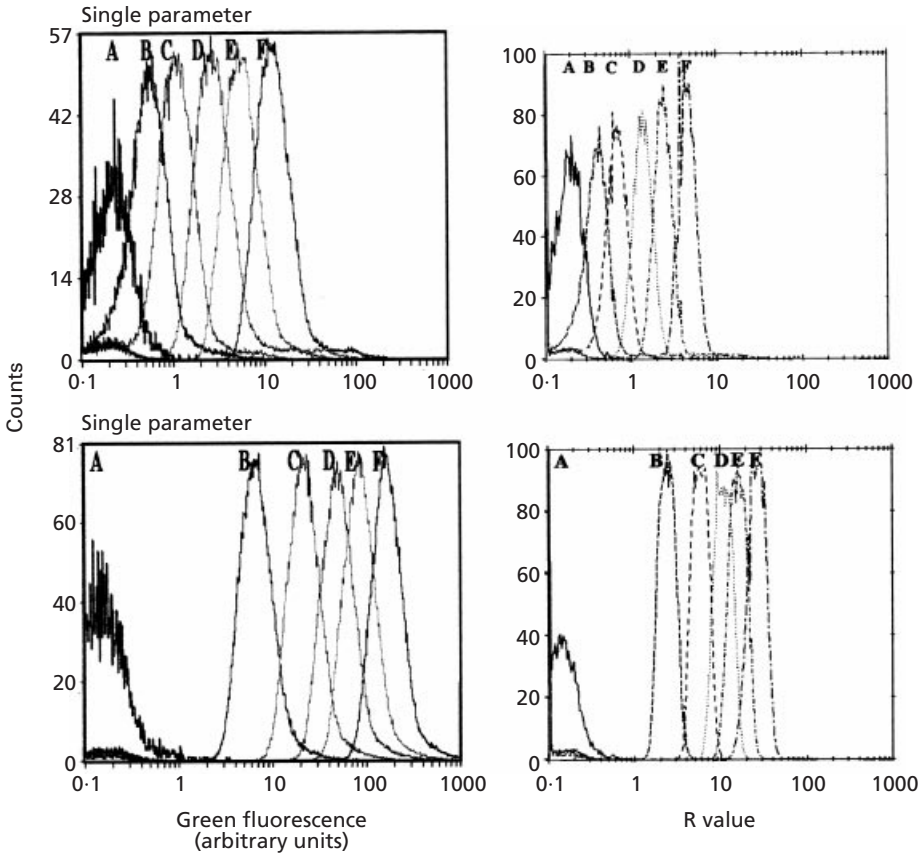

Fig. 2. Overlay of green fluorescence histograms (left panels) and respective R values (right panels) of *Z. bailii* before (A; autofluorescence) or after staining with different Rh123 concentrations (B, 10 nM; C, 25 nM; D, 50 nM; E, 100 nM and F, 200 nM) in a live cell control suspension (top panels) and a dead cell control suspension (bottom panels).

and C gated regions correspond to cells in phases G0/G1, S and G2/M, respectively. The fluorescence obtained in each gated region gives rise to three different distributions (Fig. 1c), pointing to the necessity to normalize the Rh123 fluorescence. Actually, cells with the same fluorochrome concentration but different relative volumes showed different levels of fluorescence. Normalization was achieved by representing in a monoparametric histogram the log of the green Rh123 fluorescence intensity, divided by the log of the FS signal. This ratio (R) was calculated by MULTIPLUS software (Phoenix Flow Systems).

Optimization of the staining conditions with Rh123: dye to cell concentration ratio, pH, temperature, incubation time and composition of the staining medium

To find the optimal ratio between the dye and cell concentration, the Rh123 concentration was varied from 10 to 200 nM while the cell concentration was kept constant (about 1×10^6 cells ml^{-1}). For the range of Rh123 concentration tested the mean fluorescence intensity increased stepwise with the dye concentration (Fig. 2, top panels) and no saturating kinetics were observed in the concentration range tested. This indicates that changes in the concentration of the probe produced distinct fluorescence levels for a given cell concentration. Thus, the maintenance of a constant ratio between dye and cell concentration, when comparing $\Delta\Psi m$ from different cell samples, turned out to be a critical step in the staining protocol. In addition, the mean fluorescence intensity of dead cells was seen to be much higher than for live cells, when a heat-killed cell suspension was stained with the same dye concentration (Fig. 2, bottom panels). The higher mean fluorescence level observed in dead yeast cells was also evident on EFM (Fig. 3). In fact, dead cells displayed a diffuse fluorescence in the cytoplasm whereas live cells displayed mitochondrial staining together with some slight non-specific fluorescence at the cell envelope.

The R values between green fluorescence and FS (both measured as log signals) for the live and dead cell suspensions are shown in Fig. 2. This estimation increased the homogeneity of the fluorescence and confirmed the advantage of normalizing this value.

Following these results, the Rh123 concentration selected was 50 nM rather than 25 or 10 nM. The former concentration value, although yielding a cell

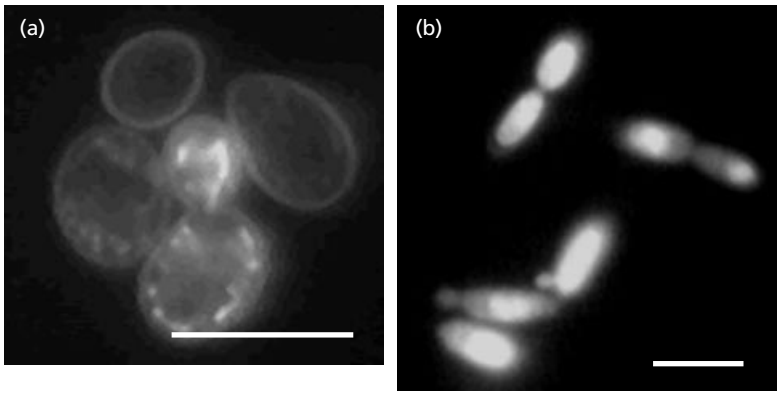

Fig. 3. Photomicrographs of *Z. bailii* live (a) and dead (b) cells, stained with Rh123 (500 nM). Bars, 10 μm.

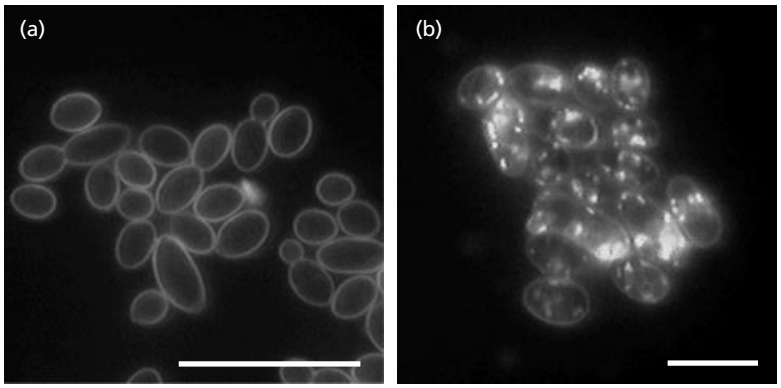

Fig. 4. Photomicrographs of *Z. bailii* cells stained with Rh123 (50 nM) after (a) 5 min incubation with Rh123 (b) 10 min incubation with Rh123. Bars, 10 μm.

fluorescence level distinct from autofluorescence, would allow a more sensitive detection of decreases in the mean fluorescence intensity and hence in $\Delta\Psi m$. Moreover, 50 nM Rh123 was not cytotoxic for either of the yeast species tested when assessed by counts of c.f.u. (data not shown).

The kinetics of Rh123 uptake by *S. cerevisiae* and *Z. bailii* was studied by FCM (data not shown). For both yeast species the uptake of the probe was very fast and the steady level of fluorescence was reached in about 10 min. Rh123 uptake was also followed by EFM (Fig. 4). By observation of the image obtained after 5 min incubation it was concluded that the observed rapid initial uptake corresponded to non-specific binding of the dye to the cell envelope. However, after 10 min Rh123 was electrophoretically accumulated into the mitochondria and gave a specific staining of this organelle. The non-specific binding of the dye, associated with the cell envelope, still observed at this incubation time is most probably negligible for the dye concentration used for FCM.

The mean fluorescence intensity of cells stained with Rh123 was constant for 30 min (data not shown). However, a decrease in fluorescence intensity was observed when cells were transferred to a dye-free medium. The observed leakage of the probe probably occurs due to the establishment of a new steady state distribution between the extra- and intra-cellular compartments.

The mean fluorescence intensity of cells stained with Rh123 at room temperature or at 37 °C was identical. Therefore, incubation at room temperature was selected.

Most of the buffers used in staining media have a complex ionic composition and different pH values that could directly interfere with Rh123 uptake. However, similar levels of fluorescence were observed (data not shown) for yeast cell suspensions from both species stained with Rh123 at pH 3·0 or 6·0, indicating that the mitochondrial accumulation of the dye is independent of the extracellular pH. Furthermore, based on the dependence of the plasma membrane potential ($\Delta\Psi p$) on the extracellular pH (van den Broeck, 1982), we concluded that mitochondrial accumulation of Rh123, under the optimized staining conditions is hardly affected by $\Delta\Psi p$.

To evaluate the influence of the medium composition on Rh123 staining, the fluorescence intensity of cells stained in mineral medium (pH 4·5, MGV medium), PBS (pH 7·0) or sterile water (pH 6·0) was compared. Higher levels of fluorescence were achieved when staining with Rh123 was carried out in sterile water (Fig. 5).

Effect of yeast cell growth phase on Rh123 staining

To evaluate the effect of cell growth phase on Rh123 staining, *Z. bailii* and *S. cerevisiae* cell suspensions prepared from cultures at early and late exponential

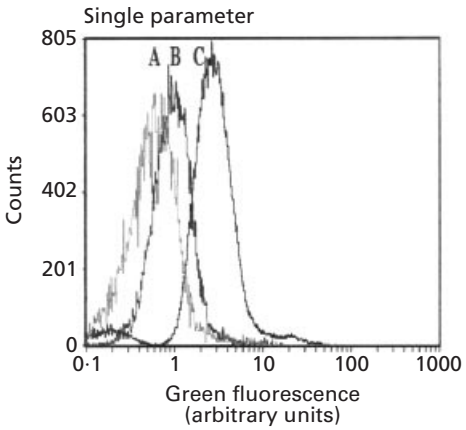

Fig. 5. Green fluorescence histograms after staining with Rh123 (50 nM) of *Z. bailii* cell suspensions in mineral medium (curve A), PBS buffer (curve B) and sterile water pH 6·0 (curve C).

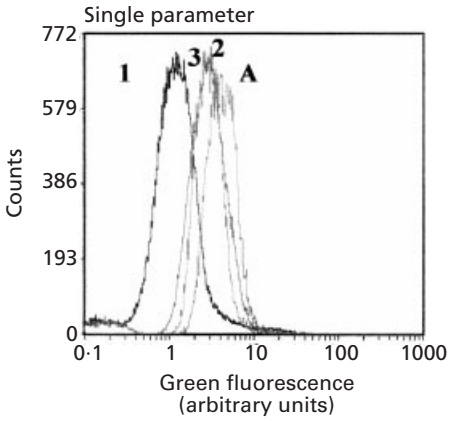

Fig. 6. Overlay of green fluorescence histograms obtained for *Z. bailii* cells stained with Rh123 (50 nM). Cells were untreated (curve A) or treated with sodium azide (curve 1) or 2-deoxy-D-glucose (curve 2) or starved (curve 3).

phase were stained under the conditions referred to above. The results obtained indicate that, whereas Rh123 staining of S. *cerevisiae* cells is growth-phase dependent, the same was not observed for *Z. bailii* cells. In *S. cerevisiae*, a concentration of 10 nM Rh123 is enough to produce the same fluorescence intensity in late exponential phase cells as 50 nM in early exponential phase cells. However, in *Z. bailii* a concentration of 50 nM of Rh123 appeared to be necessary to stain cells both from early and late exponential phases.

Definition of a simple staining protocol with Rh123

In summary, the conditions selected for the optimal staining with Rh123 and for assessment of mitochondrial membrane potential included the use of: (i) yeast cell suspensions in sterile water at a final cell concentration of 1×10^6 cells ml^{-1}; (ii) Rh123 at a final concentration of 10 nM for late exponential phase *S. cerevisiae* cells, and 50 nM for early exponential phase *S. cerevisiae* cells and *Z. bailii*, independent of the growth phase; (iii) staining for 10 min at room temperature in the dark.

Validation of the staining protocol with Rh123 for the assessment of $\Delta\Psi m$

To ascertain whether Rh123 accumulation in whole yeast cells was correlated to $\Delta\Psi m$, the effects of ionophores, specific inhibitors and substrates of mitochondrial respiration were tested. Valinomycin and FCCP [carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone] at the concentrations tested had no effect on Rh123 accumulation in yeast cells (data not shown). The same result was obtained by other authors and attributed to the possible formation of complexes between putative adducts and components of the inner mitochondrial membrane. Some of these complexes are freely accessible whatever the energy status of mitochondria but others are hidden in the energized state and freely accessible only in the de-energized state of the organelle (Salvioli *et al*., 1997).

Cells exposed to sodium azide or 2-deoxy-D-glucose, or starved cells displayed a decrease in the Rh123 accumulation compatible with a decrease in $\Delta\Psi m$ (Fig. 6). Furthermore, the two former conditions did not affect the dye accumulation in heat-killed yeast cells (data not shown). Yeast cells treated with oligomycin and nigericin showed increased Rh123 staining (data not shown). These results are consistent with previous reports by other authors. Actually, oligomycin was used by Macouillard-Poulletier de Gannes *et al*. (1998) to mimic a decrease in cellular ATP demand (transition to state 4). In a similar way, these authors observed an increase in $\Delta\Psi m$ linked to the inhibition of ATP synthase by oligomycin associated with a significant increase in $DiOC_6(3)$ fluorescence.

Nigericin collapses ΔpH and hyperpolarizes (Johnson *et al*., 1980, 1981) to a value close to that of the expected total electrochemical gradient (Nicholls, 1982). Petit *et al*. (1990) also observed an increase in the mean fluorescence value of isolated mitochondria stained with Rh123, under those conditions.

The addition of succinate, which provides reducing equivalents to the respiratory chain through succinate dehydrogenase, increased the accumulation of Rh123 in living cells.

Strain J-1-3 of *S. cerevisiae* is a null mutant for the ADP/ATP translocator and compared with the parental strain, produced a small but significant decrease of $\Delta\Psi m$ when assessed by Rh123 staining. Incubation of this mutant strain with sodium azide should abolish the mitochondrial respiratory function: $\Delta\Psi m$ should collapse and Rh123 should not accumulate in the mitochondria. Actually, under these conditions the J-1-3

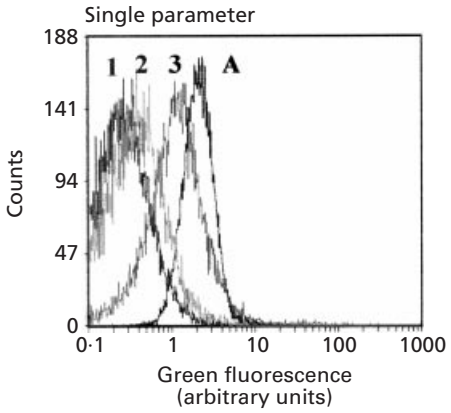

Fig. 7. Overlay of green fluorescence histograms of *S. cerevisiae* J-1-3 cells. Curve 1 shows autofluorescence. Cells were stained with Rh123 (50 nM) after (curve 2) or before (curve 3) treatment with sodium azide. Curve A shows *S. cerevisiae* wild-type cells stained under the same conditions.

mutant strain displayed a mean fluorescence intensity quite similar to the autofluorescence (Fig. 7).

DISCUSSION

In the present work, flow cytometry was exploited for the assessment, in whole yeast cells, of mitochondrial function as expressed by the membrane potential ($\Delta\Psi$m). The results showed that the relative cell volume affects the fluorescence measurements without revealing a real difference in $\Delta\Psi$m. Therefore, all the subsequent cytometric data were expressed as a ratio (R) value in an attempt to normalize the fluorescence intensity for the relative cell volume as estimated by the FS signal.

Most of the methods recently described for the assessment of $\Delta\Psi$m were derived from the protocol of Johnson *et al*. (1980), in which the Rh123 concentration used was 25 μM. More recently, Juan *et al*. (1994) used a Rh123 concentration of 250 nM to assess $\Delta\Psi$m in mouse liver cells. The Rh123 concentration selected in the present work (50 nM) is much lower than those described in the literature. At this concentration the cytotoxic effects of the probe and its non-specific binding to other intracellular organelles were eliminated or at least became negligible. The results obtained with the ADP/ATP null mutant strain of *S. cerevisiae* in the presence of sodium azide further confirmed this. In fact, in this strain under conditions where $\Delta\Psi$m is collapsed, Rh123 accumulation in the mitochondria was prevented and the level of staining corresponded almost to that of autofluorescence.

In both *S. cerevisiae* and *Z. bailii*, comparable levels of fluorescence intensity were obtained regardless of the incubation temperature (room temperature or 37 °C). In addition, and consistent with the results reported by other authors (Ronot *et al*., 1986), the uptake of Rh123 (50 nM) at room temperature is rapid and equilibrium is reached after 10 min.

In the two yeast species tested under the optimized staining conditions, an increase or decrease in the R value of stained yeast cell suspensions was observed after treatment with hyperpolarizing (substrate of mitochondrial respiration) or depolarizing (ionophores or specific inhibitors of respiration) agents, respectively. This confirmed that Rh123 fluorescence changes reflect changes of $\Delta\Psi$m, as has been described in intact animal cells (Juan *et al*., 1994).

It has been discussed in previous work that alterations of $\Delta\Psi$m may not be the only cause of Rh123 fluorescence changes. In fact, changes in mitochondrial Rh123 accumulation can be independent of $\Delta\Psi$m and reflect changes in either mitochondrial mass or the cytoplasmic concentration of the probe. An example of the former situation has been reported for an ADP/ATP translocator mutant (Petit *et al*., 1996). Such a mutant has a nearly normal $\Delta\Psi$m; however, the decrease observed in $\Delta\Psi$m by Rh123 staining is associated with a decrease in mitochondrial mass, assessed by cardiolipin staining (by nonyl acridine orange dye). This aspect further reinforces the need to complement $\Delta\Psi$m studies with a mitochondrial structure probe when changes in the cellular content of mitochondria are suspected to occur. Moreover, alterations of the Rh123 cytoplasmic concentration might cause fluorescence alterations independently of $\Delta\Psi$m (Bernardi *et al*., 2001). These alterations can be elicited either by the activity of multidrug resistance pumps for Rh123 or changes in the plasma membrane potential ($\Delta\Psi$p). Elimination of the interference of active efflux pumps on $\Delta\Psi$m determinations by the optimized Rh123 staining protocol is difficult since it would imply the knowledge for all the active systems, that use Rh123 as substrate, of the respective affinity constants. Actually, these data are only available in *S. cerevisiae*, and there only for specific active efflux systems (Kolaczkowski *et al*., 1996). That mitochondrial accumulation of Rh123 was independent of the extracellular pH eliminates $\Delta\Psi$p interference on $\Delta\Psi$m determinations.

Salvioli *et al*. (2000) observed that in a given cell only some mitochondria were polarized, indicating heterogeneous behaviour at the mitochondrial level. If observed in yeast, this further complicates $\Delta\Psi$m measurements.

The different Rh123 concentrations required to stain optimally *S. cerevisiae* glucose-grown cells from early or late-exponential phase is most probably due to the well known shift from fermentative to respiratory metabolism (Pronk *et al*., 1996), and to inherent and significant changes in the mitochondrial compartment (Visser, 1995) associated with the growth phase. The same is not found in *Z. bailii*. This observation is consistent with the higher relative proportion of respiration to fermentation (1:3) in *Z. bailii* (Fernandes *et al*., 1997) as compared with *S. cerevisiae* (1:20) (Lagunas, 1986). In addition, and in contrast to the pro-mitochondria described in glucose-grown *S. cerevisiae* cells, *Z. bailii* displayed a higher proportion of mature mitochondria (P. Ludovico, F. Sansonetty, M. T. Silva, & M. Côrte-Real,

unpublished results). The results referred to above indicate that the application of the Rh123 staining protocol to other species should take into account possible differences or changes in the mass of the mitochondrial compartment with growth conditions.

The data obtained with heat-killed yeast suspensions suggest that Rh123 can be used as a probe to discriminate live from dead cells. This is similar to what has been reported by Juan *et al*. (1994) with animal cells. Actually, heat-killed yeast cells showed a diffuse and more intense fluorescence than untreated cells. It should be expected that in the former cells, Rh123 staining would produce a lower fluorescence level. A possible explanation could be attributed to the loss of mitochondrial membrane integrity in the dead cells and consequently to a non-localized distribution of Rh123. The observed behaviour might be determined by different fluorescence-quenching effects of the probe when interacting with the denatured and precipitated cell constituents.

It has been recently reported that *S. cerevisiae* becomes committed to a programmed cell death process in response to acetic acid (Ludovico *et al*., 2001). The protocol described here will be crucial in the further evaluation of the involvement of mitochondrial function, namely of $\Delta\Psi$m changes in triggering such active cell death processes.

Presently one of the most generalized probes to assess mitochondrial membrane potential in mammalian cells is JC-1 (Seligmann & Gallin, 1986; Cossarizza *et al*., 1993). In the presence of a high $\Delta\Psi$m, JC-1 forms J-aggregates that are associated with a large shift in the wavelength of fluorescence emission. However, one disadvantage of JC-1 compared with Rh123 is related to the speed of its responses to $\Delta\Psi$m changes, which are slower than those seen under equivalent conditions when using Rh123 (Haugland, 1996). This suggests that the slower redistribution of JC-1 could result in an underestimation of the actual rate of change of $\Delta\Psi$m.

In summary, optimization of the Rh123 staining conditions described here allowed the definition of a flow cytometric protocol for the qualitative assessment of rapid changes in $\Delta\Psi$m of yeasts. However, it should be emphasized that when the Rh123 staining protocol is to be applied to a new yeast species, preliminary experiments should be carried out to establish the optimal staining conditions.

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