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Estimation of the electric plasma membrane potential difference in yeast with fluorescent dyes: comparative study of methods

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Abstract Different methods to estimate the plasma membrane potential difference (PMP) of yeast cells with fluorescent monitors were compared. The validity of the methods was tested by the fluorescence difference with or without glucose, and its decrease by the addition of 10 mM KCl. Low $CaCl_2$ concentrations avoid binding of the dye to the cell surface, and low CCCP concentrations avoid its accumulation by mitochondria. Lower concentrations of Ba^{2+} produce a similar effect as Ca^{2+}, without producing the fluorescence changes derived from its transport. Fluorescence changes without considering binding of the dyes to the cells and accumulation by mitochondria are overshadowed by their distribution between this organelle and the cytoplasm. Other factors, such as yeast starvation, dye used, parameters of the fluorescence changes, as well as buffers and incubation times were analyzed. An additional approach to measure the actual or relative values of PMP, determining the accumulation of the dye, is presented.

Keywords Yeast · Membrane potential indicators · Ion transport

Abbreviations

CCCP	Carbonylcyanide *m*-chlorophenylhydrazone
$DiSC_3(3)$	3,3′-dipropylthiacarbocyanine
$DiSC_3(5)$	3-propyl-2-[5-[3-propyl-2(3*H*)-benzothiazolylidene]-1,3-pentadienyl] benzothiazolium iodide
$DiOC_6(3)$	3,3′-dihexyloxacarbocyanine iodide
DMSO	Dimethylsulfoxide
FCCP	Carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone
MES-TEA	Morpholinoethanesulfonic acid adjusted with triethanolamine
PMP	Plasma membrane potential difference
SDS	Sodium dodecylsulfate

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Introduction

Yeast cells have the capacity to pump out protons by the activity of the plasma membrane H^+-ATPase, as proposed a long time ago for *Saccharomyces cerevisiae*. This ATPase is thus capable of generating a H^+ and an electric gradient at both sides of the plasma membrane that becomes the driving force for the uptake of cations and other molecules through their respective transporters (Peña et al. 1972; Peña 1975). The ATPase isolated from yeast and reconstituted into phospholipid vesicles, is capable of generating an electric potential difference (Malpartida and Serrano 1981; Dufour et al. 1982). The gene *PMA1* was also cloned and shown to codify for a P-type ATPase (Serrano et al. 1986). The plasma membrane potential (PMP) is the driving force for the uptake of alkaline monovalent cations through the specific uniporters Trk1p and Trk2p (Rodríguez-Navarro and Ramos 1984). These uniporters have been cloned, and found that the first one is of high affinity (Gaber et al. 1988), and the second one is of low affinity (Ko et al. 1990). Essentially, due to the high affinity of Trk1p, when

adequate concentrations of K^+ are added, they are expected to decrease the PMP as they enter the cell.

No procedures exist to measure directly the PMP of yeast, and although the use of fluorescent indicators is mostly qualitative, significant results can be obtained when differences of the fluorescence signal are large enough. Estimations in whole cells are more complex than in isolated vesicles or organelles, so discrepancies may result, due to differences in the reported methods.

Through the years, different methods using fluorescent dyes have been used to estimate changes of the electrical PMP of yeast cells with different probes and conditions (Peña et al. 1984; Gaskova et al. 1998; Madrid et al. 1998; Maresova et al. 2006; Kinclova-Zimmermannova et al. 2006; Borst-Pauwels et al. 1992). Most of these methods have not been subjected to tests regarding their validity, except (and only partially) for the procedure reported in Borst-Pauwels et al. 1992.

The methods are based on the assumption that fluorescent dyes are taken up by the cells and distributed between both sides of the plasma membrane according to the PMP (Waggoner 1979), and when entering the cells, changes in their fluorescence and spectral characteristics occur. Most of these fluorescence changes consist of either quenching, when dyes are accumulated at a high concentration or increasing when they are accumulated at lower concentrations (Sims et al. 1974).

Previous work (Peña et al. 1984) showed that the fluorescence of the dye $DiSC_3(3)$ increases according to the PMP, but it can also be concentrated by the mitochondria, where its fluorescence is quenched; this quenching can be avoided by 10 μM FCCP or CCCP. Micromolar concentrations of $CaCl_2$ also avoid the fluorescence increase observed in the absence of a substrate, which appears to be independent on the PMP, in agreement with the assumption that calcium can screen the negative charges at the surface of the cell, preventing the increase in fluorescence resulting from the binding of the cyanine to the cell surface.

Concentrations of K^+ (10 mM or less) similar to those required for its transport, and the consequent movement of positive charges into the cell, are enough to produce a decrease in the fluorescence signal. Amiodarone, which is capable of producing K^+ efflux from the cells, hyperpolarizing the plasma membrane, produces an increase of the fluorescence signal (Peña et al. 2009).

In other methods, different dyes and parameters have been employed. Gaskova et al. (1998), with this same cyanine, measured the red shift of the emission peak; also with this strategy, it was reported that the PMP of the *Δtok1* mutant was lower than that of the W303 wild type strain of *Saccharomyces cerevisiae* (Maresova et al. 2006), but we found different results (Peña et al. 2009). With another dye, $DiOC_3(6)$, the PMP of a *Δtrk1* mutant was higher than that of the corresponding wild type strain (Madrid et al. 1998). Using our method, experiments with the wild type FY833 and a *Δtrk1* mutant yielded opposite results (Peña et al. 2009).

Some other characteristics of these methods are worth mentioning; in one of them, a very low amount of cells is required, and it is assumed that in *S. cerevisiae*, CCCP 5 to 10 μM causes depolarization of the plasma membrane, while only higher concentrations affect the mitochondria (Gaskova et al. 1998). It has been demonstrated that 10 μM CCCP in *S. cerevisiae* can selectively uncouple mitochondrial oxidative phosphorylation, and inhibit K^+ transport and proton pumping only with an oxidizable substrate such as ethanol (Peña et al. 2009). When glucose is the substrate, much higher concentrations of the uncoupler are required.

In another variation of a similar method (Maresova et al. 2009), a) the assumption that 10 μM CCCP can collapse the PMP is maintained; b) an important change in the fluorescence of the cyanine can be observed only using K^+ concentrations much higher than 10 mM; c) a very low number of cells is still required to observe the changes of fluorescence, and d) differences in fluorescence increases cannot be seen when adding glucose.

In addition, these methods disregard collateral effects of the buffers employed, because of their ionic composition and concentration, and some changes may depend on the physiological state of the cells. Some of them require long incubations and more complex procedures, providing results only after the changes of the PMP of the cells have occurred.

In view of these facts, we decided to perform a series of experiments to test: a) whether our previously reported method may be valid with non-starved cells; b) the apparent useful concentrations of the dye and cells; c) the efficacy of three fluorescent indicators with different partition coefficients, and d) the effects of different buffers used by other authors. We compared a) the fluorescence changes in the presence vs. the absence of glucose; b) the effects of adding an uncoupler to prevent the accumulation by mitochondria; c) the effect of adding a divalent cation to avoid the fluorescence increase derived from the binding of the dye to the surface of the cells; d) the effects of the addition of K^+, expected to decrease the PMP, and e) the effects of amiodarone, known to produce an efflux of K^+ and expectedly an increase of the PMP.

Additionally, since $DiSC_3(3)$ appears to easily permeate the plasma membrane of yeast, another approach is presented, using its accumulation ratio to actually calculate the PMP values.

Material and methods

Cells and growth conditions

Yeast cells (*Saccharomyces cerevisiae*) from a commercial strain (La Azteca, Mexico) obtained form an isolated single colony were used.

The cells were maintained in 2% agar plates of YPD medium (1% yeast extract, 2% peptone, 2% glucose) and cultures were renewed every month. Cultures were started by placing a loophole of cells in 500 mL of liquid YPD that were grown either for 14 h or 24 h at a constant room temperature (30°C) in an orbital shaker at 250 rpm. Then, the cells were starved or not, as follows:

Non-starved cells After growth for 14 h and collection by centrifugation, the cells were washed twice with water by centrifugation and then suspended at a ratio of 0.5 g (wet weight) · mL^{-1} in water.

Starved cells To obtain starved cells, after being cultured for 24 h, they were collected by centrifugation, suspended at a ratio of approximately 8 g (wet weight) in 250 mL of water and incubated overnight in an orbital shaker at 250 rpm and 30°C. After this incubation the cells were collected by centrifugation, washed once with water and also suspended in water at a ratio of 0.5 g (wet weight) · mL^{-1}. The cells were always used during the same day of collection.

Membrane potential estimations

Experiments were variations on the procedure reported before (Peña et al. 1984), as described for each experiment. The simplest medium contained 10 mM MES-TEA, pH 6.0, to obtain a final volume of 2.0 mL. This volume is adequate to allow uniform mixing of cells. The tracings were started by adding 25 mg (wet weight) of cells, and 30 sec after, 200 nM $DiSC_3(3)$ was added. The complete medium found to give the best results with starved cells contained 200 μM $CaCl_2$, 10 μM CCCP, and 20 mM glucose. The same, or lower concentrations of $DiSC_3(5)$ or $DiOC_6(3)$ were used. Various concentrations of $CaCl_2$, or 10 mM KCl, or 10 μM CCCP were added during the course or before starting the tracing. Fluorescence was followed at 540 nm excitation, and 590 nm emission for $DiSC_3(3)$; the respective wavelength pairs were 666–702 nm for $DiSC_3(5)$, and 482–524 nm for $DiOC_6(3)$. In all cases, the slits of the instrument (SLM-Aminco spectrofluorometer updated by Olis) were fixed at 8 nm.

Potassium transport

K^+ transport was followed by means of a selective electrode using cells incubated under continuous stirring in a constant temperature vessel at 30°C. Quantification was performed by titrating the medium with known amounts of KCl. The medium contained 2 mM MES-TEA, pH 6.0, and 20 mM glucose. To start the tracings, 150 mg (wet weight) of cells was added. The desired μM concentrations of CCCP were added from a 10 mM stock solution prepared in DMSO. The final volume was 10.0 mL.

Respiration

Respiration was measured with a Clark electrode in a closed vessel with constant magnetic stirring, maintained at 30°C. Conditions were essentially the same as for the measurements of K^+, but the amount of cells was 25 mg, and the final volume was 5.0 mL.

Microscopy

Cells were observed with an Olympus FV 1000 confocal microscope with a 40X objective and 10X ocular lens. All observations were performed between 3 and 5 min after adding the cells to the same incubation mixture used for the fluorescence measurements: 10 mM MES-TEA buffer, pH 6.0, in a final volume of 2.0 mL, to which different components were added: 20 mM glucose, 10 μM $BaCl_2$, 15 μM CCCP. In order to balance the fluorescence intensity of each pair, the following concentrations of the dyes were used: Mitotracker CMXRos (Invitrogen), 280 nM; $DiOC_6(3)$, 200 nM; $DiSC_3(3)$, 200 nM, and $DiSC_3(5)$, 100 nM. The paired combinations of dyes were: Mitotracker-$DiOC_6(3)$, $DiSC_3(5)$-Mitotracker, and $DiOC_6(3)$-$DiSC_3(3)$. The last combination was used because Mitotracker fluorescence is red, and colocalize with $DiOC_6(3)$ in the mitochondria.

The wavelengths used were the same as for the fluorescence measurements; those for Mitotracker were 579 and 599 nM for excitation and emission, respectively.

All experiments were repeated at least three times.

Calcium transport

Uptake of $^{45}Ca^{2+}$ was measured by adding 50 mg of cells to 10 mM MES-TEA, pH 6.0, and 50 mM glucose in a water bath at 30°C. Final volume was 1.0 mL. After 3 min, 50 or 200 μM $^{45}CaCl_2$ was added. Aliquots of 0.1 mL were taken after 3 min, filtered through a 0.25 μm nitrocellulose filter and washed three times with 0.5 mL of 10 mM $CaCl_2$. The filters were then dried and placed in vials with scintillation liquid and counted in a Beckman scintillation counter.

Distribution coefficients of the dyes

As an index of their hydrophobicity, the distribution coefficients of the three dyes between dichloromethane and MES-TEA were measured by placing 3.0 mL of CH_2Cl_2 and 3.0 mL of 10 mM MES-TEA, pH 6.0, in glass centrifuge tubes and adding 0.2 μM of the cyanine. The tubes were vigorously agitated in a vortex and then centrifuged; 2.0 mL of the upper aqueous layer was taken, to which 0.2 mL of 10% SDS was added. Fluorescence was measured at the adequate wavelengths and compared to that of a standard prepared with the same concentration of the dye, in the presence of 1.0% SDS.

Uptake of $DiSC_3(3)$

Non starved yeast cells (25 mg, wet weight) were incubated during 5 min in a final volume of 3.0 mL of 10 mM MES-TEA, pH 6.0. Where indicated, 10 μM $BaCl_2$, 10 μM CCCP, or 20 mM glucose was also added. $DiSC_3(3)$ was added at 0.2 or 1.0 μM, final concentration. After incubation, the cells were centrifuged for 2 min, and the supernatant decanted. In another experiment, after incubating the cells for 5 min under the same conditions, either 10 mM KCl or 40 μM amiodarone was added; after 3 more min, the cells were centrifuged. The remaining cyanine was determined in the supernatant by measuring the fluorescence at 540–590 nm, after adding SDS to a final concentration of 1.0%, to increase fluorescence as reported before (Peña et al. 1984), and calculated by comparing with the fluorescence of a known concentration of the cyanine.

Results and discussion

Our previously reported method is adequate for 24-h grown starved cells

The fluorescence changes of $DiSC_3(3)$ reported before (Peña et al. 1984) are an acceptable way to estimate and continuously follow changes of the PMP of whole yeast cells. This method was devised using cells grown for 24 h and starved overnight (14 to 16 h); it requires the addition of 200 to 250 μM $CaCl_2$ to avoid the fluorescence changes of the dye due to its binding to the cell surface, as well as 10 μM CCCP or FCCP, to prevent the accumulation of the dye by the mitochondria, and the consequent quenching of fluorescence. Under these conditions, after the initial fast increase of fluorescence of the added dye in the buffer, another clear increase, higher in the presence of glucose occurs, and adding 10 mM KCl produces a large, fast, and clear decrease of fluorescence that is observed only in the presence of glucose (Peña et al. 1984). It should be pointed out that in the absence of a substrate and CCCP, endogenous substrates, mainly aerobically metabolized, may support the uptake of the cyanine and so, its concentration and quenching of its fluorescence in the mitochondria.

These assertions were tested by the separate addition of both CCCP and $CaCl_2$. Adding CCCP produced a significant fluorescence increase that afterwards remained practically constant; this initial increase of fluorescence was lower and slower in the absence of glucose (Fig. 1a). Adding Ca^{2+} (Fig. 1b) to prevent the binding of the dye to the surface of the cells resulted in a lower initial fluorescence. When both

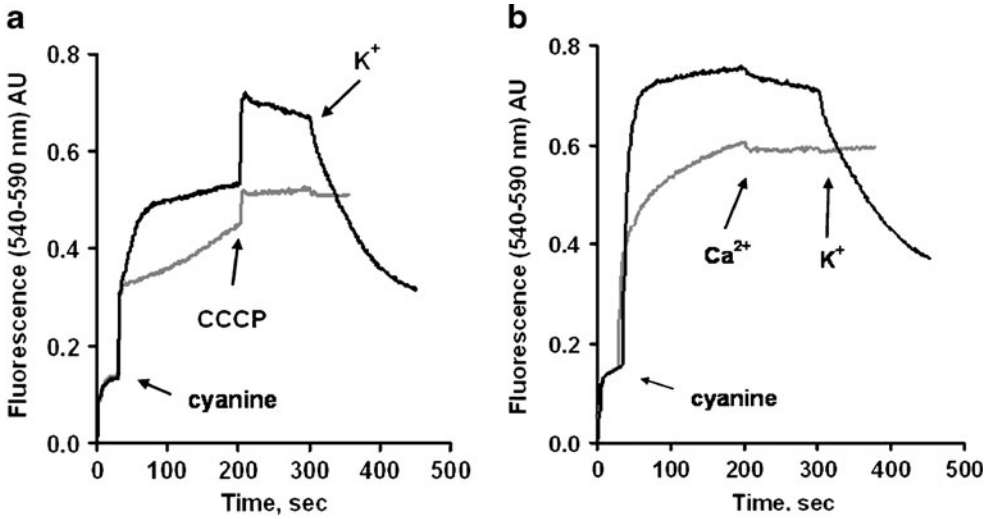

Fig. 1 Fluorescence changes (540–590 nm) of 200 nM cyanine $DiSC_3(3)$ added to starved yeast. The incubation medium contained 10 mM MES-TEA buffer, pH 6.0, without (*gray tracings*) or with 20 mM glucose (*dark tracings*). The cells (25 mg wet weight) were added at the beginning of the tracing, and 200 nM of the cyanine at 30 sec. Tracings: **a** $CaCl_2$ (200 μM) was present in the incubation mixture, and where indicated 10 μM CCCP and 10 mM KCl were added; **b** CCCP was present from the beginning, and also 200 μM $CaCl_2$ or 10 mM KCl were added where indicated. AU, arbitrary units

Ca^{2+} and CCCP were present from the beginning, a large fluorescence increase was observed after adding the cyanine to cells incubated in the presence of glucose, with only a very small increase in the absence of a substrate (data not shown). *S. cerevisiae* has an efficient K^+ transport system, and the cation, as transported, is expected to produce a significant decrease of the PMP, that according to our assumptions, should be reflected as a clear and large decrease of fluorescence, but only if the cells have been energized by glucose. In fact, Fig. 1 shows that only with glucose a large and very clear decrease was observed upon the addition of 10 mM KCl (Peña et al. 1984).

The addition of amiodarone, an agent that produces a significant K^+ efflux and an expected hyperpolarization of the plasma membrane, resulted in an increase of fluorescence (data not shown), as reported before (Peña et al. 2009; Maresova et al. 2009).

These results suggest that one of the main problems with other methods is that not adding an uncoupler at low concentrations, results of changes in the PMP are overshadowed by the accumulation of the dye in the mitochondria. Also, the fluorescence increase produced by binding of dye to the cell surface is usually disregarded, but it can be prevented by the addition of $CaCl_2$. The effects of Ca^{2+} on the surface charge of yeast cells have been documented (Gage et al. 1985; Theuvenet et al. 1984; Peña et al. 2009).

Cells grown overnight (end of the log phase)

As mentioned before, we collect the cells at the end of the log phase, while most authors use cells grown to the log phase. In order to define whether or not we might obtain different results with the latter kind of cells, experiments were carried out with yeast grown overnight (late log or early diauxic phase) without starvation.

Effects of CCCP on K^+ transport and respiration

Some authors, with cells in the log phase, assume that the PMP can be uncoupled with 10 μM CCCP, and only higher concentrations are effective to uncouple mitochondria of yeast (Gaskova et al. 1998, quoting Sigler and Höfer 1991; Maresova et al. 2006; Kinclova-Zimmermannova et al. 2006; Maresova et al. 2009). With starved cells, it has been shown that CCCP concentrations required to inhibit K^+ transport are much higher than 10 μM (Peña 1975). So, similar experiments were performed with cells grown overnight without starvation to define if this might or not be so.

K^+ transport, the fastest PMP requiring and consuming process in the cell membrane, was inhibited only at concentrations of CCCP 20 μM or higher (Fig. 2a). On the other hand, already 5 μM CCCP fully stimulates respiration (Fig. 2b). No K^+ uptake could be observed in the absence of a substrate (data not shown). If we accept that K^+ is driven inside the cells by the PMP, this experiment illustrates that this parameter is NOT uncoupled by the concentrations added of the uncoupler, but are enough to uncouple the mitochondria and to avoid the quenching of fluorescence resulting from the accumulation in this organelle.

Localization of the dyes

Since these results clearly indicate that the cyanine may be concentrated in the mitochondria, we decided to look directly at its localization within the cells and whether this can be avoided by an uncoupler. Using a confocal microscope and the appropriate wavelengths (see Material and methods), we could observe the localization of the dyes, as well as their colocalization with the dye Mitotracker red.

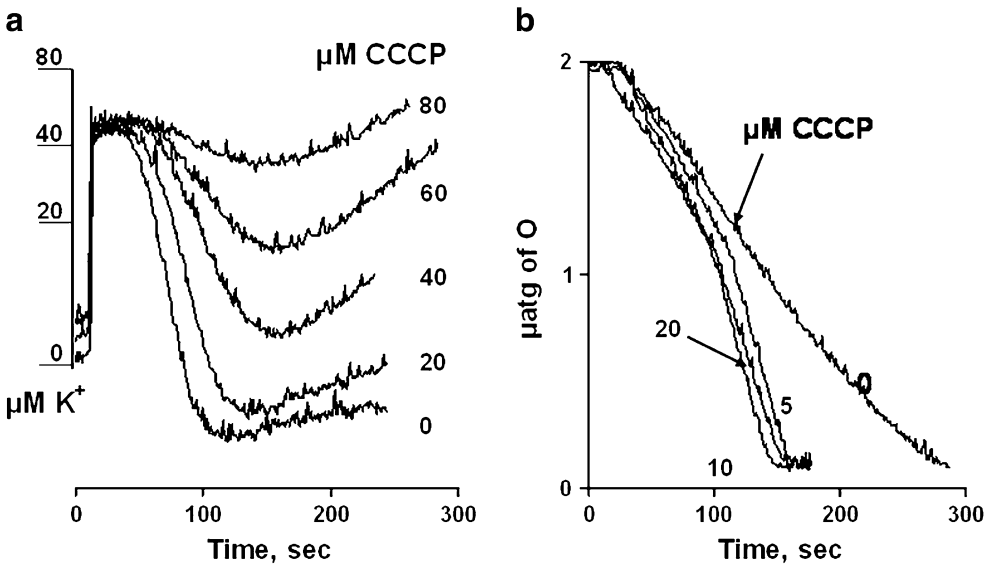

Fig. 2 Effects of various concentrations of CCCP on K^+ transport (**a**) and respiration (**b**) of non-starved yeast. K^+ transport was followed by means of a selective electrode as indicated in Material and methods. After the tracings were obtained, titration of the medium with known amounts of KCl was performed to build the concentration scale. Respiration was measured with a Clark electrode in a closed chamber at 30°C as indicated in Materials and methods. Numbers indicate the μM concentrations of CCCP added where indicated. μatg of O, microatom grams of oxygen

Given the nature of this technique, that does not allow the observation of minor changes, tests were only carried out in the presence of 10 μM $BaCl_2$ instead of $CaCl_2$ (see below), 20 mM glucose, and 200 nM of $DiSC_3(3)$ or $DiOC_6(3)$, and to balance the visualization of the Mitotracker, together with $DiSC_3(5)$, we reduced the concentration of this dye to 100 nM. Similar results were obtained with other dyes, but only the results with $DiOC_6(3)$ are shown in Fig. 3. Mitotracker (A) and $DiOC_6(3)$ (B) show a colocalization in the mitochondria, that can be appreciated in the merging (C) of images A and B. The image of Mitotracker in the presence of CCCP is not presented, because its red fluorescence was absent; however, image (D) shows that in the presence of the uncoupler, $DiOC_6(3)$ is definitely not localized in the mitochondria. Colocalization experiments with Mitotracker red and $DiSC_3(3)$ are not possible because both show similar fluorescence spectra; however, the same mitochondrial colocalization could be indirectly shown by using $DiOC_6(3)$ against $DiSC_3(3)$. Observations under these conditions showed that: 1) in the absence of CCCP, the dyes accumulated in the mitochondria, whether or not in the presence or absence of glucose, and 2) when the dye was added in the presence of CCCP, all that could be observed was a very faint fluorescence distributed in the cell, but definitely not concentrated in the mitochondria. The different behavior of the dye when entering the cells into the cytoplasm (increasing its fluorescence) and mitochondria (quenching of fluorescence) may be due to the different concentration reached (much higher in mitochondria than in the cytoplasm). When a membrane potential is established, quenching has been observed with $DiSC_3(3)$ in isolated mitochondria (Uribe et al. 1985). In previous work with ethidium bromide as an indicator of the membrane potential of isolated mitochondria (Peña et al. 1980), it was found that when concentrations of 6.6 μM are used, the generation of a membrane potential difference produces quenching of fluorescence. However, reducing the concentration of ethidium bromide to 0.33 μM, results in an increase of fluorescence when the membrane potential is generated, and vice versa.

These experiments show that, the addition of an uncoupler in the fluorescence experiments is needed in order to avoid the accumulation of the dyes by the mitochondria, and the subsequent fluorescence quenching.

Effects of CCCP and a divalent cation on the fluorescence changes in non- starved cells

The fluorescence changes of $DiSC_3(3)$ in these overnight-grown, non-fasted cells were followed. In the cells

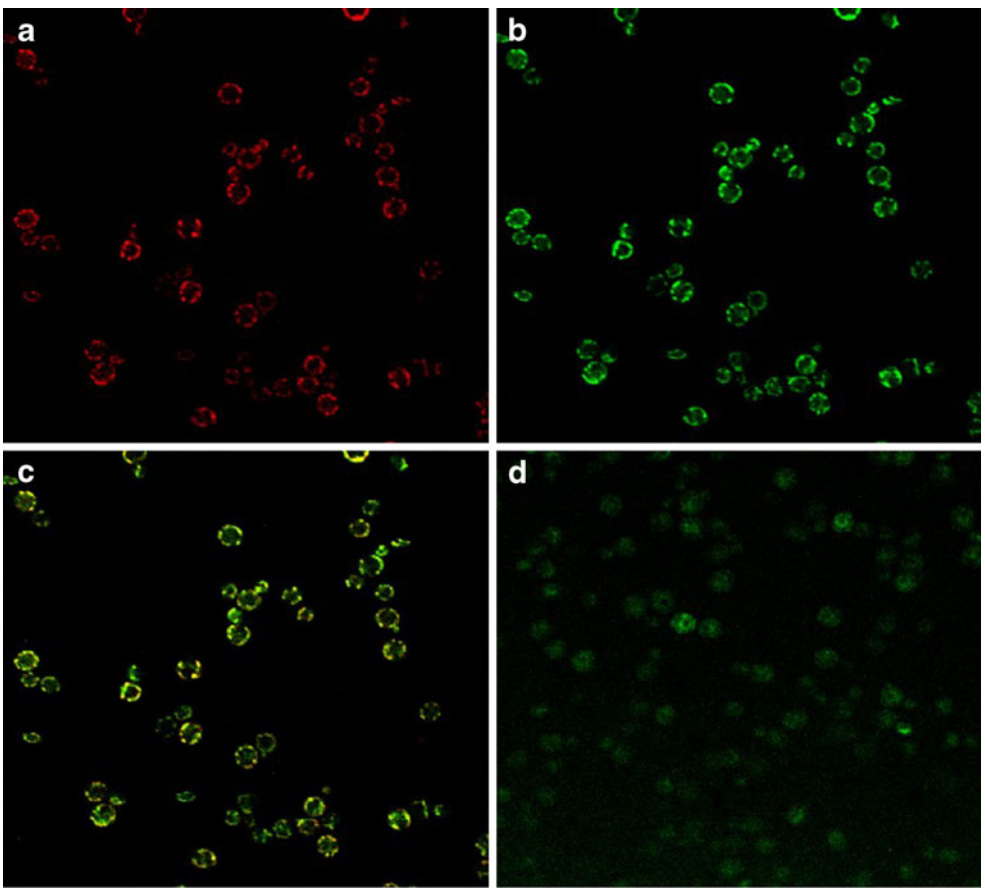

Fig. 3 Localization of Mitotracker Red and $DiOC_6(3)$ in non-starved yeast cells. The cells (25 mg) were added to the incubation mixture containing 10 mM MES-TEA buffer, pH 6.0, 20 mM glucose, 10 μM $BaCl_2$, in the absence (**a** and **b**) or the presence (**d**) of 10 μM CCCP. $DiOC_6(3)$ and Mitotracker Red were added at 200 nM and 280 nM concentration, respectively. The fluorescence images were observed between approximately 3 and 5 min, after the addition of the cells. Fluorescence was observed under the confocal microscope, at the wavelengths indicated under Material and methods. **a** Mitotracker Red; **b** $DiOC_6(3)$; **c** Merging of (**a**) and (**b**); (**d**) $DiOC_6(3)$ after the addition of CCCP

incubated in buffer only, without additions (Supplementary Fig. 1), after the initial large increase of fluorescence, another smaller increase took place when oxygen was exhausted, both in the presence or absence of glucose, but earlier in its presence. This increase could be reversed by the addition of hydrogen peroxide (H_2O_2). When CCCP was then added, a rapid increase, followed by a large decrease was observed, much faster and reaching a lower level in the absence of glucose.

As with starved cells, when the cyanine was added to cells incubated in buffer only, the results can be summarized as follows:

Addition	Fluorescence	Difference with/wo glucose	Signal stability	Effect of KCl
None	High	None	Persistent	None
$CaCl_2$	Medium	None	Persistent	None
CCCP	High	Measurable	Increase-decrease	Measurable
CCCP+ $CaCl_2$	Medium	Measurable	Increase-decrease	Measurable

In general, results were similar to those obtained with starved cells. Nonetheless, the main difference with starved cells was that, in the presence of glucose, even when CCCP and $CaCl_2$ were present, the fluorescence increase, after reaching a maximum, markedly decreased, until reaching a rather constant level. In non-starved cells, it was found that adding Ca^{2+} in the presence of CCCP and glucose produced a rapid decrease of fluorescence depending on the concentration (Fig. 4).

Transport of Ca^{2+}

One possible explanation for the fluorescence decrease produced by this cation, in the presence of CCCP and glucose, is that in non-starved cells it might be transported faster than in starved cells, resulting in a decrease of the PMP. In order to define this, the uptake of $^{45}Ca^{2+}$ was measured in both starved and non-starved cells, with 25 μM and 200 μM $CaCl_2$, (Fig. 5). It was found that in fact, the uptake of Ca^{2+} was much higher in non-starved cells.

Comparison of Ca^{2+} to Ba^{2+}

Departing from the hypothesis that the main effect of Ca^{2+} is the screening of negative charges on the surface of the cells, we decided to look at another divalent cation that might be effective in this respect, without decreasing the PMP because of its own transport. Results are not shown, but it was found that similar concentrations of Ba^{2+} to those used with Ca^{2+} as in Fig. 4, produced comparable decreases

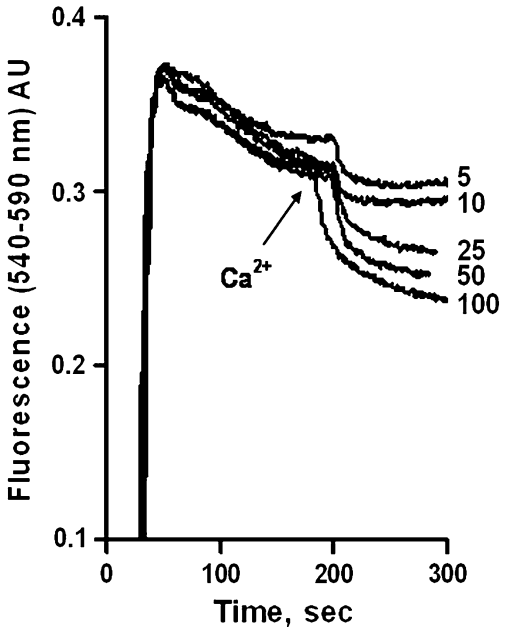

Fig. 4 Effects of the addition of different concentrations of $CaCl_2$ on the fluorescence of $DiSC_3(3)$ with non-starved yeast. The incubation medium contained 10 mM MES-TEA buffer, pH 6.0; 20 mM glucose, and 10 μM CCCP. Different μM concentrations of $CaCl_2$ were added as indicated. The cells (25 mg wet weight) were added at the beginning of the tracing, and the cyanine at 30 sec. AU, arbitrary units

of the fluorescence when added in the presence of glucose and CCCP, indicating that both cations are taken up by the cells when a substrate is present. Figure 6 shows the effects of different concentrations of Ba^{2+} in the medium, in the presence (A) or absence (B) of glucose. As with Ca^{2+}, a concentration of 100 μM resulted in practically no increase of fluorescence in the absence of glucose, but in the presence of the substrate, after an increase, a decrease was observed. However, concentrations of 5 to 10 μM Ba^{2+} were enough to prevent the fluorescence increase observed in the absence of glucose, without affecting its stability in the presence of the substrate.

From these experiments, we can conclude that, although the estimation of the PMP in non-starved cells gave results similar to those of starved cells, their higher capacity for Ca^{2+} transport reduces the PMP of the cells and the fluorescence of the cyanine. However, this can be overcome by using instead 5 to 10 μM $BaCl_2$.

Other characteristics of alternative methods

As mentioned before, other characteristics of the reported methods are the following: a) they appear to be restricted to very small amounts of cells; b) they are useful only within a limited concentration range of the cyanine; c) other dyes are used without considering their properties, and d) buffers that can affect the fluorescence changes are used.

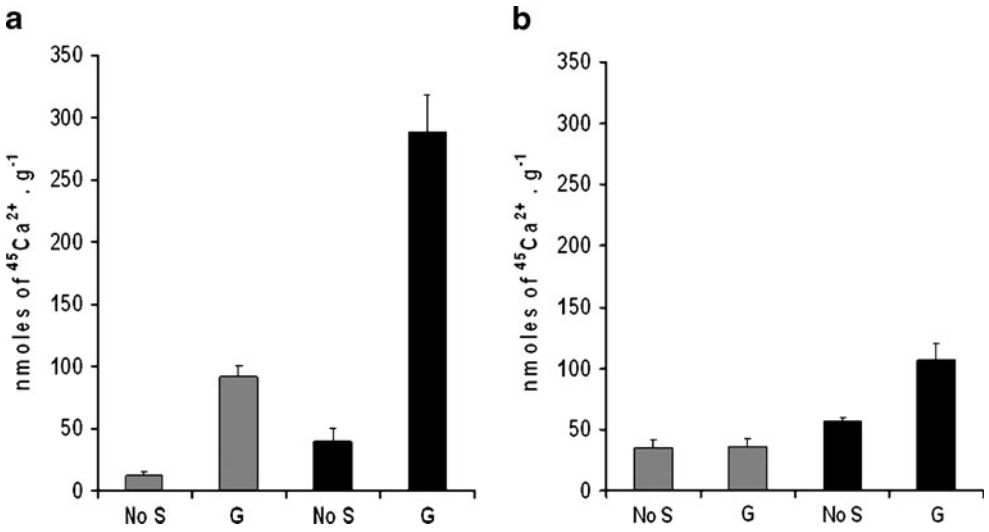

Fig. 5 Transport of $^{45}Ca^{2+}$ by non-starved (**a**) and starved (**b**) yeast cells with two concentrations of $CaCl_2$. Transport was measured by adding 50 mg of cells to 10 mM MES-TEA, pH 6.0, without substrate (No S) or with 50 mM glucose (G) in a water bath at 30°C; final volume was 1.0 mL. Gray bars: 25 μM $CaCl_2$; black bars: 200 μM $CaCl_2$

Fluorescence, the amount of cells and cyanine

Methods of other authors (Madrid et al. 1998; Maresova et al. 2006; Maresova et al. 2009) require very small amounts of cells. To test our method in this respect, experiments were carried out in two ways: 1) varying the amount of cells (25, 12.5, or 2.5 mg) with 200 nM $DiSC_3(3)$ (Fig. 7a), and 2) using two concentrations of the cyanine, 200 nM or 1.0 μM, with 25 mg of cells (Fig. 7b). In both cases, the experiments were performed in the presence of 10 μM $BaCl_2$ and 10 μM CCCP. Variations on the amount of cells (Fig. 7a) produced similar results, but of course, with different fluorescence values. With the lowest number of cells (2.5 mg), in the absence of glucose, after the initial increase of the fluorescence upon addition of the cyanine, instead of maintaining a constant value, a slow and slight decrease was obtained. Also a slight decrease was observed if the cyanine was added in the absence of cells, which is commonly due to the binding of part of the dye to the cuvette walls and to the stirring bar. Comparable results were obtained with a constant amount of cells (25 mg), and varying the concentration of the cyanine (200 nM or 1.0 μM) (Fig. 7b).

From these results we can conclude that with the method presented here, within reasonable limits, no restrictions exist regarding the amount of cells and the cyanine concentration to estimate the PMP of this yeast. Moreover, the decrease of fluorescence with the smallest cell numbers would be in favor of using larger cell numbers instead of the very low ones used in other methods (Gaskova et al. 1998; Maresova et al. 2006; Maresova et al. 2009).

Other dyes, $DiSC_3(5)$ and $DiOC_6(3)$

Two other dyes were used in order to exemplify the consequences of using dyes that in principle should behave

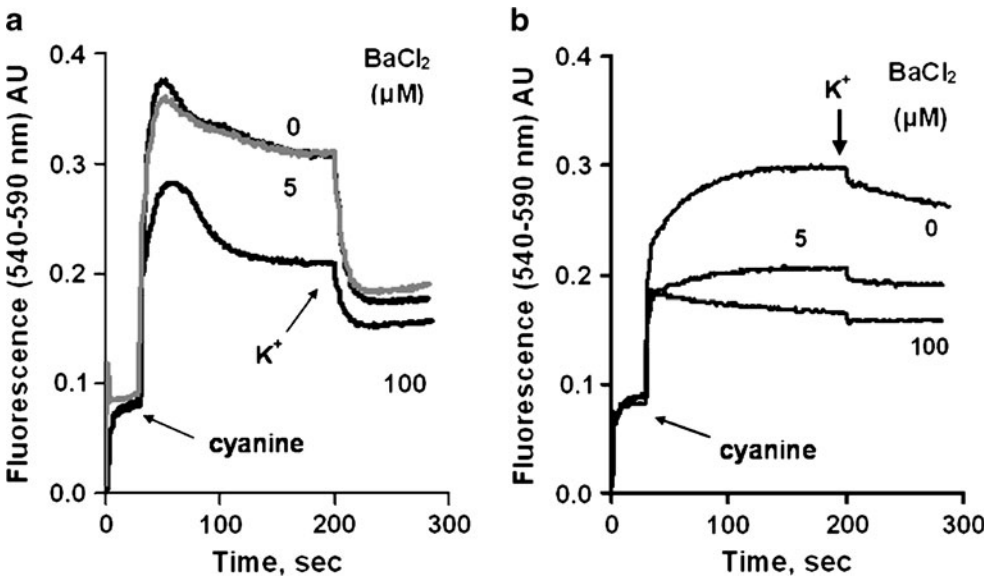

Fig. 6 Effects of the incubation with various concentrations of $BaCl_2$ on the fluorescence changes of $DiSC_3(3)$ of non-starved yeast cells. The medium contained 10 mM MES-TEA buffer, pH 6.0, with 20 mM glucose (**a**) or without a substrate (**b**) and 10 μM CCCP (in all cases). Numbers indicate the micromolar (μM) concentrations of $BaCl_2$ present during the experiment. The cells (25 mg wet weight) were added at the beginning of the tracing, the cyanine at 30 sec and where indicated, 10 mM KCl. AU, arbitrary units

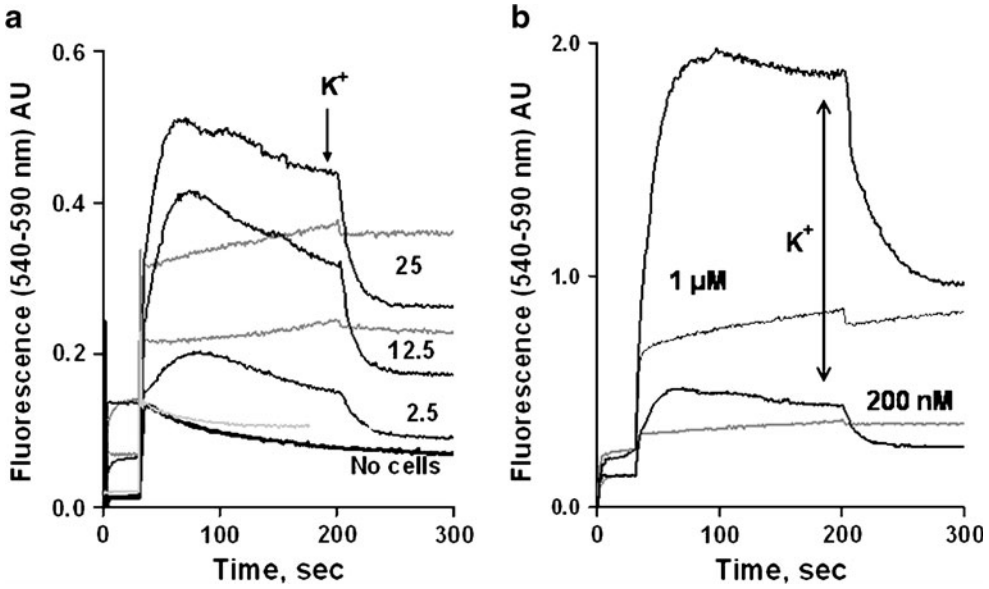

Fig. 7 Fluorescence changes of $DiSC_3(3)$ observed by varying the amount of cells (**a**), or by using two concentrations of cyanine with 25 mg of cells (**b**). The incubation medium contained 10 mM MES-TEA buffer, pH 6.0, with (*black lines*) or without 20 mM glucose (*gray lines*). **a**: upper two tracings, 25 mg of cells; middle tracings, 12.5 mg; lower pair of tracings, 2.5 mg; Lowest black line, without cells. **b**: Black lines, with glucose; gray lines, without glucose. The upper pair of tracings were obtained with 1.0 µM cyanine and the lower one with 200 nM cyanine. The cells were added at the beginning of the tracing, and the cyanine at 30 sec. For all tracings, 10 µM CCCP, 10 µM $BaCl_2$ and, later, 10 mM KCl were added. AU, arbitrary units

in a similar way. Cyanine $DiSC_3(5)$ differs from $DiSC_3(3)$ only because the two rings are separated by a pentatrienyl, instead of a propiodienyl carbon chain, indicating that it should be more hydrophobic. With this dye, similar results to those with $DiSC_3(3)$ were obtained regarding the general effects of Ba^{+2} or CCCP, glucose and KCl. A large effect of KCl was observed in the presence of both 10 µM $BaCl_2$ and CCCP (Fig. 8a). However, the effect of $BaCl_2$ in lowering the initial fluorescence (30 to 100 s) in the absence of a substrate was not as clear as with $DiSC_3(3)$, although the effect of the addition of KCl was fast and clear (detailed results are not shown).

Another dye, $DiOC_6(3)$, has been used to estimate PMP (Madrid et al. 1998). The authors actually considered the possible influence of mitochondria on the fluorescence values, taken as an indicator of the PMP; they used very low concentrations of the dye (1.0 nM) and estimated the fluorescence changes by flow cytometry. Figure 8b shows

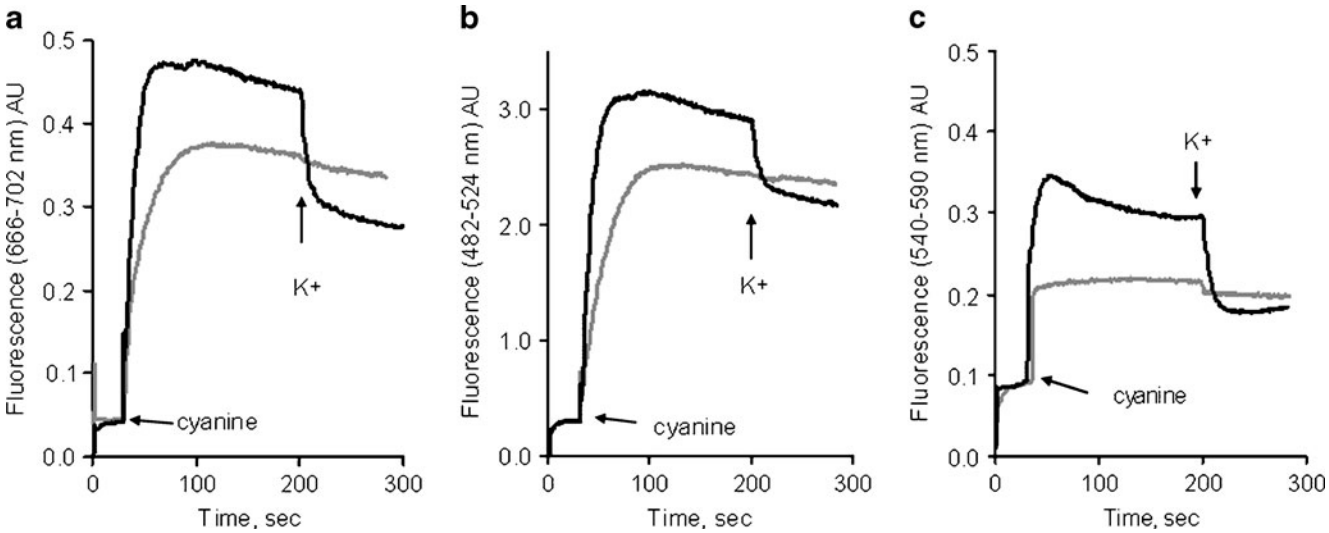

Fig. 8 Fluorescence changes of 200 nM of **a** $DiSC_3(5)$ (666–702 nm), **b** $DiOC_6(3)$ (482–524 nm) and **c** $DiSC_3(3)$ (540–590 nm) added to non-starved yeast. The cells (25 mg wet weight) were added at the beginning of the tracing, and the dyes at 30 sec. Other conditions as in Fig. 6, with 10 µM $BaCl_2$. Without (*gray tracings*), or with 20 mM glucose (*black tracings*). AU, arbitrary units

the fluorescence changes with this dye at a 200 nM concentration in the presence of 10 μM $BaCl_2$ and 10 μM CCCP. Also this dye behaved similarly to $DiSC_3(3)$, but as with $DiSC_3(5)$, $BaCl_2$ failed to clearly decrease the fluorescence in the absence of glucose, and because of this, although the effect of the addition of KCl was obvious, the changes observed were neither as clear as with $DiSC_3(3)$, included for comparison in Fig. 8c.

Madrid et al. (1998) performed experiments with $DiOC_6(3)$ using concentrations of 50 nM or even 12.5 nM, following the rationale that with low concentrations, the interaction with the mitochondria might be avoided, With this concentrations we observed minimal changes of fluorescence, regarding the effects of glucose, $BaCl_2$, CCCP, and KCl (not shown).

Hydrophobicity of the dyes

The fluorescence changes of these dyes, particularly upon binding to the cells, appear to be due to the combined contribution of their positive charge and hydrophobicity. Because of this, we decided to look at their hydrophobicity by measuring their distribution coefficients into equal volumes of dichloromethane and 10 mM MES-TEA, pH 6.0. The distribution coefficients were 26.5 for $DiSC_3(3)$, 893 for $DiSC_3(5)$, and 164 for $DiOC_6(3)$, confirming the higher hydrophobicity of these last two dyes suggested by their structure. The binding to the surface of the cells due to the cationic nature of the dyes can be avoided by a divalent cation, but not that due to their hydrophobic nature.

These are only two examples of different dyes, but still results with another one, 2-(4-dimethylaminostyryl)-1-ethylpyridinium have been reported (Borst-Pauwels et al. 1992). This dye might be tested by using it in the presence of $BaCl_2$ and CCCP.

Other buffers

In one of the methods (Maresova et al. 2009) 10 mM Na_2HPO_4 taken to pH 6.0 with citric acid is utilized. Under our experimental conditions, the use of this buffer resulted in changes similar to our MES-TEA buffer, but we found the following differences: Without glucose, a slow increase was observed, until reaching a constant value and the addition of KCl produced a clear, but small decrease of fluorescence. With glucose, the fluorescence increased rapidly to reach a maximum, and then decreased to a value similar to that without glucose. The decrease with KCl was clear, but to lower values than those in the absence of glucose (Fig. 9a). With this phosphate-citrate buffer, although we obtained results similar to our MES-TEA buffer, even in the presence of Ca^{2+} and CCCP, after some time, the difference between glucose and no substrate in the final part of the tracing was much lower than with MES-TEA.

We also used the 10 mM MES buffer adjusted to pH 6.0 with $Ca(OH)_2$ (Madrid et al. 1998) and $DiSC_3(3)$. What we found, of course without adding extra Ca^{2+} or Ba^{2+}, was that in the absence of CCCP there was a difference in the fluorescence in the presence versus the absence of glucose, and a small signal decrease was observed upon the addition of KCl (data not shown). With CCCP, the final difference of fluorescence in the presence or absence of glucose was practically absent, and only a small decrease was observed after the addition of KCl (Fig. 9b).

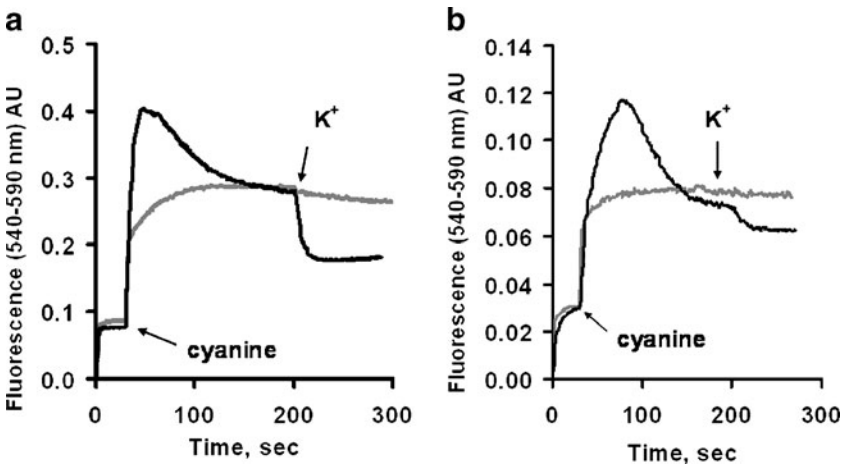

Fig. 9 Fluorescence changes of 200 nM of $DiSC_3(3)$ (540–590 nm) in different buffers. **a** 10 mM Na_2HPO_4 adjusted to pH 6.0 with citrate, or **b** 10 mM MES adjusted to pH 6.0 with $Ca(OH)_2$. Non-starved yeast. The incubation medium also contained 10 μM CCCP. Only in A, 10 μM $BaCl_2$ was added. Fluorescence changes were followed at 540 and 590 nm, excitation and emission wavelengths, respectively. Black tracings show results with 20 mM glucose, and gray lines, without substrate. The cells (25 mg wet weight) were added at the beginning of the tracing, and the cyanine at 30 sec. Where indicated, 10 mM KCl was added. AU, arbitrary units

With these buffers it is possible that part of the effects are due to citrate, Ca^{2+} or even phosphate, that may have additional effects on the PMP, since the addition of K^+ did not produce, as with the MES-TEA buffer, a similar decrease of fluorescence. The large decrease of fluorescence following the initial increase after the addition of the cyanine (Fig. 9a) was most probably due to the uptake of the rather large concentration (*ca.* 20 mM) of Na^+ in the Na_2HPO_4 buffer.

Regarding the 10 mM MES-$Ca(OH)_2$ buffer, as used in Madrid et al. 1998, the amount of the base needed to take MES to pH 6.0, is such that, the Ca^{2+} concentration in the incubation medium is around 2.5 mM, which is definitely an excess of the divalent cation. This large concentration of Ca^{2+} undoubtedly affects the results because of its already mentioned transport into the cells. Moreover, the reason why, with this buffer, the addition of K^+ did not decrease the fluorescence could be the inhibition of its transport by Ca^{2+} (Gage et al. 1985).

The accumulation of $DiSC_3(3)$

Considering that the fluorescence changes of the dyes should result from their actual concentration by the cells, we measured this parameter under our conditions (10 mM MES-TEA, pH 6.0, with or without the addition of glucose or $BaCl_2$, CCCP or KCl) in non starved cells. Two concentrations (200 nM and 1.0 μM) of the dye were used, and for comparison, the results with both are presented in Fig. 10 as the percentage of the dye taken up by the cells. We found the following: 1) the percentage of uptake under all conditions was almost identical at both concentrations of the dye; 2) under all conditions, the uptake of the cyanine with glucose was slightly higher if compared to that in its absence, but when both Ba^{2+} and CCCP were present, this difference was larger; 3) the addition of CCCP produced a large decrease of the uptake in either the presence or absence of Ba^{2+}, and 4) the uptake was significantly decreased when K^+ was added in the presence of glucose and both Ba^{2+} and CCCP.

Calculation of the PMP from cyanine accumulation

With these same data, we calculated the final internal concentrations of the dye, using the value of 0.43 mL of internal water per gram of wet weight for this yeast as measured before (Sánchez et al. 2008). From the ratio of the internal over the external concentrations and the Nernst equation, we calculated the apparent PMP values (Table 1). The analysis of the results can be summarized as follows: 1) either in the presence or absence of glucose, the uptake was higher when no CCCP was added, and an apparent PMP around −200 mV could be calculated; 2) in the presence of CCCP, even in the absence of a substrate, a basal value of PMP close to −160 mV was calculated; 3) the addition of Ba^{2+}, either in the presence or absence of a substrate did not produce any change in the calculated PMP values, indicating that this cation does not affect the uptake of the dye; 4) the lowest PMP values were obtained with CCCP only, but were consistently increased by glucose, whether in the presence or absence of Ba^{2+}; 5) without a

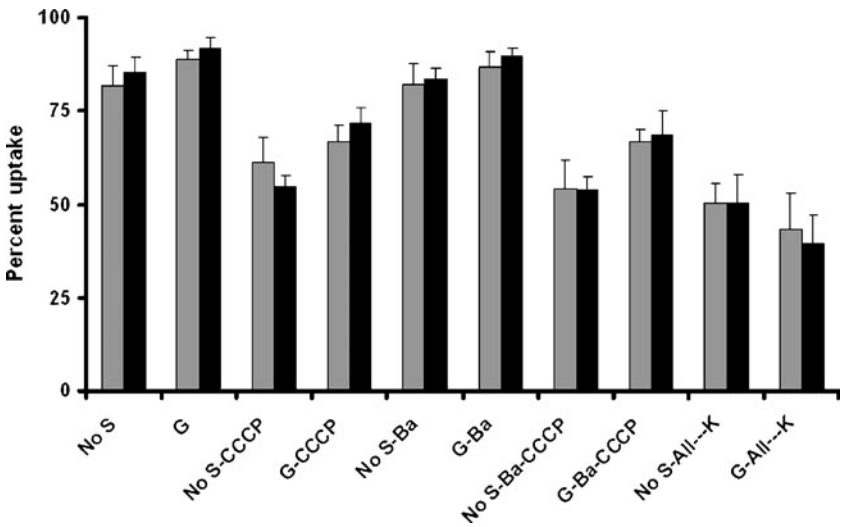

Fig. 10 Uptake of $DiSC_3(3)$ by non-starved yeast cells. The cells were incubated for 5 min in a final volume of 3.0 mL of 10 mM MES-TEA; where indicated, 20 mM glucose (G), 10 μM CCCP or 10 μM $BaCl_2$ (Ba) were added. $DiSC_3(3)$ was 0.2 μM (gray bars) or 1.0 μM (*black bars*) final concentrations. No S, no substrate. After incubation, the cells were centrifuged and the supernatant decanted. Cells were also incubated with Ba^{2+}, CCCP, and glucose for 5 min; then 10 mM KCl was added (—K), and after 3 more minutes, they were also centrifuged (*last two sets of bars*). The remaining cyanine concentration was measured in the supernatant by measuring the fluorescence at 540 nm excitation, 590 nm emission, after adding 1.0% SDS, and calculated by comparing with the fluorescence of a known concentration of the cyanine. The cyanine taken up by the cells was calculated from the difference

Table 1 Electric plasma membrane potential calculated from the accumulation of $DiSC_3(3)$ in yeast using two concentrations

	PMP (millivolts±SD)	
Condition	0.2 μM	1.0 μM
Without CCCP		
–	−188±9	−195±8
G	−203±5	−211±11
Ba	−188±10	−191±6
G+Ba	−198±9	−205±6
With CCCP 10 μM		
–	−161±7	−154±3
G	−167±5	−173±5
Ba	−153±9	−152±4
G+Ba	−167±4	−169±8
[a] Ba—K	−149±6	−149±8
[a] G+Ba—K	−142±10	−138±8

Non starved yeast cells (25 mg, wet weight) were incubated for 5 min in a final volume of 3.0 mL of 10 mM MES-TEA, pH 6.0. As indicated, 10 μM $BaCl_2$ (Ba), 10 μM CCCP, 10 mM KCl (K), or 20 mM glucose (G) were added. $DiSC_3(3)$ was either 0.2 μM or 1.0 μM, final concentration. The cells were centrifuged and the remaining cyanine was determined in the supernatant by measuring the fluorescence at 540–590 nm, after adding SDS to a final concentration of 1.0% and comparing with the fluorescence of a known concentration of the cyanine

[a] In these experiments, K^+ was added after incubation for 5 min, and the cells were centrifuged 3 min later. Values were obtained from the experiment of Fig. 10 (n=4). SD, standard deviation

substrate, necessary for its uptake, the addition of K^+ did not produce any change, and 6) in the complete system, with Ba^{2+}, CCCP, and glucose, a clear and large increase of around −30 mV was observed by the addition of KCl.

In previous experiments, the rate of uptake of the cyanine was measured, and found to be very fast at both concentrations (200 nM and 1.0 μM) and under all conditions. Even at the lower concentration of the dye (200 nM) in the absence of a substrate, the accumulation of the dye reached a constant value after approximately 5 min (not shown).

Amiodarone produces the K^+ efflux from yeast, and an increase of the fluorescence of $DiSC_3(3)$, indicating an increased PMP (Peña et al. 2009; Maresova et al. 2009). Experiments were performed with the full incubation mixture, containing glucose, $BaCl_2$, and CCCP to verify whether the calculated PMP resulting from the measurement of the accumulation of this cyanine might be increased by the addition of the drug. The calculated PMP from the accumulation of $DiSC_3(3)$ showed a relative increase from −165 ± 5 mV to −195 ± 7 mV (more negative values) when the drug was added at a concentration of 40 μM.

In the absence of CCCP, the values obtained from the accumulation of the dye obviously result in an overestimation of the PMP because of mitochondria, and this logically leads to eliminate all conditions in which the uncoupler was absent. In the presence of CCCP, in the absence of Ba^{2+}, a small but consistently higher PMP was calculated when glucose was present than in its absence, and this difference was still higher in the presence of Ba^{2+}. As expected, the lowest calculated values of the PMP were observed in the presence of both Ba^{2+} and CCCP, in the absence of a substrate, compared to those with the same additions plus glucose. With glucose, a decrease of the PMP was observed with K^+, which, by being transported inside, is expected to lower the PMP. Without glucose, when no K^+ transport could occur, the addition of this cation did not produce a decrease of the values.

We may then propose this procedure as an approximation to measure the PMP in yeast, taking advantage of the permeant character of the cyanine and the simple procedure to measure its accumulation. It appears that a high resting PMP is present even in the absence of glucose, resulting from the rather high accumulated concentration of cations, and even in the absence of a substrate, this high concentration may give rise to their constant release through ionic channels, such as Tok1p (Bertl et al. 1993), described as an outward rectifying channel or another unknown efflux system such as that proposed in Bertl et al. 2003. This might lead to a minimum PMP around minus 160 mV. Apparently, in the presence of 10 mM external K^+ and 300 mM inside, this value should be around −90 mV; however, similar changes can be obtained with 2.0 mM KCl. The resulting value would be then around −130 mV. This indicates that more than to the ratio of internal/external concentration of the monovalent cation, the changes are related to the velocity and affinity of the uptake of the cation, probably with a much faster influx than efflux. Aside from being an outward rectifying channel no information exists about the regulation of at least the Tok1p. One additional argument in this respect is that when cells are incubated in the absence of a substrate, an efflux of K^+ is produced, but it is very slow, indicating that this and/or other efflux pathways are very slow. The variations of the PMP in the presence or absence of glucose would be in a range of 15 to 30 mV, but collapsible by the uptake of K^+.

It is clear that the calculated values, even in the presence of CCCP, may be underestimated because of an unequal distribution of the dye inside the cells, which however seems to be uniform, as seen under the fluorescence microscope. On the other hand, values may as well be overestimated, because part of the dye may not be free, but bound to the cell components, due to its hydrophobic character. $DiSC_3(3)$ has the advantage of being rapidly accumulated by the cells (around 5 min), as compared to the very slow accumulation of tetraphenylphosphonium reported by some authors (Boxman et al. 1984; Eilam et al. 1985).

Fluorescence parameters measurement and instrumentation

In the method reported in Gaskova et al. 1998, the authors look at the red shift of the cyanine $DiSC_3(3)$, instead of its fluorescence intensity, which shows a large increase when taken up by the cells. The shift of the fluorescence peak observed without an uncoupler is not due to the uniform accumulation through all the cell contents, but to the much higher concentration of the dye in mitochondria. This is the reason why the addition of 10 μM CCCP produces the disappearance or decrease of the shift, without affecting the PMP, as shown by the fact that this uncoupler concentration does not inhibit K^+ uptake.

In Maresova et al. 2009, the emission fluorescence difference at two wavelengths was assumed to be equivalent to measuring the fluorescence shift. Results are not shown, but under several conditions, adding 10 μM $BaCl_2$ and 10 μM CCCP either alone or combined, incubating the cells for 5 min, and taking the fluorescence spectra, we could not detect the spectral shifts, except of 3 or 4 nm to the red, when the cells were incubated in the presence of all components, plus glucose, and 10 mM KCl. In Madrid et al. 1998, fluorescence with a flow cytometer was measured. This latter procedure has the advantage of also considering cell size, which may be important. However, besides the low values resulting from the measurements, these methods require more complex instrumentation, and results are not always possible to follow continuously.

It should be pointed out that under the conditions tested, the fluorescence changes cannot be precisely defined in numeric terms, since they vary with time within seconds, resulting perhaps in different estimations, particularly if the cells are incubated for longer times and then processed for the selected procedure.

Conclusions

The performed comparison of the methods leads to several parameters, around which the following conclusions can be presented:

1. Due to the capacity of the mitochondria to accumulate the cationic dyes and quench the fluorescence, a low concentration of an uncoupler, 10 μM CCCP or FCCP should be used without affecting the PMP.
2. The fluorescence changes resulting from binding of the dye to the surface of the cells can be avoided by the use of low concentrations of a divalent cation. Because of the variable capacity of the cells to transport divalent cations, it is preferable to use starved cells. Although with starved cells, around 200 μM Ca^{2+} allows for reasonable estimations; adequate results can also be obtained with 5 to 10 μM $BaCl_2$.
3. Not just any dye is equally useful for the estimations. Comparison of the dyes favors the use of $DiSC_3(3)$, which can be equally useful at 200 nM or 1.0 μM. In addition, with this method, a reasonable range of cell amounts can be used, but it seems desirable to use between 25 and 50 mg wet weight.
4. The buffer used should be carefully considered to avoid the presence of ions that may affect the PMP. The use of 10 mM MES-taken to pH 6.0 with TEA is recommended.
5. Although some laboratories may have adequate instruments, measurement of fluorescence intensity at around 540 and 590 nm for excitation and emission, respectively, appears to be easy with any simple spectrofluorometer. Measurement of the difference of the emitted fluorescence at two wavelengths may also be adequate. The fluorescence changes ought to be presented in full, because they do not follow a simple course, neither they reach a constant value, so they should not be presented only as the final numerical value of the fluorescence change.
6. The measurement of the accumulation of $DiSC_3(3)$ may be a useful approximation to obtain quantitative estimations of the PMP of yeast and its variations under the influence of different agents or conditions. The conditions so far explored would indicate the use of the full incubation mixture described in Table 1 with either 200 nM or 1.0 μM of the dye.
7. Any strain should be tested about the reliability of the method by simple procedures, comparing the fluorescence changes or accumulation ratios of the cyanine in presence and absence of a substrate (glucose), the capacity of low concentrations of K^+, but not of Na^+, to decrease the fluorescence signal, or the addition of agents, such as amiodarone, known to produce K^+ efflux.

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