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# The Plasma Membrane Electric Potential in Yeast: Probes, Results, Problems, and Solutions: A New Application of an Old Dye?

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## Abstract

For a long time, estimations and actual measurements of the electric plasma membrane potential (PMP) in whole yeast cells have been the subject of studies by several groups without reliable results. The conditions in the measurements, as well as precautions required to perform them, are described here. Essentially, two approaches using different dyes are reviewed: (a) qualitative estimations by following fluorescence changes under different energization conditions and (b) measurements of the PMP by the accumulation of dyes. An analysis is presented regarding the conditions recommended to obtain more consistent results when following the fluorescence changes. Also, measurements of accumulation of different dyes, and the necessary conditions to perform them, are analyzed. In particular, using acridine yellow appears to be a trustworthy method, with few reserves, both to follow in real time the qualitative changes of the PMP by fluorescence changes and to assess actual PMP values by measuring the accumulation of the dye.

**Keywords:** plasma membrane potential, yeast, acridine yellow, fluorescent monitors

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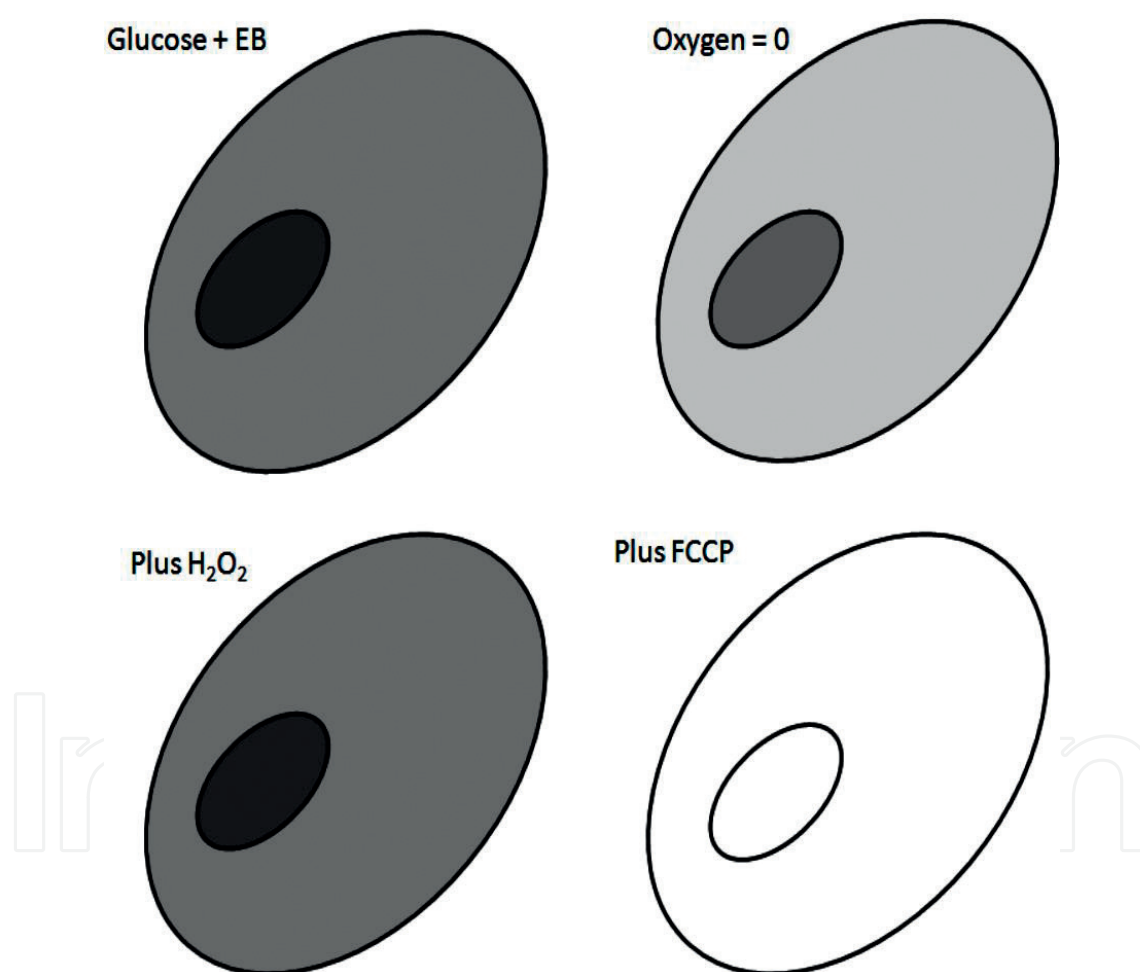
## 1. Introduction

### 1.1. Qualitative estimation of the electric plasma membrane potential (PMP) in yeast

After the original proposal for the mechanism of  $K^+$  transport in yeast [1], it was shown [2, 3] that this ion is transported because a  $H^+$ -ATPase exists in the plasma membrane, pumping protons outside, therein generating an electric membrane potential difference (PMP), negative

inside. This is then responsible mainly for the uptake of cations and other molecules required by the cells through different active uptake systems of which the most prominent is Trk1p [4]. This mechanism was also described for *Neurospora crassa* [5] and many other fungi and also for plants. These findings gave rise to a series of attempts to follow changes in the plasma membrane potential (PMP) in whole yeast cells, initially by measuring the accumulation of cationic compounds [6, 7]. Results were though far from satisfactory, mainly because of the slow rate at which these molecules entered the cells.

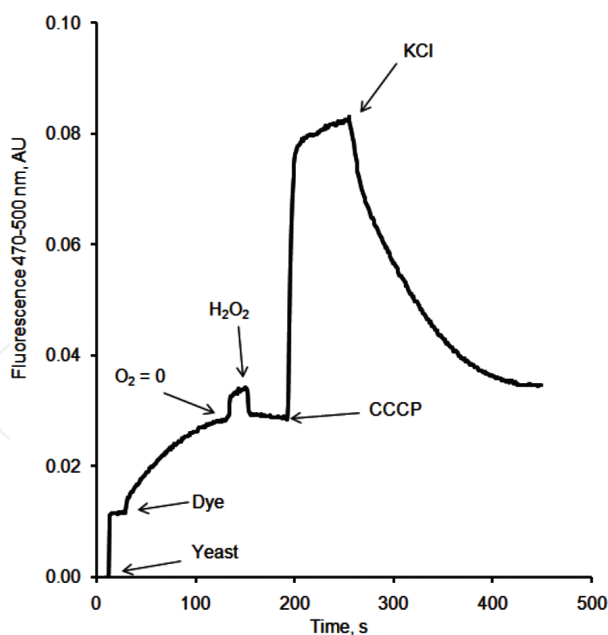
The use of fluorescent indicators originally to estimate the membrane potential in mitochondria started a long time ago [8, 9] by observing ethidium bromide fluorescence quenching under different energy states. Also, measurements of the PMP in animal cells were performed with reasonable results [10–12]. Following these studies and using whole yeast cells, fluorescence



**Figure 1.** Graphic interpretation of the fluorescence changes of ethidium bromide in yeast under different conditions [13]. (A) When glucose alone is added, fluorescence of the dye is moderately increased, but a large part of it is accumulated by the mitochondria, where its fluorescence is quenched. (B) When oxygen is exhausted, mitochondria are partially de-energized (they still can maintain most of its membrane potential by using ATP generated in glycolysis). (C) Upon the addition of  $H_2O_2$ , mitochondrial membrane potential is fully recovered, resulting in fluorescence quenching. (D) The addition of an uncoupler (FCCP) produces the full collapse of the mitochondrial membrane potential, the uniform distribution of the dye in the cytoplasm, and a large increase of fluorescence. The small oval shape represents a mitochondrion.

changes of ethidium bromide under different energization conditions were studied [13]. It was found that in order to observe the fluorescence increase derived from the accumulation of the dye, it was more convenient to use starved yeast cells, which required the addition of glucose as a substrate. **Figure 1** shows a schematic representation of the results observed and their interpretation. It was found that in the presence of glucose, fluorescence showed a rather small increase, and after a few seconds was followed by another one, coincident with the oxygen exhaustion in the medium that as expected could be reversed by the addition of  $H_2O_2$ . This led us to suspect that the initial fluorescence increase was actually composed of not only an increase due to the accumulation of the dye in the cytoplasm but also a decrease due to its accumulation in mitochondria, according to what had been described before to what was happening in the isolated mitochondria [9]. Moreover, it was found that, also as expected, the addition of an uncoupler resulted in a much larger increase of fluorescence, consistent with the idea that collapsing the membrane potential of the mitochondria resulted in an efflux of the dye and its uniform distribution in the cytoplasm, where the dye was highly fluorescent [14]. The actual fluorescence changes observed in the spectrofluorometer were similar to those shown in **Figure 2** with acridine yellow.

Several years later, a new procedure, under the same principles was proposed by following the fluorescence changes of the cyanine DiSC<sub>3</sub>(3) [14]. Since then, perhaps the most used monitor to follow changes of the PMP in yeast is DiSC<sub>3</sub>(3), a high affinity probe that in the presence of a substrate (glucose) is concentrated in the cytoplasm, and similarly to ethidium bromide, also in the mitochondria [16]. Although the latter can be avoided by using low concentrations



**Figure 2.** Fluorescence changes of 25  $\mu$ M acridine yellow at 470–500 nm when added to *S. cerevisiae* cells. Incubation was carried out in 10 mM MES-TEA buffer, pH 6.0; 10  $\mu$ M  $BaCl_2$ ; 20 mM glucose in a final volume of 2.0 mL. Where indicated, 8  $\mu$ L of 3%  $H_2O_2$ , 10  $\mu$ M CCCP or 5 mM KCl were added. Representative figure of one experiment similar to that already reported [15]. Fluorescence was followed in an SLM Aminco spectrofluorometer with continuous stirring and at a temperature of 30°C. Excitation and emission fluorescence were 470 and 500 nm, respectively. Phototube voltage was 600 V, and slits were set at 8 nm. AU, arbitrary units.

of an uncoupler, still the high affinity and binding of the dyes to the cell components turns it difficult to calculate their free concentration inside, in order to accurately measure the PMP through its accumulation. Other authors [17] also used DiOC<sub>6</sub>(3) with this same purpose with rather uncertain values. Thus, results using cationic molecules, mostly dyes, have been under discussion for many years without arriving to a general consensus about the methods, and even less regarding the results obtained, especially when it comes to the real values of the PMP in yeast.

## 2. The quantitative measurement of the PMP

DiSC<sub>3</sub>(3) has been one of the most used monitors to estimate changes of the PMP in yeast [14, 16, 18–22] by following its fluorescence changes under different energy conditions of yeast cells. Attempts have also been made to assess the actual PMP values from the internal and external concentration ratios between the cells and the media. This implies (a) measuring the concentration and the amount of the dye or any cationic agent remaining after incubation with the cells; (b) knowing the amount originally added that taken up by the cells can be obtained; (c) the internal concentration of the dye can be obtained by previously measuring the internal water volume of the cells to acquire the internal concentration. Finally, by using the Nernst equation, the value of the PMP, in millivolts can be obtained from:

$$E = -(RT/ZF) \ln C_{\text{in}} / C_{\text{out}} \quad (1)$$

where R is the gas constant, Z the charge of the ion, F is the Faraday constant,  $C_{\text{in}}$  and  $C_{\text{out}}$  the internal and external dye concentrations. Then, PMP is approximately equal to:

$$\text{PMP} = -60(\log [C_{\text{in}}] / [C_{\text{out}}]). \quad (2)$$

In this way, approximate values of the PMP have been obtained [14, 15, 17]. However, those values are subject to many errors and uncertainties that will be discussed below.

Studies with *Saccharomyces cerevisiae* [18] and *Rhodotorula glutinis* [19] proposed another approach to measure the actual PMP by following the changes of the  $\lambda_{\text{max}}$  of the fluorescence spectrum of this monitor as an indicator, which has been also used by other authors [20, 23]. Within the claims to actually measure the plasma membrane potential in yeast, some values reported are too low to explain, among others, the large accumulation of K<sup>+</sup>, which can reach internal concentrations of around 300 mM against micromolar external concentrations of the cation. Results are hard to rationalize, even considering that one part of the cation redistributes into the vacuole [24] and another is neutralized by the accumulation of bicarbonate when glucose is the substrate [25]. The use of fluorescent probes not only for the estimation of plasma membrane potential but also for many other purposes was partially reviewed by Slavik [26], and more recently, for yeast cells [16, 19]. However, this topic continues to be unsolved, and it is our belief that several aspects should be considered. The problems to estimate and measure the PMP in yeast imply a large series of factors that may affect results, such

as the following: (a) the influence of the binding of the cationic monitor to the surface of the cell; (b) the use of an adequate buffer, avoiding organic molecules and other cations that may be taken up by the cells; (c) the concentration of the dye, critical and different for each one and probably for different yeast strains or species to observe the fluorescence changes and accumulation; (d) the accumulation of the dye by the mitochondria; (e) the binding of the dye inside the cells, and (f) the use of starved cells that allows observing changes due to energization of the cells by adding a substrate.

### 3. Interaction, uptake, distribution, and binding inside the cells

#### 3.1. Binding to the surface

The first interaction of the dyes is, of course, with the negatively charged cell surface. Since the first studies performed [14], an immediate increase of fluorescence of DiSC<sub>3</sub>(3) was observed upon its interaction with the cells that could be diminished by the addition of low concentrations of a divalent cation eventually recommending the use of BaCl<sub>2</sub> to avoid binding [16].

#### 3.2. Uptake

Using starved cells, the addition of a substrate, usually glucose, is required in order to generate the PMP [14]. Most probes appear to enter the cells by free diffusion. However, ethidium bromide, at least at certain concentrations, seems to be transported into the cell through the K<sup>+</sup> transport system [27]. Monitors are generally cationic, with a delocalized electron structure that nonetheless does not eliminate their positive charge. In general, anionic molecules do not seem to enter the cells [28]. The main relevant characteristic to the topic of this review is that cationic molecules employed seem to be driven inside by the plasma membrane electric potential difference, and because of this, they can be used to follow changes of the membrane potential under varied conditions [16, 18, 19, 21, 22].

#### 3.3. The internal distribution

Once the dyes enter the cells, they are not uniformly distributed inside due to the negative inside membrane electric potential difference of the mitochondria. Experiments show [16] that in fact the changes observed in the dyes' fluorescence within yeast cells mainly when using ethidium bromide, DiSC<sub>3</sub>(3), and acridine yellow [15] depend on the addition of a substrate, generally glucose. When this is added (a) a slow increase of fluorescence is seen, then after a few seconds that is followed by another small increase, coincident with the exhaustion of oxygen that can be reversed by the addition of a small concentration of H<sub>2</sub>O<sub>2</sub>, (b) if then a low concentration of an uncoupler around 5–15 μM is added, such as CCCP or FCCP, a much larger increase of fluorescence is observed. Finally, (c) when a concentration of KCl enough to be transported inside is added, a decrease of the fluorescence is seen. One additional observation with at least two dyes, DiSC<sub>3</sub>(3) [15, 16] and acridine yellow is that they under none of these conditions enter the vacuole. **Figure 2** shows the results of one experiment in



which these fluorescence changes were observed with acridine yellow but are similar to those observed with DISC<sub>3</sub>(3) or ethidium bromide.

### 3.4. Interpretation of results

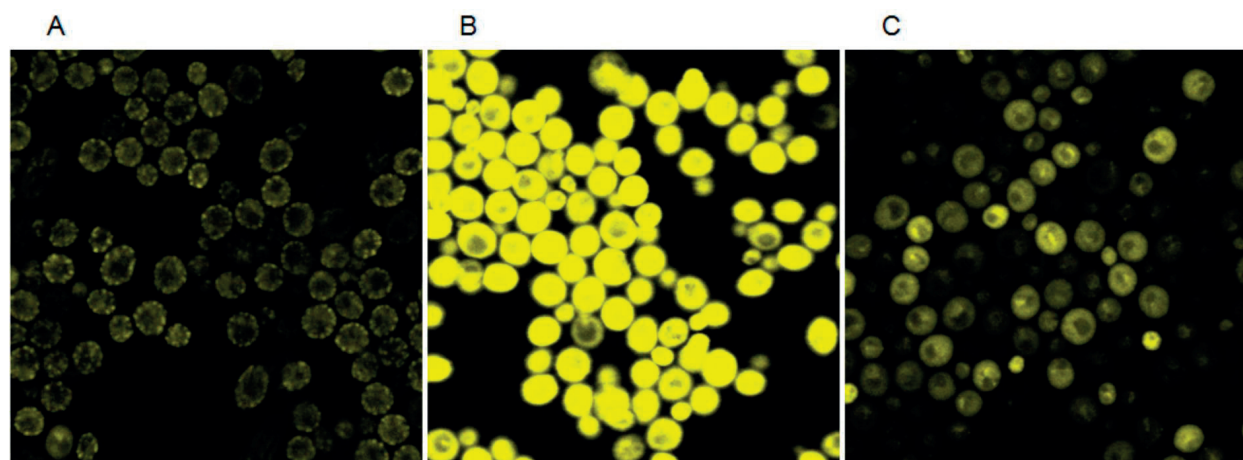
Results shown in **Figure 2** are interpreted as follows: in the presence of a substrate necessary to energize the cells, the dye is transported inside, driven by the PMP. Once inside, it is also taken by the mitochondria, where a large accumulation takes place, resulting in quenching of most of the fluorescence. This observation is supported by the small fluorescence increase when oxygen in the medium is exhausted by respiration that can be reversed by the addition of H<sub>2</sub>O<sub>2</sub>. When oxygen is exhausted, a partial deenergization of mitochondria occurs. Both these changes are small because even in the absence of oxygen, mitochondria are energized by the ATP produced in glycolysis. Adding then a low concentration (5–15 μM) of CCCP or FCCP depolarizes mitochondria, producing the efflux of the dye from the organelles and its uniform distribution in the cytoplasm, resulting in a large fluorescence increase (dequenching). After this, K<sup>+</sup> addition, since the ion must be taken up through a PMP-driven transport partially neutralizing it, produces a large decrease of the fluorescence. Another important result in this respect is that the addition of a similar concentration of NaCl does not produce the fluorescence decrease resulting from the addition of KCl because Na<sup>+</sup> affinity for the transporter is much lower than that of K<sup>+</sup> (not shown).

### 3.5. Binding to the internal cell components

One important characteristic of the monitors employed is the low fluorescence they show only in the buffered medium, which is largely increased by their interaction with the internal components of the cell [14, 15]. This characteristic is actually the basis for the fluorescence-based studies of the PMP and its changes under the different conditions. However, knowing the amount bound to the internal components of the cells becomes one of the main problems when trying to measure the actual values of the PMP by their accumulation.

## 4. Evidence from microscope images

Additional evidence is provided by looking at the cells under the microscope. **Figure 3** shows the images obtained with acridine yellow in a similar experiment to one already reported [15]. It can be clearly seen that in the presence of glucose alone the dye is concentrated in the mitochondria, but fluorescence is rather faint, which confirms that in fact, most of the dye is accumulated by these organelles; its fluorescence is quenched because of its large accumulation (**Figure 3A**). Then, particularly with acridine yellow, upon the addition of a low concentration of an uncoupler (10 μM CCCP), fluorescence of the dye increases remarkably and gets uniformly distributed in the cytoplasm (**Figure 3B**). The subsequent addition of KCl results in a general decrease of the fluorescence (**Figure 3C**). It is important to point out that the dye does not enter the vacuole.



**Figure 3.** Microscopic images following fluorescence changes of acridine yellow in yeast as a result of differences in PMP. In (A), the cells were incubated in 10 mM MES-TEA, pH 6.0, 20 mM glucose and 50  $\mu$ M acridine yellow and observed 5 min later. Then, 10  $\mu$ M CCCP was added, and 5 min later, image B was obtained. Image C was obtained 5 min after adding 10 mM KCl to the same preparation of image B. Similar figure to that of [15].

## 5. The problems and solutions tested

The general procedure to follow the fluorescence changes is as follows. The cells are added to an adequate buffer, and as a substrate, 20 mM glucose is used. A 2.0 mL final volume of the medium is important to ensure the effective continuous mixing of the incubation mixture. Adequate settings of the instrument regarding high voltage applied to the photomultiplier and the slit width should be chosen to get an adequate level of the fluorescence signal and its changes. We use an SLM Aminco spectrofluorometer with a cell holder provided with continuous magnetic stirrer and a constant temperature system. Different factors affecting the values obtained are the following:

*Buffer.* Salts that may be taken up by the yeast cells such as phosphate, sodium, or potassium salts should not be used to avoid their interference with the functioning of the cells. We prefer 10 mM MES (morpholinoethanesulfonic acid) taken to pH 6.0 with triethanolamine.

*Amounts of cells and dye.* With different monitors, different amounts of cells and dye concentrations should be assayed to find those in which the best tracings are obtained.

*External binding.* When fluorescence is followed incubating the cells in the presence of an adequate buffer, even in the absence of a substrate, an increase of fluorescence is observed, which appears to be due to the simple binding of the dyes to the negative external charges of the membrane. To avoid this, we started using a low concentration of  $\text{CaCl}_2$  [14], but later realized that it was better to use a low concentration of 10–20  $\mu$ M  $\text{BaCl}_2$  [16].

*Accumulation in the mitochondria.* Uncouplers in yeast, at least *S. cerevisiae*, show an interesting behavior. They produce a very clear stimulation of respiration at concentrations around 2–5  $\mu$ M, while to inhibit  $\text{K}^+$  transport, a process dependent on PMP, several times higher



concentrations are required [16, 27]. This indicates that low concentrations can uncouple the mitochondria, without affecting the PMP. The solution then is to use low concentrations (5–15  $\mu\text{M}$ ) of either CCCP or FCCP to eliminate the accumulation of the dyes by the mitochondria.

*Binding to the internal components.* This factor is impossible to eliminate, and it is the actual basis of the analysis of the PMP by fluorescence, since as already mentioned, the monitors used in these studies show very low fluorescence values in water and require their interaction with the internal components of the cells. However, as it will be seen ahead, binding values can be measured.

## 6. Conclusions from the fluorescence changes

Changes of fluorescence of different monitors are an excellent way to follow not only the qualitative variations of the PMP in yeast, depending on the dye and conditions used. This has been shown with ethidium bromide [13, 27]. Regarding the claims that follow the displacement of the maximal fluorescence, peaks can provide the way to determine the actual value of the PMP and should be taken with caution [19]. Two reasons allow to affirm this (a) as mentioned before, the values obtained are too low to explain the large accumulation of  $\text{K}^+$  of around 300 mM inside the cells in the presence of micromolar concentrations outside, even considering that part of it is within the vacuole [24], or partially neutralized by the simultaneous accumulation of bicarbonate [16], and (b) although the authors state that the PMP can be fully collapsed by the addition of 10–20  $\mu\text{M}$  of the usual uncouplers, CCCP or FCCP, results show that the concentrations required to stimulate respiration are 5–10  $\mu\text{M}$  and those to inhibit  $\text{K}^+$  uptake are more than 20  $\mu\text{M}$  [16]. Moreover, this anomalous incapacity of uncouplers to freely transport  $\text{H}^+$  through the yeast plasma membrane has been used to measure the internal pH of yeast cells by following the distribution of 2,4-dinitrophenol [2, 29]. When using acridine yellow [15], the fluorescence changes observed when deenergizing the mitochondria are particularly large. This means that this dye may be particularly useful to monitor the changes of the electric potential difference of mitochondria.

## 7. Actual measurement of the PMP by the probe accumulation

Initial attempts to measure the PMP based on the accumulation of cationic agents [6, 7] were unreliable because of the slow entrance of the cationic agents used and the apparently incomplete equilibrium reached between the inside and outside of the cells. Previous calculations with the simple accumulation of different dyes, either ethidium bromide [13], or  $\text{DiSC}_3(3)$  [14, 16], gave results that appeared too high, and the variations under the different conditions too small. As already pointed out, more recently [15], acridine yellow was found to require higher concentrations than  $\text{DiSC}_3(3)$  to observe the fluorescence changes usually described (**Figure 2**). Its behavior was also similar to that reported for other indicators when observed under the microscope (**Figure 3**). In summary, its activity was typical of that observed with

other dyes, such as ethidium bromide or DiSC<sub>3</sub>(3). One difference is that while DiSC<sub>3</sub>(3) had to be used at nM concentrations, this dye required a concentration of 50 or even 150  $\mu$ M to clearly observe changes in its accumulation.

### 7.1. The PMP measured by the accumulation of acridine yellow

Following a similar procedure to that used with other dyes, its accumulation was measured to calculate the PMP under different conditions. In these experiments, from the amount of dye remaining in the supernatant after centrifuging the cells, we could calculate its internal amount, and from the value of the internal water content, its internal concentration, which allowed to calculate the apparent noncorrected  $\Delta\psi$  values that are shown in **Table 1**.

These results were already encouraging and different to those obtained before with DiSC<sub>3</sub>(3) [16], but we still had to consider that at least part of the dye was not free, but bound to the internal components of the cells. Values were also interesting regarding their magnitude and reproducibility. With glucose alone, the highest accumulation was observed present in both the cytoplasm and the mitochondria. CCCP addition produced a decrease because the dye was no longer concentrated in the mitochondria, reaching a new equilibrium with the external concentration. As expected, adding 5 mM KCl, which should at least partially collapse the PMP, produced another large decrease. It is also important to emphasize that the addition of NaCl produced only a small change.

### 7.2. Binding of the dye inside the cells, a possible solution

Considering that we had no way to produce the efflux of the dye bound to the internal components of the cell, we decided to use another approach, trying to measure it. Chitosan, a cationic polymer mostly composed of glucosamine is very effective to permeabilize the plasma membrane of *Candida albicans* [30], but actually this effect was found before on *S. cerevisiae* (unpublished). We therefore used the cells incubated with glucose plus CCCP and added to

	$\Delta\psi$ , mV $\pm$ std. dev
Glucose	-168.4 $\pm$ 7.3
CCCP	-105.2 $\pm$ 1.8
CCCP + KCl	-46.0 $\pm$ 4.5
CCCP + NaCl	-95.7

Experiments were conducted using the typical incubation mixture with 10 mM MES-TEA buffer, pH 6.0, 10  $\mu$ M BaCl<sub>2</sub>, and 20 mM glucose, with 50  $\mu$ M acridine yellow and 250 mg of cells (wet weight) in a final volume of 10.0 mL. Absorbance spectra of the samples, and the mean readings between 409 and 411 nm were obtained. From these readings and the linear part of a standard curve, the external concentrations were calculated. Accumulated dye was measured 5 min after the indicated successive additions by determining the concentration, and from it the amount of dye in the supernatant after centrifuging the cells. The internal amount of dye was obtained by subtracting that amount from that originally added. Its concentration was calculated considering the value of internal water of yeast, which has been measured and equivalent to 0.47 mL g<sup>-1</sup>, wet weight (33). The values were obtained in each case 5 min after the successive addition of (a) cells; (b) CCCP (10  $\mu$ M), and (c) either KCl or NaCl (5 mM).  $\Delta\psi$  was calculated from the Nernst equation, considering the log of the quotient of the internal/external concentrations.

**Table 1.** Raw calculations of  $\Delta\psi$  from the measured accumulation of acridine yellow in yeast.

them 100 µg of chitosan. After centrifuging the cells, it was possible to calculate the amount of the dye leaking out, and of course, that remaining inside bound to the internal components. In this way the amount of dye that remained in the cells after the addition of chitosan could be subtracted from that remaining under the different conditions. Results of one typical of those experiments are the following:

Chitosan produces the permeabilization of the plasma membrane of the cells, producing the efflux of the dye, but still part of it remains inside, bound by its cationic nature. This is an interesting approach to subtract the contribution of the internal binding of the dye in the calculations of  $\Delta\psi$ , at least in this yeast. However, still one problem exists: the values of the dye remaining inside the cells when KCl was added were found to be lower than those obtained with chitosan. This may be because  $K^+$  may produce an additional displacement of the also cationic dye from its binding sites.

7.3. The exclusion of the dye from the vacuole

Results from the accumulation of the dye considered a uniform distribution inside the cells, whose total water content has been measured and estimated at 47% of water per g of wet weight [31]. Microscope images show that after the uncoupler, the dye is no longer in the mitochondria and distributes in the cytoplasm, but it is absent from the vacuole [15]. This implies that the distribution volume of the dye is smaller than that in the total cell water. Considering this, the internal water in which the dye distributes is not the total value of 0.47 mL g<sup>-1</sup> [31], but 0.355 mL g<sup>-1</sup> of cytoplasmic water, excluding the vacuole. Using this new volume, the values shown in **Table 2** were obtained, indicating a still higher value of the PMP, shown in parenthesis in **Table 2**.

	[Int] nmoles/mL	[Ext] nmoles/mL	Ratio	Log	mV
Control	1953	4.1	476	2.67	-161
CCCP	1244	20.8	60	1.77	-107
KCl	284	64.4	4.4	0.64	-39
NaCl	1023	25.9	39	1.60	-96
Chitosan	391	40.4			
<b>Correction for internal binding</b>					
Control	1562	4.1	380	2.6	-155 (211)
CCCP	853	20.8	41	1.6	-97 (196)
KCl	0.7	43.3	?	?	?

The experiment was performed as described in **Table 1**, but where indicated, 100 µg of chitosan was included, and the last sample was taken 5 min later. The amount of the dye that had not entered the cells, subtracted from the total added, allowed to get the amount that entered the cells. To obtain the internal concentration of the dye, this latter amount was divided by the internal water content of the cells, which was estimated before for this yeast strain of 0.47 mL g<sup>-1</sup> of cells (33). From the internal and external concentrations, the quotient and its log were obtained, and using the Nernst equation,  $\Delta\psi$  was calculated. Values in parenthesis were obtained considering that if the dye does not enter the vacuole, the total water in which the dye was distributed was only 0.355 mL g<sup>-1</sup>. Question marks indicate that the values were not calculated because the efflux of the dye with KCl was larger than that with chitosan.

**Table 2.** Values of the accumulation of acridine yellow obtained in a representative experiment under different conditions.

## 8. Concluding remarks

Our work is a constant attempt to clarify a long time controversy, first about the estimation of the fluorescence changes of different monitors with different methods. We could add to the list acridine yellow, long known, but also quite inexpensive, which can be used to that purpose. With this dye larger concentrations are required, which may be probably due to the fact that it has a lower binding affinity to the internal components of the cells. This can be inferred already by comparing the results of its uncorrected uptake values as shown in **Table 3**.

With these corrections, larger differences under the conditions tested could be obtained with acridine yellow, as compared to those obtained with other agents, but much higher than those reported by other authors [19]. The simple accumulation in the presence of glucose already resulted in values lower than those reported before. Then, the addition of CCCP produced an apparent large decrease of the calculated PMP, because of the release of the large accumulation by the mitochondria and then outside the cells. Interesting also are the lower values obtained in the presence of  $K^+$ , whose uptake should lower the PMP.

From previous and recent work, we can summarize the basic conditions needed to obtain reliable results, such as the buffer used, which must not contain cations or organic molecules that may interfere or modify the PMP of the cells. When using different monitors, different conditions should be tested, mainly the concentration of the dye to adjust it depending on the yeast used. Changes due to binding to the surface of the cells can be minimized by the addition of a low concentration (10  $\mu$ M)  $BaCl_2$ . The accumulation of the monitor by mitochondria can be avoided by the addition of around 10  $\mu$ M CCCP or FCCP. Finally, corrections can be applied by using the correct volumes for the distribution of the dye, as well as its efflux with a permeabilizing agent.

There is another factor influencing the results obtained. The efflux of the dye produced by chitosan was lower than that found after the addition of  $K^+$ . When the dye concentration after the addition of the monovalent cation is subtracted from that obtained after the addition of chitosan, a negative net accumulation results, meaning that the cells would have a positive PMP value, which is hard to accept. The most probable explanation is that when chitosan is present, the dye goes out of the cells, leaving inside that bound, and the dye binds inside because of its hydrophobic and also its cationic nature. So the addition of  $K^+$  not only reduces the PMP but also produces the liberation of the dye particularly from its internal binding due

	Acridine yellow*	1.0 $\mu$ M DiSC <sub>3</sub> (3)**	167 mM ethidium***
Control	-168.4 $\pm$ 7.3	-205 $\pm$ 6	-225.6 $\pm$ 21
CCCP	-105.2 $\pm$ 1.8	-169 $\pm$ 8	-211.2 $\pm$ 31
CCCP+ KCl	-46.0 $\pm$ 4.5	-138 $\pm$ 8	–

\* As described in Ref. [18].

\*\* As described in Ref. [17].

\*\*\* As described in Ref. [14].

**Table 3.** Comparison of PMP values (in mV) obtained with different dyes.

to its cationic nature. The conclusion then is that, although the accumulation of acridine yellow provides an adequate method to measure the PMP of yeast, the values obtained after the addition of positively charged ions that accumulate in large concentrations within the cell are distorted because of the displacement of the dye from what most probably are anionic sites inside the cell. It is possible that part of the  $K^+$  taken up by the cells may be bound to their negative internal components. If this were so, one would expect acridine yellow to produce an efflux of  $K^+$ . However, in other experiments (unpublished) we have found that the dye at concentrations of 60 and 120  $\mu M$ , higher than those used in those reported here does not produce the efflux of the monovalent cation. It must also be considered that although the uptake of  $K^+$  is expected to decrease the PMP, the values obtained after the addition of this cation are too low. It has to be considered that after its addition, and because of the decrease of the PMP, this results in the stimulation of the plasma membrane  $H^+$ -ATPase, originating a transient increase of ADP, that is then compensated by the acceleration of glycolysis [32], all of which must at least partially restore the PMP values. In this sense, it appears that results following the accumulation of DiSC<sub>3</sub>(3) are more in agreement with these facts.

## Appendix

In more recent experiments (unpublished), in order to correct as much as possible the values of the binding of the dye to the internal cell components, we incubated the cells under the same conditions, always in the presence of 10  $\mu M$  CCCP to avoid its accumulation by the mitochondria. Previously, we used 50  $\mu M$  acridine yellow. When incubation was performed with glucose alone, practically all of the dye was taken up by the cells and made difficult to distinguish between the dye bound inside and that taken up driven by the PMP. Because of this, in these experiments we used the dye at a 150  $\mu M$  concentration. The conditions were the following:

- A. Cells with glucose in which the dye is taken up and accumulated by the cells due partly to the PMP but also to its binding to their internal components.
- B. Cells incubated first with glucose for 10 min, adding then 10 mM KCl, in which a large efflux of the dye is observed due in part to the decrease of the PMP, but also to a large  $K^+$  accumulation, around 200–300 mM, that produces its liberation from the anionic sites of the cell. Not considering that this results in values lower than real for the PMP.
- C. Cells permeabilized with 100  $\mu g$  of chitosan for the total 3.0 mL of the incubation mixture. Chitosan liberates the free dye and that bound remains inside.
- D. Cells with glucose and with the same concentration of chitosan, but after their permeabilization adding 200 mM KCl. This concentration should displace the dye from the anionic sites to which it supposedly binds because of its cationic nature, but requires a large  $K^+$  concentration to be displaced. Then, the remaining dye inside is that due to its hydrophobicity (Table 4).



	Glucose	G + K	Chitosan	Chitosan + K200
[External]	24	85	72	118
nmoles 3.0 mL	71	255	216	355
Entered	379	195	234	95
[Internal]	108,195	55,572	50,554	20,585
[Int]/[Ext]	4551	652		
Log	3.7	2.8		
Apparent $\Delta\psi$ (mV)	-219	-169		

The experiment was conducted as described in **Table 1**, but to those treated with 100  $\mu\text{g}$  of chitosan, after 10 min, 200 mM KCl was added, and after 5 more min, they were centrifuged. Also, in these experiments, a higher acridine yellow concentration (150  $\mu\text{M}$ ) was used. Results from a typical experiment.

**Table 4.** Accumulation values of acridine yellow in yeast cells and calculations to obtain the apparent values of the PMP.

The experiment was conducted as described for **Table 1**, but to those treated with 100  $\mu\text{g}$  of chitosan, after 10 min, 200 mM KCl was added, and after 5 more min, they were centrifuged. Also, in these experiments, a higher acridine yellow concentration (150  $\mu\text{M}$ ) was used, and in all cases, 10  $\mu\text{M}$  CCCP was present. Results from a typical experiment.

The accumulation values using 150  $\mu\text{M}$  acridine yellow concentration were much higher, similar to previous experiments, and values of the PMP without any corrections were -219 and -169 mV, respectively, for the cells incubated only with glucose and with glucose plus 10 mM KCl.

From the values obtained with chitosan, we found that a total concentration of 50,554 nmoles/mL still remained inside the cells, independent from the PMP, presumably bound, both because of the cationic and hydrophobic nature of acridine yellow. The internal concentration, 50,554, was reduced to 20,585 by the addition of 200 mM KCl. This amount remaining in the cells after the addition of chitosan and KCl is that bound due to its hydrophobic nature. The difference (50,554 - 20,585) can be considered that bound because of its cationic nature and is equal to 29,269. All values are given in nmoles/mL.

These results then, allow the following corrections:

#### 1. With glucose.

A. The internal concentration reached was 108,195 nmoles/mL, but from these, 50,554 were bound independently from the PMP. Subtracting the total bound, the PMP-dependent internal concentration should be 57,641 nmoles/mL. The internal/external concentration ratio would then be 2401.7, its log would be 3.38, and the corrected PMP:  $-60 \times 3.38 = -201.8$  mV.

#### 2. With glucose + KCl.

A. The internal concentration after the addition of KCl decreased to 55,572. However, from these, 50,554 - 20,585, or 29,969 nmoles/mL was displaced by the increased internal concentration of  $\text{K}^+$ , reached due to its transport, assumed to be near 200 mM. This means that the

actual internal concentration of KCl plus that displaced would be 85,541 nmoles/mL. Then, the internal/external concentration ratio would be 1006, and the PMP would change to  $-180.2$ .

With these corrections the values obtained in three experiments (means  $\pm$  std. dev) were  $-219.5 \pm 1.8$ , with glucose, and  $-163.3 \pm 1.4$  with glucose plus 10 mM KCl. The values corrected for the binding due to the cationic nature of the dye were  $-205.3 \pm 3.2$  with glucose, and  $-183.2 \pm 3.1$  for glucose plus KCl.

As expected, values with glucose, when corrected for the amount of dye bound because of its cationic nature, are somewhat higher than those shown in **Table 1**. The value with glucose plus KCl is much higher, also as expected, because to the amount of dye remaining inside, that displaced by the large accumulation of  $K^+$  was added.

These results confirm that the PMP values obtained are higher than those suggested by other authors. In fact, only with glucose, corrected values are only around 15 mV higher; in the presence of  $K^+$ , the values are even higher. This in fact is not unexpected, because, although the addition of  $K^+$ , due to its transport mechanism should produce a decrease of the PMP, since our old studies [2, 32] it is known that  $K^+$ , by decreasing the PMP, accelerates the plasma membrane  $H^+$ -ATPase, which transiently increases the ADP levels, but this increase is rapidly compensated by accelerating glycolysis and respiration. This series of events, but mainly the acceleration of proton pumping, should compensate for the PMP decrease produced by the uptake of  $K^+$ . To our knowledge, these measurements with the shown corrections are the most accurate measurements of the PMP in *S. cerevisiae*.

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