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ORIGINAL PAPER

Complementary Methods of Processing diS-C_3(3) Fluorescence Spectra Used for Monitoring the Plasma Membrane Potential of Yeast: Their Pros and Cons

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Abstract Carbocyanine dye diS-C_3(3) was repeatedly employed in monitoring the plasma membrane potential of yeast and other living cells. Four methods of measuring and evaluating probe fluorescence signal were used in different studies, based on following fluorescence parameters: fluorescence intensity emitted within a certain spectral interval, $F(580)/F(560)$ fluorescence emission ratio, wavelength of emission spectrum maximum, and the ratio of respective fluorescence intensities corresponding to the diS-C_3(3) bound to cytosolic macromolecules and remaining dissolved in the aqueous cell medium (i.e., unbound, or free). Here we show that data corresponding to the three latter spectral assessments of diS-C_3(3) accumulation in cells is mutually convertible, which means that their alternative use cannot lead to ambiguities in the interpretation of the results of biological experiments. On the other hand, experiments based on the effortless measurements of fluorescence intensities should be interpreted cautiously because controversial results can be obtained, depending on the particular choice of cell-to-dye concentration ratio and emission wavelength.

Keywords Yeast · *Saccharomyces cerevisiae* · Plasma membrane potential · Fluorescent probe · Spectral analysis

Introduction

3,3′-dipropylthiadicarbocyanine, diS-C_3(3), has been repeatedly used to monitor the plasma membrane potential of living cells, including yeast. This small lipophilic cation is a member of the family of slow (accumulation, or redistribution) fluorescent probes that report on cell membrane potential (negative inside) by their accumulation in the cells. The accumulation of diS-C_3(3) in cells leads to its binding to cytosolic macromolecules (mainly proteins), which is followed by a shift in the emission spectrum maximum towards longer wavelengths, and an increase of its fluorescence quantum yield [1]. Besides a simple measurement of the intensity of fluorescence [2, 3] three different spectroscopic indicators can also be used to track the accumulation of diS-C_3(3) in yeast cells in aqueous suspensions: the wavelength of emission spectrum maximum [4–7], the ratio of fluorescence intensities measured at two different emission wavelengths [8–10], and the ratio of regression coefficients, B/A, obtained by linear unmixing of fluorescence spectra of diS-C_3(3) stained yeast cell suspensions into free- and bound dye fluorescence components [11].

The third option requires comment, since its aim is not only the usual qualitative assessment of whether the cell membrane potential is high or low. The spectral unmixing means the fitting of experimental spectra F(λ) to the following equation:

$$F(\lambda) = AF_F(\lambda) + BF_B(\lambda) \quad (1)$$

where $F_F(\lambda)$ and $F_B(\lambda)$ are the peak-height normalized spectra of free (dissolved in aqueous cell medium) and bound (to cytosolic macromolecules) forms of the dye, respectively, A and B are the corresponding fractions of these spectra in the overall spectrum F(λ), [1]. Based on the theory presented in [11], the ratio of these regression coefficients, B/A, can report on changes in membrane potential in an absolute scale (mV). In brief, the possibility of assessment of membrane potential changes of yeast cells is derived from the assumptions that (i) the ratio of respective diS-C_3(3) fluorescence intensities of the dye accumulated in the cells and that in their aqueous surrounding is directly proportional to the ratio of intra- to extracellular dye concentrations, and (ii) the distribution of

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the dye follows the Nernst equation. By means of the linear unmixing of synchronously scanned diS-C_3(3) fluorescence spectra it is possible to assess the amount of dye accumulated in cells without otherwise necessary sample taking and following separation of cells from the medium. For a detailed description of the principles underlying the methodology see [11].

Over time we learned that some yeast researchers consider the multiplicity of spectroscopic indicators associated with the application of a single fluorescent probe of cell membrane potential to be a drawback that hampers making overall conclusions from different studies. Here we show that alternative data on the diS-C_3(3) accumulation in cells obtained as either i) the wavelength of emission spectrum maximum λ_{max}, ii) the ratio of fluorescence intensities measured at two different emission wavelengths, or iii) the ratio of regression coefficients, B/A, is mutually convertible. This means that any alternative use of these spectroscopic indicators of the accumulation of diS-C_3(3) in cells cannot lead to ambiguities in the interpretation of the results of biological experiments.

To prove this fact, we started with numerical simulations of an explicit relationship between B/A ratio, λ_{max}, and the ratio of fluorescence intensities measured at two different emission wavelengths (λ_{em}=560 and 580 nm) using a) Eq. 1, b) model diS-C_3(3) fluorescence emission spectra shown in Fig. 1, and c) B/A values ranging from 0.1 to 10. With these simulations we were able to determine the useful range of the probe accumulation in the cells (quantified in terms of B/A values) for which both the λ_{max} and the emission ratio can be considered reliable and sensitive indicators of the level of dye accumulation in cells. Furthermore, the conclusions based on the results of these numerical simulations were tested in monitoring the depolarization of yeast plasma membranes induced by increasing the concentration of extracellular potassium in the cell suspensions. This study was carried out with the pump deficient mutant strain AD1-3 of *Saccharomyces cerevisiae*.

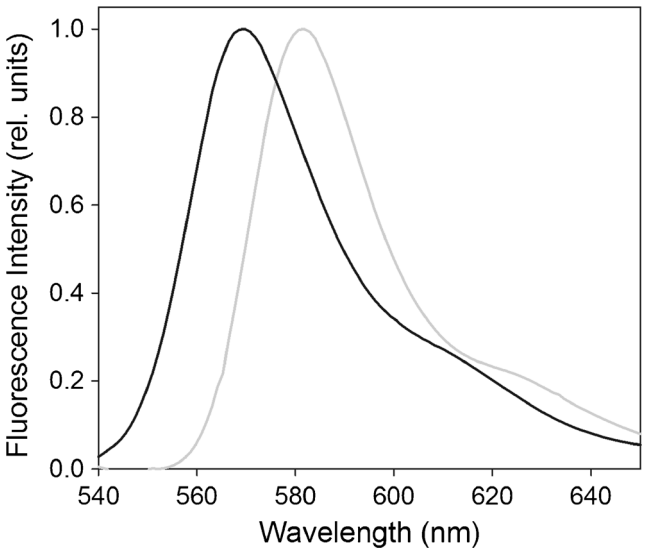

Fig. 1 Peak-height normalized emission spectra of diS-C_3(3) fluorescence. Black line - free form of dye in MES-TEA buffer; grey line—protein-bound form of dye accumulated in cells

Materials and Methods

Chemicals

Fluorescent probe diS-C_3(3), 3-propyl-2-[3-[3-propyl-2(3H)benzothiazolylidene]-1-propenyl]benzothiazolium iodide (often abbreviated as 3,3′-dipropylthiadicarbocyanine iodide), CAS no. 53336-12-2, was purchased from Fluka, and added to cell suspensions from a 20 μM stock solution in ethyl alcohol for UV spectroscopy (Lach-Ner, Czech Republic). D-glucose was purchased from Penta (Czech Republic); potassium chloride, choline chloride, peptone, MES hydrate and triethanolamine (TEA) were obtained from Sigma-Aldrich. All these compounds were of p.a. quality, except of MES hydrate (Biotech. Performance Certified) and TEA (Ultra, >99.5 %). Yeast extract was purchased from Serva (Heidelberg, Germany), and bactopeptone from Oxoid (Brno, Czech Republic).

Yeast Strain and Cell Culturing

Pump deficient *S. cerevisiae* strain AD1-3 (*MATα, PDR1-3, ura3, his1, yor1Δ::hisG, snq2Δ::hisG, pdr5Δ::hisG*), which is derived from the parent strain US 50-18C (*MATα, PDR1-3, ura3, his1*), was used in this study [12]. Yeast was precultured in YPD medium (1 % yeast extract, 1 % bactopeptone, 2 % glucose) at 30 °C for 24 h. A small volume (2–10 μl) of inoculum was added to 10 ml fresh YPD medium and the main culture was grown until it had reached the desired phase of exponential growth.

Sample Preparation

Cells were harvested and washed twice by centrifugation, first with distilled water and then with MES-TEA buffer (25 mM MES, pH 6.2). Series of samples in MES-TEA buffers containing required concentrations of K^+ (0.1, 1, 10 and 150 mM) were prepared. In the K^+ titration experiments, choline chloride was added to the cell suspensions together with KCl to maintain constant both the ionic strength and extracellular Cl^- concentration. After adding cells to a buffer (to OD ≈ 0.1, 0.2, or 0.4 at 580 nm) each sample was hand-shaken and left in this medium for 2 min before staining with diS-C_3(3), which was added to the final concentration of 40 or 200 nM.

The titration experiment was repeated four times, always with fresh cells from a new cultivation flask. Three titration series were measured with the diS-C_3(3) concentration of

40 nM and different cell concentrations (OD ≈ 0.1, 0.2, and 0.4), the remaining one with 200 nM diS-C_3(3) and OD ≈ 0.2. For each titration series the samples were prepared in duplicates, designed for a parallel analysis of diS-C_3(3) fluorescence response to K^+-induced changes of yeast plasma membrane potential using usual emission spectra and synchronously-scanned fluorescence (SSF).

Fluorescence Measurements

Samples of cell suspensions were prepared and their fluorescence measured in disposable 1×1 cm UV-grade cuvettes (Kartell, Italy). The autofluorescence spectrum of each sample was measured before adding diS-C_3(3). Upon adding the dye (to the final concentration of 40 or 200 nM), the samples were left for 20 min under occasional hand-shaking to allow dye equilibration across the plasma membrane. Then the fluorescence spectra of the stained cell suspensions were measured either using usual emission spectroscopy or the SSF technique.

Emission Spectroscopy

Fluorescence emission spectra were measured with a Fluoromax-3 spectrofluorometer (Jobin-Yvon Horiba) using the following settings: λ_{exc}=530 nm, excitation and emission slit widths were 5 and 2 nm, respectively, 1 nm scanning step, and 0.75 s integration time. Smoothed (using the SigmaPlot 11 bisquare algorithm) differential spectra, $F(\lambda_{n+2}) - F(\lambda_n)$, were used for a precise determination of λ_{max}. The $F(580)/F(560)$ ratio was calculated using the means of $F(\lambda)$ values found in three neighbouring data points (560 ± 1 nm and 580 ± 1 nm).

A pure emission spectrum of bound dye fluorescence was assessed using the method of trial and errors, which is based on subtracting a properly weighted free-dye spectrum measured in a pure aqueous solution from the diS-C_3(3) fluorescence spectra measured in a few cell suspensions [1, 13]. We used for this purpose the fluorescence emission spectrum measured with 200 nM diS-C_3(3) solution in MES-TEA buffer and the series of emission spectra found in the K^+ depolarization experiment performed with yeast cell suspensions (OD ≈ 0.2) stained by 200 nM diS-C_3(3).

Synchronously-Scanned Fluorescence (SSF)

The SSF spectra of the stained cell suspensions were measured as described in [11], also using the Fluoromax-3 spectrofluorometer. The offset between λ_{exc} and λ_{em}, was set to 13 nm, and slit widths to 2.3 nm. With 5 nm large steps and λ_{exc} spectral range from 520 to 590 nm measured SFS spectra comprised only 15 spectral data points. With such a small number of spectral points and the integration time set to 0.5 s, a single SSF spectrum was measured in less than 10 s, and the average of seven spectra in 75 s, which is fast enough for monitoring membrane potentials in multiple samples in extensive titration experiments. The advantage of averaging the repeatedly measured spectra, instead of the more common use of longer integration times, consists in the reduction of the statistical weight of sudden fluctuations of fluorescence intensity caused by tiny bubbles of dissolved air released from cell suspensions. In this way we were able to significantly reduce the incidence of flawed points in the titration curves.

This low number of data points was sufficient for the spectral unmixing of experimental SSF spectra, using the Nonlinear Regression module of SigmaPlot 11 software (Systat Software Inc., USA), and thus for the determination of the ratio of bound-to-free fluorescence intensities (B/A). The values of B/A ratio obtained by analyzing equilibrium diS-C_3(3) fluorescence spectra measured under defined experimental conditions in yeast suspension were finally converted into underlying membrane potential differences in the scale of millivolts. For a detailed description of this procedure see [11].

Results and Discussion

Three Spectroscopic Indicators of diS-C_3(3) Accumulation in Cells are Mutually Convertible

As presented in the Introduction, the accumulation of diS-C_3(3) in cells is indicated by an increase of the bound dye fluorescence component in the overall fluorescence spectra of cell suspensions. Thus the ratio of linear regression coefficients B/A, which can be obtained by linear unmixing of the measured overall spectra using Eq. 1, is the most straightforward measure of the extent of dye redistribution between the cells and their aqueous medium. However, the other two spectroscopic markers of the diS-C_3(3) accumulation in cells, i.e., the ratio of fluorescence intensities measured at two different emission wavelengths and the wavelength of emission spectrum maximum λ_{max}, can be explicitly related to the B/A ratio.

For the ratio of fluorescence intensities it is even possible to derive a simple mathematical formula describing this relationship. Let $F(\lambda)$, $F_F(\lambda)$ and $F_B(\lambda)$ are fluorescence spectra defined when introducing Eq. 1; λ_{Fmax}, λ_{Bmax} and λ_{max} are the wavelengths of emission maxima corresponding to the free- and bound- dye fluorescence, and overall fluorescence of cell suspensions, respectively. Based on Eq. 1, we can express fluorescence intensities measured at two different wavelengths, e.g., 560 and 580 nm used by Sychrova et al. [10], as follows:

$$F(580) = A\,F_F(580) + B\,F_B(580) \tag{2a}$$

$$F(560) = A\,F_F(560) + B\,F_B(560) \tag{2b}$$

Using elementary algebra we then derive a formula unambiguously relating the $F(580)/F(560)$ emission ratio to the corresponding B/A value:

$$\frac{F(580)}{F(560)} = \frac{F_F(580) + F_B(580)B/A}{F_F(560) + F_B(560)B/A} \quad (3)$$

The relationship between λ_{max} and B/A ratio is less straightforward and thus no simple formula relating λ_{max} to λ_{Fmax}, λ_{Bmax}, and B/A ratio can be derived directly from Eq. 1. However, overall emission spectra can easily be calculated with this equation for a range of B/A values. Finally, their λ_{max} wavelengths can be assessed, and the relationship between λ_{max} and B/A ratio presented in a graphical form, as a λ_{max} vs B/A plot.

Fluorescence Emission Spectra of Free- and Bound diS-C_3(3)

Peak-height normalized emission spectra of diS-C_3(3) fluorescence shown in Fig. 1 were obtained as described in Materials and Methods. These spectra are not corrected for the spectral sensitivity of fluorometer, because such a correction is not essential for understanding the fluorescent probe response to variations of cell membrane potential. Compared to the fluorescence of the free form of dye in MES-TEA buffer, the emission spectrum of the protein-bound form of dye accumulated in cells is shifted by 12.5 nm towards longer wavelengths, while these two spectra exhibit nearly identical shapes (except for a moderate decrease of FWHM of the bound form spectrum, ≈ 3 nm). A similar shape of these spectra and the substantial spectral shift between them explain why the redistribution of diS-C_3(3) from aqueous media into the cells is accompanied by significant changes of λ_{max} and $F(580)/F(560)$ emission ratios.

Explicit Relationships Between F(580)/ F(560), λ_{max}, and B/A

The $F(580)/F(560)$ fluorescence emission ratios and λ_{max} were calculated as described in Materials and Methods, for the range of B/A values from 0.1 to 10. This range covers practically all situations found in real experiments. The results were plotted as $F(580)/F(560)$ vs $\log(B/A)$ and λ_{max} vs $\log(B/A)$ graphs. The semi-logarithmic plots were chosen because the difference between logarithms of certain $(B/A)_1$ and $(B/A)_2$ values measured under two different conditions may be converted to the difference (in millivolts) of underlying membrane potentials, $\Delta\Psi_1$ and $\Delta\Psi_2$, if certain requirements concerning the dye concentration and other specific details of experimental protocol are matched (for details see [11]). Then it holds that

$$\Delta\Psi_2 - \Delta\Psi_1 = 59 \log(B/A)_2 - 59 \log(B/A)_1 \quad (4)$$

In Fig. 2 the relationship between $F(580)/F(560)$ and $\log(B/A)$ is shown, which was calculated using Eq. 3, and four fluorescence intensities found in the $F_F(\lambda)$ and $F_B(\lambda)$ spectra. Using data shown in Fig. 1 we got $F_F(560)=0.69$, $F_F(580)=0.76$, $F_B(560)=0.058$, $F_B(580)=0.99$. Considering the above mentioned possibility to convert $\log(B/A)$ values to differences in membrane potentials, the semi-logarithmic plot shown in Fig. 2 reveals that the $F(580)/F(560)$ emission ratio is a highly non-linear indicator of real membrane potentials if its apparent values are small. In particular, for the uncorrected emission spectra of diS-C_3(3) fluorescence measured with Fluoromax 3 spectrofluorometer and slit settings as specified above, this happens for $F(580)/F(560)<3$. Such condition is likely to take place when dye and cells are used at low dye-to-cell concentration ratios. Then the ratiometric approach may fail to report reliably about true differences between the cell membrane potentials of various samples. Furthermore, data shown in Fig. 2 indicates that the ratiometric method can be considerably improved by either increasing the concentration of cells in suspensions, or lowering dye concentrations, thus shifting the resultant B/A ratio towards higher values where the dependence of $F(580)/F(560)$ ratio on $\log(B/A)$ is expected to be roughly linear.

The relationship between λ_{max} and $\log(B/A)$ is shown in Fig. 3. As the use of $F(580)/F(560)$ and λ_{max} for the qualitative assessment of membrane potentials is concerned, it is clear from Figs. 2 and 3 that the dependence of λ_{max} on $\log(B/A)$ value is more uniform than that of the $F(580)/$

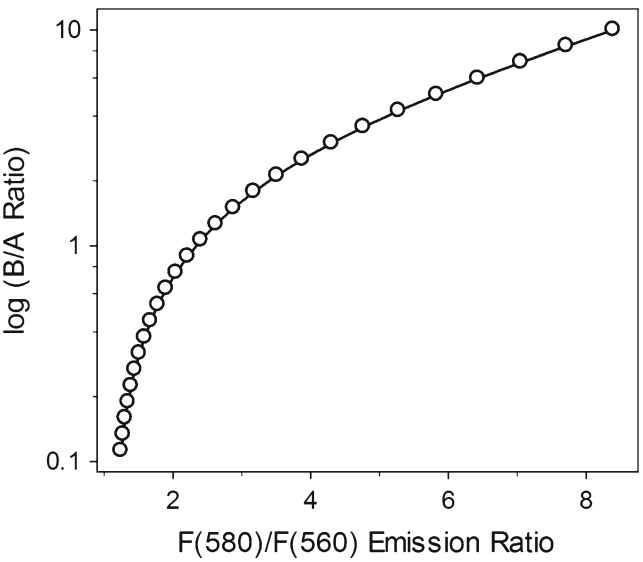

Fig. 2 Semi-logarithmic plot of B/A ratio vs $F(580)/F(560)$ emission ratio. Data calculated using Eq. 3

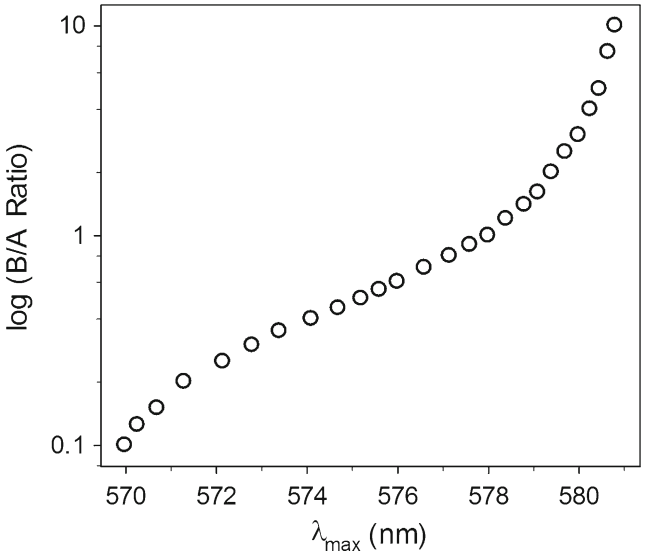

Fig. 3 Semi-logarithmic plot of B/A ratio vs λ_{max} of diS-C_3(3) fluorescence emission spectra. Circles represent λ_{max} values computed for the set of B/A values ranging from 0.1 to 10 using Eq. 1 and model spectra presented in Fig. 1

$F(560)$ emission ratio. Specifically, λ_{max} appears to be more suitable for monitoring plasma membrane potential of diS-C_3(3) stained cells in suspensions than the $F(580)/F(560)$ emission ratio if the apparent λ_{max} values occur within a "linear-response" range, from about 572 to 579 nm. On the other hand, the measurement of λ_{max} is no longer appropriate in dense suspensions characterized by a high proportion of bound dye fluorescence. Once λ_{max} is closer to 580 nm, it ceases to be a sensitive indicator of cell membrane potential. Under this condition, the measurement of $F(580)/F(560)$ emission ratio may, theoretically, be preferable. In practice, however, this option would be complicated, because of the strong light scattering by the dense cell suspensions.

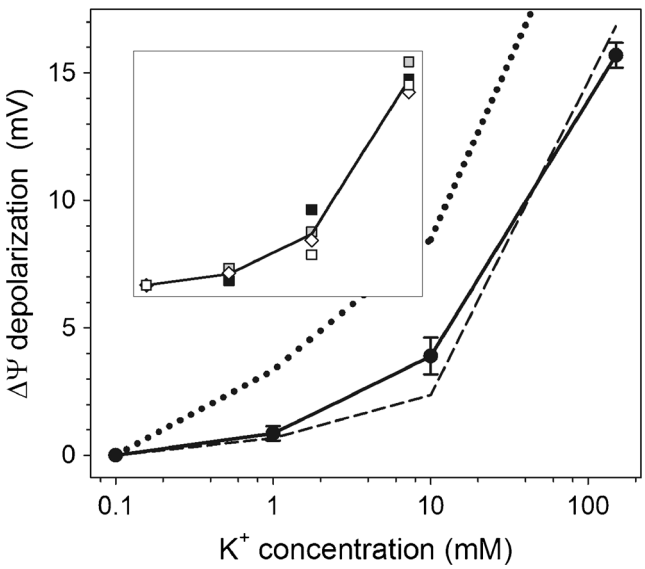

Fig. 4 Depolarization of yeast cells caused by increasing concentrations of extracellular K^+. Thick solid line and black circles represent the mean of four independent experiments, based on the B/A ratio assessment from SSF spectra. In the insert individual data are shown for particular experiments performed with the following cell suspensions: diS-C_3(3) concentration 40 nM and OD ≈ 0.1—empty squares, 0.2—grey squares, and 0.4—black squares; diS-C_3(3) concentration 200 nM and OD ≈ 0.2—empty diamonds. Dashed line represents the assessment of cell depolarization based on λ_{max} shift and dotted line is the assessment of depolarization based on $F(580)/F(560)$ emission ratio (both measured in the cell suspension of OD ≈ 0.1 stained with 40 nM diS-C_3(3))

Depolarization of Yeast Cells Induced by Increasing the Concentration of Extracellular Potassium

The suitability of different methods of diS-C_3(3) fluorescence processing as tools for the monitoring of cell membrane potential was tested based on the model of yeast depolarization caused by increasing concentration of extracellular potassium, $[K^+]_{out}$. The extent of this depolarization, as assessed using B/A ratios of regression coefficients, is shown in Fig. 4. Here the unknown membrane potential of cells suspended in a buffer with $[K^+]_{out}$=0.1 mM was chosen as the reference value against which we measure the size of the K^+-induced depolarization. Therefore, the membrane potential changes are plotted as positive values to reflect the trivial fact that the membrane potential changed from highly negative to less negative values, going from 0.1 to 150 mM $[K^+]_{out}$. The standard errors of the mean of four independent experiments carried out with various dye-to-cell concentrations (see the legend to Fig. 4) clearly illustrate the reproducibility, and thus reliability of the method based on linear unmixing of synchronously scanned diS-C_3(3) fluorescence spectra. As the physiological aspect of this result is concerned, we may conclude that the observed effect is consistent with findings published in our recent paper [14].

Since the linear unmixing of $F(\lambda)$ directly indicates the changes in the ratio of extracellular-to-intracellular diS-C_3(3) concentration [11], the data shown in Fig. 4 can be used as a benchmark for the evaluation of the results obtained with the remaining three methods. Not surprisingly, the values of both the $F(580)/F(560)$ emission ratio and λ_{max} increased considerably with increasing the cell-to-dye concentration ratio of cell suspensions used in four independent experiments, Figs. 5 and 6. However, the most important conclusion which can be obtained from these results concerns the routine use of experimental $F(580)/F(560)$ and λ_{max} values as qualitative indicators of differences between membrane potentials of yeast cells. Because of different shapes of apparent curves, the variations in membrane potential can easily be overestimated or underestimated, depending on the particular choice of the cell-to-dye concentration ratio. To illustrate this fact, we converted to B/A ratios the $F(580)/F(560)$ and λ_{max} values, measured in the titration experiment carried out with the cell suspensions of OD ≈ 0.1, stained with 40 nM diS-C_3(3), i.e. under the conditions when λ_{max} values occur within a "linear-

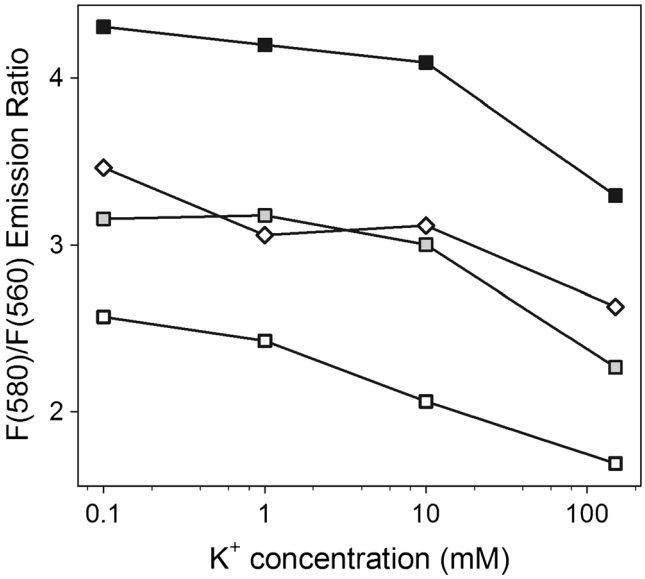

Fig. 5 $F(580)/F(560)$ emission ratio is used to indicate the depolarization of yeast cells. Four independent experiments were performed with the following parameters of cell suspensions: diS-$C_3(3)$ concentration 40 nM, and OD ≈ 0.1—empty squares, OD ≈ 0.2—grey squares, OD ≈ 0.4—black squares; diS-$C_3(3)$ concentration 200 nM, and OD ≈ 0.2—empty diamonds

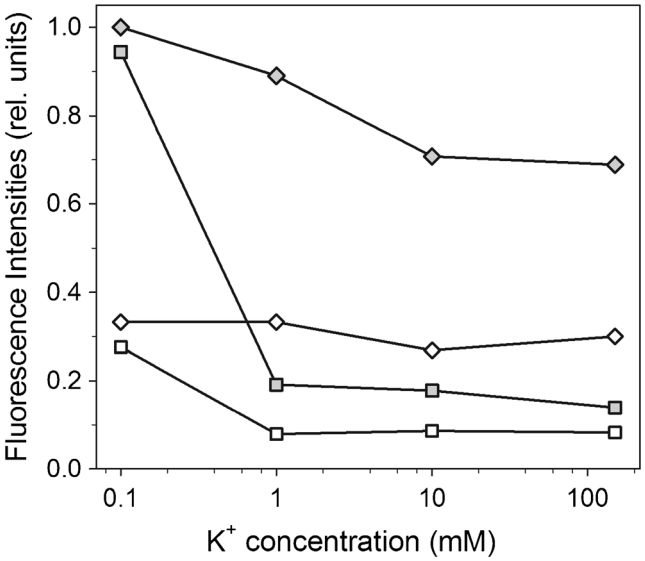

Fig. 7 Fluorescence intensities measured at emission wavelengths of 560 and 580 nm are used to indicate the depolarization of yeast cells. Data are shown for two independent experiments: diS-$C_3(3)$ concentration of 40 nM and OD ≈ 0.1—squares, diS-$C_3(3)$ concentration of 200 nM and OD ≈ 0.2—diamonds. Empty and grey symbols represent λ_{em}=560 and 580 nm, respectively

response" range of 572−579 nm. Then we used these B/A values for the estimation of underlying membrane potential changes, see Fig. 4. Using λ_{max} the assessed extent of cell depolarization was nearly identical to the above benchmark, based on the linear unmixing of SSF spectra. This result demonstrates that the easy measurement of λ_{max} can also provide reliable information on cell membrane potential, On the contrary, the values corresponding $F(580)/F(560)$ ratio were close to the non-linear part of the semi-logarithmic plot shown in Fig. 4, and thus producing the overestimated extent of cell depolarization.

Finally, we show in Fig. 7 an example of using the effortless measurements of fluorescence intensities as indicators of membrane potential differences. No doubt, a high risk of artefact is inherent to this simple experimental approach.

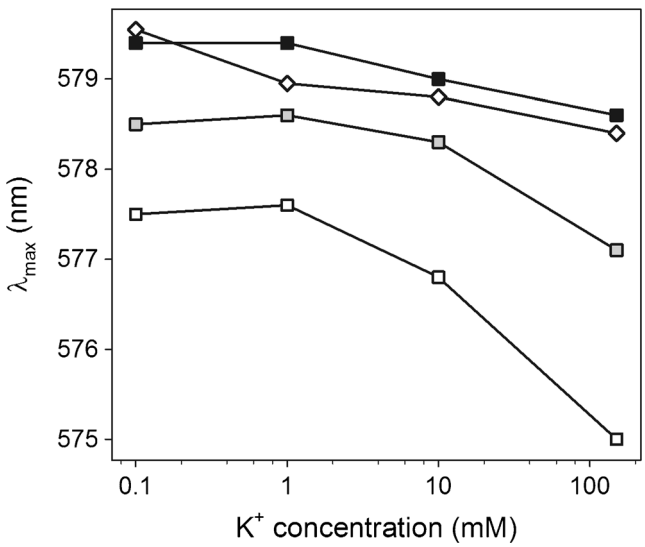

Fig. 6 λ_{max} is used to indicate the depolarization of yeast cells. Four independent experiments were performed with the following parameters of cell suspensions: diS-$C_3(3)$ concentration 40 nM, and OD ≈ 0.1—empty squares, OD ≈ 0.2—grey squares, OD ≈ 0.4—black squares; diS-$C_3(3)$ concentration 200 nM, and OD ≈ 0.2—empty diamonds

Conclusions

The theoretical examination and numerical simulations of emission spectra demonstrated that an unambiguous relationship exists between the wavelength of emission spectrum maximum of diS-$C_3(3)$ fluorescence, the ratio of fluorescence intensities measured at two different emission wavelengths, and the B/A ratio of regression coefficients. Hence these three different characteristics of diS-$C_3(3)$ emission spectra are mutually convertible. Therefore, their alternative use as spectroscopic indicators of the accumulation of diS-$C_3(3)$ in cells cannot lead to ambiguities in the interpretation of the results of biological experiments aimed at monitoring cell membrane potential. However, we have also shown that when the apparent values of both $F(580)/F(560)$ or λ_{max} are out of a specified range, their use in monitoring of cell membrane potential may be ambiguous.

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