J Fluoresc (2012) 22:1183–1188
DOI 10.1007/s10895-012-1058-9

ORIGINAL PAPER

Fluorescence Emission Spectra of Calcofluor Stained Yeast Cell Suspensions: Heuristic Assessment of Basis Spectra for their Linear Unmixing

Jaromír Plášek · Marek Dostál · Dana Gášková

Received: 17 December 2011 / Accepted: 4 April 2012 / Published online: 27 April 2012

Abstract Fluorescence emission spectra of yeast cell suspensions stained with calcofluor have recently been identified as promising markers of variations in the quality of yeast cell wall. It is shown in this paper how the raw fluorescence spectra of calcofluor can be transformed to reliable spectral signatures of cell wall quality, which are independent of actual dye-to-cell concentrations of examined cell suspensions. Moreover, the presented approach makes it possible to assess basis fluorescence spectra that allows for the spectral unmixing of raw fluorescence spectra in terms of respective fluorescence contributions of calcofluor solvated in the suspension medium and bound to yeast cell walls.

Keywords Yeast cell wall · Calcofluor · Fluorescence · Spectral unmixing

Introduction

In numerous binding assays using fluorescent substances, as well as in assays dealing with various cells that can accumulate fluorescent probes, it can be assumed that two distinct dye forms coexist in examined samples: (i) the dye solvated in the aqueous medium, and (ii) the dye bound to various binding sites found either on macromolecules in solutions or inside whole cells. Under such condition measured fluorescence emission spectra $F(\lambda)$ can be expressed in terms of the following linear combination:

$$F(\lambda) = AF_W(\lambda) + BF_B(\lambda), \quad (1)$$

where F_W (λ) and F_B (λ) are basis spectra (also called reference spectra) of solvated and bound forms of the fluorescent dye, respectively, A and B are the corresponding contributions of these basis spectra to an experimental spectrum $F(\lambda)$. Spectral mixing models that use Eq. 1 and fraction coefficients to specify how much fluorescence from each emitting species is present in $F(\lambda)$ require obviously an a priori knowledge of the basis spectra. Once the these spectra are known, any individual F (λ) spectrum measured under different experimental conditions can be then easily analyzed using linear unmixing [1–4], which means fitting $F(\lambda)$ with Eq. 1.

With both the aqueous macromolecular solutions and cell suspensions, the search for the basis spectra is partly facilitated by the trivial fact that F_W (λ) can easily be measured using pure aqueous dye solutions. Unfortunately, no suitable reference specimens representing pure F_B (λ) spectra are usually available. If the basis spectra required for the unmixing algorithms cannot be measured directly with reference specimens, the decomposition of complex spectra into the sum of their components turns to be a quite difficult task, and sophisticated statistical methods must be used. In particular, Parallel Factor Analysis that requires excitation–emission matrix fluorescence spectra measured at several different experimental conditions [5–8] was successfully used to resolve the excitation and emission profiles of various mixtures of fluorochromes, such as, for example, the solvated and complexed forms of Nile Red [9], four main fluorescence components in red wines [10], or three fluoroquinolone anitibiotics in human serum [11].

J. Plášek (✉) · M. Dostál · D. Gášková
Faculty of Mathematics and Physics, Charles University,
Ke Karlovu 3,
12116 Prague, Czech Republic
e-mail: plasek@karlov.mff.cuni.cz

Another advanced method is Non-Negative Matrix Factorization [12–14].

An obvious drawback of advanced statistical methods is the need for special software and skilled staff with expertise in this rather specific field of data analysis. These requirements appear to be unreasonably demanding in view of the nature of simple binding experiments that result only in two distinct emission components, one of them being the fluorescence of solvated dye the emission spectrum of which is exactly known. In our earlier membrane potential studies based on the use of fluorescent dye 3,3′-dipropylthiacarbocyanine iodide, diS-C_3(3), which can be used for monitoring cell membrane potential by its voltage-dependent partition between the extracellular medium and the cells, we found the reference fluorescence spectrum of intracellular bound dye by a simple trial and error method. This method has been based on subtracting properly weighted free-dye spectra F_W (λ) from complex F(λ) spectra measured in several cell suspensions of different dye-to-cell concentrations [15, 16]. Unfortunately, this simple procedure failed in the case of calcofluor spectroscopy, which we intend to use as a spectral signature of yeast cell wall quality [17]. In this paper we present another heuristic procedure capable of providing basis spectra of calcofluor bound to yeast cell walls.

Experimental

Materials

Calcofluor (Fluorescent Brightener 28, Product # F3543), chitin from crab shells, disodium phosphate and peptone, were from Sigma-Aldrich; citric acid and D-glucose from Penta (Czech Republic); yeast extract from Fluka. All these chemicals were of p.a. quality, except of peptone (Cell Culture Tested) and chitin (practical grade).

Yeast Strains and Cell Culturing

A conventional yeast *S. cerevisiae* BY4741, ATCC#201388 (MATα, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0) has been used. Yeast was pre-cultured in YEPG medium (1 % yeast extract, 1 % bactopeptone, 2 % glucose) at 30°C on a reciprocal shaker for 24 h. A volume of 150 μl inoculum was added to 20 ml fresh YEPG medium and the main culture was grown for another 20 h. Cells were harvested and washed twice by centrifugation, first with distilled water and then in 10 mM citrate-phosphate buffer (pH 6.0). Before staining with calcofluor, they were resuspended in CP buffer to the final density (OD=0.16 at 578 nm). In experiments concerning the effect of the cell wall on the fluorescence emission spectra of calcofluor, the cell wall was modified by batch-culturing the yeast under the above conditions in a low glucose (0.2 %) growth medium.

Fluorescence Spectroscopy

A stock solution of calcofluor (10^{-2} M) was prepared with distilled water of pH tuned to 10.9 using NaOH, under which condition an electrostatic repulsion between ionized calcofluor molecules boosts their solubility. Calcofluor was added to yeast cell suspensions (cell density OD=0.16) to a final concentration ranging from 2 to 10 μM. The binding of calcofluor to yeast cell walls has been practically immediate. The cuvettes with stained cell suspensions were gently shaken by hand before measuring fluorescence emission. Fluorescence spectroscopy has been performed with Fluoromax 3 (Horiba Jobin-Yvon, USA) using disposable 1× 1 cm UV grade fluorimetric cuvettes (Kartell spa, Italy). The bandwidths used for the excitation and the emission were 3 nm and 1 nm, respectively. Fluorescence emission spectra of calcofluor solution were measured with λ_{exc}=365 nm, which choice matches a typical excitation mode used in fluorescence microscopy of calcofluor stained microorganisms. Calcofluor fluorescence emission spectra of cells suspended in CP buffer were also compared with reference spectra of 10 μM calcofluor in chitin solutions (1 %w/v) in CP buffer.

Results and Discussion

Theoretical Basis of the Heuristic Assessment of Reference Spectra in Two-Component Mixtures of Fluorochromes

In cases where the use of Eq. 1 is fully justified but the difference between the reference spectra F_W (λ) and F_B (λ) is so small that suitable reference specimens cannot be obtained, an alternative spectral signature of calcofluor binding to yeast cell walls can be conveniently defined as the $F(\lambda)/F_W$ (λ) ratio, which is capable of reporting the contribution of bound dye fluorescence to the overall spectra of calcofluor stained yeast cell suspensions. To support this claim we will recast Eq. 1 to a following expression:

$$\frac{F(\lambda)}{F_W(\lambda)} = A + B\frac{F_B(\lambda)}{F_W(\lambda)}, \tag{2a}$$

or

$$R(\lambda) = A + BR_B(\lambda), \tag{2b}$$

where $R(\lambda)=F(\lambda)/F_W$ (λ), and R_B (λ)=F_B (λ)/F_W (λ). In this way any series of complex spectra measured at several different concentrations of both the fluorescent dye and its binding-sites can be converted to a new set of spectral

profiles that differ only in the actual values of A and B coefficients. To simplify future communication, we will introduce a nickname C/Q spectra for this kind of spectral ratios. In this acronym the letters C and Q correspond to the adjectives **C**omplex and a**Q**ueous, which refer to the $F(\lambda)$ and F_W (λ) fluorescence emission spectra of calcofluor, respectively.

Calcofluor Fluorescence Emission Spectra in the Series of Yeast Cell Suspensions of Various Dye-to-Cell Concentration Ratios

Fluorescence emission spectra of calcofluor measured in series of yeast cell suspensions of constant cell density (OD=0.16) and various dye-to-cell concentrations are shown in Fig. 1A. For the two highest dye concentrations (8 and 10 μM) the fluorescence emission spectra of calcofluor stained cell suspensions are very close to those of pure aqueous calcofluor solutions. Lowering the dye concentration is followed by an increase of relative spectral intensity within the long wavelength part of the emission spectrum. However, it is very difficult to trace any clear regularity in the difference between individual spectra corresponding to various dye concentrations, apart from a finding that the interaction of calcofluor with yeast cell walls is followed by moderate shifts of its emission spectrum towards longer wavelengths.

These complicated and seemingly random spectral variations amongst individual samples become clearer if we move from the ordinary emission spectra to their related C/Q spectra, Fig. 1B. In particular, the C/Q spectra exhibit a regular behaviour in a short valley-like sector around λ_{em}=380 nm, where C/Q values are nearly constant and goes up with increasing calcofluor concentration in the cell suspension. The emission spectra of calcofluor-stained yeast cell suspensions exhibit a moderate shift towards longer wavelengths compared to the fluorescence of pure aqueous calcofluor solutions. Owing to this feature it is possible to identify the C/Q value measured within the flat sector around λ_{em}=380 nm with a net contribution of the constant factor A satisfying Eq. 2b. Within the framework of this model, the observed growth of A value is consistent with a trivial fact that the concentration of free dye in cell suspensions increases under this condition.

The robustness of the determination of C/Q spectra, and thus the potential for their use as reliable spectral signatures of yeast cell wall quality was tested by assessing the difference between several spectra measured at different dye-to-cell concentrations. For this purpose we have calculated first the mean of all the particular C/Q spectra: $R^*(\lambda)=\Sigma\, R_j(\lambda)/n=R^*(\lambda)$, where $R_j(\lambda)$ represents j-th C/Q spectrum, n is the number of averaged spectra, and $R^*(\lambda)$ is their mean. Then particular C/Q spectra were fitted to their mean by nonlinear least squares method using a fit equation $R_j(\lambda)=a_j+b_jR^*(\lambda)$. For all curves shown in Fig. 1B very good fits were obtained with correlation coefficients r>0.99. Using the calculated fit

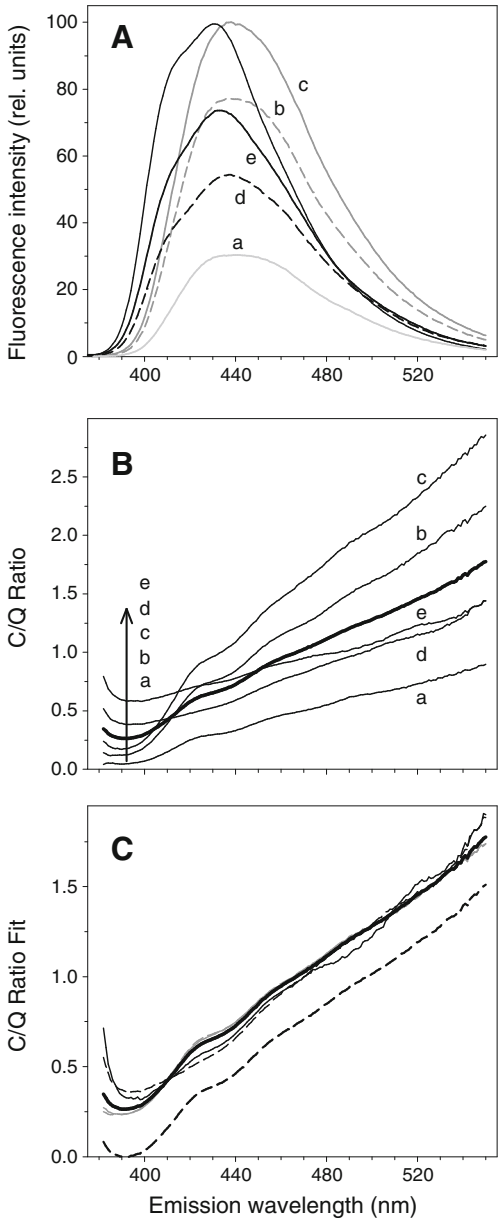

Fig. 1 Raw calcofluor fluorescence emission spectra and resultant C/Q spectra measured in the series of yeast cell suspensions in distilled water using various calcofluor concentrations (wild type yeast grown in HG medium; cell suspension density OD=0.16). **A**) Raw calcofluor fluorescence spectra: curves a) to e) – dye concentrations 2, 4, 6, 8, and 10 μM; full thin line with no index - calcofluor solution in distilled water (pH 7, dye concentration 10 μM). **B**) C/Q spectra corresponding to the above calcofluor fluorescence emission spectra; full thick line – the mean of measured C/Q spectra. **C**) Least squares fitting of measured C/Q spectra to the mean value curve: full thick line – the mean of measured C/Q spectra; thin lines – individual C/Q spectra transformed using the fit parameters; full dashed line – the mean of C/Q spectra after the subtraction of a constant term

parameters a_j and b_j, the original curves were normalized and then compared with their mean to demonstrate the similarity of C/Q curves measured at different dye-to-cell concentrations, Fig. 1C. When plotted together with R*(λ), they exhibit very low scatter around the mean curve. Increased fluctuations of the data in the peripheries of C/Q curves are a trivial consequence that F_W (λ) values are close to zero both in the onset and tail of the spectrum, and thus signal-to-noise ratio drops significantly in these spectral sectors.

If this constant factor A, which is identified with the flat sector of C/Q curves around λ_{em}=380 nm, is subtracted from the C/Q mean spectrum, see Fig. 1C, we get a corrected C/Q spectrum corresponding to A≈0. This corrected spectrum can finally be used to calculate the basis spectrum of calcofluor bound to yeast cell walls by multiplying it with F_W (λ) spectrum. However, the C/Q spectrum itself can still be used as a spectral signature of the dye binding. The clear advantage of using these spectra consists in independence of their shapes on dye-to-cell concentration ratio.

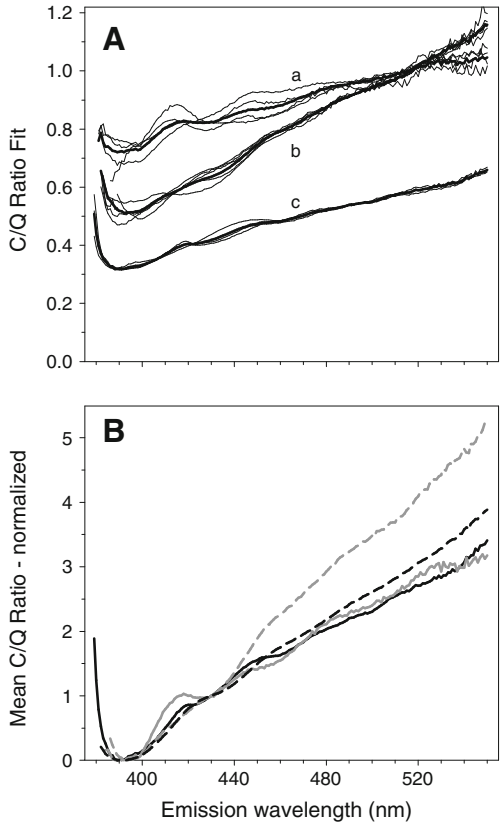

Fig. 2 The illustration of spectral fingerprinting quality of C/Q spectra measured in LG and HG yeast cells. **A**) The illustration of reproducibility of C/Q spectra measurements: a,b) yeast cells grown in the LG medium and suspended in CP buffer and distilled water, respectively, c) yeast cells grown in the HG medium and suspended in CP buffer; cell suspension OD=0.16, thick full lines – means of C/Q spectra, thin full lines – individual C/Q spectra fitted to their means. **B**) The difference between LG and HG cells, as revealed by C/Q spectra of calcofluor-stained cell suspensions: LG cells – black lines, HG cells – grey lines; cells suspensions in CP buffer (*full lines*) and distilled water (*dashed lines*)

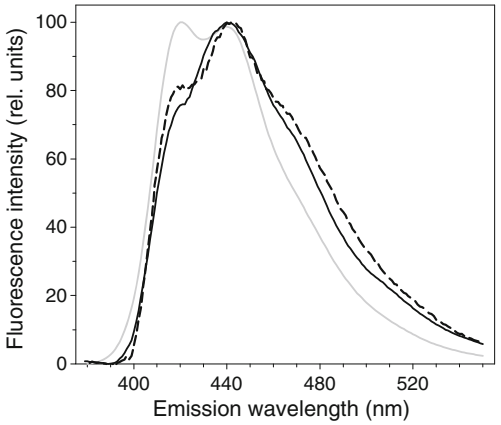

Fig. 3 Basis fluorescence emission spectra of calcofluor bound within the cell walls of yeast cells suspended in CP buffer: black full line – cells grown in HG medium; black dashed line – cells grown in LG medium; grey line – fluorescence emission spectra of calcofluor stained chitin solutions

The Illustration of Spectral Signatures of the Changes in Yeast Cell Wall Quality: the Effect of Different Glucose Concentration in the Growth Medium

The quality of yeast cell wall depends on the concentration of glucose in the growth medium, see, for example [18, 19]. Yeast cells with their walls modified in this simple way were chosen to illustrate the promise of C/Q spectra of calcofluor as the spectral signature of varying yeast cell wall quality. In particular, the C/Q spectra of calcofluor measured with yeast grown in media containing 2 % and 0.2 % glucose (further referred to as HG and LG cells, respectively), and suspended

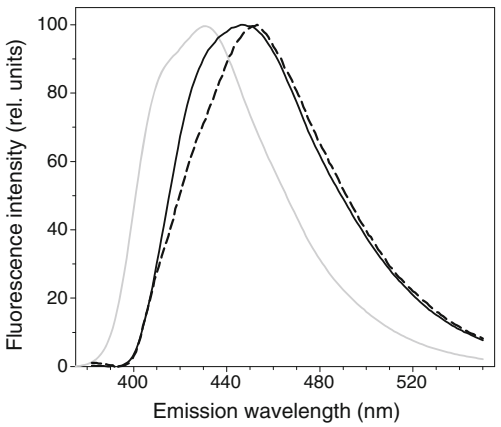

Fig. 4 Fluorescence emission spectra of calcofluor bound within the cell walls of yeast cells suspended in distilled water (pH 7): black full line – cells grown in HG medium; dashed line – cells grown in LG medium; grey line – fluorescence emission spectra of calcofluor solutions in distilled water

in both CP buffer and distilled water were used, Fig. 2. For each of these four types of cell suspensions the experiment was repeated 3–5 x with different samples. The individual spectra of each set were fitted to their means to illustrate further the reproducibility of C/Q profiles in repeated experiments, Fig. 2A.

Similar to the spectrum presented in Fig. 1C, the mean C/Q spectra shown in Fig. 2A exhibit also a flat sector around λ_{em}=380 nm and could therefore be corrected for the contribution of constant factor A, see Fig. 2B. Moreover, the C/Q spectra shown in Fig. 2B were normalized to a common value of 1 at λ_{em}=430 nm in order to enhance variations between C/Q spectral profiles characterizing different sets of cell suspensions. The difference between these spectral signatures becomes more comprehensible after the assessment of underlying basis spectra of calcofluor bound to yeast cell walls, Figs. 3 and 4, which consist in multiplying the corrected C/Q spectra by the F_W (λ) spectrum. The shape of fluorescence spectra of bound calcofluor is significantly changed on going from CP buffer to distilled water as suspending medium for both HG and LG cells. In distilled water the calcofluor emission spectra are similar to those of dye microcrystals, Fig. 4. This effect was attributed to the aggregation calcofluor in the negatively charged mannoprotein outer layer of the yeast cell wall (17). Since the amount of this negative charge decreases with the ionic strength of cell medium, the contribution of microcrystal-like spectra to the fluorescence of calcofluor stained yeast cell suspensions must be higher in distilled water than in CP buffer.

When measured in CP buffer, the basis fluorescence spectra of calcofluor bound to the walls of both LG and HG cells exhibit features similar to the spectra of calcofluor in chitin solution, Fig. 3. On the other hand, the mutual difference between HG and LG cells is nearly negligible apart from a moderately higher relative contribution of calcofluor emission from the mannoprotein outer layer in LG cells, which can also be interpreted as a lower contribution of calcofluor emission from the chitin and other polysaccharides of thin cell wall of LG cells. A manuscript on the use of C/Q spectra in the analysis of differences between yeast cell walls of selected strains and their MDR pump deficient mutants is in preparation.

Conclusions

Fluorescence emission spectra of calcofluor-stained yeast cell suspensions, which vary in a complex way with changing dye-to-cell concentration ratio, can be transformed into reliable spectral signatures of calcofluor binding to yeast cell walls. This transformation consists in dividing the cell-autofluorescence-corrected emission spectra of calcofluor-stained yeast cell suspensions by the fluorescence emission spectra of pure aqueous calcofluor solutions. In addition, these ratio spectra (called C/Q spectra in this paper) are also an appropriate tool to assess basis spectra, which allows for the spectral unmixing of respective fluorescence contributions of free (in aqueous medium) and cell-bound calcofluor to the fluorescence spectra of calcofluor-stained yeast cell suspensions. Moreover, the analysis of the series of both C/Q spectra and related spectra of cell-bound calcofluor measured in suspensions of cells grown at different glucose concentrations showed that the presented approach allows an easy and rapid detection of differences in the quality of the cell wall of yeast.

Acknowledgements This work was supported by the MSM 0021620835 grant from the Ministry of Education, Youth and Sports of the Czech Republic, and Czech Science Foundation grant 205/10/1121. We also thank Ivana Benešová for her assistance in growing yeast cell cultures and sample preparation.

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