Folia Microbiol. 44 (4), 429–434 (1999) http://www.biomed.cas.cz/mbu/folia/

2',7'-Bis-(2-Carboxyethyl)-5(6)-Carboxyfluorescein as a Dual-Emission Fluorescent Indicator of Intracellular pH Suitable for Argon Laser Confocal Microscopy

E. LANZ, †J. SLAVÍK, A. KOTYK

Institute of Physiology, Academy of Sciences of the Czech Republic, 142 20 Prague, Czech Republic
fax +420 2 475 2556

Received 28 June 1999

ABSTRACT. The widely used fluorescent probe 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) serves as a pH-sensitive indicator in classical microscopy. Characteristics of BCECF were studied and a way of employing the probe in a confocal laser scanning microscope equipped with an argon laser at 488 nm was developed, based on the fact that the emission fluorescence spectra are pH-dependent with spectral maximum shift from 518 to 529 nm. Optical filters for the dual-emission ratio method were set to 506 and 529 nm. pH values measured inside a single cell of *Saccharomyces cerevisiae* were similar to those obtained with other pH estimation methods.

Regulation of intracellular pH belongs to fundamental processes in cell physiology. The pH is one of the regulators of enzyme activity and ion channel function. Furthermore, H^+ transport across the plasma membrane is coupled with that of Na^+, K^+, HCO_3^- and other ions (Amos and Richards 1996). The technique of choice for intracellular pH measurement under various conditions in a non-invasive way is the use of fluorescent, pH-sensitive, probes.

BCECF is a widely used pH-sensitive fluorescent probe (Rink *et al.* 1982). This fluorescein derivative has been so far employed in dual-excitation ratio imaging (435 *vs.* 500 nm) mostly for classical microscopy and cuvette measurements (Slavík 1994; Tsien and Poenie 1986). Introduction of the confocal laser scanning microscope (CLSM) made this probe obsolete because most of the CLS microscopes use an argon laser with a 488 nm excitation line which does not make the above mentioned dual-excitation ratio imaging practicable. The UV and blue excitation lasers are expensive and rarely used. In this paper we show that there is a simple way of using BCECF with argon laser excitation and dual-emission ratio imaging in CLSM. Confocal laser scanning microscopy yields better quantitative results than classical microscopy because it displays only a thin slice of the sample and thus excludes some of the artifacts involved in classical microscopy, caused by light scattering and greater depth of the focused field. Moreover, the advantage over the double-excitation technique is that the dual-emission ratio imaging permits simultaneous detection of both fluorescence signals. This method is suitable not only for confocal microscopy but also for flow cytometry with argon laser excitation.

MATERIALS AND METHODS

2',7'-Bis-(2-carboxyethyl)-5(6)-carboxyfluorescein obtained from *Molecular Probes* (USA) was used in a standard way (1 mmol/L stock solution in dimethyl sulfoxide). Spectra were taken in a Perkin-Elmer LS 50 B spectrofluorimeter. Confocal microscopy images were obtained in a BIO-RAD microscope model MRC-600, equipped with an inverted fluorescence microscope Nikon, using argon laser excitation at 488 nm and two detectors with custom-made filters (from the *Academy Developmental Workshop* in Trutnov, Czech Republic) with spectral properties based on spectral analysis shown below.

Fluorescence spectra were analyzed using mathematical approaches and numerical methods. Principal component analysis makes it possible to separate the emission spectra into two components that correspond to the spectral response of each dissociated form of BCECF. To attain this separation we used NIPALS method (nonlinear iterative partial least squares), as described in *Galactic Industries Corp.* (1996; Pekeler 1996) and we modified it for pH-dependent fluorescence spectra (Kubista *et al.* 1996). In start, the input values were eleven emission spectra of BCECF with related pH, the total probe concentration and pK of the probe, which was fixed through the whole procedure.

Cells of *Saccharomyces cerevisiae* were employed as they are routinely used in this laboratory (Kotyk and Georghiou 1994; Kotyk and Lapathitis 1998; Lapathitis and Kotyk 1999; Kotyk *et al.* 1999) and had also been previously tested here for pH measurement with other fluorescent probes, such as

fluorescein and SNARF. They were grown in the usual way for 1 d in a semisynthetic medium YPG, then twice washed in distilled water, resuspended in 5 μmol/L BCECF–AM (BCECF–acetoxymethyl ester) in triethanolamine–phthalic acid buffers (TEPA) and then incubated for 15 min, which appears to be the standard BCECF-staining procedure. BCECF-AM allways freshly prepared from a 1 mmol/L stock solution in dimethyl sulfoxide stored in a refrigerator. The staining procedure was as follows. The ester BCECF-AM then penetrates plasma membrane, inside the cell it is hydrolyzed by esterases which cleave the acetoxymethyl ester chains. The resulting fluorescent acid form of the BCECF displays a reduced membrane permeation and, consequently, cells remain filled with BCECF. To observe cells in the inverted microscope the cell suspension was placed in a Petri dish.

All experiments and calibrations were done with 0.2 mol/L TEPA in the pH range of 4–9 because they do not interfere with yeasts (Kotyk and Georghiou 1991). We also studied the effect of protein addition and ionic strength on the BCECF spectra. For this purpose bovine serum albumin (BSA), KCl and NaCl were employed. The final solutions contained 114 mg/mL of BSA, fraction V (*Sigma Chemical Co.*), or 1 mol/L KCl or NaCl.

Yeasts exhibit a strong buffering power, so it was not possible to apply *in-vivo* pH; calibration using the nigericin method (Thomas *et al.* 1979). This can be solved using a method introduced by Boyarsky *et al.* (1996) but the best way to calibrate pH; values inside a yeast cell seemed to be the *in-vitro* calibration with some *in-vivo* approximation when the conditions in a cuvette were similar to those in the cell (*e.g.*, 11.4 % protein concentration was simulated with bovine serum albumin).

All experiments were done at room temperature.

RESULTS

It is generally known that BCECF excitation spectra change with pH in a way suitable for the dual-excitation ratio method (435 or 450 nm *vs.* 500 nm, emission 530 nm). However, it appears that it is not only excitation but also emission spectra that are pH-dependent. When excited at 488 nm (argon line used in CLSM or FACS), there is a spectral maximum shift in the emission from 518 to 529 nm when going from acidic to alkaline pH. This shift is large enough to allow BCECF to be used as a dual-emission pH indicator in the microscope (Fig. 1).

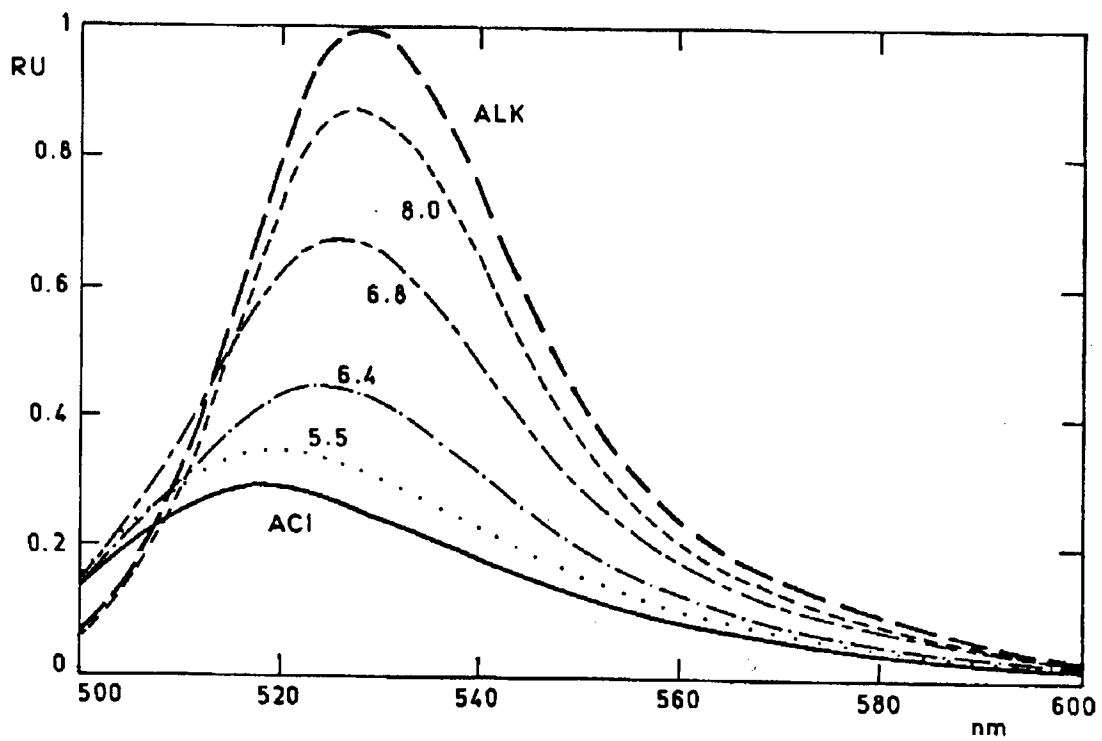

Fig. 1. BCECF emission spectra (excitation 488 nm, concentration 5 μmol/L). Two *thick lines* represent extracted spectra of both BCECF components (ACI – acidic, ALK – alkaline), evaluated from the real pH-dependent spectra using extended NIPALS algorithm. *Thin lines* are some of the real spectra of BCECF diluted in 0.2 mol/L TEPA buffer (*numbers at curves* – pH values).

Depending on pH, the probe dissociates into two forms (denoted A and B) differing in the degree of protonation in the physiological range of pH. Hence, with a pK_a of 6.98, BCECF has either four or five negative charges and each of the forms gives its characteristic spectral response. Extended NIPALS algorithm can separate the resulting experimental spectra into the two components that correspond to the spectral response of each dissociated form of BCECF.

Based on the above data, a special set of optical filters and a dielectric mirror were designed for the confocal microscope. The set would cut of the excitation laser beam (488 nm line) and divide the fluorescence into two beams corresponding to the emissions of alkaline and acidic forms of BCECF, respectively. The alkaline filter (λ_{max} 529 nm, $\Delta\lambda_{1/2w}$ = 6 nm, transmission at maximum 44.6 %) transmits the more intensive fluorescence of the alkaline form. The second filter (λ_{max} 506 nm, $\Delta\lambda_{1/2w}$ = 10 nm, transmission at maximum 44.6 %) detects the fluorescence of the acidic form.

To demonstrate the feasibility of the method *S. cerevisiae* cells were chosen and grown in a standard way. The confocal microscope BIO-RAD was set to dual emission with simultaneous detection of the emission signal after a 488 nm excitation. Two corresponding images obtained from the CLSM (each displaying space distribution of one BCECF form) were processed by LUCIA software for image analysis from *Laboratory Imaging, Ltd.*, and by a special rationing program produced in our laboratory. Images of both forms A, B and their ratio B/A proportional to pH distribution of *S. cerevisiae* resuspended in TEPA buffer at 5.5 are shown in Fig. 2. Intracellular pH was found to be 5.8 in one cell and 5.9 in the other. To calibrate quantitatively the grey values (displayed in pseudocolors) of the image with respect to pH, we chose *in vitro* calibration with solutions containing 0.2 mol/L TEPA buffers (pH of 4–9), 114 mg/mL of BSA and 5 μmol/L BCECF. Images for fluorescence calibration taken in the bulk buffer were processed in the same way as images of cells.

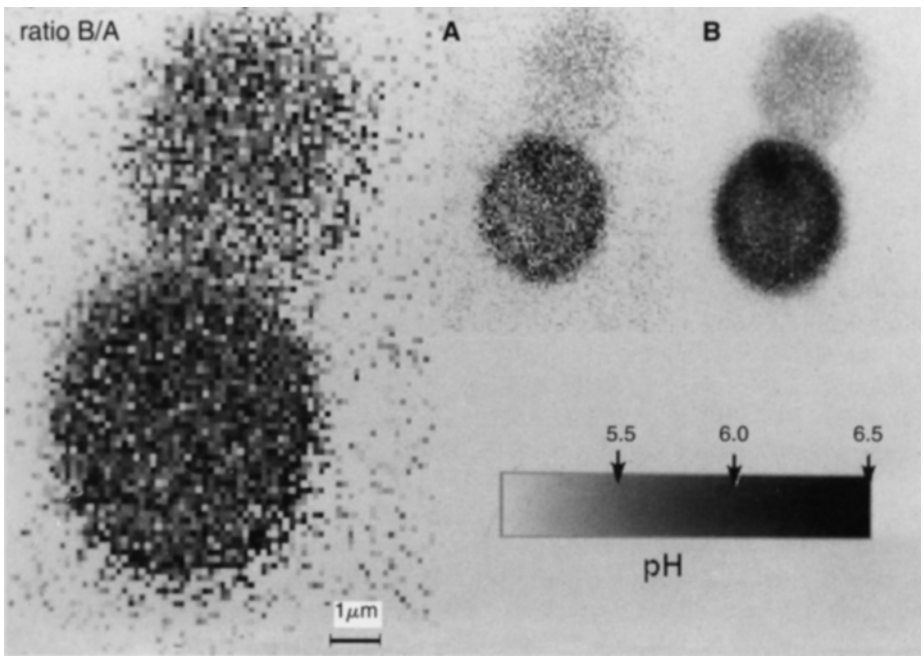

Fig. 2. *Left*: ratio image – pH map of *S. cerevisiae* cells; *right top*: fluorescence images of a cell obtained from the CLS microscope: **A** – emission of 506 nm, **B** – emission of 529 nm; excitation for both pictures was 488 nm. Intracellular pH is 5.5 resp. 5.9 at external pH 5.5.

In order to check how the real intracellular pH values comply with *in vitro* standard curve measured with TEPA buffers, we studied the possible effects of cytoplasmic composition on the calibration curve, especially the effect of proteins and ions on the spectra in the cuvette and on the calibration curve under conditions of confocal microscopy (Fig. 3) according to the effects observed in excitation spectra (Plášek *et al.* 1996; Russell *et al.* 1995; Opitz *et al.* 1996). When comparing the spectra of BCECF diluted in pure buffer with those in the presence of protein (bovine serum albumin, 114 mg/mL) there occurred a pH-dependent spectral red shift. The set of excitation and emission

spectra was processed and separated into components using NIPALS. The excitation spectrum of the acidic form shifts to the red by 5 to 8 nm but the spectral shift of the alkaline form is almost negligible at about 1–2 nm. The shift of the emission spectra is also very small (less than 1–2 nm). However, the fluorescence intensity decreases to 50–60 % after albumin addition (Fig. 4).

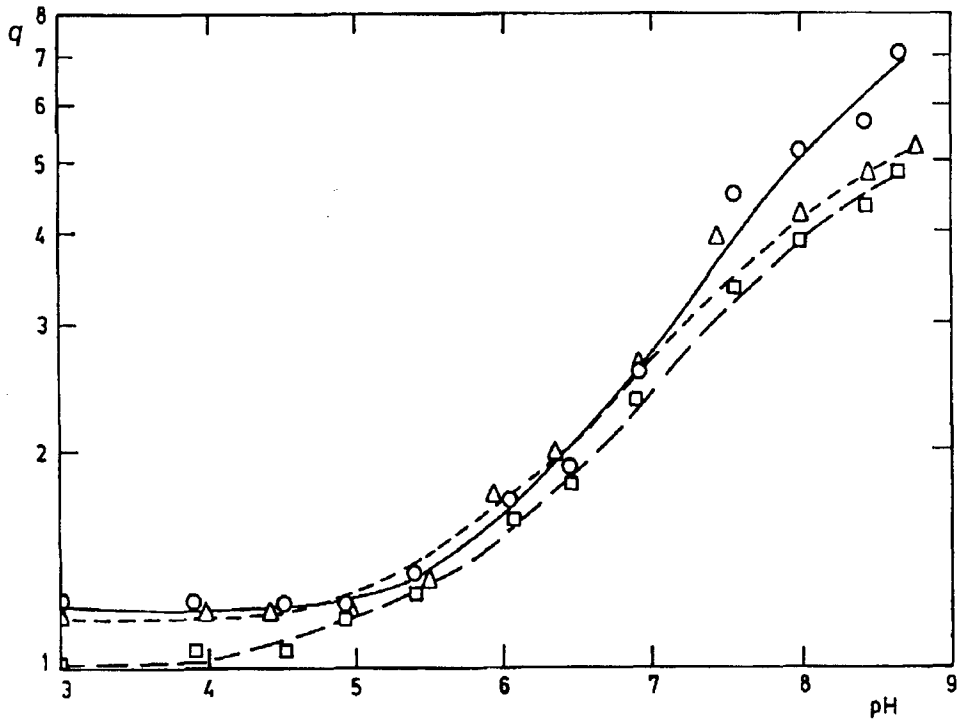

Fig. 3. Calibration curves based on BCECF emission spectra in pure TEPA buffer (○), in buffer containing 11.4 % BSA (□), and in buffer with 1 mol/L KCl (△); excitation 488 nm; q – fluorescence ratio (529/506 nm).

We also studied the effect of KCl addition (1 mol/L final concentration) to the BCECF-buffer solution, assuming that ions may also influence the shape of the spectra. There was a small, almost negligible, blue shift (1–2 nm) in all spectra (excitation, emission, at acidic or alkaline pH). Fluorescence intensity decreased by 20 % (Fig. 4). The same effect was observed with 1 mol/L NaCl.

Emission spectra of the two BCECF forms, characteristic for a solution at physiological pH, were established by NIPALS from a pH-dependent set of spectra. The fluorescence maximum of the acidic form is at 518 nm, that of the alkaline form at 529 nm, the fluorescence quantum yield of the acidic form being about 30 % of the alkaline form. The spectral shift between the emission peaks of the two BCECF forms is 11 nm which is less than in the excitation spectra (40–50 nm), but still large enough to permit the use of the probe in the dual-emission ratio method.

The alkaline and acidic optical filters were arranged to transmit fluorescence of the more intense alkaline and the less intense acidic form. The selection was done with care because the laser beam of the confocal microscope is rather intense and if not properly designed the filter system could add the initial laser beam (line of 488 nm) to the fluorescence signal. Maximum transmission of the acidic filter was at 506 nm and the contribution of the excitation laser beam 488 nm to an image in the microscope was less than 0.06 % of the initial light and thus negligible.

The pH maps obtained in a confocal microscope with *S. cerevisiae* were well defined and showed similar details and pH values (5.8 and 5.9 with a standard deviation of 0.2) as reached in the excitation ratio experiments using classical fluorescence microscope (Cimprich *et al.* 1995) but with better definition that mainly represents the absence of artifacts appearing in a "wide-field" classical microscope. It can be seen that the signal-to-noise ratio of the picture is low, which is limiting to more exact evaluation of pH. However, this problem can be overcome by using more sensitive detector, which is able to detect lower fluorescence signal (our A-detector (Fig. 2) is really old). Similar results were obtained with the *Endomyces magnusii* yeast culture.

The main advantage of our approach is the possibility to use BCECF in CLSM without expensive demands on the microscope equipment, such as an additional laser (*e.g.*, helium–cadmium laser

with maximum at 442 nm; Weinlich *et al.* 1993). Moreover, the dual-emission method enables simultaneous detection of both BCECF forms to be made, which is faster and more accurate for monitoring pH changes during physiological processes than the dual-excitation method.

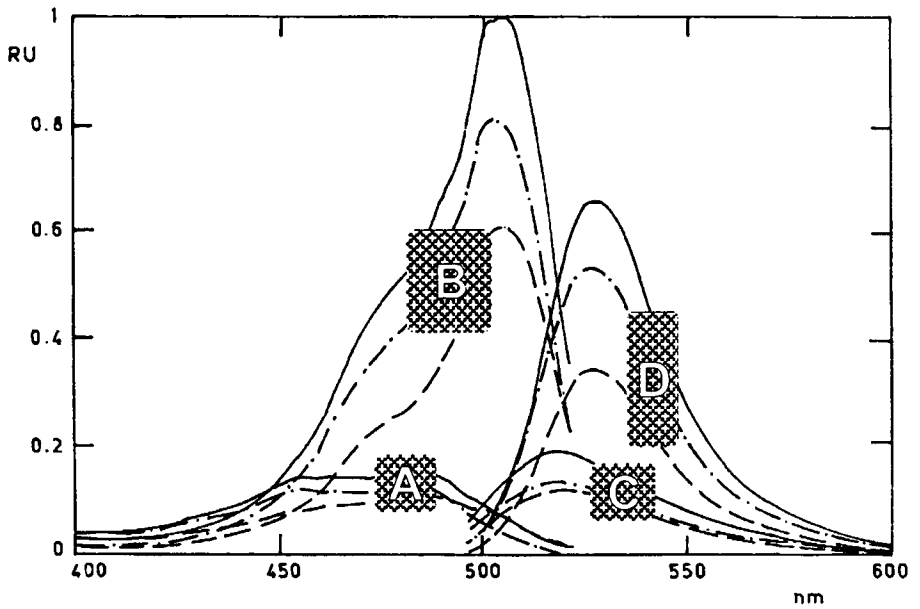

Fig. 4. Calculated (using NIPALS) excitation and emission spectra of 1 μmol/L BCECF in pure 0.2 mol/L TEPA (*solid lines*), in buffer with 11.4 % BSA (*dashed lines*) and in buffer with 1 mol/L KCl (*dot-and-dashed lines*); A — excitation spectra of the acidic form; B — excitation spectra of the alkaline form; C — emission spectra of the acidic form; D — emission spectra of the alkaline form.

DISCUSSION

Fluorescent probes have always been very useful and reliable when investigating relative changes in pH. Still, in order to obtain absolute values a careful calibration is needed. There are many factors that may influence the spectra of fluorescent probes and thus the pH-standard curve — for example, viscosity or temperature (Russell *et al.* 1995). This applies to virtually all fluorescent pH indicators. Some of them are more vulnerable than others. BCECF as a fluorescein derivative belongs to those, the fluorescence of which is predominantly pH-sensitive and less prone to other effects. It has been reported that proteins affect both excitation spectra and emission spectra. Fortunately, most of these effects are too small to have a significant influence on ratio imaging and are only observed in a spectrofluorimeter. Shifts to the red are negligible (1–2 nm) considering that the half width of the detected fluorescence in the confocal microscope is 6 nm in the one and 10 nm in the other detector. A more significant effect in the calibration is the decrease of fluorescence intensity in the presence of proteins which may be caused both by an inner filter effect and by direct quenching of BCECF by proteins. The 529/506 ratio then changes mainly on the alkaline side. The effect of KCl or NaCl addition also results in a decline of the alkaline side of the calibration curve. So it is important to calibrate *in-vivo* or to simulate living conditions (protein concentration and/or ion concentration, and temperature) in a cuvette.

The work was supported by grant of the *Grant Agency of the Czech Republic* no. 204/98/0474.

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