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# Accurate Whole-Spectrum Measurements of Intracellular pH and [$Na^+$]

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Fluorescent measurements of intracellular $H^+$ and $Na^+$ are improved by using whole spectra of the fluorescent indicators BCECF and SBFI, respectively. The extra data in whole spectra enable both an accurate calibration and a ready detection of artifacts which are not possible to identify using a more conventional data analysis that relies upon only two wavelength "windows" in the fluorescence spectra. The whole-spectrum technique is applicable to cell suspensions in a conventional fluorimeter (as is reported here with SBFI), as well as to attached cells using a fluorimeter combined with an inverted epifluorescence microscope. The spectral method was highly reproducible in that pairs of successive pH measurements differed, on average, by only 0.01 ± 0.02 U. Random uncertainty from sample to sample was estimated numerically from the standard deviation of measurements on ionophore-treated cells. When full-spectrum analysis was employed, this scatter showed a two-fold improvement over results obtained using the two-wavelength ratio method. Because SBFI has a relatively narrow dynamic range, whole-spectrum analysis has been applied to improve the accuracy of sodium determinations. The calibrated system measured $[Na^+]_i$ with excellent linearity over the range 2–150 m*M* and with an accuracy of approximately 5 m*M*.

**KEY WORDS:** BCECF; SBFI; intracellular pH; intracellular sodium.

## INTRODUCTION

Fluorescent probes which indicate an intracellular ion by a change in spectral shape upon binding are widely used, especially to measure calcium[1] and pH.[2] Somewhat more recently, the dye SBFI has been introduced for measuring intracellular sodium.[3,4] The equilibrium between ion-bound and -free forms of the dye (*B* and *D*, respectively) is described by the associated dissociation constant, $K_d$, according to Eq. (1), in the case of sodium:

$$D + Na^+ \rightleftarrows B, \qquad [D][Na^+] = K_d[B] \qquad (1)$$

For purposes of calculating a single ion concentration, the simplest procedure is to make fluorescence measurements at just two wavelengths and use the ratio of the two readings.[1] The ratio depends on spectral shape, is independent of the amount of dye in the cell, and provides sufficient information to calculate an ion concentration as long as no artifacts are present.

On the other hand, basing the measurements on the overall shape of an entire spectrum of 100–200 points instead of just two wavelengths provides increased accuracy.[5] In addition, the whole spectrum allows one to test for the quality of the results. This advantage comes into play if a portion of the signal happens to be con-

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tributed by unhydrolyzed dye ester, light scattering, background fluorescence, or other artifacts. Such contributions distort the spectrum and thus affect the two-wavelength ratio, but nothing in the calculation of pH or ion concentration from the ratio would reveal the problem. In contrast, a full-spectrum analysis will immediately show up any artifacts that introduce unwanted contributions to the signal.[5–7] Because the calculated ion concentration derives from over a hundred spectrum intensities, the data analysis differs from the two-wavelength case. It is, for example, no longer possible to draw a simple calibration curve of ratio vs ion concentration from a set of standard measurements for conversion of intensities into ion concentrations.

The analysis of a measured spectrum, $S_m(\lambda)$, is based on knowing the "basis spectra," $S_1(\lambda)$ and $S_2(\lambda)$, which are the spectra of dye forms $B$ and $D$, respectively. If all nondye components of the signal have previously been subtracted, the data curve $S_m(\lambda)$ will consist of a combination of the basis spectra, each multiplied by a weighting factor, $\alpha$ or $\beta$, which will be proportional to $[B]$ or $[D]$, respectively. The experimental approach for measuring potential basis spectra and then choosing the best pair is the main subject of this paper.

After basis curves have been obtained, data spectra can be analyzed by a least-squares calculation[8] to find the weighting factors ($\alpha$, $\beta$) that give the best fit to the data. After that, the ion concentration is given by

$$[Na^+]_i = K_d\ \alpha/\beta \tag{2}$$

or, in the case of pH, the logarithmic form of the same equation,

$$pH = pK_a + \log(\beta/\alpha) \tag{3}$$

The background spectrum, which must be subtracted before analysis, is measured on identical cells that received no intracellular dye, so that it will represent the light-scattering, water Raman peak, signal due to buffer or serum, etc. The assumption that nondye contributions have been removed underlies the analysis and it is checked in each case by using the calculated $\alpha$ and $\beta$ and the basis spectra to generate the "best-fit" calculated spectrum,

$$S_c(\lambda) = \alpha S_1(\lambda) + \beta S_2(\lambda) \tag{4}$$

Each data spectrum is compared with its calculated spectrum, and if any artifact has caused the fit to be poor, the data is not used. The correlation coefficient $r^2$ is typically above 0.998 for these curve fits, and values below 0.980 are discarded. In particular, unhydrolyzed dye could cause the spectra after addition of ionophore to diverge from the ion-bound "basis" spectrum $S_1(\lambda)$, in which case the entire preparation would be discarded.

The whole-spectrum approach is well suited for the sodium-sensitive dye SBFI because it undergoes such a small spectral change between its sodium-free and sodium-bound forms. The small dynamic range at any particular wavelength makes a two-wavelength ratio method more subject to any experimental "noise" in the measurements. The total SBFI intensity is also very sensitive to temperature and to changes in total ionic strength,[4] so that the use of this dye at a single wavelength is essentially out of the question.

To use the whole-spectrum analysis, one must first obtain the basis spectra $S_1(\lambda)$ and $S_2(\lambda)$. In other systems, such as with calcium-binding dyes, cells tolerate $[Ca^{2+}]$ extremes that fully saturate and desaturate the dye, so the basis spectra can be measured directly.[9] For sodium and hydrogen ions, however, ionic extremes lead to dye leakage or cell death, so an alternative has been devised which allows the use of defined but less severe conditions.

## MATERIALS AND METHODS

*Reagents.* BCECF, SBFI, their respective "-AM" acetoxymethyl esters, and Pluronic F-127 were obtained from Molecular Probes (Eugene, OR). HEPES, DMSO (grade 1), fetal bovine serum (FBS), and all routine biochemicals were obtained from Sigma (St. Louis, MO).

*Buffered Solutions.* The normal buffer was Hanks balanced salt solution (HBSS) with 5.6 m*M* dextrose and 20 m*M* HEPES at pH 7.3. For pH calibrations, cytosol-like buffer (130 m*M* KCl, 20 m*M* HEPES buffer, 1 m*M* $MgCl_2$) was adjusted to pH values from 6.5 to 7.5 at 37°C. For the calibration of intracellular SBFI, two buffers were intermixed which contained 1.2 m*M* $CaCl_2$, 0.6 m*M* $MgCl_2$, 20 m*M* HEPES ($Na^+$ or $K^+$ salt), and either 130 m*M* NaCl or 130 m*M* KCl adjusted to pH 7.3. The proportions of the two in each mixture were chosen so as to generate a series of sodium ion concentrations.

*Cells.* A stable cell line (OvCa) derived from Chinese hamster ovarian carcinoma cells was originally obtained from Dr. George Yerganian of Boston Children's Hospital, Boston, MA. Cells were grown in McCoy's 5A modified medium (Sigma) containing 10% heat-inactivated fetal bovine serum (FBS). The Jurkat human T cell line was the kind gift of Dr. Arthus Weiss (Stanford University, Stanford, CA) and was carried in RPMI 1640 medium with 10% FBS. For adherent-cell experiments, OvCa cells were plated 48 h prior to measure-

ment at a concentration of $7 \times 10^4/cm^2$ on 35-mm coverslip dishes (MatTek, Ashland, MA).

*Dye Loading.* BCECF-AM (10 μg) was dissolved in 25 μl of DMSO, 6% (w/v) Pluronic F127.[10] Cells were incubated with 4 μ*M* dye in medium for 2–4 min at room temperature, then washed and incubated in HBSS/10% FBS for 20 min at 37°C and 5% $CO_2$ to ensure complete hydrolysis of the dye ester by intracellular esterases. Cells were washed and kept on ice in suspension ($1 \times 10^7$/ml) and used immediately after preparation for coverslip plates.

SBFI-AM (14 μg) was dissolved in 25 μl of DMSO, 12.5% (w/v) Pluronic F127. Jurkat cells were incubated with 6 μ*M* dye in medium for 1 h at 37°C and 5% $CO_2$. The cells were resuspended in HBSS/10% FBS and incubated at 37°C for 15 min, which we have established in control experiments will ensure complete intracellular hydrolysis of the dye ester in this cell type. The cells were then counted, pelleted, and resuspended in HBSS/10% FBS at $10–20 \times 10^6$ cells/ml and kept on ice. Cell viability was >95%, by trypan blue exclusion.

*Fluorescence Spectroscopy.* These dyes require measurement of excitation spectra to see ion-dependent changes. For cell suspensions this was done with a Model RF-M2001 fluorometer from Photon Technology International (East Brunswick, NJ). The light source was a 75-W xenon arc lamp, with excitation monochromator slits of 4 nm for BCECF or 6 nm for SBFI. For adherent cells, the same source illuminated coverslip plates on a Nikon diaphot inverted fluorescence microscope which had an identical photon-counting detector. For suspended cells, signals were fed through an emission monochromator with 8-nm slits. For adherent cells, the filter cube on the microscope had a 510-nm dichroic mirror and a 520-nm emission interference filter (10-nm bandwidth; Omega Optical, Brattleboro, VT). Excitation wavelengths were scanned in 1-nm increments from 400 to 540 nm (BCECF) or from 300 to 400 nm (SBFI), with a 0.4-s per step integration time.

An aliquot from the cell suspension on ice was diluted 10-fold into cytosol-like buffer at 37°C in the cuvette, or for attached cells, the medium on the coverslip dish was changed to cytosol-like buffer at 37°C. Cells were allowed approximately 3 min to come to equilibrium before the first spectrum was measured. In the instrument, the excitation shutter was closed except during data acquisition. No statistically significant photobleaching of BCECF samples was observed under these conditions, and SBFI bleaching was minimized.

For all spectra involving cells, equivalent baselines were obtained with the appropriate combinations of buffer and dye-free cells, and these were subtracted from the measured spectra before any comparisons or other analyses were performed. All spectra were measured at 37°C. Data analysis was carried out[8] on a desktop computer.

*Spectra at Known $pH_i$.* To bring intracellular pH into equilibrium with extracellular pH, cells were incubated for 1–2 min with cytosol-like buffer, and 10 μg/ml nigericin and 5 μg/ml valinomycin were added.[11] After 3–5 min the spectral shape was stable, and any further small changes could be attributed to dye leakage from the cells.

*Spectra at Known [Na⁺].* To calibrate SBFI, a set of spectra was first obtained on the extracellular dye at various known [Na⁺]. Two buffers containing equal (4 μ*M*) concentrations of dye were prepared, one containing 150 m*M* [Na⁺] and the other 150 m*M*[K⁺]. Mixtures of these allowed spectra to be obtained which corresponded to various [Na⁺], at constant total ionic strength and pH (7.3). Each was then diluted 50% (v/v) with ethanol to create the low-polarity cytosol-like buffer, which was found to mimic intracellular conditions for Indo-1 and Snarf dyes.[5,9,12–14] For intracellular SBFI, aliquots of cells were diluted 10- or 20-fold into cuvettes containing various mixtures of NaCl and KCl (130 m*M* total) and Hepes buffer (pH 7.3, 20 m*M*, which contributed approximately another 20 m*M* to the total [Na⁺] or [K⁺]). Gramicidin (2 μ*M*; Sigma Chemicals) was added and sequential spectra were taken for 5–15 min, depending on the magnitude of the difference between intracellular and extracellular [Na⁺] which had to be equilibrated.[10] The final sodium concentration outside the cells was calculated taking into account the ratio of $Na^+$ to $K^+$ in the starting buffer, as well as the contribution from the FBS and the HBSS in which the cell suspension was kept before dilution.

*Calculation of Measured [Na⁺] or pH.* After subtraction of background signal the remaining measured spectrum $S_m(\lambda)$ was due to the dye itself. Starting with two basis spectra, $S_1(\lambda)$ and $S_2(\lambda)$, the admixture which best fit the data was found by a least-squares calculation, as described previously.[8,12,14] The weighting factors $\alpha$ and $\beta$ found by this calculation were used to determine [Na⁺] or pH, as shown in Eqs. (2) and (3), respectively.

*Obtaining Basis Spectra.* The analysis required that the intracellular basis spectra $S_1(\lambda)$ and $S_2(\lambda)$ represent (1) identical amounts of dye and (2) dye in its fully bound and fully free states. These spectra had to be obtained for intracellular dye without exposing cells to the extremes that would be needed to remove all intracellular sodium or protonate all BCECF. To do this, $S_1(\lambda)$ and $S_2(\lambda)$ were calculated from two measured spectra, $S_{m1}(\lambda)$ and $S_{m2}(\lambda)$, which were taken using iono-

phores[10,11] to set up known concentrations $[Na^+]_1$, $[Na^+]_2$ (for SBFI) or $pH_1$, $pH_2$ (for BCECF).

For the calculation of the basis spectra $S_1(\lambda)$ and $S_2(\lambda)$ from the measured spectra $S_{m1}(\lambda)$ and $S_{m2}(\lambda)$, Eq. (4) was used to relate each measured spectrum to the two basis spectra:

$$S_{m1}(\lambda) = \alpha_1 S_1(\lambda) + \beta_1 S_2(\lambda), \quad (5.1)$$

$$S_{m2}(\lambda) = \alpha_2 S_1(\lambda) + \beta_2 S_2(\lambda) \quad (5.2)$$

This situation was the opposite of the analysis of a usual data spectrum because, in this case, the $\alpha$, $\beta$ factors were known and the purpose was to calculate $S_1(\lambda)$ and $S_2(\lambda)$. The weighting factors $\alpha_1$, $\beta_1$ corresponded [by virtue of Eq. (2)] to the known concentration $[Na^+]_1$ and the pair $\alpha_2$, $\beta_2$ corresponded to $[Na^+]_2$:

$$\alpha_1/\beta_1 = [Na^+]_1/K_d, \qquad \alpha_2/\beta_2 = [Na^+]_2/K_d \quad (6)$$

We further required that the spectra $S_{m1}(\lambda)$ and $S_{m2}(\lambda)$ had to be measured on the same amount of dye (set arbitrarily equal to unity) or else corrected for small differences in the concentration, so we had

$$\alpha_1 + \beta_1 = 1, \qquad \alpha_2 + \beta_2 = 1 \quad (7)$$

Mathematically, the procedure was to use the last four equations to eliminate the constants $\alpha_1$, $\beta_1$, $\alpha_2$, and $\beta_2$, so that the first two equations could be solved for $S_1(\lambda)$ and $S_2(\lambda)$. This process, or the analogous process using logarithmic equations in the case of pH, was carried out by the computer for each value of $\lambda$, and the result was a pair of basis spectra $S_1(\lambda)$ and $S_2(\lambda)$:

$$S_1(\lambda) = \{(K_d+[Na^+]_2)S_{m2}(\lambda) - (K_d+[Na^+]_1)S_{m1}(\lambda)\}/\{[Na^+]_2-[Na^+]_1\} \quad (8)$$

$$S_2(\lambda) = \{[Na^+]_2(K_d+[Na^+]_1)S_{m1}(\lambda) - [Na^+]_1(K_d+[Na^+]_2)S_{m2}(\lambda)\}/K_d\{[Na^+]_2-[Na^+]_1\} \quad (9)$$

## RESULTS

In order for the whole-spectrum system to be calibrated and ready to analyze data, we had to obtain an appropriate $K_d$ or $pK_a$ and a pair of basis spectra that would give the most accurate results when used to analyze data.

*Reproducibility.* The repeatability of fluorescence measurements on a given sample was essentially as good as the pH meter, so the accuracy of the system was found to be limited by sample-to-sample spectral anomalies. For example, when BCECF spectra were measured on 14 different attached cells or cell clusters with sequential readings about 1 min apart, the values calculated for $pH_i$ differed by only 0.01 ± 0.02 U (mean ± SD).

*BCECF Calibrations.* Potential pairs of basis spectra were calculated, using the method described above, from nine pairs of spectra which were obtained at known pH values between 6.5 and 7.5. In five of the cases there were small but obvious experimental irregularities in the starting curves that led to flawed basis spectra, and these then imposed a noticeable systematic error if they were used for subsequent calculations of pH.

To select the best pair of basis spectra, the four remaining pairs were tested by using them to analyze 89 other data curves which had also been measured at known $pH_i$. This created four sets of calculations, each with 89 calculated $pH_i$ values that were compared with their corresponding $pH_e$. In all four cases, the average value of $(pH_i - pH_e)$ for the 89 comparisons was better than 0.03 U. This suggested that *systematic* errors were <0.03 U, in the sense that a general shift in calculated pH values associated with the choice of one set of basis curves over another would be a systematic error.

As a related measure of the uncertainty in the calculation of $pH_i$, we took each of the 89 spectra and compared the four pH values that were calculated when the four different pairs of basis spectra were used. Changing the calculation by changing basis spectra not only had a small systematic effect on $pH_i$, but also had a random effect that varied with which of the 89 spectra was being analyzed. The sum of the two effects was about 0.05 U, since the standard deviations in the sets of four $pH_i$ values ranged from 0.01 to 0.13, with an average of 0.05.

Taken together, these findings suggest that the systematic error associated with a particular calibration of the system (in particular, the choice of basis curves) is of the order of 0.02 U and that, in our hands, the random error in the analysis of a given sample may be of the order of 0.03 U. The random error appeared to arise from changes in the non dye-related signal or other sample-to-sample variations, since reproducibility on a particular sample was about ±0.01 U.

Finally, the "linearity" of the system was confirmed. One set of basis curves was chosen and 42 different spectra, measured on 4 different days, were analyzed to confirm that the system obeyed Eqs. (2) and (3) over the range of interest. The 42 calculated values of $\alpha$ and $\beta$ were plotted as $\log(\beta/\alpha)$ *vs* $pH_e$ [cf. Eq. (3)]. The linear regression slope was 0.99 ± 0.03 (slope ± SE), which was not significantly different from the theoretical value of 1.00 [Eq. (3)]. When the theoretical slope was used in Eq. (3), the difference $[pH_e - \log(\beta/\alpha)]$ gave a value for the apparent $pK_a$. The average of the 42 apparent values for $pK_a$ was 7.01 ± 0.08

**Table I.** Summary of Results

| Sample | Analysis | Average ΔpH[a] | ±σ[b] | n[c] |
|---|---|---|---|---|
| Repeat measurements on same cell | Spectrum | 0.01 | 0.02 | 14 pairs |
| Cell suspensions | Spectrum | 0.03 | 0.05 | 89 |
| Adherent cells | Spectrum, adjustable baseline | −0.01 | 0.06 | 69 |
| Adherent cells | Spectrum, fixed baseline | −0.01 | 0.07 | 69 |
| Adherent cells | Ratio of 2 wavelengths | −0.01 | 0.11 | 69 |

[a]Average discrepancy between $pH_i$ and $pH_e$ in the presence of ionophore, except in the case of repeated measurements on the same cell, in which case the discrepancy is between successive measurements, and no ionophore was used.
[b]Average standard deviation of $pH_i$ values.
[c]Number of measurements.

(mean ± SD). When the experiment was repeated with different instrument settings, the value obtained for $pK_a$ was 6.96 ± 0.06 ($n$ = 32). These results for $pK_a$ are in excellent agreement with the published value of 6.97.[2]

*Microfluorometer Spectroscopy.* Excitation spectra of single cells or small groups of cells were measured by quantitative epifluorescence microscopy. This introduced a small complication since spectrum pairs for basis curve calculation could not be obtained on the same cell at two different pH values, due to movement when the medium was changed. Because the two spectra that were used to calculate basis spectra had to be measured on different ionophore-treated cells, which generally had slightly different concentrations of dye, the height of one curve of each pair usually had to be adjusted. The multiplier for this purpose was chosen so as to make the pair intersect at the previously determined isofluorescence wavelength. Cell morphology (shape) was not a factor since calculated pH values did not vary with cell number or shape in the ionophore-treated cells.

A total of 71 spectra was obtained on clusters of one to four cells in the presence of ionophores. Two of the spectra were used to obtain basis curves, and the other 69 spectra came from 39 different cell clusters, in a total of nine petri dishes having nine different values of $pH_e$. Three different methods of data analysis were then compared statistically by examining the differences between the calculated $pH_i$ and $pH_e$. The results are summarized in Table I.

In the first case, analysis was carried out by the ratio (two-wavelength) method, at 490 and 440 nm. The basis spectra provided the values for maximum and minimum intensities at the two wavelengths as required for the ratio formula.[1] The mean for the difference ($pH_i$ − $pH_e$) was only −0.01, which indicates negligible systematic error. However, the standard deviation, which we take as a measure of the random error, was ±0.11 U.

When full-spectrum analysis was used, accuracy was improved. The ΔpH was still only −0.01, but the scatter in the calculated values was reduced to ±0.07 U (standard deviation of ΔpH). The improvement in accuracy arose from (1) a small across-the-board reduction in ΔpH values and (2) the fact that 10 of the worst curves were detected by the curve-fitting program as poor measurements and discarded. (Curve fits with $r^2 <$ 0.9980 were eliminated.)

The third method of analysis improved on the whole-spectrum approach by also adjusting the strength of the light-scattering baseline that should be subtracted at the same time as it calculated α and β. This mathematics was originally developed to analyze the binding of a dye to more than one cation.[15] For the 69 BCECF spectra which were analyzed, the adjustable baseline procedure resulted in a small improvement, as the new ΔpH value was −0.01 ± 0.06 U (mean ± SD).

*SBFI.* Figure 1 demonstrates a family of SBFI curves, with the extracellular dye shown in Fig. 1A and intracellular curves shown in Fig. 1B. The former were measured in low-polarity cytosol-like buffer containing 50pct (v/v) ethanol to mimic intracellular polarity.[5,9,12–14] The latter were measured in the presence of 2 μ$M$ gramicidin to equilibrate intracellular and extracellular [$Na^+$]. The free dye spectra showed an isofluorescence point at 345 nm. This corrected for overall intensity changes that resulted from temperature, photobleaching, or other variations between samples. With this factor included, the

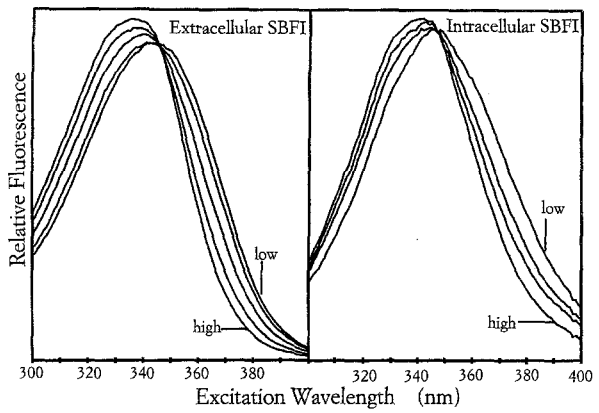


**Fig. 1.** Excitation spectra of SBFI (dye only, since background signals have been subtracted). A shows 4 μ*M* extracellular dye in low-polarity cytosol-like buffer containing 0, 0.9, 2.7, 8.8, and 46.7 m*M* sodium ion (total monovalent cation was held constant at approximately 150 m*M*). B shows intracellular SBFI measured in the presence of 2 μ*M* gramicidin to equilibrate $[Na^+]_i$ with extracellular sodium ion. Extracellular buffers contained 12.5, 30.7, 80.7, and 58 m*M* sodium plus 20 m*M* Hepes, pH 7.3 (see text).

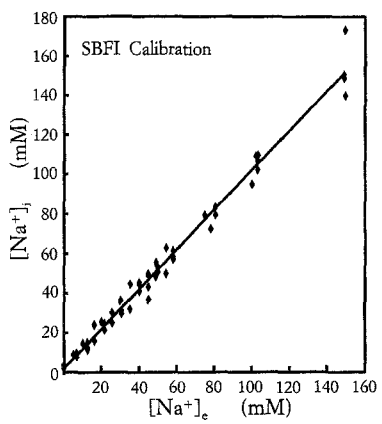


**Fig. 2.** Calibration of intracellular sodium ion assay showing values calculated from whole SBFI spectra for cells treated with 2 μ*M* gramicidin to equilibrate intracellular sodium ion with various extracellular concentrations from 5 to 150 m*M*. The linear regression line (shown) differs insignificantly in slope from a theoretical line of unit slope.

families of intracellular and extracellular curves were qualitatively the same, as has been reported previously.[4] This indicated that the changes in overall intensity were not occurring during the acquisition of a spectrum and causing a distortion of its shape.

An intracellular curve at 12.5 m*M* $[Na^+]_i$ and another at 60 m*M* $[Na^+]_i$ were found which crossed at 347 nm without adjustment. These were used (together with a value of 19 m*M* for $K_d$) to generate basis curves for the analysis of other data spectra. Analogous to the pH experiments, the basis curves were then tested by using them to analyze other data curves having known $[Na^+]_i$ (measured in the presence of 2 μ*M* gramicidin). To determine the accuracy of the spectral analysis for $[Na^+]$, the calculated $[Na^+]_i$ values were graphed versus $[Na^+]_e$ over the range 5–150 m*M*. The results are shown in Fig. 2 along with the linear regression line, which had a slope of 1.005, an intercept of 1.89 m*M*, and a correlation coefficient $r^2 = 0.984$.

The agreement of the linear regression with the theoretical slope of 1.00 shows that the assay had excellent linearity between 5 and 150 m*M*. Even though the SBFI dye has a very narrow dynamic range, the accuracy of our measurements over this range was ±2–10 m*M*. The standard error in estimates of $[Na^+]_i$ from the best-fit line was ±5 m*M* at $[Na^+]_e$ = 50 m*M*. At the extremes, it was ±8 m*M* at $[Na^+]_e$ = 2 m*M* and ±12 m*M* at 140 m*M*.

## DISCUSSION

There are at least three general approaches to cation measurements with spectrum-shifting dyes. In the simplest, a ratio of measurements at just two wavelengths is used, and a standard curve is drawn from a set of measurements on ionophore-treated cells. The ratio that is graphed usually contains contributions from both the dye and various background signals, and the calibration curve converts measured ratios into ion concentrations as long as the background signal is always the same. In the second system, one requires the standard curve to have the theoretical sigmoidal shape for a simple binding reaction that is characterized by a single dissociation constant.[1] In this case, it is important to identify and subtract contributions from sources other than the dye. The third method utilizes the shape of the spectrum. There is no standard curve, and a least-squares analysis finds the mixture of bound- and free-dye basis spectra that best matches the shape of each data curve.[8]

Resistance to artifacts is a virtue of the whole-spectrum approach. Especially for a dye such as SBFI, which exhibits a relatively small change in fluorescence, it is useful to seek increased accuracy by using data points from the entire spectrum. The improvement is due partly to the larger number of measurements, and in this sense, it is analogous to making a two-wavelength measurement with longer measurement times to average out noise in the system. More importantly, however, the spectrum which is analyzed can be required to have the

proper shape, i.e., it can be expressed as a composite of the known "basis" spectra for ion-bound and -free dye. This information about the spectral shape can let one detect even previously unsuspected artifacts and reject those data. The computer finishes each analysis by synthesizing the composite curve that best fits the data, and this immediately indicates if there was an artifact that introduced some new spectral shape.

The system relies on a pair of basis spectra (that can be used day after day), as well as a method for obtaining nondye signals for each sample and subtracting them before the analysis. When basis spectra have been obtained that can accurately analyze measured spectra, they are independent of changes in cell sample (i.e., factors which affect the background signal but not the dye signal). They can be used for samples of different cell types and densities as long as appropriate no-dye baseline spectra are subtracted. The calibration is good until such time as changes in the instrument (such as slit width) affect the measured dye spectra.

The basis spectra have to represent identical amounts of dye or the multipliers $\alpha$ and $\beta$ will not correctly represent relative concentrations of bound and unbound dye. This is easy when cells are in suspension and measured calibration spectra, $S_{m1}(\lambda)$ and $S_{m2}(\lambda)$, can be obtained on the same sample. It has not proven possible for adherent cells, and it has proven ineffective for cells containing SBFI, so in these cases, we have mathematically adjusted the two calibration spectra to correct for different amounts of dye. The adjustment technique focuses on the isofluorescence point as the most sensitive indicator of whether or not the two curves at different $pH_i$ or $[Na^+]_i$ values represent the same or different amounts of dye.

The accuracy that was found in the spectral analysis is as good as, or better than, that reported using two-wavelength ratios and comparing with a standard curve derived from measurements in cytosol-like buffer with nigericin.[16] In nigericin-"clamped" cells, reproducibility of ratio measurements was within 0.01–0.025 pH U, and the cell population (50 cells in a single dish) had a standard deviation of 0.04–0.08.[16] The larger standard deviation between cells in different dishes and on different days was not given.[16] Our standard deviation of 0.06–0.07 for adherent cells, on the other hand, already includes contributions from day-to-day variance in instrumentation setup, dye-loading protocol, cell density, passage number, etc.

SBFI is very sensitive to changes in temperature, total ionic strength, etc.,[4] so even spectra from the same sample usually failed to cross at the correct isofluorescence point. Nevertheless, both for these and for adherent cells containing BCECF, excellent basis spectra were obtained by requiring the measured starting spectra to cross at the isofluorescence wavelength which was found for extracellular dye. The test of the basis spectra was the excellent accuracy of $pH_i$ or $[Na^+]_i$ values calculated with them when tested on ionophore-treated samples, where $pH_i$ or $[Na^+]_i$ was known due to equilibration with the extracellular value.

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## REFERENCES

1. G. Grynkiewicz, M. Poenie, and R. Y. Tsien (1985) *J. Biol. Chem.* **260**, 3440–3450.
2. T. J. Rink, R. Y. Tsien, and T. Pozzan (1982) *J. Cell Biol.* **95**, 189–196.
3. A. Minta and R. Y. Tsien (1989) *J. Biol. Chem.* **264**, 19449–19457.
4. A. T. Harootunian, J. P. Kao, B. K. Eckert, and R. Y. Tsien (1989) *J. Biol. Chem.* **264**, 19458–19467.
5. E. Popov, I. Gavrilov, E. Pozin, and Z. Gabbasov (1988) *Arch. Biochem. Biophys.* **261**, 91–96.
6. C. S. Owen and R. L. Shuler (1989) *Biochem. Biophys. Res. Commun.* **163**, 328–333.
7. N. Kurebayashi, A. B. Harkins, and S. M. Baylor (1993) *Biophys. J.* **64**, 1934–1960.
8. C. S. Owen (1988) *J. Biol. Chem.* **263**, 2732–2737.
9. C. S. Owen, N. L. Sykes, R. L. Shuler, and D. Ost (1991) *Anal. Biochem.* **192**, 142–148.
10. M. Borin and W. Siffert (1990) *J. Biol. Chem.* **265**, 19543–19550.
11. J. Thomas, R. Buchsbaum, A. Zimniak, and E. Racker (1979) *Biochemistry (USA)* **18**, 2210–2218.
12. C. S. Owen (1991) *Cell Calcium* **12**, 385–393.
13. C. S. Owen (1992) *Anal. Biochem.* **204**, 65–71.
14. C. S. Owen, P. Carango, S. Grammer, S. Bobyock, and D. B. Leeper (1992) *J. Fluoresc.* **2**, 75–80.
15. C. S. Owen (1993) *Anal. Biochem.* **215**, 90–95.
16. M. A. Schwartz, B. Both, and C. Lechene (1989) *Proc. Natl. Acad. Sci. USA* **86**, 4525–4529.