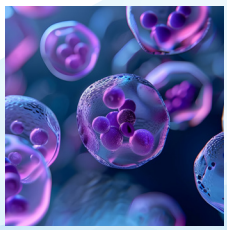
HORIBA

Fluorescence

In-Situ Calibration on Intracellular $[Ca^{++}]_i$, $[Na^+]_i$ and pH_i



Technical Note
FL TN 08-17-24

Karoly Csatorday[1], Ram V. Sharma and Ramesh C. Bhalla
[1]Photon Technology International, University of Iowa, College of Medicine, Iowa City, IA 52242
An expansion of the poster presented at the THIRD INTERNATIONAL SYMPOSIUM ON CALCIUM AND GENE HYPERTENSION Portland, OR, July 10-14, 1991

Calcium

The fluorescence excitation spectrum of the calcium sensitive dye Fura-2 (Grynkiewitz et al. (1985) J. Biol. Chem. 260, 3340-3450) shifts to the shorter wavelengths upon chelating Ca^{++} ions.

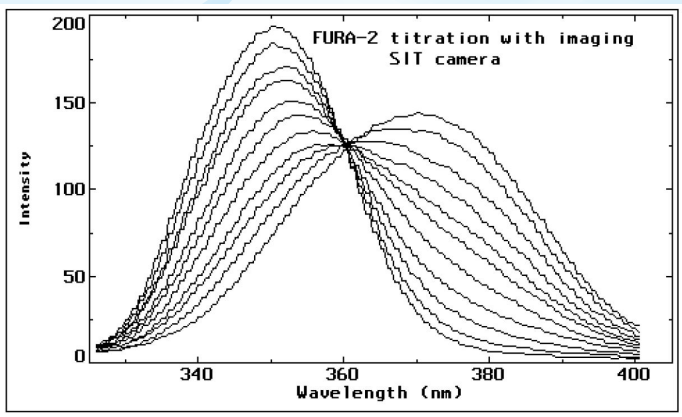


Figure 1: Fluorescence excitation spectra were obtained using a Nikon Diaphot inverted microscope. The UV transmission characteristics determine the peak position of the short wavelength form. In this case the peak is at 348 nm.

The ratio of intensities at 340 and 380 nm reflects the equilibrium between the Ca-bound and Ca-free Fura-2 molecules and can be expressed as Ca^{++} ion concentration via the analytical expression

$$[Ca^{++}] = K_d \times b \times (R - R_{min})/(R_{max} - R)$$

where k_d is the dissociation constant and Rmin and Rmax are the 340/380 ratios of the Ca-free and Ca-bound forms respectively. b is the ratio of fluorescence excitation intensities of the two latter forms at 380 nm. K_d has to be determined independently, whereas the other variables are readily accessible from the measurement.

Alternately, by measuring the 340/380 ratios as a function of known free Ca^{++} concentration, a calibration function called a look-up table (LUT) may be constructed.

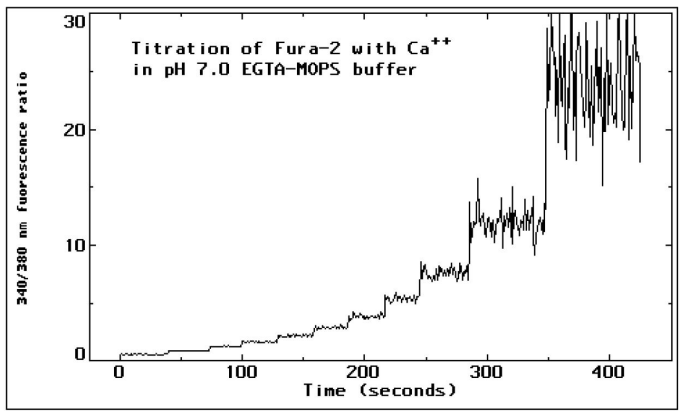


Figure 2: The measured 340/380 fluorescence ratio trace obtained by stepwise titration of Fura-2 with calcium is mapped into concentration space using the analytical formula and the calibration look-up table.

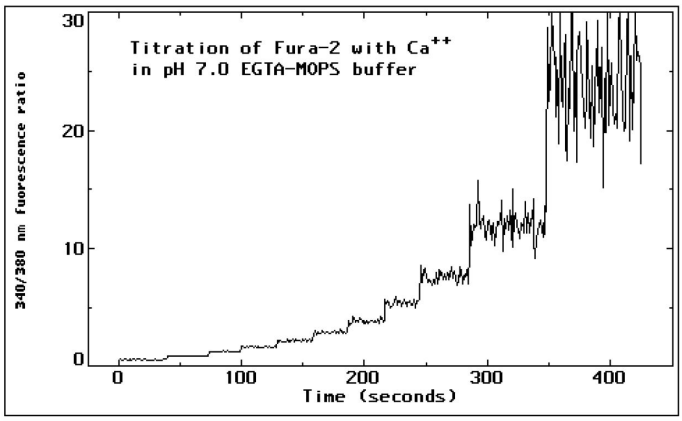


Figure 3: The two traces have been shifted for easy comparison. Congruence of the two traces attests to the viability of the calibration technique via (LUTs).

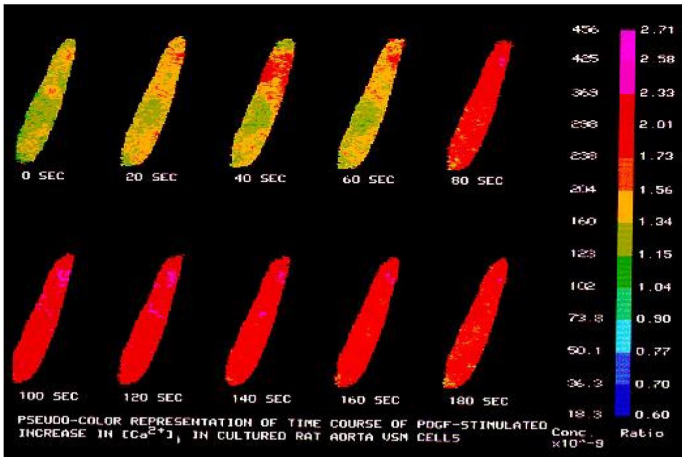


Figure 4: Ratioing fluorescence intensities can be performed on fluorescence images as well. Cells loaded with fluorescent dye are illuminated by alternating 340 and 380 nm light and the resultant fluorescent images are captured by a sensitive video camera and ratioed pixel to pixel. The ratio image is displayed subsequently in pseudo-color with the ratio scale mapped to calcium concentration. (This figure Copyright © 1993 The American Physiological Society. Used by permission.)

SBFI

A number of researchers attempting to study intracellular Na^+ transients have faced difficulties using the sodium-sensitive dye SBFI. In a study of its behavior in rat aortic vascular smooth muscle cells we reproduced and confirm the results on its spectroscopy in Fluorescence Ratio Imaging of Free Na^+ in Individual Cells by Harootunian A.T. Kao J.P.Y. Eckert B.K. and Tsien R.Y., J. Biol. Chem. 1989, 264, 19458-19467. The imaging hardware of the PTI ImageMaster™ allowed the acquisition of fluorescence excitation spectra directly, using a sensitive video camera that was also used to image the samples

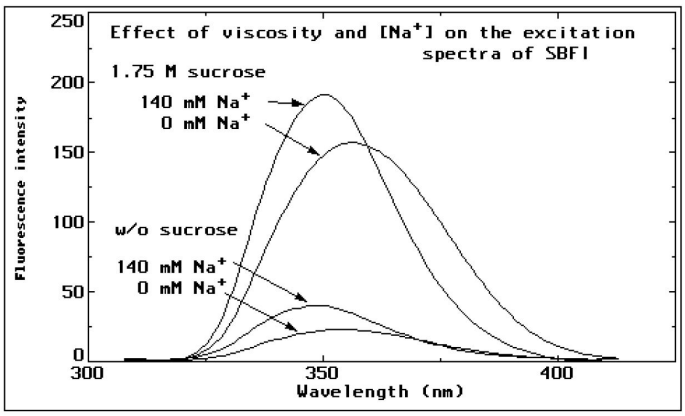
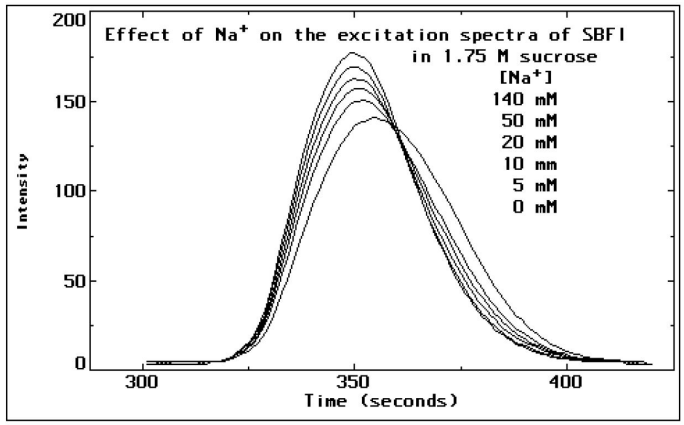
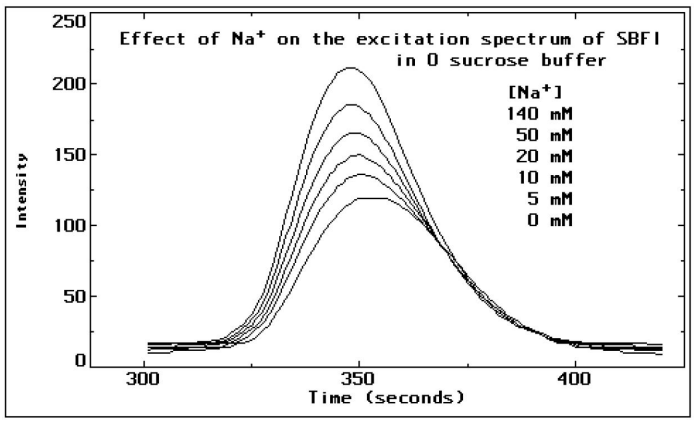


Figure 5: The quantum yield of SBFI fluorescence is 4-6 fold greater in the presence of 1.75 M sucrose than in a sucrose-free buffer.





Figures 6 and 7: The spectral shift that occurs upon Na binding is greater in 1.75 M sucrose than in a sucrose-free buffer, facilitating the ratiometric measurement of [Na+]i.

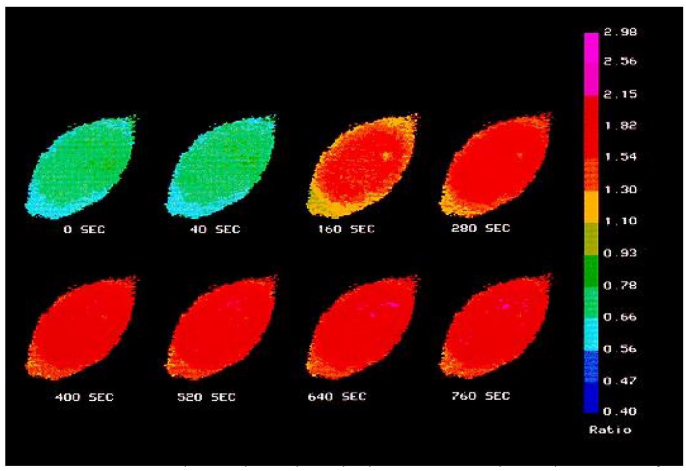


Figure 8: SBFI was observed to indicate higher Na ratios in the nuclear region of VSM cells than in the cytoplasmic region. The effect persisted even in the presence of a mixture of gramicidin/monensin/nigericin, which should abolish Na+, K+ and pH gradients. This leads to the conclusion that the two distinct regions should be calibrated using two different calibration functions, one for the nuclear and another for the cytoplasmic region.

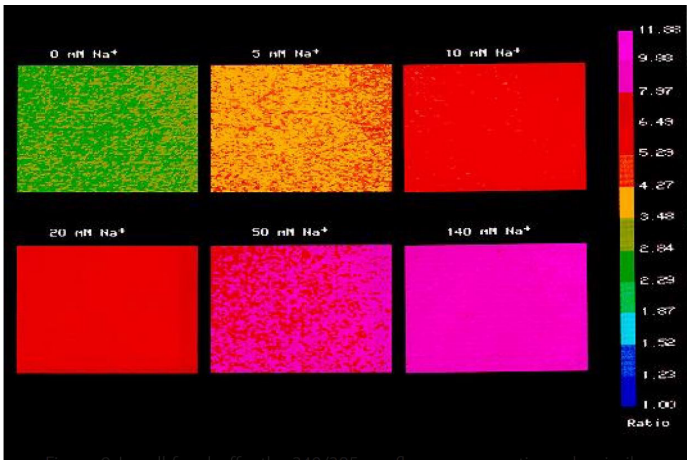


Figure 9: In cell-free buffer the 340/385 nm fluorescence ratio under similar conditions of [Na^+] underwent a much greater change than within VSM cells. Thus, such a buffer-based calibration which, if carefully done, can be used in Fura-2 experiments, but will not be adequate to successfully approximate intracellular [Na^+] concentrations

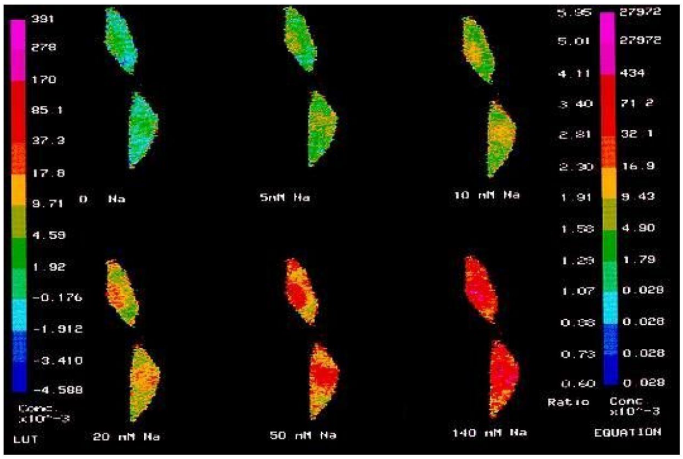


Figure 10: For this composite image, rat aortic vascular smooth muscle cells were clamped at calibrating Na^+ concentrations using gramicidin, monensin and nigericin. The ratio scale was calibrated using a LUT constructed on the basis of Region of Interest photometry from the nuclear region (left scale) as well as based on the concentration equation (right scale).

R_{max} and R_{min} were 4.3 and 1.1 respectively, and b was measured from the fluorescence excitation spectra to be 1.32. A match in the two scales was achieved in the physiologically meaningful region using a K_d of 22 mM.

Note: Increasing the number of calibrating points in the look-up table would provide an even closer match in the extreme regions of the scale.

pH

Not having to worry about properly cooling a reflector allows you to concentrate on your work. And, there is no chance of a catastrophic failure ruining an experiment or damaging equipment.

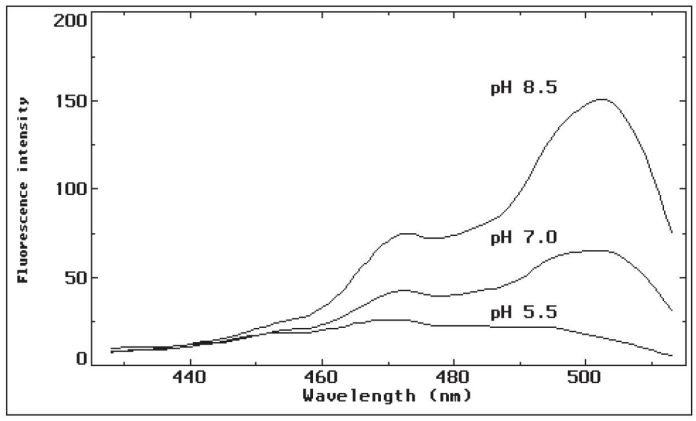


Figure 11: BCECF-loaded VSM cells clamped at calibrating pH values with 10 µM nigericin to abolish transmembrane [H^+] gradients were imaged using the technique of fluorescence video microscopy. With the video camera serving as a fluorescence detector, the fluorescence excitation spectra of the pH-sensitive dye BCECF within the cells may be acquired by scanning the excitation monochromators of the ImageMaster™.

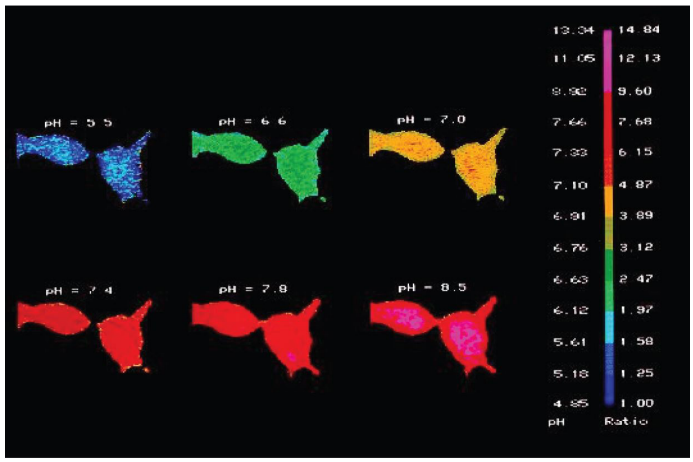


Figure 12: Fluorescence excitation spectra of BCECF at three different pH values were obtained with a SIT video camera. Ratioing the fluorescence images obtained with 500 and 440 nm excitation, a set of pseudocolor images are obtained that serve to calibrate the pH scale.

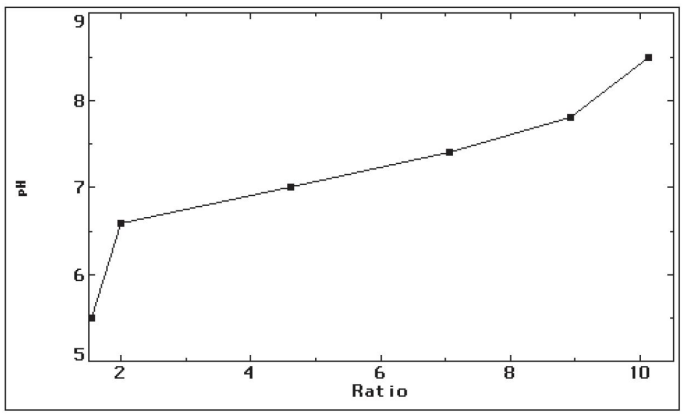


Figure 13: The calibration function is linear in the pH range 6.5-7.5. Changes outside this range may be calibrated by increasing the number of calibrating points in the LUT to span the pH range of interest.

HORIBA

info.sci@horiba.com

horiba.com/fluorescence

USA: +1 732 494 8660
UK: +44 (0)1604 542 500
China: +86 (0)21 6289 6060
France: +33 (0)1 69 74 72 00
Italy: +39 06 51 59 22 1
Brazil: + 55 (0)11 2923 5400
Germany: +49 (0) 6251 8475 20
Japan: +81 (0)3 6206 4721
Other: +1 732 494 8660