HORIBA Scientific

Advantage of Using a Scanning Monochromator for Ratio Fluorometric Experiments

OSD-AN-118

ELEMENTAL ANALYSIS
FLUORESCENCE
GRATINGS & OEM SPECTROMETERS
OPTICAL COMPONENTS
FORENSICS
PARTICLE CHARACTERIZATION
RAMAN
SPECTROSCOPIC ELLIPSOMETRY
SPR IMAGING

Ten advantages of using a scanning monochromator in ratio fluorometric experiments studying intracellular cation concentrations.

ADVANTAGE #1

Avoid the gross overestimation of $[Ca^{++}]_i$ due to the presence of unhydrolyzed dye.

Measuring the fluorescence excitation spectrum of the indicator dye in situ confirms its adequacy for a meaningful measurement. For example, to study changes in the intracellular concentration of calcium, cells are loaded with the membrane-permeable acetoxymethyl form of the calcium-sensitive dye Fura-2. Intracellular esterases then cleave the acetoxymethyl group, thus allowing the chelation of Ca^{+}. The unhydrolyzed portion of Fura-2/AM is calcium-insensitive and will show up as a red-shifted peak or shoulder at 390-400 nm. The presence of unhydrolyzed dye affects calibration significantly. In order to convert measured 340/380 nm fluorescence ratios into calcium concentrations using the analytical expression:

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

Grynkiewicz et al. (1985)
J.Biol. Chem. 260, 3340-3450

R_{max} and R_{min} have to be determined from the measurement. When at the end of a measurement the intracellular dye is saturated with calcium by using a Ca^{++} ionophore and saturating concentrations of calcium, the calcium-insensitive AM form will not undergo a wavelength shift as does the sensitive form. The contribution at 380 will be due mostly to the Ca-insensitive form resulting in drastically diminished 340/380 ratios. Figure 1 illustrates the effect of residual unhydrolyzed dye on the 340/380 nm ratio. Compared to the sample not containing unhydrolyzed dye (R_{max}=24), the 340/380 nm ratio drops to R_{max}=8, clearly an artifact. The consequent drop in dynamic range will lead to a gross overestimation of any observed Ca^{++} concentration change. (A simple substitution into the calibration equation will show that for a ratio change in the physiological range from 1 to 2, the extent of overestimation of $[Ca^{++}]_i$ will be 3.7-fold.)

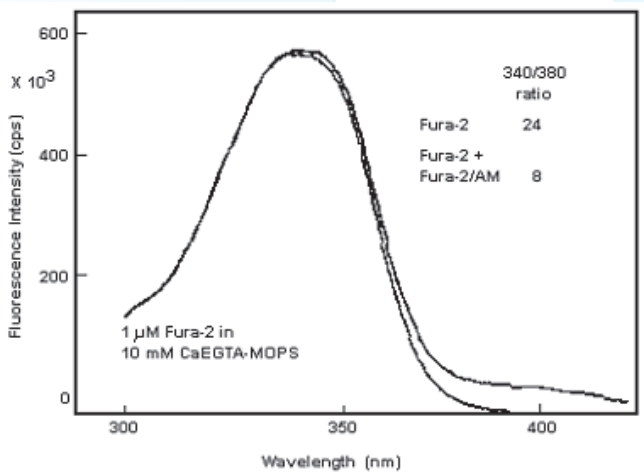

Figure 1

ADVANTAGE #2

Avoid the over- or under-estimation of $[Ca^{++}]_i$ due to a change in the background signal during the experiment.

A diminished value of the R_{max} ratio can also be due to an increase in the level of the background signal. This happens quite often as a result of the addition of various chemicals during the course of the measurement. On the other hand, the addition of detergents such as digitonin or triton as part of the calibration routine to solubilize membranes, can actually lower the background due to the solubilization of suspended light-scattering particles in

the medium. If real-time background subtraction is carried out and the background level changes, the data will be in error.

A quick check of the excitation spectrum of the Ca-saturated dye in the spectral region where it does not absorb (420-450 nm) will provide a measure of the change in background signal and allow for subsequent circumspect determination of the true R_{max}.

ADVANTAGE #3

The narrow bandpass of monochromators allows the correct value of R_{max} to be determined for calibration.

The excitation monochromators provide continually adjustable bandpass from 0.25 nm to 25 nm with the standard 1200 lines/mm grating. The narrower the bandwidth of the excitation, the greater dynamic range of measurement may be obtained. For measurements of intracellular [Ca^{++}] or pH using Fura-2 or BCECF respectively, we typically use 2 or 3 nm bandpass on the monochromators of the PTI DeltaScan illuminator. Most filter systems use interference filters with 10 nm bandpass or more. Figure 2 illustrates the effect of increasing bandwidth of the excitation light on the 340/380 nm fluorescence ratio.

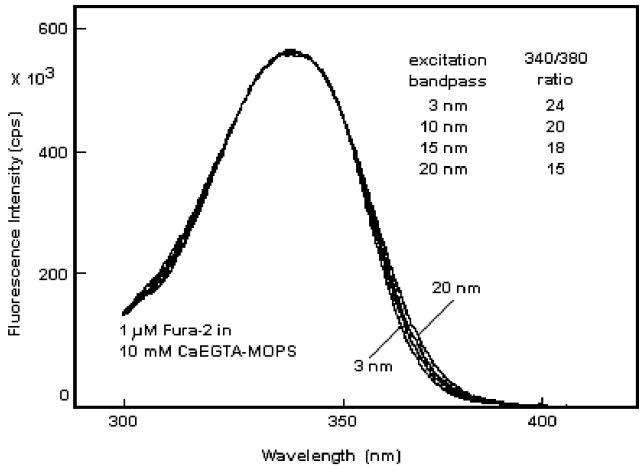

Figure 2

ADVANTAGE #4

Excitation at the exact isobestic point.

In certain experimental protocols it is desirable to choose one of the excitation wavelengths at the isobestic point of the dye. In the case of Fura-2, the fluorescence intensity at the isobestic point is invariant with $[Ca^{++}]_i$ and therefore provides a measure of events independent of the $[Ca^{++}]_i$ such as light scatter, dye leakage or shape changes. The wavelength of the isobestic point may be anywhere in the range 356-362 nm, depending on the intracellular environment, and the exact positioning is critical. Even an error of 1 nm will result in a [Ca^{++}]i-dependent contribution, defeating the purpose of the measurement.

ADVANTAGE #5

Choice of any combination of excitation wavelengths and bandpass, depending on the dye or dyes being used.

ADVANTAGE #6

Allows the selection of the best microscope objective for the experimental task at hand, in terms of percent transmission of excitation light.

Microscope objectives are highly sophisticated assemblies of optical elements with multiple optical surfaces. Light transmission, especially in the UV region, can be adversely affected. By measuring the excitation spectrum of the dye to be used, the transmission properties of available objectives can be checked. Depending on the quality of the microscope objectives available for work with Fura-2, for example, you may have to measure 345/380, 347/380 or 352/380 ratios instead of the ubiquitously quoted 340/380 in order to obtain acceptable signal-to-noise ratios in the measurements. (Many objectives have negligible transmission at 340 nm.)

ADVANTAGE #7

By measuring the fluorescence excitation spectra of the sample loaded with multiple fluorescent probes, the optimum wavelengths of excitation and emission may be established.

Interest in studying several intracellular parameters simultaneously has spawned suggestions of using combinations of two or even more dyes. For work with multiple probes it is essential that the amount of spectral overlap between the probes, and consequently interference between them, be assessed. Since the fluorescence quantum yield of the dyes may be significantly different (e.g. Fura-Red and BCECF, used for calcium and pH measurements respectively), depending on their relative concentrations, the optimal wavelengths of excitation and observation may be, and usually are, different from those used with the individual dyes. It is vital that the two parameters [Ca^{++}] and pH be measured independently of each other in spite of the spectral overlap between their fluorescence excitation and emission spectra.

Figure 3 indicates the results of an improper choice of measurement parameters to obtain a meaningful titration of two dyes simultaneously. The pH titration in the range pH 7.0-7.2 was clearly unsuccessful. Using the very same buffers with the properly chosen measurement conditions, established on the basis of measuring the excitation spectra of the dye pair, a successful simultaneous double titration is easily obtained (Figure 4).

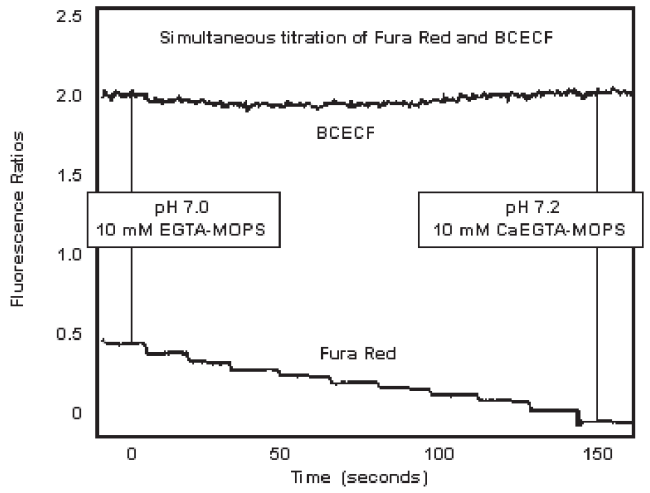

Figure 3

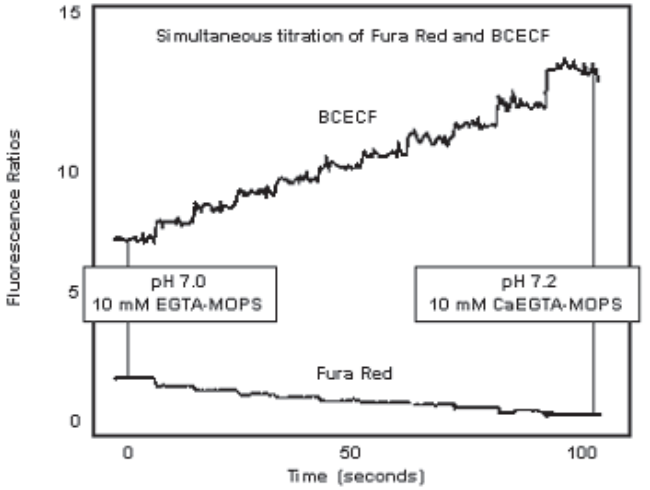

Figure 4

ADVANTAGE #8

Scanning allows the detection of the inner filter effect that is indicative of the cells having been overloaded with indicator dye. A cell overloaded with Fura-2, for example, may exhibit significant buffering of intracellular calcium.

The inner filter effect means that a significant portion of the excitation light is absorbed by the sample, and successively deeper layers of the sample will be excited by a different spectral distribution of light that is depleted in light of wavelengths of maximum absorption. Consequently, the 340/380 nm ratios will be drastically altered. The effect is easily detected by the characteristically distorted excitation spectrum while the value of the 340/380 nm ratio or the appearance of the fluorescent cell through the microscope does not indicate anything seriously amiss (Figure 5). In the figure, the fluorescence excitation spectra of two adjacent Fura-2-loaded cells obtained with the PTI ImageScan is presented. The fluorescence detector used was a sensitive SIT camera.

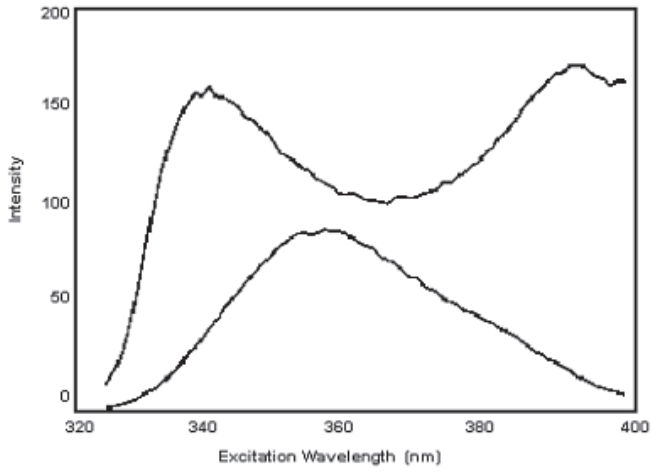

Figure 5

ADVANTAGE #9

Detection of electronic excitation energy transfer between indicator molecules, allowing the calculation of intermolecular distances.

When electronic excitation energy transfer occurs between donor and acceptor, the contribution from the fluorescence excitation bands of the donor molecule can be detected in the acceptor's fluorescence excitation spectrum. The spectral overlap integral between donor fluorescence and acceptor absorption allows the calculation of intermolecular distances between donor and acceptor molecules.

ADVANTAGE #10

Monochromators do not exhibit degradation of performance over prolonged use, whereas optical filters tend to solarize, degrade, crack, smudge and get lost over time.

This technical note was first published by Photon Technology International and reprinted with permission.

HORIBA Scientific

info.sci@horiba.com

www.horiba.com/scientific

USA: +1 732 494 8660
UK: +44 (0)20 8204 8142
China: +86 (0)21 6289 6060
France: +33 (0)1 69 74 72 00
Italy: +39 2 5760 3050
Brazil: + 55 11 2923 5400
Germany: +49 (0) 6251 8475-0
Japan: +81 (75) 313-81231
Other: +1 732 494 8660