Published in final edited form as:

Cell Calcium. 1995 July ; 18(1): 64–75. doi:10.1016/0143-4160(95)90046-2.

Possibility of simultaneously measuring low and high calcium concentrations using Fura-2 and lifetime-based sensing

H. SZMACINSKI, J.R. LAKOWICZ

Center for Fluorescence Spectroscopy, Department of Biological Chemistry, University of Maryland School of Medicine, Baltimore, Maryland, USA

Abstract

We characterized the fluorescence probe Fura-2 for calcium measurements using frequency-domain phase-modulation fluorometry. By the use of different excitation wavelengths from 345 to 380 nm, the apparent calcium dissociation constants can be altered from 41 nM to 1.92 μM Ca^{2+} . This change in apparent K_d results from changes in the relative extent of excitation of the calcium-bound and calcium-free forms, and the excitation wavelength-dependent contribution of each form to the intensity decay. These results indicate that lifetime-based measurements with Fura-2 can be used for imaging of calcium over a wide range of concentrations. An additional favorable feature of Fura-2 is that the calcium-free form can be almost exclusively excited at wavelength of 390 nm or longer, and can thus be used as a reference providing the lifetime in the absence of calcium, without removing the calcium. Additionally, exposure of Fura-2 to intense illumination shifts but does not distort the frequency response. For cellular imaging, these favorable properties of Fura-2 may allow calibration of the calcium concentrations without the use of ionophores.

In the last 10 years, substantial progress has been made towards creating fluorescent indicators which can provide rapid quantitative measurements of the free intracellular Ca^{2+} concentration in a variety of physiological systems and in the cytoplasm of living cells [1,2]. Their structures share nearly identical binding sites for Ca^{2+} as the selective chelator BAPTA, (1,2-*bis*(2-aminophenoxy)ethane- $\text{N},\text{N},\text{N}',\text{N}'$ -tetraacetic acid), as introduced by Tsien [3]. There are two designs for the fluorescent Ca^{2+} indicators. First, the aromatic BAPTA ring has been incorporated into heterocyclic systems, resulting in indicators such as Quin-2 [3], Fura-2 and Indo-1 [4], Fluo-3 and Rhod-2 [5], and the newest – STBT (styryl-benzothiazole) [6] and BTC (benzothiazol-coumarin) indicators [7]. These probes exhibit a significant shift in their absorption and/or excitation spectra and increase of quantum yields on Ca^{2+} binding. Fluo-3 and Rhod-2 can be excited with visible wavelengths and display changes in quantum yield, but there are no shifts in the absorption or emission spectra. The properties and biological applications of several of the most used Ca^{2+} indicators are well described elsewhere [8,9].

The second design of Ca^{2+} indicators uses high quantum yield fluorophores with visible excitation wavelengths such as fluorescein and rhodamine derivatives, conjugated to BAPTA

through an amide linker. This synthetic method resulted in probes like the Calcium Color series (Calcium Green, Orange, and Crimson) [10,11], Calcium Green-2 (two fluorescein dyes conjugated to BAPTA), and Calcium Green-5N (fluorescein dyes conjugated to modified BAPTA) [12]. This approach is very versatile since a large number of dyes with desired fluorescence properties are known, the fluorescence properties of the dye are retained, and conjugation has little effect on the fluorophore. These conjugated probes display long excitation and emission wavelengths and good quantum yields, but no spectral shifts upon Ca^{2+} binding. The absence of a wavelength-ratiometric Ca^{2+} probe with visible wavelength excitation is the main reason that Fura-2, in spite of UV excitation and associated problems [13–19], is still the most widely used Ca^{2+} indicator [20].

The lack of wavelength-ratiometric probes is a major limitation for quantitative Ca^{2+} imaging. This is because most biological imaging applications rely on fluorescence intensity measurements. In fluorescence microscopy it is difficult to use the intensity data because of the unknown concentrations of the probe at each point in the image, and changing probe concentrations due to diffusion and/or photobleaching. This problem can be circumvented using fluorescence lifetime imaging microscopy (FLIM) or lifetime-based sensing, which does not require ratiometric probes [10,21]. Lifetime-based sensing or imaging requires that both forms of indicator (free and ion bound) be fluorescent but with different lifetimes. The mean lifetime does not depend on total intensity of the probe, but reflects the fractional contribution of the Ca^{2+} -free and Ca^{2+} -bound forms of the probe, and thus the Ca^{2+} concentration. Fluorescent lifetime indicators are already known for Ca^{2+} , Mg^{2+} , H^+ , Na^+ and K^+ [21]. Among them are the calcium ratiometric probes Quin-2, Fura-2 and Indo-1. Quin-2 displays an excellent lifetime change on calcium binding [22–24], and has been used for intracellular Ca^{2+} imaging of COS cells [25], but requires complex corrections because of photodecomposition [25]. Indo-1 also displays a useful change in lifetime on calcium binding on the long wavelength side of its emission spectrum and can be used for lifetime imaging with a single excitation wavelength [26].

A significant limitation of all Ca^{2+} indicators is the limited range of Ca^{2+} concentrations which can be measured with a single fluorophore. This limitation can be overcome using fluorescence lifetime imaging, a wavelength ratiometric probe, and various excitation wavelengths. The goal of this report is to describe the possibility for using Fura-2 with phase-modulation methods to measure a wide range of Ca^{2+} concentrations. It has already been reported that the lifetime of Fura-2 is sensitive to calcium [21,27] but, since the first report in 1991, no attempt has been made to use the lifetime of Fura-2 for calcium imaging. We now show that, by selection of the excitation wavelengths, the apparent calcium dissociation constants (K_d) of Fura-2, as measured by phase or modulation, can range from 41 nM to 1920 nM, which is about a 50-fold increase in the range of measurable calcium concentrations (about 15-fold towards higher and 3-fold towards lower) compared to that possible with wavelength ratiometric intensity measurements and a single K_d value near 140 nM. This range of K_d values is the result of different relative contributions of the Ca^{2+} -bound and Ca^{2+} -free forms of the probe to the intensity decays at different excitation wavelengths. We also found that the intensity decays of Fura-2 are relatively insensitive to intense illumination, which suggests that lifetime imaging using Fura-2 will not be sensitive

to photobleaching and/or phototransformation of the probe. Finally, we describe some possible effects of local viscosity on the intensity decays of Fura-2.

Materials and methods

Fura-2 and Calibrated Calcium Buffer Kit II were obtained from Molecular Probes (Eugene, OR, USA). Absorption spectra were measured using a Perkin-Elmer Lambda 6 UV/vis spectrophotometer. The samples were freshly prepared before each measurement and were carried out at room temperature (20°C). Fluorescence intensity decays were measured with frequency-domain instrumentation described in [28]. The light source was the cavity-dumped and frequency-doubled output of pyridine 2 dye laser from 345 to 380 nm. The emission was observed through long wave pass filter (above 445 nm) or interference filters with a bandpass of 10 nm. The frequency-domain data were used to determine the intensity decay law using the multi-exponential model:

$$I(t) = \sum_{i=1}^n \alpha_i e^{-t/\tau_i} \quad \text{Eq. 1}$$

where α_i is the pre-exponential factor, τ_i is the decay time, and n number of exponential components. The mean decay time is given by:

$$\bar{\tau} = \alpha_i \tau_i^2 / \sum_j \alpha_j \tau_j \quad \text{Eq. 2}$$

The intensity decays were also fit to a global model in which the decay times were assumed to be independent of Ca^{2+} concentration (k), but the values α_{ik} to reflect changes in the fractional amounts of each species. In this case the intensity decay of each Ca^{2+} concentration is given by:

$$I_k(t) = \sum_{i=1}^3 \alpha_{ik} e^{-t/\tau_i} \quad \text{Eq. 3}$$

where the subscript k indicates the Ca^{2+} concentration. All calcium concentrations refer to free calcium. For the intensity decay measurements we used magic angle conditions to eliminate the effects of Brownian rotation.

In phase-modulation fluorometry, the sample is excited with an intensity modulated light source. The emission is delayed in time relative to the modulated excitation. At each modulation frequency ($\omega = 2\pi f$) this delay is described as the phase shift (θ_ω), which increases from 0° to 90° with increasing modulation frequency (ω in radians/s). The finite time response of the sample also results in demodulation of the emission by a factor m_ω which decreases from 1.0 to 0.0 with increasing modulation frequency. The phase angle (θ_ω) and the modulation (m_ω) are separate measurements, each of which are related to the intensity decay parameters, α_i and τ_i , and modulation frequency ω by:

$$\theta_{\omega} = \arctan(N_{\omega}/D_{\omega}), m_{\omega} = (N_{\omega}^2 + D_{\omega}^2)^{\frac{1}{2}} \quad \text{Eq. 4}$$

where

$$N_{\omega} = \frac{1}{J} \sum_{i=1}^n \frac{\omega \alpha_i \tau_i^2}{1 + \omega^2 \tau_i^2}$$

$$D_{\omega} = \frac{1}{J} \sum_{i=1}^n \frac{\alpha_i \tau_i}{1 + \omega^2 \tau_i^2}$$

$$J = \sum_{i=1}^n \alpha_i \tau_i \quad \text{Eq. 5}$$

The values α_i and τ_i are determined by minimization of the goodness-of-fit parameter:

$$\chi_R^2 = \frac{1}{v} \sum_{\omega, k} \left(\frac{\Phi_{\omega} - \Phi_{\omega}^c}{\delta \Phi} \right)^2 + \frac{1}{v} \sum_{\omega, k} \left(\frac{m_{\omega} - m_{\omega}^c}{\delta \omega} \right)^2 \quad \text{Eq. 6}$$

where the subscript c indicates calculated values for known values of α_i and τ_i , and $\delta \Phi$ and $\delta \omega$ the experimental uncertainties in the measured phase and modulation values. The sum can extend over a single set of data (ω), or over multiple data for both different frequencies (ω) and calcium concentrations (k). For the latter global analysis, we assumed the values of τ_i were global, i.e. were the same at all calcium concentrations.

Results and discussion

Absorption spectra

Absorption spectra of Fura-2 with various calcium concentrations are shown in Figure 1. The absorption spectra free and Ca^{2+} -bound forms of Fura-2 show a spectral shift with isobestic point at 352 nm. The spectral shift and relative heights can be different in the excitation spectra (not presented) because of different quantum yields for free Fura-2 and the Ca^{2+} -bound form, 0.23 and 0.49, respectively [4]. The relative emission spectra (not shown) of free and Ca^{2+} -bound form depend on the extinction coefficients at the excitation wavelength and relative quantum yields. Excitation with wavelengths longer than the isobestic point (352 nm) preferentially excites Ca^{2+} -free form of Fura-2, and excitation below 352 nm preferentially excites the Ca^{2+} -bound form of Fura-2. We used excitation wavelengths from 345 to 380 nm.

Frequency-domain intensity decays

We examined the frequency-domain intensity decays of Fura-2 with various Ca^{2+} concentrations. Three representative frequency-domain intensity decays of free, saturated with Ca^{2+} (40 μM) and a partially saturated (100 nM free calcium) Fura-2 are shown in Figure 2. The shift in the frequency-response to lower frequencies in the presence of Ca^{2+} indicates an increase in the mean lifetime upon Ca^{2+} binding. These results indicate that Fura-2, with a single excitation wavelength, can be used for fluorescence lifetime imaging of Ca^{2+} .

We measured the frequency responses of six samples at various calcium concentrations from 0 to 40 μM with an excitation wavelength of 354 nm. The fluorescence intensity decays of Fura-2 were found to be multi-exponential at all calcium concentrations. Acceptable fits could be obtained with three decay times at all calcium concentrations, except for free and calcium saturated samples, where the two exponents model was adequate. We used global multi-exponential intensity decay analysis (Eq. 2) to determine the components associated with each form of Fura-2 and reveal their contribution at various calcium concentrations. The results of this global analysis are summarized in Figure 3. The three decay times were found to be 0.29, 1.09 and 1.72 ns; the amplitudes are dependent on the calcium concentration (Fig. 3). The components with $\tau_F = 1.09$ ns and $\tau_B = 1.72$ ns appear to be associated with calcium free and bound forms, respectively. The amplitude of the third component ($\tau_3 = 0.29$ ns) is insensitive to calcium, with only a small decrease at very high calcium concentrations. A form of Fura-2 insensitive to calcium has been reported earlier based on intensity and spectral measurements [29].

It is important to note that the intensity decay contributions of the free (τ_F) and Ca^{2+} -bound (τ_B) components at each calcium concentration are strongly dependent on the excitation wavelength. The fractional contribution of the free form at each calcium concentration, except of calcium saturated sample, can be significantly enhanced using excitation wavelengths longer than 352 nm. The use of excitation wavelengths above 352 nm results in a shorter mean lifetime and higher apparent dissociation constants for calcium. At excitation wavelengths longer than 354 nm we can expect a shift of α_F and α_B plots presented in Figure 3 towards higher calcium-concentrations. One can imagine a family of such plots which span low and high calcium concentrations obtained for various excitation wavelengths. The plots of amplitude α_F and α_B versus Ca^{2+} concentration may be regarded as the calibration curves, but such detailed information usually is not available in fluorescence lifetime imaging. Such data require multi-exponential intensity decay analyses, which in turn require variable frequency lifetime imaging and time consuming analysis [25,30,31]. Fortunately, the Ca^{2+} concentration can be determined with phase or modulation data measured at a single modulation frequency.

For lifetime imaging, one expects to obtain data using a single light modulation frequency because multi-frequency lifetime imaging is presently time consuming. To determine the effect of different excitation wavelengths on the apparent K_d values, we measured fluorescence phase angles and modulations with a modulation frequency of 102.465 MHz using various excitation wavelengths from 345 to 380 nm. The calcium-dependent phase angles and modulations for various excitation wavelengths are shown in Figure 4, top and

bottom, respectively. Importantly, the mid-point of these calcium-dependent data shift from 41 nM to 1.92 μ M depending on the excitation wavelength. It is possible that a still wider range of apparent K_d values could be obtained with a wider range of excitation wavelengths, which was not available with our dye lasers. These data indicate that the apparent K_d value of the Fura-2 can be selected to match the needs of the cell biology experiment simply by changing the excitation wavelength. Similar responses may be expected in the frequency range from 70 to 250 MHz, as can be seen by examination of Figure 2.

One may question the use of Fura-2 as a lifetime-based calcium indicator because of the relatively small change in lifetime. An accuracy of 0.3° in phase angle at the steepest part of the curves (Fig. 4, top) will yield $[Ca^{2+}]$ values which are accurate to ± 9 nM at 345 nm, ± 30 nM at 365 nm and ± 130 nM at 380 nm excitation. Similar accuracy can be obtained from modulation with $m = 0.5\%$. These results show that even with moderate changes in lifetime, the phase-modulation method can provide accurate measurements of intracellular calcium.

Apparent dissociation constants

Table 1 summarizes the apparent dissociation constants calculated from the phase angle and modulation data in Figure 4. The apparent dissociation constants for Fura-2 were calculated using plots of $\log [(X - X_{\min})/(X_{\max} - X)]$ VS $\log [Ca^{2+}]$ (Hill plot), where X refers to phase, modulation or fluorescence intensity. The apparent dissociation constants (Table 1) are equal to Ca^{2+} concentration at the zero intercept of the Hill plots using the respective measurable parameter X (phase angle, modulation or intensity). The practical range of concentration that can be measured is within a factor of 0.1–10 of the apparent dissociation constant.

The data in Figure 4 and Table 1 show that phase-modulation measurement using Fura-2 allows accurate quantitation of low calcium concentrations as well as large Ca^{2+} fluxes. Low Ca^{2+} concentrations (below 10 nM) can be measured using an excitation wavelength near 345 nm (Fig. 4, curve A) or shorter. Alternatively, high Ca^{2+} concentrations (up to 20 μ M) can be obtained by using excitation wavelengths near 380 nm (Fig. 4, curve F). These illustrations show that Fura-2 can be used for a significantly wider range of Ca^{2+} concentrations than provided by the intensity-ratiometric method with a dissociation constant about 140 nM. Low calcium concentrations have been usually investigated using Quin-2 because of its strong affinity to calcium with a K_d of 60 nM [32].

For comparison with Fura-2, the dissociation constant of other calcium indicators are included in Table 1. Current fluorescent indicators can cover a wide range of calcium concentrations, but, when using intensity or wavelength ratio measurements, each indicator must be used for the particular range determined by its dissociation constant. It is important to note that only lifetime measurements with wavelength-ratiometric indicators provide an extended range of analyte concentrations. For calcium probes which do not display a spectral shift, the value of apparent dissociation constant from phase or modulation measurements may differ significantly from its true K_d , but the apparent K_d value cannot be changed by choice of excitation or emission wavelength, since the fractional intensities of each form of these indicators (free or Ca^{2+} -bound) are independent of the excitation and

emission wavelength. The shift of the apparent dissociation constant relative to the true K_d for non-ratiometric probes depends mostly on ratio of lifetimes τ_B/τ_F . If the ratio of τ_B/τ_F is higher than 1.0, apparent dissociation constants from phase angle and modulation are expected to be lower than the true K_d (see Calcium Color series in Table 1), and the reverse is true if τ_B/τ_F is less than 1.0.

Simultaneous measurements of low and high calcium concentrations using only one fluorescent indicator should be advantageous over the use of two different indicators. To measure calcium transients in skeletal muscle fibers, two calcium indicators were proposed, Fura-2 for low calcium and the absorbance indicator antipyrylazo III for high calcium [33]. To measure high $[Ca^{2+}]_i$, mag-Fura-2 has been used, which has an affinity for Ca^{2+} of 20–50 μM [34–36]. However, mag-Fura-2 also binds Mg^{2+} with an affinity 1.5 mM which is close to the normal intracellular free Mg^{2+} (0.4–1.0 mM). Mg^{2+} binding to mag-Fura-2 produces changes in fluorescence that are indistinguishable from Ca^{2+} binding. This may compromise interpretation of the data obtained with mag-Fura-2 [35]. A much smaller effect of Mg^{2+} has been observed on the fluorescence of Calcium Green-5N (CG5N) [37]. Intensity methods using CG5N for measuring large changes in Ca^{2+} are problematic because it lacks the ratiometric capabilities. We expect that CG5N is very likely to display calcium-dependent lifetime as it is structurally similar to Calcium Green [10]. However, its apparent dissociation constant from phase angle and modulation may be shifted significantly towards lower values compared to the actual K_d of 4.3 μM [37] because of significant enhancement of quantum yield on calcium binding and expected high value of τ_B/τ_F , as has been observed for Calcium Green [10]. A new indicator from Molecular Probes is an excitation ratiometric calcium indicator BTC (benzothiazocoumarin) with K_d of 7 μM , which is supposed to be useful in Ca^{2+} concentrations in the micromolar range [7]. Our preliminary data (not published) show that the lifetime BTC is sensitive to calcium, with mean lifetimes of 0.7 and 1.4 ns for excitation at 515 nm for the calcium free and bound forms, respectively. Using BTC with its absorption shifts and phase-modulation method, one may obtain similar results as for Fura-2, probably for the higher calcium concentrations.

Photobleaching of the fluorescence of Fura-2

Significant difficulties in wavelength-ratiometric or lifetime imaging are changes in the excitation spectra, emission spectra or intensity decay due to the intense illumination present in a fluorescence microscope. In principle, if the probe photobleaches and becomes non-fluorescent, then the measured calcium concentration should be independent of the extent of photobleaching. However, in practice, one often observes not perfect photobleaching, but rather phototransformation to a different species with different spectra or sensitivity to calcium.

To determine the extent of photobleaching and/or phototransformation of Fura-2, we investigated the effect of intense illumination on a small sample volume of calcium free and bound form of Fura-2. The frequency-domain intensity decays before and after illumination (about 25% decrease in intensity) are shown in Figure 5. For clear presentation, data points before photobleaching are not present in Figure 5, only fitted functions as dashed lines. The differences in frequency responses between the before and after photobleached samples

indicate a modest change in intensity decay. These results show that intense illumination of Fura-2 is associated with phototransformation or photodecomposition. We used a global two exponential analysis separately for the intensity decay of the free and bound forms. The two decay time model was adequate in this case because the Fura-2 was either Ca^{2+} -free or saturated with Ca^{2+} , which is different from Figure 3 where intermediate calcium concentrations were needed. The two decay times were global for the before and after photobleaching data, and the amplitudes were nonglobal. The detailed analysis of intensity decays is summarized in Table 2. Photobleaching of Fura-2 results in an increased contribution of the short decay time, here referred to as τ_1 which appears to be insensitive to calcium (in Fig. 3 this component was called $\tau_3 = 0.29$ ns). Its fractional contribution (α_1) displays an increase of 0.132 and 0.192 for the calcium free and bound forms, respectively. Formation of fluorescent photoproducts of Fura-2 has been reported earlier by Becker and Fay [38], who observed no differences in the photobleaching in the presence and absence of calcium and for different excitation wavelengths. They concluded that the calcium-sensitive species diminished in concentration producing an insensitive fluorescent specie(s). The lifetime data can be explained in a similar manner. However, the effect of illumination seems to be more complex because an insensitive form exists before photobleaching. Its increase with continued illumination may be due phototransformation (calcium-sensitive form transforms to a fluorescent insensitive form), or due to photodecomposition (calcium-sensitive form changes to a non-fluorescent form). These effects may not alter the calcium-sensitive lifetime of Fura-2 since both forms (calcium free and bound) decrease to a similar extent. The mean lifetime is shorter after illumination, but relative phase angles and modulations remain without significant changes. The photobleaching behavior of Fura-2 is advantageous compared with Quin-2 where we have sensitivity to illumination, which requires complex corrections and, in some cases, cannot be properly corrected [25].

An important characteristic of the phase and modulation data is the uniform shift of the frequency response upon intense illumination. This suggests that one may be able to avoid calibration using EGTA or ionomycin. In cellular applications of Fura-2, the effect of photobleaching can be estimated by measuring intensity decay with long excitation wavelength (390–400 nm). Excitation at long wavelength can be regarded as a type of calibration, similar to that with using the EGTA, to measure properties of the calcium free form (e.g. phase angle and modulation). This effect can be seen by examination of the dependence of the phase-angle of Fura-2 on excitation wavelengths (Fig. 6). Excitation at 380 nm can be used for calibration if the calcium concentration is below 100 nM (Fig. 6). In this case, phase angle (Fig. 6) and modulation (not shown) values will reflect only the free form. Higher calcium concentrations will require longer excitation wavelengths for better discrimination between calcium free and bound forms. This kind of calibration does not require buffering with EGTA or using ionomycin after completion of the experiments with living cells.

Lifetime and anisotropy of Fura-2 in viscous solution

A further complication of using fluorescent calcium indicators is their possible interaction with intracellular biomolecules. It is likely that such interactions alter the absorption and emission spectra, and may alter the K_d values. Measurements of the fluorescence anisotropy

can reveal whether the probe is bound to a macromolecule, which would increase its rotational correlation time. Surprisingly, little information is available about the anisotropy properties of Fura-2. We measured emission spectra, excitation polarization spectra and intensity decays for calcium free and bound form in a mixture of buffer/glycerol 6:1 (v/v) (viscosity about 16 cP at 20°C). The emission maxima were at 506 and 493 nm for the calcium-free and bound forms, respectively. The mean lifetimes of the free and Ca²⁺-bound forms were found to be 1.72 and 2.1 ns, respectively. In this solvent, the increase in lifetime on calcium binding resulted in a 6° and 5.5% change in phase angle and modulation, respectively. Such small changes make difficult the use of Fura-2 as a lifetime indicator in viscous environments. The fluorescence enhancement in viscous solutions has been observed from excitation spectral changes for both forms of Fura-2 [39–41]. The spectral observations in viscous solution, relative to those in water solutions, indicate that the quantum yield of the free form increases more than that of the Ca²⁺-bound form [39,40]. These observations are in agreement with our lifetime measurements. In this viscous solvent, the mean lifetime of the free form increases from 1.09 ns to 1.72 ns, and the Ca²⁺-bound form from 1.68 to 2.10 ns, compared to the lifetimes in a buffer solution.

Two methods have been proposed to correct the viscosity effect on calcium measurements using the wavelength-ratiometric method. Poenie [39] proposed a constant correction factor, and Busa [40] suggested the use of an optimal excitation wavelength pair at which viscosity effect on the excitation spectra is minimal. With lifetime measurements, we suggest measuring the lifetime (or only phase and modulation at single modulation frequency) of free form to estimate effect of viscosity. The free form can be observed with excitation from 390–400 nm (Fig. 6). If the lifetime of the free form is 1.7 ns or longer, then the probe is probably in a viscous environment, where the lifetime is less sensitive to calcium.

Another viscosity control measurement can be achieved by fluorescence anisotropy. We measured the excitation anisotropy spectra of Fura-2 in a mixture of buffer/glycerol 6:1 (v/v) at 20°C. There was no significant dependence on excitation wavelength in the range from 310–420 nm. The anisotropy values are 0.31 and 0.28 for calcium free and bound forms, respectively. The high anisotropy value and relatively short lifetime are promising for use of Fura-2 in intracellular viscosity measurements. The calcium-free form of Fura-2 with its mean lifetime of about 1 ns may allow measurement of the viscosity in the range from 1 to about 12 cP with anisotropy changes from about 0.05 to 0.32. Of course, the possible binding of Fura-2 to proteins in the cytoplasm cannot be excluded, and may lead to significant overestimation of actual cytosolic viscosity.

Excited state reaction of Fura-2

We measured the emission wavelength-dependent intensity decays of Fura-2 for its free and calcium bound forms (Fig. 7). For both forms, we observed a dependence on emission wavelength, which was somewhat stronger for calcium bound form. The solid lines in Figure 7 represent a global double-exponential analysis. In these global analyses, the decay times were assumed to be the same at all emission wavelengths, and the amplitudes were assumed to be wavelength-dependent. We note that this analysis provides only an approximate representation of what is likely to be more complex decay law. The fractional intensity of the

short decay-time component (f_1) decreased with the emission wavelength (Table 3). However, we note that in the presence of an excited state reaction, the f_1 values do not represent the relative intensities of the individual states. For the Ca^{2+} -bound form, we observed a negative pre-exponential factor at 580 and 600 nm (Table 3), which demonstrates the presence of excited-state reaction [42,43]. Such a component clearly demonstrates that this fraction of the emission results from an excited-state process, that is formation of a product subsequent to formation of the initially excited-state. In frequency-domain measurements, excited-state reaction is suggested by the failure to reach a plateau for the phase angle at high frequencies (Fig. 7, bottom, 580 nm) [44]. The presence of an excited-state reaction could result in difficulties in use of Fura-2 because the rate and extent of the reaction may depend on the viscosity, ionic strength, pH and other additional conditions in the immediate environment of the fluorophore. A similar dependence on emission wavelength was observed for Indo-1 [26]. Recently, the complex forming reaction between Fura-2 and Ca^{2+} in the ground and excited states has been investigated by steady-state and time-resolved measurements [45].

Conclusions

Fluorescence lifetime imaging using Fura-2, when performed with excitation wavelengths ranging from 345 to 380 nm, allows measurements of Ca^{2+} concentrations ranging from 4 nM to about 20 μM .

Acknowledgements

This work was supported by grants RR-08119 and RR-07510 from National Institutes of Health.

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Fig. 1. Absorption spectra of Fura-2 in buffer (100 mM KCl, 10 mM MOPS, 0–10 mM Ca-EGTA, pH 7.2) at 20°C. Arrows indicate the excitation wavelengths: A – 345, B – 354, C – 365, D – 370, E – 375, and F – 380 nm.

Fig. 2. Frequency-domain intensity decays of Fura-2 at three representative calcium concentrations. The excitation wavelength was 354 nm and emission above 445 nm was observed using a longpass filter.

Fig. 3. Calcium-dependent amplitudes of the intensity decay of Fura-2 recovered from a global multi-exponential analysis for a number of calcium concentrations. Intensity decays were collected with excitation at 354 nm, and emission above 445 nm, at 20°C.

Fig. 4. Calcium-dependent phase angles (top) and modulations (bottom) for Fura-2 at various excitation-wavelengths. The modulation frequency was 102.465 MHz, emission above 445 nm, 20°C. Apparent dissociation constants for each excitation wavelength are given in Table 1. The excitation wavelengths are also shown in Figure 1.

Fig. 5. Frequency-domain intensity decays of Fura-2 before (dashed line and after about 25% photobleaching (filled circles). See data in Table 2.

Fig. 6. Excitation wavelength-dependent phase angles for Fura-2 at various Ca^{2+} concentrations. The modulation frequency was 102.47 MHz, emission above 445 nm, 20°C.

Fig. 7. Emission wavelength-dependent intensity decays of Fura-2 free anion (top) and Ca²⁺-bound (bottom). Excitation was 354 nm, 20°C.

Table 1

Apparent dissociation constants for Fura-2 and other calcium probes determined from intensity (I), phase angle (θ) and modulation (m) measurements. Numbers in square brackets refer to references.

<i>Probe</i>	λ_{exc} (nm)	K_d^I (nM)	K_d^θ (nM)	K_d^m (nM)
Fura-2	345	135 [4]	66 $\pm$ 3	41 $\pm$ 7
	354	156 $\pm$ 19	108 $\pm$ 15	67 $\pm$ 9
	365		258 $\pm$ 8	160 $\pm$ 10
	370		546 $\pm$ 34	341 $\pm$ 26
	375		1068 $\pm$ 52	691 $\pm$ 106
	380		1920 $\pm$ 171	1393 $\pm$ 360
Quin-2 [16]	342 ^a	48, 60 [32]	29	10
Indo-1 [19]	345	250 [4]	1130	953
CaG [10]	514	128, 189 [12]	40	14
CaO [10]	565	269, 328 [12]	250	150
CaC [10]	590	283, 205 [12]	150	100
Fluo-3 ^b	-	400 [5]	-	-
STBT ^c	-	1500 [6]	-	-
CG5N ^d	-	4300 [37]	-	-
Mag-Fura-5	-	6500 [2]	-	-
BTC ^e	-	7000 [7]	-	-

^a At longer excitation wavelengths higher values of apparent K_d s are expected from phase angle and modulation measurements.

^b The quantum yield of calcium-free form is too low for lifetime based sensing.

^c The lifetimes of the Ca^{2+} -free and bound forms are 70 and 100 ps, respectively.

^d The quantum yield of CG5N increases upon Ca^{2+} binding, similarly to the other Calcium Series probes. The apparent dissociation constants from phase angle and modulation are expected to be shifted towards lower values, as for CaG.

^e Displays an absorption spectra shift and increase of mean lifetime upon Ca^{2+} binding. Phase-modulation measurement may provide similar properties to Fura-2.

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Table 2

Intensity decay analysis before and after photobleaching of Fura-2.

Condition	τ_1	τ_2	f_1^a	α_1	f_1^a	α_1	f_1
Free form							
Before	0.33	1.21	0.321	0.116	-	-	-
After	0.33	1.21	0.453	0.186	0.132	0.070	0.070
Ca ²⁺ -bound form							
Before	0.27	1.73	0.166	0.030	-	-	-
After	0.27	1.73	0.358	0.080	0.192	0.050	0.050

^aFractional intensity $f_1 = \alpha_1 \tau_1 / (\alpha_1 \tau_1 + \alpha_2 \tau_2)$.

^bThe decay times were global parameters.

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Table 3

Emission wavelength-dependent intensity decays of Fura-2^a.

λ_{obs} (nm)	Free anion (no Ca^{2+}) ^c $\tau_1 = 0.21$ ns, $\tau_2 = 1.15$ ns		Ca^{2+} -bound (40 μ M Ca^{2+}) $\tau_1 = 0.16$ ns, $\tau_2 = 1.78$ ns	
	α_1	f_1	α_1	f_1
460 ^b	0.464	0.134	0.494	0.080
500	0.347	0.086	0.189	0.021
540	0.292	0.068	0.114	0.012
580	0.161	0.033	-0.138	-0.014
600	0.088	0.013	-0.225	-0.026

^aExcitation wavelength was 354 nm, T = 20°C.

^bWith bandpass of 10 nm.

^cGlobal fits for free anion and Ca^{2+} bound forms. For these two global fits we used $|a_1| + a_2 = 1$ and $f_1 + f_2 = 1.0$, respectively.