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Fluorescence lifetime imaging of calcium using Quin–2

J.R. LAKOWICZ¹, H. SZMACINSKI¹, K. NOWACZYK¹, M.L. JOHNSON²

¹Center for Fluorescence Spectroscopy, Department of Biological Chemistry, University of Maryland at Baltimore, Baltimore, Maryland, USA

²Department of Pharmacology, University of Virginia, Charlottesville, Virginia, USA

Abstract

We describe the use of a new imaging technology, fluorescence lifetime imaging (FLIM), for the imaging of the calcium concentrations based on the fluorescence lifetime of a calcium indicator. The fluorescence lifetime of Quin–2 is shown to be highly sensitive to $[Ca^{2+}]$. We create two-dimensional lifetime images using the phase shift and modulation of the Quin–2 in response to intensity-modulated light. The two-dimensional phase and modulation values are obtained using a gain-modulated image intensifier and a slow-scan CCD camera. The lifetime values in the 2D image were verified using standard frequency-domain measurements. Importantly, the FLIM method does not require the probe to display shifts in the excitation or emission spectra, which may allow Ca^{2+} imaging using other Ca^{2+} probes not in current widespread use due to the lack of spectral shifts. Fluorescence lifetime imaging can be superior to stationary (steady-state) imaging because lifetimes are independent of the local probe concentration and/or intensity, and should thus be widely applicable to chemical imaging using fluorescence microscopy.

Measurement of the intracellular concentrations of Ca^{2+} is of interest for understanding its role as a second messenger, and the response of cells to various stimuli. Equally important is the imaging of the local calcium concentration to study cell function, as exemplified by Ca^{2+} imaging of neuronal cells [1]. At present, most measurements of intracellular calcium or Ca^{2+} imaging are performed using fluorescence indicators [2, 3]. These dyes (Quin–2 and Fura–2) change intensity in response to Ca^{2+} . The second generation dye Fura–2 is currently preferred for a variety of chemical and biochemical reasons, as discussed elsewhere [3, 4]. The primary advantage of Fura–2 over Quin–2 appears to be the shift in its excitation wavelength in response to Ca^{2+} [5–7], which allows calculation of the calcium concentration from the ratio of the fluorescence intensities at two excitation wavelengths, thus providing a measure of the $[Ca^{2+}]$ which is independent of the probe concentration. While Quin–2 can also be used as a ratiometric probe, this use is not favored due to the need for 340 nm excitation, and its weak absorption at longer wavelengths. Nonetheless, Quin–2 has a number of advantages for measurements of Ca^{2+} , such as minimal interference for Mg^{2+} , a known stoichiometry for Ca^{2+} , and a favorable dissociation constant [8].

Please send reprint requests to : Dr Joseph R. Lakowicz, Center for Fluorescence Spectroscopy, Department of Biological Chemistry, University of Maryland at Baltimore, 660 West Redwood Street, Baltimore, MD 21201, USA.

Permanent address : Dr Kazimierz Nowaczyk, University of Gdansk, Institute of Experimental Physics, Gdansk, Poland 80952

In the present report we describe a new imaging methodology, in which the fluorescence lifetimes at each pixel are used to create the image contrast. An immediate advantage of this technique is that the Ca^{2+} probe need not display any shift in excitation or emission. Instead, our fluorescence lifetime imaging (FLIM) methodology requires that the probe lifetime change in response to Ca^{2+} . Since we now know that the lifetimes of Quin-2 are strongly dependent on Ca^{2+} , as was confirmed in a recent report [9], the FLIM method allows use of Quin-2 for measurements of $[\text{Ca}^{2+}]$ without the need for wavelength-ratio methods.

In the present report we describe how the Ca^{2+} -dependent lifetime properties of Quin-2 can be used to obtain Ca^{2+} images from the local lifetimes. The lifetime data are obtained using the frequency-domain (FD) method [10-13]. In this method the sample is excited with an intensity-modulated light source. The lifetime is obtained from the phase and modulation of the emission relative to the modulated excitation. Use of FD technology, as applied to a gain-modulated image intensifier [14-16], allows simultaneous acquisition of the lifetime information at all pixels in the image. This feature avoids the need for pixel-by-pixel scanning of the lifetime [17, 18], and will become increasingly important when the FLIM methodology is applied to cellular systems where the cells can move or change during the time required for data acquisition, or when the temporal changes in intracellular calcium are of interest.

Materials and Methods

Instrumentation for FLIM

Concept of Fluorescence Lifetime Imaging—The concept of FLIM is illustrated in Figure 1. Suppose the sample is composed of two regions, each with an equal intensity of the steady-state fluorescence. Assume further that the lifetime of the probe is several-fold higher in the central region of the object (τ_B). In the present example, we assume the longer lifetime is due to the presence of the Ca^{2+} -bound form of Quin-2. The lifetime of the probe in the outer region (τ_F) is shorter due to the presence of free Quin-2. The intensities of the central and outer regions could be equal ($I_1 = I_2$) due to probe exclusion or other mechanisms. Observation of the intensity image will not reveal the different calcium concentrations in regions 1 and 2. However, if the lifetimes were measured in each region, then the distinct calcium concentrations would be detected. The FLIM method allows image contrast to be created by the local decay times, which can be presented on a grey or color scale (Fig. 1, lower left) or as a 3D projection in which the height represents the local decay time or calcium concentration (lower right).

It is interesting to note that the concept of FLIM is an optical analogue of magnetic resonance imaging (MRI). In MRI, one measures the proton relaxation times at each location, and the numerical value of the relaxation time is used to create contrast in the calculated image. Also, in MRI the local chemical composition of tissue determines the proton relaxation times, and not the proton concentration. The contrast in FLIM is determined by similar principles in that the local environment determines the fluorescence lifetime, which is then used to calculate an image which is independent of probe concentration.

Instrumentation—A detailed description of the FLIM apparatus is given elsewhere [14, 15]. The light source is a ps dye laser system, consisting of a mode-locked Antares NdYAG laser, which synchronously pumps cavity-dumped dye lasers containing either rhodamine 6G or pyridine 1 (Fig. 2). The pulse repetition rate was 3.81 MHz, and we either used the fundamental or higher harmonics of the pulse train [12, 13]. The detector was a CCD camera from Photometries (series 200) with a thermo-electrically cooled PM-512 CCD (Fig. 2). The gated image intensifier (Varo 510-5772-310) was positioned between the target and the CCD camera. The intensifier gain was modulated by a RF signal applied between the photocathode and microchannel plate (MCP) input surface. Phase delays were introduced into this gating signal using calibrated coaxial cables.

The target consisted of rows of cuvettes, each containing Quin-2 and various Ca^{2+} concentrations (Fig. 2). The laser beam passed through the center of the cuvettes. To create the FLIM images we use the image intensifier as a 2D phase-sensitive detector, in which the signal intensity at each position (r) depends on the phase angle difference between the emission and the gain modulation of the detector. This results in a constant intensity which is proportional to both the concentration of the fluorophore (C) at location r ($C(r)$) and to the cosine of the phase angle difference,

$$I(\theta_D, r) = k C(r) \left[1 + \frac{1}{2} m_D m(r) \cos [\theta(r) - \theta_D] \right] \quad \text{Eq. 1}$$

In this expression θ_D is the phase of the gain-modulation and $\theta(r)$ is the phase angle of the fluorescence, $m(r)$ is the modulation, and m_D the gain modulation of the detector. In this expression $\theta_D = 0$ corresponds to the detector being in phase with scattered light. This procedure of phase sensitive gain modulation of the image intensifier is analogous to the method of phase-sensitive or phase-resolved fluorescence [19]. However, these earlier measurements of phase-sensitive fluorescence were performed electronically on the low-frequency cross-correlation signal [19], whereas our present measurements are performed electro-optically on the high frequency modulated emission. It is not possible to calculate the lifetimes from a single phase sensitive intensity. However, the phase of the emission can be determined by examination of the detector phase angle dependence of the emission (Eq. 1), which is easily accomplished by a series of electronic delays in the gain modulation signal or by optical delays in the modulated excitation. At present this is a lengthy process. The future use of electronic phase shifts could reduce the time for data acquisition to one minute or less.

The desired information ($\theta(r)$ or $m(r)$) is thus obtained by varying θ_D (Eq. 1), which in turn allows determination of $\theta(r)$ or $m(r)$. In our apparatus we collect a series of phase-sensitive images, in which θ_D is varied over 360 degrees or more. The phase intensities at each pixel are used to determine the phase at each pixel. This results in phase angle or lifetime images. The phase angle of the fluorescence is related to the apparent phase lifetime τ_θ and the light modulation frequency (ω , in radians/s) by:

$$\tau_{\theta}(r) = \frac{1}{\omega} \tan \theta(r) \quad \text{Eq. 2}$$

It is also possible to obtain the spatially-dependent lifetimes from the modulation of the emission at each pixel. The modulation (m_{EM}) of the emission, relative to the modulation of the excitation (m_{EX}), is related to the apparent modulation lifetime τ_m by:

$$m(r) = \frac{m_{EM}(r)}{m_{EX}(r)} = \frac{1}{\sqrt{1 + \omega^2 \tau_m^2(r)}} \quad \text{Eq. 3}$$

$$\tau_m(r) = \frac{1}{\omega} \sqrt{\frac{1}{m^2(r)} - 1} \quad \text{Eq. 4}$$

The term ‘apparent’ is used to describe the phase and modulation lifetimes because the lifetimes calculated according to Equations 2 and 4 are only true lifetimes if the intensity decay is a single exponential process. The expressions in Equations 2-4 are correct if the phase and modulation of the excitation are determined using scattered light (which has a zero lifetime and a relative modulation of 1.0). In the present measurements we used the phase and modulation lifetime of the sample with 602 nM Ca^{2+} as the reference, with a phase lifetime of 10.11 ns and a modulation lifetime of 11.46 ns, as shown previously for lifetime standards [20]. The value of the modulation, corrected for the non-zero lifetime of the reference (τ_R), is given by:

$$m(r) = \frac{m_{\text{obs}}(r)}{\sqrt{1 + \omega^2 \tau_R^2}} \quad \text{Eq. 5}$$

where $m_{\text{obs}}(r)$ is the observed modulation of the emission relative to the reference sample. Similarly the phase angle of the sample, corrected for the phase angle of the reference, is given by:

$$\theta(r) = \theta_{\text{obs}}(r) + \theta_R \quad \text{Eq. 6}$$

where $\theta_{\text{obs}}(r)$ is the phase angle observed relative to the reference, and $\theta_R = \text{atan}(\omega \tau_R)$ is the phase angle of the reference relative to the excitation. For clarity we note that the phase of the detector is always shifted relative to that of the modulated excitation (θ_I) due to time delays throughout the apparatus. In the present report, the detector phase angles are given as $\theta'_D = \theta_D + \theta_I$, where θ_I is the phase shift intrinsic to the apparatus. Calculation of the phase lifetime requires correction of the apparent phase angles ($\theta'(r)$) for this instrumental shift. This correction can be determined by taking the phase of one of the samples as a known value.

The data sets for FLIM are rather large (512×512 pixels, and 520 kbyte storage for each image), which can result in time-consuming data storage, retrieval and processing. In order

to allow rapid calculation of images we developed an algorithm which only uses each image one time [15]. This algorithm calculates the phase and modulation at each pixel, using the phase-sensitive images obtained with various detector phase angles (Eq. 1). For completeness we note that the FLIM algorithm is being modified because of an intensity-dependent bias in the calculated lifetimes. Additional detail will be presented elsewhere [15].

Materials

Phase and modulation values from the FLIM apparatus were obtained from the phase sensitive images in two ways. These values were calculated on a pixel-by-pixel basis using an algorithm which will be described elsewhere [15]. This calculation is referred to as CCDFT. Alternatively, we used averaged values of the phase sensitive intensities, for about 5×10 pixels from the central portion of the illuminated spot. This calculation is called the Cosine fit.

Frequency-domain data were fit to single and double exponential models,

$$I(t) = \sum_{i=1}^2 \alpha_i e^{-t/\tau_i} \quad \text{Eq. 7}$$

where α_i are the pre-exponential factors and τ_i are the lifetimes, as described previously [21, 22]. Lifetimes recovered from the FLIM measurements were compared with those obtained using standard frequency-domain (FD) measurements and instrumentation [11-13]. For the FLIM measurements polarizers were not used to eliminate the effects of Brownian rotations, which are most probably insignificant for these decay times and viscosities.

Quin-2 was obtained from Sigma (Q-4750, lot 110H0366, FW 541.5) and used without further purification. Ca^{2+} concentrations were obtained using the Calibrated Calcium Buffer Kit II, obtained from Molecular Probes, Eugene, OR, USA. All $[\text{Ca}^{2+}]$ refer to the concentration of free calcium. For all measurements the temperature was 20°C, except for the FLIM measurements, which were done at room temperature near 25°C. The excitation wavelength was 342 nm, and the emission observed through a Coming 3-72 filter. The recommended excitation wavelength for Quin-2 is 339 nm [8]. We did not use this precise value because of inadequate output of our frequency-doubled pyridine-1 dye laser at this wavelength. The FD lifetime and FLIM measurements were also performed using Quin-2 from Molecular Probes. While the results were qualitatively similar, the Molecular Probes sample displayed spectral properties which suggested an impurity, as judged by emission spectra with an unusual shape on the short wavelength side of the emission. Also, the frequency-domain data for the Molecular Probes Quin-2 required three lifetimes to fit the frequency response, whereas two lifetimes were adequate to fit the Ca^{2+} -dependent lifetime from the Sigma Quin-2.

Results

Frequency-domain characteristics of Quin-2

Absorption and emission spectra of Quin-2 are shown in Figure 3. These spectra agree with previously published spectral data for Quin-2 [8, 9], and show that our sample of Quin-2 displayed the expected dependence on Ca^{2+} . Also, the intensity increases 4.6-fold upon complexation with Ca^{2+} , which is within the expected range [2, 8]. Based on the increased yield, one might expect the lifetime of Quin-2 to increase on binding Ca^{2+} . However, an increased lifetime cannot be predicted with certainty because of the unknown static versus dynamic quenching processes operative in Quin-2 and its Ca^{2+} complexes.

Frequency-domain lifetime data for Quin-2 with various amounts of Ca^{2+} are shown in Figure 4. One notices that the frequency-response shifts to lower frequencies with increasing amounts of Ca^{2+} , indicating that the mean lifetime is increasing. At intermediate concentrations of Ca^{2+} the shape of the frequency-response is more complex than at the extreme of low and high Ca^{2+} concentrations. This is because the Quin-2 decay in the free and Ca^{2+} -bound states is dominantly a single exponential, and the decay becomes doubly exponential under conditions of partial Ca^{2+} saturation where the emission from both species contribute to the measured phase and modulation data.

The Ca^{2+} -dependent lifetime data for Quin-2 are summarized in Tables 1 and 2. The decay is nearly a single exponential at 0 and 40 μM Ca^{2+} , as seen from the reasonable values of χ_R^2 for the single exponential fits. Also, the value of χ_R^2 did not decrease substantially when these data were analyzed using the double exponential model (Table 1). In contrast, the value of χ_R^2 for the single exponential fits is markedly elevated for partial Ca^{2+} saturation at $[\text{Ca}^{2+}] = 38 \text{ nM}$. In this case the single lifetime analysis results in an elevated value of $\chi_R^2 = 1205$ (Table 1). However, the frequency-response of Quin-2 at 38 nM Ca^{2+} is well fit by the double-exponential model, yielding $\chi_R^2 = 3.4$. This result suggests that the two decay times were due to free and bound Quin-2. We assign the 1.3 and 11.6 ns decay times to free and bound Quin-2, respectively. Analysis of the 38 nM frequency-response with three decay times resulted in only a modest improvement in χ_R^2 (Table 1), which based on our experience is not adequate to require the use of three lifetimes for the subsequent analysis.

We reasoned that the same two species should be present at all Ca^{2+} concentrations, but in different relative proportions. Hence, we attempted a global fit of the Quin-2 frequency-responses at all Ca^{2+} concentrations using just two lifetimes, while allowing the pre-exponential factors to vary in response to Ca^{2+} . This fit was successful, resulting in a reasonable global χ_R^2 value of 4.0 (Table 2). The goodness-of-fit is seen from the agreement of these global calculated frequency-responses (solid lines) with the data in Figure 4. This result suggests that Quin-2 exists in only two forms (free and bound), and that other complexes do not form or are not significantly fluorescent, as has been reported to be the case for Quin-2 [8]. In support of this claim we note that the double-exponential analysis

yielded essentially the same two lifetimes at all $[Ca^{2+}]$, as seen from the mean lifetimes ($\bar{\tau}$) calculated from the global and single $[Ca^{2+}]$ analysis (Table 2).

The response of Quin-2 to Ca^{2+} is summarized in Figure 5. The intensities and lifetimes both increase with increasing concentrations of Ca^{2+} . These data were used to calculate the dissociation constant using

$$[Ca^{2+}] = K_D \left(\frac{F - F_{min}}{F_{max} - F} \right) \quad \text{Eq. 8}$$

$$[Ca^{2+}] = K_D \left(\frac{\bar{\tau} - \bar{\tau}_{min}}{\bar{\tau}_{max} - \bar{\tau}} \right) \quad \text{Eq. 9}$$

where f_i refers to the intensities and the $\bar{\tau}_i$ refers to the mean lifetime obtained using

$$\bar{\tau}_i = f_1 \tau_1 + f_2 \tau_2 \quad \text{Eq. 10}$$

The dissociation constant obtained from the intensity data agrees with the published value of 60 nM in the absence of Mg^{2+} [8]. However, use of the mean lifetime in Equation 8 results in a lower apparent value for the Quin-2 Ca^{2+} dissociation constant K_D . This occurs because the fluorescence yield of the Ca^{2+} -bound Quin-2 is about 5-fold larger than the free form, and the lifetime is about 9-fold larger for 342 nm excitation, so that a given percentage of Ca^{2+} -Quin-2 contributes a disproportionately large fraction to the total emission and distorts the measured lifetime towards that of the bound form. This sensitive of the mean lifetime to lower concentration of Ca^{2+} can be an advantage or a disadvantage depending upon the calcium concentration being measured.

The multi-exponential lifetime analysis provides an opportunity to directly determine the fractional saturation of Quin-2 by Ca^{2+} . This can be understood from the following considerations. Let τ_F and τ_B be the lifetimes of free and bound Quin-2, respectively. The reciprocal lifetime (or decay rate) is the sum of the rate processes which depopulate the excited state. Hence, the decay rate of Ca^{2+} -bound Quin-2 is given by

$$k_B = \frac{1}{\tau_B} = k_e + k_{nr} \quad \text{Eq. 11}$$

where k_e is the rate of emission and k_{nr} is the non-radiative decay rate. Since the lifetime of Quin-2 appears to vary in proportion to its steady-state fluorescence intensity, it is probable that the radiative rate is not altered by binding to Ca^{2+} . Hence, the shortened lifetime of free Quin-2 is probably due to a non-emissive quenching process competitive with emission (k_q). For this condition the decay rate of free Quin-2 is given by

$$k_F = \frac{1}{\tau_F} = k_e + k_{nr} + k_q \quad \text{Eq. 12}$$

Since the rate of emission (k_e) is the same for both forms, the intensity decay for a mixture of free and bound Quin-2 is given by

$$I(t) = f_F k_e e^{-t/\tau_F} + f_B k_e e^{-t/\tau_B} \quad \text{Eq. 13}$$

where f_F and f_B represent the fractional population of each form. Evidently, the normalized pre-exponential factors (α_1 and α_2) are equal to the fractional population of free and bound Quin-2. These values of α_i (Table 2) were used to calculate the Ca^{2+} -dependent saturation of Quin-2 (Fig. 5). If our analysis is correct, the dissociation constant should be obtained when the fractional saturation is 50%. This value of 74 nM is in good agreement with the expected value, so it appears that the fractional saturation of Quin-2 with Ca^{2+} can be obtained from the pre-exponential factors. We note that the steady state intensity of a fluorophore is proportional to the product $k_e \tau_i$, where τ_i is τ_F or τ_B , so that the higher intensity of Ca^{2+} -bound Quin-2 appears to be due to its longer lifetime and not a change in the emissive rate.

Fluorescence lifetime imaging of Quin-2

As presently implemented, FLIM measurements are performed using a single modulation frequency. Hence, it is of interest to examine the Ca^{2+} -dependent phase and modulation data at selected frequencies, in order to select an optimal frequency consistent with the lifetimes displayed by the samples and the useful frequency range of the instrumentation. Phase and modulation data for Quin-2 at selected frequencies are shown in Figure 6. Substantial Ca^{2+} -dependent phase angle changes are seen from 34 to 72 MHz. Somewhat smaller changes in phase and modulation are seen at lower (16 MHz) and higher (104 MHz) frequencies (Fig. 4), but these changes are more than adequate with the current precision of our FLIM instrumentation. Conveniently, this range is consistent with that of our FLIM instrument, which operates to about 150 MHz. For the subsequent FLIM experiments we selected 49.335 MHz, which is in the center of the Ca^{2+} response curve and provides maximal changes in phase and modulation (Fig. 4).

Ultimately, we expect FLIM to be useful in fluorescence microscopy where it is not practical to perform single-wavelength intensity measurements. However, the FLIM technology is new and has not yet been adapted for use in microscopy. In the present study we imaged a row of four cuvettes, each with a different Ca^{2+} concentration (Fig. 7). This allowed control measurements in which the same samples were measured with our standard frequency-domain instrumentation. Such control measurements are important because the current FLIM apparatus uses homodyne detection, which is less robust with regard to rejection of harmonics and/or non-linear effects. Hence, it is important to perform comparative measurements to verify the accuracy of the phase and modulation data obtained from the FLIM apparatus.

To compare the FLIM-measured phase and modulation values, with those measured using FD methods, we used the average phase sensitive intensities observed for various detector phase angles. These averaged values were observed from the central region of the illuminated area of the cuvette. These data were fit using the Cosine program, to obtain the

phase and modulation values. Using the phase (72.3) and modulation (0.27) value of Ca^{2+} -saturated sample as the reference, we computed the phase and modulation values of the other three Ca^{2+} concentrations (Table 3). These values are in excellent agreement with those obtained by the FD method. Inspection of Figure 7 reveals that the phase increases and the modulation decreases with increasing amounts of Ca^{2+} . We note that there is no loss of generality in selecting one of the Quin-2 samples as the reference. We could have used scattered light, or a reference fluorophore of known lifetime [20] and obtained similar results.

We stress the importance of verifying the phase and modulation values obtained from the FLIM apparatus. To the best of our knowledge, a gain-modulated image intensifier has not previously been used to measure fluorescence phase or modulation. Also, without careful control of the electrical settings of the modulation, it is easy to be outside the useful range for gain modulation and to introduce harmonics and/or distortions into the phase-sensitive intensities. Such technical issues can be better controlled in future FLIM instruments.

The FLIM images are calculated from a series of phase-sensitive images. Four such images are shown in Figure 8. One notices that the phase-sensitive images vary dramatically with the phase angle shift between the detector gain and the emission. For instance, the phase sensitive intensity of the 0 nM Quin-2 sample is larger than that of the 17nM sample for $\theta'_D = 0^\circ$, whereas these phase-sensitive intensities are nearly equal for $\theta'_D = 152^\circ$ and 252.5° . A series of phase sensitive images can be used to calculate a phase lifetime image (Fig. 9). To calculate this image we use an algorithm which determines the best-fit phase angle for each pixel across the phase-sensitive image planes [15]. This image can be presented as a phase angle image, or transformed into phase lifetimes using Equation 2. In Figure 9, the height of the surface is the phase angle (top) or phase lifetime (bottom). In calculating this surface we only performed calculations for regions of the image where the steady state intensity was 5% or greater than the peak intensity. Also shown is a line (on the background panels) representing the phase angles drawn through the center of the four cuvettes. The phase angles increase with increasing concentrations of Ca^{2+} . The phase lifetimes obtained from the FLIM measurements are in excellent agreement with those measured using the standard FD instrumentation (Table 3). Calculation of a Ca^{2+} concentration image is a simple transform of these data according to the calibration curve in Figure 6. Lifetime and/or Ca^{2+} -images can also be calculated from the modulation at each pixel (Fig. 10). Notice that the modulation decreases with increasing $[\text{Ca}^{2+}]$, whereas in Figure 9 the phase angles increase with increasing $[\text{Ca}^{2+}]$. However, in both cases the apparent lifetimes increase with calcium concentration, and these lifetimes are in agreement with the expected values (Table 3).

Examination of Figure 10, and to a lesser extent Figure 9, reveals peaks on the sides of the lifetime surfaces (bottom), or equivalently rounded edges on the modulation surface (top). These structures are surprising because each cuvette is a homogeneous solution and is expected to display a single phase angle or modulation value. We are currently investigating this phenomenon, which presently appears to be the result of a computational effect rather than an electro-optic phenomenon in the FLIM apparatus. More specifically, these peaks appear to be due to the use of data files with near zero intensity, such as $\theta'_D = 196^\circ$ for 0 nM Ca^{2+} (Fig. 7).

In Figures 9 and 10 we presented the lifetime images (bottom panels). However, it is important to remember that for multi-exponential decays, the apparent phase and modulation lifetimes calculated from Equations 2 and 4, are only apparent values. Hence, different phase and modulation lifetimes would be observed for each modulation frequency. The effects of multi-exponential decays on the phase and modulation data have been described elsewhere [21-23]. For this reason it will be preferable to make the calibration curves in terms of phase or modulation versus $[Ca^{2+}]$, instead of phase or modulation lifetimes. Importantly, it would be possible using the present apparatus to measure the phase and modulation values of a range of frequencies where Quin-2 displays Ca^{2+} -sensitive values. Least squares analysis of the data, with or without the lifetimes being constrained as global parameters, would allow the fractional saturation of Quin-2 and hence the calcium concentration to be determined from the pre-exponential factors (Eqs 7 & 13).

Alternative presentations of the Ca^{2+} images are shown in Figures 11 and 12. In these figures we used a color scale to indicate the various concentrations of Ca^{2+} . Such images may be most appropriate for Ca^{2+} imaging of cells, particularly if colors are assigned for each interesting calcium concentration range, resulting in easier visualization of the Ca^{2+} concentrations with physiological significance. In Figure 11 the color scales indicate the phase or modulation values. The changing colour from left to right reveals the increase in phase angle, and decrease in modulation, with increasing concentrations of Ca^{2+} . The lowest row of color spots indicates an intensity image in which the colors indicate the total fluorescence intensity. The intensities are brightest in the center of the image, and are nearly the same for all four samples.

A different color-code was used in Figure 12. In this case the color scale indicates the apparent phase and modulation lifetime in each sample. In contrast to the phase-modulation image (Figure 11), both apparent lifetimes increase with calcium concentration. Closer inspection of Figure 12 will reveal that the apparent modulation lifetimes are larger than the apparent phase lifetimes. This is a known consequence of single-frequency lifetime measurements of a multi-exponential decay. In fact, the sensitivity of the apparent lifetime to the multi-exponential nature of the decay suggests that multi-frequency FLIM may allow resolution of fluorophores in different environment based on the amplitudes in the multi-exponential decay.

Phase suppression imaging of Ca^{2+}

A unique property of FLIM is the ability to suppress the emission for any desired lifetime and/or calcium concentration. Suppression of the emission with any given decay time can be accomplished by taking the difference of two phase sensitive images obtained for detector phase angles of θ_D and $\theta_D + \Delta$. In the difference image $I = I(\theta_D + \Delta) - I(\theta_D)$ components are suppressed ($I = 0$) with a phase angle θ_S which is given by

$$\theta_S = \theta_D + \frac{\Delta}{2} \pm n \cdot 180 \quad \text{Eq. 14}$$

Regions of the image with a decay time of $\tau_s = \omega^{-1} \tan \theta_s$ have an intensity of zero in the difference image. This concept is shown schematically in Figure 13 for $\theta_s = 180^\circ$.

Components with a lifetime τ_2 larger than the suppressed lifetime appear negative in the difference image ($I_2 < 0$) and components with a shorter lifetime are positive ($I_1 > 0$).

This relationship is reversed if one calculates $I(\theta_D) - I(\theta_D + \pi)$. A more complete description of this suppression method will be presented elsewhere [15].

The use of difference images to suppress the emission for various concentrations of Ca^{2+} is shown in Figure 14. In the top panel we chose to suppress the emission from areas with $[\text{Ca}^{2+}] = 80 \text{ nM}$. If one examines only the grey-scale representation, and sets negative intensities to the background color, then only regions with $[\text{Ca}^{2+}] = 80 \text{ nM}$ are observed. Remarkably, the sample with $65 \text{ nM } \text{Ca}^{2+}$ still shows positive intensity in the difference image. Alternatively, one can suppress the emission from regions with $[\text{Ca}^{2+}] = 17 \text{ nM}$ (bottom). In this case, the grey-scale image only shows regions with $[\text{Ca}^{2+}] = 17 \text{ nM}$. The sample with 0 and $17 \text{ nM } \text{Ca}^{2+}$ both show negative intensities in the difference image, or no intensity in the grey-scale representation. This ability to selectively visualize regions with high or low Ca^{2+} may be useful in evaluation of the role of Ca^{2+} in the control of cellular processes. Acquisition and computation of complete FLIM images is presently time consuming. In contrast, phase suppression images require only the difference of two images without further numerical analysis, making it easier to acquire and display real time images.

Discussion

Fluorescence lifetime imaging provides a new opportunity for the use of fluorescence in cell biology. This is because the lifetimes of probes can be sensitive to a variety of chemical or physical properties, many of which are of interest for studies of intracellular chemistry and physiology. An advantage of FLIM is the insensitivity of lifetime measurements to the local probe concentration and photobleaching. Consequently, one does not require dual-wavelength ratiometric probes. Instead, one needs a change in lifetime, which may occur in any Ca^{2+} probe which changes intensity in response to Ca^{2+} .

At present, the selection of fluorophores for FLIM is not straightforward. This is because most sensing work does not rely on lifetimes and the probe lifetimes are often unknown. For instance, the Ca^{2+} probes Fura-2 [24] and Indo-1 (Lakowicz et al., unpublished observation) showed only small changes in phase angles in response to Ca^{2+} . Nonetheless, one can expect lifetime probes to become available as the available sensors are tested. It should be noted that it may be easier to obtain lifetime probes for pH, Cl^- , Na^+ and K^+ than wavelengths shifting probes. For instance, it is known that Cl^- is a collisional quencher and alters the lifetime of quinine [25]. Hence, the chloride intensity indicator SPQ (6-methoxy-N-(3-sulfopropyl) quinolinium) is probably also a lifetime probe for Cl^- , as suggested by Tsien [26], and confirmed by the experiments of Illsley and Verkman [27]. Hence, elimination of the requirement for dual-wavelength excitation and/or emission, may result in the rapid introduction of many FLIM probes. We also note that long-wavelength lifetime probes for Ca^{2+} have already been identified, and will be the subject of a future publication [28]. These probes can be used with excitation wavelengths of up to 630 nm , which will be advantageous due to reduced autofluorescence from and phototoxicity to the cells.

And finally, we note that the apparatus required for FLIM is a modestly straightforward extension of that already in use in fluorescence microscopy. Slow-scan CCD cameras are in use and are the preferred detector for fluorescence microscopy [29]. Laser light sources are also increasingly used because of their intensity and ease of manipulation. The image intensifier is commercially available, and is easily gain-modulated with low voltages [14]. Phase angle or lifetime image files are easily rewritten in the format of the image processing software packages, so that these powerful image manipulation programs remain available after obtaining the lifetime images. Consequently, FLIM technology is easily introduced into most fluorescence microscopes and allows for chemical imaging of cells.

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Abbreviations:

EGTA	[ethylene bis(oxyethylenenitrilo)]-tetraacetic acid
FD	frequency-domain
FLIM	fluorescence lifetime imaging
Quin-2	2-{{[2-bis-(carboxymethyl)-amino-5-methylphenoxy]-methyl}-6-methoxy-8-bis-(carboxymethyl)-aminoquinoline

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Fig. 1.

Intuitive presentation of the concept of Fluorescence Lifetime Imaging (FLIM). It is assumed that the object has two regions which display the same fluorescence intensity ($I_1 = I_2$) but different decay times, $\tau_B > \tau_F$; a – object; b – color or grey-scale Ca^{2+} image; c – lifetime contour Ca^{2+} image

Fig. 2.

Schematic diagram of a FLIM experiment. The 'object' consists of a row of cuvettes, each with a different $[Ca^{2+}]$ and lifetime. This 'object' is illuminated with intensity modulated light. The spatially and temporarily-varying emission is detected with a gain-modulated image intensifier, which acts like a phase-sensitive detector and is imaged onto a CCD camera. A series of phase-sensitive images are used to compute the phase angle, modulation and/or lifetime images. The light source is a cavity-dumped dye laser

Fig. 3. Absorption (A) and emission spectra of Quin-2 (B) in the presence of increasing amounts of Ca^{2+} . For the emission spectra the excitation wavelength was 342 nm. The dashed line (lower panel) shows the transmission of the Corning 3-72 emission filter used to isolate the emission during the FLIM or FD measurements

Fig. 4. Frequency-response of Quin-2 in the presence of increasing amounts of Ca^{2+} . See Table 1 for additional detail and data

Fig. 5.

Above: Ca^{2+} -dependent lifetime, intensity and fractional saturation of Quin-2. The fractional saturation was obtained from $\alpha_2/(\alpha_1 + \alpha_2)$

Right: The dissociation constants (K_D) were obtained from plots at $\log \{(F - F_{\min})/(F_{\max} - F)\}$, $\log \{(\bar{\tau} - \bar{\tau}_{\min})/(\bar{\tau}_{\max} - \bar{\tau})\}$ or $\log \{(\alpha_2 - \alpha_{2\min})/(\alpha_{2\max} - \alpha_2)\}$ versus $\log [\text{Ca}^{2+}]$ where $\bar{\tau} = f_1\tau_1 + f_2\tau_2$ from the double exponential fits (Table 2)

Fig. 6.
 Ca^{2+} -dependent phase and modulation values for Quin-2 at 34.155, 49.335 and 72.105 MHz

Fig. 7.

Phase-sensitive intensities of Quin-2 collected with the FLIM apparatus. Θ_i is the instrumental phase shift between the modulated excitation and the intensifier gain modulation. The value of Θ_i was determined from the known phase of the reference sample ($\Theta_R = 72.3^\circ$ for 602 nM Ca^{2+}) using $\Theta_i = \Theta'_R - \Theta_R$, where $\Theta'_R = 78.7^\circ$ is the observed phase of the reference

Fig. 8.
Phase-sensitive images of Quin-2 measured at various detector phase angles

Fig. 9. FLIM images of Ca^{2+} obtained from the phase angle image. The phase lifetimes were obtained from the calibration curve in Figure 6

Fig. 10.

FLIM images of Ca²⁺ obtained from the modulation image. The modulation lifetimes were obtained from the calibration curve in Figure 6

Fig. 11.

Ca²⁺ imaging using phase and modulation color scales. The color changes in the cuvettes from left to right indicate increasing phase angles and decreasing modulation. The lowest row of images shows the intensity images

Fig. 12.
Phase and modulation lifetime images of Ca^{2+} . The color scale represents the apparent lifetimes

Fig. 13.

Intuitive descriptions of phase suppression. In a difference image with $I = I(\theta_D + 180) - I(\theta_D)$ a component with $\theta = \theta_D$ is completely suppressed. Components with longer lifetimes (phase angles) appear to be negative, and those with shorter lifetimes (phase angles) appear to be positive

Fig. 14. Ca^{2+} images with suppression of regions with $[\text{Ca}^{2+}] \geq 80 \text{ nM}$ (top) and $\text{Ca}^{2+} \leq 17 \text{ nM}$ (bottom). The upper and lower suppression images were calculated from the phase sensitive images $I(\theta'_D)$ using $I(348.2^\circ) - I(152.4^\circ)$ and $I(152.4^\circ) - I(304.6^\circ)$.

Table 1Multi-exponential decay analysis for Quin-2 at selected $[Ca^{2+}]^a$

$[Ca^{2+}]$	$\bar{\tau}(ns)$	$\tau_i(ns)$	α_i	f_i	χ_R^2
0	–	1.32	1.0	1.0	4.6
		0.55	0.110	0.047	
	1.35	1.38	0.880	0.953	2.6
38 nM	–	5.16	1.0	1.0	1205.3
		1.25	0.661	0.178	
		9.47	11.25	0.339	0.822
		0.73	0.236	0.038	3.4
		1.56	0.443	0.152	
		9.55	11.46	0.321	0.810
40 μ M	–	11.58	1.0	1.0	1.6

^aThe excitation wavelength was 342 nm, a Corning 3-72 emission filter, 20°C. The values of τ_1 are assigned to the free Quin-2 and τ_2 to Ca^{2+} -Quin-2, the value of $\bar{\tau}$ was obtained from $\bar{\tau} = f_1\tau_1 + f_2\tau_2$

Table 2Calcium-dependent lifetimes of Quin-2^a

$[Ca^{2+}]$	<i>Global analysis</i> ^b				<i>Single $[Ca^{2+}]$ analysis</i> ^b	
	α_1	α_2	f_1	f_2	$\bar{\tau}(ns)$	χ^2_R
0 nM	0.998	0.002	0.982	0.018	1.48	1.35
4 nM	0.966	0.034	0.758	0.242	3.78	2.6 (4.6) ^c
8 nM	0.934	0.066	0.615	0.385	5.25	3.50
17 nM	0.818	0.182	0.335	0.665	8.12	3.1 (269.1)
38 nM	0.669	0.331	0.184	0.816	9.67	4.95
65 nM	0.522	0.478	0.109	0.891	10.44	3.5 (777.8)
100 nM	0.412	0.588	0.073	0.927	10.81	8.07
225 nM	0.219	0.781	0.030	0.970	11.24	2.2 (1360.4)
602 nM	0.092	0.908	0.012	0.988	11.44	9.55
1.35 μ M	0.049	0.950	0.006	0.994	11.48	2.8 (1205.3)
40 μ M	0.000	1.000	0.000	1.000	11.55	10.50
						2.4 (624.2)
						10.96
						2.9 (367.4)
						11.40
						2.7 (76.3)
						11.64
						2.3 (15.3)
						11.70
						1.5 (6.1)
						11.58
						1.6 (1.6)

^aSee Table 1 for additional details^bFor the global analysis, τ_1 and τ_2 were held equal at all $[Ca^{2+}]$. In the single $[Ca^{2+}]$ analysis, τ_1 and τ_2 were allowed to vary for each $[Ca^{2+}]$ ^cThe values in parenthesis are the χ^2_R values for the single lifetime fits to the frequency-response at a single concentration of calcium^dBest single lifetime fit

Table 3

Phase and modulation of Quin-2^a

[Ca ²⁺] (nM)	Method ^b	Phase		Modulation	
		θ°	τ_θ (ns)	m	τ_m (ns)
0	FD	21.6	1.27	0.921	1.36
	Cosine	24.3 ± 6.0	1.45 ± 0.40	0.639 ± 0.08	3.87 ± 0.80
	CCDFT	24.5 ± 4.0	1.47 ± 0.27	0.611 ± 0.09	4.16 ± 1.00
17	FD	40.6	2.77	0.447	6.46
	Cosine	40.4 ± 8.1	2.74 ± 0.80	0.408 ± 0.07	7.19 ± 1.9
	CCDFT	40.6 ± 6.0	2.76 ± 0.60	0.391 ± 0.08	7.67 ± 1.9
65	FD	59.1	5.39	0.314	9.76
	Cosine	57.2 ± 6.1	4.99 ± 1.2	0.320 ± 0.04	9.52 ± 1.3
	CCDFT	57.3 ± 7.0	5.01 ± 1.4	0.310 ± 0.04	9.86 ± 1.4
602	FD	72.3	10.11	0.271	11.46
	Cosine	<72.3> ± 5.7	10.11 ± 3.8	<0.27> ± 0.03	11.46 ± 1.5
	CCDFT ^c	<72.3> ± 7.0	10.11 ± 5.0	<0.27> ± 0.04	11.46 ± 1.8

^aExcitation wavelength 342 nm, Corning 3-72, emission filter, at 25°C and 49.53 MHz^bFD, standard frequency-domain measurements at 49.335 MHz; Cosine fit to averaged phase-sensitive intensities, measured at 49.53 MHz to Equation 1; CCDFT as calculated from our algorithm [15]^c< > used as reference values