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Recent Developments in Monitoring Calcium and Protein Interactions in Cells Using Fluorescence Lifetime Microscopy

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Time-resolved fluorescence lifetime microscopy (TRFLM) allows the combination of the sensitivity of fluorescence lifetime to environmental parameters to be monitored in a spatial manner in single living cells, as well as providing more accurate, sensitive, and specific diagnosis of certain clinical diseases and chemical analyses. Here we discuss two applications of TRFLM: (1) the use of non-ratiometric probes such as Calcium Crimson, for measuring Ca^{2+}; and (2) quantification of protein interaction in living cells using green and blue fluorescent protein (GFP and BFP, respectively) expressing constructs in combination with fluorescence resonance energy transfer microscopy (FRET). With respect to measuring Ca^{2+} in biological samples, we demonstrate that *intensity*-based measurements of Ca^{2+} with single-wavelength Ca^{2+} probes such as Calcium Crimson may falsely report the actual Ca^{2+} concentration. This is due to effects of hydrophobicity of the local environment on the emission of Calcium Crimson as well as interaction of Calcium Crimson with proteins, both of which are overcome by the use of TRFLM. The recent availability of BFP (P4-3) and GFP (S65T) (which can serve as donor and acceptor, respectively) DNA sequences which can be attached to the carboxy- or amino-terminal DNA sequence of specific proteins allows the dual expression and interaction of proteins conjugated to BFP and GFP to be monitored in individual cells using FRET. Both of these applications of TRFLM are expected to enhance substantially the information available regarding both the normal and the abnormal physiology of cells and tissues.

KEY WORDS: Fluorescence lifetime imaging microscopy; calcium; green fluorescent proteins; blue fluorescent proteins; Calcium Crimson; fluorescence resonance energy transfer.

INTRODUCTION

TRFLM is a noninvasive and powerful technique for the measurement of fluorescence lifetimes in two or three dimensions in biological specimens.[1] The measurement of fluorescence lifetimes offers many benefits, among which is that such measurements are independent of local intensity and concentration of the fluorophore and provide visualization of the molecular environment in a single living cell. TRFLM uses a nanosecond-gated multichannel plate image intensifier providing a two-dimensional map of the spatial distribution of the fluorescent lifetime in the sample under observation.[2] Picosecond laser pulses from a tunable dye laser are delivered to fluorophore inside living cells on the stage of a fluorescent microscope. Images of the fluorescence emission at various times during the decay of the fluorescence are collected using a high-speed gated image

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intensifier and the lifetimes are calculated on a pixel-by-pixel basis.

Fluorescence lifetime probes are sensitive to numerous chemical and environmental parameters such as pH, oxygen, and Ca^{2+} and thus TRFLM can directly image the local environment (chemical and structural) immediately surrounding the probe. With respect to measuring Ca^{2+} in biological samples, TRFLM allows the use of recently developed nonratiometric probes for Ca^{2+} such as Calcium Green, Calcium Crimson, and Calcium Orange,[3,4] because the Ca^{2+} concentration is derived from the lifetime and not the intensity of emission. TRFLM also provides the opportunity to study the rotational mobility of a fluorescence lifetime probe using time-resolved emission anisotropy, providing the lifetime of the excited state is long enough.

TRFLM can also be used to quantify the interaction, binding, or association of two types of molecules using fluorescence resonance energy transfer (FRET) spectroscopy.[5,6] FRET occurs through dipole–dipole interactions (nonradiative transfer of energy from one molecule to another) and is sensitive to the inverse sixth power of the distance between the two fluorophores. FRET occurs when the donor and acceptor molecules are within 10–100 Å of each other. The advantage of FRET measurements based on lifetimes as opposed to intensities is that the donor–acceptor distance is more precisely measured. By combining TRFLM with FRET, it is possible to obtain quantitative temporal and spatial information about the binding and interaction of proteins, lipids, enzymes, DNA, and RNA *in vivo*.

RESULTS

Effect of Protein, Hydrophobicity and Cellular Contents on Intensity-Based Measurements of Ca^{2+} Using Calcium Crimson

The role of Ca^{2+} as an important signaling molecule has become well established, and observation of dynamic changes in Ca^{2+} in individual cells has been made much more accessible by the development of Ca^{2+}-sensitive fluorescent probes and technical advances in digital video microscopy.[7,8] Currently available Ca^{2+}-sensing probes generally fall into two classes—"ratiometric," where inverse changes in the intensity at two different wavelengths of excitation or emission are reflective of a given Ca^{2+} concentration, and "non-ratiometric," where an increase or decrease in intensity is used to report Ca^{2+} levels in cells. Ratiometric probes have the advantage of being able to correct for differences in pathlength and probe concentration but, like their nonratiometric counterparts, are difficult to calibrate *in situ*.[9–11]

An alternative approach for quantifying cellular Ca^{2+} is based on the measurement of fluorescent lifetimes.[12] Determination of Ca^{2+} using lifetime measurements is independent of probe concentration, photobleaching, and probe leakage. Thus, the use of fluorescent lifetimes to determine Ca^{2+} has multiple advantages over intensity-based measurements. Another potential benefit of lifetime measurements for the determination of Ca^{2+} is that they are the only approach by which one can quantitatively estimate Ca^{2+}. This is based on the recent findings of Sanders and colleagues, who demonstrated that fluorescent lifetime pH calibration curves of carboxyl SNAFL-1 (Molecular Probes) using buffers of different pH and *in situ* clamping of intracellular and extracellular pH using nigericin gave identical calibrations curves, whereas these same calibration curves obtained by ratio imaging did not. These results suggested straightforward calibration procedures in buffer might suffice for quantitating ion concentrations *in situ* when employing measurements of fluorescent lifetime.

To determine whether this finding also holds for Ca^{2+}-sensing probes, we examined the effect of hydrophobicity, protein concentration, and potential interaction of cellular constituents on the Ca^{2+}-sensing capabilities of Calcium Crimson, using both intensity-based and fluorescent lifetime analysis. Calcium Crimson was chosen because previous studies have indicated that its fluorescent lifetime is sensitive over a broad range of Ca^{2+}, unlike other nonratiometric Ca^{2+}-sensitive probes.[13] Table I demonstrates the effects of the hydrophobicity of the probe environment, and interaction with protein and cellular constituents, on Calcium Crimson's Ca^{2+}-sensing capabilities as reported by changes in emission intensity. At constant Ca^{2+}, the hydrophobicity, concentration of protein, and cellular constituents all had a substantial effect on Calcium Crimson's accuracy in reporting Ca^{2+}. Increases in the concentration of protein (either pure BSA or cell extracts) generally quenched Calcium Crimson intensity by as much as 30% at high concentrations (relative to buffer alone). In contrast, increases in the hydrophobicity of Calcium Crimson's environment caused an increase in intensity by as much as 36% (relative to buffer alone).

Similar studies were carried out using fluorescent lifetime measurements. The principle of lifetime measurements for assessing ion concentrations is based on the fact that the lifetime of the ion-bound form of the probe differs from the lifetime of the free form of the probe.

Table I. The Effect of Protein, Hydrophobicity, and Cellular Contents on Intensity-Based Measurements of Ca^{2+} Using Calcium Crimson[a]

BSA (%, w/v)	Pixel intensity (% change)	Ethanol (%, v/v)	Pixel intensity (% change)	Cellular content (%, v/v)	Pixel intensity (% change)
0	0	0	0	0	0
0.5	−17	3	14	7.5	0
1.0	−25	6	19	10	−8
2.0	−27	10	17	13	−1
5.0	−30	13	36	20	−17

[a]Solutions of 25 μ*M* Calcium Crimson salt in calcium standard buffers obtained from Molecular Probes Inc. were titrated to a final Ca^{2+} concentration of 280 n*M* following the addition of BSA, ethanol, or cell contents at the indicated concentrations. Intensity measurements (0 to 255) were then obtained using identical settings of the camera, neutral density filters, number of frames averaged, and background. Results are the averages of three independent experiments.

Table II. Effects of Hydrophobicity and Interaction with Protein and Cellular Constituents on the Fluorescent Lifetimes of the Ca^{2+}-Bound and Ca^{2+}-Free Forms of Calcium Crimson

BSA	Lifetime		Ethanol	Lifetime		Cellular content	Lifetime	
(%, w/v)	τ_1	τ_2	(%, v/v)	τ_1	τ_2	(%, v/v)	τ_1	τ_2
0	2.1	4.9	0	2.0	4.8	0	0.99	4.40
0.5	nd	nd	3	2.1	4.8	7.5	1.0	4.42
1.0	1.8	5.0	6	2.3	5.0	10	0.95	4.43
2.0	1.8	5.0	10	2.4	4.9	13	0.92	4.43
5.0	1.8	5.0	13	2.5	4.9	20	1.0	4.48

[a]Solutions of 25 μ*M* Calcium Crimson salt in calcium standard buffers obtained from Molecular Probes Inc. were titrated to a final Ca^{2+} concentration of 280 n*M* following the addition of BSA, ethanol, or cell contents at the indicated concentrations. Lifetime measurements were then obtained using identical settings of the system as described under Materials and Methods. Results are the averages of three independent experiments. nd, not done.

For example, each Ca^{2+}-sensitive fluor will have two lifetimes, that of the Ca^{2+}-free form and that of the Ca^{2+}-bound form. These two lifetimes are characteristic for each of the Ca^{2+}-sensitive fluors; as the concentration of Ca^{2+} changes, the amplitudes (relative contribution of each lifetime to the observed lifetime), *but not the lifetime of the Ca^{2+}-bound and Ca^{2+}-free forms of the probe*, changes. Thus, because the lifetime of the Ca^{2+}-bound and Ca^{2+}-free form remains constant (independent of the concentration of Ca^{2+}), Ca^{2+} can be determined by changes in the respective amplitudes of the lifetimes of the Ca^{2+}-bound and Ca^{2+}-free forms of the probe. In addition, since the lifetime of the Ca^{2+}-bound and Ca^{2+}-free states remains constant independent of the Ca^{2+} concentration, if a change in the lifetime is observed, it would most probably represent effects of probe interaction with its environment (i.e., an effect of environment would be reflected as different lifetimes than those observed with pure probe in solution at equivalent Ca^{2+}). We have previously documented that the lifetimes of the Ca^{2+}-bound and Ca^{2+}-free states (τ_1 and τ_2) remain constant independent of Ca^{2+}.[14] However, for quantitative estimation of Ca^{2+} using lifetime measurements to be useful, the lifetime of the Ca^{2+}-bound and Ca^{2+}-free forms must remain constant independent of the probes environment. Therefore, we determined the effects of hydrophobicity and interaction of Calcium Crimson with protein and cellular constituents on the fluorescent lifetimes of the Ca^{2+}-bound and Ca^{2+}-free states. These results are presented in Table II. The Ca^{2+}-bound form of Calcium Crimson is represented by the longer τ component, and the Ca^{2+}-free form of Calcium Crimson is represented by the shorter τ component. In addition, we also performed *in situ* calibration of Calcium Crimson in cells, by selectively lysing the plasma membrane (retaining the cytosolic components) and/or nuclear membrane and examining whether any spatially distinct differences in the lifetimes of the Ca^{2+}-bound and Ca^{2+}-free states were observed between the cytoplasm and the nucleus (Fig. 1). As can be seen, very little change in τ_1 or τ_2 occurred as a function of protein or cellular constituent concentration, hydrophobicity, or cellular location. These findings indicate that the lifetimes of Calcium Crimson are relatively insensitive to both Ca^{2+} and environmental factors and suggest that calibration of Calcium Crimson *in situ*, like carboxy-SNAFL-1 (Molecular Probes), can be accomplished simply by the use of buffers.

The quenching of the intensity of Calcium Crimson by increasing concentrations of BSA and cellular constituents may be the result of competition for divalent cations by nonspecific binding sites. However, the fact that there was little change in the amplitudes of the lifetimes of the Ca^{2+}- bound or Ca^{2+}-free forms of Calcium Crimson argues against nonspecific binding as a mechanism of quenching. The amplitudes of the Ca^{2+}-bound and Ca^{2+}-free forms of Calcium Crimson were 0.51 and 0.49 at 1% BSA and 0.47 and 0.53 at 5% BSA, respectively.

Using Green- and Blue- Fluorescent Proteins (GFP and BFP)—Conjugated Proteins for the Study of Protein–Protein Interaction

Recently, the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* has been found to be fluorescent with fluorescein-like characteristics and is attract-

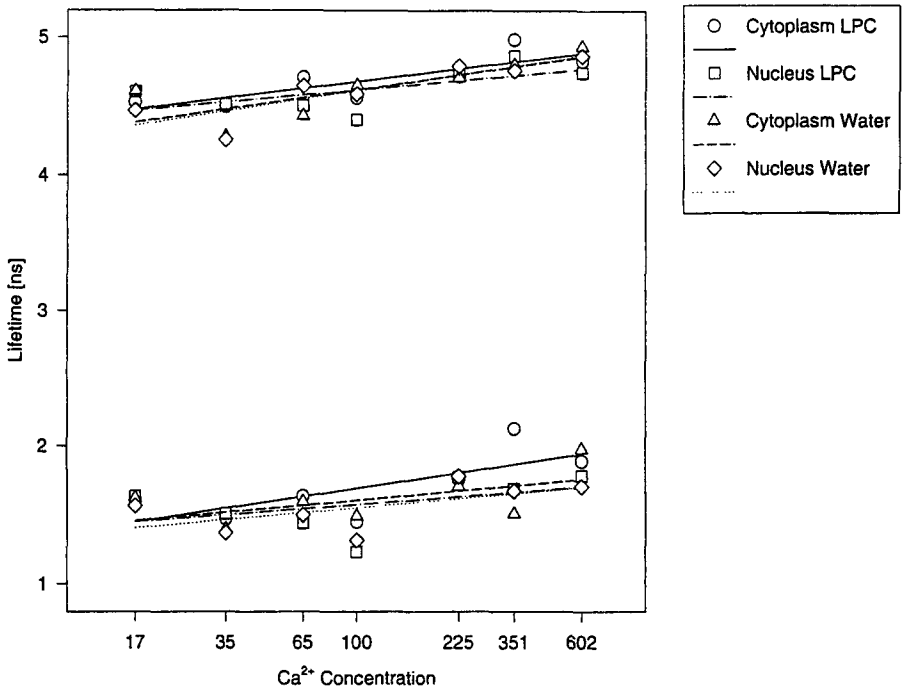

Fig. 1. *Mean Lifetime values of Calcium Crimson in the cell cytoplasm and nucleus as a function of Ca^{2+} concentration.* Cytosolic and nuclear Ca^{2+} were calibrated using a 100 μM concentration of the salt form of Calcium Crimson in Ca^{2+}–EGTA buffers applied following permeabilization of cells with 100 μg/ml lysophosphotidyl choline (cytosolic) or following permeabilization of cells by osmotic shock (60 s in distilled H_2O). Osmotic shock resulted in stripping of cell plasma and nuclear membranes and loss of cytosolic components. The lifetimes of Calcium Crimson in both the cytoplasm and the nucleus were compared under all extraction conditions. The instrumentation used for measuring fluorescent lifetimes has been described previously.[1] Briefly in the reverse mode configuration, the start signal generated by the first single photon (fluorescence from the excited fluorophores in the specimen) is detected by an MCP-PMT (R3809U, Hamamatsu Photonics, NJ), and sent to a time-to-amplitude converter (TAC) (Model TC 864, Oxford–Tennelec Instruments, TN). The stop signal is generated from the excitation pulse detected by a pin photodiode (Model DET2-SI; Thorlabs, Newton, NJ) or a SYNC pulse from the CD driver, which is delayed for a defined period that is greater than the time required for detection of the first emitted single photon. The TAC in turn provides a voltage pulse whose amplitude is proportional to the time elapsed between start and stop signal. The amplitude of this pulse is stored in a multichannel analyzer (MCA). This cycle is repeated many times, yielding a histogram of the individual lifetimes of excited fluorophore molecules accumulated in the MCA. The histogram has an exponential form and is directly related to the fluorescence decay curve of the sample. To achieve single-photon detection and avoid pileup, the level of incident fluorescence intensity delivered to the MCP-PMT is limited so that the count rate at the constant fraction discriminator (CFD) is no more than 10% of the exciting laser pulse repetition frequency. Lifetime values were determined by deconvolution of the fluorescence decay with the system response obtained by placing a light-scattering medium on the microscope stage and then acquiring data using the same settings (time scale, count rate, etc.) with which the experimental data were accumulated. For each Ca^{2+} concentration, two decay curves were collected form the cytoplasm and nuclear regions of the cells from two cells before and after permeabilization. The data were analyzed using one- and two-component curve-fitting software.

ing tremendous attention as a strong visible fluorescent reporter without the need of additional cofactors. To date, GFP has been used as a reporter of gene expression, a tracer of cell lineage, and a fusion tag to monitor protein localization within living cells. GFP engineering is rapidly creating different color mutants, from wild-type green to blue emitters and red-shifted excitation derivatives.[15] The availability of several different colored mutants of GFP opens the attractive possibility of FRET imaging in living cells or tissues. Recently, preliminary experiments performed employing bright BFP (P4-3) and GFP (S65T) demonstrated the possibility of using these proteins for FRET experiments.[16] BFP (P4-3) has an absorbance peak at 380 nm and an emission peak at 450 nm, while GFP (S65T) has an absorbance peak at 489 nm and an emission peak at 511 nm. GFP (S65T) has an absorbance spectrum which overlaps with BFP's emission spectrum, providing a donor and acceptor pair for FRET experiments. In principle, the spatial and temporal dynamics of protein–protein interactions could be monitored by fusion proteins with different blue and green proteins and FRET. In addition, because the spectra of GFP and BFP are distinct, it is possible to use both of these mutants to distinguish two different proteins in the same cells. FRET imaging using fluorescent protein techniques represents a new generation of powerful assays to study protein–pro-

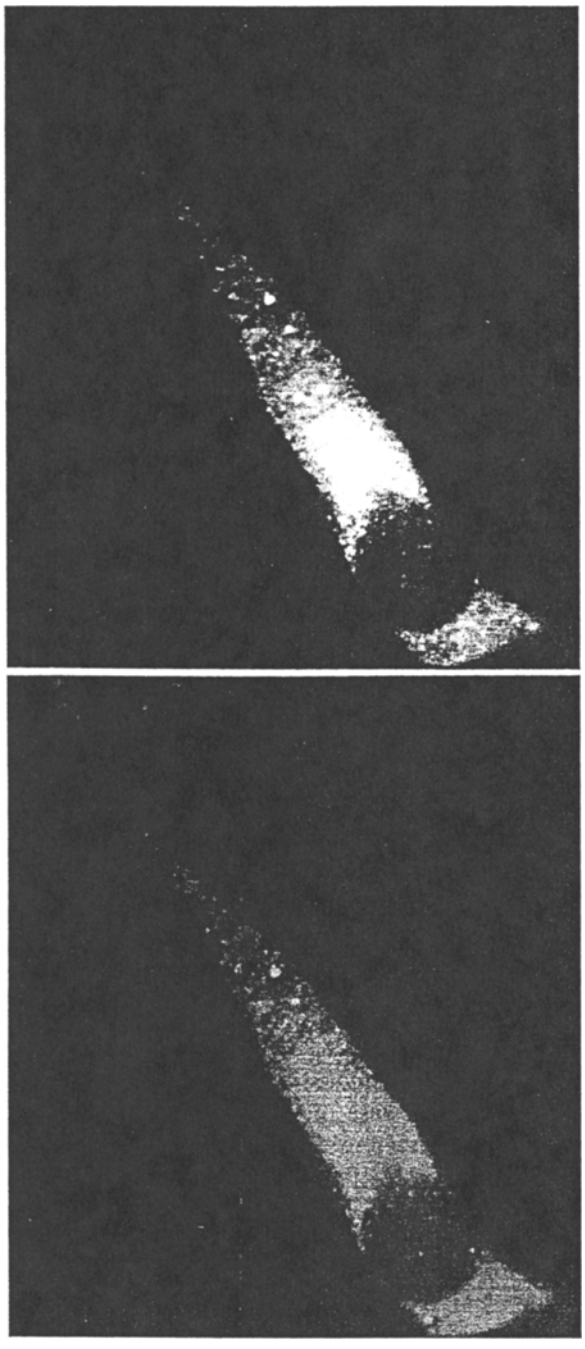

Fig. 2. *Expression and localization of GFP–Bax in 3T3 cells.* Cells were transfected with DOTAP and 5 μg of pNBX construct (GFP–BAX chimeric protein) in J2 3T3 cells. Cells were selected at 1.2 mg/ml G418 and plated onto glass coverslips. J2 3T3 cells transfected with pNBX were visualized 24 h after replating using FITC (top; BAX) or TMRM (bottom; tetramethyl rhodamine methyl ester—mitochondria) filter sets at 100 × magnification.

tein interactions *in vivo* and *in situ* for wide arrange of biomedical applications.

The Bcl-2 protein is an important regulator of apoptosis (programmed cell death).[17] Recently, it has been reported that Bcl-2 interacts with Bax, a protein that stimulates apoptosis. Thus, the interaction of these two proteins with each other is thought to play a crucial role in programmed cell death or apoptosis. However, the mechanism and detailed process of Bcl-2-Bax protein–protein interaction at the intact cellular level in relation to the apoptotic response is not clear. We are currently conducting studies examining the interaction of these regulators of apoptosis (Bcl-2 and Bax) using FRET microscopy, employing a mammalian expression vector pS65T (green), a "bright" variant with the serine at position 65 mutated to threonine to label Bax, and PY-4 (Y66H/Y145F) (blue) to label Bcl-2. Compared to the wild-type protein, the emission peak of S65T is about sixfold higher; even more efficient humanized GFP mutants have recently been developed.

The cDNA of the human Bax protein was first amplified by polymerase chain reaction (PCR). A 0.55-kb PCR product was digested with *Bql*II and *Eco*RI and subsequently subcloned (in-frame with GFP coding region) into the pS65T vector. The cDNA of the jellyfish *Aequorea victoria* blue-shifted variant (Y66H/Y145F) protein was first amplified by the PCR and subsequently subcloned into the pS65T vector. (The pS65T vector was earlier digested with *Pin*AI and *Bgl*II enzymes so as to remove the GFP coding region.) This construct was named pNBL. The cDNA of human Bcl-2 protein was then amplified by PCR, and a 0.73-kb PCR product was digested with *Xho*I and *Kpn*I and subsequently subcloned (in-frame with BFP coding region) into the pNBL vector. This construct was then digested with *Nhe*I and *Kpn*I enzymes to release a fragment of 1.5 kb (which represents the blue variant of GFP in-frame with Bcl-2 cDNA). This 1.5-kb DNA fragment was subcloned into a mammalian expression vector pREP10, containing a hygromycin resistance gene for use as a selectable marker. The GFP and BFP constructs were then transfected into a variety of cells and selected by growing transfected cells in the presence of 1–2 mg/ml G-418 and 0.4 mg/ml hygromycin. Figure 2 demonstrates the expression of GFP-Bax and its localization to mitochondria, as has been shown by others.[18]

DISCUSSION

Ionized calcium (Ca^{2+}) is an important signal transduction element in cells ranging from bacteria to spe-

cialized neurons.[19] With the introduction of fluorescent probes (e.g., Fura-2, Fluo-3), Ca^{2+} imaging has been intensively undertaken in a number of cell types.[20] Quantitative Ca^{2+} imaging has been pursued using fluorescent ratiometric probes (e.g., Fura-2, Indo-1).[21] However, in practice, there are still some problems regarding quantitative estimation of Ca^{2+} using these probes. Existing ratiometric probes are usually excited at ultraviolet (UV) wavelengths, which could generate photobleaching and other photodamage in living cells. Although some confocal microscopes have modified optics and are adapted for use with UV excitation, the UV laser wavelengths available are limited (e.g., it is not possible to perform Ca^{2+} measurements using Fura-2 which requires two UV excitation wavelengths) and the interactions between the UV excitation and the natural chromophores in living cells can still generate photobleaching and photodamage as well as autofluorescence. Moreover, since there is no proper ratio probe for visible confocal laser scanning microscopy studies, nonuniform loading of Ca^{2+} fluorescent probe concentration can affect quantitative confocal Ca^{2+} imaging. To address these problems, new types of fluorescent probes have been developed.[22] Some of these can be used as fluorescence lifetime probes for Ca^{2+} imaging. Fluorescence lifetime probes are equilibrium probes that respond to calcium concentrations independent of probe concentration and photobleaching.[23] Fluorescence lifetime probes eliminate the need for ratiometric measurements using UV-wavelength Ca^{2+}-sensitive probes. These nonratiometric probes are extremely important for imaging the calcium concentration in cells using confocal laser scanning microscopy for three-dimensional Ca^{2+} imaging.

Lifetime measurements are relatively independent of the intensity and concentration of the probe; measurement of ion concentrations using intensity measurements may not be. For example, increases in probe concentration could be mistaken for an increase in Ca^{2+} when in fact the Ca^{2+} level remains the same. This is particularly worrisome when Ca^{2+} levels are being estimated using single-wavelength Ca^{2+} probes such as Calcium Crimson. In addition, the environment of the probe can also interfere with its ability to determine Ca^{2+} concentrations accurately. Intensity images of cells labeled with the Calcium Crimson show that the nuclear region of the cell appears much brighter than the cytoplasm and would thus be assumed to have a higher level of Ca^{2+}. However, lifetime images demonstrate a fairly uniform lifetime of Calcium Crimson throughout the cell, indicative of a fairly uniform level of Ca^{2+}.[24] This demonstrates one of the advantages of TRFLIM—that *lifetime measurements, especially of single wavelength probes, are more accurate than intensity-based measurements.* Lifetime measurements also appear to be insensitive to environmental effects normally seen in intensity-based Ca^{2+} measurements.

TRFLM can also be used to measure other types of cellular ions. The fluorescence lifetime of a probe can be dependent on pH, oxygen, intracellular ion concentrations (e.g., Mg^{2+}, Na^{+}, K^{+}) and a variety of other substances. For example, the fluorescence lifetime of the electron carrier nicotinamide adenine dinucleotide (NADH) increases after binding to proteins, allowing one to image the free and protein-bound NADH in cells.[25] Moreover, the time resolution of present calcium imaging systems is insufficient to study fast changes (e.g., receptor mediated) in living cellular calcium levels in real time.[26,27] Such changes are expected to be very rapid and not assessable using current available instrumentation. Use of TRFLM would enable examination of these transient calcium signal changes on a nanosecond time scale in living cells.

FRET imaging[26,27] is a microscopic technique for quantifying the distance between two molecules conjugated to different fluorophores. In principle, if one has a donor molecule whose fluorescence emission spectrum overlaps the absorbance spectrum of a fluorescent acceptor molecule, then they will exchange energy between one another through a nonradiative dipole–dipole interaction.

To date there are only a few reports in the literature using time-resolved FRET imaging. Studies of epidermal growth factor (EGF) receptors have been carried out using frequency-domain lifetime imaging microscopy and FRET.[28] EGF receptor clustering during signal transduction was monitored and a stereochemical model for the tyrosine kinase of the EGF receptor has been investigated. Time-resolved FRET imaging has also been applied to study the extent of membrane fusion of individual endosomes in single cells.[29] Using time-domain FLIM and FRET, the extent of fusion, and the number of fused and unfused endosomes were clearly visualized and quantitated.

Successful undertaking of FRET imaging requires that several points be considered. First, the donor and acceptor fluorophore concentrations need to be strictly controlled. This is currently a challenge when using GFP and BFP constructs, as each construct needs to be under the control of an inducible promoter, so that proper expression and appropriate donor/acceptor ratios can be obtained. To achieve a high S/N, high fluorescence signals are preferred. This is currently a concern with BFP, which has a low quantum yield. Conversely, too high a concentration of dye can cause self-quenching and dis-

ordered biological function.[30] Second, photobleaching needs to be prevented. Almost all fluorescent molecules are sensitive to photobleaching, although the GFPs (but not the BFPs) are relatively resistant to photobleaching. Photobleaching can alter the donor–acceptor ratio and therefore the value of FRET. Third, ideally, the donor emission spectrum should substantially overlap the absorption spectra of the acceptor. Fourth, there should be relatively little direct excitation of the acceptor at the excitation maxima of the donor. Fifth, the emission of both the donor and the acceptor occur in a wavelength range in which the detector has maximum sensitivity. Sixth, there should be little if any overlap of the donor absorption and emission spectrum, thus minimizing donor–donor self-transfer. Seventh, the emission of the donor should ideally result from several overlapping transitions and thus exhibit low polarization. This will minimize uncertainties associated with the K^2 factor.

The use of GFP and BFP provides a unique opportunity for FRET measurements. By inserting the GFP and/or BFP cDNA into the cDNA of the molecules of interest using molecular biology techniques, direct labeling of the donor and acceptor molecules can be achieved. Thus, it is possible to obtain directly labeled donor and acceptor molecules where the label is actually part of the molecule itself. Future challenges include regulating the expression of the donor and acceptor tagged molecules such that quantitative FRET measurements are possible. In addition, further mutations of the GFP molecule will hopefully result in better donor/acceptor pairs, more spectrally distinct fluorescent proteins, donor fluorophores with higher quantum yields, and acceptor fluorophores with higher extinction coefficients.

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