PERSPECTIVE

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Time-resolved fluorescence microscopy†

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In fluorescence microscopy, the fluorescence emission can be characterised not only by intensity and position, but also by lifetime, polarization and wavelength. Fluorescence lifetime imaging (FLIM) can report on photophysical events that are difficult or impossible to observe by fluorescence intensity imaging, and time-resolved fluorescence anisotropy imaging (TR-FAIM) can measure the rotational mobility of a fluorophore in its environment. We compare different FLIM methods: a chief advantage of wide-field time-gating and phase modulation methods is the speed of acquisition whereas for time-correlated single photon counting (TCSPC) based confocal scanning it is accuracy in the fluorescence decay. FLIM has been used to image interactions between proteins such as receptor oligomerisation and to reveal protein phosphorylation by detecting fluorescence resonance energy transfer (FRET). In addition, FLIM can also probe the local environment of fluorophores, reporting, for example, on the local pH, refractive index, ion or oxygen concentration without the need for ratiometric measurements.

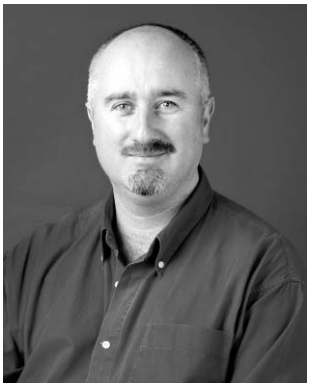
† Dedicated to Professor Hiroshi Masuhara on the occasion of his 60th birthday.

Klaus Suhling is a lecturer in the Department of Physics at King's College London. Trained as a physicist, he has held various post-doctoral positions in biology, chemistry and physics laboratories, most recently at Imperial College London. The common theme of most of his work has been fluorescence spectroscopy and imaging. His research at the interface of biology, chemistry and physics involves the development and use of fluorescence lifetime imaging (FLIM) and related techniques to understand the properties and interactions of macromolecules in the biological and biomedical sciences.

Paul French was born in Felixstowe, UK, in 1962. He received his BSc degree in Physics and his PhD degree for work on femtosecond dye lasers from Imperial College, University of London, in 1983 and 1987, respectively. His subsequent research career at Imperial College London has concentrated on ultrafast laser technology and its applications, particularly in biomedical optics, although he worked on ultrafast all optical switching in optical fibres at AT&T Bell Laboratories from 1990 to 1991. He is currently a Professor of Physics at Imperial College London and is Head of the Photonics Group. His research portfolio includes ultrafast laser and fibre technology, high-speed 3-D imaging and multi-dimensional fluorescence imaging with a particular emphasis on fluorescence lifetime imaging.

David Phillips is the Dean of the Faculties of Life Sciences and Physical Sciences at Imperial College London. He is a leading figure in the world of photochemistry and laser-based research and has published over 500 papers in this area. The main achievements of his group in the past decade have been in the field of photodynamic therapy (PDT): his group has synthesised new dyes, studied the binding of these dyes to proteins, and investigated the photophysics and photochemistry in detail, including measurement of singlet oxygen. To visualise the distribution of the dyes in tissue and in single cells, a variety of microscopies including confocal and picosecond time-resolved imaging has been developed in his laboratory.

Klaus Suhling



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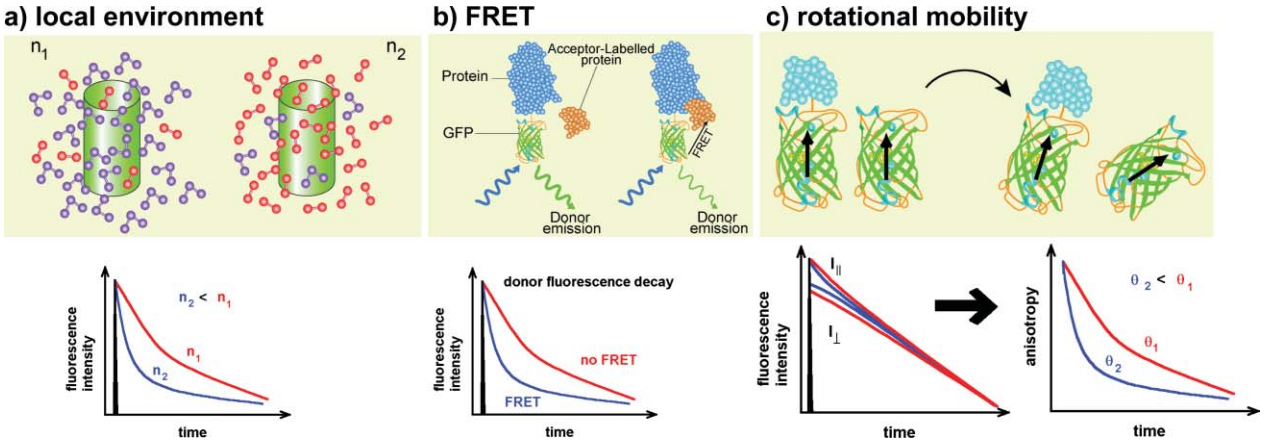

Fig. 1 Schematic representation of the photophysical phenomena that FLIM can study, and their effect on the fluorescence decay. (a) The fluorescence decay is a function of the environment of the fluorophore. For example, the fluorescence lifetime of GFP is a function of its local refractive index.[80] A low refractive index around GFP leads to a longer lifetime, whereas a high refractive index environment causes a shorter lifetime. The fluorescence lifetime of other probes can be sensitive to pH, ions, oxygen *etc.* (see text). (b) FRET occurs when a suitable donor and acceptor are in close proximity, usually below 10 nm. Thus imaging of FRET can measure the proximity of fluorescent or fluorophore-tagged proteins in live cells with 10–100 times the resolution limit in far-field optical microscopes. With FLIM, FRET between a donor and acceptor can be identified by a shortened fluorescence decay of the donor. (c) Polarization-resolved FLIM to perform time-resolved fluorescence anisotropy imaging[55,56] reveals the rotational mobility of a fluorophore. This is affected by the viscosity of its surroundings, or by binding and conformational changes that affect the rotational mobility. The latter is characterized by the rotational correlation time which can be calculated from the difference between the polarization-resolved fluorescence decays $I_{\parallel}$ and $I_{\perp}$. A fast rotational motion leads to a rapid depolarization. Furthermore, the initial anisotropy may be used to probe homo-FRET. In each of these applications, the fluorescence lifetime [or in (c), the rotational correlation time] is encoded in a false colour scale for each pixel of the image to generate contrast.

Introduction

Hiroshi Masuhara was a pioneer of the use of microscopy to study complex systems, mainly those of technological significance. In honour of his great contribution to his field, we offer here a review of fluorescence lifetime imaging (FLIM) instrumentation and highlight some cell biological studies.

Optical imaging techniques, in particular fluorescence imaging techniques, are powerful tools in the biological and biomedical sciences today, because they are minimally invasive and can be applied to live cells and tissues.[1,2] Microscopy, in particular, relies on the contrast that can be achieved from absorption, polarization, phase *etc.* in conventional microscopy, and fluorescence parameters in a fluorescence microscope. Conventionally, fluorescence intensity is measured, and three-dimensional images can be recorded using confocal [one or two-photon excitation] techniques. However, fluorescence intensity is dependent upon a variety of environmental influences, such as quenching by other molecules, aggregation, energy transfer, and refractive index effects, and can thus be difficult to quantify or interpret. FLIM provides contrast according to the fluorescence decay time, the inverse of the sum of the rate parameters for all depopulation processes.[3]

The fluorescence decay time, or as commonly used, lifetime τ is the average time a fluorophore remains in the excited state after excitation, and it is defined as

$$\tau = \frac{1}{k_r + k_{nr}} \quad (1)$$

where k_r is the radiative rate constant, and k_{nr} the non-radiative rate constant. $\tau_0 = k_r^{-1}$ is the natural or radiative lifetime which is related to the fluorescence lifetime τ *via* the fluorescence quantum yield ϕ:

$$\phi = \frac{\tau}{\tau_0} = \frac{k_r}{k_r + k_{nr}} \quad (2)$$

Eqn. (1) and (2) assume no quenching processes. If there is quenching by another molecule present, Q, then in addition to the terms in the denominator there will appear the term k_Q[Q] where k_Q is the quenching rate constant, and [Q] the quencher concentration.

While the fluorescence decay time depends on the intrinsic characteristics of the fluorophore itself, it also depends in a measurable way upon the local environment. In general, the local viscosity, pH, or refractive index [see Fig. 1(a)], as well as interactions with other molecules *e.g.* by collision or energy transfer [Fig. 1(b)], can all affect the fluorescence lifetime.[4,5] Thus, as well as being able to distinguish spectrally similar fluorophores,[6] imaging of the fluorescence lifetime can be used to probe the surroundings of a fluorophore (see Table 1). In the last decade or so, since the first reports on FLIM,[7,8] the technique has been improved, developed further and applied to an increasing number of studies in cell biology.[9,10]

FLIM instrumentation

FLIM is a time-resolved image acquisition method, the technologies for which can be divided into two categories: (i) confocal scanning[11,12] or multiphoton excitation[13,14] FLIM where the image is acquired pixel-by-pixel using a non-imaging detector, *e.g.* a photomultiplier, and (ii) wide-field camera-based FLIM.[15] The time-resolved information is obtained either in the time domain by exciting the sample with a short optical pulse and observing the decay of the fluorescence intensity (with time-correlated single photon counting (TCSPC), gating, or a streak camera), or in the frequency domain by modulating the excitation source and/or the detector to calculate the fluorescence decay time from the demodulation and the phase shift of the fluorescence.

In the time domain, a fluorescence decay curve can be directly acquired after excitation of the sample with an ultrashort light pulse, usually using a sampling technique.[8,16–19] In wide-field time-gated FLIM, 'snapshots' of the fluorescence emission are taken at various nanosecond delays after the excitation using high-speed gated image intensified cameras.[15,17] This approach is fast, since all the pixels are acquired in parallel—a 100 Hz FLIM frame rate has been reported[20]—but it lacks single photon sensitivity and accuracy, and its temporal resolution is limited to ≈80 ps[21] (see Table 2). In confocal scanning or multiphoton excitation microscopes (which provide inherent optical sectioning) FLIM is essentially a series of single channel fluorescence lifetime measurements where the fluorescence decay can be acquired by TCSPC.[22,23] TCSPC is a mature and reliable

Table 1 Advantages and disadvantages of FLIM

Advantages	Disadvantages
The fluorescence lifetime is a molecular property generally independent of variations in fluorophore concentration, illumination intensity, light pathlength, scatter, or photobleaching	Photophysical and time-resolved fluorescence spectroscopy expertise required for data interpretation (*e.g.* fluorescent proteins usually have complex fluorescent decays)
FLIM can robustly and quantitatively probe the fluorophore's local environment directly, *e.g.* refractive index, viscosity, pH, ions *etc.* without the need to compromise the cell with biochemical assays	Interpretation of specific changes in fluorescence lifetime in terms of the underlying cell biochemistry may not be straighforward
FLIM of FRET by imaging the fluorescence decay of the donor is more robust than fluorescence intensity-based FRET and allows to distinguish between effects due to FRET efficiency and probe concentration. Quantitative FRET studies between spectrally similar donor and acceptor, *e.g.* GFP and YFP are also possible[88]	Complex and expensive equipment required
FLIM can experimentally distinguish spectrally similar probes (if their fluorescence lifetimes are different) with the same detector	

Table 2 Summary of some of the advantages and disadvantages of various implementations of FLIM. Note that in wide-field FLIM, optical sectioning to remove out-of-focus blur can be achieved with structured illumination,[140,141] or multiple beam scanning techniques.[48] Although the wide-field point-spread function (PSF) is bigger than the confocal PSF, the poor spatial resolution of intensified CCD cameras in wide-field microscopy is mainly due to the microchannel plate and phosphor screen technology. In principle, this drawback could be overcome by photon counting imaging with centroiding to sub-CCD pixel accuracy[142,143]

Implementation	Advantages	Disadvantages
Time-gated wide-field time domain	Fast	Low sensitivity, need strong signal
	All pixels acquired in parallel	Consecutive acquisition of time gates vulnerable to photobleaching and sample movement
		Poor spatial resolution due to phosphor screen on intensified CCD camera
		Need pulsed laser
Wide-field frequency domain	Fast	Cannot easily resolve two very different lifetimes
	Easy to modulate cw laser	Vulnerable to photobleaching and sample movement
	Can resolve two similar lifetimes	Complex data and error analysis
	No deconvolution (temporal) of instrumental response and fluorescence decay necessary	Poor spatial resolution due to phosphor screen on intensified CCD camera
	All pixels acquired in parallel	Usually lower temporal resolution and lower signal-to-noise ratio than time domain methods
Confocal/multiphoton scanning with time-correlated single photon counting	Single photon sensitivity	Slow, each photon has to be timed individually
	Unlimited dynamic range associated with photon counting techniques	Need pulsed laser
	Linear recording characteristics independent of excitation intensity fluctuations and photobleaching	
	Easy visualization of fluorescence decays and well-defined Poisson statistics	
Inherent optical sectioning		
	Best signal to noise ratio	
	High temporal resolution	
Confocal/multiphoton scanning with time-binning photon detection	Fastest scanning technique	Need pulsed laser
	Inherent optical sectioning	Slower than wide-field imaging
	Single photon sensitivity	Less accurate than time-correlated single photon counting
Streak camera FLIM	Very high temporal resolution	Expensive
	Fast	
	Easy visualization of fluorescence decays	

technique which records the arrival time of single photons after an excitation pulse. The ease of reproducibility of measurements is due to the unique combination of advantages such as the unlimited dynamic range associated with photon counting techniques, linear recording characteristics independent of excitation intensity fluctuations and photobleaching, well-defined Poisson statistics, excellent signal to noise ratio and a high temporal (picosecond) resolution (see Table 2). As each photon is timed individually in each pixel of the image, the collection of many photons for a high statistical accuracy can be time-consuming.[24] The maximum photon flux that can be timed using a single channel (one detector, time to amplitude converter (TAC) and analogue to digital converter (ADC)) is limited by photon pile-up and the dead time of the electronics to $\approx 10^6$ photons s^{-1}.

A similar but rather faster approach is to bin all incoming photons within preset time windows after excitation.[16,25] This time-binning method is significantly faster than TCSPC because it is not necessary to reduce the fluorescence signal to the level of single photon timing. However, it is less accurate than TCSPC (see Table 2). The use of streak-camera based FLIM has also recently been reported.[26–28] The technique works in line-scanning mode, is fast, has the highest temporal resolution of any FLIM technique, and it has been commercialised.

In the frequency domain periodically modulated excitation beams and detectors may be used to measure the phase shift and demodulation of fluorescence signals with respect to their excitation signals, both in wide-field microscopy using modulated intensified cameras[29–32] and in confocal/multi-photon

laser scanning microscopy using single channel detectors.[33–37] Frequency domain techniques have been used since the 1920s to measure nanosecond fluorescence decays. With this approach a fluorescence lifetime may be calculated from both the phase shift and demodulation (at several modulation frequencies if necessary).[38] For a simple mono-exponential fluorescence decay profile, both calculations should yield the same value. For more complex decays, *e.g.* in the case of some fluorescent proteins such a CFP, the phase shift lifetime is shorter than the demodulation lifetime.[29]

There is a lively debate as to the relative merits of time or frequency domain approaches to FLIM. In principle they are, of course, related by a Fourier transformation and have experimentally been demonstrated to be equivalent.[39] To non-specialists, the easy visualization of fluorescence decays in the time domain may be an advantage over the frequency domain, where the analysis of complex fluorescence decay profiles, such as stretched exponentials, is less tractable than in the time domain.[40] However, for some applications the frequency domain instrumentation is considered easier to implement since ultra-short pulsed laser sources are not required, especially for longer lifetimes, although practitioners are increasingly using mode-locked lasers for frequency domain measurements—particularly in multiphoton microscopes.[41,42] Frequency domain techniques are more photon efficient than time-gating techniques and require no deconvolution of the instrumental response and the fluorescence decay (see Table 2). However, a recent study shows that the signal-to-noise ratio is higher for time domain measurements than for frequency domain measurements.[43]

One potential pitfall of the time domain approach is that there needs to be sufficient time ($\approx 5\tau$) between excitation pulses for the sample fluorescence to completely decay in order to obtain accurate fluorescence lifetime values. In practice this implies using mode-locked lasers with pulse-pickers, cavity-dumpers, lower repetition rate pulsed diode lasers[44] or appropriate fitting procedures to take residual fluorescence into account.[45] This is not an issue for the frequency domain approach.

The FLIM techniques continue to be improved, particularly by the reduction of acquisition times,[25] the extension to include spectrally-resolved imaging[46,47] and rapid optical sectioning capabilities.[48,49] The relative merits of the various FLIM implementations are summarized in Table 2, and it depends on the operator's preference (and finances!) for fast data acquisition or accuracy, high temporal and spatial resolution which system to choose.

Time resolved fluorescence anisotropy imaging (TR-FAIM)

Polarization-resolved FLIM captures a further parameter of the multidimensional fluorescence emission contour and allows complementary information about the fluorophore's environment to be obtained. Upon excitation with linearly polarized light, rotational diffusion of the fluorophore in its excited state results in a depolarization of the fluorescence emission.[4,5] Steady-state anisotropy imaging has, for example, been used to obtain contrast between fluorescein and GFP in a cell due to their different anisotropies, *i.e.* their molecular sizes.[50] In additon, energy migration or homo-FRET [resonance energy transfer between the same type of fluorophore] can be detected with this method, since it also leads to a depolarization of the emitted fluorescence.[51,52] The technique has been used to study the proximity of isoforms of the GPI-anchored folate receptor bound to a fluorescent analogue of folic acid to detect lipid rafts.[53] Fluorescence decays measured at polarizations parallel and perpendicular to that of the excitation, *i.e.* TR-FAIM, can measure the rotational mobility of the fluorophore in its environment [Fig. 1(c)]. Thus, the viscosity of the fluorophore's environment, binding events or hindered rotation can be examined with this method.[51,54–56] The time-resolved fluorescence anisotropy $r(t)$ can be defined as

$$r(t) = \frac{I_{\parallel}(t) - B - G(I_{\perp}(t) - B)}{I_{\parallel}(t) - B + 2(GI_{\perp}(t) - B} \quad (3)$$

where $I_{\parallel}(t)$ and $I_{\perp}(t)$ are the fluorescence intensity decays parallel and perpendicular to the polarization of the exciting light. G accounts for different transmission and detection efficiencies of the imaging system at parallel and perpendicular polarization, and B accounts for a non-zero background.[54] For a spherical rotor, $r(t)$ decays as a single exponential and is related to the rotational correlation time θ according to

$$r(t) = (r_0 - r_\infty)\exp\left(-\frac{t}{\theta}\right) + r_\infty \quad (4)$$

where r_0 is the initial anisotropy and r_∞ is the limiting anisotropy which accounts for a restricted rotational mobility. For a spherical rotor in an isotropic medium, θ is directly proportional to the viscosity η of the solvent and the volume V of the rotating molecule:

$$\theta = \frac{\eta V}{kT} \quad (5)$$

where k is the Boltzmann constant and T the absolute temperature.

Clayton *et al.* used time-resolved fluorescence anisotropy imaging not only to map the viscosity of fluorescein solutions, but also to study the average distance between GFPs expressed in bacteria.[56] They detected a reduced initial anisotropy which was thought to be caused by GFP-GFP FRET due to the close proximity of the proteins in bacteria. Moreover, as the rotational diffusion can be slowed down by binding, TR-FAIM has potential to visualise the binding of ligands and receptors in the cell. The system used in the authors' laboratory for TR-FAIM is shown in Fig. 2.

Applications of FLIM to cell biology

Förster resonance energy transfer (FRET)

The most widespread application of FLIM in cell biology is the identification of FRET upon the interaction between suitably (and stochiometrically) labelled specific proteins, lipids, enzymes, DNA and RNA, as well as cleavage of a protein, or conformational changes within a protein.[57–59] FRET is a bimolecular fluorescence quenching process where the excited state energy of a donor fluorophore is non-radiatively transferred to a ground state acceptor molecule by a dipole–dipole coupling process.[60] The FRET efficiency, E, varies with the inverse 6th power of the distance between donor and acceptor, and is usually negligible beyond 10 nm. FRET can therefore be used as a 'spectroscopic ruler' to probe intermolecular distances on the scale of the dimensions of the proteins themselves.[61–63] This is a significant advantage over co-localization studies with two fluorophores which is limited by the optical resolution (approximately 200 nm laterally, 500 nm axially[11,12]).

For FRET to occur, the emission spectrum of the donor and the absorption spectrum of the acceptor must overlap,[64] and the transition dipole moments of the donor and acceptor must not be perpendicular—otherwise the transfer efficiency is zero, irrespective of the donor–acceptor distance or the spectral overlap.[65,66] The critical transfer distance R_0, where FRET and fluorescence emission are equally likely, can be calculated from the spectral overlap. We note here that free Photochem-CAD software to calculate the R_0 for any donor/acceptor pair can be downloaded from http://chemdept.chem.ncsu.edu/%7Ejslftp/.[67] Imaging FRET[68] can thus be used to map interactions between proteins, lipids, enzymes, DNA and RNA, as well as follow cleavage of a protein, or conformational changes within a protein.[1,2,69,70] FRET, as a fluorescence quenching process, reduces the quantum yield and the fluorescence lifetime of the donor. If the acceptor is fluorescent (which incidentally

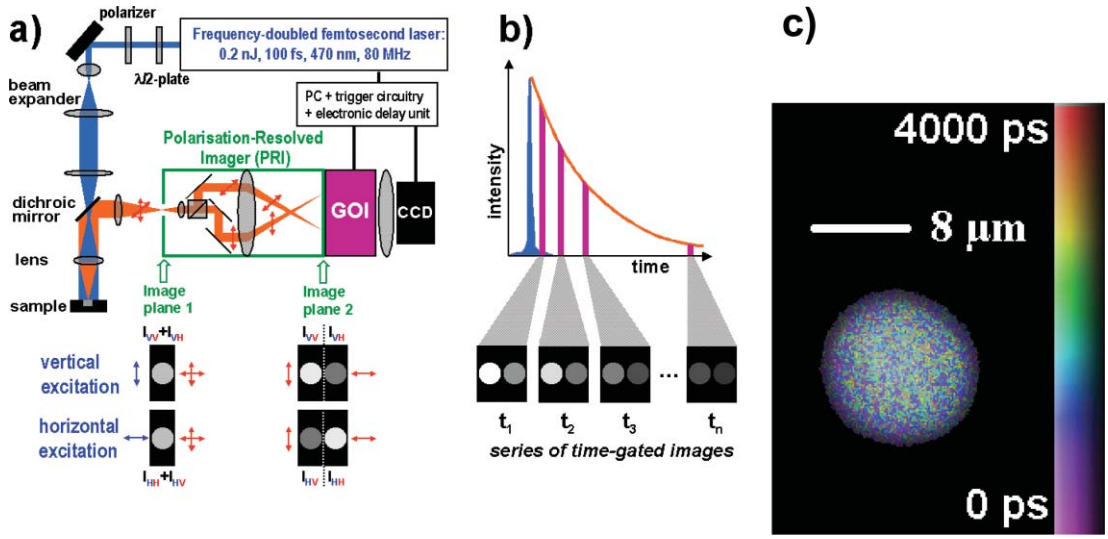

Fig. 2 (a) Experimental set-up of the wide-field TR-FAIM instrument (polarization-resolved time-gated FLIM). The Polarization-Resolved Imager (PRI) contains a polarizing beamsplitter and adjustable mirrors. Using a C-mount adapter, the PRI is mounted onto the gated optical image intensifier (GOI), the output phosphor screen of which is imaged with a CCD camera. The PRI splits a single image in image plane 1 into two spatially identical images differing only by their polarization (image plane 2), which are thus recorded simultaneously. (b) A series of such polarization-resolved fluorescence intensity image pairs are acquired at various delays after the excitation pulse to sample their fluorescence decay profiles. (c) A rotational correlation time image of a B cell stained with the fluorescein derivative CFSE staining the cytoplasm. The rotational correlation time reports on the viscosity of the cytoplasm. Its average value is 4.50 ± 0.87 ns at 20 °C, which corresponds to an average cytoplasmic viscosity of 14 cp. The image was obtained on a wide-field time-gated FLIM microscope with a ×63 water immersion objective.[54]

is not a necessary requirement for FRET to occur), FRET leads to sensitized acceptor emission. To identify and quantify FRET, the fluorescence decay of the donor can be measured in the absence and presence of the acceptor. The advantage of time-resolved over intensity-based measurements is the ability to directly distinguish between effects due to FRET or probe concentration. For example, a low donor fluorescence intensity can be caused by either a low donor concentration or efficient quenching—but only in the latter case is the fluorescence decay shortened [see Fig. 1(b)].

Before the availability of fluorescent proteins, intracellular fusion of endosomes was studied with FLIM of FRET.[71] The endosomes were sequentially loaded using calcein as the donor, and a sulforhodamine acceptor, and FRET occurred upon the fusion of the endosomes containing the donor and those containing the acceptor. Another example before the common use of fluorescent proteins in cell biology is the study of the dimerisation of epidermal growth factor (EGF) receptors by covalently binding fluorescein and rhodamine to EGF, and observing FRET upon their dimerization.[72] A similar concept was used to study the role of the protein kinase C (PKC) family of proteins in cellular signal transduction. This was done using purified PKC βI, tagging it with the fluorophore Cy3 and microinjecting it into cells.[73] When the investigators co-injected an antibody to PKC βI tagged with Cy5, they found that FRET was abrogated for intranuclear PKC βI, concluding that this protein is fragmented there. A limitation of this approach is that the microinjection into cells is not a routine procedure for many cell types and is somewhat invasive.

Now, fluorescent proteins can be directly tagged to a specific protein using genetic means, which is usually minimally invasive.[74,75] The excitation and emission spectra of the green fluorescent protein (GFP) are in the visible range, but the photophysics of the fluorescence proteins is complex.[76] The widely used mutant enhanced GFP (F64L, S65T), for example, has at least two emitting states.[77–80] Nonetheless, FLIM of GFP, and their spectral variants cyan or cerulean fluorescent protein (CFP) and yellow fluorescence protein (YFP), with average fluorescence lifetimes in the 2–3 ns region,[81] has proved valuable.

FLIM of FRET between the donor PKCα-GFP and the cytoskeletal linker ezrin stained with a Cy3-labelled antibody as the acceptor showed that, on activation, PKCα colocalizes and interacts with ezrin at the plasma membrane.[82] Association of the protein CD44 with ezrin upon PKC activation was observed by a similar methodology, using FLIM of FRET between GFP-tagged CD44 and a Cy3-conjugated antibody.[83] These studies serve to demonstrate an approach that could be exploited for studying the constitutive or transient association of numerous other receptors with ERM proteins, other cytoskeletal linkers, or adaptor proteins.

FLIM of the GFP fluorescence decay has been used to report on the phosphorylation of GFP-tagged PKCα in live cells by identifying a shortened GFP fluorescence lifetime due to FRET between the donor PKCα-GFP and a Cy3.5-labelled phosphorylation specific antibody as the acceptor.[84] A disadvantage of this application is that it requires use of an antibody against a specific phosphorylated form of the protein, which is not always available. However, this requirement was circumvented in imaging the phosphorylation of GFP-tagged ErB1 receptors, by identifying a shortened GFP fluorescence lifetime due to FRET between the donor ErB1-GFP and a Cy3-labelled antibody to phosphotyrosine.[85,86] Dephosphorylation has also been studied with FRET.[87]

FLIM of FRET between the spectrally similar donor GFP and acceptor YFP has been used to monitor caspase activity in individual cells during apoptosis.[88] Here, a tandem GFP YFP construct incorporating the DEVD caspase recognition sequence is used as a monitor of apoptosis such that FRET occurs between them. The GFP and YFP fluorescence decays are collected simultaneously, and using appropriate data analysis, FRET is identified by a rise time due to sensitised acceptor emission. As the protein undergoes proteolytic cleavage, GFP and YFP move away from each other and FRET is disrupted. In this case, detecting FRET by FLIM is particularly advantageous over detecting FRET by imaging fluorescence intensities, since the donor and acceptor emission spectra overlap so that their fluorescence intensities cannot be easily separated.

FLIM of FRET has also been used to probe NADH[89,90] and the the supramolecular organization of DNA.[91–94]

FLIM of Ca^{2+}, oxygen, pH concentration

Instead of using intensity-based imaging of ratiometric probes, the fluorescence lifetime of the Ca^{2+} sensor Quin-2,[95,96] calcium

crimson[97] or CalciumGreen and Fluo-3[98] has been used to image the Ca^{2+} concentration in cells. The use of FLIM in these cases is more robust and reliable than fluorescence intensity-based imaging methods, since FLIM is unaffected by variations of illumination intensity, variations of fluorophore concentration or photobleaching–provided the probes do not aggregate, and the photoproducts do not fluoresce (see Table 1). Moreover, FLIM of a long-lived ruthenium-based oxygen sensor with an unquenched decay time of 760 ns has been used to map oxygen concentrations in macrophages.[99] Note that intensity-based fluorescence imaging of oxygen in cells would require a calibration of the intensity of the probe unquenched by oxygen as well as knowing its concentration in the cell. This is not practically possible. FLIM has also been used to map the pH in single cells[34,100,101] and skin.[41,42] Here the pH sensor 2′,7′-bis-(2-carboxyethyl)-5-(and-6) carboxyfluorescein (BCECF) was used to image pH in the skin stratum corneum. The authors used two-photon excitation FLIM to non-destructively obtain pH maps at various depths, which is difficult to achieve by non-optical methods. Moreover, as the authors point out, intensity-based fluorescence imaging of the pH probe could not have been used for their study as the observation of a variation in fluorescence intensity could be ascribed to either a change in pH or a variation of the local probe concentration.

FLIM of tissue

FLIM of autofluorescence has been used to provide intrinsic contrast in unstained tissue[21,102–105] and teeth.[106,107] The combination of multiphoton excitation for deep, sectioned, tissue imaging with FLIM yields contrast not available with fluorescence intensity-based imaging. FLIM has also been employed to study aggregation of sensitisers in photodynamic therapy.[108–113]

Perspectives

FLIM to probe local viscosity and membrane fluidity and polarity?

The fluorescence lifetime of so-called molecular rotors, which provide a non-radiative de-excitation pathway by internal twisting in competition with radiative de-excitation, is strongly dependent on the viscosity of their surroundings, as this affects the internal twisting.[114,115] This property has been used to measure the fluidity of cell membranes[116] and binding in bulk solution.[117] The use of molecular rotors has been extended to imaging[118] and could be combined with FLIM to image the viscosity distribution in cells.

Ratiometric imaging of excited state dimer (excimer) formation of the aromatic hydrocarbon probe pyrene has also been used to study lateral diffusion in membranes.[119] An excimer is only formed between an excited state and ground state molecule, and it occurs when the monomers are in close proximity. It is characterized by a red-shift in the emission spectrum, as well as a bi-exponential fluorescence decay, with one component originating from the monomer, and the other one from the excimer. As excimer formation is a diffusion-controlled process it can be used to study the membrane fluidity, and FLIM would facilitate mapping of excimer distributions. In addition, membrane probes such as Laurdan may also be used to study membrane fluidity and cholesterol content.[120]

FLIM to probe local refractive index?

Recently, a comprehensive comparison of the fluorescence emission of the enhanced green fluorescent protein GFP (*i.e.* GFP F64L, S65T) in solution and in an intracellular environment was reported.[121] Careful analysis of the GFP fluorescence decays, obtained by TCSPC, revealed that the average fluorescence lifetime of GFP was about 8% shorter in the cytoplasm of rat basophilic leukemia mast cells than in aqueous solution—an experimental observation in agreement with those of other workers.[81,122,123] It may be interpreted, at least in part, in terms of the refractive index of the GFP environment.

In general, k_r in eqn. (1) is a function of the refractive index in the vicinity of the fluorophore, recently reviewed by Toptygin.[124] A simple model for this phenomenon is given by the Strickler–Berg equation[125]

$$k_r = k_0 n^2 = 2.88 \times 10^{-9} n^2 \frac{\int I(\tilde{\nu})\,\mathrm{d}\tilde{\nu}}{\int I(\tilde{\nu})\tilde{\nu}^{-3}\,\mathrm{d}\tilde{\nu}} \int \frac{\varepsilon(\tilde{\nu})}{\tilde{\nu}}\,\mathrm{d}\tilde{\nu} \qquad (6)$$

where n is the refractive index, defined as the ratio of the speed of light in vacuum c_0, to that in a medium $n = c_0/c$, I the fluorescence emission, ε the extinction coefficient and $\tilde{\nu}$ the wavenumber. k_r explicitly depends on the refractive index due to the polarizability of the host medium surrounding the fluorophore where the absorption and emission processes occur. Many experimental observations demonstrate the influence of the refractive index on fluorescence lifetimes, as discussed in ref. 80 and 124.

We recently studied the fluorescence decay of GFP in aqueous solution with added glycerol, polyethylene glycol, NaCl or glucose and fructose.[80] Quantitative analysis showed that τ_{av}^{-1} varied approximately linearly with the square of the refractive index in accordance with the Strickler–Berg equation, irrespective of whether the refractive index is increased by adding glycerol, polyethylene glycol, NaCl or glucose and fructose,[80] as shown in Fig 3.

The observation that, in reverse micelles, the average GFP fluorescence lifetime is lower in the water pool than in bulk water, and that it increases with increasing water pool size[79] is consistent with the decreasing refractive index of the reverse micelle as the water pool is increased.[126] In biological cells, the cellular refractive index is higher to that of the surrounding medium—a fact exploited in light microscopy to generate contrast. It is therefore consistent that the fluorescent lifetime of GFP is shorter in cells than in buffer.[81,121–123]

Despite this consistent interpretation, a cell is a much more complex system than a carefully controlled homogeneous solution. The lifetime of GFP in cells may also be affected by other factors, but our work shows that the refractive index effect on the GFP fluorescence decay cannot be neglected.

Using the fluorescence lifetime of GFP as a refractive index sensor may allow FLIM to directly image the refractive index around specific GFP-tagged proteins in live cells. A biologically relevant and specific interpretation of a local refractive index may not be straightforward, but local refractive index variations could indicate heterogeneity. This may be of use, for example, in understanding complex supramolecular processes such as the assembly of an immunological synapse.[127–129] On average, the cell membrane has a higher refractive index than the cytoplasm, $n_{membrane} = 1.46$–1.60, and $n_{cytoplasm} = 1.35$.[130,131] Different domains within a cell membrane may differ in refractive index, and contrast in the fluorescence lifetime image of GFP-tagged membrane proteins may reflect heterogeneity of the composition of the membrane. Indeed, using GFP-tagged MHC proteins in the target cell of with NK cell immune synapse, we have observed a small GFP fluorescence lifetime contrast at the intercellular contact.[132]

Outlook

FLIM of cell biology will not only be improved by progress in instrumentation, but also by advances in the techniques, methods and fluorescent labels used. New fluorescent proteins that can be activated by light,[133] or probes that allow FRET to be switched on and off by light may allow a greater versatility in studying protein–protein interactions.[134] It is likely that for imaging of cell biology, the use of quantum dots with a high fluorescence quantum yield, low photobleaching susceptibility and narrow, size-dependent emission spectra which can be excited with a

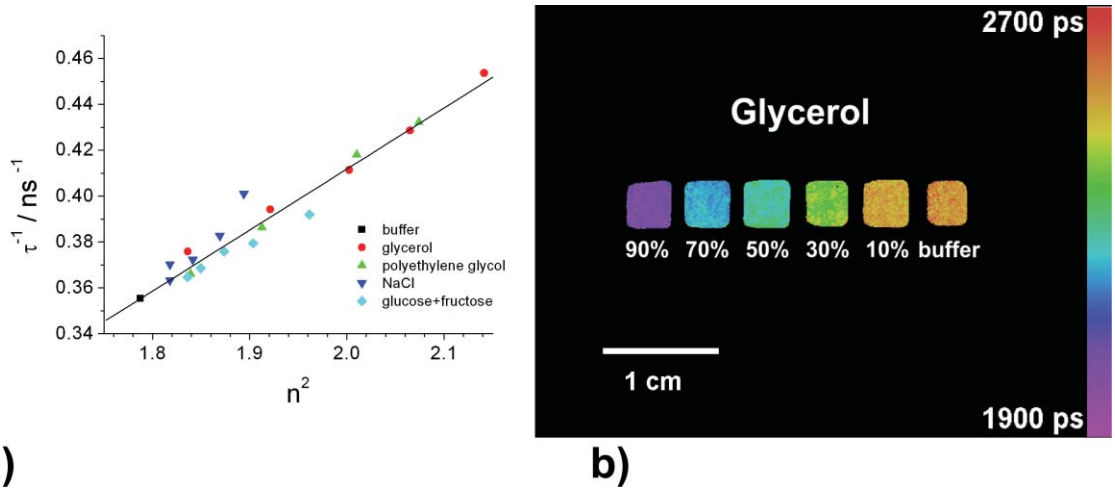

Fig. 3 (a) The inverse average fluorescence lifetime τ_{av}^{-1} of GFP *versus* the square of the refractive index of the solution. τ_{av}^{-1} varies linearly with the square of the refractive index inaccordance with the Strickler–Berg formula [eqn. (6)], irrespective of whether the refractive index is increased by adding glycerol, polyethylene glycol, NaCl or glucose and fructose. (b) Fluorescence lifetime image of GFP in mixtures of aqueous buffer and glycerol in a multiwell plate.[80,144]

single wavelength will become more widespread.[135] However the extent to which they are useful for FLIM is still unclear,[136] as their non-radiative rate constant appears to fluctuate.[137,138] On the other hand, genetically encoded fluorophores have already been designed for a wide range of wavelengths.[74] In time the complex photophysics of these probes may be fully understood. However, preferably for FLIM a fluorescent protein will be discovered or engineered to have a single excited state and a single fluorescence lifetime. The CFP variant has recently been improved along those lines with a higher quantum yield and a nearly monoexponential decay.[139] Ideally, in the future this single fluorescent lifetime would be made sensitive to a biophysical parameter of choice!

The application of new physical techniques to important problems in cell biology is often the path to unexpected discoveries. For example, micrometer-scale supramolecular organization of T cell receptors and integrins at immunological synapses were discovered by the use of 3-D fluorescence microscopy.[127–129] There is clearly a tremendous way to go before we are close to saturating the capabilities of fluorescence imaging for cell biology. Imaging fluorescence parameters such as lifetime, spectrum and polarization, as well as imaging more rapidly in three dimensions at higher resolution, are certain to reveal exciting new aspects of inter- and intra-cellular communication.

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