Journal of Pathology
J Pathol 2000; **191**: 229–234.

Review Article

Methods for imaging the structure and function of living tissues and cells: 2. Fluorescence lifetime imaging

Paul J. Tadrous*
Department of Histopathology, Imperial College School of Medicine, The Hammersmith Hospital, Du Cane Road, London, W12 0NN, UK

*Correspondence to:
Dr P.J. Tadrous, Department of Histopathology, Imperial College School of Medicine, The Hammersmith Hospital, Du Cane Road, London, W12 0NN, UK.
E-mail: pjtadrous@ieee.org

Received: 16 November 1999
Accepted: 1 February 2000

Abstract

This second article in the series shows how fluorescence lifetime imaging allows natural biochemical and physiological properties of tissues to act as contrast agents and so provide a basis for distinguishing normal and diseased tissue components. When combined with methods for imaging through non-transparent tissues and tomographic reconstruction it shows promise as a new optical biopsy technique. In addition to this, specially designed vital fluorescent probes of specific biochemical, secondary messenger and receptor activity in living cells may be imaged using FLIM. This is the youngest of the techniques covered in these review articles on imaging, the first FLIM images of cells having been produced in 1994. Copyright © 2000 John Wiley & Sons, Ltd.

Keywords: fluorescence lifetime imaging (FLIM); fluorescence resonance energy transfer (FRET)

Introduction

This review of methods capable of *in vivo* histological imaging, which began in last month's issue with a review of optical coherence tomography [1], continues with fluorescence lifetime imaging (FLIM), a relatively new technique which can harness the natural biochemical content of cells and tissues, and turn this information into image contrast—histological special staining without stains! In addition, specially designed vital fluorophores may be used to image specific biomolecular activities in cells, such as specific receptor behaviour or secondary messenger systems. In this way, FLIM exceeds the capabilities of even the most sophisticated of histological special stains and can be applied to live cells or unstained, unfixed and unprocessed tissues. Table 1 lists the salient features of FLIM.

Principles

In addition to brightness and wavelength, fluorophores possess a property known as the fluorescence lifetime (FL) which is a measure of the decay rate of the fluorescence emission after the excitation radiation has ceased (Figure 1). The fluorescence decay is mediated by a number of atomic and molecular interactions which can conveniently be classified into two groups:

1. Intramolecular interactions account for the so-called *radiative decay time* or τ_r. This is a molecular constant for a given fluorophore and is generally not sensitive to the surrounding physicochemical environment.
2. Intermolecular events (such as the binding of surrounding ions, collisional quenching and macromolecular associations) account for another source of energy transfer and determine the *non-radiative decay time*. Thus FL is an indicator of molecular environment [2].

Perhaps of even more significance is the fact that FL, being essentially a property of individual fluorescent molecules, is independent of the local concentration of fluorophore. The only difference concentration will make is that the absolute fluorescence brightness peak will be higher or lower; the decay rate will not change.

However, since FLs are measured in picoseconds or nanoseconds, special techniques (known as *time-resolved fluorescence methods*) are needed for their measurement. One of these methods measures the FL directly by illuminating the sample with a pulse of radiation and measuring the fluorescence decay with a fast recorder (*time-domain measurements*). A second method involves a continuous source of stimulant radiation which is designed to vary in intensity at extremely high frequency. The resulting fluorescence is thus forced to vary at the same frequency, but, because of the FL, there will be a delay in the emission (fluorescence) light train compared with the stimulation light train. In other words, the emission light train and excitation light train will be out of phase in a manner determined by the FL. Thus, measuring this phase difference allows FL to be calculated. These phase methods are called *frequency domain measurements* [3].

Such measurements may be made on a point-by-point (single-pixel) basis and scanned across the 2D image plane by using a confocal scanning light microscope (CSLM), or the whole 2D field may be imaged

Copyright © 2000 John Wiley & Sons, Ltd.

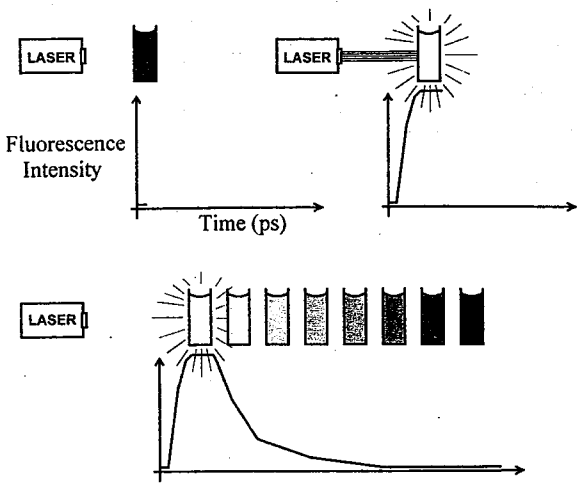

Figure 1. Fluorescence lifetime. *Top left:* a vial containing a solution of a fluorophore is about to be illuminated by an excitation laser. *Top right:* upon excitation, fluorescent light is almost instantaneously emitted from the vial. *Bottom:* upon cessation of the excitation light, the fluorescence decays gradually with a characteristic decay profile. This is the FL of the fluorophore in this solution and this decay characteristic will change with the composition of the solution containing the fluorophore (e.g. viscosity, acidity, etc.) but NOT with the concentration of the fluorophore *per se*

using a conventional fluorescence microscope coupled to a modulated laser light source and a modulated image intensifier/CCD camera detector [2,3].

Sources of contrast

In a FLIM image, pixel values vary according to FL alone, not intensity, local probe concentration, etc. Thus, the source of contrast is variation in the chemical composition of the cell or tissue; which chemical(s) depends on the type of fluorophore used, as discussed below.

Limitations

FLIM is a relatively new cellular imaging modality, the first FL images of cells having been achieved in 1994 [4]. Thus, it is still developing and the following comments represent the present state rather than absolute limitations.

Optical transparency is not required to take FL measurements [5], but when image formation is via confocal or conventional microscopes, the transparency limitations of these image forming devices are imposed.

The limit of resolution is limited, at best, to that of the image-forming device, such as the confocal microscope.

Several images need be collected for each FL image in order to reduce noise generated by the sensitive imaging system and also for correction of background, etc. There is also a large image processing requirement to calculate the FL image from the phase data. This limits the rate of image frame acquisition to anything from one frame/s at best, to around one frame/hour [4,6].

Although, in theory, the FL is independent of factors such as fluorescence intensity, photobleaching and fluorophore concentration, in order to realise this in practice there must be stringent attention to details of the apparatus and the photochemistry of the probe, with appropriate calibration checks and controls [4]. As the novelty of FLIM subsides and our experience grows, this limitation may become less of a problem.

The equipment (lasers, linear CCDs, modulated photomultipliers) although commercially available, is expensive, requires technical expertise to set up and is not easily portable/accessible for non-invasive *in vivo* use. These limitations may regress with technical advances in laser, fluorophore and fibre-optic design [2].

Advantages

The ability to image, in living cells and tissues, the distribution of specific chemical/metabolic components at microscopic resolution and to follow the changes in these components over time.

Chemical components, such as ion concentration or viscosity, may be calibrated for quantitative measurement [4].

Changes in chemical micro-environment may have drastic effects on the FL of a fluorophore even when there is only a minor effect on fluorescence wavelength (cf. ratiometric optical probes—i.e. fluorescence wavelength imaging) [2].

Conversely, fluorophore concentration, fluorescence intensity, light path length and photobleaching do not affect the FL [2–4,7].

The advantages of CSLM and two-photon excitation fluorescence microscopy (to be covered in the final article in this review series) are available [3,8] including 3D FLIM [6].

FLIM can be used in combination with methods of imaging within opaque/highly scattering tissue [5,9]. This opens up the possibility of FLIM optical computed tomography.

Vital staining fluorophores are not necessary for FLIM, as useful information can be gained by imaging the effects of local environment on natural cell constituents which are autofluorescent (such as the flavoproteins).

Multiple FLs (from distinct fluorophores) can be measured simultaneously by being resolved (extracted) from a single measurement using frequency-domain mathematics [3,10]. This means that multiple chemical components can be measured from a single image capture. A further unique property of the FLIM method described by Lakowicz *et al.* [7] is the ability to suppress the emission signal for any desired lifetime.

Copyright © 2000 John Wiley & Sons, Ltd.

Uses and modifications

FLIM as an alternative to ratiometric imaging

Readers may be familiar with the ratiometric imaging of specific ions in living tissues and cells by means of optical probes. An example is the probe fura-2, which is an EDTA-like fluorophore with a specific affinity for calcium ions [11]. Upon binding calcium, fura-2 undergoes a change of its excitation wavelength. Thus, taking two images of a cell loaded with fura-2 (using different illumination wavelengths) and dividing (pixel-by-pixel) one image by the other gives a ratio image. The ratio image is a visualization of the bound : unbound fura-2 ratio in different parts of the cell and is thus a measure of calcium ion concentration [12,13].

Using such methods, one may, for example, obtain real-time video footage of 'waves' of calcium ion concentration changes that take place in muscle cells [11] and neurons [14] during depolarization. Less well known are the calcium 'waves' that have been observed in traditionally non-electrically active organs, such as the liver [15] (thought to underlie bile peristalsis or metabolic variations across the acinus), and the relatively recent development of optical probes of secondary messenger activity, such as the diamino-fluoresceins used for nitric oxide visualization in living cells [16].

In addition to the wavelength changes described, the same optical probes undergo a change in the FL upon binding their specific biomolecule/ion. Thus, FLIM may be used instead of traditional wavelength imaging with the same repertoire of optical probes and hence, can measure the same wide range of secondary messengers and physiological ions, such as calcium [2–4,7]. However, using FLIM with fura-2, a much larger dynamic range of calcium concentrations can be imaged compared with ratiometric imaging of the same calcium probe [17]. Also, since FLIM does not have the restriction of requiring imaging at two separate wavelengths, there is no need for complicated muti-beamsplitting multi-camera or rotating filter wheel arrangements, as may be required for wavelength imaging [18].

Other probes can be used for FLIM imaging of sodium [19], magnesium [8], potassium [8] and oxygen [3,9]. Saunders *et al.* [20] show how the same pH-sensitive probe used in ratiometric (wavelength) imaging—SNAFL-1—can be used for FLIM of pH, with the added benefits of lesser dependency on local hydrophobicity or protein concentration.

FLIM using inherent autofluorescence

Application of extrinsic fluorophores is not always necessary, as intrinsic fluorescent biomolecules may be used, such as the reduced pyridine nucleotides NAD(P)H, which have been measured by FL in living human skin [21]. Furthermore, the FL of free NADH is distinguishable from protein-bound NADH [7]. This may be used as a measure of cell oxidative stress, as free NAD(P)H increases in these circumstances [22].

The intrinsic fluorescence signals of different types of tissue (glandular, fibrous, muscular, fatty, elastic) have different FLs, such that FLIM may act as the vital equivalent of traditional 'special stains' [23–25].

Furthermore, it is of great interest that malignant breast tissue (freshly excised, unfixed, infiltrating ductal carcinoma) has quite a different FL signature at 340 nm emission compared with benign breast biopsies, including fibro-adenoma, with high statistical significance even at small sample size [23]. Although these are very early days, such studies must surely be followed up and expanded; these new imaging modalities, which image according to intrinsic life chemical processes, may provide our best ever chance of getting a truly useful marker of benign versus malignant cells and tissues.

FLIM for molecular state and structural information

Physicochemical properties, such as viscosity, heat and local order [3], may also be detected using FLIM. In imaging chromosomes, the effect of local G–C : A–T ratios on fluorophores may provide FL contrast [7] and may form a basis for imaging particular regions of certain chromosomes in live cells. Another example is the study of the conformational state of tRNA using $tRNA^{Phe}$ which contains the naturally occurring fluorophore Y_t-base [7]. Fluorophores may be combined with biomolecules to study their micro-environment; for example, a GFP-cyclin-B1 fusion construct was used to study the tubulin-bound (as opposed to free) cyclin-B1 distribution in live Vero cells in culture [6] (Figure 2). Furthermore, the free cyclin-B1 in the nucleoplasm has a different FL from the free cyclin-B1 in the cytoplasm, as indicated in the slight purple tinge to the pseudocolour of the nucleus compared with the cytoplasmic interstices, which appear orange. In the latter study, Squire and Bastiaens [6] also demonstrated the practice of 3D FLIM using a computer controlled z-stage and 3D Fourier-domain processing (including deblurring by deconvolution as shown in Figure 2).

Fluorescence resonance energy transfer (FRET)

When two different fluorophores, such as rhodamine and fluorescein, are held close together by molecular bonding, some of the excitation energy absorbed as excitation light falls on one fluorophore (fluorescein) and is transferred to the other fluorophore (rhodamine), causing it to emit fluorescence. In practice, it appears as though rhodamine is fluorescing at the excitation wavelength of fluorescein. This phenomenon is called fluorescence resonance energy transfer (FRET). When the molecular bonding holding the two fluorophores is broken, they part company and FRET ceases, resulting in a change in the wavelength of the emitted light. In the example of fluorescein : rhoda-

Copyright © 2000 John Wiley & Sons, Ltd.

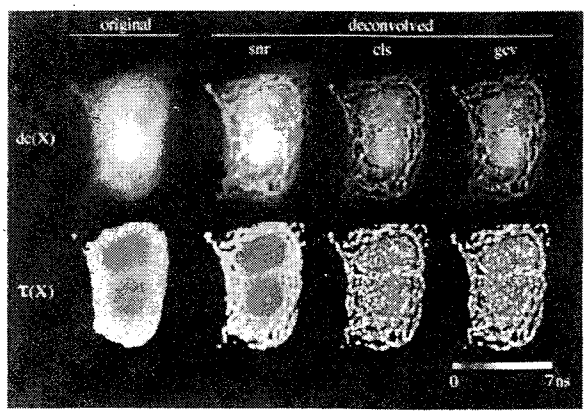

Figure 2. Pseudocolour FL maps of a single, binucleate, live Vero cell in culture (the different columns represent a deconvolution series). The top row shows fluorescence intensity images of the cell, whereas the bottom row shows FL images ('maps') of the same cell. The cell contains GFP-cyclin-B1 present in both free (cytoplasmic interstitium and nucleoplasm) and tubulin-bound (cytoplasmic microtubules) form. The intensity images cannot distinguish the two forms, whereas the FL images clearly distinguish the bound (yellow—associated with cytoplasmic filamentous structures) from the free (orange–red) cyclin-B1. Furthermore, the free cyclin-B1 in the nucleoplasm has a slight purple tinge distinguishing it from the cytoplasmic free form. (Reproduced with permission from the *J Microsc* 1999; **193**:36–49, courtesy of Dr Philippe Bastiaens of the ICRF and copyright © 1999 Blackwell Science Ltd.)

mine, the emitted light would revert back to the green colour characteristic of fluorescein. In addition to the wavelength change, FRET also entails a change in the FL. An example of wavelength (colour) dependent imaging, is the study of Adams *et al.* [26], who made use of FRET by genetically combining fluorescein to the catalytic subunit and rhodamine to the regulatory subunit of a cAMP-dependent protein kinase. In the inactive state, the two fluorophores are so close together that their fluorescent energies interact in such a way that when illuminated at the excitation wavelength of fluorescein, some of this incident light energy is transferred to rhodamine; this causes rhodamine fluorescence, which does not normally occur with this excitation light. In the presence of cAMP the kinase is activated (cleaved), the subunits (and therefore fluorophores) part company and there is a resultant shift of the fluorescence wavelength back to that which is characteristic of fluorescein, due to lack of resonance energy transfer. The genetically modified protein kinase thus acts as an optical probe which is specific for cyclic AMP activity in living cells.

Another application of FRET is in the realm of reporter gene technology [27]. This exciting visualization technology encompasses the use of genetic engineering to place the gene of an optically active molecule, such as a fluorophore, under the control of a specific promoter. The promoter chosen is one which controls the gene product whose expression is under study. For example, to visualize the expression of the GAS-1 gene in a live cell under various conditions, one would first engineer a fusion gene construct of an optically active molecule under the control of the GAS-1 promoter, and insert this into the cells under study. When the cells receive the signal to transcribe GAS-1 (via activation of its promoter) the optically active gene is also transcribed in tandem, providing a visual marker of GAS-1 expression in the cells. One embodiment of reporter gene technology has been to use the β-lactamase gene as the optically active reporter. β-lactamase is not optically active in its own right, but the concept [28] is also to load cells with a cephalosporin, modified so that it is covalently bonded to two fluorophores. These fluorophores are held in close apposition, but are bound either side of the β-lactamase cleavage site. Thus, when β-lactamase is expressed, the cephalosporin is cleaved and there is a loss of FRET, with a resulting colour change.

Gadella and Jovin have used FLIM to study FRET [29]. By using fluorescein-labelled epidermal growth factor (fl-EGF) and rhodamine-EGF (rh-EGF), they were able to image EGF-receptor dimerization on the surface of live human epidermoid carcinoma cells under various conditions. They imaged the FL changes that occur when the two fluorophores are brought together on the surface of the cells upon binding EGF receptor, which dimerizes, bringing the fl-EGF and rh-EGF into such close proximity that FRET occurs (Figure 3). They were thus able to identify a subclass of receptors on quiescent cells which exist in a pre-dimerized state; they postulated that dimerization *per se* is not the activating event in these receptors, but rather a stereochemical rotation of the pre-dimerized receptors ('twist') which occurs upon ligand binding.

The potential use of FLIM in imaging secondary messenger systems and receptor pathways by means of such 'designer probes' should by now be obvious.

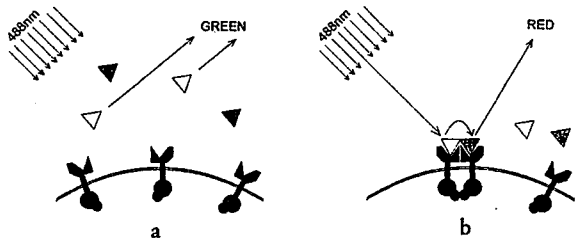

Figure 3. FRET. The Figures show part of a cell membrane containing transmembrane epidermal growth factor receptors (EGF-R—black). The white triangles are fluorescein-labelled epidermal growth factor (fl-EGF) and the shaded triangles, rhodamine-labelled EGF (rh-EGF). (a) Prior to receptor binding, the 488nm light is absorbed by the fl-EGF which emits a green fluorescence. (b) Upon binding of the EGFs to the receptors, the receptors dimerize, bringing fl-EGR and rh-EGF into close apposition. When the fl-EGF absorbs the 488 nm light, some of that energy is transferred to the rh-EGF, causing a red emission from rhodamine (with a different FL). Hence, either the change in FL, or the amount of colour-shift in the emitted fluorescence may be used as an indicator of the amount of receptor dimerization on the surface of the cells

Copyright © 2000 John Wiley & Sons, Ltd.

Current research and future expectations

FLIM tomography

The passage of excitation and fluorescent light through multiply scattering or heterogeneous media (such as turbid biological tissues) can be described by the diffusion approximation to the radiative transport equation. One method of imaging through turbid media involves making measurements of such multiply scattered light at different angular positions about a specimen, and then using an inverse solution to the equation to see how the measurements differ from those that would be predicted if the specimen were uniform and internally structureless. By such methods, Sevick-Muraca *et al.* [9] have conducted studies and derived CT-like spatial cross-section FL maps of a simulated tissue phantom. There are alternative ways of imaging into turbid media and this research is in an early stage, but the prospects for live tissue imaging with biochemical contrast is a real possibility in the near future.

Technical advancement in hardware

As stated, FLIM currently involves some expensive and bulky components, with multi-hundred thousand pound laser sources and light modulating devices, occupying whole optical benches. There is a drive, however, to alleviate both cost and bulk by turning to the latest advances in laser diode technology for a portable and relatively cheap alternative. Laser diode FLIM systems are already in use. Customized software and image processing may be achieved in compact and custom built VLSI modules. These continuing advances in hardware may see a more widespread adoption and versatility of FLIM outside the specialist optical physics laboratory.

The nature of the signal

The ability of one FLIM experiment apparently to distinguish benign from malignant fresh human breast tissue has already been mentioned [23]. However, in this study, as in a number of the other studies mentioned which obtain natural tissue component contrast, the exact nature of the chemical difference which accounts for the different FLs is not known. Clearly, much work needs to be done to clarify the exact nature of the observed influences on FL. However, in so doing, important avenues of research may be uncovered and we may gain insight into the mechanisms of disease; hence the need for pathologists and other biomedical and biomolecular scientists to take an active interest in this new imaging technology.

Fourier micro-holography (μHol) for FLIM DNA sequencing in living cells

The principles of optical μHol were discussed in the mid 60s [30] and X-ray μHol for living specimens was reviewed in the early 80s [31]. However, X-rays destroy the specimen after imaging. A recent review of modern μHol is presented by Boyer *et al.* [32] where the submicron resolution, the intrinsic 3D nature of the images and the potential for combining FLIM with μHol for DNA sequencing is discussed.

Conclusion

In times past, we were content with soaking our dead tissue sections in vegetable extracts and metal salt solutions to provide an optical density contrast image of the specimen, according to ill-understood binding affinities of different tissue components. Then it was discovered that using solutions of labelled antibodies could highlight more specific chemical components and that labelled nucleic acids could yield contrast according to DNA/RNA sequences present in cells. FLIM seems to promise the best of both worlds, with tissue component contrast and biochemically specific imaging—all in unstained and unfixed tissues. Furthermore, there is the tantalizing prospect of imaging cells and tissues according to their behaviour, which has no correlate in current staining technologies. At present, FLIM cannot reliably and controllably deliver all these capabilities, but the preliminary studies reviewed above suggest that it deserves serious investigation and development in both physical and biomedical fields to assess fully its limitations and potential benefits in the study of disease.

Table 1. FLIM saliency table

Principles	Diffraction limited imaging with measurement of the fluorescence emission decay rates of intrinsic or applied fluorescent molecules. These decays are influenced by the biochemical environment of the fluorophore.
Contrast	Can differentiate different tissue types according to their biochemical composition analogous to 'special stains' of histology. Metabolic status, macromolecular structural conformation, specific ions and chemical messengers may also provide FLIM contrast.
Spatial resolution	0.2 μm at current best (resolution of the optical imaging device used e.g. confocal microscope)
Temporal resolution	Full frame image capture is a reality. Video real-time not yet achieved. One frame per second has been achieved.
Peculiarities	Current systems are not portable or generally available outside expert centres. FLIM has great potential for chemically specific *in vivo* histology and is a possible tomographic modality.

Copyright © 2000 John Wiley & Sons, Ltd.

Appendix—Abbreviations

cAMP	cyclic Adenosine MonoPhosphate
CCD	Charge Coupled Device (a type of image sensor)
CSLM	Confocal Scanning Light Microscopy
CT	[X-ray] Computed Tomography (not to be confused with OCT)
EGF	Epidermal Growth Factor
EGF-R	EGF Receptor
EDTA	Ethyline Diamine Tetra Acetic acid
fl-EGF	fluorescein-labelled EGF
FL	Fluorescence Lifetime
FLIM	Fluorescence Lifetime IMaging
FRET	Fluorescence Resonance Energy Transfer
GAS-1	Growth Arrest Specific gene-1
GFP	Green Fluorescent Protein
LM	Light Microscopy
μHol	micro-Holography
NADH	Nicotinamide Adenine Dinucleotide (reduced form)
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced)
NO	Nitric Oxide
OCT	Optical Coherence Tomography
rh-EGF	rhodamine-labelled EGF
SNAFL-1	5′(and 6′)-carboxySemiNaphthoFLuorescein one
VLSI	Very Large Scale Integration

Acknowledgements

I am grateful to Dr Philippe Bastiaens of the Cell Biophysics Laboratory of the ICRF, London, UK and to Blackwell Science Ltd., Oxford, UK for permission to reproduce Figure 2. I also thank Prof. I. C. Talbot of St Mark's Hospital, London for proofreading the original drafts of this review series.

References

1. Tadrous PJ. Methods for imaging the structure and function of living cells: 1. Optical coherence tomography. *J Pathol* 2000; **191**: 115–119.
2. Szmacinski H, Lakowicz JR, Johnson MJ. Fluorescence lifetime imaging microscopy: homodyne technique using high-speed gated image intensifier. *Methods Enzymol* 1994; **249**: 723–748.
3. French T, So PTC, Dong CY, Berland KM, Gratton E. Fluorescence lifetime imaging techniques for microscopy. *Methods Cell Biol* 1998; **56**: 277–304.
4. Lakowicz JR, Szmacinski H, Nowaczyk K, Lederer WJ, Kirby MS, Johnson ML. Fluorescence lifetime imaging of intracellular calcium in COS cells using quin-2. *Cell Calcium* 1994; **15**: 7–27.
5. Bambot SB, Rao G, Romauld M, *et al.* Sensing oxygen through skin using a diode laser and fluorescence lifetimes. *Biosens Bioelectron* 1995; **10**: 643–652.
6. Squire A, Bastiaens PIH. Three dimensional image restoration in fluorescence lifetime imaging microscopy. *J Microsc* 1999; **193**: 36–49.
7. Lackowicz JR, Szmacinski H, Nowaczyk K, Berndt KW, Johnson M. Fluorescence lifetime imaging. *Anal Biochem* 1992; **202**: 316–330.
8. French T, So PTC, Weaver Jr DJ, *et al.* Two-photon fluorescence lifetime imaging microscopy of macrophage-mediated antigen processing. *J Microsc* 1997; **185**: 339–353.
9. Sevick-Muraca EM, Reynolds JS, Troy TL, Lopez G, Paithankar DY. Fluorescence lifetime spectroscopic imaging with measurements of photon migration. *Ann N Y Acad Sci* 1998; **838**: 46–57.
10. Pepperkok R, Squire A, Geley S, Bastiaens PIH. Simultaneous detection of multiple green fluorescent proteins in live cells by fluorescence lifetime imaging microscopy. *Curr Biol* 1999; **9**: 269–272.
11. Hayes A, Cody SH, Williams DA. Measuring cytosolic Ca2+ in cells with fluorescent probes: an aid to understanding cell pathophysiology. *Int Rev Exp Pathol* 1996; **36**: 197–212.
12. Moreton RB. Optical methods for imaging ionic activities. *Scanning Microsc Suppl* 1994; **8**: 371–390.
13. Balsover SR, Silver RA, Whitaker M. Ratio imaging measurements of intracellular calcium and pH. In *Electronic LM*, Shotton D (ed). Wiley-Liss: New York, 1993; 181–210.
14. Fetcho JR, O'Malley DM. Imaging neuronal networks in behaving animals. *Curr Opin Neurobiol* 1997; **7**: 832–838.
15. Nathanson MH, Burgstahler AD, Mennone A, Fallon MB, Gonzalez CB, Saez J. Ca2+ waves are organised among hepatocytes in the intact organ. *Am J Physiol* 1995; **269**: G167–G171.
16. Kojima H, Nakatsubo N, Kikuchi K, *et al.* Detection and imaging of nitric oxide with novel fluorescent indicators: diaminofluoresceins. *Anal Chem* 1998; **40**: 2446–2453.
17. Szmacinski H, Lakowicz JR. Possibility of simultaneously measuring low and high calcium concentrations using fura-2 and lifetime-based sensing. *Cell Calcium* 1995; **18**: 64–75.
18. Morris SJ. Real-time multi-wavelength fluorescence imaging of living cells. *Biotechniques* 1990; **8**: 296–308.
19. Szmacinski H, Lakowicz JR. Sodium green as a potential probe for intracellular sodium imaging based on fluorescence lifetime. *Anal Biochem* 1997; **250**: 131–138.
20. Saunders R, Draaijer A, Gerritsen HC, Houpt PM, Levine YK. Quantitative pH imaging in cells using confocal fluorescence lifetime imaging microscopy. *Anal Biochem* 1995; **227**: 302–308.
21. Masters BR, So PC, Gratton E. Multiphoton excitation fluorescence microscopy and spectroscopy of *in vivo* human skin. *Biophys J* 1997; **72**: 2405–2412.
22. König K, So PTC, Mantulin WW, Tromberg BJ, Gratton E. Two-photon excited lifetime imaging of autofluorescence in cells during UVA and NIR photostress. *J Microsc* 1996; **183**: 197–204.
23. Das BB, Liu F, Alfano R. Time-resolved fluorescence and photon migration studies in biomedical and model random media. *Rep Prog Phys* 1997; **60**: 227–292.
24. Dowling K, Dayel MJ, Lever MJ, French PMW, Hares JD, Dymoke-Bradshaw AKL. Fluorescence lifetime imaging with picosecond resolution for biomedical applications. *Optics Lett* 1998; **23**: 810–812.
25. Jones R, Dowling K, Cole MJ, *et al.* Fluorescence lifetime imaging using a diode-pumped all-solid-state laser system. *Electronics Lett* 1998; **35**: 1–2.
26. Adams SR, Harootunian AT, Buechler YJ, Taylor SS, Tsien RY. Fluorescence ratio imaging of cyclic AMP in single cells. *Nature* 1991; **349**: 694–697.
27. Rutter GA, Kennedy HJ, Wood CD, White MRH, Tavaré J. Real-time imaging of gene expression in single living cells. *Chem Biol* 1998; **5**: R285–R290.
28. Zlokarnik G, Negulescu PA, Knapp TE, *et al.* Quantitation of transcription and clonal selection of single living cells with β-lactamase as reporter. *Science* 1998; **279**: 84–88.
29. Gadella Jr TWJ, Jovin TM. Oligomerization of epidermal growth factor receptors on A431 cells studied by time-resolved fluorescence imaging microscopy. A stereochemical model for tyrosine kinase receptor activation. *J Cell Biol* 1995; **129**: 1543–1558.
30. Rogers GL, Palmer J. The possibilities of X-ray holographic microscopy. *J Microsc* 1969; **89**: 125–135.
31. Solem JC, Baldwin GC. Microholography of living organisms. *Science* 1982; **218**: 229–235.
32. Boyer K, Solem JC, Longworth JW, Borisov AB, Rhodes CK. Biomedical three-dimensional holographic microimaging at visible., ultraviolet and X-ray wavelengths. *Nature Med* 1996; **2**: 939–941.

Copyright © 2000 John Wiley & Sons, Ltd.