Journal of Fluorescence, Vol. 4, No. 1, 1994

Emerging Biomedical and Advanced Applications of Time-Resolved Fluorescence Spectroscopy

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Received October 18, 1993

Time-resolved fluorescence spectroscopy is presently regarded as a research tool in biochemistry, biophysics, and chemical physics. Advances in laser technology, the development of long-wavelength probes, and the use of lifetime-based methods, are resulting in the rapid migration of time-resolved fluorescence to the clinical chemistry lab, the patient's bedside, and even to the doctor's office and home health care. Additionally, time-resolved imaging is now a reality in fluorescence microscopy and will provide chemical imaging of a variety of intracellular analytes and/or cellular phenomena. Future horizons of state-of-the-art spectroscopy are also described. Two photon-induced fluorescence provides an increased information content to time-resolved data. Two photon-induced fluorescence, combined with fluorescence microscopy and time-resolved imaging, promises to provide detailed three-dimensional chemical imaging of cells. Additionally, it has recently been demonstrated that the pulses from modern picosecond lasers can be used to quench and/or modify the excited-state population by stimulated emission since the stimulated photons are directed along the quenching beam and are not observed. The phenomenon of light quenching should allow a new class of multipulse time-resolved fluorescence experiments in which the excited-state population is modified by additional pulses to provide highly oriented systems.

KEY WORDS: Time-resolved fluorescence spectroscopy; two photon-induced fluorescence; light quenching.

INTRODUCTION

In this closing talk, at the Third Conference on Methods and Applications of Fluorescence Spectroscopy in Prague, Dr. Jan Slavik asked for a summary of the "trends in fluorescence spectroscopy." Such a summary talk on trends necessarily involves predictions of future developments. In our opinion, the uses of time-resolved fluorescence spectroscopy are no longer focused completely on biomedical, biophysics, and physical chemistry research, as has been summarized in recent publications [1–3]. There can be no doubt that fluorescence methods have contributed immensely to our present understanding of biological micromolecules and their assemblies. In addition to these research applications of fluorescence, there is a continuing use of fluorescence detection to replace analytical methods based on radioactivity, as can be judged from the recent books and conferences on fluorescence sensing methods [4–8]. These emerging applications of fluorescence can be seen by the growth and introduction of improved methods for immunoassays, enzyme-linked immunoassays (ELISA), protein and DNA staining, and protein and DNA sequencing. A major driving force in this evolution is the introduction of long-wavelength probes which allow excitation with simple and robust light sources such as laser diodes. Importantly, the use of red–near-infrared (NIR)

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1053-0509/94/0300-0117$07.00/0

excitation improves detection limits because of the lower levels of autofluorescence observed with these long excitation wavelengths. Consequently, several laboratories are directing their efforts to develop long-wavelength probes which can be excited with laser diodes form 635 to 820 nm.

APPLICATIONS OF FLUORESCENCE SENSING AND IMAGING

Schemes for Fluorescence Sensing

At present, most fluorescence assays are based on the standard intensity-based methods, in which the intensity of the probe molecule changes in response to the analytes of interest. However, there has been the realization that lifetime-based methods possess intrinsic advantages for chemical sensing. Figure 1 summarizes some of the known schemes for fluorescence sensing. If the intensity of a probe varies in response to an analyte, or if the amount of signal is proportional to the analyte, then it appears simple and straightforward to relate this intensity to the analyte concentration (left). Intensity-based methods are initially the easiest to implement because many probe fluorophores change intensity and/or quantum yield in response to analytes. Additionally, collisional quenching processes, such as quenching by oxygen, iodide, chloride, etc., result in changes in intensity without significant shifts in the emission spectrum. While intensity measurements are simple and accurate in the laboratory, these are often inadequate in real-world situations. This is because the sample may be turbid, the optical surfaces may be imprecise or dirty, and the optical alignment may vary from sample to sample (Table I). In the case of fluorescence microscopy, it is often impossible to know the probe concentration at each point in the image because the intensity changes continually due to photobleaching, phototransformatiuon, and/or diffusive processes.

In principle, the problems of intensity-based sensing can be avoided using wavelength-ratiometric probes, that is, fluorophores which display spectral changes in the absorption or emission spectrum upon binding or interaction with the analytes (Fig. 1). In this case, the analyte concentration can be determined independently of the probe concentration by the ratio of intensities at two excitation or two emission wavelengths.

Wavelength-ratiometric probes provide a straightforward means to avoid the difficulties of intensity-based sensing. However, few such probes are available, and it is clear that they are difficult to create [9]. For instance, in spite of the enormous interest in measurements of intracellular Ca^{2+} concentration, there appears to be no practical wavelength-ratiometric indicator for Ca^{2+} which allows visible wavelength excitation. The two most widely

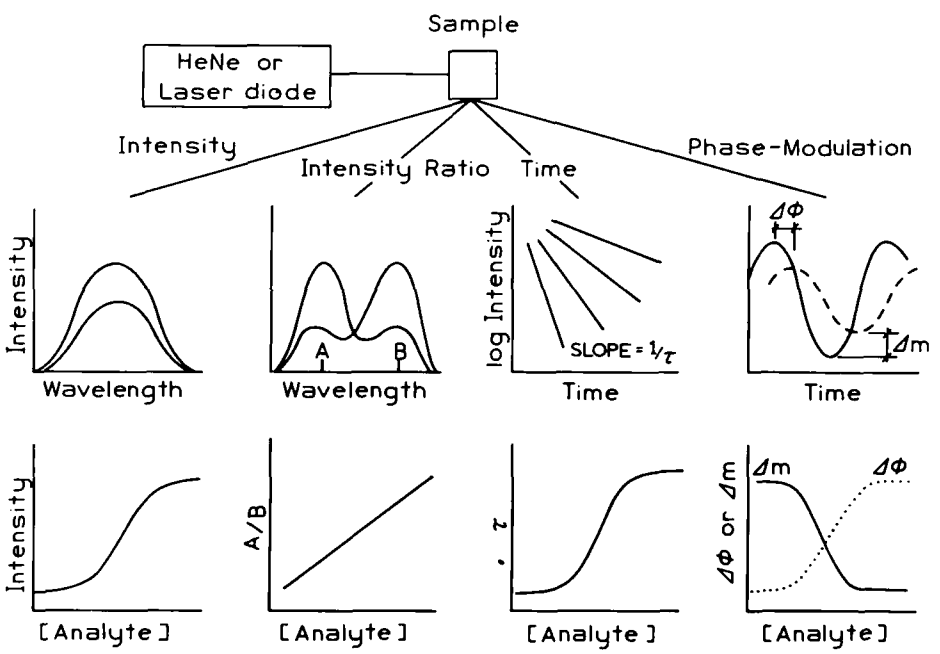


Fig. 1. Schemes for fluorescence sensing: intensity, intensity ratio, time domain, and phase -modulation, from left to right.

Table I. Characteristics of Intensity, Intensity Ratio, and Time-Resolved Sensing Methods

Methods	Comments
Intensity-based sensing	Many probes are available Dependent on probe concentration Sensitive to absorption, scatter, and sample autofluorescence Sensitive to light lasers, photobleaching, etc. Difficult to measure small changes in intensity
Wavelength-ratiometric sensing	Limited number of probes and analytes Requires UV excitation with most present probes, except pH probes
Lifetime-based sensing Time domain and phase modulation	Independent of probe concentration Insensitive to simple absorption and scatter Insensitive to light losses and optical imperfections
Phase-modulated sensing	Simple and robust instrumentation Easy to measure small changes High accuracy Probe development simpler than wavelength-ratiometric probes

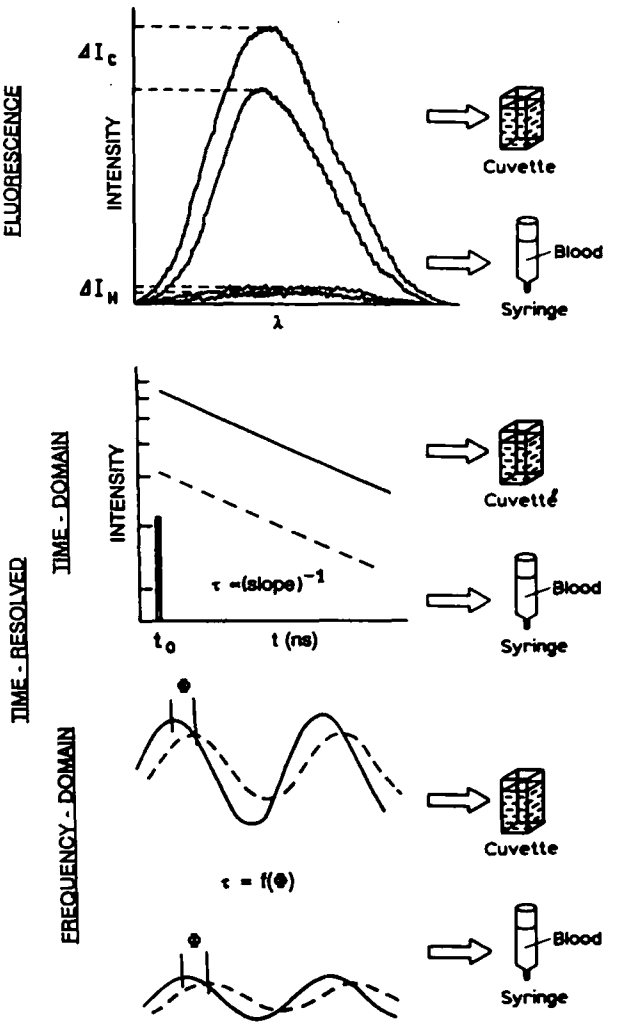


Fig. 2. Intensity, time-domain, and frequency-domain sensing, as applied in the laboratory, a cuvette, and a blood sample in a clinical setting.

used probes, Fura-2 and Indo-1, both require UV excitation, with the associated problems of complex UV laser sources and the high amounts of autofluorescence which are excited at these wavelengths. Attempts to make long-wavelength Ca^{2+} probes have resulted in probes which may change intensity, but do not display spectral shifts in either the excitation or the emission spectra, such as Fluo-3 and Calcium Green [10]. Wavelength-ratiometric probes for pH have recently become available.

The difficulties of intensity-based measurements and of the scarcity of probes may be circumvented by the use of time-resolved or lifetime-based sensing. Several years ago we decided that it would probably be easier to identify and/or synthesize probes which display changes in lifetime in response to analytes, rather than to design and synthesize probes which display spectral shifts. Our opinion was based on the knowledge that a wide variety of quenchers and/or molecular interactions resulted in changes in the lifetimes of fluorophores, while changes in spectral shape were the exception rather than the rule. This prediction proved to be correct, as we now know that probes such as the Calcium Green series [11] and the analogous Mg^{2+} probes all display changes in lifetime in response to binding their specific cations [12]. Additionally, the pH probes of the SNAFL and SNARF series also display changes in lifetime upon pH-induced ionization [13]. Of course, collisional quencher such as Cl^-, O_2, etc., also cause changes in lifetimes, as summarized in Ref. 14.

It is important to notice that a change in lifetime is not a necessary result of a change in fluorescence intensity. For instance, the Ca^{2+} probe Fluo-3 displays a large increase in intensity upon binding Ca^{2+}, but there is no change in lifetime. This is because the Ca-free form of the probe is effectively nonfluorescent, and its emission does not contribute to the lifetime measurement. To obtain a change in lifetime, the probe must display detectable emission from both the free and the cation-bound forms. Then the lifetime reflects the fraction of the probe

Table II. Blood Gases (pO_2, pCO_2, and pH) of Critically Ill Patients

Methods	Comments
Present status	Requires arterial blood Performed in a central clinical lab Time delays, changing patient status Complex instrumentation Requires handling of blood (AIDS) Estimated U.S. market, $400,000,000 per year
Optical measurements of blood gases	Available at bedside in the CCU Continuous readout of pO_2, pCO_2, and pH Rapid response and improved patient care No handling of blood by health-care workers

complexed with cations. Of course, this consideration does not apply to collisional quenching, when the intensity decay of the entire ensemble of fluorophores is decreased by diffusive encounters with the quencher.

Instrument Complexity and the Spectral Properties of Fluorophores

It is well-known that the lifetimes of fluorophores are typically in the range of 1 to 10 ns and that it is easily possible to spend $50,000 to $500,000 for lifetime instrumentation. How, then, can one rationally propose such measurements at the patient's bedside? Such instruments and measurements are possible if we reverse the usual paradigm of designing the instrument to suit the spectral properties of the probe molecules. When adopting this approach, one may be forced to use complex and expensive laser sources, as well as sophisticated schemes for generation of pulsed or amplitude-modulated light. Simple and robust instrumentation can readily be designed if we first decide on the laser source, the probe molecule, and the measurement scheme. For instance, laser diodes provide an ideal source of light from 635 to 800 nm. Importantly, the output of the laser diodes can be amplitude-modulated at any desired frequency up to several gigahertz [15], and these devices have been used in phase-modulation fluorometry [16,17]. A bedside or even a handheld lifetime instrument can be readily designed if we synthesize and develop functional and specific probes which can be excited with laser diode sources. At present, there are many dyes in this range of wavelength, but only a handful which can be covalently attached to macromolecules and, to the best of our knowledge, none which are specifically sensitive to Ca^{2+}, Mg^{2+}, or other analytes.

Prior to describing the possible applications of laser-diode fluorometry, it is important to understand the two

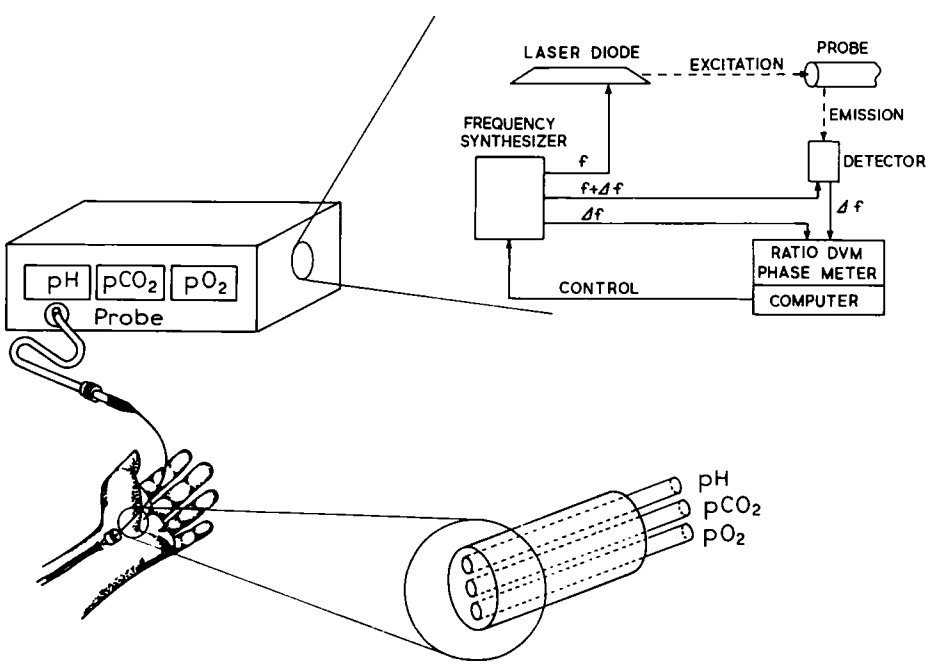


Fig. 3. Phase-modulation blood gas instrumentation based on a laser diode light source.

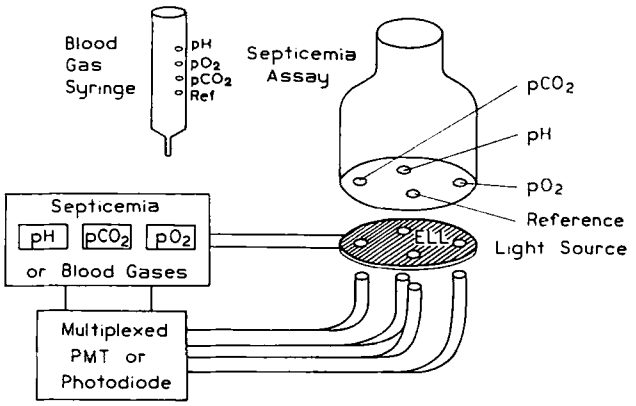


Fig. 4. Blood gas septicemia apparatus based on an electroluminescent light source.

Table III. Blood Glucose Determination in Diabetics

Methods	Comments
Present status	Invasive i.e., requires withdrawing of blood Infrequent measurements result in blood glucose variations Long-term health consequences result from inadequate control of blood sugar
Noninvasive optical measurement of blood glucose	Does not require withdrawing of blood Can provide continuous monitoring Improved control of blood glucose Fewer long-term health effects of poorly regulated blood glucose Can be based on laser diode lifetime measurements

methods now used to measure fluorescence lifetimes: the time-domain [18–20] and frequency-domain or phase-modulation methods [21]. In time-domain (TD) fluorometry, the sample is excited by a pulse of light, followed by measurement of the time-dependent intensity. In frequency-domain (FD) fluorometry, the sample is excited with amplitude-modulated light. The lifetime can be found from the phase angle delay and demodulation of the emission relative to the modulated incident light. We do not wish to fuel the debate of TD versus FD methods, but it is clear that phase and modulation measurements can be performed with simple and low-cost instrumentation and can provide excellent accuracy with short data acquisition times.

Lifetime-Based Sensing

Why can we expect lifetime-based sensing to be superior to intensity-based sensing? We feel that this is the case because real-world sensing applications occur in environments which are not equivalent to optically clear and clean cuvettes. Instead, there are numerous factors which can affect the intensity values, such as imperfections or misalignment of surfaces and light losses in optical fibers, to name just a few. Additionally, many desired applications, such as homogeneous immunoassays or transdermal sensing measurements, require quantitative measurements in highly turbid or absorbing media (Fig. 2, top). Such factors preclude quantitative measurements of intensities or even intensity ratios.

Lifetime-based sensing can be mostly insensitive to these real-world effects. This is because these factors are not expected to alter the rate at which the intensity decays (Fig. 2, middle). In our opinion, phase-modulation sensing provides additional advantages (Fig. 2, bottom). The instruments take advantage of radiofrequency methods to reject noise and filter signals, resulting in reliable data even in electrically noisy environments. Standard phase-modulation instruments provide 50-ps resolution with just seconds of data acquisition, so that small changes in lifetime can be easily measured. The merits and disadvantages of various sensing schemes are summarized in Table I.

Phase-Modulation Sensing of Blood Gases and/or Blood Septicemia

Optical detection of blood gases (pH, pCO_2, and pO_2) is the Holy Grail of optical sensing. This is because current methods do not fully satisfy the needs of the intensive-care patient. In these unfortunate cases the blood gases change on the time scale of minutes in response to the patient's physiological status. Measuring a blood gas requires taking a sample of arterial blood, placing it on ice, transporting it to a central laboratory, and measuring the pH using an electrode and O_2 and CO_2 by Clark and Severinghous electrodes, respectively (Table II). Even for a stat request, it is difficult to obtain the blood gas report in less than 30 min, by which time the patient's status is often quite different. Additionally, handling of blood by health-care workers is undesirable with regard to the risk of AIDS and other infectious diseases. At present, determination of blood gases is time-consuming and expensive, with a cost of at least $400,000,000 per year in the United States.

How can phase-modulation fluorometry contribute to this health-care need? It now seems possible to con-

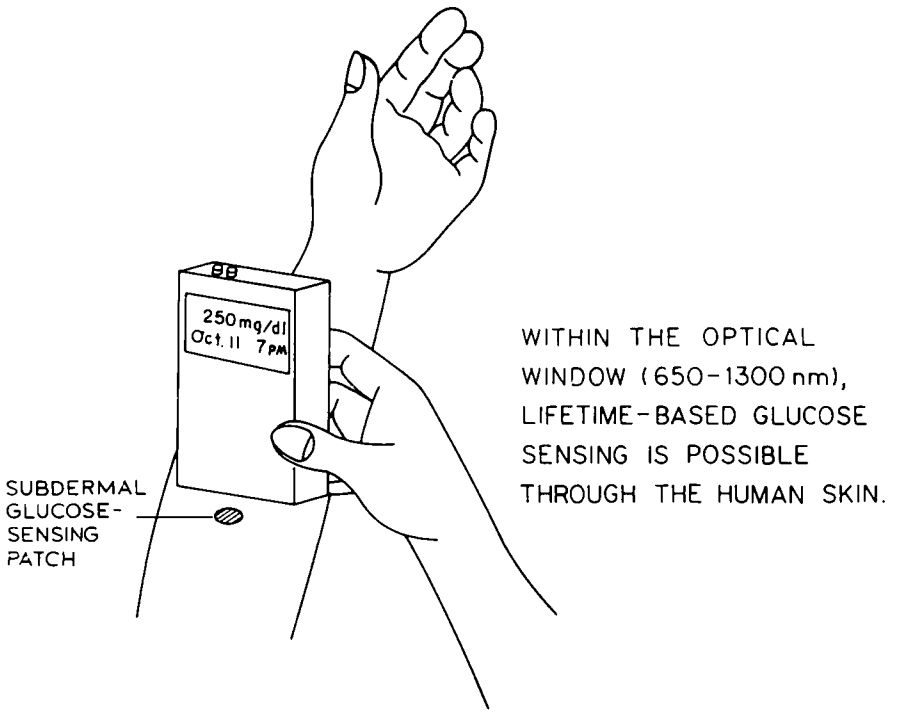


Fig. 5. Noninvasive sensing of glucose.

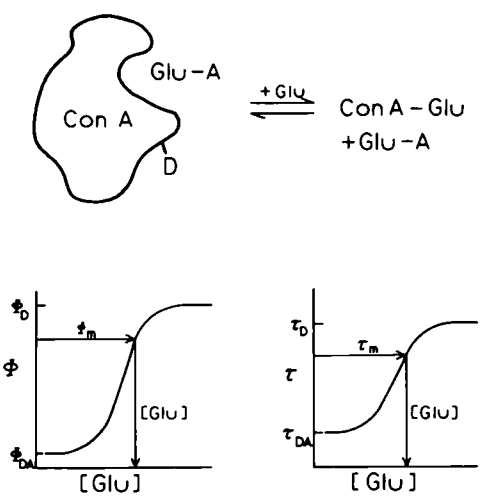


Fig. 6. Glucose sensing by fluorescence resonance energy transfer (FRET).

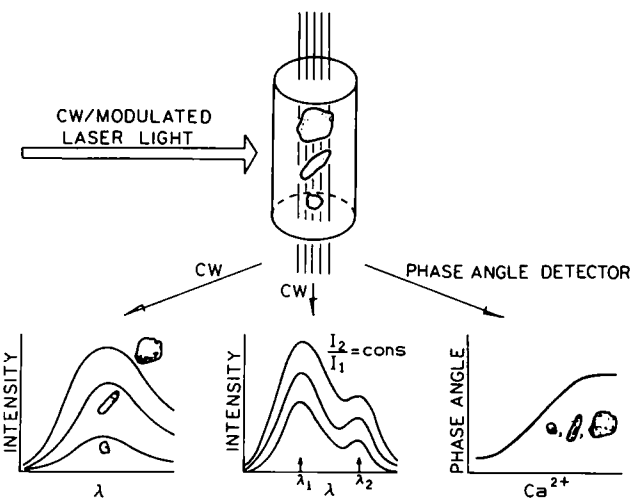


Fig. 7. Cell-by-cell lifetime measurements in flow cytometry.

struct a lifetime-based blood gas catheter (Fig. 3) or, alternatively, an apparatus to read the blood gas in the freshly drawn blood at the patient's bedside. To be specific, fluorophores are presently known to accomplish the task using a 543-nm Green HeNe laser [13,14], and it seems likely that the chemistries will be identified for a laser diode source. The use of longer wavelengths should minimize the problems of light absorption and autofluorescence of the samples, and the use of phase or mod-

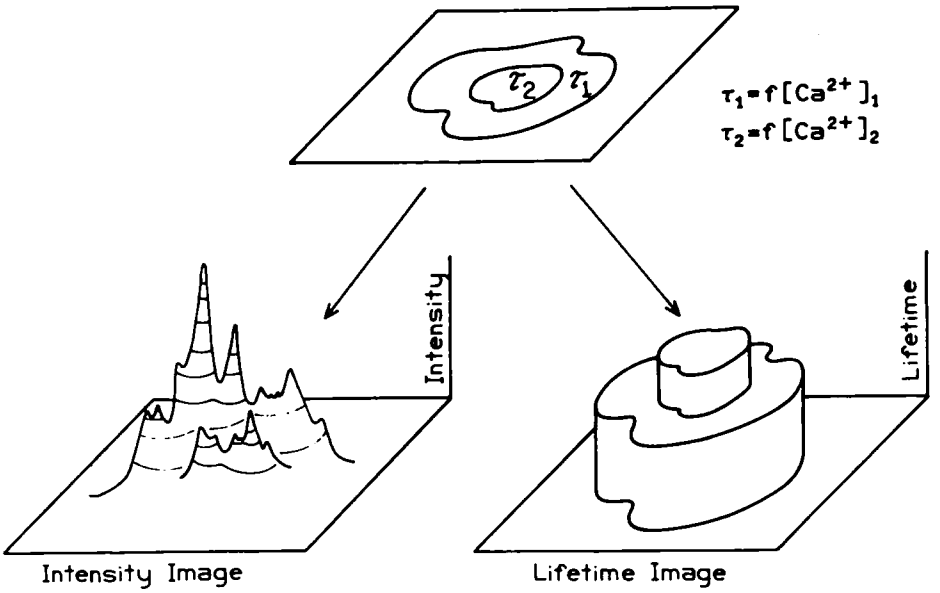


Fig. 8. Fluorescence lifetime imaging microscopy (FLIM).

ulation sensing should provide the robustness needed in a clinical environment. For the more technically oriented researcher, we note that the use of both phase angle and modulation measurements, which are simultaneously available, can provide error checking in critical applications.

One can also imagine a blood septicemia assay based on phase-modulation fluorometry (Fig. 4). It is known that for certain long-lived fluorophores, it is possible to use a simple electroluminescent device as the amplitude-modulated light source [22]. In this case, the probe chemistry is not yet completely developed, but we now know how such an apparatus can be constructed and have identified preliminary probes for this purpose. Importantly, such a blood septicemia assay (Fig. 4) would allow for the simultaneous measurement of pH and pO_2, as well as pCO_2, and should be insensitive to optical alignment of the sample vials.

Noninvasive Transdermal Glucose Sensing

Noninvasive glucose measurements can also be performed with phase-modulation fluorometry. The blood gas applications described above requires drawing the blood, i.e., an invasive as well as an unpleasant procedure. Similarly, present measurements of blood glucose also require fresh blood. Insulin-dependent diabetics often require to be measured four times per day. The unpleasantness and pain of this procedure result in the minimum number of blood glucose measurements by diabetics. However, erratic blood glucose control may be responsible for the adverse long-term health effects of blindness and heart disease, possibly due to the irreversible glycosylation and modification of blood proteins and blood vessels. Continuous noninvasive monitoring of glucose can provide the input needed for continuous insulin injection, that is, the "insulin pump," or improved information to the diabetic of the effects of food intake on his/her glucose levels (Table III).

Noninvasive monitoring of glucose now appears possible based on our current understanding of lifetime-based sensing and the optical properties of tissues. Recall as a child when you placed a flashlight (a white light source) behind your hand and noticed the red transmitted light. We should have all recognized that this observation enables noninvasive sensing using long-wavelength light sources and time-resolved detection. In modern times, the red wavelengths of laser diodes are only weakly absorbed by skin but, of course, are highly scattered. Consider the implantation of a glucose sensing patch below the skin, in which the decay time of the laser diode-excitable probe is sensitive to glucose (Fig. 5). Because the skin transmits the red light, the sensor will be excited. Because the decay times are not dependent on total intensity, they can be measured in this scattering medium, most probably by the phase-modulated method. The times required for light migration in tissues are typically on the 200-ps time scale [23] and, thus, can be

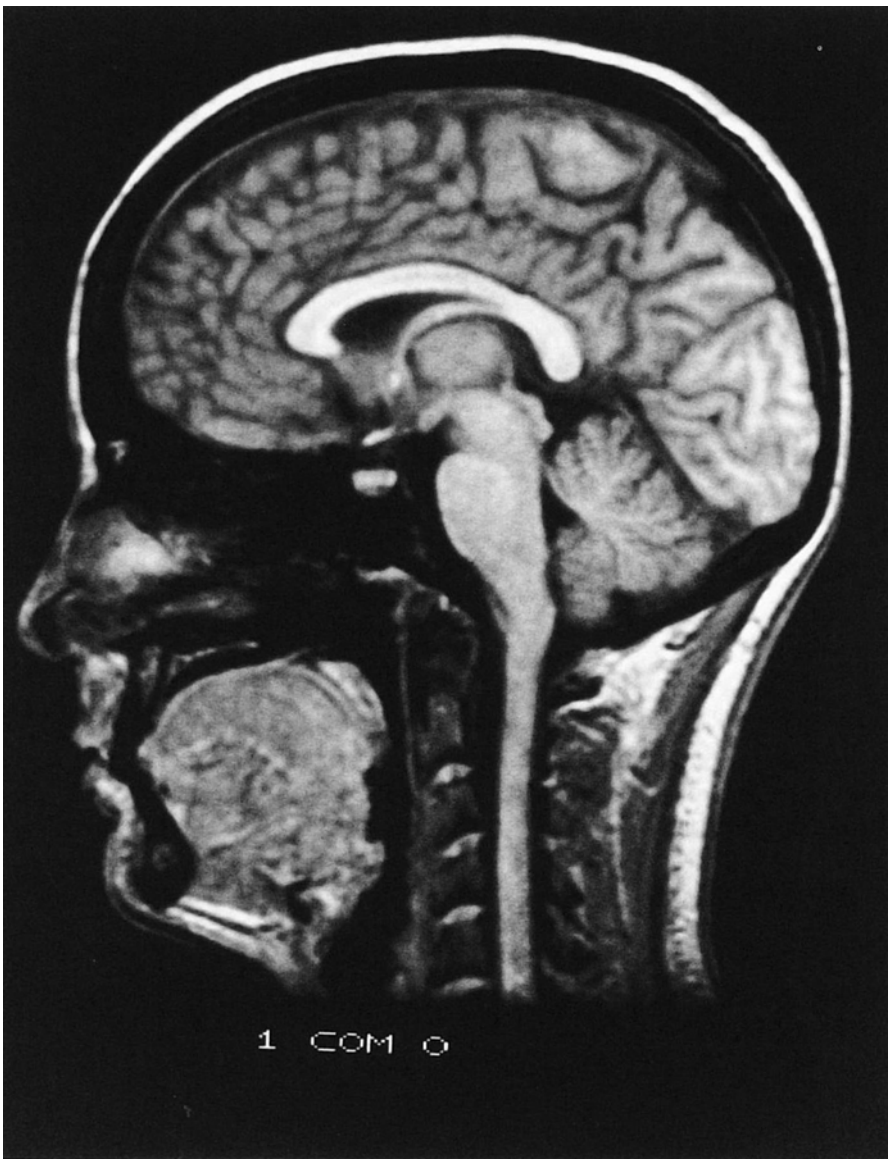


Fig. 9. Sagittal MRI images of a human head. (Used with permission from General Electric Medical System Group.)

readily accounted for when measuring ns lifetimes. Additionally, tissue glucose levels are thought to follow blood glucose to within a 30-min delay [24], so that the patch can be under the skin and need not penetrate the venous system. Also, there are probably better locations for this glucose patch than in the forearm (Fig. 5).

What mechanisms can be used to create a lifetime-based glucose sensor? In our opinion, the mechanism should be fluorescence resonance energy transfer (FRET). The phenomenon of FRET results in transfer of the excitation from a donor fluorophore to an acceptor chromophore, which need not itself be fluorescent. FRET is a through-space interactor which occurs over distances of 20 to 60 Å. The characteristic distances for FRET can be reliably calculated from the spectral properties of the donor (D) and acceptor (A). Importantly, FRET can be reliably predicted to occur for any D–A pair, so that the system can be wavelength-adjusted to match the wavelengths of laser diode sources. The extent of FRET depends on the proximity of the donor and acceptor.

Based on the above considerations, a glucose lifetime sensor can be based on a protein which reversibly

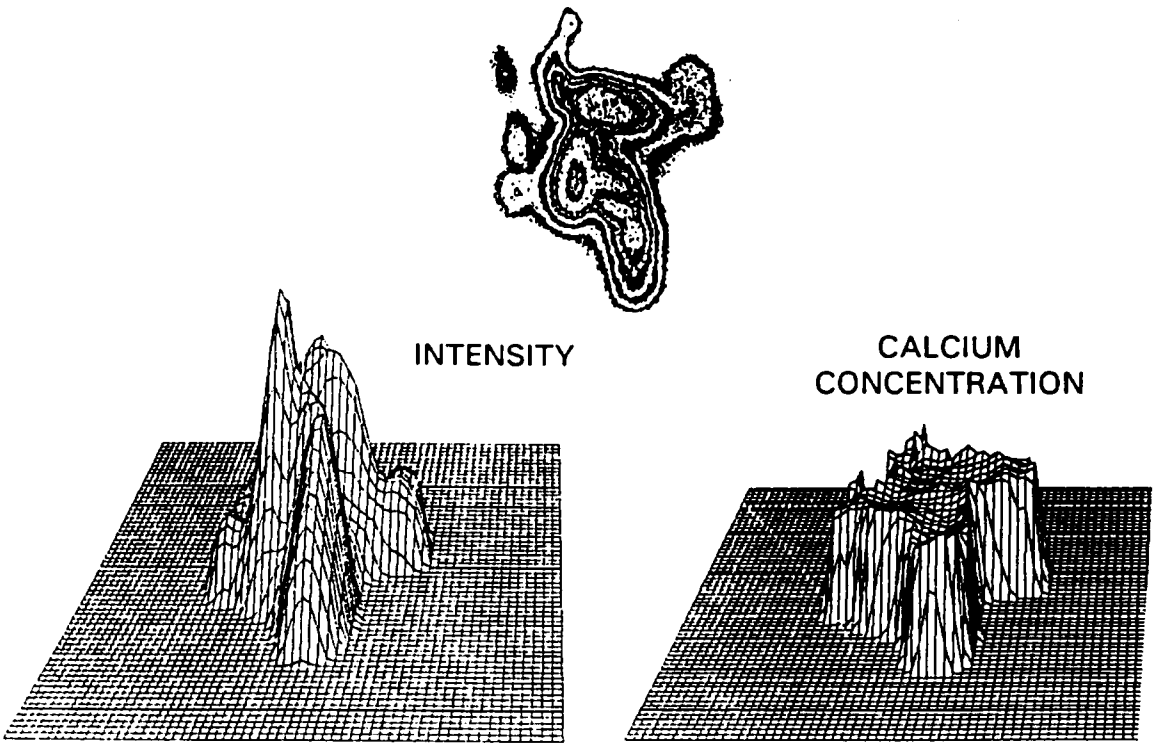


Fig. 10. Intensity (top and left) and calcium images (right) of Quin-2 fluorescence in COS cells.

binds glucose (Fig. 6), such as concanavalin A (Con A). The acceptor should be attached on a polymeric medium such as dextran, which will bind the Con A but not diffuse out of the semipermeable patch. Glucose will competitively display the Con A from the dextran acceptor, resulting in an increase in donor lifetime in rough proportion to the glucose concentration. It should be noted that this sensor would not require any external connections, would not consume glucose, and can potentially be replenished if needed by injection rather than removal. Such implantation devices have now been accepted as a means of birth control. Hence, it seems that individuals with diabetes are likely to accept such an implant if it results in improved or more convenient control of his/her blood glucose.

The glucose and blood gas sensing applications should not be regarded as a "Star Wars" approach, which will only increase the cost of health care without significant benefit. In these two cases, the costs of the new technology will probably be less than existing methods. More importantly, improved monitoring of blood gas is likely to decrease the time spent in the intensive care unit (ICU), and control of blood glucose reduce the long-term consequences of diabetes. In both cases, the improved care should decrease the total cost of health care and maintenance. Also, the technology for these applications is available today and requires only that these concepts be developed into the actual applications.

Intracellular Chemical Analysis and Flow Cytometry

Flow cytometry and/or fluorescence-activated cell sorting (FACS) is presently widely used in the diagnosis of cancer and other diseases [25,26]. Most applications of flow cytometry are based on the presence or absence of cell surface antigens or the presence of one or two copies of the DNA, as determined by measurement of the fluorescence intensity of cells labeled with fluorescent antibodies or nucleic acid stains. The immunological or cell-division emphasis of flow cytometry may be a consequence of the difficulty in measuring the precise intensity values during the 10-μs passage of the cell through the laser beam (Fig. 7). Also, there is considerable cell-to-cell variation in the extent of staining or uptake of probe molecules.

The difficulties of intensity-based flow cytometry are illustrated by the present difficulties of cell-by-cell

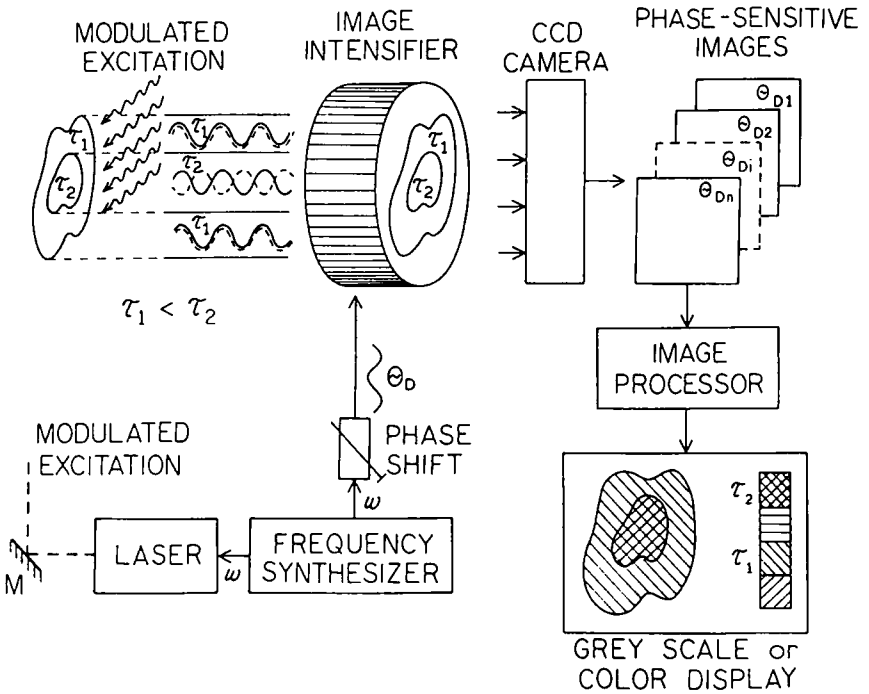


Fig. 11. Apparatus for fluorescence lifetime imaging microscopy (FLIM).

measurements of intracellular calcium. This can be accomplished using the calcium probe Indo-1 [27–31] but requires a UV laser source, which is not routinely available in flow cytometry (Indo-1 is an emission wavelength-ratiometric probe). Flow cytometers routinely have argon ion laser sources with outputs of 488 or 514 nm. Measurement of intracellular ions other than Ca^{2+} is nearly impossible. (The SNAFL and SNARF probes should allow pH measurement from the wavelength-ratiometric data.)

The advantages of lifetime-based sensing can be particularly attractive for flow cytometry, where the size, shape, and degree of labeling can vary between these cells. For instance, the probe Calcium Green displays a lifetime change from 1 to 4 ns upon binding Ca^{2+}, and Calcium Green can be excited with an argon ion laser [11]. Consequently, intracellular Ca^{2+} measurements could be readily accomplished if cell-by-cell lifetime measurements were possible in flow cytometry. At first, this task seemed nearly impossible in that lifetime measurements almost always require continuous data acquisition from minutes to hours, and even lifetime measurements in 1 s would not be adequate for the 10-μs flow cytometry signal from each cell.

The problem has now been solved, and it is possible to measure the phase angle of the probe as the cells pass through the laser beam [32,33]. While these measurements have not yet been applied to Ca^{2+}, the method has been validated with standard beads and stained cells. In our opinion, this new flow cytometry parameter, and our increasing knowledge of lifetimes of probes, will result in the increased use of flow cytometry for studies of intracellular physiology, in addition to the current emphasis on immunology, cell activation, and ploidy.

Fluorescence Lifetime Imaging Microscopy (FLIM)

Fluorescence microscopy is routinely used to study the location and movement of intracellular species. In general, the fluorescence image reflects the location and concentration of the probe or that amount of probe remaining in a photobleached sample (Fig. 8, lower left). Consequently, quantitative fluorescence microscopy is very difficult, except for those cases where wavelength-ratiometric probes are available.

Consider now that the lifetime of the probe is different in the two regions of the cell (Fig. 8, top). If one could create a contrast based on the lifetime at each point in the image, one would resolve two regions of the cell, each with an analyte (Ca^{2+}) concentration which was revealed by the lifetime image.

A sagittal magnetic resonance (MRI image) of a human is shown in Fig. 9. Why have we shown this

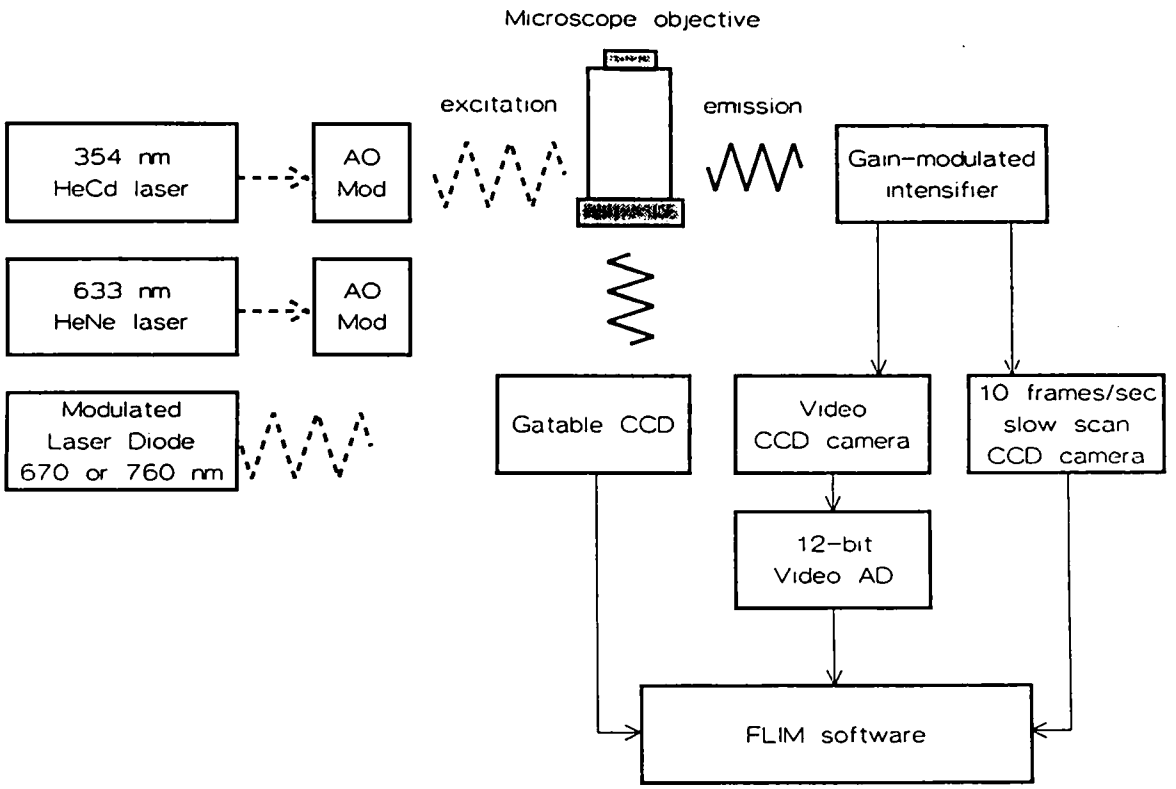


Fig. 12. Future apparatus for FLIM.

Table IV. Biomedical Applications of FLIM

Type of imaging	Analyte or property
Chemical imaging	Ca^{2+}, Mg^{2+}, Cl^-, pH, O_2, Na^+, and K^+
Ligand binding to proteins	NADH, TNS
Chromosome imaging	Acridine lifetimes depend on DNA base composition
Microviscosity imaging	Identify viscosity-lifetime probes
Proximity imaging by energy transfer	Protein–protein binding Protein–membrane association

Table V. Additional Applications of FLIM

Type of imaging	Application
Photon migration imaging	Optical tomography Clinical sensing Mammography
Imaging of air flow by O_2 partial pressure	Aerodynamics research
Temperature imaging	Semiconductor manufacturing
Remote sensing	Lifetime-based LIDAR

image? The image is shown to illustrate the fact that lifetime, or in this case, relaxation time imaging, is currently in widespread use. The contrast, or black–gray–white scale, in the MRI images is based on the proton relaxation times, which are analogous to a fluorescence lifetime. MRI images are not routinely based on the total signal, which is analogous to the local intensity in the fluorescence microscopic images. MRI provides useful medical images because the contrast reflects the different chemical and physical properties of the organ. In the same way, the contrast in FLIM can provide chemical images of cells based on the local lifetime, which can be affected by cations, anions, pH, O_2, temperature, viscosity, or polarity. In this sense, FLIM is the microscopic analogue of MRI.

The creation of such fluorescence lifetime images, in which the contrast is based on lifetimes, appeared to be a daunting challenge. Imagine performing 2.62 ×

Table VI. Product Predictions Based on Advanced Optical Devices

Optical monitoring of bioreactors
A bedside blood gas analyzer, Green HeNe, then laser diode
Doctor's office and/or bedside clinical chemistry and diagnostics
Noninvasive optical transdermal glucose sensor
Noninvasive diagnostics based on laser diodes
Fluorescence microscope analogous to MRI images
Optical mammography
Optical tomography and imaging

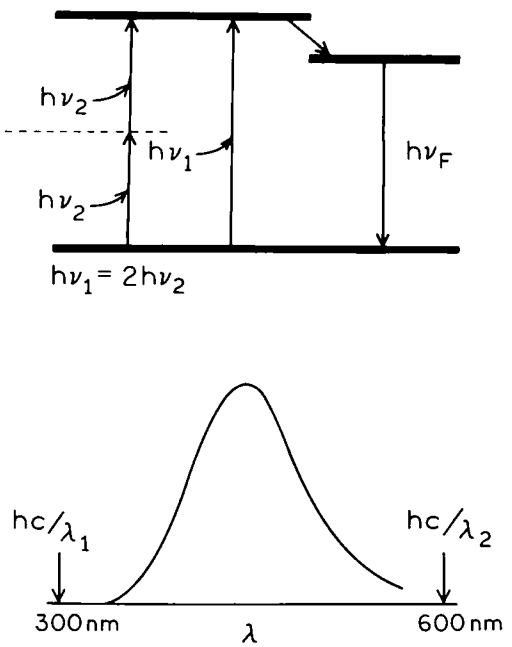


Fig. 13. Jablonski diagram for two -photon-induced fluorescence.

10^5 lifetime measurements for a typical 512 × 512 image. Given the difficulties of measuring even a single lifetime in a cuvette, such a task seems nearly impossible. However, image intensifiers and CCD camera technology now make this possible [34,35]. Figure 10 (right) shows the Ca^{2+} lifetime image of COS cells based on the probe Quin-2 [36], along with the intensity image (left). The intensity images show the expected spectral variations due to probe localization, and the Ca^{2+} (phase angle) image shows the expected uniform concentration of intracellular calcium. As predicted, the lifetime imaging provides chemical imaging, which within limits is insensitive to the local probe concentration.

The cellular FLIM images in Fig. 10 were obtained using moderately complex instrumentation, which consists of a picosecond dye laser, a gain-modulated image intensifier, and a slow-scan scientific grade CCD camera (Fig. 11). However, the FLIM instruments in the future can be compact, and mostly solid-state devices. This possibility is shown in Fig. 12, where we show that the light source can be a laser diode, assuming the probes are available. The image intensifier is a moderately simple device but is delicate and requires high voltages. Reports have appeared on gatable CCD detectors [37]. Present gatable CCDs are too slow (50-ns gating time). This time response is likely to improve, and probes can be developed with longer decay times. Then the FLIM apparatus will consist of only modest additions to a standard fluorescence microscope.

What type of chemical imaging will be possible using FLIM technology? Based on our current understanding of FLIM, and factors which affect fluorescence lifetimes, we can predict that lifetime imaging will allow images of a variety of cellular properties (Table IV). We also believe that FLIM technology can play an important role in biomedical imaging, process control, and engineering research (Table V).

The Need for Development of New Fluorescence Probes

In our opinion, the applications of fluorescence to analytical chemistry, clinical chemistry, flow cytometry, and imaging are limited not by the instrument technology, but by the available probes. There are only a limited number of conjugatable long-wavelength probes, and none which display specific analyte sensitivity. What is needed is an arsenal of probes, all of which can be excited with laser diodes and which are specifically sensitive to cations, anions, and other analytes. While several laboratories are working on this topic, the total effort is minor in comparison to the number of scientists engaged in instrument development, technology development, theory, or applications. The development of this arsenal of probes is crucial for the practical application of fluorescence to real-world sensing applications. As these new probes are developed, one can predict a number of healthcare products, as summarized in Table VI.

ADVANCED APPLICATIONS OF FLUORESCENCE SPECTROSCOPY

Since time-resolved fluorescence is now an existing technology, and one which is migrating toward analytical and clinical applications, what areas remain for research? One could describe many possibilities, such as

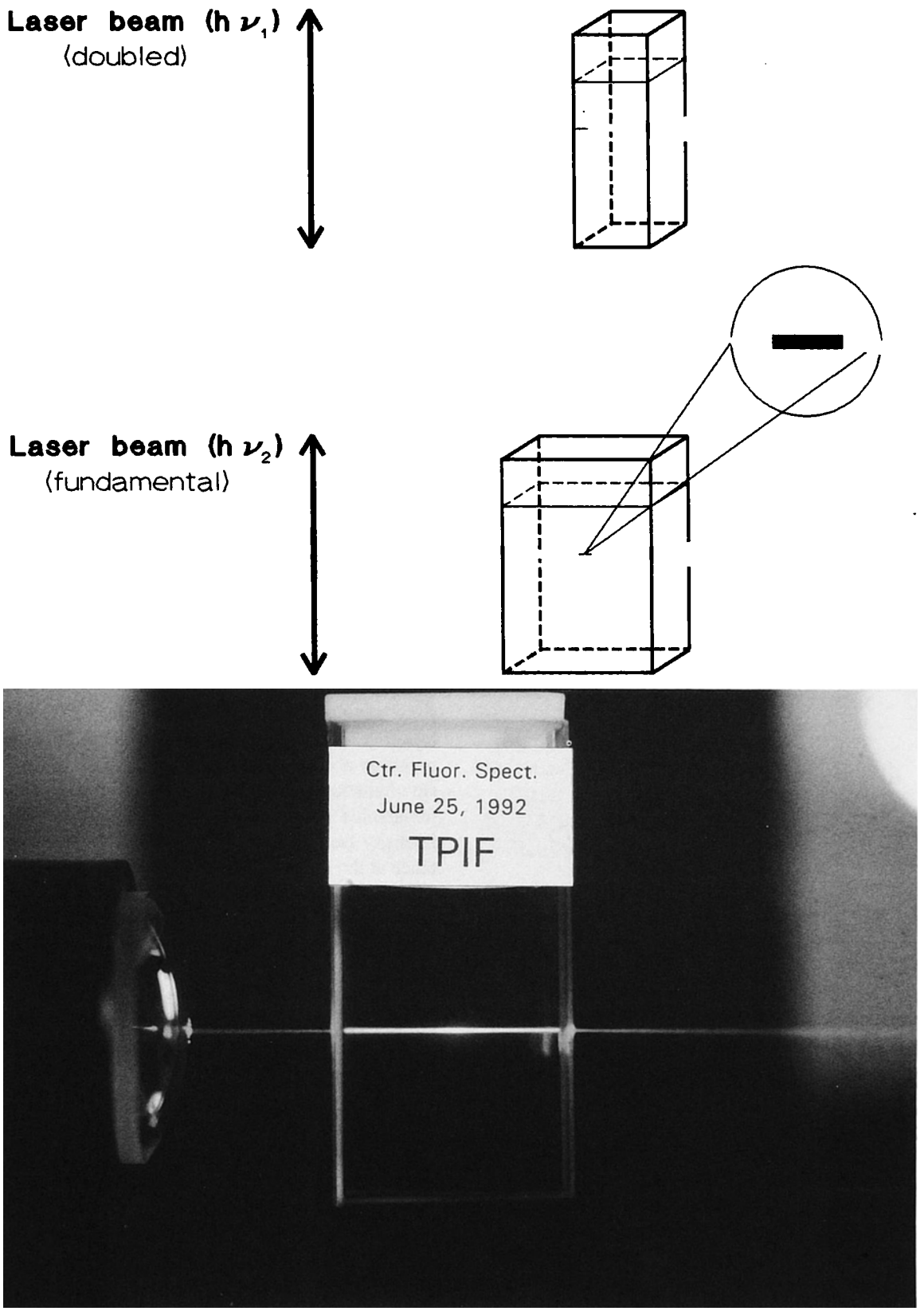


Fig. 14. Schematic of two photon-induced fluorescence (top) and two photon-induced fluorescence of a coumarin dye (bottom). The red excitation is seen to excite blue fluorescence.

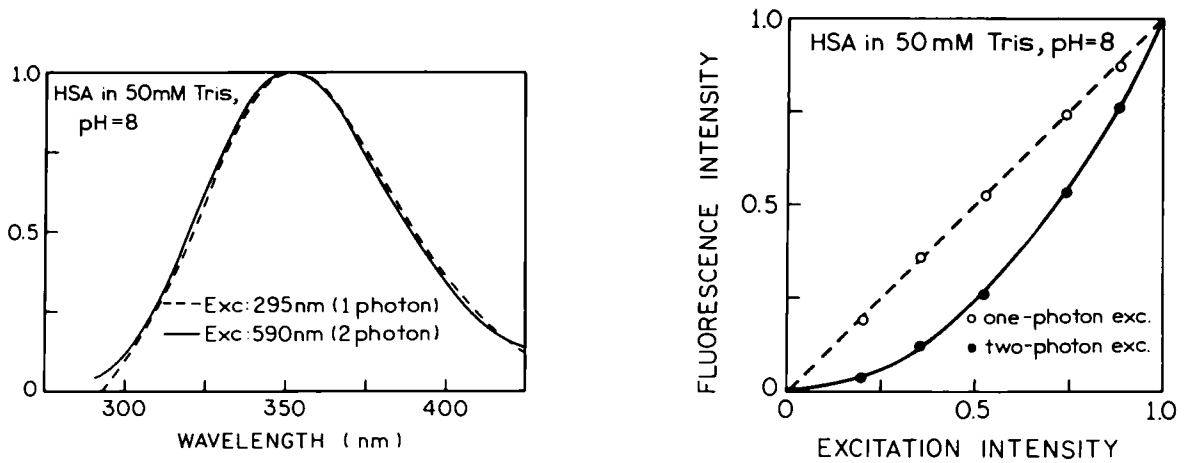


Fig. 15. Emission spectra of HSA for one- and two-photon excitation (left) and the dependence of the emission intensity on the incident light intensity (right).

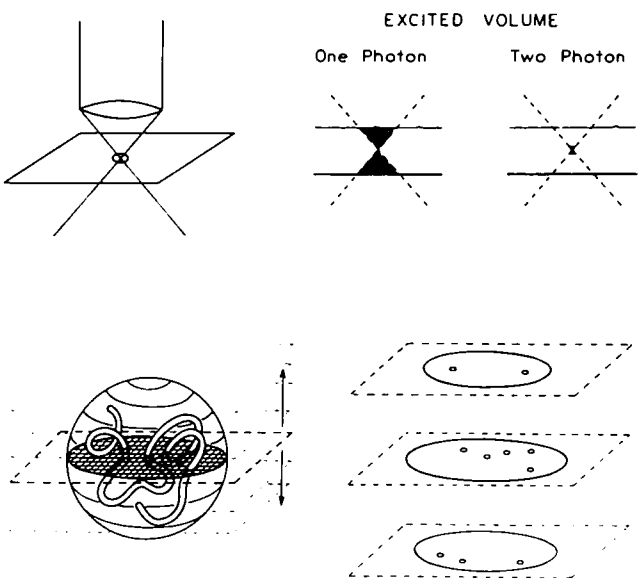


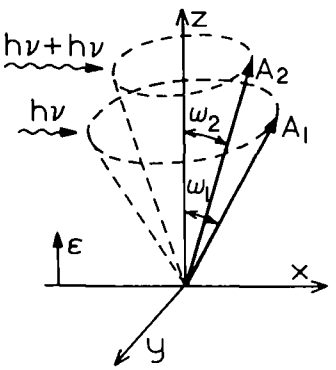
Fig. 16. Intrinsic "confocal" excitation using TPIF in microscopy (top) and three-dimensional optical imaging (bottom).

the present efforts to perform medical imaging based on the time-dependent migration of light in tissues [38–43], the uses of FRET to recover the structure and dynamics of biological molecules [44], and the use of oriented systems to the information content of the fluorescence spectra data [45]. In the present article, we want to emphasize two areas of special promise, both of which take advantage of the intense pulses available from modern laser sources. These topics are two photon-induced fluorescence (TPIF) and light quenching (LQ).

Two Photon-Induced Fluorescence (TPIF)

Most of us are familiar only with one photon-induced fluorescence (OPIF), which is the common occurrence in our experiments. With intense laser sources, it is possible to observe the emission resulting from the simultaneous absorption of two long-wavelength photons (Fig. 13). For instance, tryptophan in proteins, which normally absorbs light at 290 ns, can be excited by the simultaneous absorption of two 580-nm photons. This remarkable phenomenon occurs only with a high light intensity because the two photons must be in the same place at the same time to allow simultaneous absorption.

A schematic of TPIF is shown in Fig. 14 (top). With one photon-induced fluorescence (OPIF), the violet light is absorbed according to Beer's law with light absorption starting immediately at the surface of the sample. With TPIF, the absorption occurs primarily at the point of highest intensity, in this case, where the red incident light is focused at the center of the sample. The actual phenomenon of TPIF is shown at the bottom of Fig. 14, which shows a solution of a coumarin dye which absorbs near 350 nm. This sample is illuminated with picosecond pulses from a dye laser, which is focused in the center of the cuvette. The red excitation light is visible in Fig. 14 along with the blue fluorescence induced by simultaneous absorption of two red photons. Notice that the fluorescence is seen only in the middle of the cuvettes, where the laser beam is focused and most intense. Because this is a two-photon phenomenon, the intensity is proportional to the square of the light intensity. This dependence in the square of the intensity is



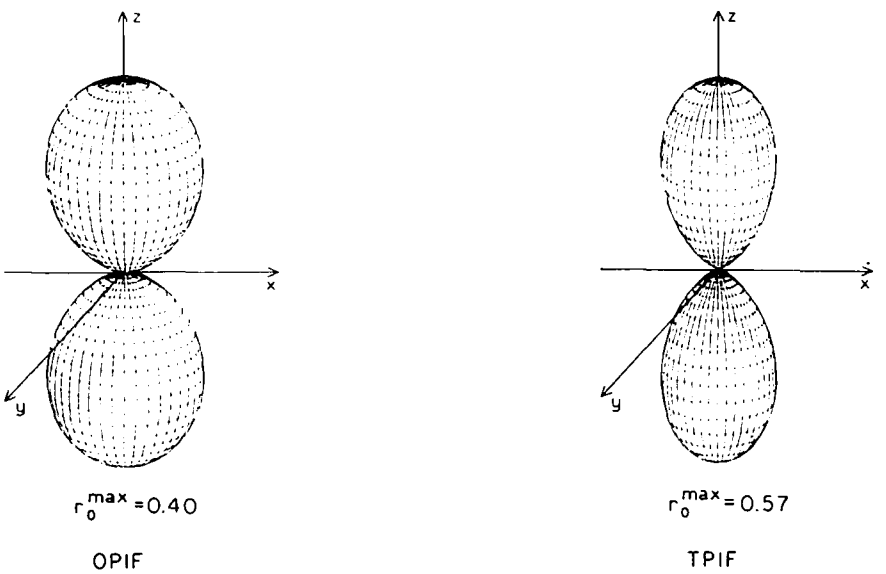


Fig. 17. Increased photoselected orientation for TPIF. The lower surfaces represent the orientation of the excited state, assuming that $r_0 = 0.4$ and no rotational diffusion.

shown in Fig. 15, which shows the spectra of human serum albumin (HSA) excited at 295 or 590 nm. Also shown is the emission intensity of HSA, which is linearly dependent on the incident light intensity at 295 nm and quadratically dependent on the intensity at 590 nm [46]. TPIF has also been observed for fluorophores bound to membranes [47] and nucleic acids [48].

The fact tht TPIF depends on the intensity squared provides an important opportunity for fluorescence microscopy. In fluorescence microscopy, confocal optics are often used to eliminate fluorescence from outside the focal plane of the lenses [49,50]. Removal of this out-of-focus light provides a remarkable improvement in image quality because in OPIF the fluorescence occurs from the entire thickness of the sample. Much of this emission is devoid of spatial information and serves only to degrade image contrast and resolution.

Professor Webb and colleagues at Cornell University recognized that the intensity-squared dependent of TPIF provided the opportunity for intrinsic "confocal" excitation [50,51]. The sample can be excited only at the desired depth (Fig. 16), and the signal comes only from this region. Perhaps more importantly, the fluorophores which are not in the focal plane are not excited,

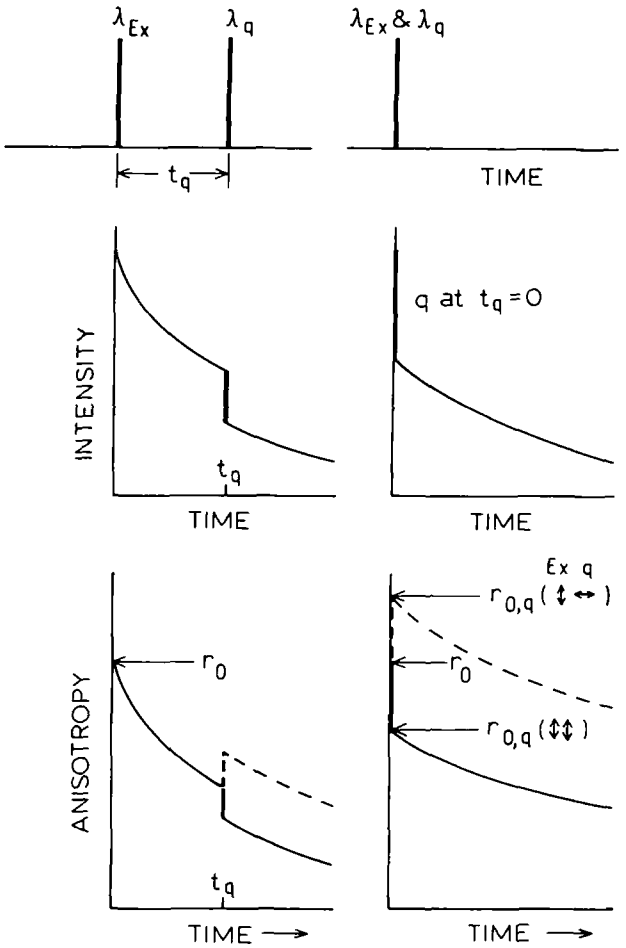


Fig. 18. Schematic of light quenching by a time-delayed quenching pulse.

consequently are not photobleached, and are thus available for imaging when the focal plane is moved (Fig. 16). There are additional advantages of two-photon microscopy, such as the greater availability of optical components and increased transmission of the optics for the longer wavelengths. It is possible that the sample autofluorescence will be lower with two photon excitation, but at present, we do not know if the endogenous fluorophores in cells will display high or low cross sections for two-photon excitation. It is already known that some Ca^{2+} probes display good two-photon absorption [52], and Dr. Webb and co-workers, have reported lifetime images with two-photon excitation [53]. Hence, we can now imagine the creation of three-dimensional (3D) chemical images of cells (Fig. 15), which could display the local Ca^{2+} concentration as seen for the two-dimensional (2D) FLIM imaging in Fig. 10.

TPIF and Time-Resolved Fluorescence

We note that we were surprised by the ability to observe TPIF with picosecond laser sources. The two-photon microscopy work used femtosecond lasers, and it was generally assumed that these exotic lasers were necessary to obtain adequate intensity for TPIF. (At the same average power a 10-fs pulse is expected to result in 10^6-fold more TPIF then a 10-ps pulse.) These observations allow the study of TPIF in many other laboratories.

In the next several years, we expect to see increasing activity in studies of the time-resolved emission resulting from TPIF. One reason will be the increased photoselected orientation which results from two-photon excitation (Fig. 17). For OPIF, the probability of absorption depends on $\cos^2\theta$ where θ is the angle between the electric vector of the polarized excitation and the transition moment of the fluorophores. The fact that the maximal observable anisotropy for a random distribution of fluorophores is 0.4 is a direct consequence of the $\cos^2\theta$ dependence of light absorption.

Simultaneous absorption of two photons results in an apparent $\cos^4\theta$, which results in a more highly oriented excited-state population. This fact is illustrated at the bottom of Fig. 17, which shows the excited-state population immediately following light absorption. Consequently, the maximum anisotropy for TPIF is 0.57, which provides for improved resolution of complex rotational motions of macromolecules in solution. Another important aspect of TPIF is that the cross section (absorption spectra) for two-photon absorption spectra can be different from that for one-photon absorption spectra (after correcting for λ and 2λ). Such differences have already been observed for indole and tryptophan [54], which appear to be the result of different relative cross sections for the ^1L_a and ^1L_b transitions for OPIF and TPIF. Hence, there can be different information available from the OPIF and TPIF spectral data.

Light Quenching of Fluorescence

Measurements of time-resolved fluorescence, and particularly the recent interest in TPIF, require the use of intense laser sources. The use of these picosecond laser sources allows observation of the phenomenon of stimulated emission. If a fluorophore is illuminated at a wavelength which overlaps its emission spectrum, the fluorophore can be stimulated to return to the ground state (Fig. 18). Since the stimulated photon travels parallel to the "quenching beam," and since the emission

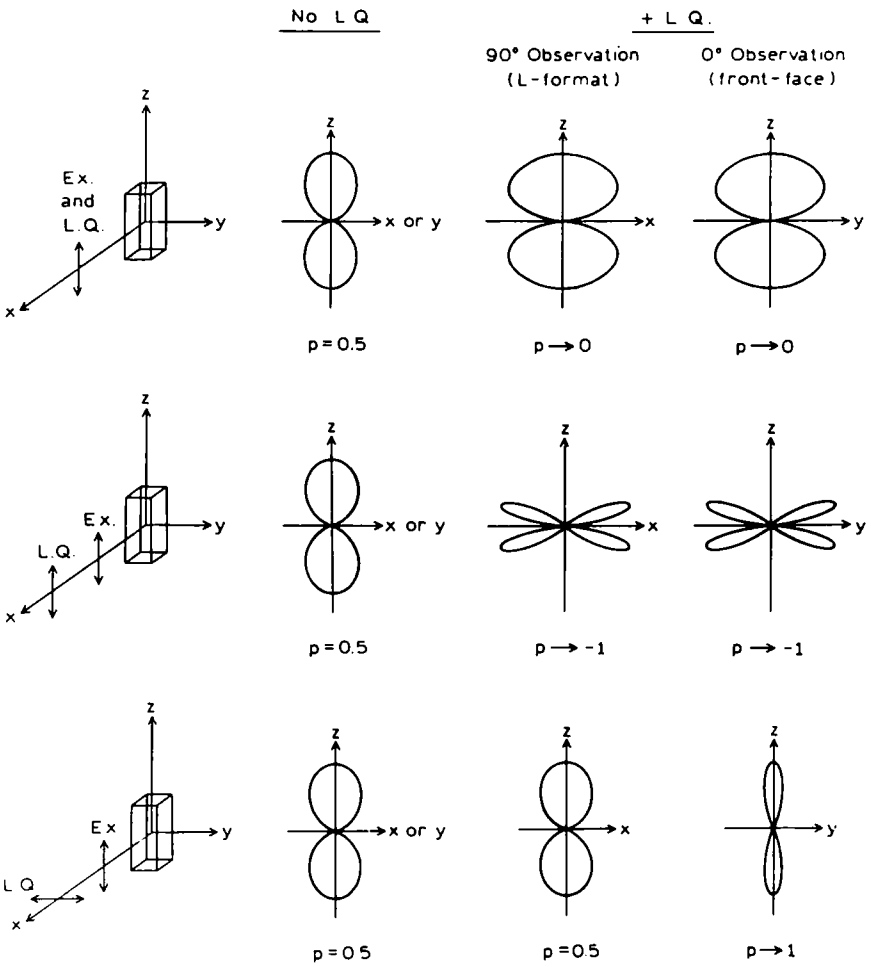


Fig. 19. Effects of polarized light quenching on the orientation of the excited-state population.

is generally observed at right angles to the illumination, the emission appears to be quenched.

Of course, light quenching or stimulated emission was predicted by Einstein in 1917 for atoms in the gas phase [55]. Historically, light quenching has been observed only using the very intense pulses from Q-switched ruby lasers [56–58]. The fact that we now know that light quenching can be observed with modern picosecond lasers results in numerous opportunities for novel fluorescence experiments [59–61].

Consider that the sample is excited with one pulse, followed by a second longer-wavelength quenching pulse (Fig. 18). The quenching pulse can result in an instantaneous change in the excited-state population. It is important to recognize that this change in population should be nondestructive; we are not depleting the ground state or bleaching the sample. Hence, the experiment may be repeated numerous times for improved signal-to-noise, if needed, to measure small effects.

One remarkable opportunity of light quenching, and there are other opportunities which are not described in this article, is that light quenching displays the same $\cos^2\theta$ dependence as does light absorption [57,61]. This means not only that the total excited-state population is altered by the quenching pulse, but that selectively oriented parts of the excited-state population are quenched. Consequently, depending on the polarization of the quenching light, the polarization of the emission can be altered from 1.0 to -1.0 (Fig. 19), resulting in a high degree of orientation of the excited-state population. In contrast, one-photon excitation of randomly oriented fluorophores can result only in polarization values from 0.5 to -0.33. It may even be possible to break the Z-axis symmetry, which heretofore has been pervasive in

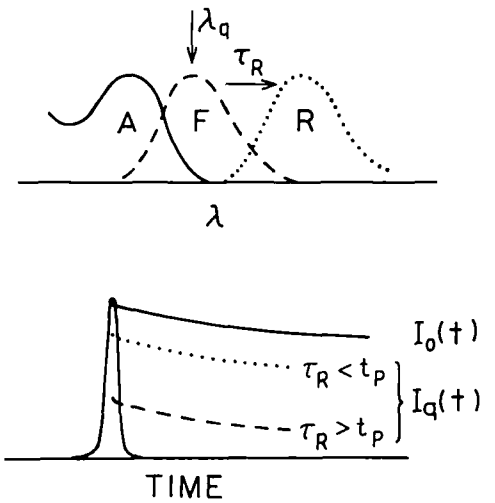


Fig. 20. Light quenching and time-dependent spectral relaxation.

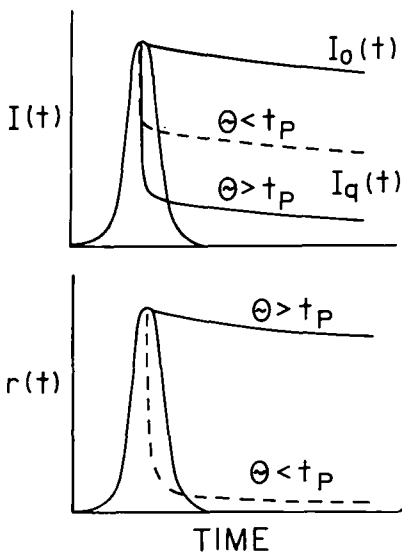


Fig. 21. Light quenching and rotational diffusion.

the optical spectroscopy of randomly oriented solutions. In our opinion, the phenomenon of light quenching can result in a new class of fluorescence experiments in which the sample is excited with one pulse and the excited-state population is modified by the quenching pulse(s) prior to measurement.

It is important to notice that interesting information may be available by studies of the extent of light quenching which occurs within a single laser pulse. For instance, suppose that one studies light quenching of a solvent-sensitive fluorophore, which displays spectral

Table VII. Enabling Technologies for the Biomedical Applications of Time-Resolved Fluorescence Spectroscopy

Lasers	Laser diodes Two-photon excitation with picosecond–femtosecond lasers
Image intensifiers	Gatable on nanosecond time scale Red-sensitive for optical tomography
CCDs	Fast frame rates Gatable on nanosecond time scale
Computers	Allows processing of numerous images from CCD images
Probe chemistry	Need long-wavelength probes to take advantage of laser diodes and low autofluorescence

relaxation away from the quenching wavelength (Fig. 20). Then the extent of quenching would depend on the instantaneous fluorescence intensity at the incident wavelength during the duration of the incident pulse. This concept is illustrated in Fig. 20, where F represents the initially excited state (Frank–Condon State), R the solvent relaxed state, and $\tau_R > t_p$ (Fig. 20, bottom), where the emission spectrum overlaps with the incident wavelength (Fig. 20, top). The amount of quenching is expected to be less if the relaxation is rapid, $\tau_R < t_p$, so that the emission spectrum is already shifted to longer wavelengths during the duration of the incident pulse. It is important to note that, in principle, such experiments contain information on processes which occur on the time-scale of the pulse width t_p. Hence, with the availability of picosecond and femtosecond lasers, one can use the amounts of light quenching observed, under stationary conditions, to study processes which occur on the picosecond or femtosecond time scale.

Similar reasoning indicates that single-pulse light quenching experiments can reveal the rates of fluorophore motion or more rapid rotations of the electronic moments from the extent of light quenching. This possibility is illustrated in Fig. 21, where we assumed that the incident light is vertically polarized and the absorption and emission transitions are parallel. Suppose that the rotational correlation time (Θ) is long relative to t_p, $\Theta > t_p$ (Fig. 21, bottom; solid line). Then the excited fluorophores remain favorably oriented to be quenched by the incident beam during the duration of the pulse (top; lower solid line). In contrast, assume that the molecules rotate significantly during the time t_p. In this case, the excited fluorophore can rotate during the pulse (bottom; dashed line), resulting in less light quenching (top;

dashed line). The "rotation" process can be actual rotation diffusion of the fluorophore, which can be studied with subnanosecond and picosecond lasers, or more rapid electronic processes, which can be studied with femtosecond lasers. As in the case of spectral relaxation, the time resolution of these light quenching experiments is determined by the pulse width, and not the detector or associated electronic circuits. At this time, we can only imagine, and not predict, the types of information which can be obtained by such experimentation.

In closing, we wish to reiterate our opening statement. Time-resolved fluorescence is now positioned to move out of the research laboratory and into the world of clinical chemistry, manufacturing process control, and numerous other sensing applications. Advances in laser sources, CCD detection, and other technologies is resulting in the possibility of simple instrumentation for previously complex measurements (Table VII). The increasing availability of intense picosecond and femtosecond lasers, and laser systems which provide multiple time-delayed pulses, will result in the increased use of two-photon excitation and stimulated emission to control and/or modify the excited-state population.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Jan Slavik, organizer of the conference in Prague, for the invitation to write this article. The concepts and results described in this article were developed over a number of years. Dr. Joseph R. Lakowicz wishes to thank the National Institutes of Health (RR-08119, RR-07510, GM 39617, GM 35154) and the National Science Foundation (MCB 8804931, BIR 9319032, DIR 8710401) for their continued support. We also thank the General Electric Medical System Group for the MRI image.

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