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Emerging Applications of Fluorescence Spectroscopy to Cellular Imaging: Lifetime Imaging, Metal-Ligand Probes, Multi-Photon Excitation and Light Quenching

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EMERGING APPLICATIONS OF FLUORESCENCE SPECTROSCOPY TO CELLULAR IMAGING: LIFETIME IMAGING, METAL-LIGAND PROBES, MULTI-PHOTON EXCITATION AND LIGHT QUENCHING

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Abstract

Advances in time-resolved fluorescence spectroscopy can be applied to cellular imaging. Fluorescence lifetime imaging microscopy (FLIM) creates image contrast based on the decay time of sensing probes at each point in a two-dimensional image. FLIM allows imaging of Ca^{2+} and other ions without the need for wavelength-ratiometric probes. Ca^{2+} imaging can be performed by FLIM with visible wavelength excitation. Instrumentation for FLIM is potentially simple enough to be present in most research laboratories. Applications of fluorescence are often limited by the lack of suitable fluorophores. New, highly photostable probes allow off-gating of the prompt autofluorescence, and measurement of rotational motion of large macromolecules. These luminescent metal-ligand complexes will become widely utilized. Modern pulse lasers allow new experiments based on non-linear phenomena. With picosecond and femtosecond lasers fluorophores can be excited by simultaneous absorption of two or three photons. Hence, Ca^{2+} probes, membrane probes, and even intrinsic protein fluorescence can be excited with red or near infrared wavelengths, without ultraviolet lasers or optics. Finally, light itself can be used to control the excited state population. By using light pulses whose wavelength overlaps the emission spectrum of a fluorophore one can modify the excited state population and orientation. This use of non-absorbed light to modify emission can have wide reaching applications in cellular imaging.

Key Words: Time-resolved fluorescence spectroscopy, fluorescence lifetime imaging microscopy (FLIM), calcium imaging, fluorescent probes, metal-ligand probes, pulse lasers, two-photon-induced fluorescence.

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Recent Advances in Fluorescence Microscopy and Spectroscopy

Fluorescence Lifetime Imaging Microscopy

Fluorescence microscopy is widely used in cell biology to study the spatial distribution of ions and macromolecules [1, 10, 20, 32]. At present almost all microscopic imaging is accomplished by intensity imaging. However, it is well known that quantification of intensities is difficult in a microscope due to the unknown probe concentration at each point in the image, unknown quantum yield, photobleaching and other confounding factors. To circumvent these difficulties we developed a method for imaging based on the decay time or lifetime at each point in the image. We chose the lifetime as the contrast mechanism because we knew that decay times were mostly independent of the probe concentration and photobleaching, and that lifetimes could be measured under optically compromised conditions [18, 27]. Lifetime imaging can be advantageous compared to intensity imaging if one wishes to localize intracellular ions. Many ion indicators are now known to display ion-sensitive lifetimes. If one images the fluorescent intensity one observes where the indicator is localized in the cell. If one measures the lifetime at each point in the cell, that is creates a lifetime image, one learns the local ion concentrations independent of indicator localization.

Assume that the lifetime of the probe is different in the two regions of the cell (Fig. 1, 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. The creation of such fluorescence lifetime images, in which the contrast is based on lifetimes, appeared to be a daunting challenge. Consider the difficulties of performing 2.62×10^5 lifetime measurements for a typical 512 x 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 (charge-coupled device) camera technology now makes this possible [15, 16]. Fig. 2, (right) shows the Ca^{2+} lifetime image of

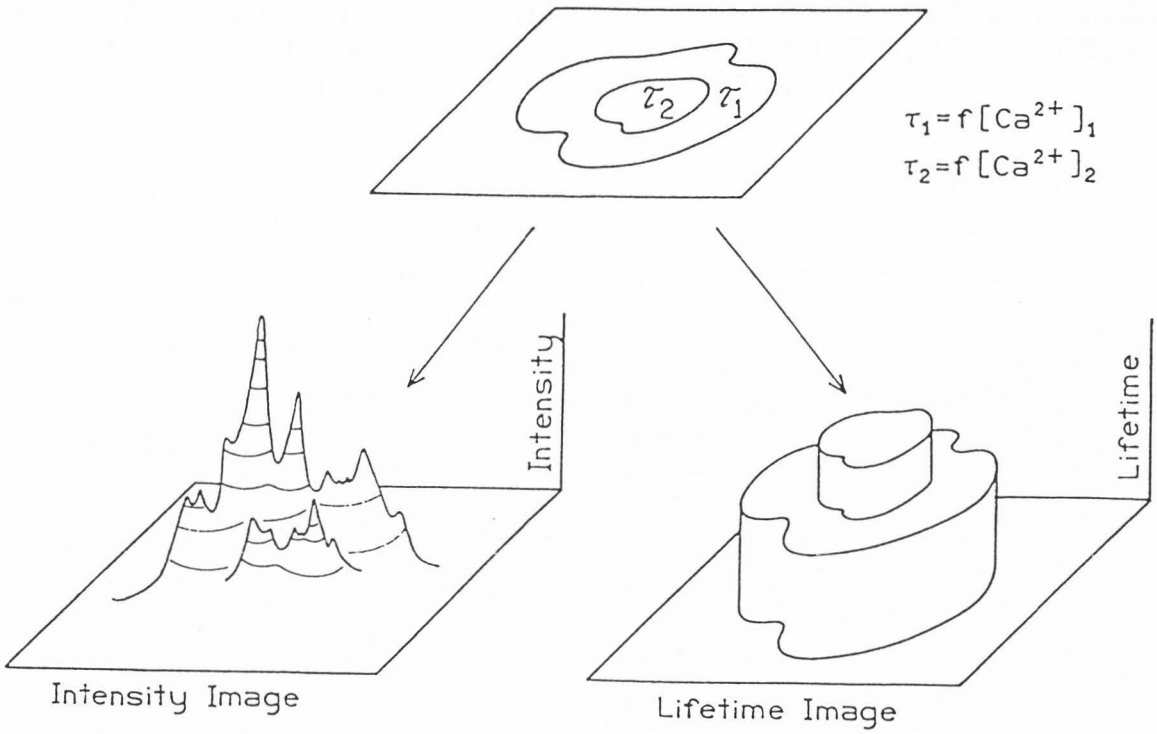

Figure 1. Fluorescence lifetime imaging microscopy (FLIM).

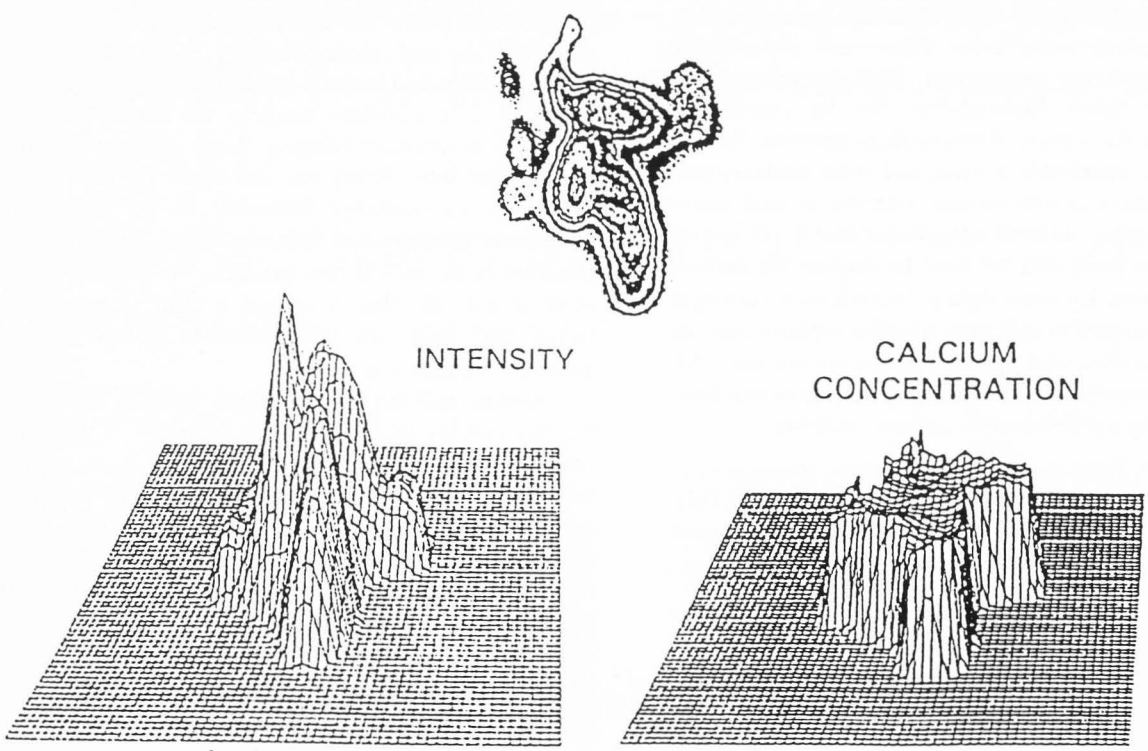

Figure 2. Intensity (top and left) and calcium images (right) of Quin-2 fluorescence in COS cells.

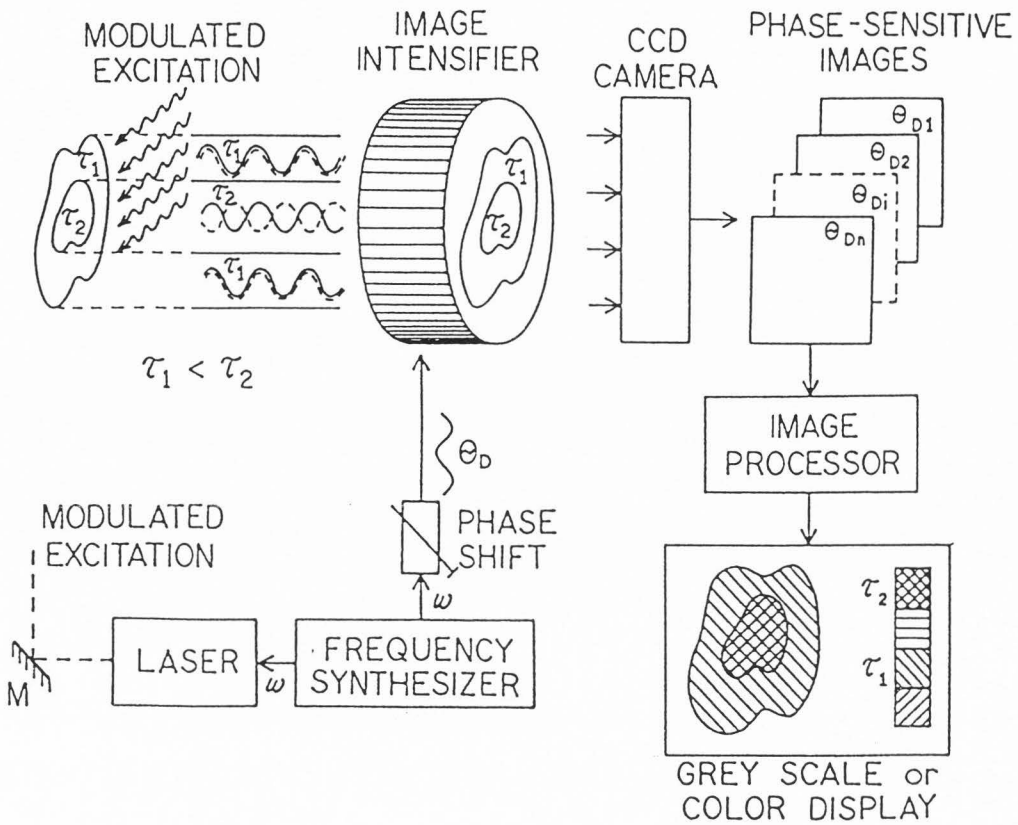

Figure 3. Apparatus for fluorescence lifetime imaging microscopy (FLIM).

COS cells based on the probe Quin-2 [19], along with the intensity image (left). The intensity images show the expected spatial 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. 2 were obtained using moderately complex instrumentation, which consists of a ps dye laser, a gain-modulated image intensifier, and a slow-scan scientific grade CCD camera (Fig. 3). However, the FLIM instruments in the future can be compact, even mostly a solid state device. This possibility is shown in Fig. 4, where we show that the light source can be a laser diode, assuming the fluorescent probes are available. An image intensifier is a moderately simple device, but is delicate and requires high voltages. Reports have appeared on gatable CCD detectors [24]. 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, which is described in the next section. 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 imaging of a variety of cellular properties such as proximity, binding and microviscosity (Table 1). Proximity imaging can be possible based on the phenomena of fluorescence resonance energy transfer (FRET). Energy transfer occurs when a fluorescent donor is within a given distance of an acceptor. The distances for FRET are typically 30-60Å, which is typical of the size of biological macromolecules. FRET decreases the lifetime of the donor; thus allowing the proximity of a donor and acceptor to be determined from the donor decay time. Lifetime-based fluorescence probes are also known for a wide variety of ions [26], including Ca^{2+}, Mg^{2+}, K^+, pH, Cl^- and O_2. Hence, FLIM can have wide-ranging applications in cell physiology.

Probe Chemistry - Development of Long-Lived Metal-Ligand Probes

The application of fluorescence to analytical chemistry, clinical chemistry, flow cytometry, and imaging are

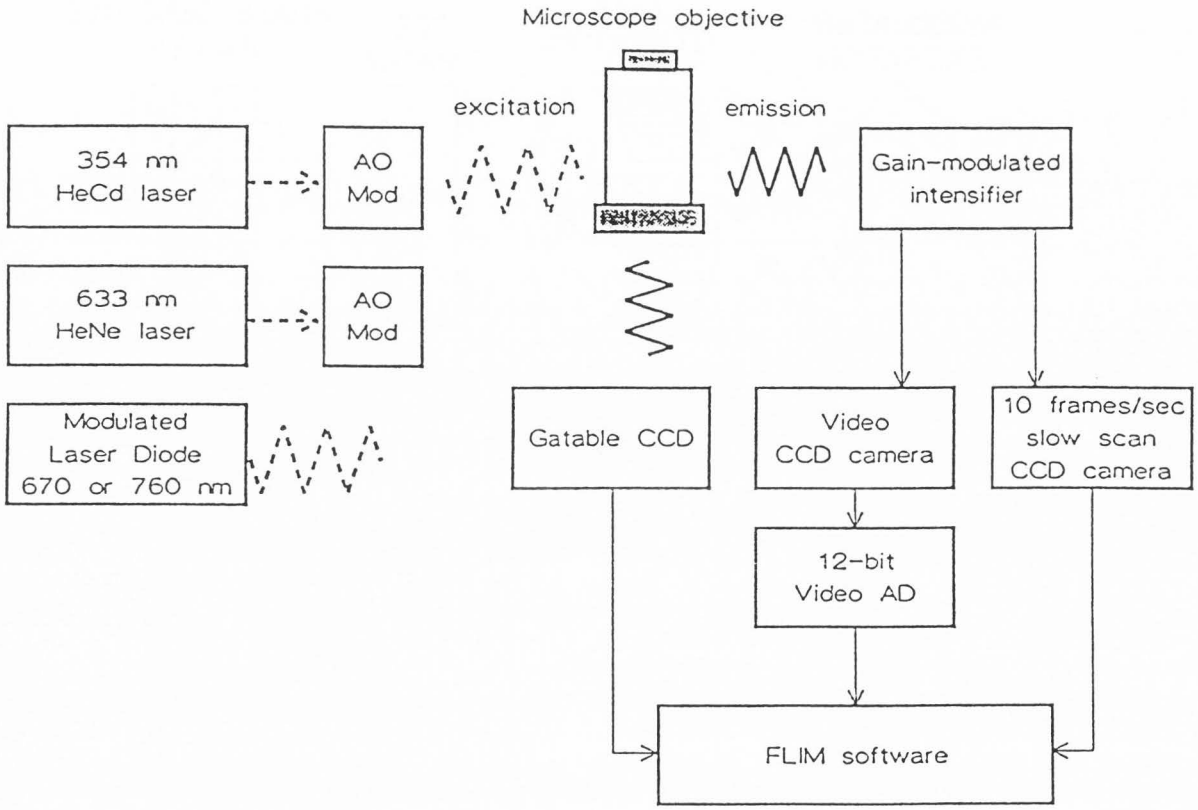

Figure 4. Future apparatus for FLIM.

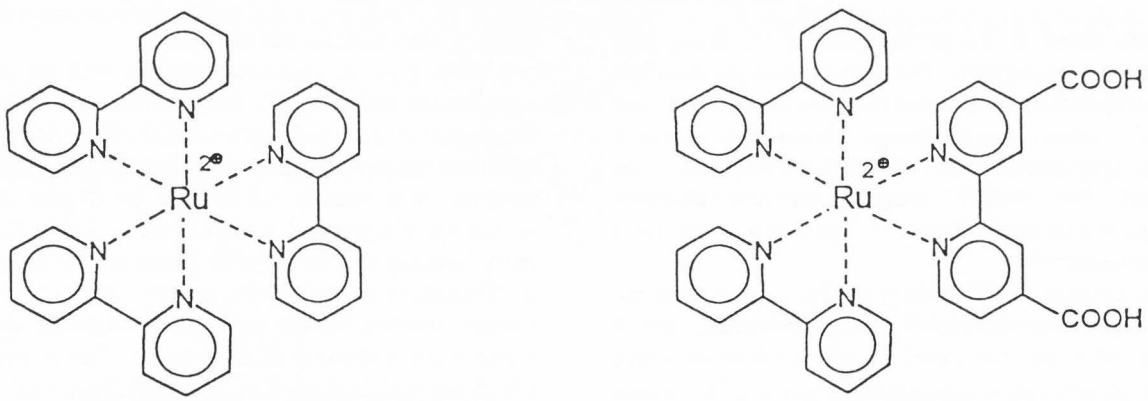

Figure 5. Chemical structure of $[Ru(bpy)_3]^{2+}$ and of $[Ru(bpy)_2(dcpby)]$.

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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 in this topic, the total effort is minor in comparison to the number of scientists engaged in instrument development, technology development, theory or applications.

In an attempt to circumvent the limitations of available probes we have been developing long-lived metal ligand complexes for measurement of the hydrodynamics of high molecular weight biomolecules [30, 31].

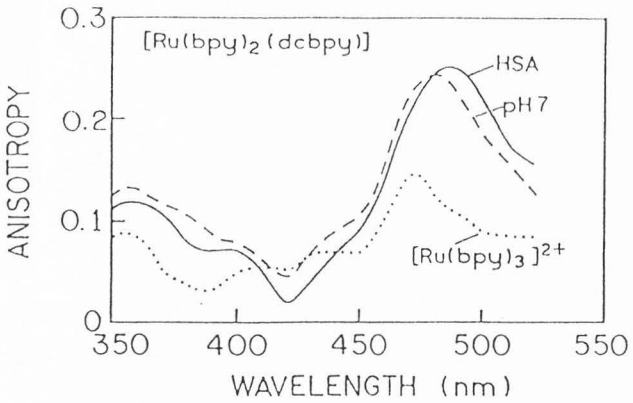

Figure 6. Excitation anisotropy spectra (bottom) of [Ru(bpy)$_2$(dcpby)], free (— — —) and when conjugated to HSA (————). The anisotropy spectra are in glycerol:buffer (9:1, v/v) at -55°C. This viscous solvent is used to prevent rotational diffusion during the excited state lifetime. The dotted lines (·····) represent the symmetrical [Ru(bpy)$_3$]Cl$_2$.

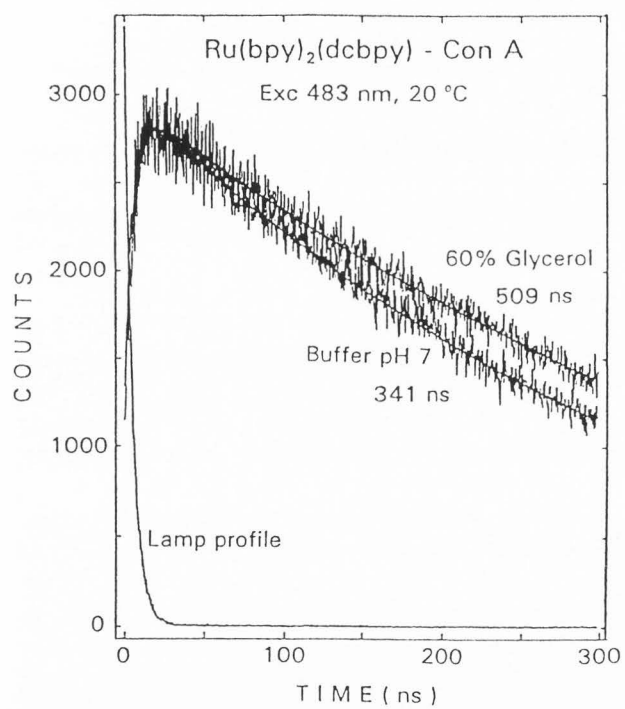

Figure 7. Intensity decays of [Ru(bpy)$_2$(dcbpy)] conjugated to ConA.

For instance, almost all known fluorophores display lifetimes ranging from 1 to 10 ns. This decay time limits the timescale of the events which alter the emission. Metal-Ligand complexes (MLC) of the type [Ru(bpy)$_3^{2+}$]

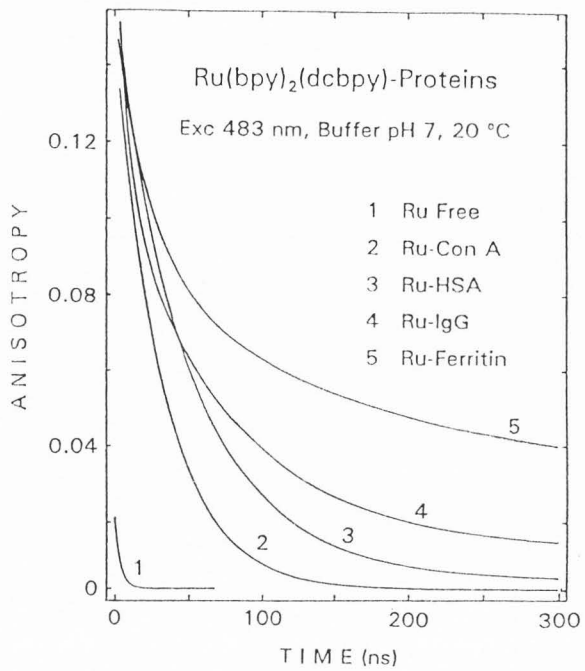

Figure 8. Anisotropy decays of [Ru(bpy)$_2$(dcbpy)] in buffer and conjugated to proteins.

(Fig. 5) display lifetimes ranging from 100 ns to microseconds, and thus allow the detection of slower processes.

The usefulness of such metal-ligand complexes (MLCs) is derived from their favorable anisotropy properties. Suitable non-symmetrical complexes, such as [Ru(bpy)$_3$(dcbpy)]$^{2+}$ (Fig. 5, right) display high anisotropy in the absence of rotational diffusion (Fig. 6). The high anisotropy makes this probe useful for measuring rotational motion of proteins. A valuable feature of the MLCs is their long decay times, over 300 ns when conjugated to a protein (Fig. 7). The long decay times in turn allow measurement of long rotational correlation times, as shown for MLC-labeled proteins in Fig. 8.

The polarized emission from metal-ligand complexes offers numerous experimental opportunities in biophysics and cellular imaging. A wide range of lifetimes, absorption, and emission maxima can be obtained by careful selection of the metal and the ligand. For instance, long wavelengths are desirable for clinical applications, such as fluorescence polarization immunoassays. Absorption wavelengths as long as 700 nm can be obtained using osmium [11], and lifetimes as long as 100 μs can be obtained using rhenium as the metal in such complexes [25]. The rhenium complexes also display good quantum yields and high initial anisotropies in aqueous solution. At present it is difficult to obtain long lifetimes, long wavelengths and high quantum yields all in a single

Table 1. Cell biology 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 2. 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 timescale Red-sensitive for optical tomography
CCDs	Fast frame rates Gatable on nanosecond timescale
Computers	Allow processing of numerous images from CCD images
Probe chemistry	Need long-wavelength probes to take advantage of laser diodes and low autofluorescence

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metal-ligand complex. Additional research is needed to identify which of these metal-ligand complexes displays the most favorable spectral properties for a particular application, and to synthesize conjugatable forms of the desired probes.

Advanced Applications of Fluorescence Spectroscopy

Two Photon-Induced Fluorescence (TPIF)

We are all familiar 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. For instance, tryptophan in proteins, which normally absorbs light at 290 nm, can be excited by the simultaneous absorption of two 580 nm photons (Fig. 9), which shows the emission spectra of human serum albumin (HSA) excited at 295 or 590 nm. This remarkable phenomenon occurs only with high light intensity because the two photons must be in the same place at the same time to allow simultaneous absorption. It should be emphasized that there is no direct relationship between the absorption spectra and absorption cross sections for one- and two-photon excitation. In principle, one- and two-photon absorption occurs to excited states of different symmetry due to the selection rules for optical absorption.

Because two-photon excitation requires two-photons, the fluorescence intensity is proportional to the square of the light intensity. This dependence on the square of the intensity is shown in Fig. 10. The emission intensity of HSA, is linearly dependent on the incident light intensity at 295 nm, and quadratically dependent on the intensity at 590 nm [13]. Two-photon induced fluorescence has also been observed for fluorophores bound to membranes [14] and nucleic acids [12].

The fact that 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 [22, 34]. Removal of this out-of-focus light provides remarkable improve-

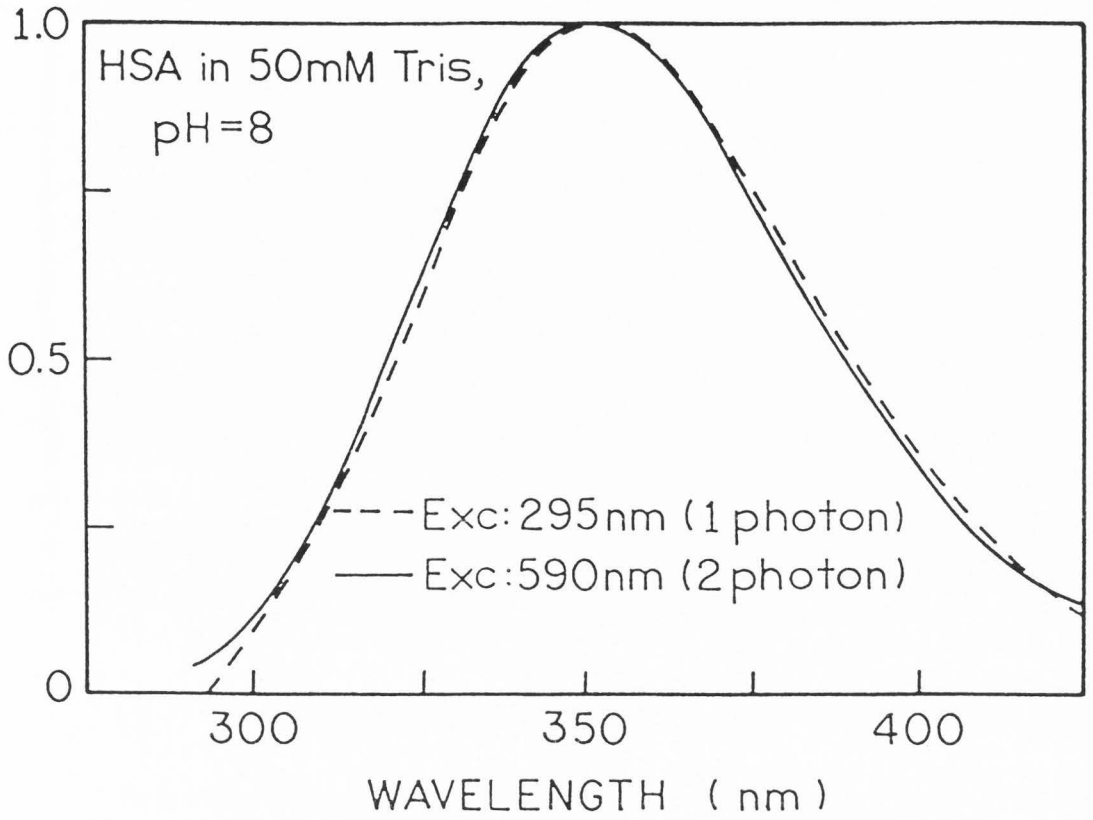

Figure 9. Emission spectra of HSA for one- and two-photon excitation.

ment in image quality because for one-photon excitation the fluorescence occurs from the entire thickness of the sample. Much of this emission is devoid of spatial information and only serves 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 [2, 33]. The sample can be excited only at the desired depth based on the position of the focal point (Fig. 11), and the signal comes dominantly from this region. Perhaps more importantly, the fluorophores which are not in the focal plane are not excited, consequently, are not photobleached and are thus available for imaging when the focal plane is moved (Fig. 11). 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 auto-fluorescence 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 [23], and Dr. Webb has reported lifetime images with two-photon excitation [23]. Hence, we can now imagine the creation of three-dimensional (3D) chemical images of cells (Fig. 10), which could display the local Ca^{2+} concentration as seen for the two-dimensional (2D) FLIM imaging in Fig. 2.

Three-Photon Excitation

We have recently extended the use of high intensity laser pulses to allow three-photon excitation. Three-photon excitation has been observed for the scintillator 2,5-diphenyloxazol (PPO) [7], for a tryptophan derivative [8] and for the calcium probe Indo-1 [9, 29]. Three-photon excitation was accomplished with femtosecond pulses of a mode-locked Ti:Sapphire laser at wavelengths above 800 nm. The emission spectra of Indo-1 were the same for excitation at 351 and 860 nm (Fig. 12). At 885 nm the intensity of Indo-1 depended on the cube of the laser power, but at a slightly shorter wavelength of 820 nm the intensity depended on the square of the laser power (Fig. 13). This indicates that the mode of excitation (two- or three-photon) can change with small changes in wavelengths. Remarkably, the intensity of Indo-1 with three-photon excitation is within a factor of 10 of that observed with two-photon excitation (Fig. 13), suggesting that three-photon excitation can be practical in fluorescence microscopy.

An important aspect of three-photon excitation is that it can be accomplished with wavelengths near 800 nm, which is the peak of the Ti:Sapphire laser tuning

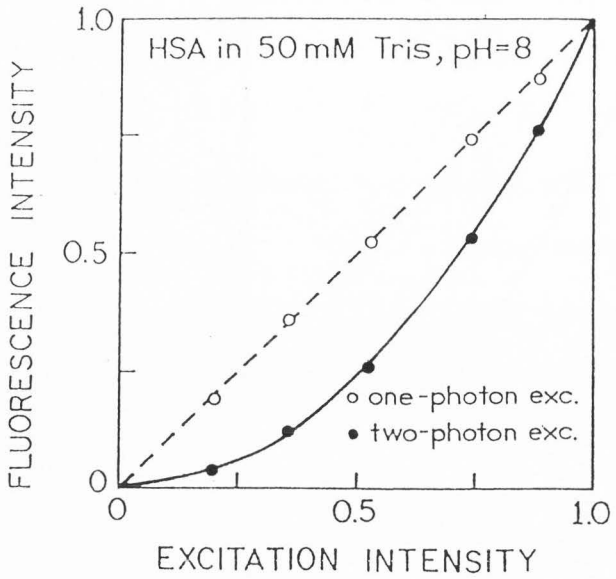

Figure 10. Dependence of the emission intensity of HSA on the incident light intensity (right). The excitation intensities are normalized to one at the highest values.

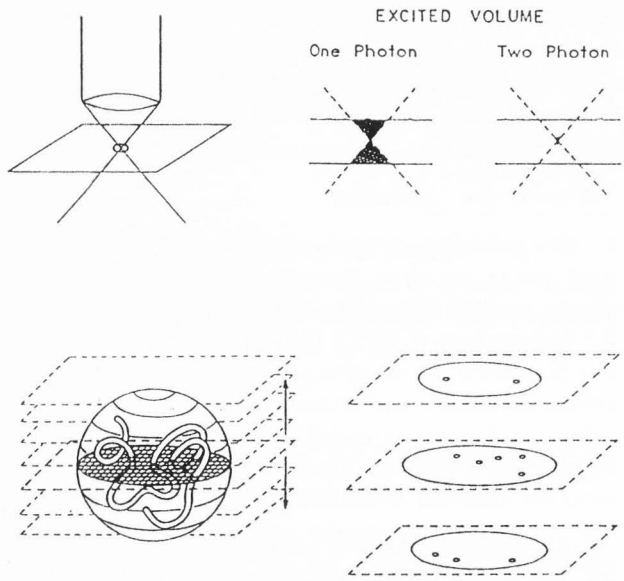

Figure 11. Intrinsic "confocal" excitation using TPIF in microscopy (top) and three-dimensional optical imaging (bottom).

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curve. Also, the spatial profile of the excited fluorophores can be smaller than available with two-photon excitation. This is shown experimentally in Fig. 14, which shows the width of the excited volume of Indo-1 with one-, two- and three-photon excitation. It

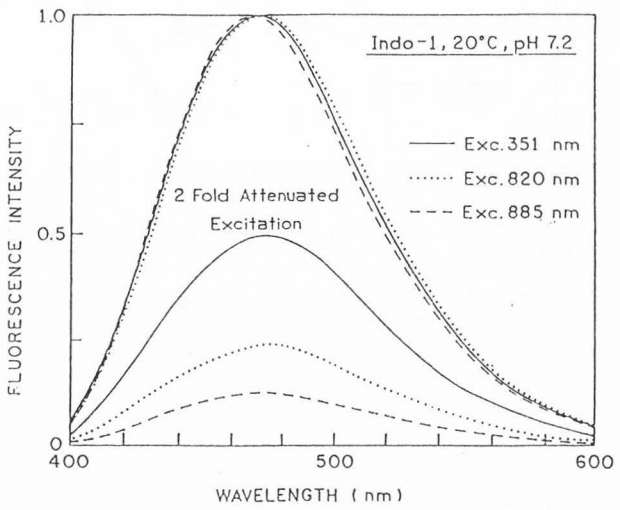

Figure 12. Emission spectra of Indo-1 with excitation at 351, 820 and 885 nm.

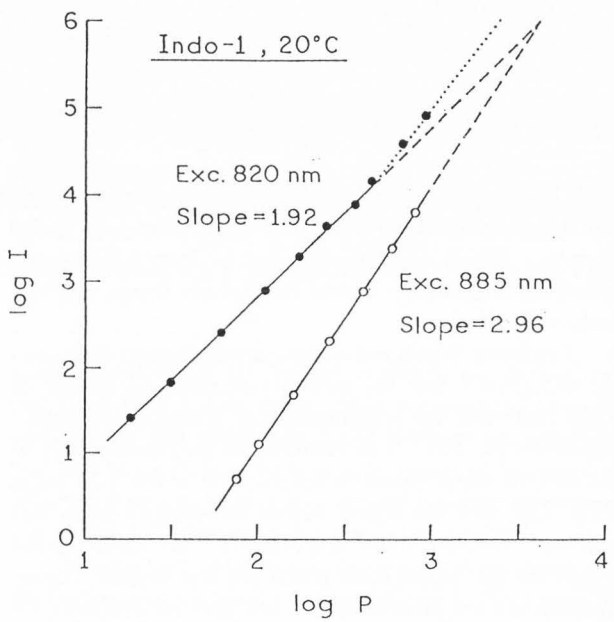

Figure 13. Dependence of the emission intensity of Indo-1 on laser power at 820 and 885 nm.

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seems probable that simple Ti:Sapphire lasers will be available in the near future, making two- and three-photon excitation possible in many microscopy laboratories.

Light Quenching of Fluorescence

Measurements of time-resolved fluorescence, and particularly the recent interest in two- and three-photon

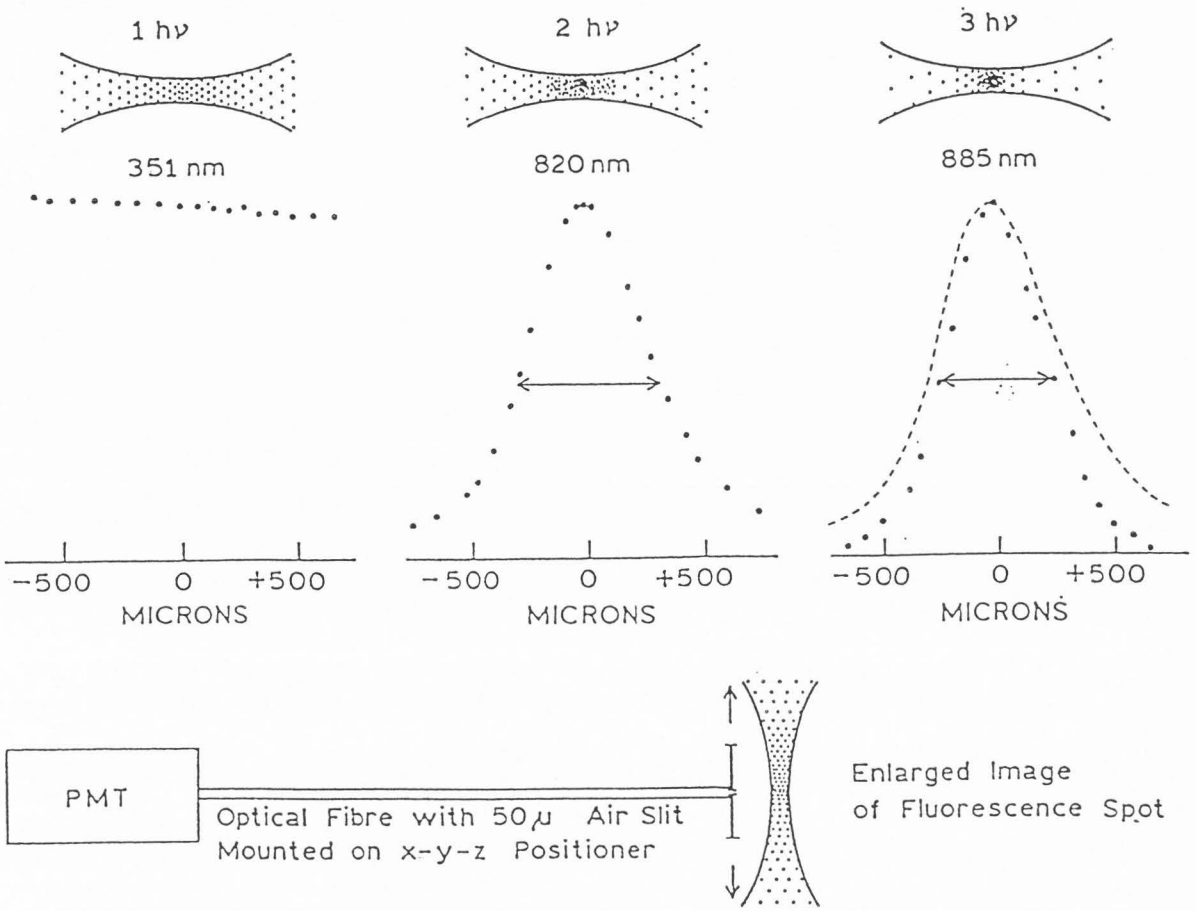

Figure 14. Spectral profile of the excited volumes of Indo-1 with one-, two- and three-photon excitation.

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excitation, require the use of intense laser sources. The use of these intense laser sources allows observation of the phenomena of stimulated emission. If a fluorophore is illuminated at a wavelength which overlaps its emission spectra, the fluorophore can be stimulated to return to the ground state (Fig. 15). Since the stimulated photon travels parallel to the "quenching beam," and since the emission 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 [3]. Historically, light quenching has only been observed using the very intense pulses from Q-switched Ruby lasers [4, 21]. The fact that we now know that light quenching can be observed with modern ps lasers results in numerous opportunities for novel fluorescence experiments [5, 6, 17].

Consider that the sample is excited with one pulse, followed by a second longer wavelength quenching pulse (Fig. 15, left). 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 non-destructive since 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 [6, 21]. This means that not only is the total excited state population 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. 16), resulting in a high degree of orientation of the excited state population. In contrast, one-photon excitation of randomly oriented fluorophores can only result in polarization values for 0.5 to -0.33. It may even be possible to break the Z-axis symmetry, which heretofore has been pervasive in

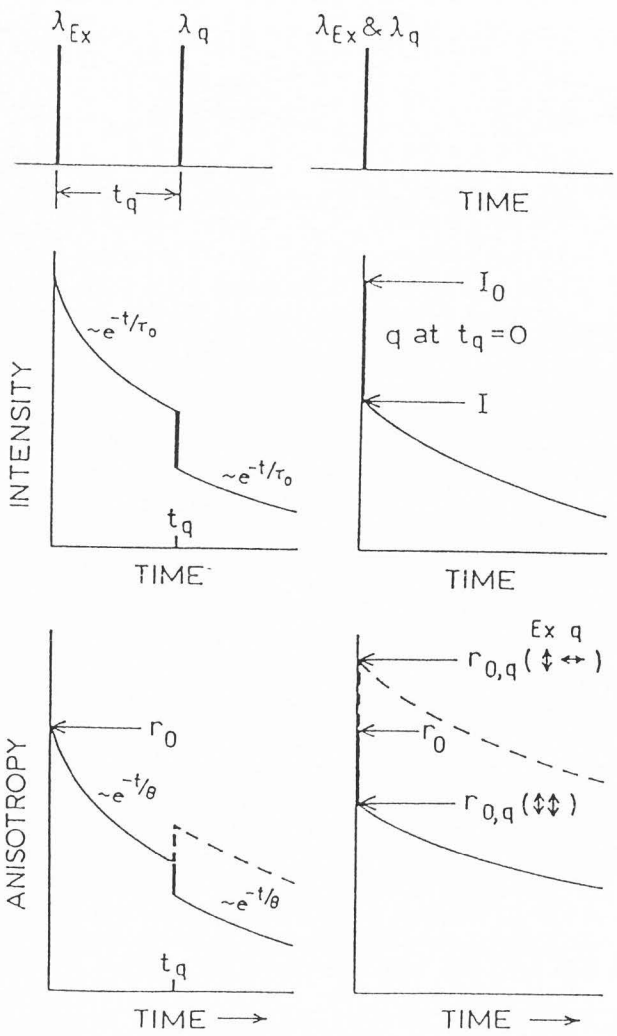

Figure 15. Schematic of light quenching by a time-delayed quenching pulse. The dotted lines refer to light quenching with perpendicular polarized light.

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the optical spectroscopy of randomly oriented solutions. In our opinion, the phenomena 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.

In closing, we wish to reiterate that time-resolved fluorescence is now moving out of the research laboratory and into the world of cellular imaging 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. These enabling technologies are summarized in Table 2. 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.

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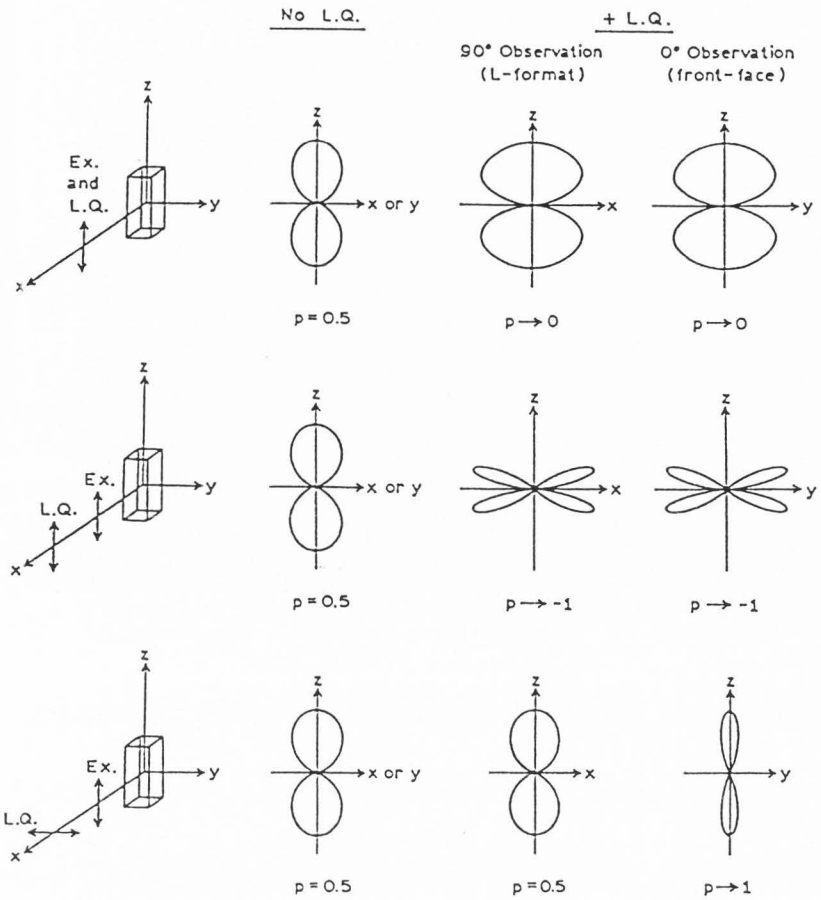

Figure 16. Effects of polarized light quenching on the orientation of the excited state population.

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