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**Fluorescence spectroscopic properties of normal and abnormal
biomedical materials**

Pradhan, Asima, Ph.D.

City University of New York, 1991

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**FLUORESCENCE SPECTROSCOPIC PROPERTIES OF NORMAL AND
ABNORMAL BIOMEDICAL MATERIALS**

by

ASIMA PRADHAN

**A dissertation submitted to the Graduate Faculty in Physics
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy, The City University of New York.**

1991

@ 1991

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This manuscript has been read and accepted for the Graduate Faculty in Physics in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

FLUORESCENCE SPECTROSCOPIC PROPERTIES OF NORMAL AND ABNORMAL BIOMEDICAL MATERIALS

by

Asima Pradhan

Advisor: Professor R.R.Alfano

Steady state and time-resolved optical spectroscopy and native fluorescence is used to study the physical and optical properties occurring in diseased and non-diseased biological human tissue, in particular, cancer of the human breast, artery and the dynamics of a photosensitizer useful in photodynamic therapy. The main focus of the research is on the optical properties of cancer and atherosclerotic tissues as compared to their normal counterparts using the different luminescence based spectroscopic techniques such as steady state fluorescence, time-resolved fluorescence, excitation spectroscopy and phosphorescence.

The excitation and steady-state spectroscopic fluorescence using visible excitation wavelength displays a difference between normal and malignant tissues. This difference is attributed to absorption of the emission by hemoglobin in normal tissues. This method using 488nm fails to distinguish

neoplastic tissue such as benign tissues and tumors from malignant tumors.

The time-resolved fluorescence at visible, near-uv and uv excitation wavelengths display non-exponential profiles which are significantly different for malignant tumors as compared to non-malignant tissues only with uv excitation. The differences observed with visible and near-uv excitation wavelengths are not as significant. The non-exponential profiles are interpreted as due to a combination of fluorophores along with the action of non-radiative processes. Low temperature luminescence studies confirm the occurrence of non-radiative decay processes while temporal studies of various relevant biomolecules indicate the probable fluorophores responsible for the observed signal in tissues. Phosphorescence from human tissues have been observed for the first time and lifetimes of a few hundred nanoseconds are measured for malignant and benign tissues.

Time-resolved fluorescence studies of normal artery and atherosclerotic plaque have shown that a combination of two excitation wavelengths can distinguish fibrous and calcified atherosclerotic plaque from normal artery.

A minor effort of the study involves the high intensity effects on the optical properties of the dye, doxycycline (a particular photosensitizer of the tetracycline group) occurring during relaxation when excited at different laser intensities. This study has been performed by observing the fluorescence lifetimes and quantum yields of DOTC at different excitation intensities. The results obtained support the sequential excited state absorption model.

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For more than half a century, fluorescence, absorption and excitation spectroscopy have been widely used as probes to acquire fundamental knowledge about various physical, chemical and biological processes. Fluorescence spectroscopy has found applications in several fields such as analytical chemistry, physics, biology, optoelectronics, information processing and remote sensing. Over the past decade it has been extended to the medical community, both on a fundamental level and for tissue diagnostics. Irradiation of a heterogeneous medium such as tissue by external quasimonochromatic or monochromatic light gives rise to fluorescence which is intrinsically dependent on its constituent fluorophores. The heterogeneous medium in which the fluorophore finds itself has many constituents in different environments differing in their optical responses. To be specific, the response of native fluorophores such as flavins, porphyrins, nucleotides (NADH), tryptophan, tyrosine, collagen, elastin in biological media such as tissues differs significantly in different environments. Many of these are responsible for spectral fluorescence differences observed in different states of biomedical materials. Optical spectroscopy and laser techniques offer a unique and sensitive approach to reveal changes in the physical and chemical properties that occur in healthy and abnormal biological tissues.

Although the physical principle behind fluorescence spectra is rather simple, it had not been extensively applied for probing biomedical systems for diagnostic purposes. In view of the possible side effects of X-rays and

nuclear radiation new techniques should be looked into for non-invasive diagnosis. Optical techniques should in principle be safer than X-rays and nuclear approaches and are in some cases non-invasive.

Fluorescent dyes used in complex molecular systems have enabled researchers to obtain information about conformational changes in muscle and nerves, polarity of the surrounding environment, dynamical conformation of molecules in membranes and secondary structure of DNA and RNA [1]. Hematoporphyrin derivative (HPD) is now used as a fluorescent marker for cancer detection [2]. However, such extrinsic markers interact with the native cellular environment. The majority of the molecular systems of tissues are made of proteins, nucleic acids, and enzymes, lipids and other constituents. These proteins, nucleic acids and enzymes are fluorescent and non-fluorescent chromophores which could change their optical properties depending upon the environmental conditions [3]. The intrinsic fluorescence of certain chromophores and their sensitivity to the surroundings can be used to characterize the physiological state of the abnormal tissue systems as compared to the corresponding normal counterparts. Use of native fluorescence as a marker instead of external probes is advantageous since it detects microscopic structure and changes in tissues without interfering with the surrounding. In the late 1950's and 60's, fluorescence from proteins, nucleic acids and free nucleotide coenzymes had been studied in detail [4]. There are a number of natural fluorophores in cells and tissues fluorescing in the visible and the UV regions such as flavins, tryptophan, tyrosine,

NADH, collagen, elastin and porphyrins. Spectral properties of these fluorophores have been studied earlier and their sensitivity to changes in environment have been noticed [1,3,5].

Diseased tissues are different from non-diseased tissues morphologically with no gross visible changes sometimes. Steady state fluorescence spectroscopy is a sensitive index of such morphological changes in and around the fluorophores. As mentioned earlier, major progress has been made in using fluorescence spectroscopy from the fluorophores emitting in the visible, to detect disease. Alfano and Yao first used luminescence spectroscopy as a diagnostic tool [6]. This work was later extended by Alfano and coworkers to establish a difference between normal and tumor rat tissues [7] and by Kitrell and coworkers in the diagnosis of fibrous arterial atherosclerosis [8]. The study on rat tissues has now been extended to human tissue.

Steady state fluorescence studies on human breast and lung tissues was started by Alfano et al [27]. However, visible excitation was found to be an unreliable diagnostic tool since the marker was hemoglobin. Since the amounts of blood in diseased and normal tissues depends on the method of excision and selection, this method failed as a diagnostic method in the visible region. With uv excitation and elimination of the effects of blood this method later showed significant differences suitable as a diagnostic tool. Native fluorescence has also been measured from different organs of live and sacrificed mice with nitrogen laser by Andersson et al [9]. Native fluorescence

studies have also been performed using pulsed laser. Buccal carcinoma have been studied by a Chinese group [10] with optical fibers coupled to a spectrometer excited by a xenon-ion laser operating at 365nm. Premalignancy studies have also been pursued using this technique. Alfano and group found differences in the fluorescence spectra of normal and cancer breast tissues [11] photoexcited by the second and third harmonics of an Nd-glass laser. This work is described in this thesis. Different tissue types were found to display different spectroscopic signatures since the buccal carcinoma and breast malignancies showed different spectral profiles. Normal and diseased bladder wall and gastrointestinal tract have also been studied by Feld's group [12,13,14]. Using a nitrogen-pumped dye laser fluorescence spectra of adenoma and normal mucosa have been found to be different. Fluorescence contour mapping of tissue where a measurement of fluorescence emission as a function of the excitation wavelength has also been performed by Feld's group along with a study of chromophores and proteins to establish the molecular details of the physicochemical properties of tissues [15]. Fluorescence contour mapping has been performed on human colon. Kapadia et al tested the hypothesis that adenomatous transformation of colonis mucosa results in an alteration of laser induced fluorescence spectra that enables differentiation from normal or hyperplastic tissue [16]. This quantitative analysis of the spectra enables the detection of adenomatous transformation in colonic mucosa.

Diseased artery as compared to normal artery have also been studied

using native fluorescence. Kittrel et al made the first attempt to distinguish between normal and fibrous arterial atherosclerosis [8] in 1985 by comparing the fluorescence intensity ratio at 600nm relative to 580nm obtained on irradiation at 480nm of a Perkin-Elmer spectrofluorometer. Sartori [17] et al were able to correlate the intimal thickness with the ratio of fluorescence intensities at 530/550nm. Ratio of fluorescence intensities at 448/514nm and 538/514nm measured by Deckelbaum were capable of discriminating normal and atherosclerotic aorta [18]. Much later, in 1987, Andersson et al [19,20] also used the nitrogen laser and found differences between the normal artery wall and atherosclerotic lesions, both for large arteries such as aorta, arteria femoralis and pulmonaris, as well as for coronary arteries. Differences in fluorescence from plaque and normal artery wall excited at 476nm from an argon ion laser have also been observed by Feld and co-workers [21]. However, different types of plaque have not been distinguished from normal artery. Deckelbaum et al found the steady state technique to be unable to distinguish calcified atherosclerotic plaque from normal artery. To solve this problem it became necessary to probe other techniques such as time-resolved fluorescence, which is studied in this thesis.

Time-resolved fluorescence spectroscopy and excitation spectroscopy are two methods that can be used to diagnose disease and to study the various physical processes occurring in tissues. Picosecond time-resolved fluorescence is used to perceive dynamical changes and processes which take place in the picosecond and nanosecond time scales in human tissues.

Excitation spectra is used to identify the different chromophores which are responsible for the fluorescence spectra.

This thesis theme involves using optical spectroscopy in the study of the physical and optical processes occurring in diseased and non-diseased biological tissue, in particular cancer of the breast, and new photosensitizers used in therapy. Much of this study occurred during the explosive research around the work. The key focus of the thesis is on the optical properties of diseased tissue (cancer tissue and atherosclerotic plaque) and its normal counterpart using the different fluorescence based spectroscopic methods such as steady state fluorescence, time-resolved fluorescence, phosphorescence and excitation spectroscopy. The goal of the thesis is to obtain the time-resolved and steady state characteristics of emission from tissues and, possibly, identify the different chromophores responsible for the signatures observed and obtain information about the physical processes occurring in tissues. A minor study involves the high intensity effects on the optical properties of the dye tetracyclines (particular group of sensitizers) occurring during relaxation when excited at different laser intensities. This study is done by observing the fluorescence lifetimes and quantum yields of doxycycline at different excitation intensities. This research is important to obtain an idea of the amount of phototoxicity in tissue during photodynamic therapy.

The thesis involves 9 chapters. Chapter 2 describes the various steady-state and time-resolved experimental methods used in this study. The steady state and transient spectral work of breast tissues is described in Chapter 3

while Chapter 4 deals with the time-resolved fluorescence. Chapter 5 describes the phosphorescence and enhanced fluorescence from breast tissues at low temperatures. Chapter 6 concentrates on time-resolved fluorescence from diseased and normal aorta. Chapter 7 describes the intensity dependence emission dynamics of doxycycline. Chapter 8 is a conclusion and Chapter 9 deals with future research direction that is not solved and left as an open question. Appendix I describes the step by step procedure of using the Nd-glass laser system. Appendix II displays the expressions for $S_1(t)$ in Chapter 7.

CHAPTER 2: EXPERIMENTAL METHODS

Spectroscopy has evolved along two directions. In the first approach, scientists study the characteristic frequency spectrum of the material to derive information about its structure and internal processes. In the other approach, the material is subjected to a pulse excitation and then the characteristic relaxation time from the excited state to its original state is measured to obtain information about the various physical processes occurring.

In this thesis, both approaches have been used and this chapter describes the various methods used in the research.

(1) STEADY STATE FLUORESCENCE SPECTROSCOPY:

A typical experimental arrangement used to measure the fluorescence spectra from the various biological samples is shown in Figure 1. A cw argon-ion laser is used to excite the sample. The laser wavelengths used to excite the sample is selected from 457.9nm, 488nm and 514.5nm. Depending on the fluorescence intensity of the sample the laser power focussed on the sample can be changed from 1 to 100 mW. For most biological samples, a power of 10 mW is strong enough. It is better to keep the same level of power on samples. If the fluorescence intensity is different for a pair of normal and cancer tissues, neutral filters which have almost the same transmission over the visible spectral region can be placed in the fluorescence path to keep the

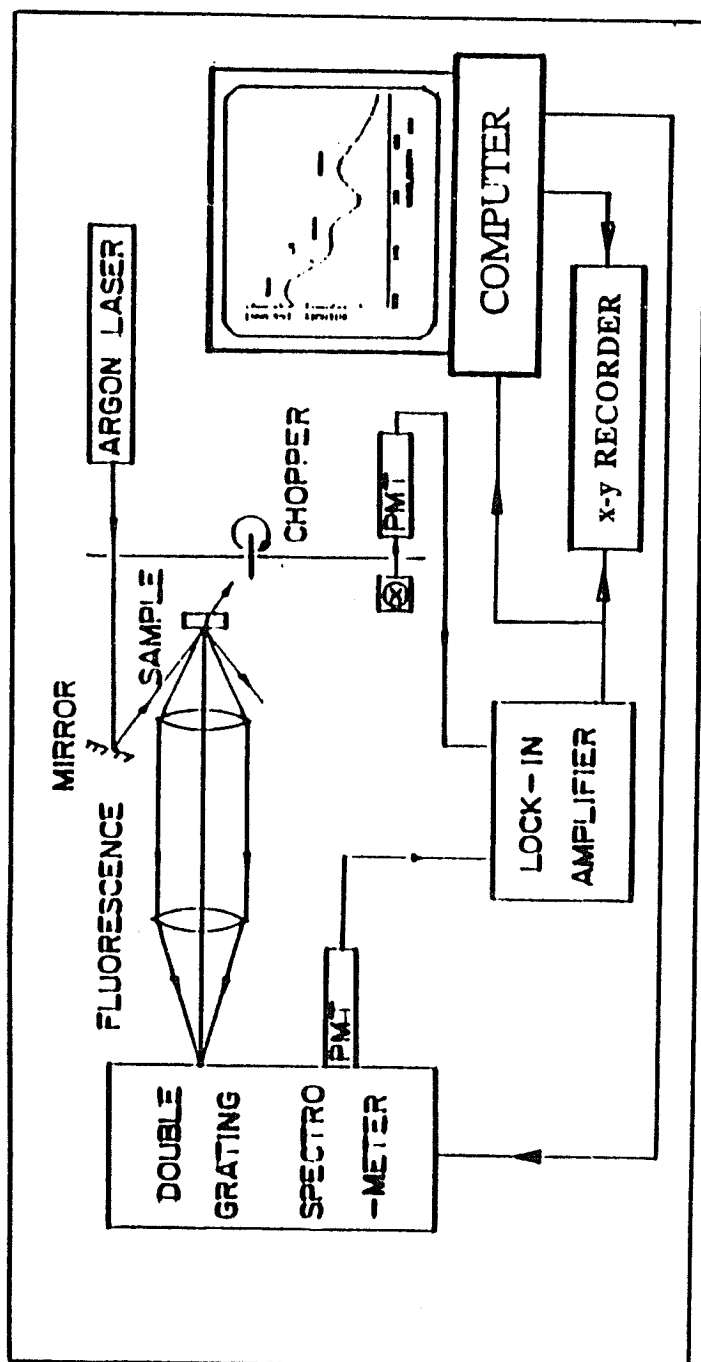


Figure 1. Experimental set-up for laser steady state spectrum.

same sensitivity. However, this approach can sometimes alter the spectral profiles. Thus it may be better to change the gain of the detection electronics.

The laser beam is focussed onto the front of the sample to a spot size of about 200 μm . To avoid the influence of the weak emission lines surrounding the strong laser line used to excite the sample, a narrow band filter of 458nm, 488nm or 514.5nm is inserted in the laser path to eliminate spectral lines from the discharge of the laser. The laser is chopped at about 200Hz. The laser reflection from the cuvette surface is strong so that one should be careful and avoid directing the reflection into the collection optics.

The fluorescence from the sample's surface is collected and focussed into the entrance slit of a double 1/2m grating spectrometer (Spex Industries) blazed at 500nm. A photomultiplier tube (Model RCA 7265, S-20) is located at the exit slit of the spectrometer to measure the fluorescence intensities at different wavelengths. The spectral resolution of the spectrometer is 2nm. The output of the photomultiplier tube is connected to a lock-in-amplifier (EG&G Princeton Applied Research), and then to a X-Y recorder and a personal computer to display each spectrum.

(2) EXCITATION FLUORESCENCE SPECTROSCOPY:

Excitation spectroscopy is a useful tool to investigate medical and biological samples to identify the emitting species. The excitation spectrum is determined from the measurement of the intensities at a select fluorescence wavelength as a function of the excitation wavelength.

The apparatus used to obtain the excitation spectra from the samples is a Perkin-Elmer LS-50 Fluorescence Spectrometer shown in Figure 2. The excitation source in this spectrometer is a special xenon flash tube which produces an intense, short duration pulse of radiation over the spectral range of the instrument. A small lamp close to the excitation source maintains an even triggering of the xenon flash tube at line frequency. Energy from the source is focussed by the ellipsoidal mirror and reflected by a toroidal mirror onto the entrance slit of the excitation monochromator. The monochromator consists of the entrance slit, with the center wavelength being determined by the setting of the grating, the angle of which is controlled by a stepper motor. The majority of the excitation beam is transmitted to the sample area via the focussing toroidal mirror, a small proportion is reflected by a beam splitter onto the reference photomultiplier.

Energy emitted by the sample is focussed by the toroidal mirror onto the entrance slit of the emission monochromator. The monochromator consists of the entrance slit, a spherical mirror, a 1200 lines per millimeter grating and the exit slit. A narrow wavelength band emerges from the exit slit, with the center wavelength being determined by the setting of the grating, the angle of which is controlled by a stepper motor. The excitation and emission monochromators can be scanned over their ranges independently, synchronously or driven to selected points in their ranges. Synchronous scanning can be either a fixed wavelength difference or a fixed difference between the excitation and emission monochromators. The spectral ranges of

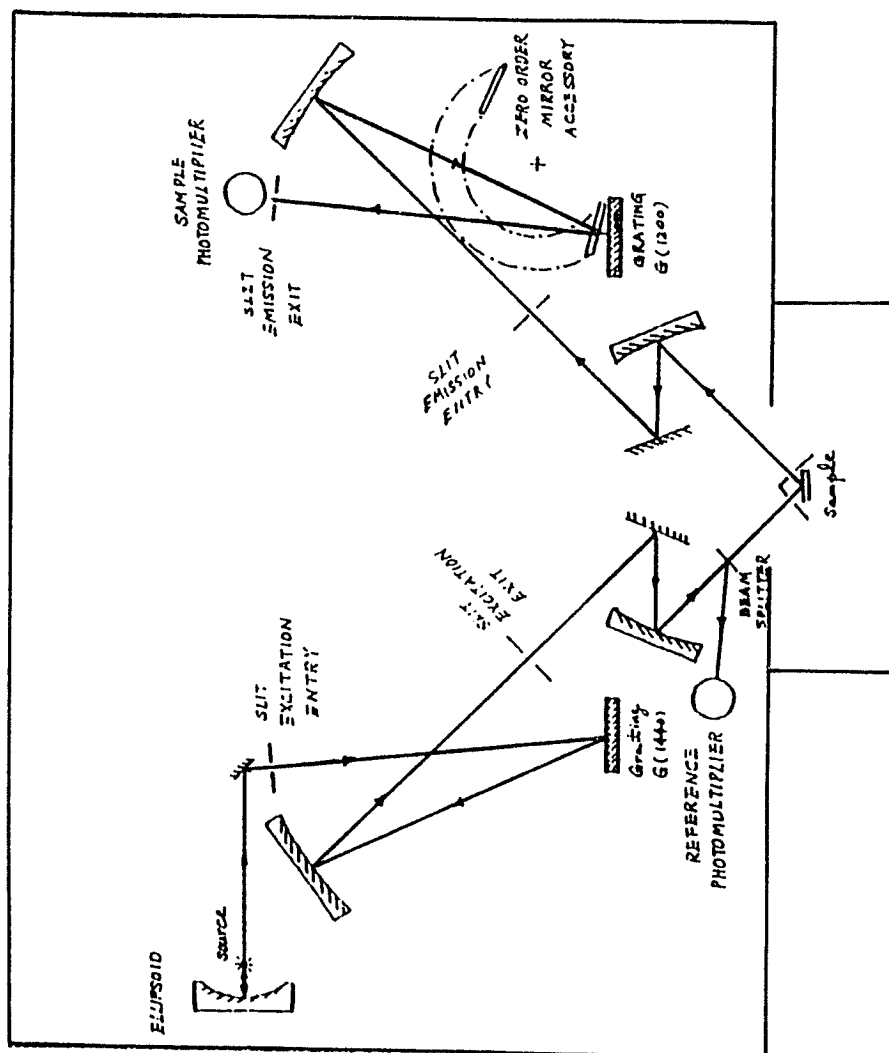


Figure 2. Experimental set-up of lamp excitation spectrum-Fluorescence Spectrometer.

the monochromators are about 230nm to 800nm for excitation and about 230nm to 900nm for emission.

The excitation spectra are stored and processed by a personal computer.

(3) TIME-RESOLVED FLUORESCENCE:

To obtain information on the time resolution of the excitations in biological samples, the temporal behaviour of the fluorescence intensities emitted at different wavelengths needs to be investigated. In time-resolved fluorescence spectroscopy the samples are excited by an optical pulse which is much shorter than the lifetime of the excited state of the samples. The fluorescence intensity at different wavelengths can be measured as a function of time by various novel techniques.

3(a) Picosecond Nd-Glass Laser System

A picosecond mode-locked Nd-glass laser has been used in most of the experiments of this thesis. Figure 3 displays the experimental set-up described below. The laser oscillator consists of a Nd³⁺-doped phosphate laser rod surrounded by a flashlamp and a saturable absorber dye between two high reflectivity mirrors. The spectral width of the gain profile is due to inhomogeneous broadening and its FWHM is 30 cm⁻¹. The laser rod is cut at Brewster's angle for the 1054 nm emission. The rod is pumped optically by discharging a bank of capacitor (5 kV) through a helical water cooled halogen

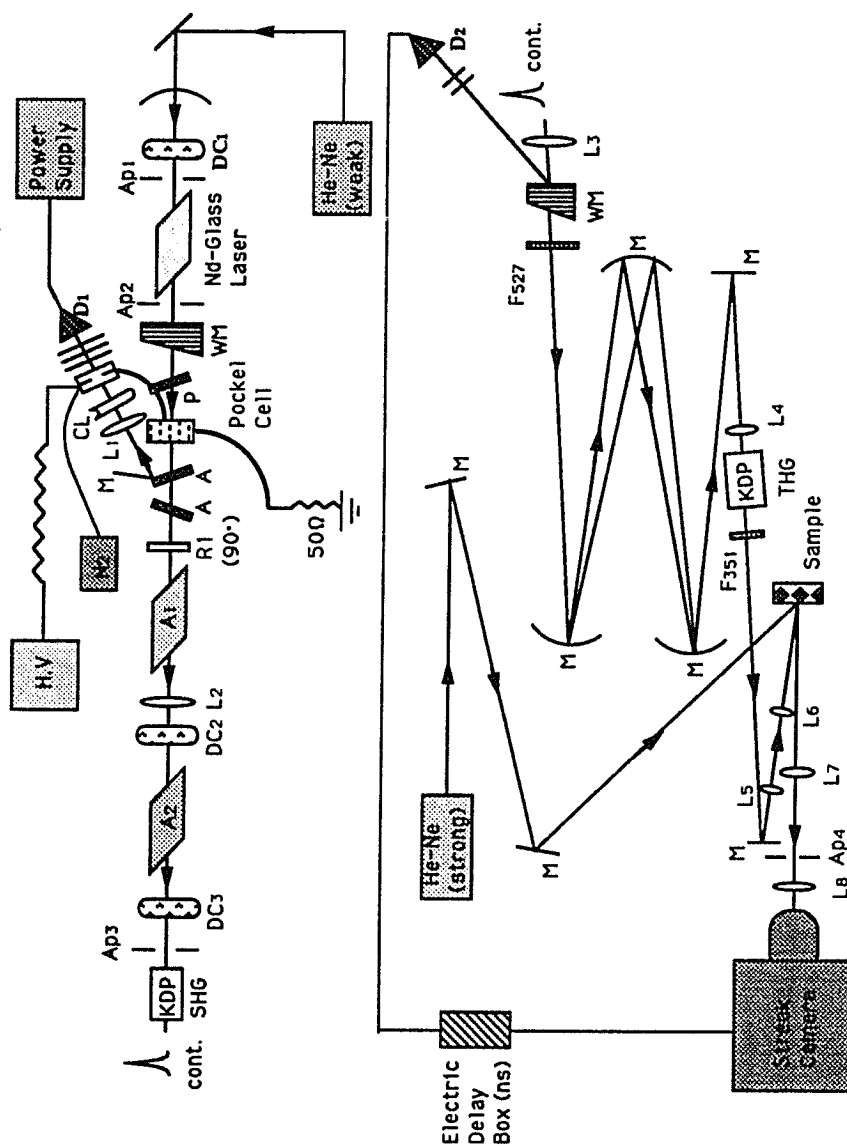
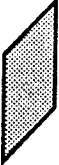


Figure 3. Experimental set-up for time-resolved fluorescence studies using a pico-second mode-locked Nd-Glass laser.

Notations for Fig. 3

	Lens
	Mirror (Cavity mirrors : dielectric)
	Wedged Mirror
	Aperture
	Amplifier ($A_1=1/2''$, $A_2=3/4''$) and laser
	Dye Cell (diameter= 5 cm)
	Cylindrical Lens
	Filters [$F_{527}=(2)3-67$; $F_{351}=(2)0-51$]
	Spark Gap
	Photodiode (D1 : Hadron 105C Photodiode, D2 : Hamamatsu C1083 Pin Diode)
	Cable

lamp. The laser is operated only in the pulse mode at one shot every two minutes. The longitudinal modes determined by the round trip time ($2l/c \sim 7$ nsec) within the laser cavity and limited by the gain profile $G(w)$ can be phase locked using a saturable absorbing medium. The saturable absorber used is a dye "Kodak 9860" dissolved in 1,1' dichloroethane. This passive mode locking of the longitudinal modes generates a train of ~ 100 pulses separated from each other by a round trip time (~ 6 psec) duration at 1054nm. The train of pulses leaks out of the cavity from a 50% transparent mirror at the end of the cavity. A single pulse is selected from the mode-locked pulse train with a tranverse laser-triggered spark gap connected to a KD*P Lasermetric Pockels cell with double-shielded coaxial cable. The pulse selection is accomplished by applying a high voltage pulse to the Pockels cell which is placed in between crossed polarizers. When the voltage is applied to the Pockels cell, one half wave retardation is induced by the electro-optic effect. This rotates the plane of the polarization by 90 so that the pulse is transmitted through the second polarizer and along the main optical path of the laser. The voltage pulse is generated and timed by a short transmission line terminated at one end by a high impedance current limiting resistor (10 megohms) connected to a high voltage power supply (15.8 KV for the Nd⁺³ glass laser, which corresponds to twice the one half wave retardation for 1054nm). The other end of the short transmission line is terminated at a high pressure laser triggered spark gap (pressure ~ 100 psi). The length of the transmission line is so adjusted as to produce a voltage pulse whose duration

(5 nsec) is less than the time between consecutive pulses when it is discharged into a matched impedance load. When there is no voltage on the Pockels cell the train of pulses is deflected out of the main optical path by the second polarizer which is crossed. This train is focussed into the the spark gap where one of the pulses initiates an avalanche breakdown which allows the charge that has been stored in the short transmission line to discharge into another transmission line whose length is adjusted so that the voltage pulse arrives at the Pockels cell shortly before another pulse from the laser train. This voltage pulse then induces a retardation. The pockels cell is connected to another long transmission line (20 nsec) which is terminated into a matched resistive load (50 ohms) to prevent reflections which would otherwise partially reopen the Pockels cell. Figure 4(a) shows the pulse propagation along the two possible optical paths. A photodiode placed behind the spark gap connected to an oscilloscope displays the pulse train with the selected pulse missing. Figure 4(b) displays a photographic trace of a pulse train with the selected pulse missing. This oscilloscope trace is used to adjust the perfect the selection by proper alignment.

The single pulse is then amplified by two stages of amplifiers. The two amplifiers are two $\text{Nd}_{:3}$ glass rods. The flashlamps that excite the rods are triggered simultaneously with the laser oscillator flashlamp via common electrical pulses so that maximum gain in the amplifier is available when the pulse arrives at the amplifier. This timing is relatively easy since the amplifier gain follows the flashlamp intensity which has a broad peak of one

millisecond width. Total amplification is a factor of 20. The amplified pulse is then frequency doubled after passing through a KDP second harmonic generator crystal with 10% efficiency.

Part of the 1054nm pulse is then split by a beam splitter and directed onto a silicon photodiode. The electrical pulse output of the silicon photodiode is used as the trigger signal for a streak camera. The output of the silicon photodiode is directed to a variable delay line where various transmission lines can be switched in and out of the electrical circuit. This provides for various delays (adjustable in 250 ps steps) to be added to the arrival time of the photodiode output at the streak camera. It is necessary to easily adjust the timing of the triggering since different streak rates have different times between the trigger pulse arrival and the start of the camera streak (intrinsic delay). The second harmonic pulse is separated from the 1054 nm pulse after the beam splitter by appropriate filters (SP540 and 4-96 to cut off the 1054nm pulse). This 527nm pulse is then reflected by a series of mirrors or a 'white cell' which provides for multiple reflections from a pair of concave mirrors. The optical delay is typically 30ns. After passing through the time delay unit and other optical units, the single pulse is focussed onto the sample site with energy of about 150 μ J. The measured energy of the 1054nm pulse and its second and third harmonics are listed in Appendix I. The variation of the pulse energy of the single pulse was within 15%. The pulse duration measured by streak camera for a pulse selected out of a train was 8 ± 1 ps.

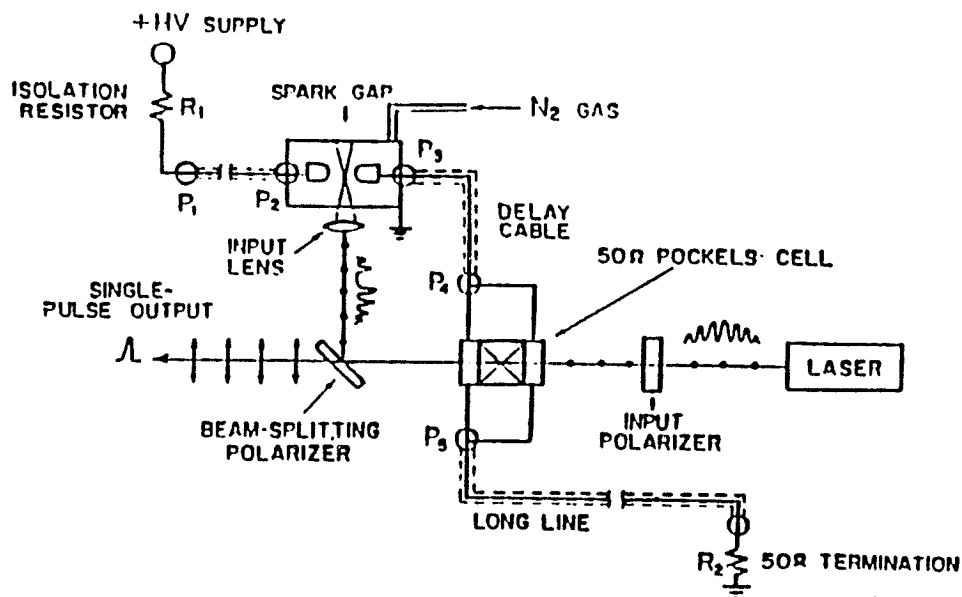


Figure 4(a). Pulse selection set-up.

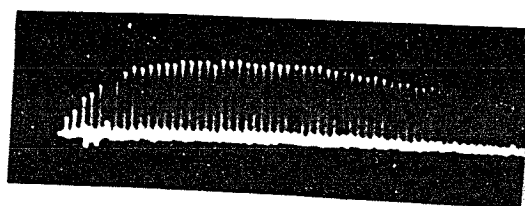


Figure 4(b). Photograph of a pulse train with single pulse missing.

For biological application, the visible and uv regions of the laser are usually used to excite the samples. A visible 8ps, 527nm pulse is generated by the first KDP crystal using the second harmonic of the mode-locked Nd-glass laser. This second harmonic laser pulse can be separated with SP540 and 4-96 filters from the 1054nm fundamental wavelength pulse of the Nd-glass laser. A uv 8ps, 351nm pulse is generated by the second KDP crystal using the third harmonic of the mode-locked Nd-glass laser. This third harmonic laser pulse can be separated with 7-51 and 7-60 filters from the 527nm second harmonic and the 1054nm fundamental laser pulses.

The single pulse energies of the second and third harmonics are about 180 J and 30 J at the sample site, respectively.

Streak Camera

The time-resolved detection system is the streak camera. Streak cameras have the ability to determine the optical temporal profile directly, with excellent time resolution and superior sensitivity, the capability for two-dimensional analysis, such as spatially time-resolved measurements. The streak camera is an ideal tool to investigate biological processes which involve emission of light.

A streak camera (Model # C979, Hamamatsu) converts time information from a luminous event into spatial information. Figure 5 displays the working of a streak camera. The heart is the streak tube. The tube combines the operating principles of a photomultiplier and an oscilloscope. During the basic operation of the streak camera, photons strike a

photocathode which produces an emission of electrons proportional to the incident light intensity. The electrons are accelerated into the tube by an accelerating mesh and are electrostatically swept at a known rate and a known distance, converting temporal information into spatial information. These electrons strike a microchannel plate capable of producing electron multiplication through secondary emission. The streaked luminous output formed on the phosphor screen is measured by a photodetection system where temporal information of the event is displayed.

The photocathode of the streak tube is a multialkali with a uv glass faceplate. It has a spectral response similar to but slightly better than an S-20 surface and a useful diameter of 5mm, which yields an effective diameter of 15mm at the output phosphor screen after magnification. 50 photons/electron are produced from a P-11 phosphor under 3KV electron bombardment. The multichannel plate has a center to center distance of 14 μ m. At a maximum rate of applied voltage (900V), the MCP gain reaches a value of about 10^4 . In the streak mode, a 1.5KV acceleration mesh over a distance of 3mm extracts photoelectrons from the photocathode. The applied deflecting voltage is 2KV p-p, and a central 1KV linear portion deflects the photoelectrons at variable speeds of 15, 7.5, 3 and 1.5mm/ns. The deflection sweep circuitry requires a minimum triggering voltage of 2V into 50 ohms. After triggering, the typical delay to initiate a streak is about 50ns at the fastest sweep rate.

The streak camera is combined with a direct video readout consisting of a silicon intensifier target vidicon camera, an analyzer, monitor, and a

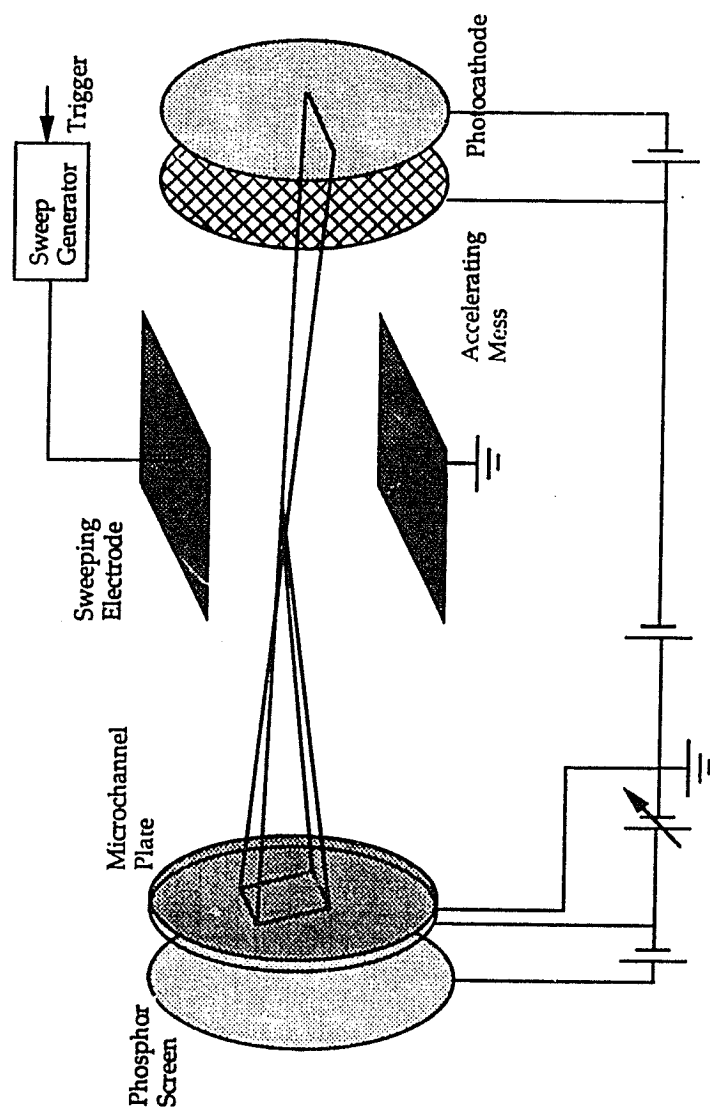


Figure 5. Schematic diagram of a streak camera

computer for analysis.

The SIT vidicon camera, which has an S-20 photocathode, monitors the streak image on the phosphor screen. The video signal is analyzed by a Model C1000 (manufactured by Hamamatsu, Inc.). The TA microcomputer converts the image information from analog to digital and corrects for dark current and sensitivity variations in the vidicon. It integrates and outputs the video intensity along each horizontal scanning line or a portion of it for various window settings.

To calibrate the time axis and the intensity linearity of the combined system in the streak or operate mode, a single 8ps 527nm laser pulse is passed through a pair of etalon mirrors of transmission coefficient T (for each mirror) and separated by an air spacing of d . The calibrating pulses produced in this manner make up a train of pulses separated in time by $\Delta\tau = 2d/c$, where c is the velocity of light. The intensity profile of the train is a decreasing exponential with each subsequent peak reduced by $(1-T^2)$. For each round trip of the pulse between mirrors, a light pulse of intensity $I_k = I_0 (1-T)^{2k}$ is produced, for $k = 0, 1, 2, \dots, n$. Since

$$I_k/I_{k+1} = 1/(1-T)^2 = \text{constant},$$

the envelope formed by the peaks of the pulses follows a single exponential decay given by

$$I = I_0 \exp [(t/\Delta\tau) \ln(1-T)^2],$$

where the time between peaks is $t = k\Delta\tau$. The peaks are used to calibrate the time axis and correct for the intensity variations. The sweep rate per channel

$\Delta T/\Delta x$ versus the channel number x is used to calibrate the time base of the streak camera where Δx is the number of channels between peaks and $\Delta \tau$ is fixed for a given etalon spacing. The data acquired is then corrected for intensity and the non-linearity of the streak rate.

The streak camera can also be operated in the focus mode or non streak mode by switching off the sweep generator and applying an appropriate voltage across the deflection plate so that the electronic image of the input slit is brought into the center of the field.

To measure fluorescence lifetimes, the streak camera is switched to the streak mode. The data seen on the screen displays the intensity versus channel. This data is stored in a personal computer. Step by step operation of the set-up is described in detail in the Appendix I.

3(b) Femtosecond CPM Mode-locked Dye Laser System:

Time-resolved measurements have also been performed using the femtosecond colliding mode-locked dye laser for 310nm excitation. The schematic diagram of a colliding mode-locked laser is displayed in Figure 6. A series of reflection mirrors form a ring cavity whose key elements are a saturable gain dye and a saturable absorber dye. The choice of the gain medium depends on the wavelength of interest. In these experiments the design employs rhodamine 6G as the gain medium and diethyloxycarbocyanin iodide as the saturable absorber. They are placed at two focal points in the cavity, respectively. A CW argon-ion laser operating at 514.5nm is used to pump the gain medium. As the lasing process builds

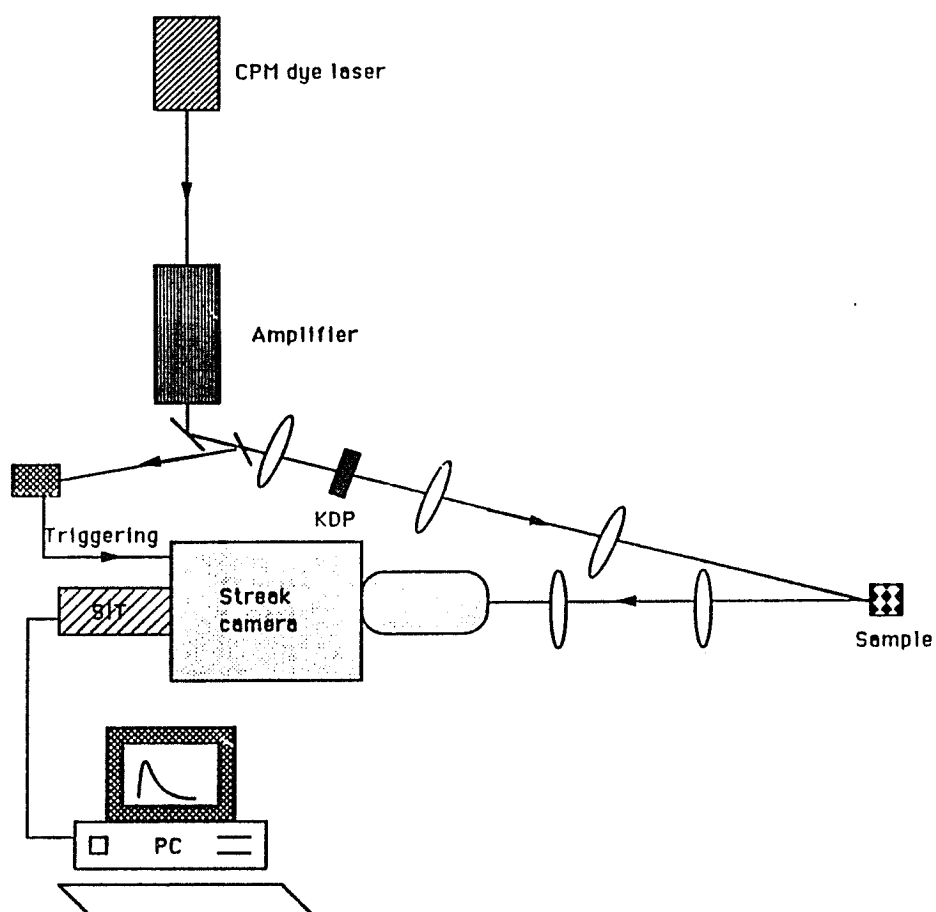


Figure 6. Experimental fluorescence set-up using a femtosecond colliding pulse mode-locked laser system.

up, two counter-propagating laser beams are formed in the cavity.

A sequence of four prisms, each cut for minimum deviation at Brewster's angle, is incorporated in the cavity. The prism sequence enable intracavity group velocity dispersion (GVD) to be adjusted continuously from positive through zero to negative values. In addition to compensating for GVD, the prisms also make compensation for self-phase modulation possible. Using such a cavity and carefully balancing the four basic pulse shaping mechanisms- self phase modulation, group velocity dispersion, saturable absorber and saturable gain within a single resonator- pulses as short as 27fs have been generated directly from the CPM Rh6G dye laser oscillator. The pulses used in these experiments were about 80-100fs. The 620nm pulse from the CPM is frequency doubled to 310nm and focussed onto the tissue samples. The fluorescence is collected at two wavelength bands 340nm and 440nm by using appropriate narrow band filters and focussed onto the streak camera by two lenses.

Typical energy used is about 0.5 J. The pulses are amplified by a sulphur rhodamine gain medium pumped by a copper vapour laser. The repetitive rate of the pulses used after amplification is 6KHz. The pulse energy is 0.5 J at the sample site. Beam diameter at the sample site is a few hundred microns.

The fluorescence lifetimes are measured using a synchroscan streak camera with a temporal resolution of 16ps. The results are averaged over tens of thousand pulses. The signal is then analyzed using a temporal analyzer

and data stored in a personal computer.

(4) QUANTUM YIELD SET-UP: (Picosecond Nd-Yag Laser System)

The Nd-Yag laser system uses active mode-locking and the laser has a repetition rate of 10Hz. The third harmonic 351nm 20ps pulse is focussed onto the sample and the fluorescence from the sample collected and focussed by two lenses onto a S-20 photomultiplier tube (Pacific Photometric Instrument: Model # 62/2F). Two 0-51 filters are placed in front of the photomultiplier tube. The photomultiplier tube is coupled to a boxcar integrator for signal detection taking an average over 30 shots. Figure 7 displays the experimental set up.

(5) PULSED FLUORESCENCE SPECTROSCOPY

Fluorescence spectra of biological samples have been measured using picosecond pulses of Nd glass laser. The experimental set-up used is the same as the one used for time-resolved fluorescence. The only difference is in the detection system as seen in Figure 8. Instead of the streak camera, here, a Jarrel Ash spectrometer and a PAR OMA II is used as the detection system to obtain the transient fluorescence spectra.

(6) PHOSPHORESCENCE SET-UP

The third harmonic of the Nd-glass laser described earlier in this chapter is used to measure phosphorescence spectra and lifetimes. The sample is placed in a glass dewar cooled by flowing nitrogen gas and the temperature monitored with a copper-constantan thermocouple. The spectral profiles are obtained using a Jarrel Ash spectrometer and a SIT connected to

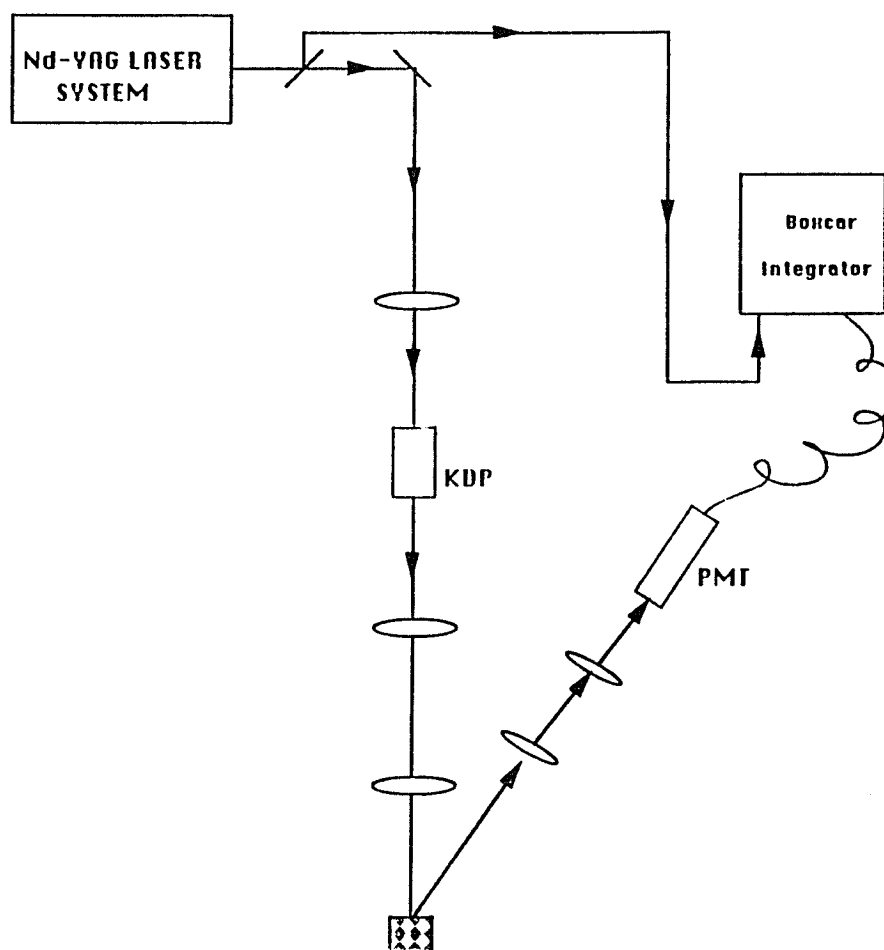


Figure 7. Experimental set-up of quantum yield set-up.

a OMA II. The excitation wavelength was cut off by placing two 0-51 filters in front of the spectrometer. The phosphorescence spectra was recorded after the emission path was blocked for a fraction of a second, at low temperature (-120 K). This produces a delay $> 100\text{nsec}$ and eliminates the probability of any fluorescence showing up. Phosphorescence lifetimes were obtained from photographs of temporal trace using a S-20 photomultiplier tube (Pacific Photometric Instrument: Model # 62/2F) connected to a 7844 oscilloscope with two 0-51 filters to cut off the excitation pulse.

CHAPTER 3: FLUORESCENCE SPECTROSCOPY

Fluorescence is a well-known phenomenon. The molecule is photo-excited to an upper electronic energy level when a photon interacts with matter provided the wavelength of the photon is short enough or the energy is high enough to raise it to the higher level. Figure 9 describes the energy levels and various processes occurring in an emitting species found in nature. Absorption usually takes place from the ground state to a higher vibrational level of the first excited state. Relaxation to the ground state takes place in several non-radiative and radiative ways. There is non-radiative relaxation to the lowest vibrational of the excited singlet state (S_1). Relaxation from there to the ground state can occur both radiatively (as fluorescence) and non-radiatively (as internal conversion). Intersystem crossing from the singlet excited state to the triplet state occurs even though it is a forbidden process. This leads to either phosphorescence or non-radiative relaxation from the triplet state to the ground singlet state. Most fluorescing species found in nature have broad absorption bands so that the excitation wavelength is not critical. Generally, the visible and uv regions of the light spectrum induces fluorescence. Due to vibrational relaxation, the fluorescence is always red shifted as compared to the absorption. The fluorescence spectral distribution reflects the different transitions from the first excited state to the different vibrational levels of the ground state. The fluorescence spectrum is characteristic for each molecule and is largely independent of the excitation

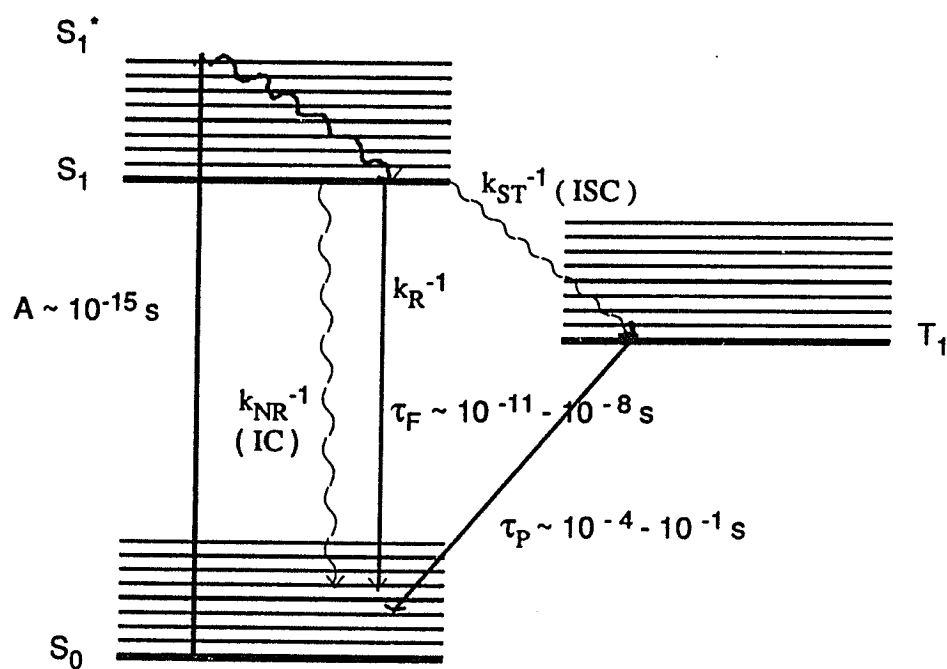


Figure 9. Energy level diagram of fluorescence from organic molecule emitting species found in nature.

wavelength. Thus fluorescence spectrum can be used to determine molecular structure as well determine which molecule is responsible for the particular fluorescence spectrum.

It is well known that all molecules that absorb do not fluoresce. This implies that the fluorescence efficiency of most absorbing molecules is low. When a molecule does not fluoresce, it is due to other competing processes which tend to decrease the quantum yield of fluorescence. The quantum yield of fluorescence thus determines the internal radiationless processes occurring in a molecule. Some molecules also do not fluoresce after absorption due to dissociation. If the excitation energy is lower or the excitation wavelength is higher then the fluorescence is not quenched. In solutions, the solvent tends to influence fluorescence yield by affecting the dissociation process. Interaction with other molecules or solutions also change the fluorescence yield and spectrum. Concentration effects of either the molecule in a solution or another species in the same solution give rise to fluorescence efficiency and spectral changes. Many compounds are ionizable and can exist in aqueous solution in various stages of dissociation, depending on the hydrogen concentration. There have been several studies on the pH dependence of biological molecules in solution or bound in proteins. The fluorescence spectra is influenced both in intensity and in position, by the pH, whereas the absorption spectra is less affected. Intrinsic and extrinsic fluorescence have both been used extensively over the years to determine the structure and environment of several fluorescing and non-fluorescing biological molecules.

The advantage of intrinsic fluorescence is the fact that the surrounding environment remains unchanged during the studies.

Intrinsic fluorescence from proteins have been observed since the late 1950's and have been used to study protein structure since the 1960's. Shore and Pardee [23] were the first to describe the phenomenon of fluorescence in the ultraviolet. Udenfriend and Knevel [1,24] have also presented studies of protein fluorescence. Visible fluorescence has been studied by Chance [1,3]. Chemical events in cells and subcellular systems have been monitored by fluorescence studies. Chance and colleagues have studied the fluorescence from intact tissues to understand the oxidation-reduction processes occurring. Brumberg and his colleagues [25,26] were the first to note that cellular fluorescence provides a sensitive index of the functional state of a living cell. Fluorescence microscopy and microspectrofluorometry have also been used to localize and identify natural and foreign substances in living tissue [27]. Loeser adapted quartz rod illumination for carcinogen and antibiotic localization in tissue [28].

In the early 1980's diagnostic studies were started by Alfano and co-workers [1] using visible fluorescence to detect tooth decay. Thus an awareness of the importance of fluorescence spectroscopy in the diagnostic field of medicine was felt. This also led to the studies of distinguishing cancerous rat tissues from normal tissue using visible wavelengths of light in 1984 [7]. This method was extended to human tissue which is elaborated in this chapter.

This chapter along with the following two chapters deals with the spectroscopic properties of normal and benign tissue, benign tumor and malignant tumor of the breast. This chapter consists of 4 sections. The first section defines the main tissue types and describes the pathology of cancer tissues. Section II describes the steady state fluorescence work. Section III deals with excitation spectroscopy and section IV describes the pulsed laser spectroscopy.

SECTION I

Classification of Tissue and Pathology

Human tissue are broadly classified into four main types: Epithelial, muscular, nervous and connective according to the type of cell of which each is composed.

(a) Epithelial tissue: Epithelial cells serve as a protective covering. They are arranged in a series of layers or strata to form the outer part of the skin. They also line the digestive, respiratory and urinary tracts. The various external secretory glands (digestive, mammary, sweat, etc.) are composed of epithelial cells. Thus, breast tissues are composed of epithelial cells. The various types of epithelial cells are : columnar type, cuboidal and squamous. The shapes of the columnar and cuboidal cells are evident from the names. Squamous cells are scale-like or plate-like. The epithelial cells composing glands form 'glandular tissue'. The simplest type of these secreting cells form a single tube and are called simple tubular. In other glands, the cells

surround a central spherical cavity into which the glandular secretion is received. The channel or duct from the cavity leads to the surface. Breast tissue contains such ducts. Several lobules are formed in breast tissues by means of separation of the connective tissue (See fig.10).

(b) Muscular tissue form the muscles. The two types of muscular tissues are striated and unstriated. Skeletal or voluntary muscle are striated while involuntary muscle of the internal organs except the heart, are unstriated.

(c) Connective tissue: Connective tissue serves as a strengthening, connecting, and supporting material. It binds together masses of different types of cells and forms a supporting framework for various organs. It also serves to fill in spaces between neighbouring organs and parts and replaces tissue destroyed by injury or disease. Connective tissue consists of slender fibers embedded in a homogeneous jellylike matrix or ground substance. The ground substance dominates and cells are relatively few. The fibers in some cases possess elastic properties and contain a protein, elastin. In other parts, they are bundles of strong fiber with no elastic properties. These contain a protein known as collagen. Adipose or fatty tissue is a modified connective tissue.

(d) Intercellular substances: Between the cells forming a solid tissue there is a semifluid viscous and structureless material resembling a soft jelly. In most tissues this acts as a glue to hold cells together and to permit water, dissolved substances and certain material to pass between cells. In some other

tissues such as connective tissue, this intercellular substance is more abundant and forms a matrix in which cells and fibers are embedded. Tissues are made up of cells in between which are connective tissue which contains the tissue fluid and ground substance. The tissue fluid contains various large proteins, ions, gases, amino acids, sugars, vitamins, lipids and hormones, which are exchanged between cells. The body contains 55% water, 19% proteins, 19% fats, 1% carbohydrates and 7% inorganic materials. This implies that the tissue fluid contains more amounts of proteins and lipids than other substances.

Figure 10 displays a diagram of the breast. The breast tissue contains main ducts which branch out into several smaller ducts that lead into lobules. Approximately 15 lobules serve each duct. The lining of the ducts and the lobules are epithelial tissue. The space in between lobules is filled with fat and connective tissue in varying proportions.

(i) Normal breast tissue are generally glandular and lipomatous in nature.

(ii) The benign tissue includes fibrocystic mastopathy. These benign tissues are the fibrous tissue of the breast which appear white in color.

(iii) A neoplastic tumor is an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissue.

Benign tumor includes neoplasms that do not usually recur after excision. Chances of transformation to malignancy is also less. The cells in a benign tumor are well-differentiated, that is, they closely resemble the parent

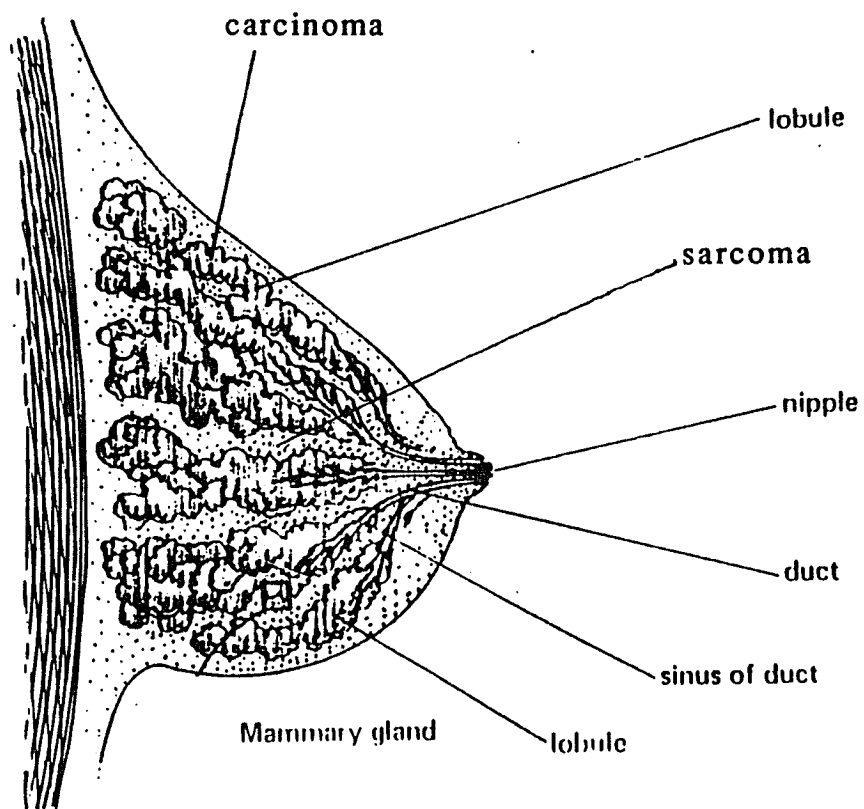


Figure 10. Diagram of breast showing ducts and lobules and origin of malignant tumors.

tissue. There are some cases of cystosarcoma phylloides, though, which may already be cancerous. Benign tumors of the surface epithelia are called papillomata and those of the solid epithelia are adenomata. In the connective tissues, the benign tumors are named after the tissue of origin, e.g. fibroma and osteoma. The benign tumors that are of concern in these studies are generally fibroadenomas and some are adenofibromas.

A malignant tumor always invades the surrounding tissue. It eventually enters channels like the lymphatic and blood vessels, and groups of cells are carried to other parts, where they set up secondary growths, or metastases. Microscopically, they show less accurate reproduction of the parent tissue than do benign tumors. Malignant epithelial tumors are all carcinomata. The cancers originating from mesoepithelial tissue (which include the tiny muscles and the connective tissue) are known as sarcomas. Most breast cancers originate from the cells which line the ducts (ductal carcinoma). Breast malignancy can generally be categorized as invasive or non-invasive. Invasive cancer penetrates the basement membrane extending through the duct wall into the surrounding fibrous supporting tissue. These cancers are more commonly seen. The non-invasive cancers have cells that have not penetrated the limiting basement membrane and remain confined within the walls of the ducts. Breast cancers originate from the epithelia and the mesoepithelial tissue. Figure 10 shows the regions of a carcinoma and a sarcoma. Again, the tumor may arise from the lobules or the ducts of the breast. These are called lobular and ductal, respectively. The malignancy

usually originates from the cells which line the ducts, as seen in Figure 10. Ductal carcinomas arise either in the main ducts or the smaller ducts, more often deep in the smaller branches.

Breast tissue, like any other tissue of the human body, contains elastin and collagen as the structural proteins and other fluorophores such as NADH (nicotinamide adenine dinucleotide-reduced form), NADPH (Nicotinamide adenine dinucleotide phosphate-reduced form), NAD^+ (nicotinamide adenine dinucleotide-oxidized form), tryptophan, tyrosine, flavins (FAD-flavin adenine dinucleotide and FMN-flavin mononucleotide), porphyrins and other biomolecules. The framework of the tissue is given by the structural proteins, collagen and elastin. Malignant breast tissues also contain the same fluorophores. Cancer tissues seem to lose contact inhibition and nothing seems to stop them from growing. Another view is that cancer cells escape the immune system. Hence at the time of this research, it is accepted that cancer tissues contain similar fluorophores as normal tissues. Breast malignancies are known to induce productive fibrosis [29,30] or produce collagen directly [31]. Studies have also shown that there is increase in elastic tissue in infiltrating ductal and lobular carcinomas [32,33]. A correlation of the fluorescence lifetimes of these fluorophores and the tissue lifetimes may be able to show that the differences observed are in fact due to the higher collagen content of malignant tumors. Studies with rat kidney fibroblasts [34] have shown a 2-3 fold decrease in the absolute concentrations of NAD^+ and NADH following murine sarcoma virus transformation. Studies of NADH

oxidation-reduction changes have been performed by Chance [1] in intact cells only. Spectroscopic diagnosis of colonic dysplasia [35] has also shown a decrease in the NADH peak in adenomatous tissue as compared to the normal tissue. Similar changes may be expected in human breast tissues but is yet to be studied.

Flavins and the different forms of NAD are coenzymes which help in oxidation-reduction processes of enzymes and proteins. Tryptophan and tyrosine are amino acids that make up proteins and are two of the three fluorescing amino acids, the other being phenylalanine. Elastin and collagen are structural proteins as mentioned earlier. Porphyrins are metal-containing organic rings that are derivatives of a parent tetrapyrrole compound, porphin. These are present in various heme proteins like hemoglobin, myoglobin and in cytochromes.

The normal breast tissues studied in this research were mainly glandular, with some lipomatous tissues. In the earlier stage of the research, the normal tissue may have contained some fibrous parts which were not distinguished from normal tissue. However, in the later stage of the research, these were identified as benign tissue. The benign tissues included fibrocystic mastopathy. Benign tumors studied were generally fibroadenomas and some adenofibromas. Most of the breast malignancies studied in this research were infiltrating ductal carcinomas with varied differentiation.

In the aorta, the intima or the inner layer consists typically of a smooth

layer of endothelial cells situated on a delicate basement membrane. The middle layer is elastic in nature with elastin fibers separated by alternating layers of smooth muscle cells. The outer layer consists mainly of connective tissue with some elastic and nerve fibers. The two kinds of atherosclerotic lesions studied here are fibrous and calcified plaque. These are lesions confined to the intima. The fibrous plaque consists of connective tissue including collagen, elastic fibers, proteoglycans and variable number of smooth muscle cells. The calcified plaque consists of deposits of calcium. Aorta is rich in elastin while coronary artery is more muscular. Figure 11 displays the cross-sectional view of an artery with a fibrous plaque, respectively.

SECTION II

Steady State Fluorescence

(a) Results

The steady state fluorescence spectra of 23 pairs of normal and malignant tumor human breast tissues have been measured and analyzed [27]. Most of the pairs were from the same patients, one from a malignant site and the other from a normal area while a few were isolated samples from different patients. The normal samples here were generally glandular and fatty tissue. The malignant tumors were generally infiltrating ductal carcinomas of the breast with isolated cases of papillary carcinoma and mucinous carcinoma. The spectra were measured using 488nm, 457.9nm and

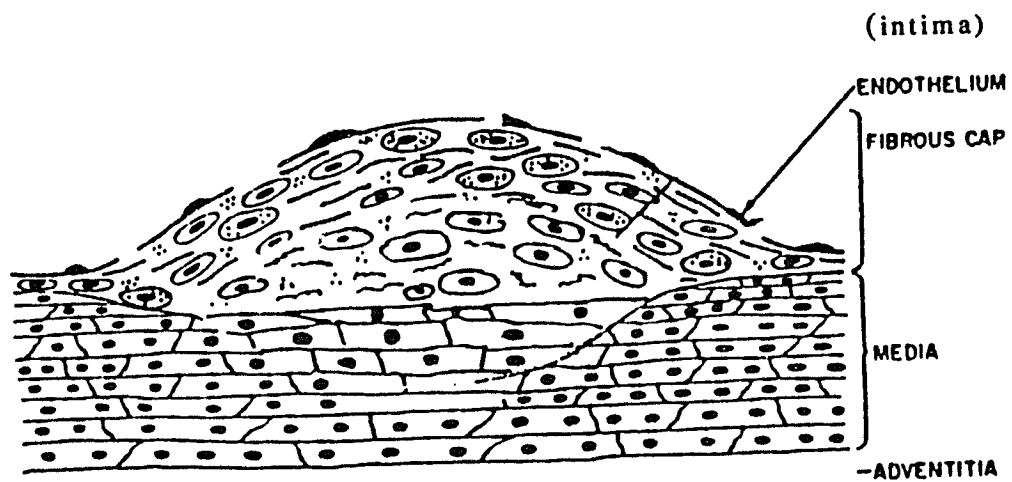


Figure 11. Cross-section of an artery.

514.5nm wavelengths of an argon-ion laser. The experimental set-up has been described in Chapter 2.

The fluorescence spectra were measured from 500nm to 700nm when excited with 488nm, from 460nm to 700nm with 457.9nm excitation and 520nm to 700nm with 514.5nm excitation. Typical fluorescence spectral curves of each type are shown in Figures 12, 13 and 14. The significant difference between normal breast tissue spectra and the tumor lies in the shape of the spectral profiles. The principal peaks in both normal and tumor spectra are located at around 515-520nm with 488nm excitation (Figs. 12 (a) and (b)), 500-510nm with 457.9nm (Figs.13 (a) and (b)) and 535-555nm with 514.5nm excitation (Figs.14 (a) and b)). In the case of normal tissue, there are two subsidiary peaks at around 555nm and 600nm with 488nm as seen in Fig. 12 (a) with dips at 540nm and 580nm. The tumor tissue profile shows a smooth monotonic decrease with no clear subsidiary peaks (Fig. 12(b)). The tumor tissue spectra also consistently display about four times stronger fluorescence intensity as compared to the normal tissue. The normal tissues are typically of two types: one with Raman peaks (Figs.12, 13 and 14) and the other without Raman peaks (Figs.15,16 and 17). The Raman lines in the normal breast sample are shifted by about 1037,1243, 1588, 2761 cm^{-1} with 488nm excitation whereas the corresponding tumor does not show any such peaks.

In the case of 457.9nm excitation a distinct difference is noticed, too, in Figs.13 (a) and (b). Here, the main peaks are located at 517 and 511nm for the normal and tumor tissues respectively. The subsidiary peak of the normal

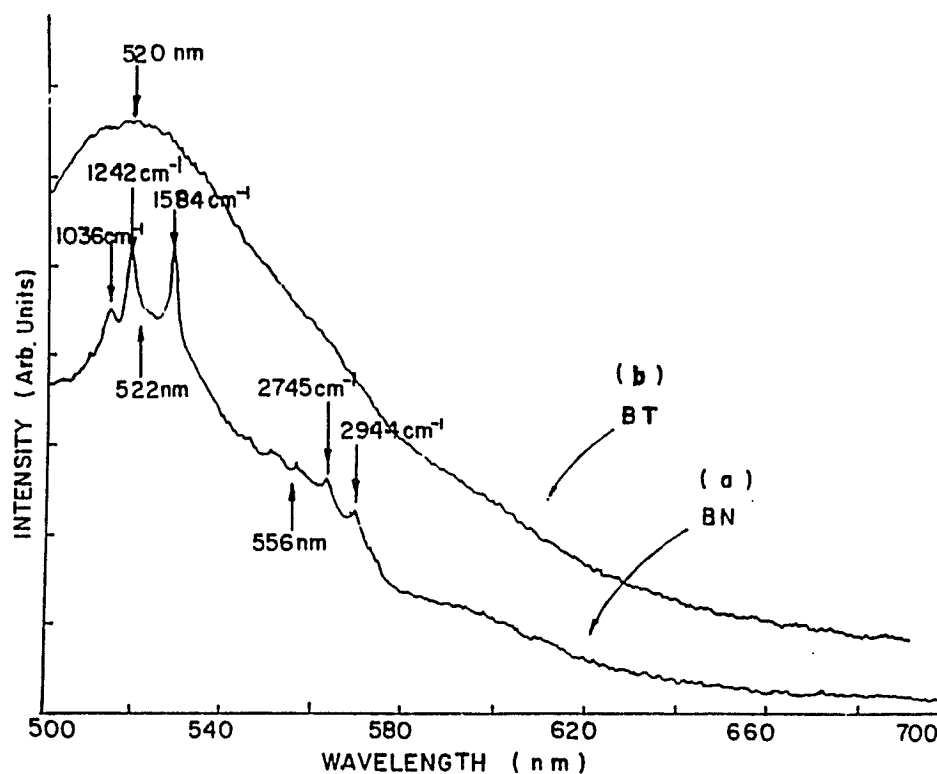


Figure 12. Fluorescence spectrum excited at 488 nm from (a) human breast tissue with Raman peaks and (b) human tumor breast tissue.

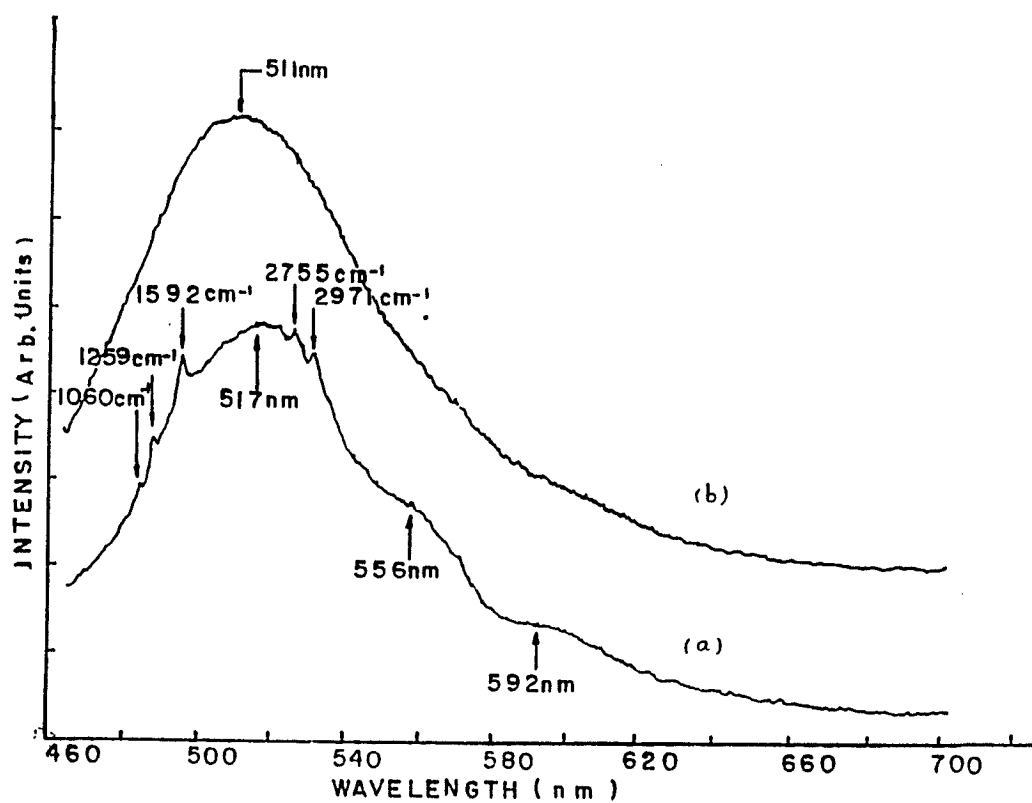


Figure 13. Fluorescence spectrum excited at 457.9 nm from (a) human breast tissue with Raman peaks and (b) human tumor breast tissue.

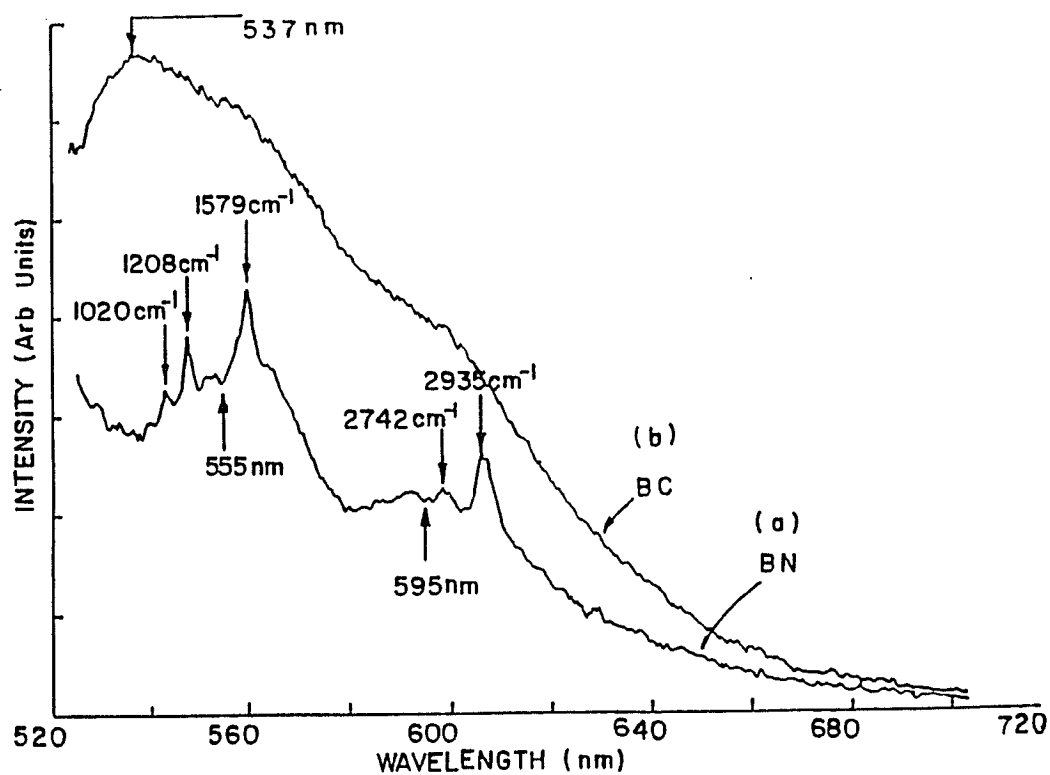


Figure 14. Fluorescence spectrum excited at 514 nm from (a) human breast tissue with Raman peaks and (b) human tumor breast tissue.

tissue are at 556 and 592nm with dips at 540nm and 580nm. The normal tissue displays Raman lines shifted by 1060, 1259, 1592, 2755 and 2971 cm^{-1} . The tumor is again distinctly different, with a smoother profile and no Raman peaks.

Differences observed with 514.5nm excitation are also similar as seen in Figs.14 (a) and (b). The main peaks, in this case are at 555nm and 537nm for normal and tumor respectively. The subsidiary peak of the normal is located at 595nm with a prominent dip at 580nm. The Raman lines of the normal tissue are shifted by 1020, 1208, 1579, 2742 and 2935 cm^{-1} . No subsidiary peaks are observed in the tumor tissue of Fig.14 (b).

The second type of normal tissue (type II) displayed profiles which did not exhibit Raman peaks. These profiles also displayed more prominent subsidiary peaks than the ones with Raman peaks. Figures 15, 16 and 17 display the spectral profiles without Raman peaks at 488nm, 514.5nm and 457.9nm, respectively.

(b) Statistics of Spectral Results:

To ascertain the consistency of the observed results of Figs.12-17, statistics of the data have been taken. Two different algorithms have been used to obtain the statistics. Since the spectral profiles showed distinct differences in shape, the two algorithms were based on the spectral shape of the profile. The criteria of selection in the two cases are: (1) visual algorithm and (2) sum of deviation algorithm.

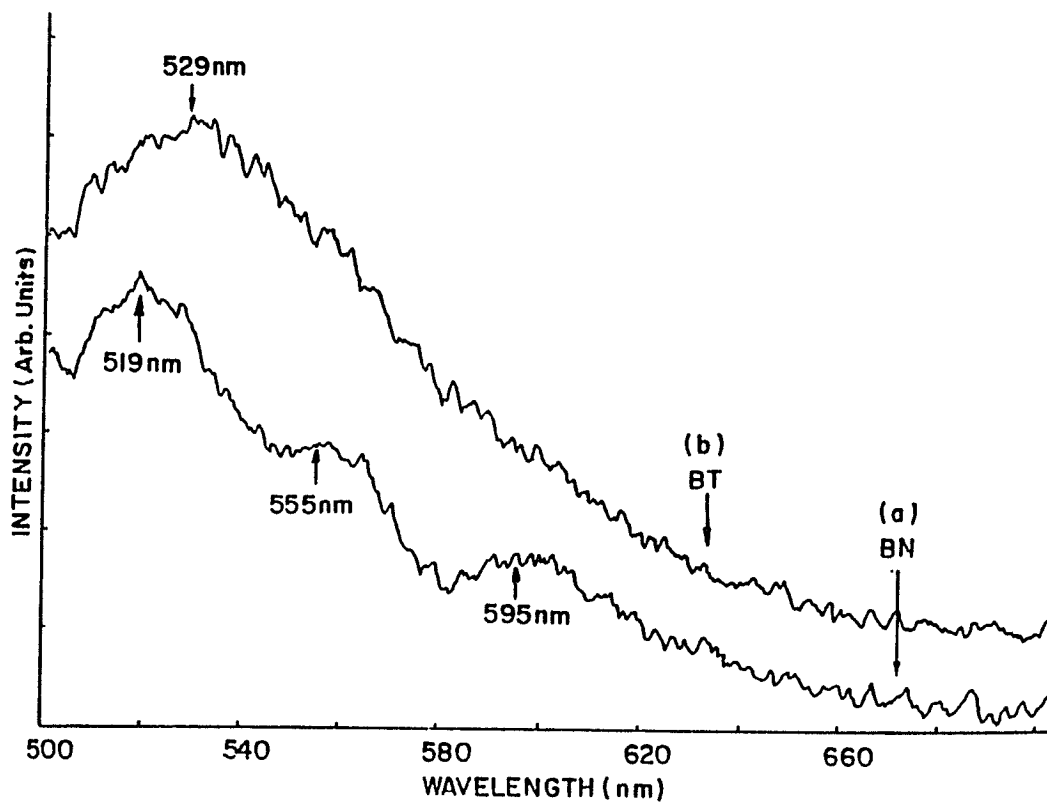


Figure 15. Fluorescence spectrum excited at 488 nm from (a) human breast tissue without Raman peaks and (b) human tumor breast tissue.

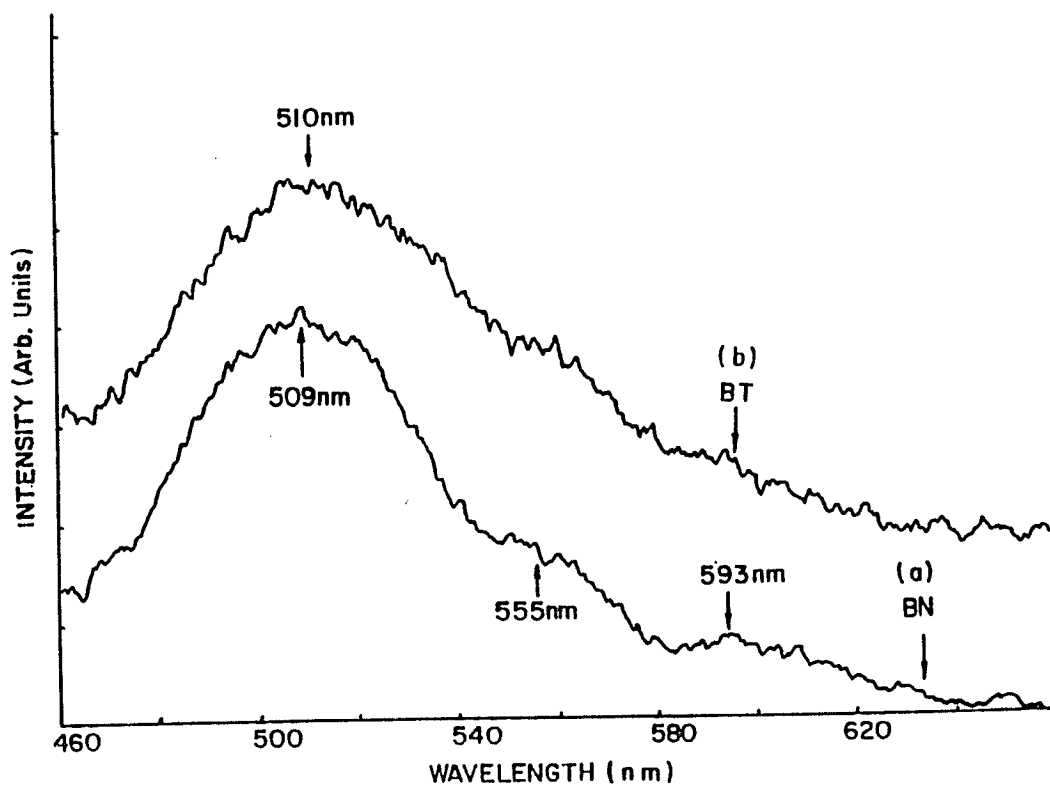


Figure 16. Fluorescence spectrum excited at 457.9 nm from (a) human breast tissue without Raman peaks and (b) human tumor breast tissue.

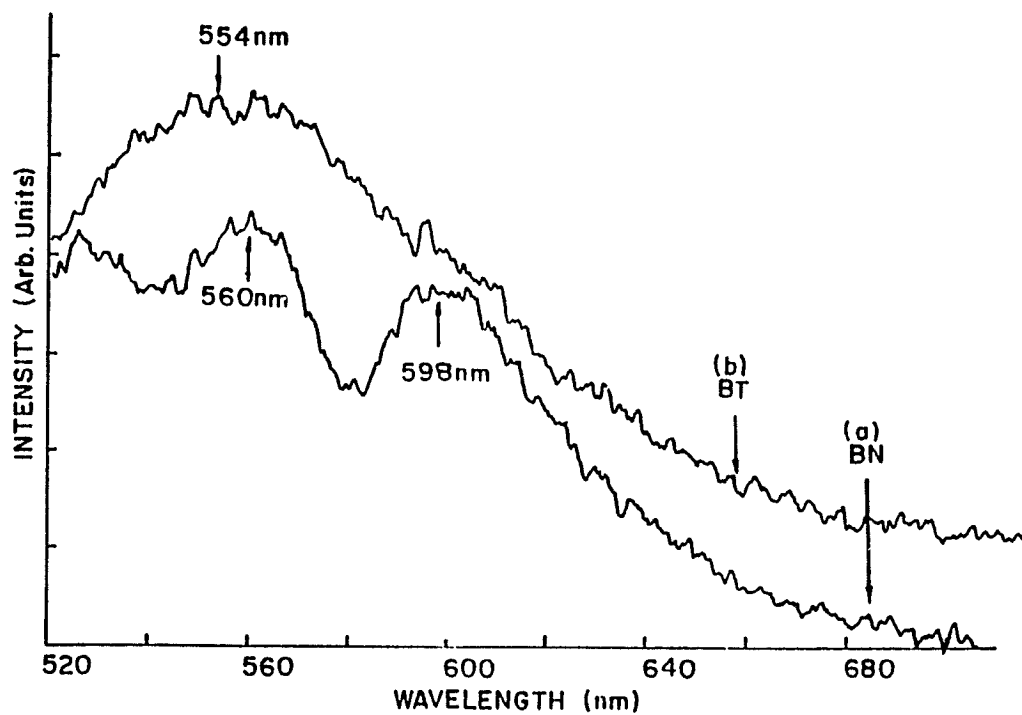


Figure 17. Fluorescence spectrum excited at 514 nm from (a) human breast tissue without Raman peaks and (b) human tumor breast tissue.

1. Visual:

The first method of statistics to separate tissues is based on the visual shape criteria to distinguish normal breast tissue spectral profile from cancerous tissue profile with 488nm excitation. It has been seen in Figs.12 and 15 that the normal spectra consist of two dips at 540nm and 580nm while the tumor spectra display smooth profiles with no dips. This comparison of normal and tumor tissue spectra is selected as the criteria by which the statistics is taken. The spectrum with dips is considered to be that of a normal sample while a smooth spectrum with no dips is considered to be that of a tumor sample. Consistency with and deviations from this criteria are the basis of the statistics. As defined in Table 1, the sensitivity is the percentage of cancer detection in agreement with pathology while the specificity is the normal tissue detection in agreement with pathology. False negative is defined as the percentage of cancer detection not in agreement with pathology and false positive is the percentage of normal detection not in agreement with pathology. The percentage of normal and cancer detection neither in agreement with nor opposing pathology is defined as equivocal.

A statistics of the results from 43 samples have been taken. Table 1 displays the statistical results: 17 out of 20 normal samples showed consistent results with pathology. The specificity was 0.85. These are categorized as negative. 19 out of 23 tumor samples showed a positive result, which agreed with pathology. The sensitivity was 0.83. One pathologically normal sample

Table 1. Statistics of spectral shape diagnosis for breast tissue.

Statistics of spectral shape diagnosis for breast tissue

Cancer	Number of samples	23
	Sensitivity	19/23= 83%
	False negative	1/23= 4 %
Benign & normal	Number of samples	20
	Specificity	17/20= 85%
	False positive	1/20= 5%
Equivocal		5/43= 12 %

<u>Sensitivity</u>	Percentage of cancer detection in agreement with pathologists	$= 100 \times \frac{\text{number of correctly diagnosed cancer samples}}{\text{total number of cancer samples}}$
<u>False negative</u>	Percentage of cancer detection not in agreement with pathologists	$= 100 \times \frac{\text{number of incorrectly diagnosed cancer samples}}{\text{total number of cancer samples}}$
<u>Specificity</u>	Percentage of normal detection in agreement with pathologists	$= 100 \times \frac{\text{number of correctly diagnosed N samples}}{\text{total number of normal samples}}$
<u>False positive</u>	Percentage of normal detection not in agreement with pathologists	$= 100 \times \frac{\text{number of incorrectly diagnosed N samples}}{\text{total number of normal samples}}$
<u>Equivocal</u>	Percentage of normal and cancer detection neither in agreement with pathologists nor opposing pathologists	$= 100 \times \frac{\text{number of undiagnosable N \& C samples}}{\text{total number of normal and cancer samples}}$
<u>Method</u>	Comparison of spectral profiles of normal and cancer samples excited by 488nm.	

was detected as a tumor by this system. This was categorized as a false positive. Two normal sample could not be identified as either normal or tumor since different positions of the sample showed different results. These were categorized as equivocal. Similarly, there were three tumor samples categorized as equivocal. The false negative and false positive were 0.04 and 0.05 respectively and equivocal was 0.12. These statistics (Table 1) were performed initially to determine the accuracy of this method of diagnosis. The results demonstrate that the fluorescence technique may be useful to diagnose cancer.

2. Sum of Deviation:

A second algorithm to separate tissue types was used to ascertain the reliability of the optical diagnostic technique. This algorithm used the sum of deviation as the basis of taking the statistics. This method involved a subtraction of each data point of the individual spectral profiles of the normal and tumor tissue samples from each data point of the average tumor curve. This curve is divided by the number of data points. A t-test is performed to see if the difference in the sum of deviation of normal and cancer samples is statistically significant.

This analysis was performed on 11 pairs of samples. The peak values of the fluorescence intensities were adjusted to 100. Raman peaks were observed in 9 out of 11 normal cases while none of the cancer samples displayed Raman peaks. The peak value was adjusted to cut off the Raman

peaks. The X and Y axes were divided into 450 points. Averages of 11 normal spectra and 11 tumor spectra were taken. A sum of the absolute value of deviation of each curve from the Y value of the mean curve, was calculated. Thus the sum of deviation of each normal and cancer curve was given by

$$[Y_i - Y_i (\text{mean curve})]/450$$

Distribution of these numbers were compared between the curves from normal and cancer tissues and the difference was analyzed by the Student t-test. All the data processing were performed using a Macintosh Plus with Data Scan and Statview 512 + (Brain Power, CA) and Microsoft Excel. The spectroscopic difference between normal and cancer profiles can be magnified by subtracting the mean curve of one from that of the other. Figure 18 (a) and (b) represent the average curves of normal and cancer breast tissues, respectively with 488nm excitation. The sharp dips of Figure 18 (c) at 520nm and 530nm represent the unsubtracted Raman peaks of the normal curves. The peaks at 540nm and 580nm represent the subsidiary peaks of the normal curves. The 600-700nm range shows little difference. The individual normal and cancer spectra have been subtracted from the mean normal curve and the mean cancer curve to obtain the sum of deviations in the 500-600nm range and the 500-700nm range. The normal tissue gave a higher deviation score as compared to the cancer deviation from the cancer mean curve. The sum of deviations from the normal mean curve gave less distinct differences. The difference between the sum of deviation of normal and cancer was statistically significant by the Student t-test (Table 2, $p=0.0002$ for the 500-

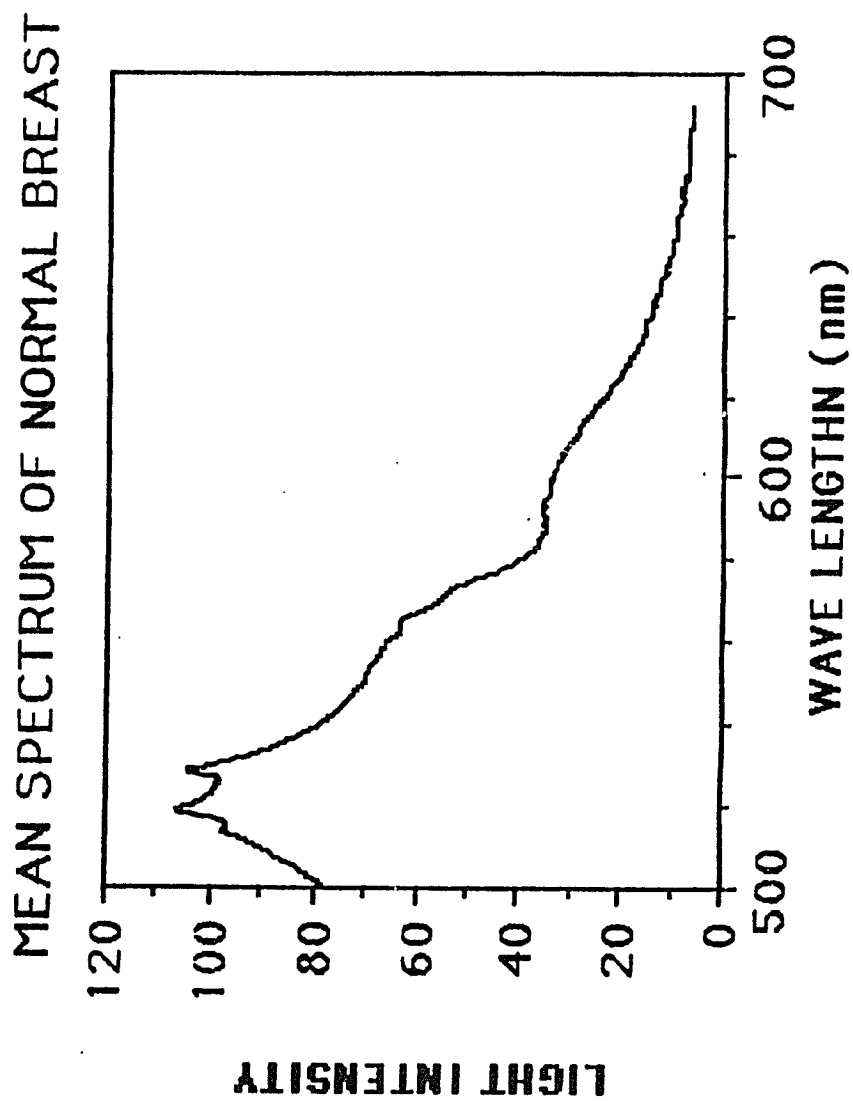


Figure 18 (a). Average fluorescence spectra of 11 curves each of normal breast tissues

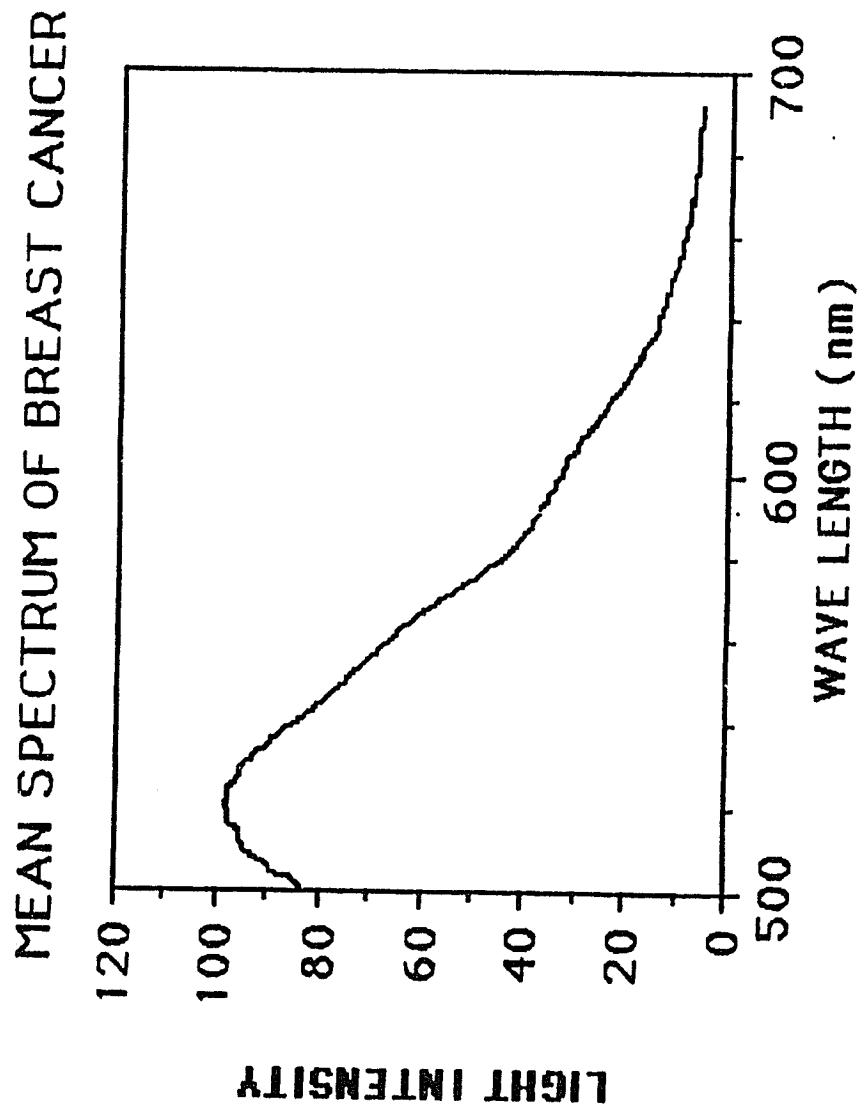


Figure 18(b). Average fluorescence spectra of 11 curves each of normal breast tissues

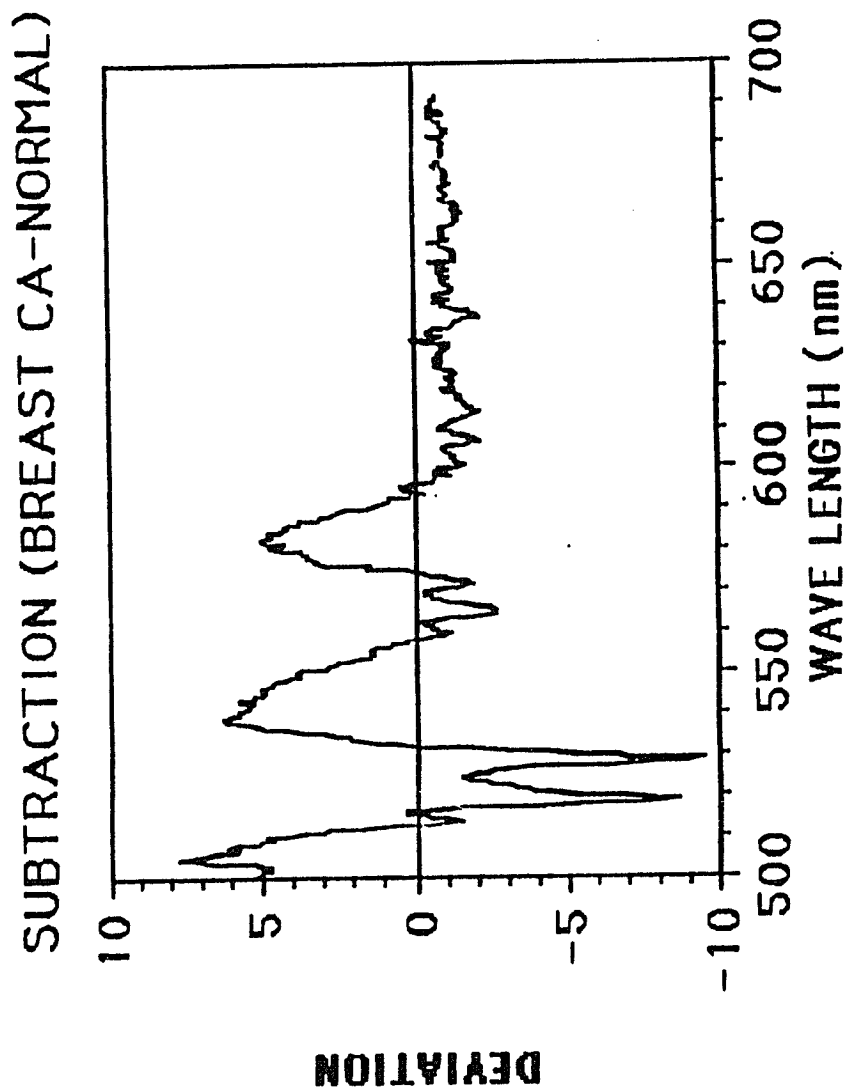


Figure 18(c). Spectral difference between normal and cancer human breast tissues excited at 488nm.

Table 2. Sum of deviation from mean cancer spectra.

Samples	Calculated at 500-600 nm	Calculated at 500-700 nm
Normal breast	1,405±494(1)	2,205±758(2)
Breast cancer	694±264(1)	1,148±319 (2)

Sum of deviation for each spectra from the mean cancer curve was analyzed by Student-t test in 11 paired samples. (1) $p=0.0002$. (2) $p=0.0008$.

600nm range, $p=0.0008$ for the 500-700nm range).

A histogram (figure 19) of the deviations of cancer and normal spectra from the mean cancer curve also shows that the normal curves have a higher deviation from the mean cancer than the individual cancer curves from the mean cancer curve in the 500-600nm range.

This study on 11 pairs of normal and cancer spectra reveals a measurable deviation of normal profiles when compared to the mean cancer profile. In contrast, when the curves were compared to the mean normal curve, no significant deviation was observed. This was caused by the large deviation of the individual normal curves as compared to the mean normal curve. This is due to the fact that the normal curves have variations within the group, which is caused by the prominent dips in type II and smaller peaks in type I. The cancer spectra, on the other hand, are devoid of such peaks and are consistently smooth. Hence there is less deviation of the individual cancer curves from the mean cancer curve whereas the normal curves show large deviations from the mean cancer curve.

These statistical studies using the visual and sum of deviation criteria shows the reliability of optical spectroscopy in diagnosis but further work described in section g showed that the diagnostic fingerprint was not reliable.

(c) Discussion:

Before the technique could be taken as foolproof, further research was

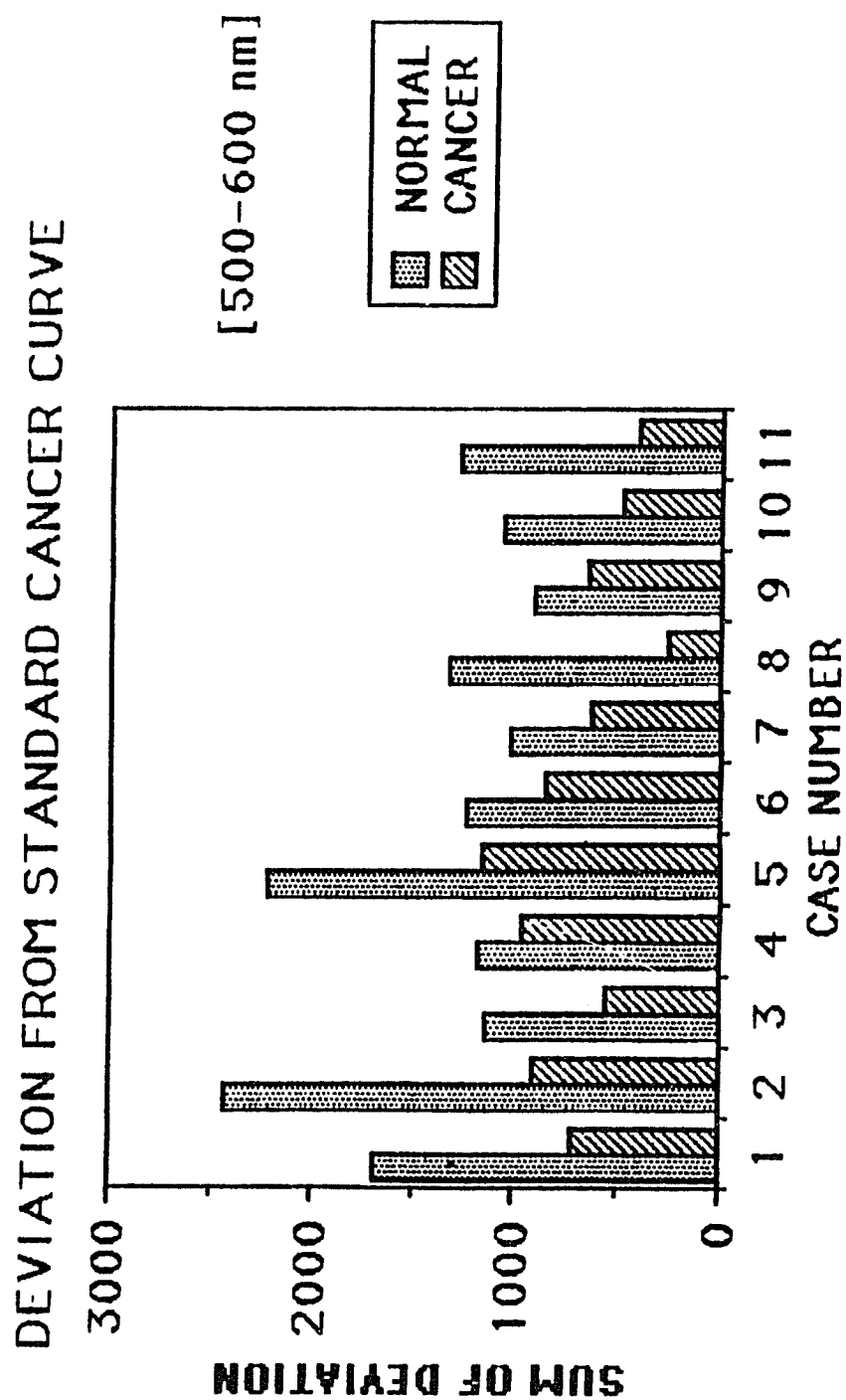


Figure 19. Histogram of the deviations of cancer and normal spectra from the mean cancer curve for 488nm excitation.

necessary to understand the features of the spectra and to determine the fingerprint. The fluorescence spectra of normal and cancer breast tissues peaked around 500-520nm with 488nm. It is known that flavins, which are natural fluorophores present within intact cells emit in the visible near 510-530nm. Fluorescence of flavins in solution have been measured and the spectra with 488nm excitation resembles that of cancer tissue spectra (Figure 20). Thus the fluorescence obtained from normal and cancer breast tissue with 488nm excitation can be ascribed to flavins. But the normal spectra have peaks at 550nm and 590nm. It was noticed that the normal tissue spectra were of two types, one with Raman peaks and the other without. It was also noticed that the normal tissue with Raman peaks have more fat and yellow in color and the corresponding spectra do not have distinct secondary peaks. The tissue without Raman peaks appear red due to the presence of blood and the corresponding spectra have distinct dips at 540 and 580nm.

Absorption spectra (Figure 21) of oxyhemoglobin displays peaks at 540 and 580nm. The origin of these peaks is partially attributed to the self-absorption of fluorescence arising from heme-based molecules located in the tissues. Figure 21 displays the absorption spectra of (a) hemoglobin (Hb), (b) carboxy-hemoglobin (HbCO) and (c) oxy-hemoglobin (HbO₂). Hb displays one peak around 555nm, HbCO displays two peaks at around 540nm and 570nm and HbO₂ displays peaks at around 540nm and 580nm. The effects of these absorption peaks on the fluorescence spectra of the tissues is to produce dips at those wavelengths. Tissues contain more oxyhemoglobin and its absorption

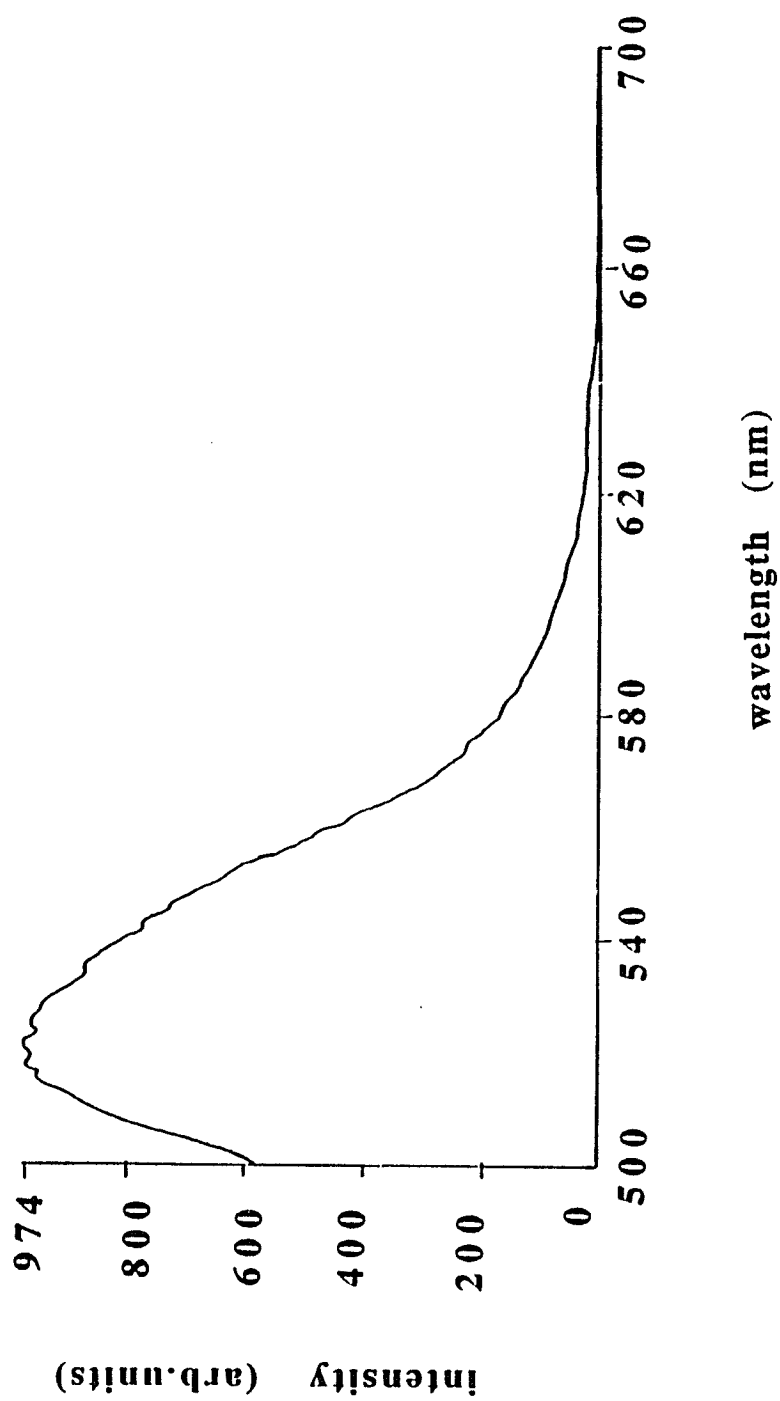


Figure 20. Fluorescence spectra of flavin mononucleotide (FMN) excited at 488nm.

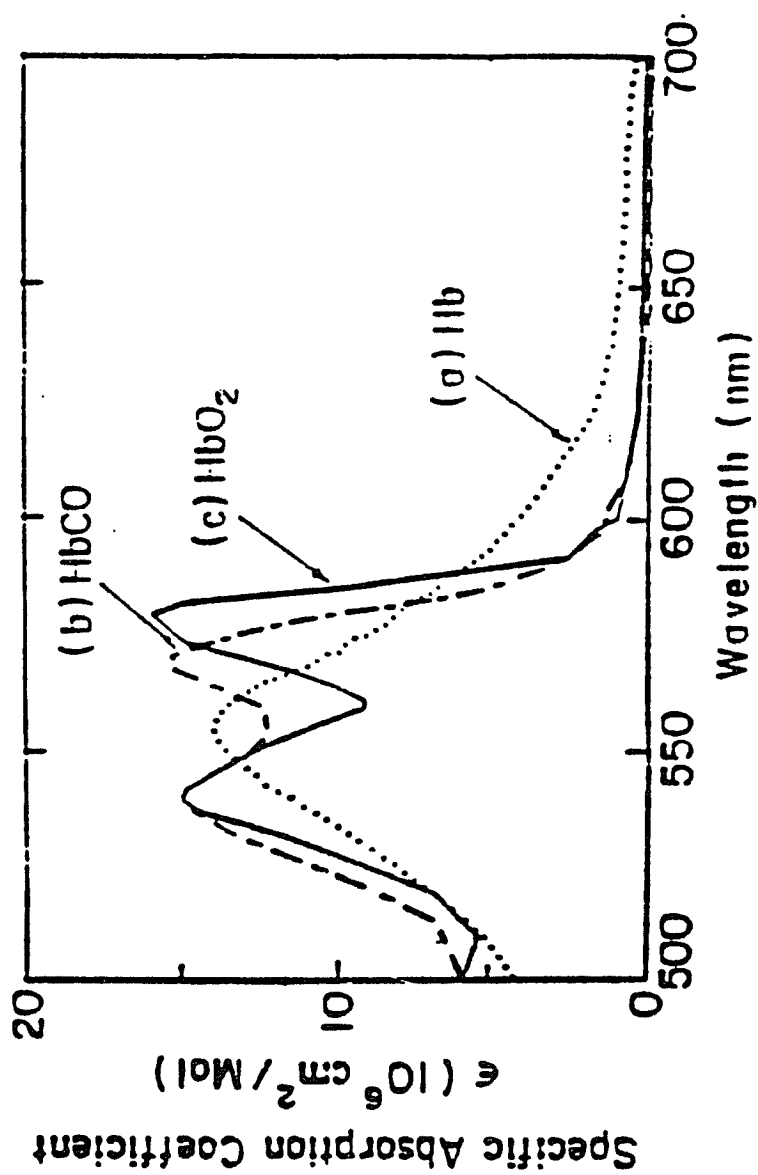


Figure 21. Absorption spectra from heme groups of (a) hemoglobin, (b) carboxyhemoglobin and (c) oxyhemoglobin.

peaks are closest to the dip positions of the normal tissue. It appears that oxyhemoglobin is the natural marker in tissues. A chemical analysis and a mathematical model have been used to prove that the dips observed in the normal spectra are due self absorption by hemoglobin.

(d) Chemical Analysis and Self Absorption:

It is known that hemoglobin exhibits absorption peaks at 540nm and 580nm. As seen from the experiments, the normal samples appear to contain more hemoglobin and the spectral profiles display dips at 540nm and 580nm with 488nm excitation. The dips coinciding with the absorption peaks of hemoglobin imply that the fluorescence may be absorbed at these emission wavelengths by the hemoglobin chromophore.

To explore the origin of the dips which may be due to hemoglobin, the normal breast tissue samples were chemically treated with ammonium chloride solution (the concentration of NH_4Cl was 0.8mg/mliter with original pH 7). This is a well-known procedure to extract blood from tissues [37] using ammonium chloride solution. The spectra of the normal tissues were measured after they were placed in the NH_4Cl solution for various lengths of time. The spectra were measured after they were removed from the solution. The experimental results are shown in Figure 22. The spectrum from the normal tissue before the tissue was placed in the solution, is shown in Fig.22 (a). The spectrum of the normal tissue removed from the solution after two hours is shown in Figure 22 (b). It should be pointed out that the spectral

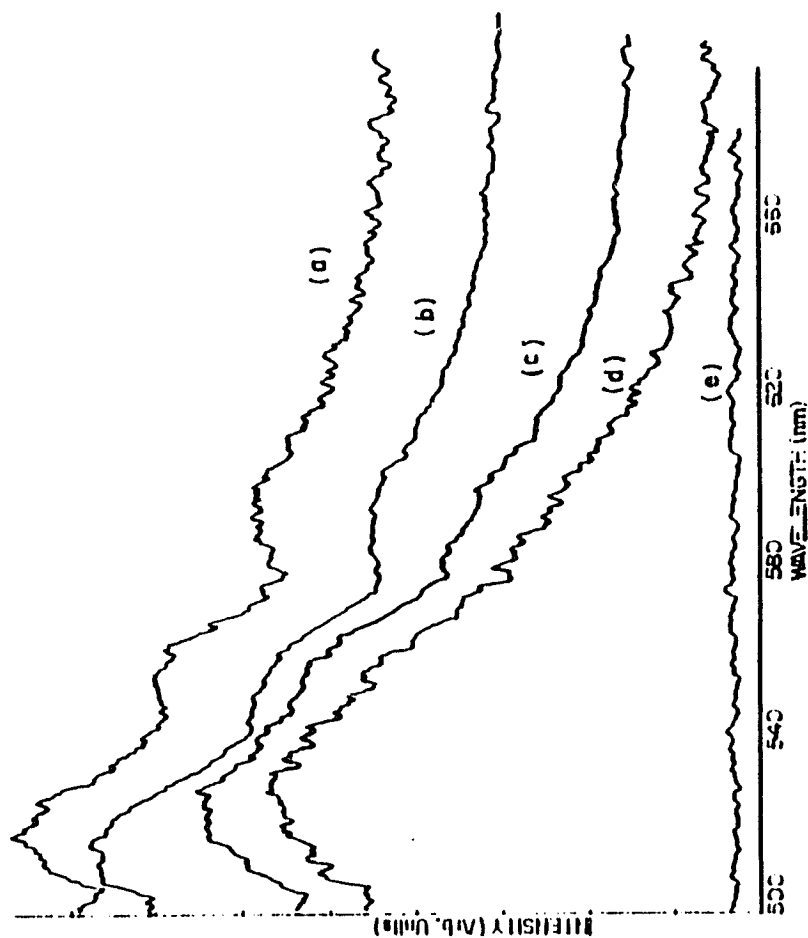


Figure 22. Steady state fluorescence spectra excited at 488nm to explore the origin of the spectral differences between normal and cancer human breast tissues: (a) the spectrum emitted from the original normal tissue (no chemical treatment); (b) the spectrum emitted from the normal tissue with blood extracted using NH_4Cl solution for 2 hours, the intensity is reduced by 4; (c) the spectrum emitted from the normal tissue with blood extracted by putting the tissue in NH_4Cl solution for 21 hours, the intensity reduction is by 4; (d) the spectrum emitted from the cancer tissue (no chemical treatment); (e) the spectrum emitted from NH_4Cl solution.

intensity of the tissue with blood extracted out is four times stronger compared with the original tissue spectrum shown in (a). Figure 22 (c) shows the spectrum after being placed in the solution for 21 hours. The spectral intensity of (c) is almost the same as (b). For reference, the spectrum of the spectrum of the cancer tissue is shown in Figure 22 (d). At the bottom of the figure, (e) displays a spectral curve of the NH_4Cl solution with no emission.

One notices that the normal breast tissue spectra have been significantly changed after extracting the hemoglobin from it. The original fluorescence spectra shows two subsidiary peaks at 555nm and 595nm. However, the spectral curves of the chemically treated tissues become smoother as the lengths of time in solution is increased. In addition, the spectral maximum of the tissues with blood extracted moves toward the peak of the cancer tissue spectrum. Both characteristics may be attributed to the reduction of tissue self-absorption on fluorescence due to reduction of hemoglobin. The cancer tissues, on the other hand, are grayish or whitish in appearance, implying the presence of much less blood. This is because the cancer samples used are taken from the core of the cancer where very little blood enters. The smooth profile of the cancer spectra is thus consistent with the self absorption model.

(e) Mathematical Analysis of Self Absorption on Fluorescence Spectrum:

To prove that hemoglobin is indeed the absorbing chromophore which affects the fluorescence spectrum in the normal tissue, a mathematical analysis was performed. It was assumed that self-absorption of hemoglobin

occurs, which affects the fluorescence by absorption. A mathematical model based on Foerster's theory of self absorption to calculate the effect of self absorption from heme-based molecules has been presented. Self absorption affects both the fluorescence spectral profiles and the recombination lifetimes of organic molecules [38]. A simple model is developed following Foerster's theory [39] where various molecules fluoresce and absorb this emission within the overall fluorescence profile. Secondary fluorescence emission and scattering are neglected in this simple approach. Monochromatic light of uniform intensity I_0 and wavelength λ_0 illuminates the entire front face area. After propagating a distance x , the exciting light intensity is reduced by the Beer-Lambert Law [40] to

$$I_x(\lambda_0) = I_0(\lambda_0) e^{-\gamma_0(\lambda_0)x} \quad (1)$$

where γ_0 is the absorption coefficient at the wavelength λ_0 .

The amount of excitation absorbed within a thickness dx is:

$$dI_x = -I_0 e^{-\gamma_0 x} \gamma_0 dx \quad (2)$$

Here $\gamma_0 = \gamma_{\text{chromophore}} + \gamma_{\text{fluorophore}}$ is the absorption from chromophore and the fluorophore.

Eqn.2 reduces to

$$dI_x = -I_0 e^{-\gamma_0 x} \gamma_{\text{ch}} dx + I_0 e^{-\gamma_0 x} \gamma_{\text{fl}} dx$$

$$= dI_{ch} + dI_{FI}$$

The fluorescence emitted from dx at a distance x from the surface is proportional to only dI_{FI} . The fluorescence with bandwidth $d\lambda_f$ is given by

$$d^2 I_f(\lambda_f, x) = \frac{F_R \Omega}{4\pi n^2} I_0 L(\lambda_f) \Phi \gamma_{FI} e^{-(\gamma_0 + \gamma_f)x} dx d\lambda_f \quad (3)$$

where F_R is the reflection loss, $\Omega/4\pi$ is the solid angle, Φ is the quantum efficiency, n is the index of refraction, $\gamma_f(\lambda_f)$ is the self absorption coefficient at luminescence wavelength λ_f , and $L(\lambda_f)$ is the spectral distribution which is independent of the exciting wavelength.

The fluorescence intensity detected from the front surface of a sample of thickness d within bandwidth $d\lambda_f$ is obtained by integrating Eqn. (3) from 0 to d which gives

$$dI(\lambda_f) = \frac{F_R \Omega}{4\pi n^2} I_0 L(\lambda_f) \Phi \frac{\gamma_{FI}}{\gamma_0 + \gamma_f} (1 - e^{-(\gamma_0 + \gamma_f)d}) d\lambda_f \quad (4)$$

The fluorescence profile from various fluorophore components is $L_i(\lambda_f)$, where L_i is given by the Lorentzian line-shape function for the i th fluorophore

$$L_i(\lambda_f) = \frac{1}{2\pi} \frac{d\lambda_{f,i}/\lambda_f^2}{[(\frac{d\lambda_{f,i}}{2\lambda_f^2})^2 + (\frac{1}{\lambda_f} - \frac{1}{\lambda_{m,i}})^2]} \quad (5)$$

with parameters $\lambda_{m,i}$ is the center wavelength of the i th peak, $d\lambda_{f,i}$ is the bandwidth of the i th peak, and λ_f is the fluorescence wavelength.

Consequently, the fluorescence profile from several fluorophore components becomes

$$dI(\lambda_f) = \sum_i \frac{F_R \Omega}{4\pi n^2} I_0 L_i(\lambda_f) \Phi \frac{\gamma_{F1}}{(\gamma_0 + \gamma_f)} (1 - e^{-(\gamma_0 + \gamma_f)d}) d\lambda_f \quad (6)$$

The following parameters are defined for computational convenience:

$$A(\Phi, \gamma_0, \eta) \equiv (F_R \Omega / 8\pi^2 n^2) I_0 \Phi \gamma_{F1}, \quad B_{i,\lambda_f} = d\lambda_{f,i}, \quad D = (1 - e^{-\gamma_0 d}) / \gamma_0, \quad \gamma = \gamma_0 + \gamma_f$$

The fluorescence profile including self-absorption becomes

$$I_D(\lambda_f) = A \sum_i B_{i,\lambda_f} L_i(\lambda_f) \frac{1 - e^{-\gamma d}}{\gamma} \quad (7)$$

Without self-absorption ($\gamma_f(\lambda_f) = 0$), the intensity of fluorescence reduces to

$$I_F(\lambda_f) = AD \sum_i B_{i,\lambda_f} L_i(\lambda_f) \quad (8)$$

where I_F is defined as the intrinsic fluorescence profile.

The intensity of the fluorescence at λ_f depends on the self-absorption spectra $\gamma_f(\lambda_f)$. Eqns. (7) and (8) give

$$I_F(1 - e^{-\gamma d}) - \gamma D I_D = 0$$

The numerical solution for $\gamma_f(\lambda_f)$ can be obtained from solving eqn.9. The fluorescence spectrum with and without self-absorption can be calculated from the above equations.

The density of normal breast tissue was estimated to be 0.87 gms/cc. Using the above density value and the RBC concentration in normal breast tissue, $C_{rbc} \sim 4.2 \times 10^{-3}$ cc/gm, the concentration of hemoglobin in the breast tissue was estimated to be $C_{heme} \sim 2.2 \times 10^{-8}$ Mol/cc [41,42,43]. From these data, the amount of hemoglobin in various organs are in the range $\sim 10^{-7}$ Mol/cc. It appears in our model that the concentration of hemoglobin $C_{hemo} \sim 10^{-6}$ Mol/cc for $d=1\text{mm}$ or $C_{hemo} \sim 10^{-7}$ Mol/cc for $d=1\text{cm}$. in tissues are needed to reasonably fit the data.

To analyze the effects of self absorption on the fluorescence spectra from normal tissue, the spectra from cancerous tissue were taken as 'intrinsic', since they have no structure and are assumed to have weak self absorption from heme groups. The fluorescence spectra of normal tissues are calculated using Eqn.(7) from the experimental profiles of the cancerous tissue. These theoretical spectral profiles are summarized in Figure 23 and 24 using three kinds of heme absorption spectra (Figure 21). It is evident from here that the complex fluorescence spectral profile from normal tissues arises in part by self absorption arising from hemoglobins.

Part of the two peaks observed in normal tissue may also arise from two different molecules fluorescing and phosphorescing at these wavelengths.

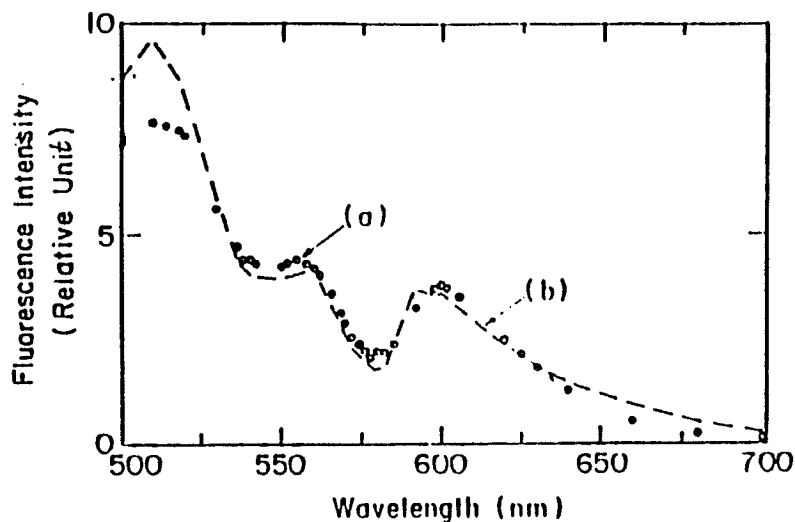


Figure 23. Experimental (a) and calculated (b) fluorescence spectral profiles emitted from normal tissue, for fitting parameters: $d=2\text{mm}$, and using the self absorption spectrum of HbO_2 (in figure 18) at the concentration $C_{\text{HbO}_2} = 1.0 \times 10^{-6}\text{M/cc}$. In the calculation, the intrinsic spectral profile was used with fitting parameters $d\lambda_{f,i} = 110\text{nm}$, $\lambda_{m,i} = 520\text{nm}$.

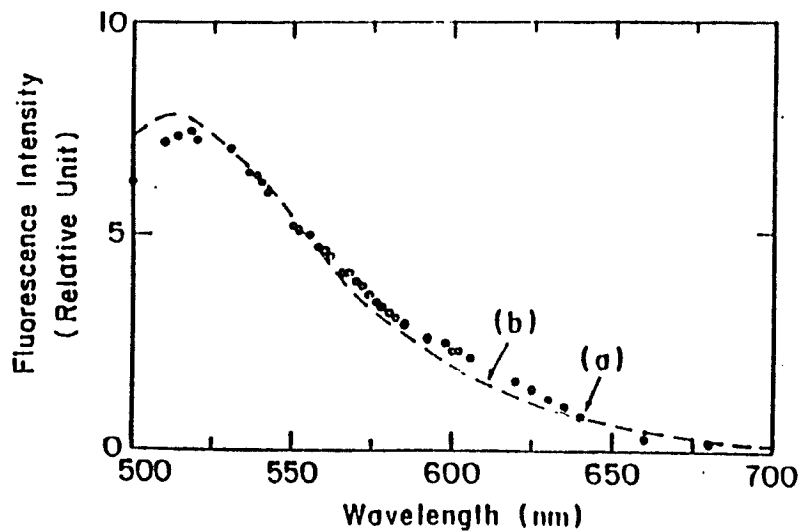


Figure 24. Experimental (a) and Calculated (b) spectral profiles from the cancerous tissue with fitting parameters $d\lambda_{f,i} = 110\text{nm}$, $\lambda_{m,i} = 520\text{nm}$.

Possible sources of emission at the two secondary peaks may be melanin (which fluoresces at 540nm), phospholipids (which fluoresce around 540-560nm), carotenoids or porphyrins (fluorescence peak at 600nm). Partial contribution from these fluorophores could cause the peaks in the normal breast tissues. The fluorophore responsible for the main peak of the normal tissue could give rise to the emission profile of the tumor tissue with no contribution from the other fluorophores responsible for the secondary peaks. But this has not yet been confirmed or pursued. It has been established that the dips are partially caused by self-absorption due to hemoglobin.

(f) Raman Peaks in Fluorescence of Normal Tissue:

It was noticed that the Raman peaks were observed only in normal samples with more fat and less blood. Also, the cancer samples did not display any Raman peaks. The positions of the Raman shifts indicate that these could be due to different molecules or the stretching and bending of certain bonds. The porphyrin ring of the heme proteins exhibit vibrational mode frequencies in the range of 1000-1650 cm^{-1} . Amide I, II, III exhibit peaks at 1650, 1550 and 1250 cm^{-1} respectively and may contribute to these peaks observed. Thus, the porphyrin rings and amide group could give rise to the Raman peaks observed between 1037 cm^{-1} and 1600 cm^{-1} in the normal breast tissues. The 2761 and 2963 cm^{-1} are most likely CH_3 modes. The fluorescence intensity of four fold or more in typeII normal tissue and cancer tissue overwhelms the observation of any Raman bands in those tissues. Further

work in the IR region is being performed to determine the source of these Raman peaks by other members of the group at IUSL.

(g) Disadvantages of the Steady State Fluorescence Technique with Visible Excitation:

The fact that hemoglobin is the marker distinguishing normal and cancer breast samples makes this method less reliable for diagnosis. This was observed from the equivocal results obtained (figs.25 (i) and (ii)). Figure 25 (i) displays the fluorescence spectra with 488nm excitation of (a) human tumor breast tissue with Raman peaks and (b) the same tumor tissue without Raman peaks at a different location. Figure 25 (ii) shows the fluorescence spectra with 488nm excitation of (a) human normal breast tissue without secondary peaks and (b) human tumor breast tissue. The presence of blood in cancer samples during cutting could lead to wrong and inaccurate diagnosis and results. In the case of normal samples, there are parts of the sample which are white in color and again, the results would be opposite. This drawback was observed when the method was extended to distinguish benign tissues and tumor from malignant tumors.

The spectra from both benign and malignant tissues were monotonically decreasing with peaks at 500-520nm with 488nm excitation. A few of the benign samples displayed subsidiary peaks due to the presence of blood in the tissues. The benign samples obtained appeared white. Figure 26 displays the two smooth profiles of the average of 7 benign tumors and 5

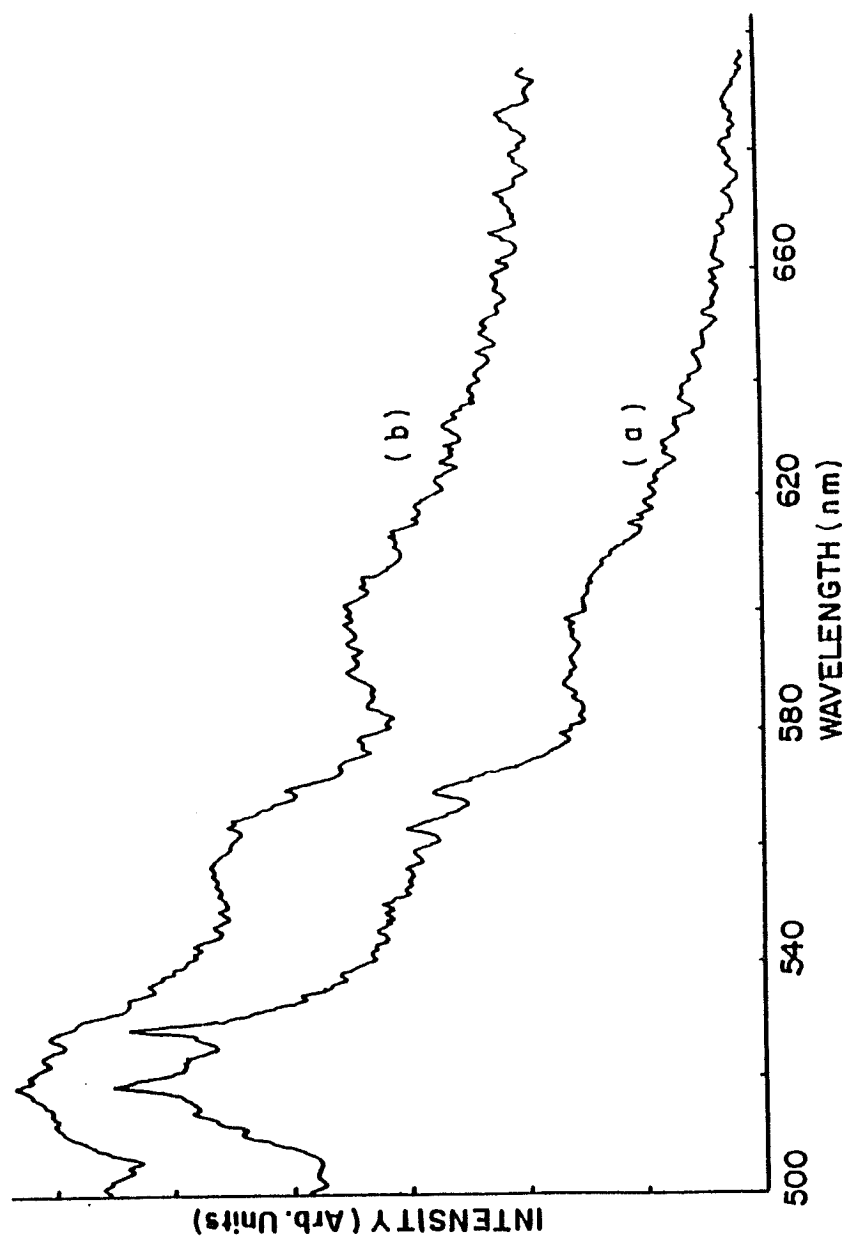


Figure 25. (i) Fluorescence spectra excited at 488nm from (a) human tumor breast tissue with Raman peaks and (b) the same tumor tissue without Raman peaks at a different location.

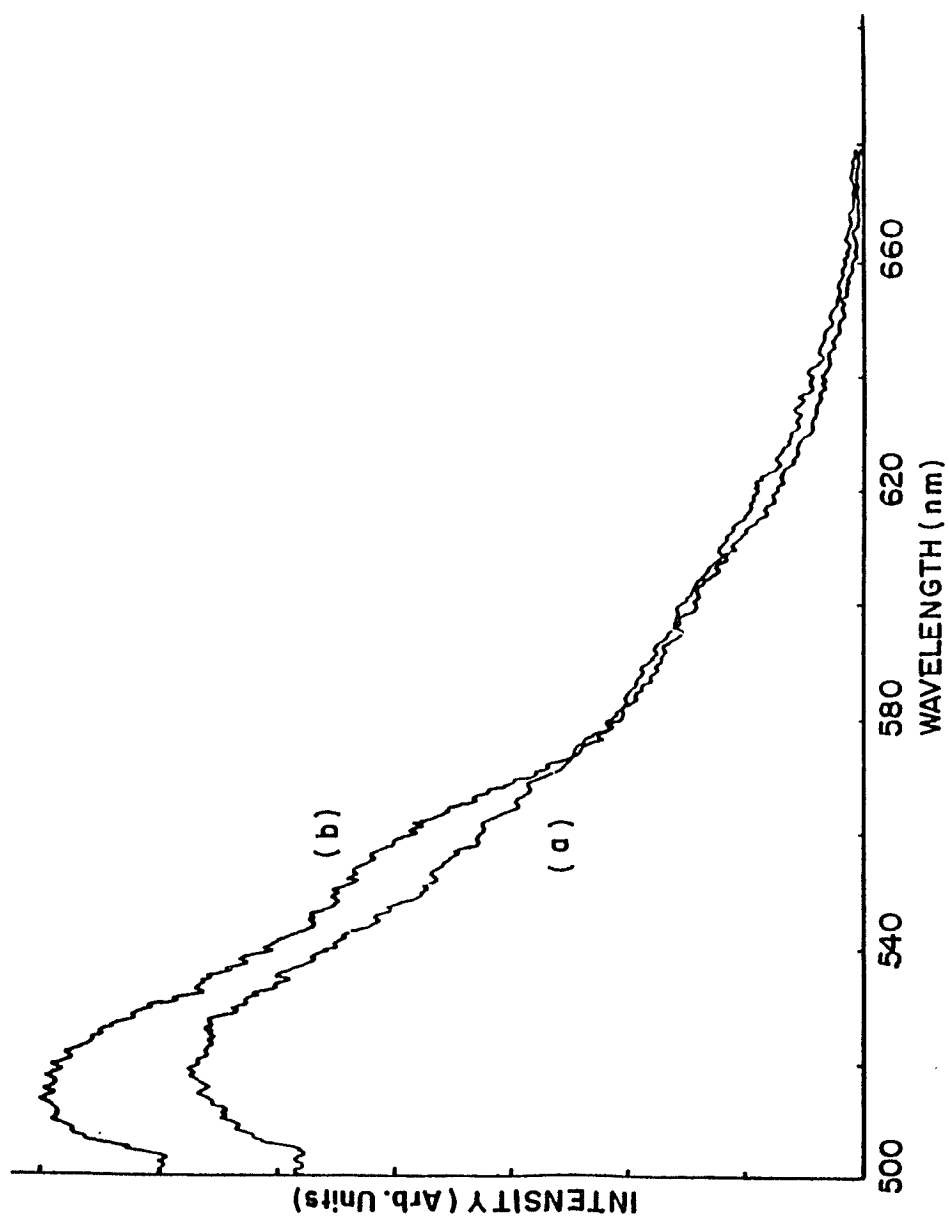


Figure 25 (ii) Fluorescence spectra excited at 488nm from (a) human normal breast tissue without secondary peaks and (b) human tumor breast tissue.

malignant tumors and the difference between the two. Distinguishing benign tumors from malignant tumors is important if optical methods are to be used to diagnose cancer. However, it was seen that this method was not useful to distinguish the two types of neoplasms. This created a problem that was not anticipated and hence this method could not be used for diagnosis using visible excitation.

Steady state fluorescence spectra of non-malignant and malignant breast tissues have also been measured with 351nm excitation using a Perkin-Elmer spectrofluorometer. The spectrum spans a bandwidth of 370nm to 600nm. Non-malignant and malignant tissues do not show any significant reliable spectral differences with this excitation wavelength.

Studies in this area have been pursued by other members of IUSL, extending to the UV region. Fluorescence spectra of human breast normal tissues, benign tissues and tumors and malignant tumors have been measured with 300nm excitation. The ratio of intensities, 340/440nm was found to be significantly different for malignant tumors as compared to normal tissues, benign tissues and tumors. This study shows that using the key excitation wavelength and emission wavelengths gives rise to an important diagnostic approach [44].

SECTION III

EXCITATION SPECTROSCOPY

Introduction:

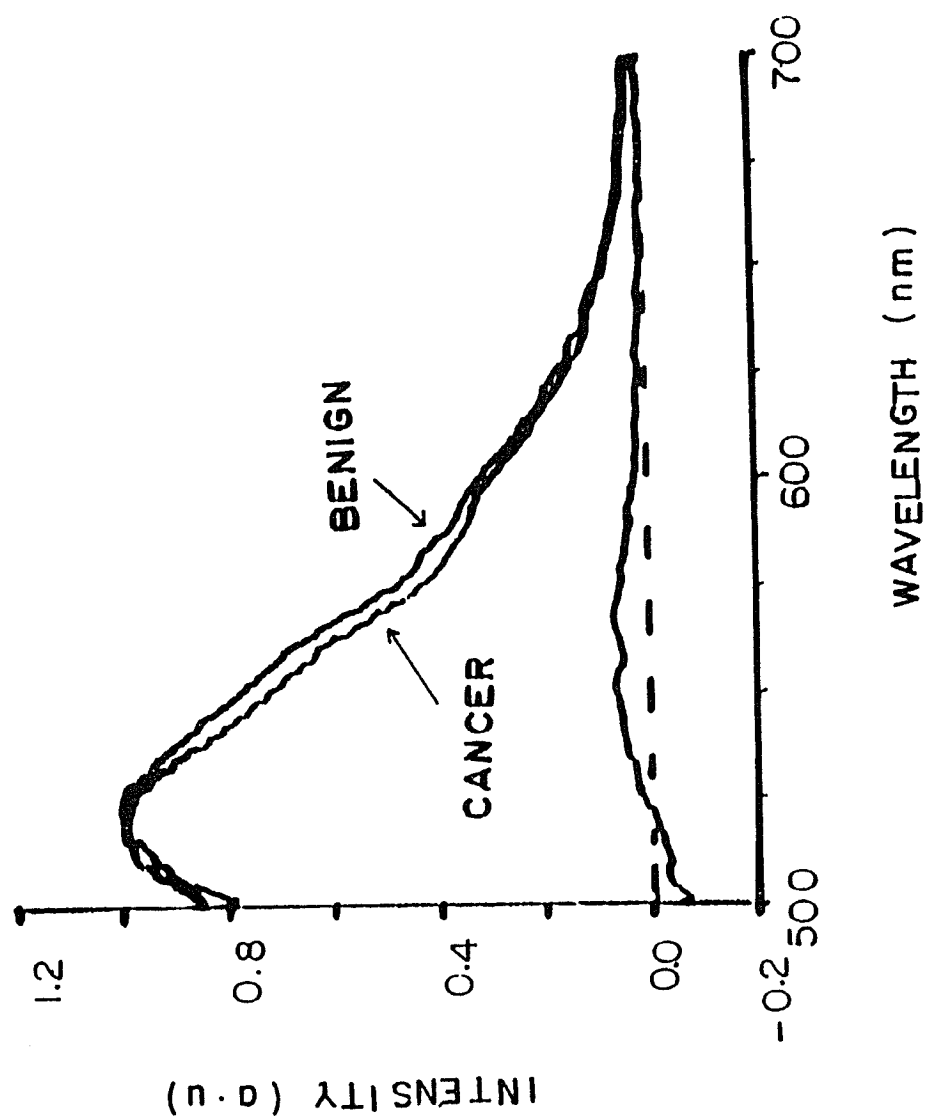


Figure 26. Fluorescence spectra of average of 7 benign tumors and 5 malignant tumors and their differences for 488nm excitation.

Excitation spectra is an important spectroscopic method to investigate the relative intensity efficiency for different wavelengths of excitation radiation to produce a fluorescence wavelength from a sample and possible tool for diagnosis. This method has been extensively used by chemists and biologists in studying photochemistry and photobiology [45]. It is also a useful tool to probe biological and medical samples to identify the emitting species. The shape of the excitation spectra usually follows that of the absorption spectra of the molecule that is emitting at the measured emission wavelength. On the other hand, the fluorescence emission spectrum shows the relative intensity of radiation emitted at various wavelengths upon photoexcitation. The excitation spectra is obtained by scanning the excitation wavelengths at one fixed emission wavelength.

Results:

The fluorescence spectra shown in Figs. 12, 13 and 14 of normal breast tissues displays three peaks, around 520nm, 550nm and 600nm. These peaks could be due to three different emitting molecules or due to only one molecule with environmental influences distorting the smooth profile as has been discussed earlier. The smooth profile of the cancer sample could be due to the same molecule as that of the normal sample or a different one with different environmental conditions. To determine this effect, the excitation spectra of the normal and cancer samples have been measured at emission wavelengths of 520nm, 550nm and 600nm.

The excitation spectra of normal and cancer breast tissues are displayed in Figures 27 (a) and (b), 28 (a) and (b) and 29 (a) and (b) respectively for the three emission wavelengths. The excitation spectra consist of two bands, one in the UV and the other in the visible. The UV bands in the normal tissue spectra in curves (a) of Figs 27 and 28 consist of sharp peaks located at 336nm, 352nm and 371nm. The major peak is located at 352nm. However, there are no clear sharp peaks in the UV bands of the tumor tissue spectra, as can be seen in curves (b) of the figures. The main peak in the visible band of the excitation spectra in the curves (a) of Figures 27 and 28 for the normal tissue is located at 473nm. The intensities of the visible band are approximately four times weaker than that of the UV bands. However, in the case of tumor tissues, in curves (b) of Figures 27 and 28 the intensities of the visible bands are higher than that of the UV bands. Figures 29 (a) and (b) display the excitation spectra of the normal and cancer tissues at 600nm emission. Differences are noticed here, too. The 600nm excitation spectra appears to be different from the other two emissions. This also is different from the excitation spectra of FMN. This could imply that the third peak observed in the emission spectra may be from a different source such as porphyrins, which fluoresce in that wavelength range.

Discussion:

The excitation spectra of normal and cancer samples at 520nm and 550nm emission both display differences in the visible region. The normal

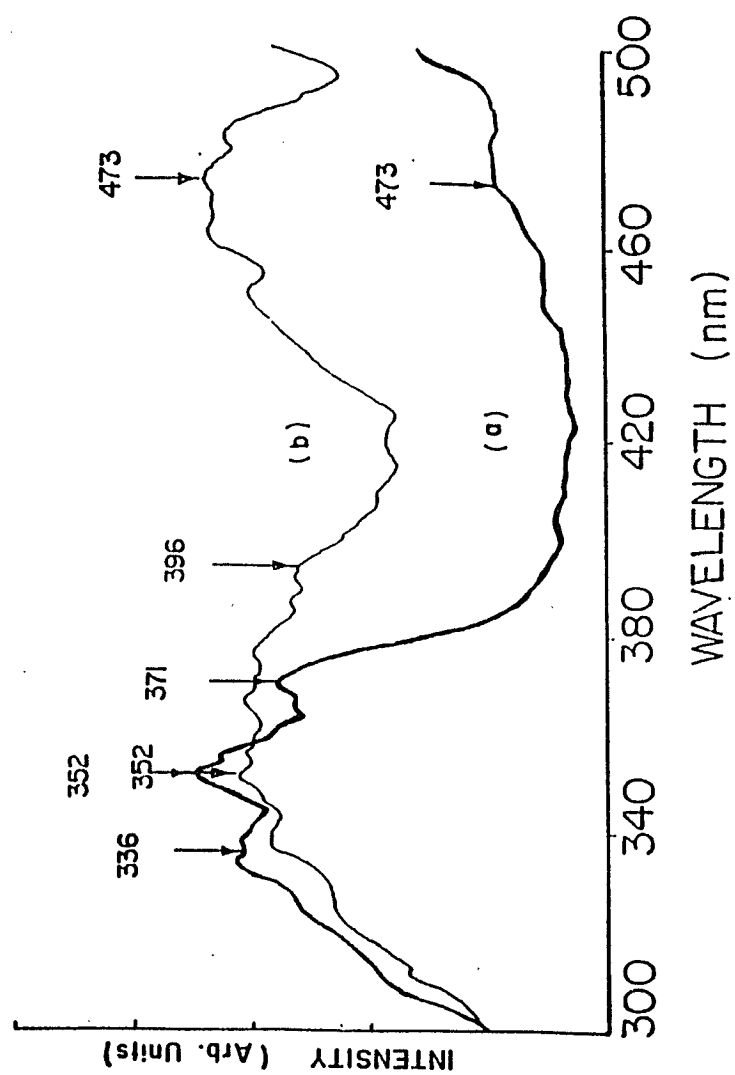


Figure 27. Excitation spectra measured at 520 nm from (a) human normal breast tissue and (b) human tumor breast tissue.

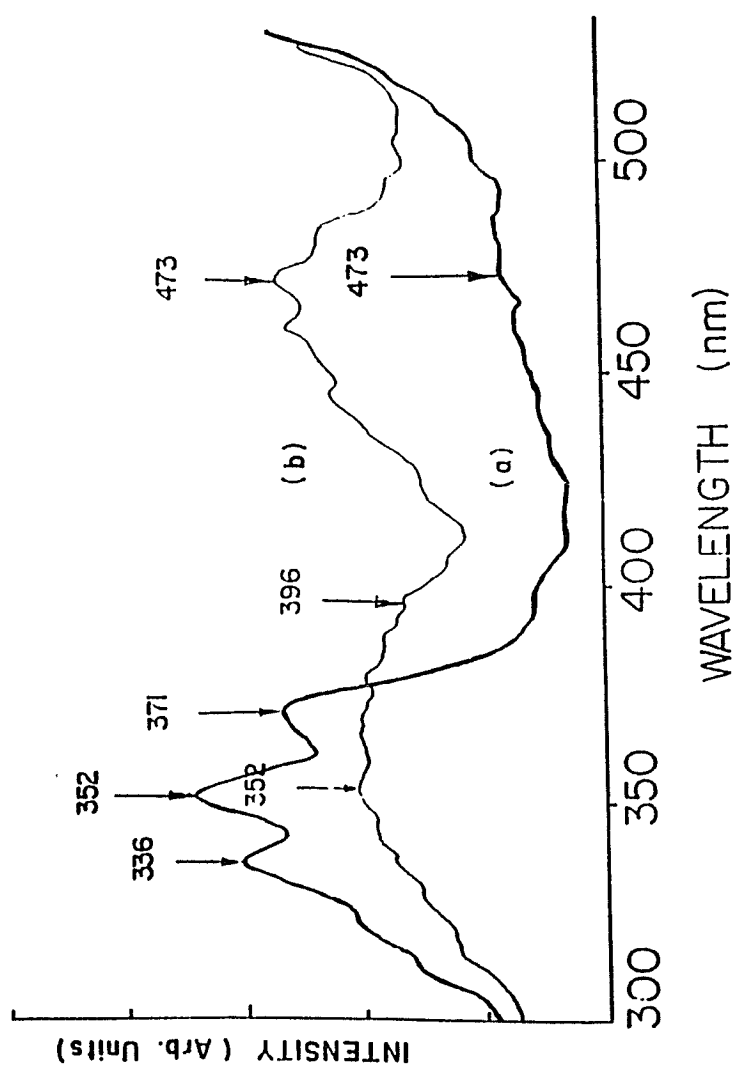


Figure 28. Excitation spectra measured at 550 nm from (a) human normal breast tissue and (b) human tumor breast tissue.

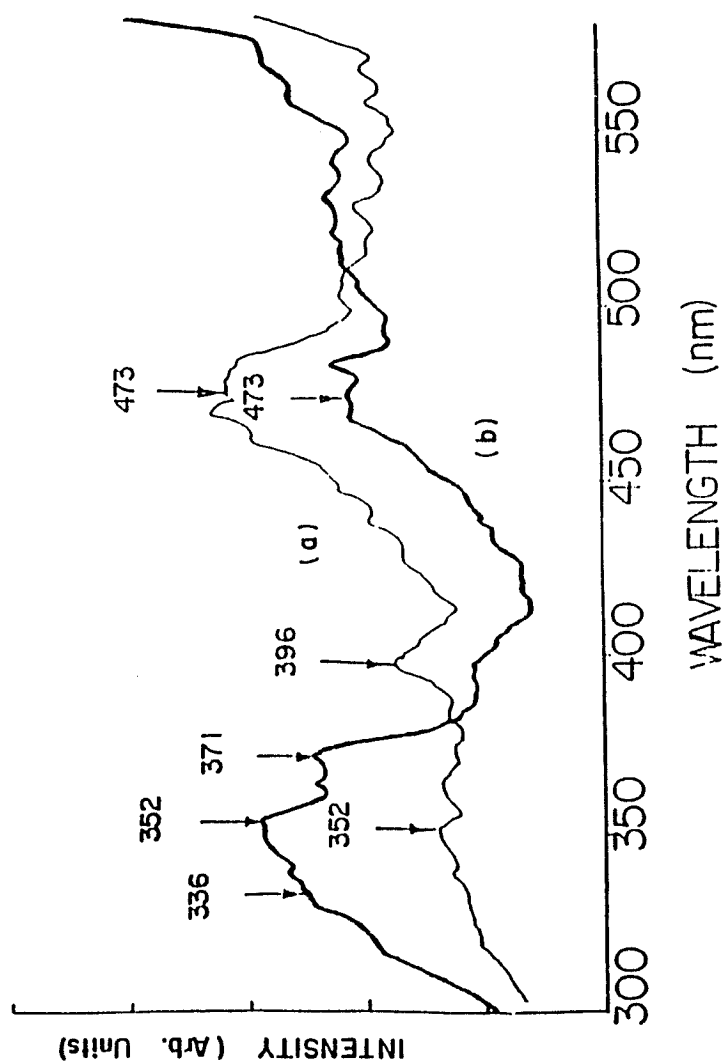


Figure 29. Excitation spectra measured at 600 nm from (a) human normal breast tissue and (b) human tumor breast tissue.

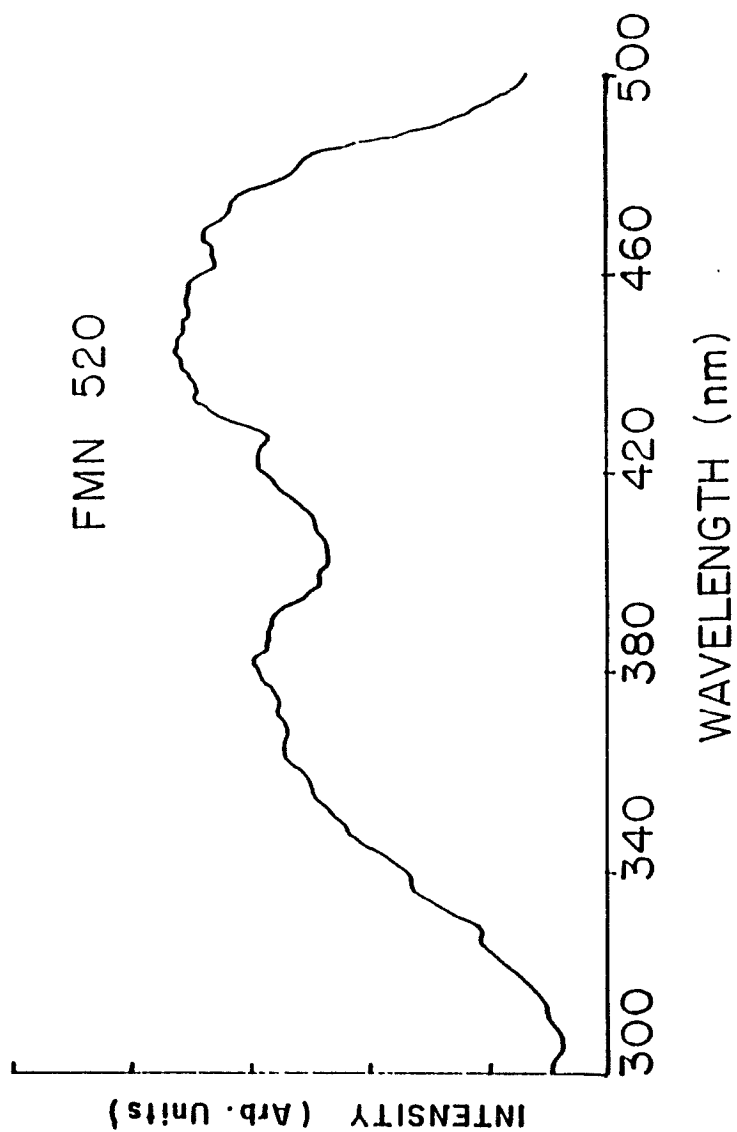


Figure30. Excitation spectra measured at 520 nm from Flavin Mononucleotide (FMN)

tissue fluorescence is weaker with visible excitation than the cancer fluorescence. This result is consistent with the self absorption of hemoglobin explained in Section II(d) and (e). In this case, the visible excitation light may be absorbed by hemoglobin since it has a strong absorption band at 400-480nm. The 520nm and 550nm emission are weaker than that of the cancer due to self absorption of hemoglobin in the 520-580nm range. Flavins are thought to be the main emitting molecules with 488nm excitation in the normal and cancer tissues centered at 520nm. This led to the measurements of the excitation spectra of flavin mononucleotide (FMN) in solution (fig.30). The excitation spectra was found to be strikingly similar to that of the tumor tissue with two broad bands in the visible and the UV. These flavins are free and in solution whereas in tissues they are known to be bound to proteins and present as flavoproteins. This similarity between free flavin and tumor tissue could imply that flavins in tumor tissues could be more free than the bound flavins of normal tissues. The 600nm band could be due to a different molecule since the excitation spectra of breast tissues centered around 600nm emission appear to be different from those centered around 520nm and 550nm emissions.

The excitation spectra study confirms that the self absorption theory of hemoglobin (blood) plays a major role in the shape of the visible spectrum. It also shows that flavins are most likely the emitting species in the visible region in the normal and cancer tissues with 488nm excitation and the differences in the two could be due to the different environments in which

flavins are present in the tissues.

SECTION IV

PULSED LUMINESCENCE SPECTROSCOPY

The fluorescence spectra of normal and cancer breast tissues have been measured using visible excitation and continuous wave light. Pulsed luminescence spectroscopy has been performed with the aim of detecting any long-lived emission. With steady state methods, such an event cannot be isolated. Electronic or mechanical gating can be used to observe delayed fluorescence or phosphorescence in the 100 nsec region. This idea led to the study of emission spectra of normal and cancer breast tissues with pulsed laser. The pulsed laser luminescence from these tissues at low temperatures is discussed in Chapter 5.

To study the transient fluorescence spectra the normal and cancer breast tissue samples were excited with a pulsed laser at 527nm and 351nm and fluorescence investigated from 400 to 740nm. Thus, the fluorescence was also probed with near-UV excitation.

Results:

The typical pulsed fluorescence spectra from normal and cancer human breast tissue photoexcited by the third harmonic pulse at 351nm [11] of a picosecond Nd-glass mode-locked laser are shown in Figure 31. The cancer breast tissue spectrum in the 430-590nm region is shown in Figure 31 (a). The

main peak is located at 464nm. The normal tissue spectrum which peaks at 500nm is shown in Figure 31 (b). The spectral intensity from the normal and cancer breast tissue photoexcited at 351nm in the 600-700nm region is small as shown in Fig.33. No peaks are observed in this region (600-700nm) for cancer or normal tissues. A note of caution here: care has been taken to eliminate the second order and higher order spectra of the grating occurring at 702nm from the leakage of the excitation wavelength at 351nm, which results from scattering inside the grating spectrometer. Any peak that did show up, disappeared after one or more Corning 3-73 filters, which have high transmission in the 600-760nm region and no transmission in the UV band, are inserted in the fluorescence pathway. Figure 32 shows the typical normal and cancer breast tissue spectra excited at 527nm [11]. Figures 32 (a) and (b) represent the cancer and normal breast tissue spectra in the 493-653nm region, respectively. The onset of the fluorescence is convoluted with the onset transmission characteristics of the 3-67 filters which are placed in the emission path to cut off the scattering from the excitation light. One notices that the normal tissue spectrum has a dip at 576nm while the cancer tissue spectrum is smooth. The normal and cancer tissue spectra with 527nm excitation in the region from 620nm and 760nm are displayed in Figure 33. We notice that no structure was observed from either normal or cancer tissue in this region (620-760nm).

Discussion:

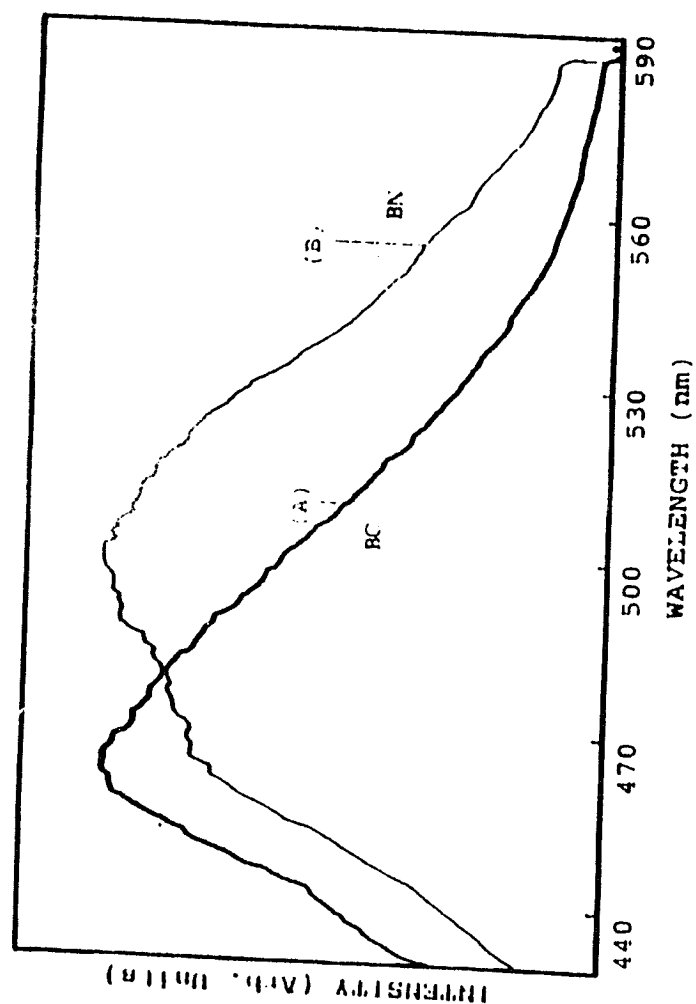


Figure 31. Pulsed fluorescence spectra in the 430-590 nm region excited by the third harmonic at 351 nm of a picosecond mode-locked Nd-glass laser from (A) the cancer human breast tissue and (B) the normal human breast tissue.

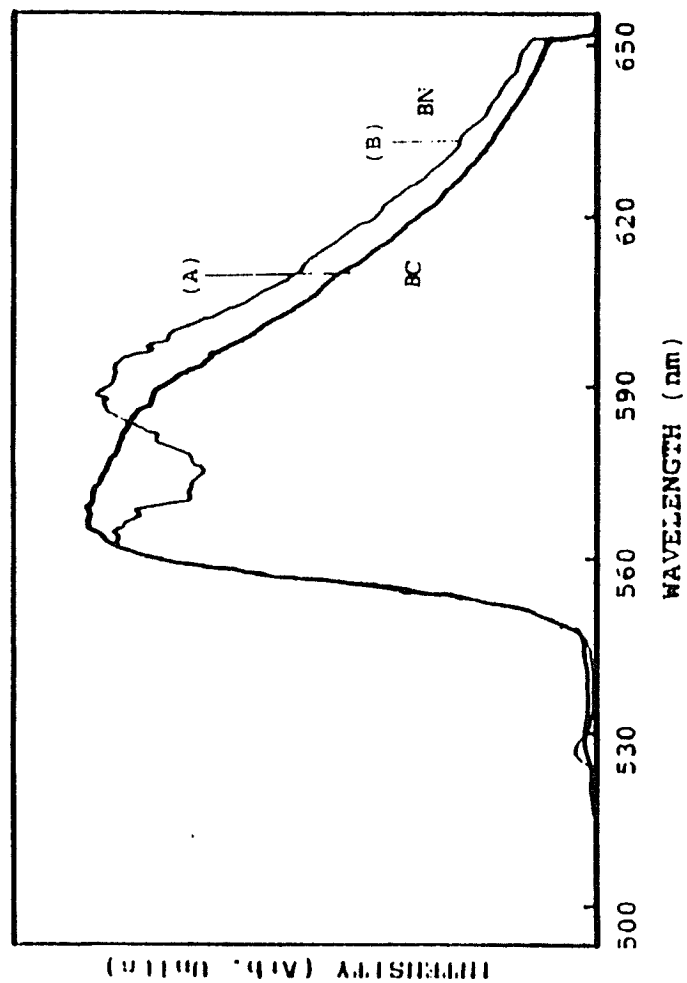


Figure 32. Pulsed fluorescence spectra in the 493-653 nm region excited by the third harmonic at 527 nm of a picosecond mode-locked Nd-glass laser from (A) the cancer human breast tissue and (B) the normal human breast tissue.

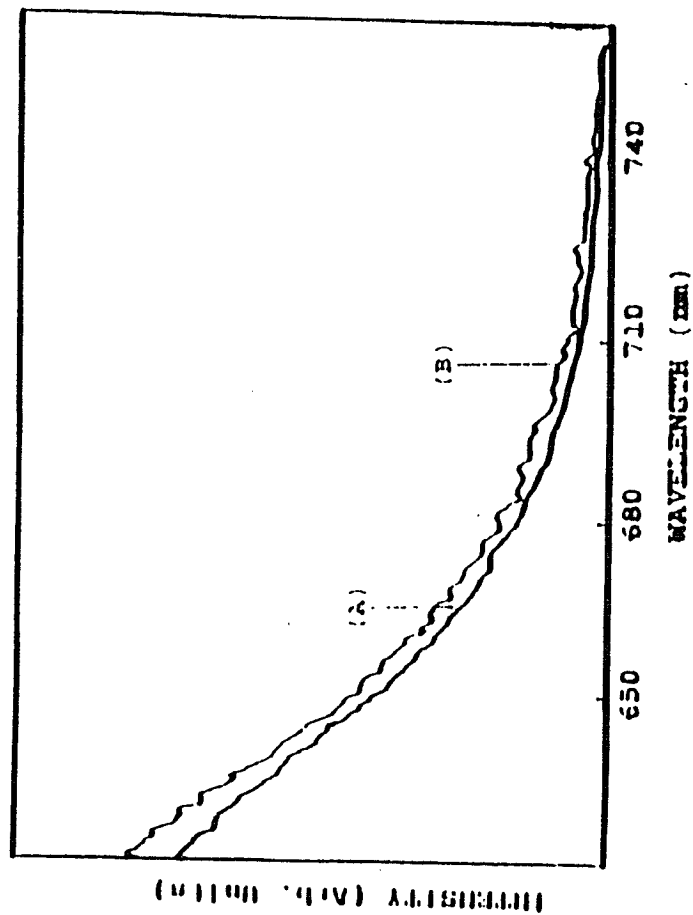


Figure 33. Pulsed fluorescence spectra in the 620-760 nm region excited by the third harmonic at 527 nm of a picosecond mode-locked Nd-glass laser from (A) the cancer human breast tissue and (B) the normal human breast tissue.

The fluorescence from normal and cancer breast tissue with 351nm excitation peaked around 460nm. It is known that certain coenzymes, NADH, fluoresce around 460nm and their absorption peak is around 350nm. Since the excitation is 351nm, NADH fluorescence most likely dominates in the 460nm emission region. Flavin fluorescence may be present but NADH fluorescence will be stronger here.

From the figures 31 and 32 one notices that the spectral profiles and peak locations of the normal and cancer breast tissues are different. The experimental results with 351nm excitation indicate a 36nm blue shift from the normal to the cancer spectra. The cancer tissue spectra are smoother than the normal spectra. The normal tissue spectra have a shoulder and a wide valley around 490nm. The cancer tissues emit more light in the shorter wavelength direction compared with the normal tissues. The observed spectral difference between normal and cancer tissue can be attributed to the greater self-absorption of hemoglobin in the normal tissue. The normal tissue has a strong absorption band from 380-480nm due to hemoglobin. This strong absorption band can give rise to the stronger self-absorption of fluorescence in the shorter wavelength direction of the intrinsic fluorescence in the normal tissue. Therefore the net fluorescence peak shifts toward the longer wavelength. The converse of this statement is that the cancer tissue spectra shift to the shorter wavelength. As for the shoulder on the spectral curves of normal tissue, it may arise due to self-absorption.

No characteristic spectral peaks occurred in the region between 600-

760nm, which was observed by Yang et al [10] when either the normal or the cancer tissues were photoexcited by picosecond pulses at 351nm and 527nm.

Pulsed fluorescence from breast tissues again shows that the differences in normal and cancer tissues arises from self absorption effects from blood. Thus the pulsed laser spectroscopy does not give any additional information to the spectral characteristics of the normal and cancer tissues.

Summarizing, the native fluorescence from breast tissues is most likely from molecules such as flavins and NADH, when excited by 488nm of the CW laser and 351nm of the pulsed laser, respectively. The absorption of the fluorescence by hemoglobin (the main absorbing chromophore) gives rise to the observed large differences between normal and cancerous breast tissues. CW and transient pulse laser fluorescence both show results consistent with this theory of self absorption of blood.

This chapter draws attention to the fact that the environment of a certain molecule in a heterogenous biological system such as a human tissue can be probed by spectroscopic methods using native fluorescence of these molecules. This method can also be used as a diagnostic tool if a reliable marker at the key excitation wavelength is found.

Studies with fluorescence spectroscopy as a tool have been pursued by other members of IUSL, extending to the UV region. Fluorescence spectra of human breast normal tissues, benign tissues and tumors and malignant tumors have been measured with 300nm excitation [44]. The ratio of

intensities, 340/440nm was found to be significantly different for malignant tumors as compared to normal tissues, benign tissues and tumors. In order to obtain further information on the chromophores responsible for the emission and to have a better picture of the heterogeneous nature of the tissue fluorescence, the dynamics of the physical processes occurring in tissues needs to be studied. This idea is the main focus of the next chapter, which deals with time-resolved fluorescence from tissues with different excitation wavelengths, in particular 300nm.

CHAPTER 4: TIME-RESOLVED FLUORESCENCE

The fluorescence spectrum provides a sensitive tool for detecting small changes in the environment of chromophores in or associated with the proteins that make up a tissue. The time evolution of emission can also be utilized to evaluate the heterogeneous nature of the fluorescence from biological systems. The fluorescence lifetimes of human tissue, tissue proteins and individual moieties in different environments offers a potential way to help understand and interpret the dynamics and changes of fluorophores occurring inside the tissues in the presence of disease.

The dynamic behaviour of systems can be probed by measuring the lifetime of the fluorescence decay. The system may be characterized by its response to either a periodic perturbation of a purely sinusoidal amplitude of a certain frequency or an instantaneous perturbation. The former is the phase-shift method of determining the lifetime while the latter is the pulse method. The phase shift method has been used earlier but has its limitations [47,48,49]. The introduction of nanosecond impulses attracted considerable interest from scientists working in photophysics, photochemistry and photobiology.

By 1970, there was convincing evidence of the versatility and sensitivity of pulse fluorometry. In early 1970, picosecond spectroscopy techniques enabled scientists to study ultrafast processes in molecules directly and more

reliably. The physical processes of biomolecules and molecules present in complex biological systems occur in the nanosecond and picosecond range. Biomolecules fluoresce while some of their electronic states are being excited. If there occurs fast non-radiative processes (See Fig.9, k_{NR}) the quantum yield may be extremely low and fluorescence (See Fig.9, k_R) being weak, lasts for a very short time $\sim$ a picosecond. Thus fluorescence kinetics provide valuable information on the relaxation of the electronically excited states of biomolecules.

Fluorescence lifetimes of several chromophores such as amino acids and peptides, nucleic acids, enzymes and hemoproteins have been measured by other researchers [50]. The photophysics, photochemistry and photobiology of a few of these biomolecules are being studied for the understanding of the dynamics of various processes in biological systems.

Biological tissues are complex systems with several molecules in different environments. These heterogeneous systems have to be probed by picosecond time-resolved spectroscopy to obtain detailed information about the biomolecules and their environment. Steady state fluorescence spectroscopy gives an idea about the molecules and the environment. Composite heterogeneity of tissue fluorescence is difficult to separate using steady state methods, but using time-resolved spectroscopic techniques one may be able to isolate the participating biological fluorophores.

Molecules in simple systems obey exponential decays of fluorescence. Deviation from exponential forms of emission implies that there may be other

emitting species or other competing physical processes such as excitation energy transfer, energy transfer from molecules in the short and long ranges, depolarization effects due to fast molecular motion, proton transfer or diffusion-controlled processes. The molecule in the tissue may transfer energy to another molecule. It may have its own restricted motion. The fast component is sensitive to the environment. Thus it will change due to the different physical processes. A temporal analysis thus makes it possible to detect small changes in the environment of chromophores in tissues due to these physical processes. The aim of the study in this chapter is to characterise the fluorescence decay properties of normal and abnormal breast tissue and to try to obtain an insight into the various dynamics of the systems.

In this chapter, the fluorescence lifetimes of normal, benign and malignant breast tissues have been studied with different excitation wavelengths ranging in the visible, near-UV and the UV regions. The temporal results have shown one key excitation wavelength where the emission properties are significantly different for normal and malignant tissues. The temporal profiles are analyzed. Fluorescence lifetimes of various relevant biomolecules such as NADH, tryptophan, tyrosine, flavins, elastin and collagen have been measured to interpret the results obtained from tissue fluorescence.

The first three sections have been devoted to the time-resolved fluorescence of human breast tissues at the three excitation wavelengths. The

fourth section deals with the fluorescence rotational depolarization studies from breast tissues to understand the physical processes occurring in the tissues with UV excitation.

SECTION I

Time-resolved Fluorescence with 527nm excitation:

Fluorescence lifetimes of normal and cancer breast tissue have been measured using the second harmonic of a 8ps Nd-glass laser pulse. The fluorescence lifetimes were measured using a 10ps streak camera which has been described in Chapter 2.

The time-resolved fluorescence with 527nm excitation was performed on two types of breast tissues: normal, which are usually glandular tissues and lipomatous tissues, and malignant tissues, usually ductal carcinomas (See Chapter 3, Section I). The normal tissue may have some fibrous parts which were not distinguished as different from normal tissue. These parts were, in the later stage of the research, identified as benign tissue. The samples were obtained from biopsies, were fresh and not homogenized. They were obtained in chunks of about 5x5x5 mm³.

The fluorescence lifetimes are measured in two wavelength bands, namely > 550 nm, and 600 ± 20 nm. These bands were selected from the spectral properties of the samples which showed peaks at those wavelengths. The fluorescence lifetimes observed were non-exponential and have been fitted to a double exponential

$$I(t) = A_f \exp(-t/t_f) + A_s \exp(-t/t_s)$$

using the least square method of fitting. A_f is the pre-exponential factor for the fast component whose lifetime is given by t_f and A_s is the pre-exponential factor of the slow component lifetime t_s .

Results:

Figure 34 (a) and (b) show the temporal profiles of normal and cancerous breast tissues with 527nm excitation in the emission region > 550nm [51]. Both profiles display a fast component and a slow component when fitted to a double exponential decay. The fast and slow components of normal breast tissue in the >550nm band are 190ps and 1700ps as is displayed in Figure 34 (a). The corresponding lifetimes of the tumor tissue are 110ps and 1600ps respectively displayed in Figure 34 (b).

Table 3 displays three pairs of normal and cancer samples studied using this excitation and emission ranges >550nm and 600nm and the results are consistent. The fast component of the cancer appears to be faster than that of the normal. The slow components of both appear to be similar. The decay times are accurate within 25ps for the fast component and 100ps for the slow component. The results of emission greater than 550nm again display a fast component for cancer tissue faster than that of the normal tissue.

Discussion:

The fluorescence lifetimes for 527nm picosecond excitation indicate a

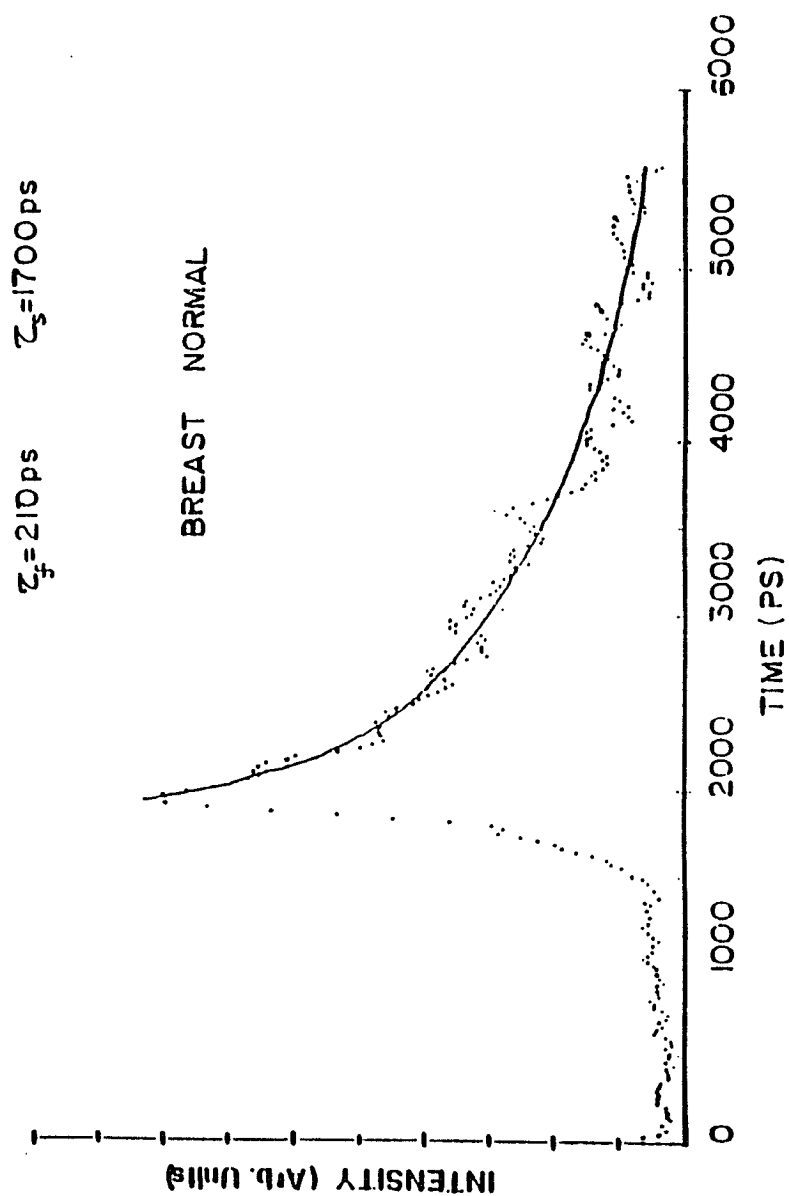


Figure 34 (a). Fluorescence lifetimes of human breast tissues excited at 527nm by the second harmonic of a Nd-glass laser of normal with $t_f = 190 \text{ ps}$, $t_s = 1.7 \text{ ns}$ in the $600 \pm 20 \text{ nm}$ emission band.

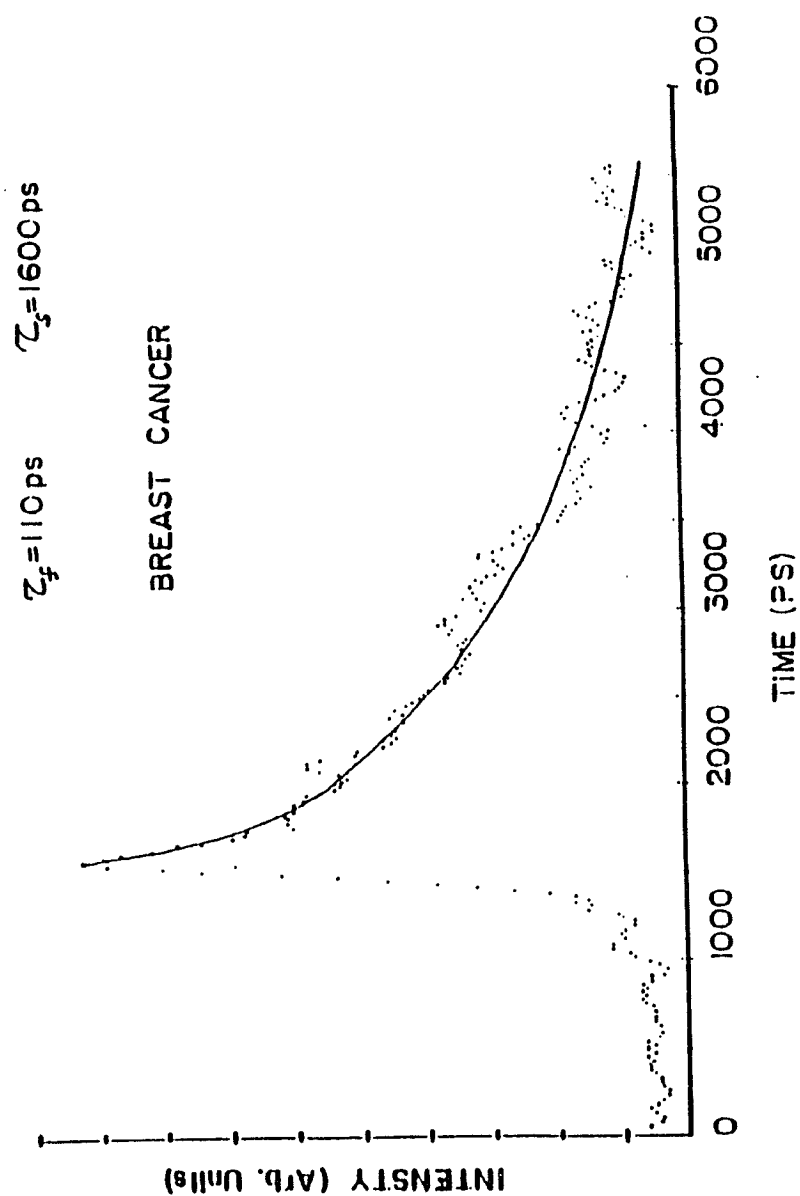


Figure 34 (b). Fluorescence lifetimes of human breast tissues excited at 527 nm by the second harmonic of a Nd-glass laser of malignant with $t_f = 110 \text{ ps}$, $t_s = 1.6 \text{ ns}$ in the $600 \pm 20 \text{ nm}$ emission band.

Table 3: Fluorescence lifetimes of normal and malignant human breast tissues with 527nm excitation.

SAMPLES	tf (ps)	ts (ns)	Wavelength
Breast Normal	190	1.6	> 550nm
Breast Malignant	110	1.6	> 550nm
Breast Normal	150	1.5	> 550nm
Breast Malignant	107	1.3	> 550nm
Breast Normal	210	1.7	600 + 20 nm
Breast Malignant	110	1.6	600 + 20 nm

difference in the fast component of lifetimes between normal and cancer breast tissues. The slow component does not change. It is known that the fluorescence lifetimes decrease with increase of non-radiative processes (See Fig.9).

A molecule is photoexcited from the ground state, S_0 to a higher vibrational level of the first excited state, S_1^* due to interaction with a photon of energy, $h\nu_L$.



From the higher vibrational level of S_1 , there first occurs non-radiative vibrational relaxation to the S_1 state. From S_1 there can occur radiative relaxation (k_R) to the S_0 state:



and non-radiative relaxation with rate constant, k_{NR} :



For delta pulse excitation, the rate of decay from the S_1 state depends on both the radiative (k_R) and non-radiative (k_{NR}) decay rates:

$$d[S_1]/dt = -(k_R + k_{NR}) [S_1] \text{ for } t > 0 \quad (4)$$

The solution is an exponential decay with initial condition $S_1(0) = [S_1]_0$:

$$[S_1] = [S_1]_0 \exp \{-(k_R + k_{NR})t\} \quad (5)$$

where k_R is the radiative decay rate and k_{NR} is the non-radiative decay rate.

The fluorescence intensity $I_F(t)$ is given by

$$I_F(t) \sim k_{rad} S_1 = k_{rad} [S_1]_0 \exp \{-(k_R + k_{NR})t\} \quad (6)$$

Here, fluorescence lifetime is given by

$$t_F = 1/(k_R + k_{NR}) \quad (7)$$

Hence the lifetime decreases if the non-radiative rate k_{NR} increases.

Typical $k_R \sim 10^8 s^{-1}$ which depends on the absorption coefficient.

Including k_v in the problem, with delta pulse,

$$dS_1/dt = -k_R S_1 - k_{NR} S_1 - K_{ST} S_1 + k_v S_1^* \quad (8)$$

$$S_1^* = [S_1]_0 \exp(-k_v t) \text{ with initial condition } S_1^*(0) = [S_1]_0$$

$$k_F = \Sigma k_i = k_R + k_{NR} + K_{ST} \quad (9)$$

The solution is:

$$S_1 = [S_1]_0 [\exp(-k_F t) - \exp(-k_v t)] \quad (10)$$

for $k_v \gg k_F$.

The various channels by which non-radiative processes occur have decay rates given by k_{NR} . These may be due to collision by diffusion and quenching of the emission by another species. Other forms of non-radiative decay are energy transfer with fast rotational motion of the molecule or transfer with diffusion while the molecule itself relaxes to the ground state. These are processes where the non-radiative decay parameter obeys a certain time dependence.

The time-resolved results may thus imply that there are more non-radiative processes that occur in cancer tissue because of local disorder of molecular configuration surrounding the fluorophores. The disorder causes more non-radiative pathways for excited fluorophores.

The non-exponential decay can be explained as either due to different

emitting species, time-dependent energy transfer processes or due to one molecule with a non-radiative competing process giving rise to the fast component.

The fluorescence lifetimes of benign tissue, benign tumor and malignant tumors were also tested using 527nm excitation. The fluorescence profiles of these samples were double exponentials, too. But these samples did not show any significant difference between the benign and malignant tissues. The five benign tissues studied showed fast lifetimes ranging from 65ps to 370ps and slow lifetimes ranging from 1300ps to 2500ps. The two benign tumor samples displayed fast lifetimes of 125ps and 160ps and slow lifetimes of 1600ps and 2000ps. The single malignant tumor measured displayed lifetimes of 125ps and 1000ps.

Table 4 lists the lifetimes of the various benign tissue and benign and malignant tumors that have been studied with 527nm excitation. The table displays the lifetimes of 5 benign tissues, 2 benign tumors and 1 malignant tumor. It is seen that the fast component of benign tissues, tumors and malignant tumors varies over a large range. The slow component also does not show any differences for the three types of tissue. The ratio of slow to fast components varies from 0.76 to 1.93.

The relaxation lifetimes of at least 5 benign and malignant tissues and tumors were also studied using the 'magic angle', 54.7 degrees to verify the presence of any rotational motion (for details, see Section IV).

Table 5 lists the tissues which display no change with polarization in

Table 4: Fluorescence lifetimes of benign tissue and tumor of the human breast in wavelength band > 550nm and excitation 527nm.

SAMPLES	tf (ps)	ts (ns)	as/af
Benign Tissue	100	1.7	1.6
Benign Tissue	150	2.0	1.5
Benign Tissue	85	1.4	1.7
Benign Tissue	65	1.3	-
Benign Tissue	370	2.5	0.9
Benign Tumor	125	1.6	0.7
Benign Tumor	160	2.0	1.9
Malignant Tumor	125	1.0	1.7

Table 5: Fluorescence lifetimes and ratio of amplitudes with no polarizer and polarizer at 54.7° in emission path with 527nm excitation.

SAMPLE	tf (ps) No pol.	ts (ns) No pol.	af/as No pol.	tf (ps) 54.7°	ts (ns) 54.7°	af/as 54.7°
Benign Tissue	85	1.4	0.58	110	1.1	0.23
Benign Tissue	370	2.5	1.13	180	2.6	1.14
Benign Tissue	100	1.7	0.6	100	1.4	0.27
Benign Tumor	160	2.0	0.5	73	1.6	0.53
Benign Tissue	150	2.0	0.65	200	2.0	0.76

the emission path. The fast and slow components of the fluorescence lifetime and the ratio of slow to fast amplitudes with no polarizer in the emission path and with polarizer at 54.7° in the emission path. Out of the four benign tissues studied, two show a decrease in the fast component while the other two show an increase in the presence of the polarizer as compared to the fast component in the absence of the polarizer. The benign tumor shows a decrease in the fast component. The slow component does not display any significant change and the ratio of amplitudes displays an arbitrary change. The decay profiles did not change significantly when the emission at 54.7° was observed. This proved that the emission was entirely from fluorescence with no rotational factor. The decay depolarizes quickly due to high scattering.

The fluorescence lifetimes of benign and malignant tissues do not show any distinguishing factors with 527nm excitation. Thus, the fluorescence lifetimes were measured at different wavelengths, to find the key wavelength (next section).

SECTION II

Time-resolved Fluorescence Using 351nm Excitation:

For a reliable diagnostic approach, it is important to differentiate benign tumors from malignant tumors. The time-resolved fluorescence profiles of benign tumors and malignant tumors did not display any

differences with 527nm excitation. Thus the method was extended to the near-UV region. The third harmonic of the Nd-glass laser (351nm) was used as the excitation source and studies were performed to see if this was the key wavelength for diagnostic purposes. Further, the pulsed laser emission spectra displayed more significant peak shifts between normal and cancerous tissues with 351nm excitation as compared to the 527nm excitation. To further enhance these results and obtain an additional diagnostic technique, the time-resolved fluorescence from human breast tissues was extended to the near-UV region.

Results:

Fluorescence lifetimes of benign and malignant tissues and tumors were extended to the near-UV region, with 351nm excitation. The samples were excited with the third harmonic of the Nd-glass laser, as described in Chapter 2. The fluorescence lifetimes were measured in the wavelength bands $>360\text{nm}$, $400 \pm 20\text{nm}$, $450 \pm 20\text{nm}$ and $500 \pm 20\text{nm}$ to span the entire fluorescence band of the breast tissue spectrum (Fig. 35). Figure 35 displays the fluorescence spectrum of a malignant breast tumor with 351nm excitation using a Perkin-Elmer spectrofluorometer. The spectrum consists of two bands, one with peak at 390nm and another with peak around 440nm. The spectrum spans a bandwidth of 370nm to 600nm. The fluorescence spectrum of a benign tissue also shows a similar profile (not shown here). The fluorescence lifetimes of benign tissues and tumors and malignant tumors were again

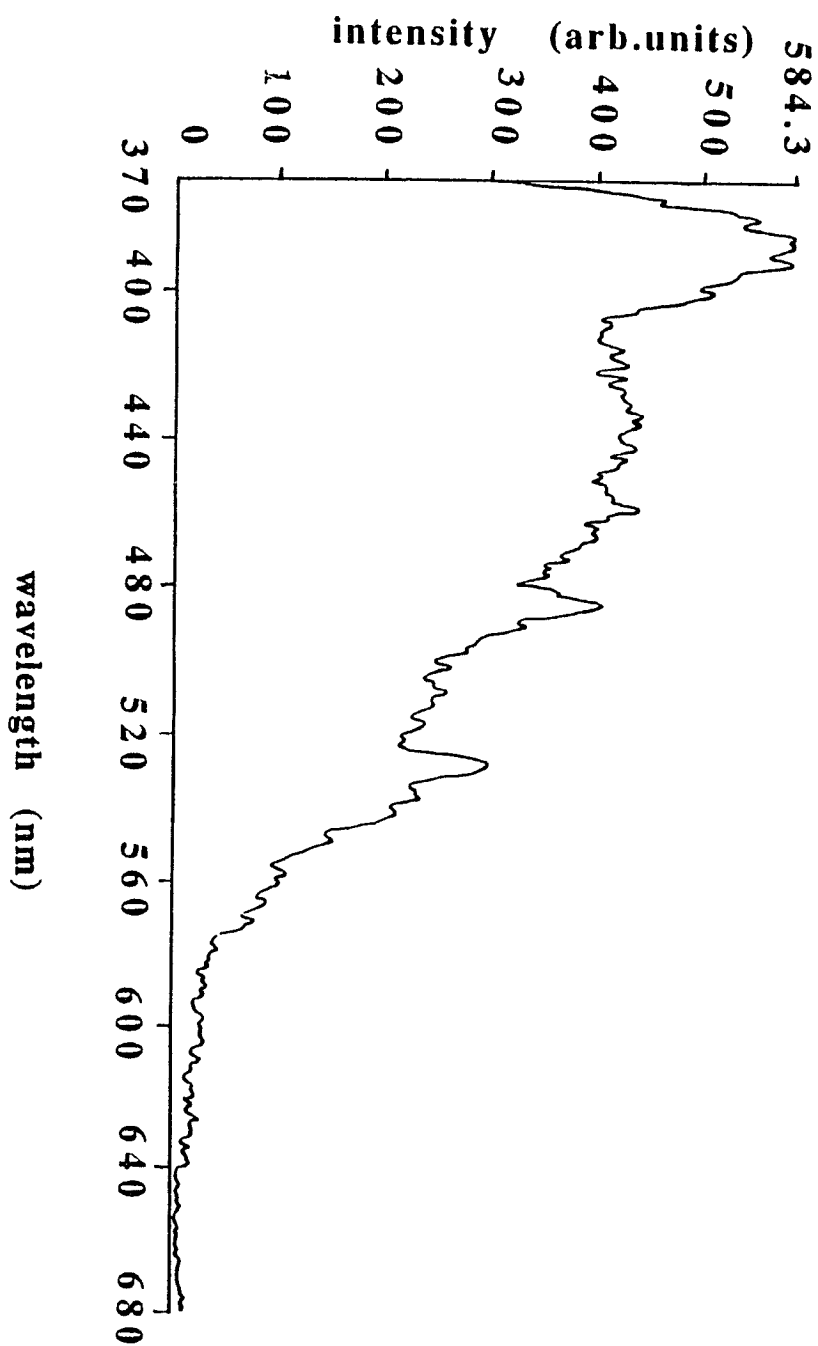


Figure 35. Fluorescence spectra of malignant breast tissue with 351nm excitation using steady state lamp excitation.

fitted to double exponentials with the fast component ranging from around 50ps to 300ps and the slow component varying from 1.5ns to 4ns. This was true for all the wavelength bands except in the 450nm band.

The 450nm band did show a consistent difference between the benign tissues and tumors and the malignant tumors in the values of the fast components. The fast components of the benign tumors and tissues displayed lifetimes of 211 ± 58 ps in the six samples studied. In the case of malignant tumors the fast components were 77 ± 32 ps in the four samples studied. Different positions in the same sample displayed consistent results. The slow components (2 ± 0.1 ns for benign and 2.5 ± 0.4 for malignant) and the ratio of slow to fast components of amplitude (1.9 ± 0.8 for benign and 1.7 ± 0.3 for malignant) did not display any differences. The data are listed in Table 6. Table 6 displays the fluorescence lifetimes and ratio of amplitudes of 2 benign tissues, 4 benign tumors and 6 malignant tumors of human breast tissue with 351nm excitation and emission band 450 ± 20 nm. The fast components in this band ranged from 42ps to 117ps for malignant samples while the range for non-malignant samples was from 134ps to 318ps. The slow component ranges from 1.8ns to 2.7ns for all tissues with no differences. The ratio of amplitudes also showed no differences between non-malignant and malignant samples.

Figure 36 (a), (b) and (c) display the fluorescence decay profiles of benign and malignant tissues respectively in the 450nm band. The temporal profiles of benign tissue shown in Fig.36(a), benign tumor shown in Fig.36(b) and malignant tumor in Fig.36(c) all display double exponentials. The benign

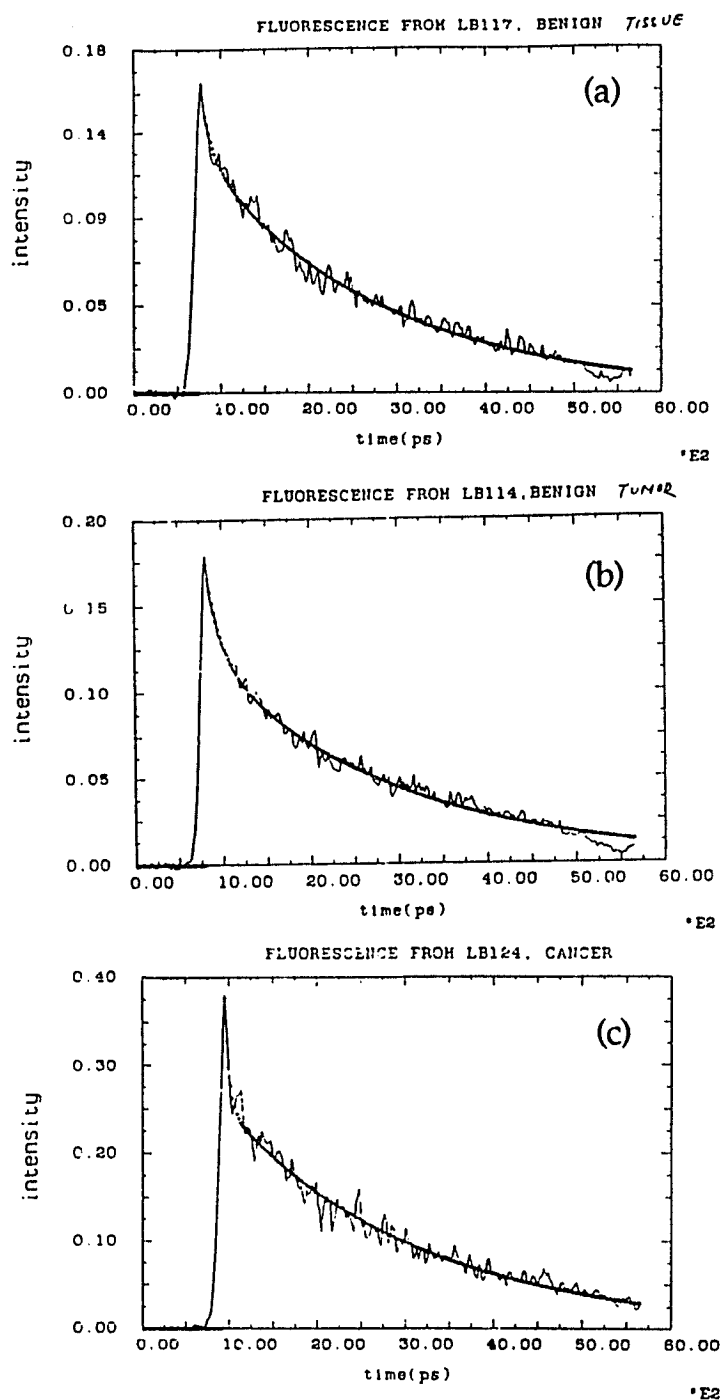


Figure 36. Time-resolved fluorescence profiles with 351nm excitation, of (a) benign tissue with $t_f = 134$ ps, $t_s = 2.0$ ns, (b) benign tumor with $t_f = 167$ ps, $t_s = 2.2$ ns and (c) malignant tumor with $t_f = 52$ ps, $t_s = 2.2$ ns.

Table 6: Fluorescence lifetimes and amplitude ratios of benign tissue, tumor, and malignant tumor of human breast excited by 351nm and in the wavelength band $450 \pm 20\text{nm}$.

SAMPLES	tf (ps)	ts (ns)	as/af
Malignant Tumor	117	2.5	1.8
Malignant Tumor	99	2.7	1.3
Malignant Tumor	52	2.7	1.9
Malignant Tumor	42	3.0	2.1
Malignant Tumor	52	2.2	2.0
Malignant Tumor	101	1.9	1.4
Benign Tumor	236	2.1	1.3
Benign Tumor	189	2.2	1.3
Benign Tumor	167	2.2	2.4
Benign Tumor	318	2.1	1.9
Benign Tissue	134	2.0	3.5
Benign Tissue	206	1.7	1.1

tissue shows a fast component of 134ps and a slow component of 2.0ns. The benign tumor shows a fast component of 167ps and a slow component of 2.2ns. The malignant tumor shows a fast component of 52ps and a slow component of 2.2ns. The fast component of the malignant tumor is sharper than that of the benign tissue and benign tumor.

Discussion:

A Student 't' test was performed for all three parameters (t_f , t_s , a_s/a_f) in the 450 ± 20 nm emission band with 351nm excitation to compare the benign and malignant samples and see test for a significant difference. The results showed that the p-value was < 0.001 only in the case of the fast component of fluorescence lifetimes. Thus it was concluded that the fast component of the 450 ± 20 nm band showed significant differences between benign and malignant samples. The average fast component of the benign tissues and tumors was found to be 211 ± 58 ps while that of the malignant tumors was 77 ± 32 ps.

The samples studied were generally fibrocystic changes classified as benign tissue, fibroadenomas and adenofibromas in the benign tumor group and infiltrating duct carcinomas in the malignant group. There were exceptions such as a medullary carcinoma which is a special cancer. It did not show any deviation from the malignant group. Another special type of benign tumor, cystosarcoma phylloides, displayed fast component of fluorescence lifetimes of 467ps and 229ps at different positions of the sample which is

consistent with the benign results.

As in the case of 527nm excitation double exponential decays are observed with 351nm excitation too. The question now is to interpret our results. As mentioned earlier in the 527nm excitation case, the non-exponential profile may be due to either two or more fluorophores or the deviation from single exponential profile could be due to some competing non-radiative decay process. The fast component of the fluorescence profile could be due to certain physical processes such as time-dependent energy transfer, depolarization or some diffusion-controlled process.

Fluorescence with 351nm excitation displays non-exponential temporal profiles of the human breast tissues. The fluorescence profiles have been fitted to double exponentials with the fast component of the order of a few hundred picoseconds and the slow component of the order of a few nanoseconds. The fluorescence profiles in the 450nm band are found to be different for benign (211 ± 58 ps) and malignant (77 ± 32 ps) tissues. The fluorescence decay of a single molecule is usually an exponential. Combination of fluorophores gives rise to multi-exponential forms. The non-exponential profiles of human breast tissues thus indicates the presence of more than a single fluorophore. The single exponential form could also be distorted by competing non-radiative processes. Thus the deviation from single exponential could arise from the action of several fluorophores and non-radiative processes. The observed differences between non-malignant and malignant tissues could arise from the environmental differences of a particular fluorophore in the tissues. The

differences could be due to a higher proportion of a certain fluorophore in the malignant tissue as compared to the non-malignant tissues.

The following subsections describe the experiments performed to analyze the profiles and observed differences.

Confirmation of non-radiative decay:

It is well known that non-radiative processes slow down and radiative processes are enhanced at low temperatures. To investigate whether non-radiative processes do occur in human breast tissues, the samples have been studied at low temperatures. This is described in detail in Chapter 5. The ratio of fast to slow components decreased at low (120 K) temperatures and the fluorescence intensity increased. These measurements prove the presence of non-radiative decay processes occurring with the radiative decay at room temperature. Detailed results are presented in Chapter 5.

Contribution from Fluorophores:

The double exponential decay of the breast tissue temporal profile could arise from different fluorophores or a complex time-dependent rate process. Biological tissues are complex systems with several biomolecules. The fluorescence signal from the tissue could arise from several molecules. The temporal profiles of a single fluorophore or combination of a group of fluorophores contributing to the tissue emission have been described in this subsection. The initial selection of the biomolecules is by comparison of the

spectral profiles of the breast tissues and the different fluorophores that are known to be present in the tissue. The temporal profiles are then compared with those of the tissues, indicating the possible contribution of certain fluorophores.

Tissues are made up of cells in between which are connective tissue which contains the tissue fluid and ground substance. The tissue fluid contains various proteins, ions, gases, amino acids, sugars, vitamins, lipids and hormones, which are exchanged between cells. The body contains 55% water, 19% proteins, 19% fats, 1% carbohydrates and 7% inorganic materials. This implies that the tissue fluid contains more amounts of proteins and lipids than other substances. The framework of the tissue is given by the structural proteins, collagen and elastin. These two proteins are found all over the tissue and may be responsible for the fluorescence at around 400nm.

Breast tissue, like any other tissue of the human body, contains elastin and collagen as the structural proteins and other fluorophores such as NADH, tryptophan, flavins, porphyrins and other biomolecules. Malignant breast tissues also contain similar fluorophores. As described in Section I of this chapter, cancer tissues seem to lose contact inhibition and nothing seems to stop them from growing. Another view is that cancer cells escape the immune system. Hence at the time of this research, it is accepted that cancer tissues contain the same fluorophores as normal tissues.

Breast malignancies are known to induce productive fibrosis (an increase in fibrosis implies an increase in the collagen content since the

fibrous tissue is mainly composed of collagen) [29,30] or produce collagen directly [31]. Studies have also shown that there is increase in elastic tissue in infiltrating ductal and lobular carcinomas [32,33]. A correlation of the fluorescence spectra and lifetimes of these fluorophores and the tissue lifetimes may be able to show that the differences observed are in fact due to the higher collagen content of malignant tumors. Studies with rat kidney fibroblasts [34] have shown a 2-3 fold decrease in the absolute concentrations of NAD⁺ and NADH following murine sarcoma virus transformation. Studies of NADH oxidation-reduction changes have been performed by Chance [1] in intact cells only. A lack of literature on spectroscopic and other changes of NADH in human breast tumors suggests that this is an important aspect that should be studied in detail. Spectroscopic diagnosis of colonic dysplasia [35] has also shown a decrease in the NADH peak in adenomatous tissue as compared to the normal tissue. Similar changes may be expected in human breast tissues but is yet to be studied.

The fluorescence spectra of a malignant breast tissue excited by 351nm is displayed in Fig. 35. The profile shows two bands at 380nm and 460nm. These two bands could be due to two fluorophores or due to one fluorophore with the dip at 410nm caused by absorption due to hemoglobin. The fluorescence peaks of elastin and collagen are at 410nm and 390nm (Figs. 37 (b) and (c)) excited by 351nm. Coenzyme NADH fluoresces at 460nm with its absorption peak at 350nm (fig. 37 (a)). From the emission spectral peaks, it is assumed that NADH, elastin and collagen are the possible contributing

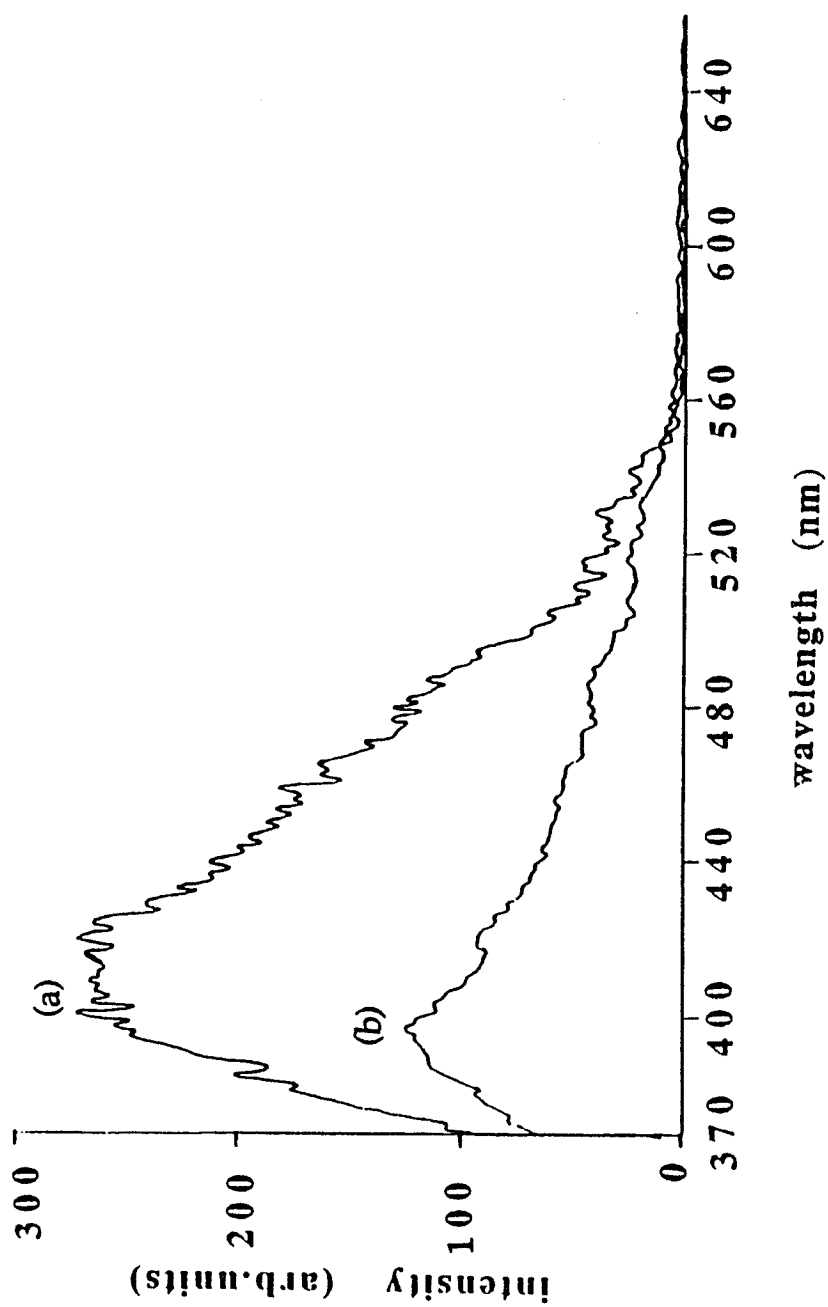


Figure 37
Fluorescence spectra with 351nm excitation of 10^{-4} M concentration of
(a) elastin with peak at 410nm and (b) collagen with peak at 390nm

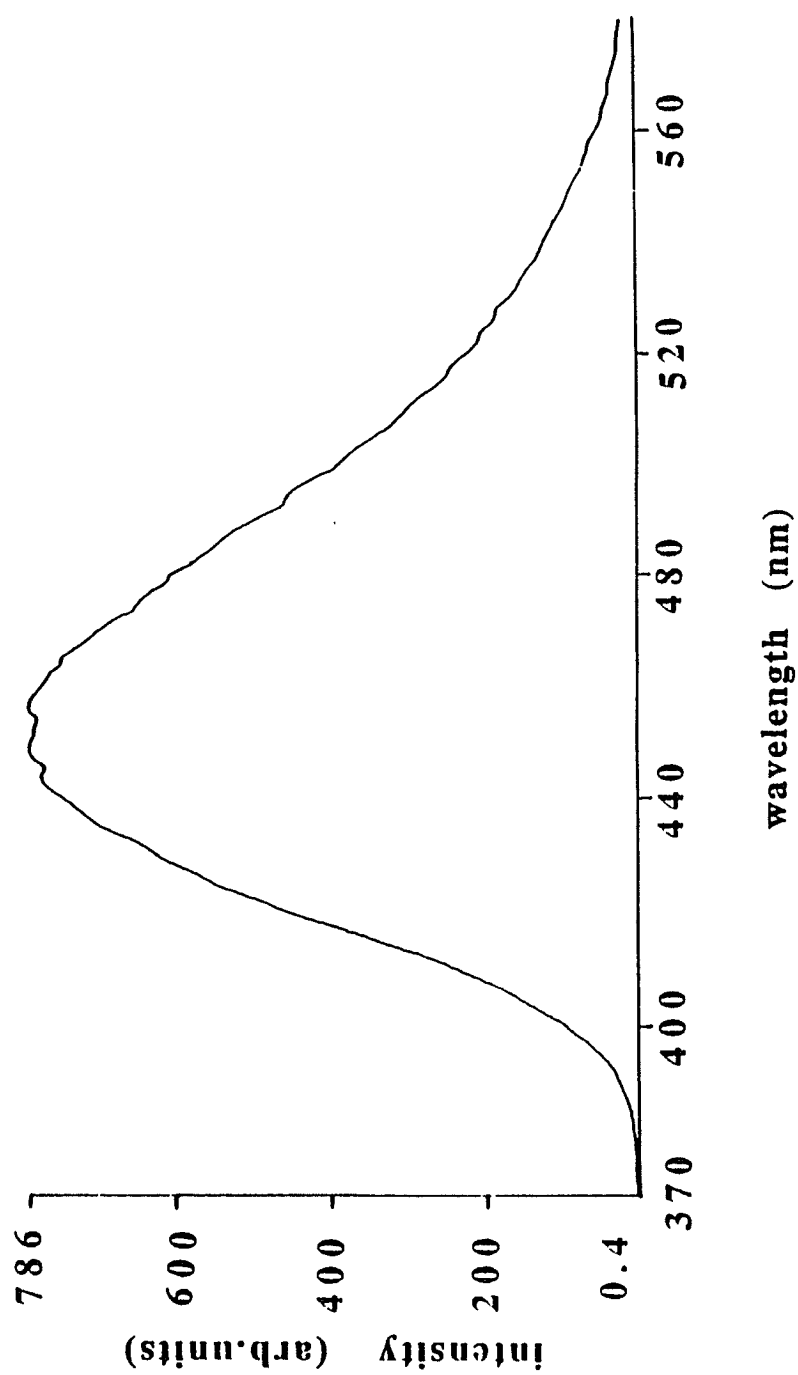


Figure 37(c). Fluorescence spectra with 351nm excitation of 10^{-4} M concentration of NADH with peak at 460nm,

fluorophores. Also, since the excitation for the breast tissue is at 351nm, it is most likely that NADH molecules are excited and responsible for the 450nm band. Elastin and collagen also have weaker fluorescence at 450nm. Hence, the 450nm band fluorescence could arise from a combination of elastin, collagen and mostly NADH. Figures 37 (d) and (e) display the fluorescence spectra of NADH + elastin and NADH + collagen, respectively with 351nm excitation and Figure 37 (f) is a combination of NADH (10^{-4}M) and saturated solutions of elastin and collagen. Figure 37 (f) appears to be similar to the tissue spectral profile in Figure 35, ignoring the dips arising from the effect of blood. The absorption spectrum of blood spanning 200nm to 600nm is shown in Fig.38. The absorption spectrum has peaks at around 350nm, 420nm, 540nm and 580nm. The fluorescence spectrum of NADH + elastin + collagen spans 370nm to 580nm with a smooth profile peaking at 420nm. The malignant breast tissue spectrum (Fig.32) spans the same range but displays two bands with peaks at 390nm and 440nm. The fluorescence spectrum of tissue is affected by absorption due to hemoglobin which gives rise to the two bands. The absence of hemoglobin would most probably smoothen the profile as has been exhibited with 488nm excitation. The tissue profile would then be similar to that of NADH with elastin and collagen. A biochemical analysis of the human tissue will determine the proportions of these fluorophores in human breast tissues since this is not yet known. The spectral studies of the fluorophores obtained from the tissue can be compared with the normal and diseased tissue spectrum for a better analysis.

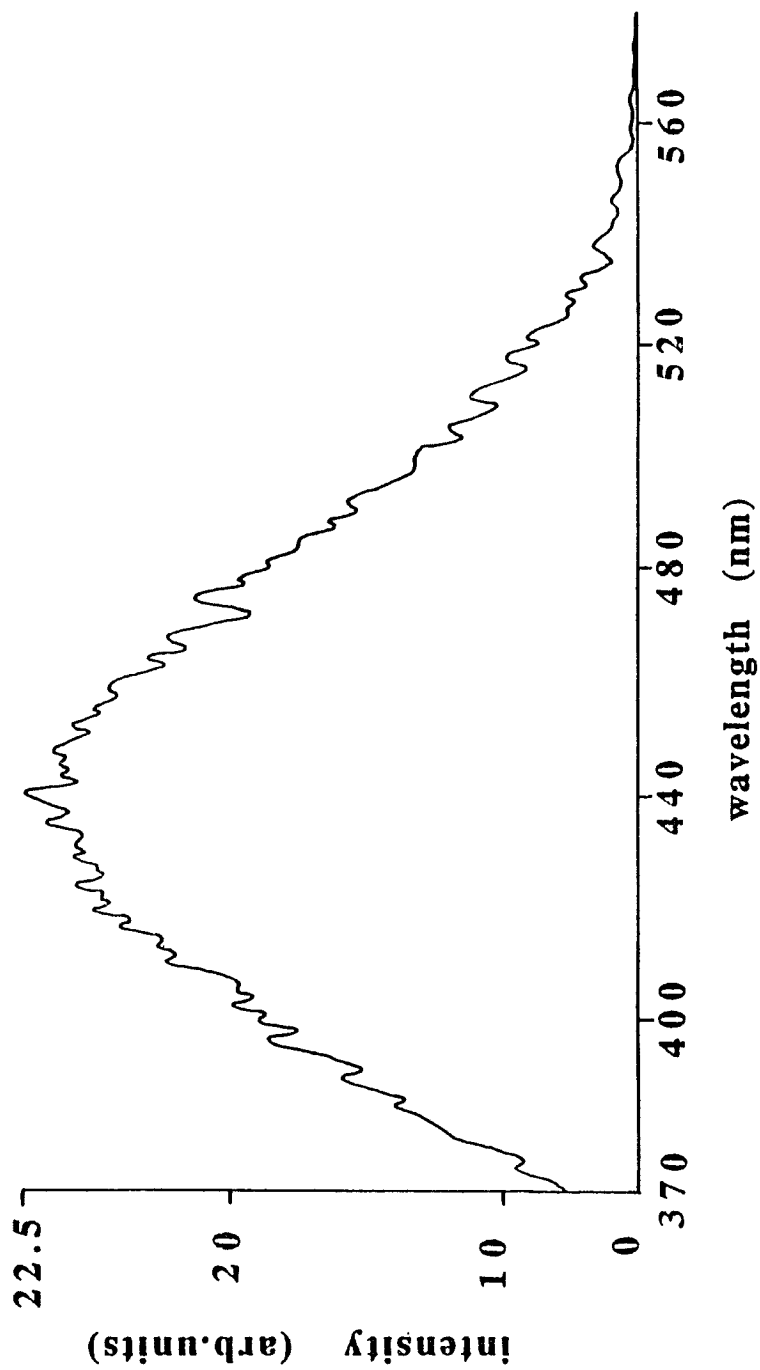


Figure 37(d). Fluorescence spectra with 351nm excitation of 10⁻⁴M concentration of NADH + elastin,

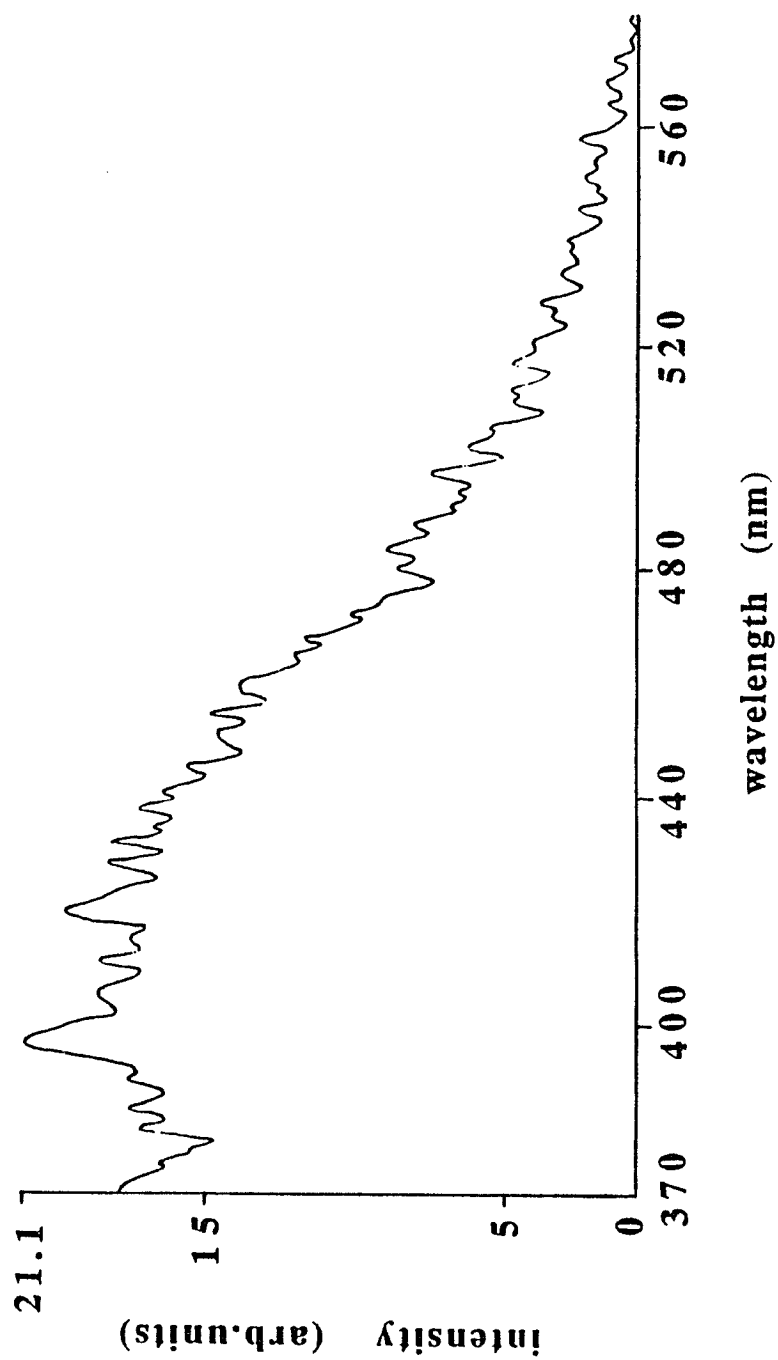


Figure 37(e). Fluorescence spectra with 351nm excitation of $10^{-4}M$ concentration of NADH + collagen

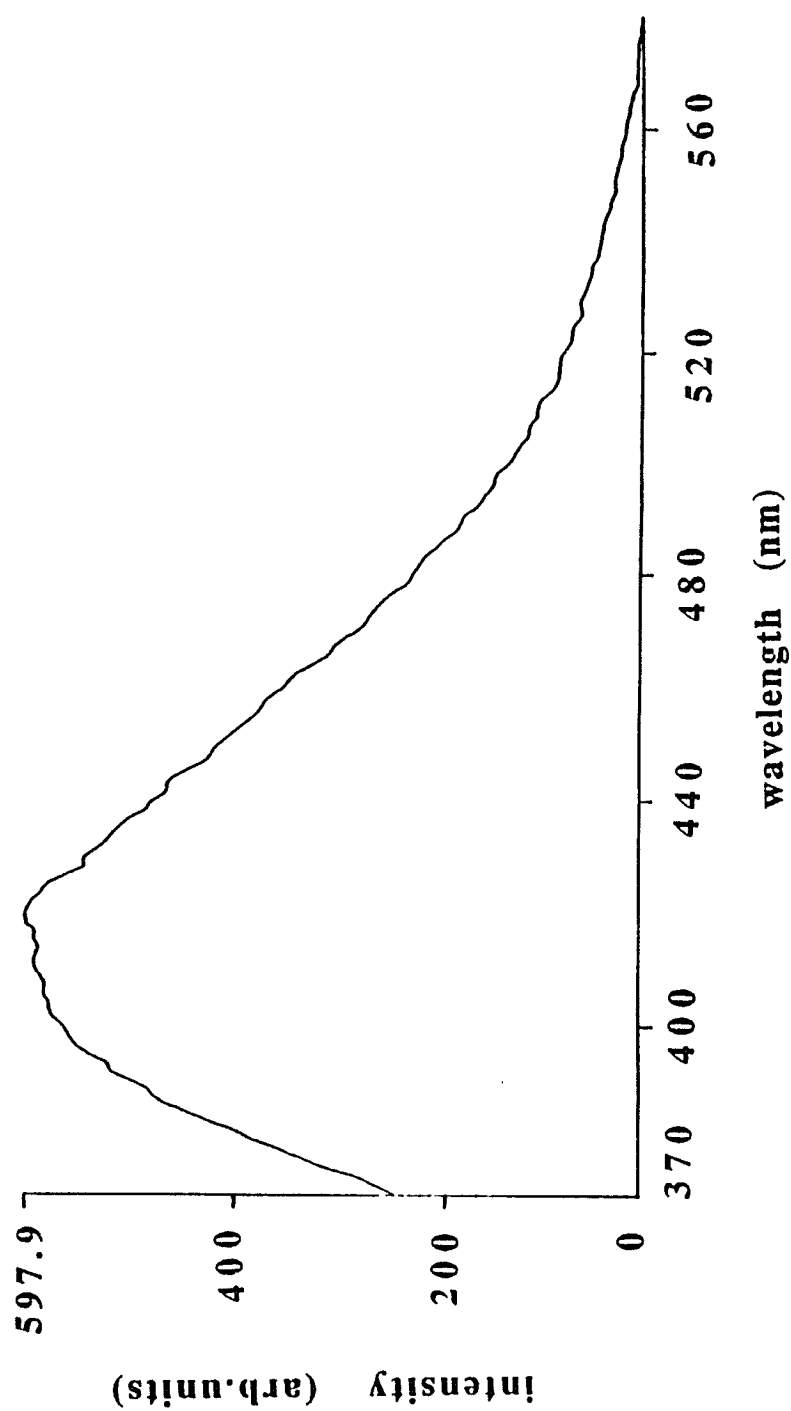


Figure 37(f). Fluorescence spectra with 351nm excitation of 10^{-4} M concentration of NADH + elastin + collagen.

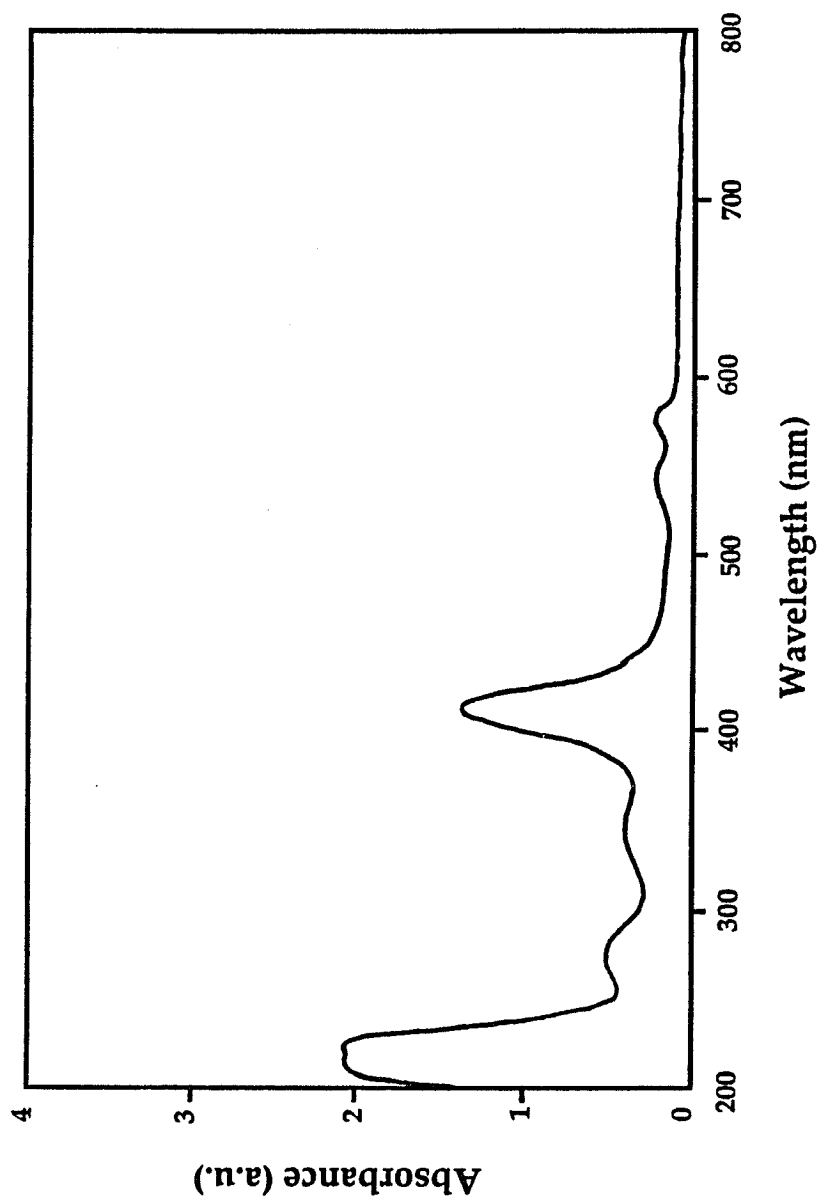


Figure 38. Absorption spectrum of blood in the 200 - 800 nm range.

To confirm whether the signatures are caused by the above mentioned fluorophores, the time-resolved fluorescence of these substances have been measured in solution and in tissue environment. The biomolecules studied are prepared in distilled water with a concentration of 10^{-4}M . NADH displayed strong fluorescence at this concentration. However, elastin and collagen both displayed weak fluorescence at this concentration. Hence elastin and collagen were measured as saturated solutions in water. The fluorescence of the molecules are also measured in tissue environment. This is done by extracting the tissue fluid after disrupting the tissue and then through centrifuging to isolate the cytoplasmic part from the nuclear material and the membranes. The fluorescence lifetimes of the molecules are listed in Table 7. The fast and slow components and the ratio of fast to slow amplitudes are displayed for the fluorophores in water solution and in cytoplasmic extract. FMN shows a single exponential decay with a lifetime of 3ns displayed in Figure 39 (a). NADH also displays a single exponential decay but with a much shorter lifetime of 354ps in water solution displayed in Figure 39 (b). In cytoplasmic extract, NADH displays a double exponential decay with lifetimes of 117ps and 776ps. Except NADH and FMN, all the others show double exponential decays. A combination of NADH and FMN (1:1) display a double exponential with 304ps and 2.6ns displayed in Figure 39 (c) while the lifetimes in cytoplasmic extract are 139ps and 1.3ns. Elastin (Figure 39 (d)) and collagen (Figure 39 (e)) display lifetimes 46ps and 2.1ns and 76ps and 2.5ns respectively. In cytoplasmic extract, the lifetimes of elastin are 111ps and 2.1ns

Table 7: Fluorescence lifetimes and ratio of amplitudes of biomolecules with 351nm excitation.

MOLECULE	tf (ps) in water	ts (ns) in water	as/af in water	tf (ps) in extract	ts (ps) in extract	as/af in extract
FMN	-	3.0	-	-	-	-
NADH	354	-	-	117	0.7	2.1
NADH+FMN (1:1)	304	2.6	3.0	139	1.3	3.7
Elastin	47	2.1	1.8	111	2.1	2.1
Collagen	76	2.5	1.7	289	3.6	1.2
NADH + Elastin	292	1.6	0.4	-	-	-
NADH + Collagen	224	2.5	0.8	-	-	-
NADH + Elastin+ Collagen	292	2.3	0.9	-	-	-

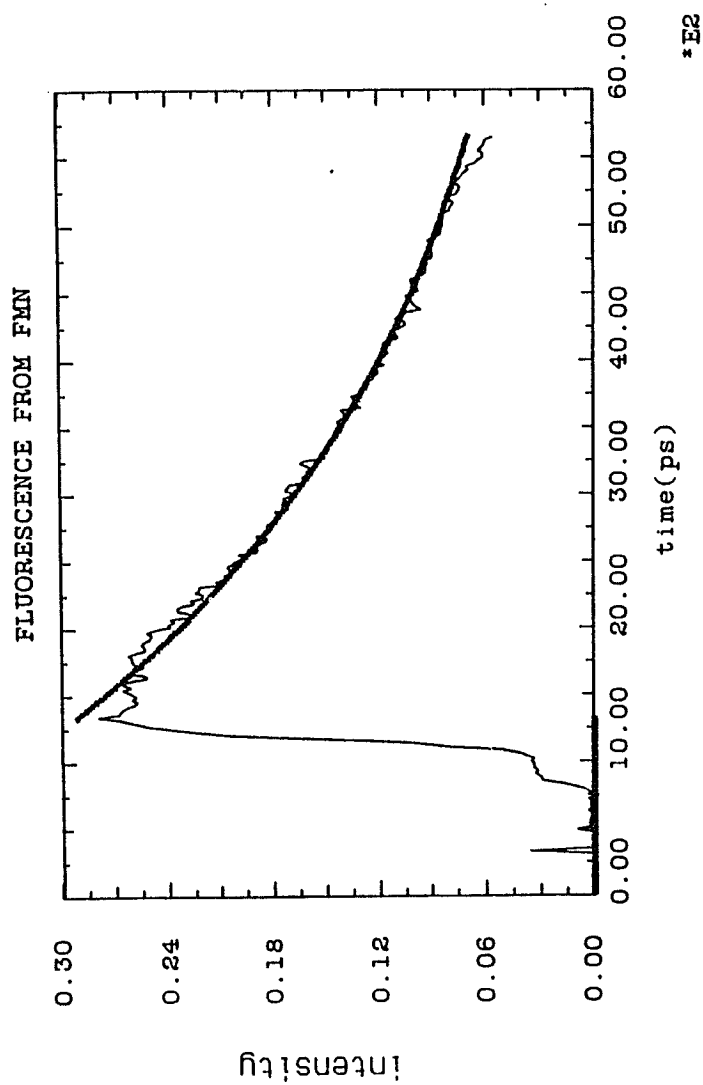


Figure 39(a). Fluorescence lifetimes with 351nm excitation of 10^{-4}M concentration of flavin mononucleotide with $t = 2.99\text{ns}$

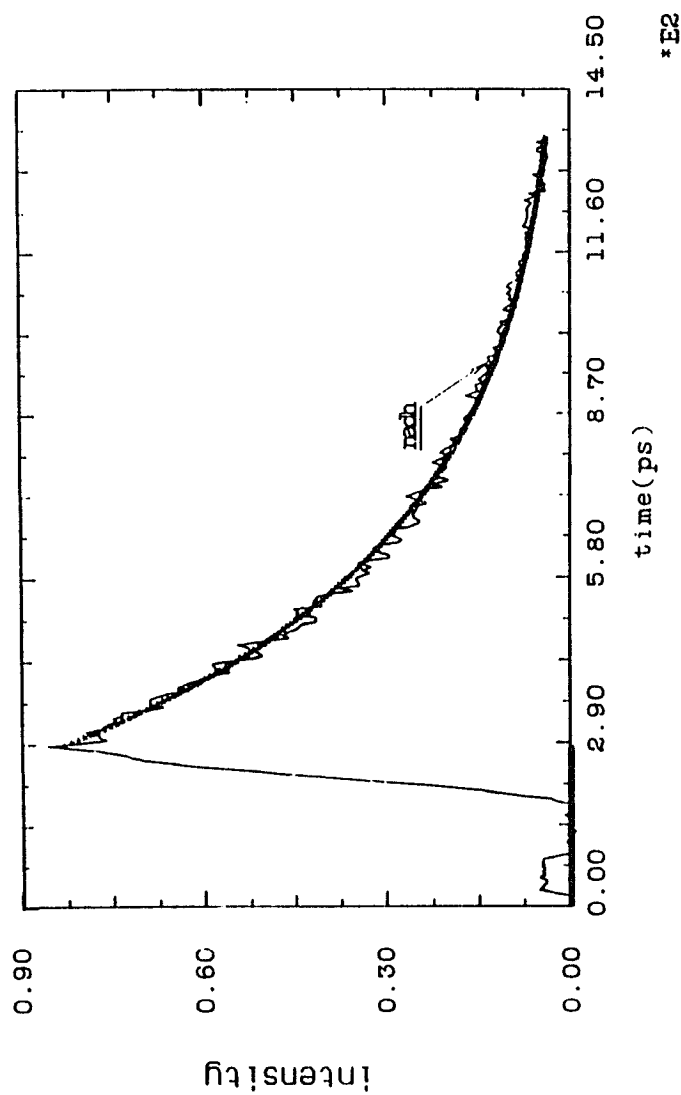


Figure 39(b). Fluorescence lifetimes with 351nm excitation of 10^{-4}M concentration of NADH with $t = 354\text{ps}$,

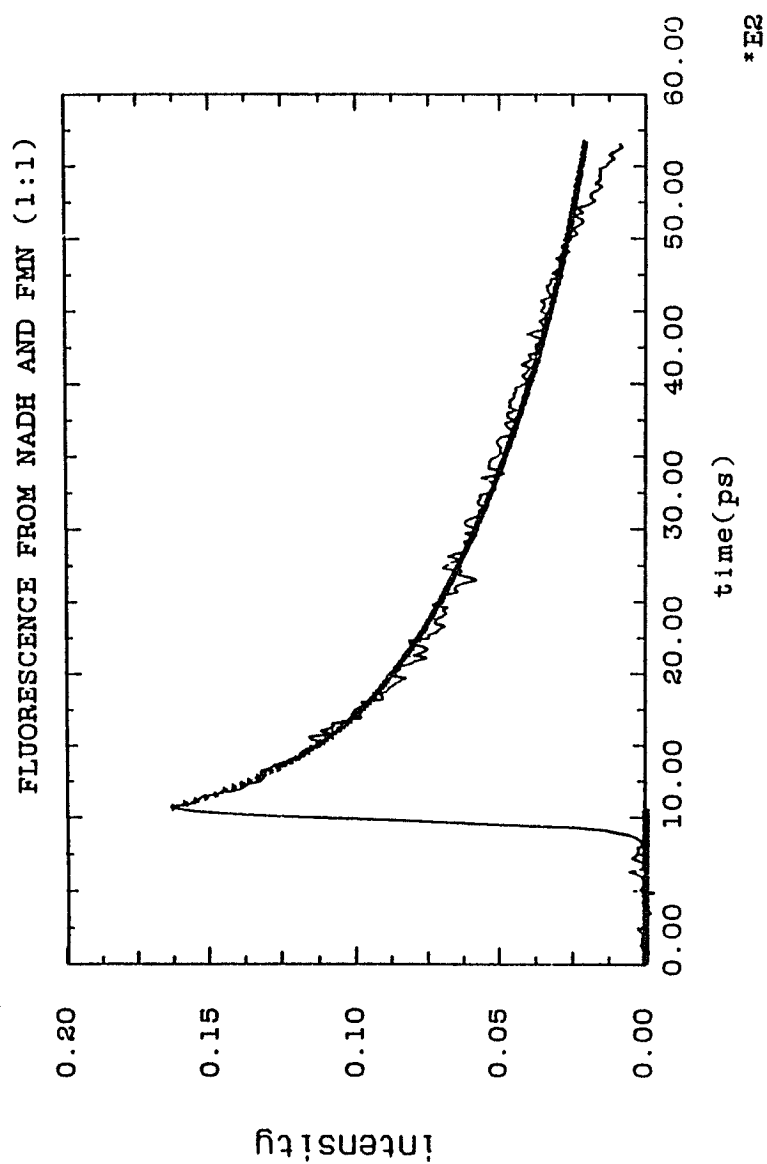


Figure 39(c). Fluorescence lifetimes with 351nm excitation of 10^{-4}M concentration of NADH + FMN (1:1) with $t_f = 303\text{ps}$, $t_s = 2.6\text{ns}$,

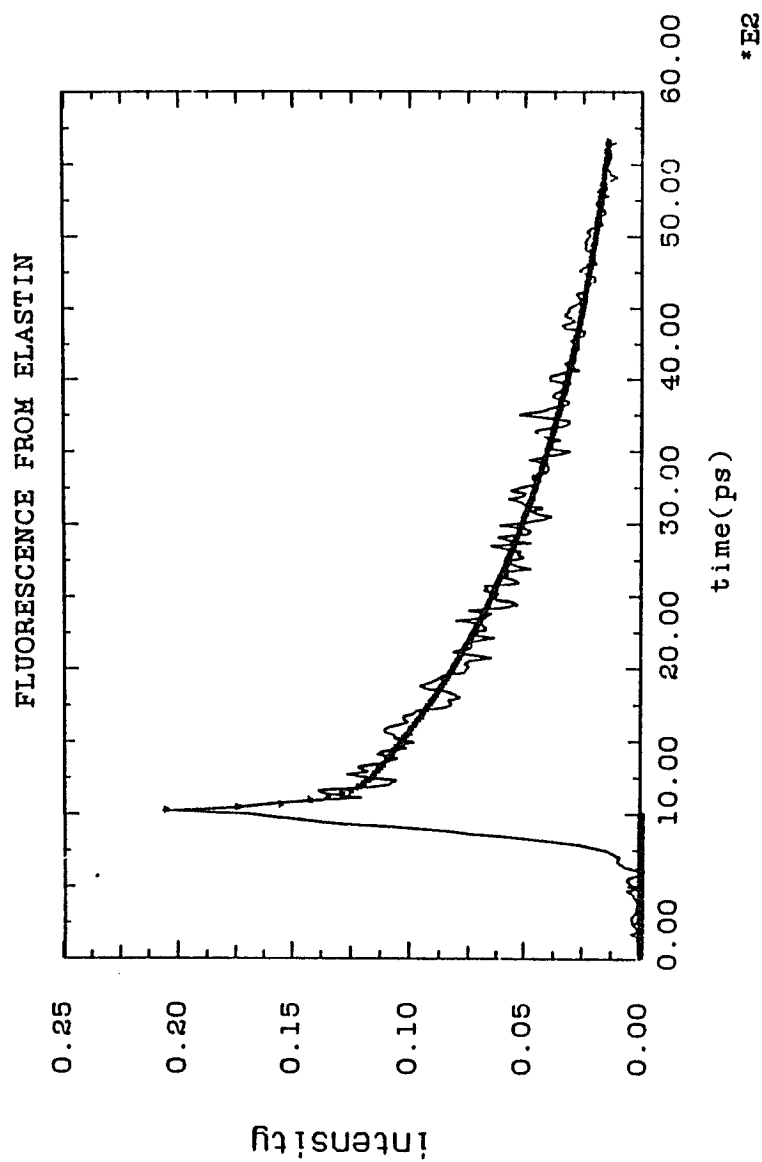


Figure 39(d). Fluorescence lifetimes with 351nm excitation of 10^{-4}M concentration of elastin with $t_f = 47\text{ps}$ and $t_s = 2.1\text{ns}$,

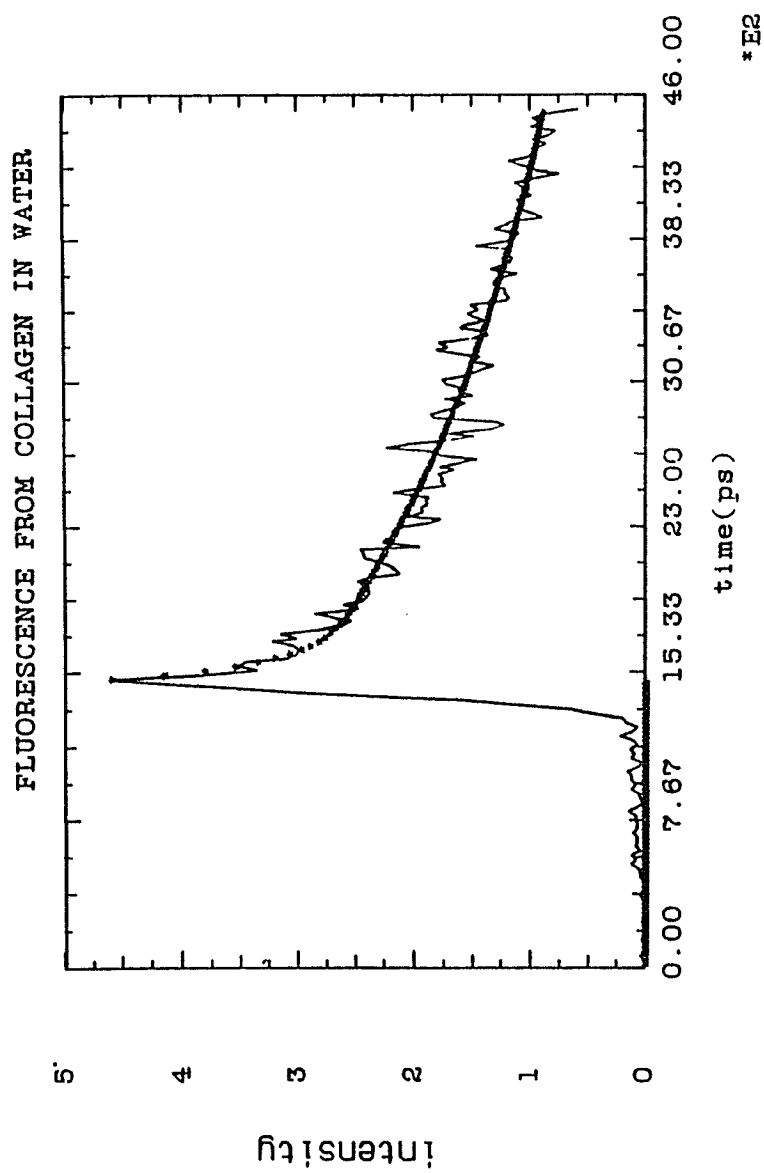


Figure 39(e). Fluorescence lifetimes with 351nm excitation of 10-4M concentration of collagen with $t_f = 76\text{ps}$ and $t_s = 2.53\text{ns}$,

and those of collagen are 288ps and 3.6ns. The fluorescence lifetimes of the molecules slow down in tissue environment. NADH with elastin displays a fast component of 292ps and a slow component of 1.6ns (Figure 39 (f)) while NADH with collagen displays a fast component of 224ps and a slow component of 2.5ns (Figure 39 (g)). The ratio of amplitudes of both combinations, however display a significant difference. The ratio decreases (0.8) for NADH with collagen as compared to that of NADH with elastin (0.4). The ratio of fast to slow amplitudes decreases due to the presence of collagen. A combination of NADH with saturated solutions of elastin and collagen displays lifetimes of 292ps and 2.3ns and a ratio of 0.9. Figure 39 (h) show the fluorescence lifetimes of the combination of NADH (10^{-4}M), elastin (saturated solution) and collagen (saturated solution). The fluorescence spectra spans 370nm to 580nm. In the tissue fluorescence spectrum (Figure 35), a dip is seen at around 400nm and the second band is weaker. This is most probably due to the absorption of fluorescence by hemoglobin. The combination of the three molecules displays a temporal profile similar to the tissue profile when compared to Figure 36 (b). The list of lifetimes and the fluorescence spectral results imply that the molecules responsible for the benign breast tissue signal with 351nm excitation may be combination of elastin, collagen and NADH. The lifetimes of malignant tumor ($77 \pm 32\text{ps}$ and $2.5 \pm 0.4\text{ns}$) correspond to that of collagen in water (76ps and 2.5ns. This would imply that there is more collagen content in the cancer environment. There may also be contribution from elastin (46.8ps and 2.1ns). Since NADH

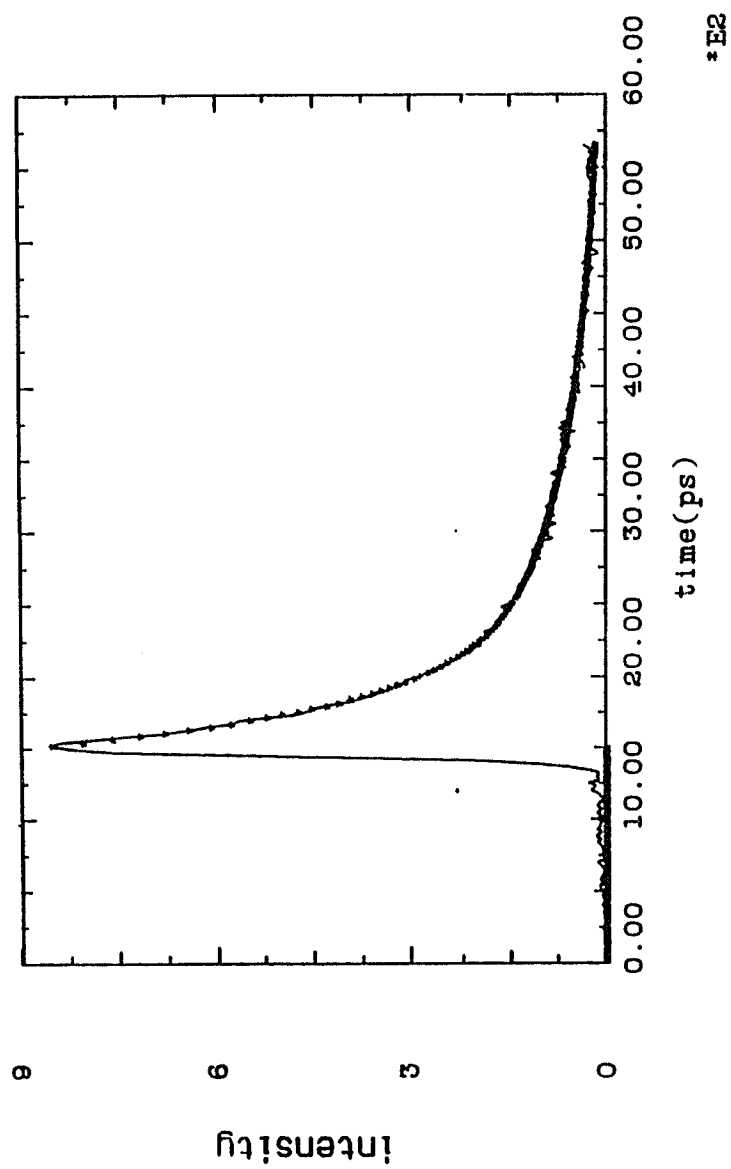


Figure 39(f). Fluorescence lifetimes with 351nm excitation of 10^{-4}M concentration of NADH + elastin with $t_f = 292\text{ps}$ and $t_s = 1.6\text{ns}$,

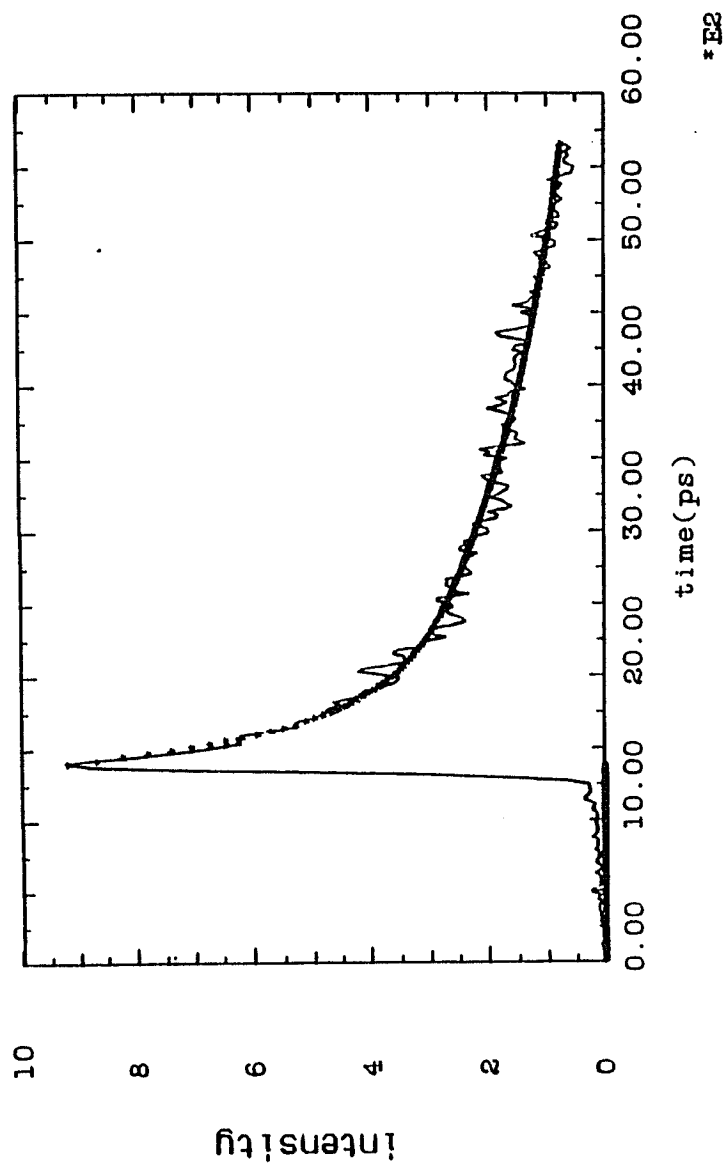


Figure 39(g). Fluorescence lifetimes with 351nm excitation of 10^{-4}M concentration of NADH + collagen with $t_f = 224\text{ps}$ and $t_s = 2.5\text{ns}$ and

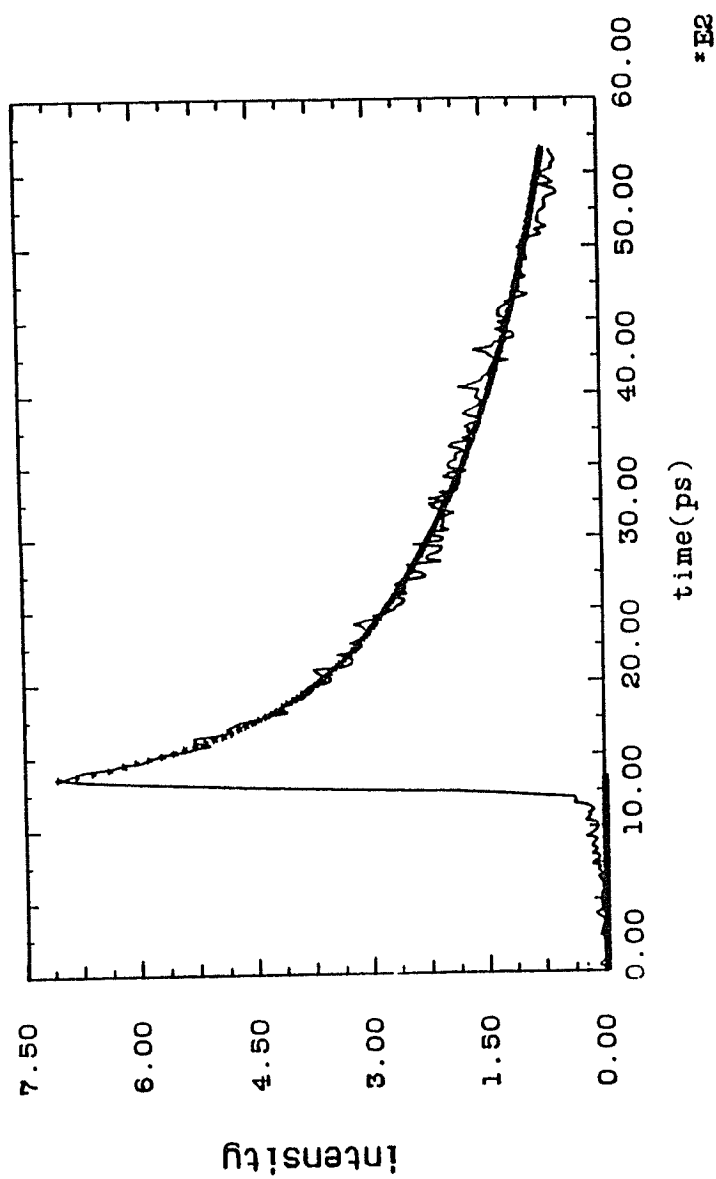


Figure 39(h). Fluorescence lifetimes with 351nm excitation of 10⁻⁴M concentration of NADH + elastin + collagen with $t_f = 292\text{ps}$ and $t_s = 2.3\text{ns}$.

shows a single exponential fluorescence decay, it cannot be the only source of the signal in the 450nm band, where the benign and malignant samples display differences. However, the fast component shows differences and NADH may be responsible for most of the fast component. It is to be noticed that NADH in the cytoplasmic extract displays a double exponential profile with the fast component (139.4ps) consistent with the fast component of the tissue lifetime (100~200ps). It may be that the fast component only contributes to the tissue fluorescence and the slower component does not show up due to the presence of a longer slow component in the tissue profile. The NADH in water solution decays with a lifetime of 354ps which is slower than that of the benign and malignant tissues in the 450nm band. This may imply that there occurs non-radiative processes in the NADH environment inside a tissue, which shortens the lifetime. Thus, NADH may be responsible for the observed differences. However, there has been no mention of NADH changes in the literature of breast tissue. From the literature, it is known that the collagen and elastin content of breast malignancies increases. The lifetime measurements also correspond to this, since the fluorescence lifetimes of malignant tumors appear to be similar to that of elastin and collagen. Decrease of the concentrations of NADH are also seen in rat tumors [35]. Thus, the ratio of NADH to collagen content or NADH to elastin content may be the cause of the differences. Much more work has to be done to clearly determine the source of difference in tissues. This will be possible by performing a biochemical study of the various parts of the tissue to determine

the amounts of NADH, elastin and collagen present in the breast tissue.

Time-Rate Process:

The non-exponential forms of the breast tissue profiles have been fitted to double exponentials assuming contribution from more than one fluorophore. The non-exponential form could also arise from certain competing non-radiative processes with time-dependence. These time-rate processes may be certain diffusion quenching by another species or energy transfer to another species with either fast rotational motion of the fluorophore or energy transfer with diffusion.

In diffusion controlled collision processes, the rate parameter is time-dependent with a $t^{1/2}$ dependence. When energy transfer to another species occurs in a solution with high viscosity, the molecule remains stationary during the transfer process while the Brownian molecular rotation is much faster than the transfer process. In this case the transfer rate is time-dependent. Another case of time-dependent energy transfer is when the diffusion is considered along with the transfer.

In all three cases the rate parameter shows a $t^{1/2}$ dependence so that

$$I(t) = \exp(-at-bt^{1/2})$$

with $a = 1/\tau_f$ and b is different for the three different cases.

The time-dependent rate process is used to fit the non-exponential decay profile of the breast tissue with 351nm excitation. Figure 40 displays the fitting with several values of a and b . The series with b fixed at $4 \times 10^{-10} \text{ps}^{-1/2}$

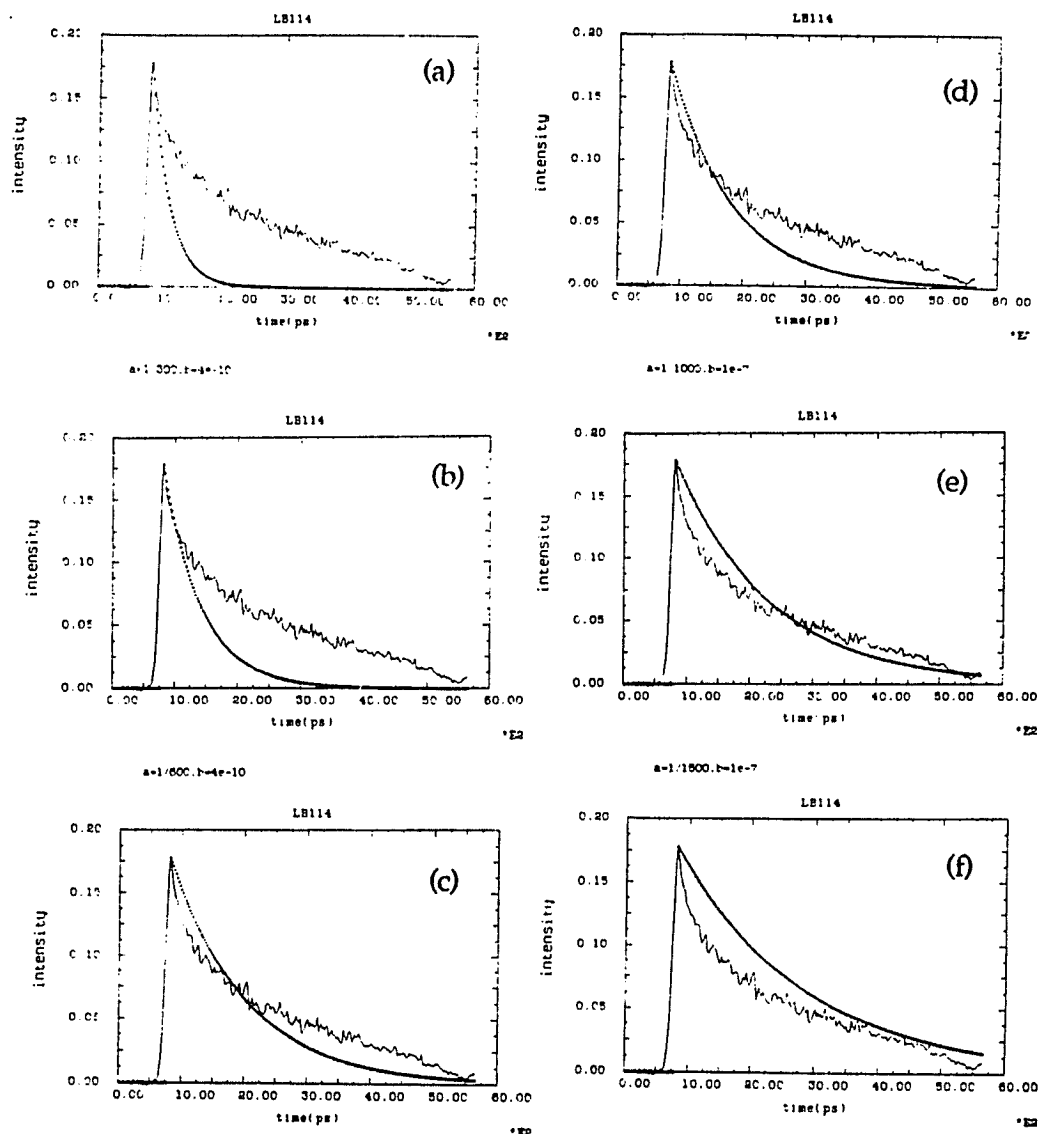


Figure 40. Time-resolved fluorescence profile of a benign breast tissue with 351nm excitation fitted to a time-rate equation with : $b = 4 \times 10^{-10}$ and (a) $a = 1/300$, (b) $a = 1/600$, (c) $a = 1/1200$; $b = 1 \times 10^{-7}$ and (d) $a = 1/1000$, (e) $a = 1/1500$, (f) $a = 1/2000$; $a = 1/300$ with least square fitting.

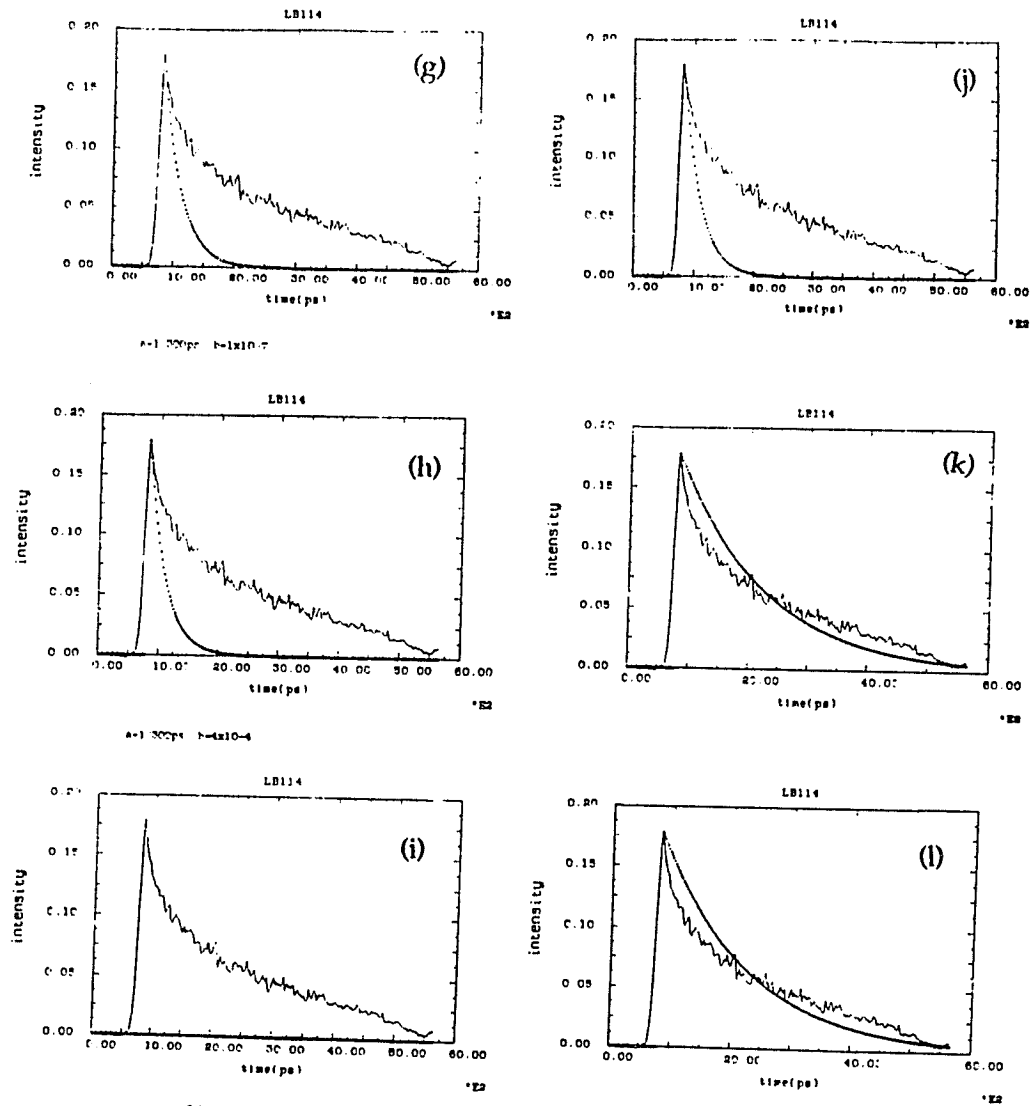


Figure 40. Time-resolved fluorescence profile of a benign breast tissue with 351nm excitation fitted to a time-rate equation with : $b = 4 \times 10^{-10}$ and (g) $b = 1 \times 10^{-7}$, (h) $b = 4 \times 10^{-4}$, (i) $b = 4 \times 10^{-4}$; $b = 4 \times 10^{-4}$ and (j) $a = 1/300$, (k) $a = 1/2003$; (l) $a = 1/2003$ and $b = 3.98 \times 10^{-4}$ with least square fitting.

and $a = 1/300, 1/600$ and $1/1200\text{ps}^{-1}$ in Figures 40 (a), (b) and (c) shows a slowing of the theoretical curve. Similarly, with $b = 1 \times 10^{-7}\text{ps}^{-1/2}$ and $a = 1/1000, 1/1500$ and $1/2000\text{ps}^{-1}$, a slowing down is observed in Figures 40 (d), (e) and (f). Keeping b fixed at $4 \times 10^{-4}\text{ps}^{-1/2}$ and $a = 1/300$ and $1/2003\text{ps}^{-1}$, the decay slows down (Figures 40 (g) and (h)). With a fixed at $1/300\text{ps}^{-1}$ and $b = 1 \times 10^{-7}, 4 \times 10^{-4}$ and $4 \times 10^4\text{ps}^{-1/2}$, the decay appears sharper with increasing b as seen in Figures 40 (i), (j) and (k). A least square fitting shows the best fit to be $a = 1/2003\text{ps}^{-1}$ and $b = 3.98 \times 10^{-4}$ (Fig.40l). These results indicate that the model does not fit the experimental results. It appears that the time-dependence of the non-radiative decay does not display a $t^{1/2}$ dependence. Further work in the future will be able to establish the form of the time-dependence of the non-radiative decay rate.

In conclusion, it appears from this study at 351nm that the fluorescence from human breast tissues originate from a group of molecules, namely, NADH, elastin and collagen. The study also confirms the occurrence of non-radiative processes. The time-resolved fluorescence profile is a non-exponential. The non-radiative time rate process with $t^{1/2}$ dependence does not seem to fit with experiment. The differences between benign and malignant tissues at 450nm appears to be due to more non-radiative processes occurring in the cancer environment of NADH, elastin and collagen. It appears that NADH is the molecule responsible for the differences, but the exact cause is not known. Due to the complexity and heterogeneous nature

of a tissue, it is difficult to point out the main source of the signal with certainty. This study confirms the contribution of several molecules and the occurrence of non-radiative processes.

SECTION III

Time-Resolved Fluorescence from Malignant and Non-malignant Breast Tissues With UV (310nm) Excitation:

The fluorescence lifetimes of benign and malignant tumors have so far been studied using visible and near-UV excitation. To complete the work, the study has been extended to the UV excitation of human breast tissues. Studies of fluorescence spectra have been pursued by other members of this group [44], extending to the UV region. Fluorescence spectra of human breast normal tissues, benign tissues and tumors and malignant tumors have been measured with 300nm excitation. The ratio of intensities, 340/440nm was found to be significantly different for malignant tumors as compared to normal tissues, benign tissues and tumors. In order to obtain further information on the chromophores responsible for the emission and to have a better picture of the heterogeneous nature of the tissue fluorescence, the dynamics of the physical processes occurring in tissues needs to be studied using 310nm pulse excitation.

The UV excitation excites several group of molecules, namely tryptophan, tyrosine, elastin, collagen, NADH, etc which may show

differences between benign and malignant tissues. This may also provide some insight on the physical properties of breast tissues. This section describes the use of time-resolved fluorescence using 310nm pulse excitation.

Results:

The fluorescence lifetimes from human breast tissues were obtained by exciting the sample with 100fs second harmonic (310nm) light [52] from a colliding pulse mode-locked dye laser, described in Chapter 2. The tissue fluorescence is collected in emission bands of $340 \pm 5\text{nm}$ and $440 \pm 5\text{nm}$ using narrow band filters. These bands are chosen from the fluorescence spectral bands. Time-resolved fluorescence measurements have been performed on 18 samples - 12 benign tissue and tumor and 6 malignant tumors. The malignant tumors were all infiltrating duct carcinomas, and the benign samples were composed of fibroadenoma, lipoma and/or fibrocystic changes.

Typical time-resolved fluorescence profiles are displayed in Figs. 42 (a), (b) and (c). The two bands chosen for the investigation were the two bands obtained from steady state measurements at 300nm (Fig.41). The temporal curves for 340nm bandwidth appear to contain two components, one fast and the other, slow. There is a marked difference between the curve profiles of benign and malignant tissues. The fast component seems to be more dominant in benign tissues as compared to the malignant tumors.

The fluorescence decay curves of the 440nm band also show double exponential profiles shown in Figure 43 (a), (b) and (c). Unlike the 340nm

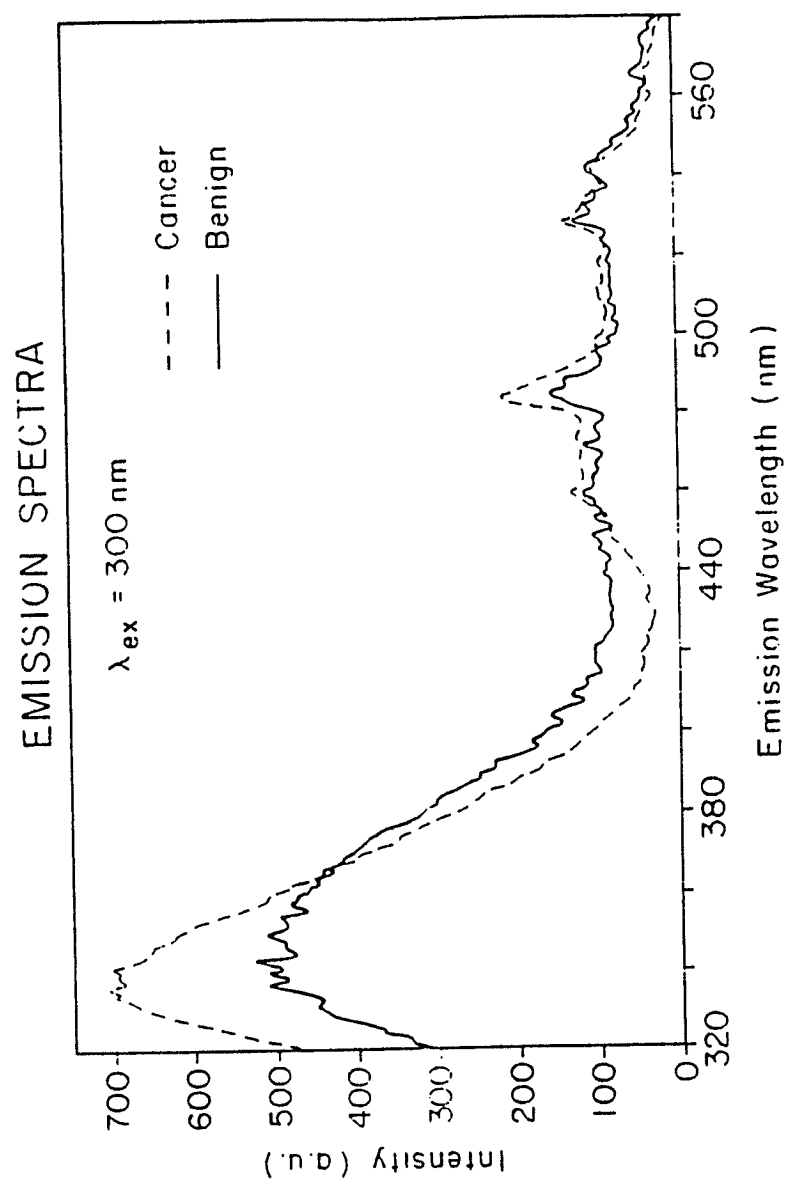


Figure 41. Fluorescence spectra from cancer and benign tumor breast tissues excited at 300nm.

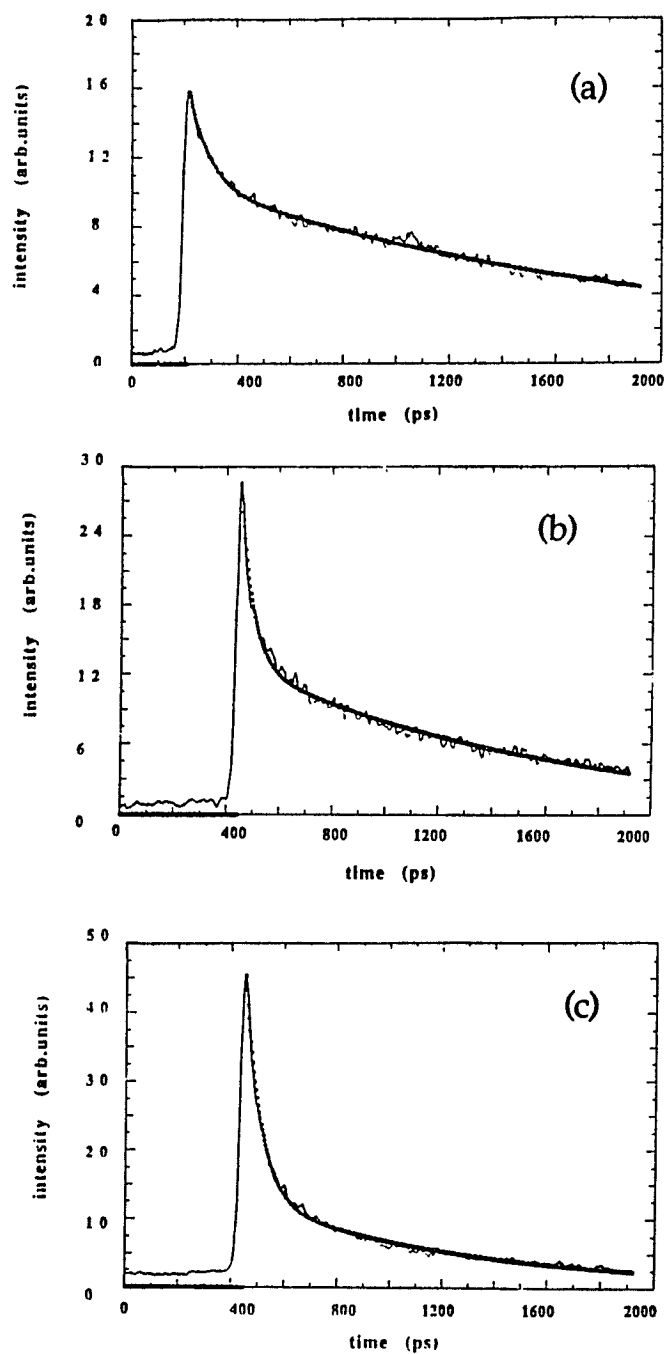


Figure 42. Time-resolved fluorescence profiles with 310nm excitation and 340nm emission. (a) Malignant tumor: $t_f = 79$ ps, $t_s = 1.99$ ns, $a_f/a_s = 0.5$, (b) Benign tissue: $t_f = 46$ ps, $t_s = 1.14$ ns, $a_f/a_s = 1.2$, (c) Benign tumor: $t_f = 59$ ps, $t_s = 0.889$ ns, $a_f/a_s = 2.58$.

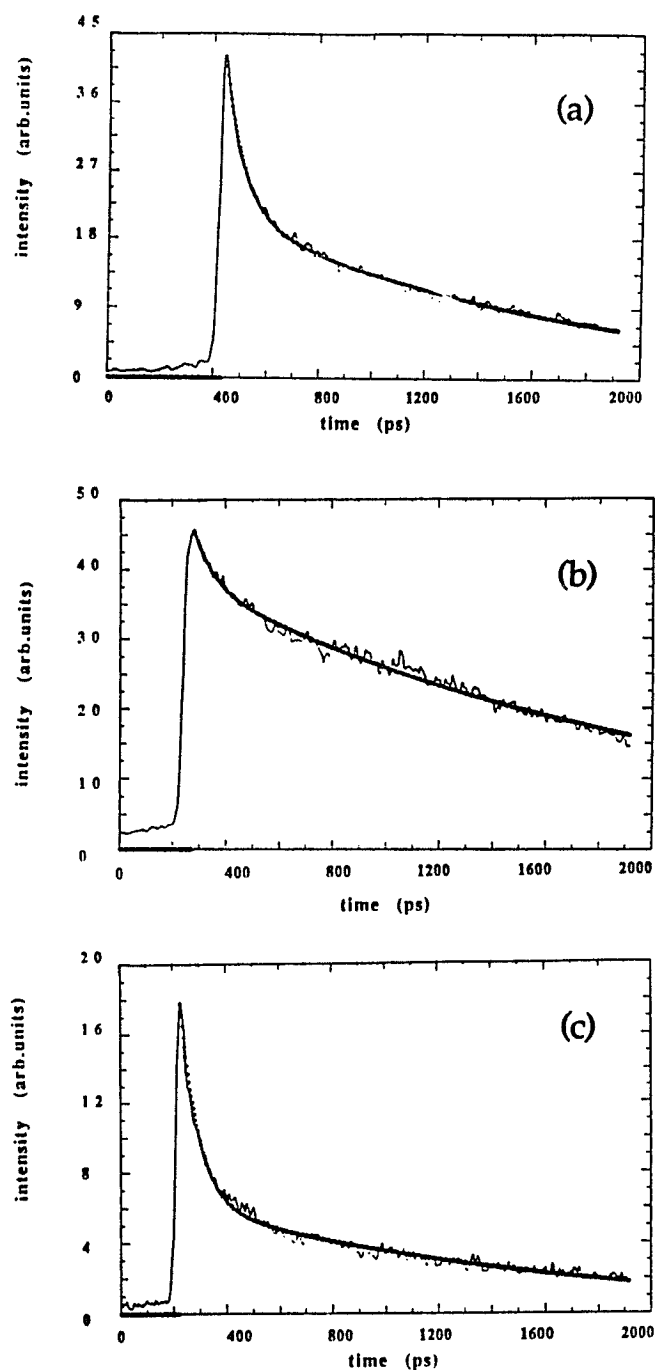


Figure 43. Time-resolved fluorescence profiles with 310nm excitation and 440nm emission. (a) Benign tissue: $t_f = 69$ ps, $t_s = 1.22$ ns, $a_f/a_s = 0.97$, (b) Benign tumor: $t_f = 79$ ps, $t_s = 1.93$ ns, $a_f/a_s = 0.21$, (c) Malignant tumor: $t_f = 67$ ps, $t_s = 1.35$ ns, $a_f/a_s = 1.8$

band, there is no marked dominance of either curve.

To obtain the relaxation times and amplitudes of the fluorescence decay the commonly used impulse response model of time-resolved fluorescence

$$I(t) = a_s \exp(-t/\tau_s) + a_f \exp(-t/\tau_f)$$

is used again to fit the experimental results by the nonlinear least square method.

In the 340nm band, while there is no significant difference in the fast decay times, the slower decay components for the malignant samples are found to be about twice as large as in the case of benign samples. The average τ_s for benign and malignant tissues are 1.2 ± 0.3 ns and 1.9 ± 0.2 ns, respectively. The slower component is more dominant in the malignant samples while the reverse is true in the case of benign samples. The average ratio of amplitudes a_f/a_s for benign and malignant tissues are 1.3 ± 0.5 and 0.4 ± 0.2 respectively. As shown in the two histograms (Figs. 44 (a) and (b)), the relative amplitudes and the slow component of lifetimes can separate cancerous from non-cancerous breast tissues.

In the histograms the height of each block represents either the ratio of amplitudes a_f/a_s , or τ_s . The number of blocks represents the number of samples studied. Figure 44 (a) displays the histogram of the ratio of fast to slow components of non-malignant and malignant human breast tissues in the 340nm band with 310nm excitation. A significant difference is noticed between the two types, with the ratios of the 12 non-malignant samples

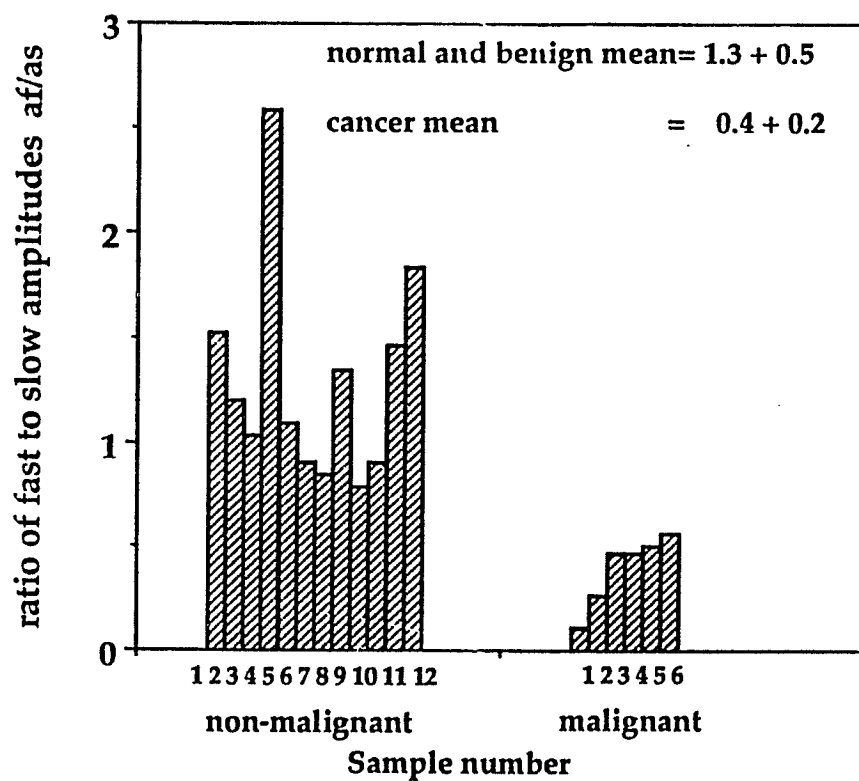


Figure 44 (a). Histogram of ratio of amplitudes of fast to slow components of fluorescence decay from non-malignant and malignant breast tissues in the 340nm emission band.

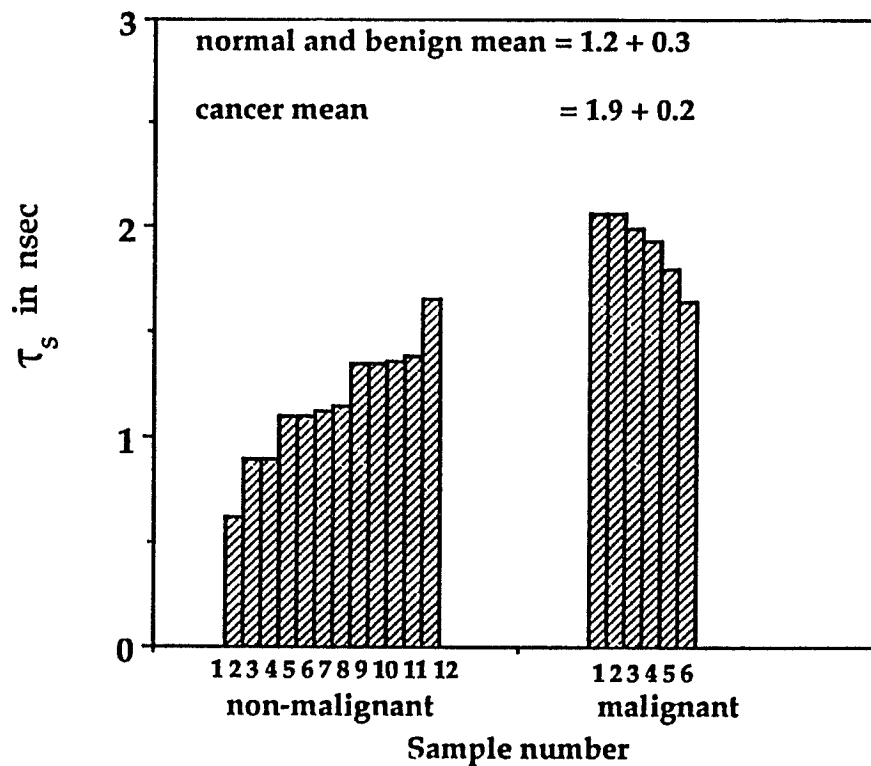


Figure 44 (b). Histogram of the slow component of fluorescence lifetimes from non-malignant and malignant breast tissues in the 340nm emission band.

Table 8: Fluorescence lifetimes and amplitude ratios of normal, benign tissue, benign tumor and malignant tumor human breast tissues excited at 310nm with 340nm and 440nm emission.

SAMPLE	tf (ps) 340nm	ts (ns) 340nm	af/as 340nm	tf (ps) 440nm	ts (ns) 440nm	af/as 440nm
Benign Tissue	102	1.1	0.8	49	1.4	0.5
Benign Tissue	60	1.1	0.9	-	-	-
Benign Tissue	50	1.3	1.1	72	1.6	0.5
Benign Tissue	60	1.1	1.0	80	1.0	0.7
Benign Tissue	46	1.1	1.2	69	1.2	0.9
Normal Tissue	85	1.4	1.8	89	1.7	0.8
Normal Tissue	114	0.6	1.5	-	-	-
Normal Tissue	81	1.34	0.9	-	-	-
Normal Tissue	75	1.6	0.8	-	-	-
Normal Tissue	103	1.3	1.3	-	-	-
Benign Tumor	49	0.9	1.5	79	1.9	0.2
Benign Tumor	59	0.9	2.6	65	1.4	0.8
Malignant Tumor	77	1.8	0.5	93	1.8	0.7
Malignant Tumor	20	1.6	0.4	52	1.7	0.6
Malignant Tumor	110	1.9	0.3	-	-	-
Malignant Tumor	79	1.99	0.5	67	1.3	1.8
Malignant Tumor	65	2.1	0.1	-	-	-
Malignant Tumor	54	2.1	0.5	39	1.6	0.2

ranging from 0.8 to 2.6 whereas the ratios of the 6 malignant samples ranges from 0.1 to 0.6. Figure 44 (b) displays the histogram of the slow component of the lifetimes, τ_s in the 340nm band with 310nm excitation. It also shows a difference between the non-malignant and malignant samples. As seen in the histogram, τ_s varies from 0.6 to 1.6ns for the non-malignant samples and from 1.6 to 2.0ns for the malignant samples.

The fluorescence kinetics for the 440nm band also show a double exponential decay (figs. 43 (a), (b) and (c)). However, no significant and consistent differences in the lifetimes or amplitudes between benign and malignant tissues can be found.

A list of the lifetimes and ratio of amplitudes in both bands are given in Table 8. The fast and slow components and the ratio of amplitudes, a_f/a_s , of 5 benign tissues, 2 benign tumors, 5 normal tissues and 6 malignant tumors have been measured and listed in the table for emission bands 340nm and 440nm. Differences in τ_s and a_f/a_s of non-malignant and malignant tissues are exhibited in the 340nm band with no differences observed in the 440nm band.

From the results of the 340nm band, a statistical study of these samples were performed using a Student 't' test. A 'p' value of < 0.001 was obtained between benign and malignant samples for τ_s and a_f/a_s . This work shows that the differences observed are significant. Yet the number of samples measured is still small to make any definite conclusive remark on the diagnosis aspect of the study.

Discussion

Thus, it is seen that 310nm excitation also displays double exponential fluorescence profiles. Again, as in the previous cases, the decay could be due to two different emitting species or due to environmental changes of one emitting species. The fluorescence spectral results indicates emission from molecules such as tryptophan, tyrosine or a combination of its residues. Table 9 lists the fluorescence lifetimes of tryptophan, NADH, elastin and collagen in water with 310nm excitation. The fluorescence profiles of the four fluorophores are fitted to double exponentials. The lifetimes of tryptophan (Fig. 45(a)) are 148ps and 2.2ns, NADH (Fig. 45(b)) are 44ps and 2.6ns, elastin (Fig.45(c)) are 169ps and 1.9ns and collagen (Fig.45(d)) are 172ps and 1.9ns. It is well known [53] that, for λ_{exc} greater than 295nm, fluorescence from tryptophan is more dominant than from tyrosine, both of which are present in most proteins. A study of the fluorescence and excitation spectrum of both these molecules shows that tryptophan is indeed a more dominant fluorophore at this excitation wavelength. Figure 46 displays the excitation spectra of tryptophan (a) with 2% filter in the emission path and tyrosine (b). The emission wavelength is 340nm with excitation ranging from 250nm to 325nm. Tryptophan absorption at 310nm appears to be stronger than that of tyrosine by at least ten times. This shows that tryptophan is the dominant fluorophore with 310nm excitation and 340nm emission.

Tryptophan and its residues in different microenvironments have long been known to show double exponential decay kinetics [53,54,55,56].

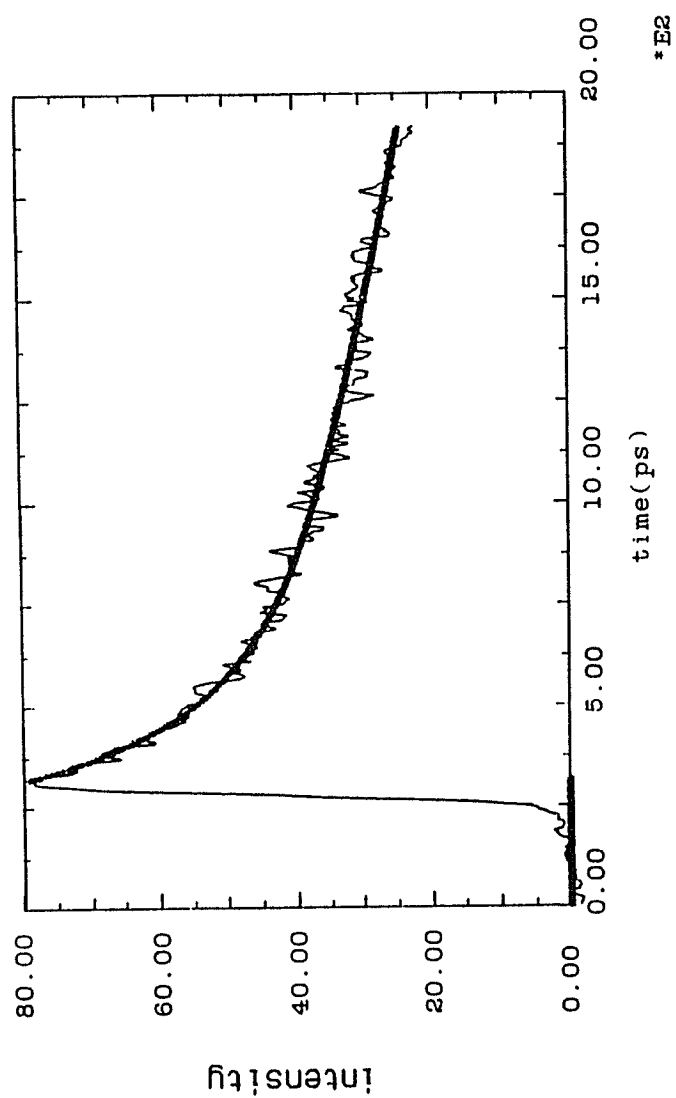


Figure 45 (a). Fluorescence lifetimes of tryptophan in water with $t_f=148\text{ps}$,
 $t_s=2.2\text{ns}$.

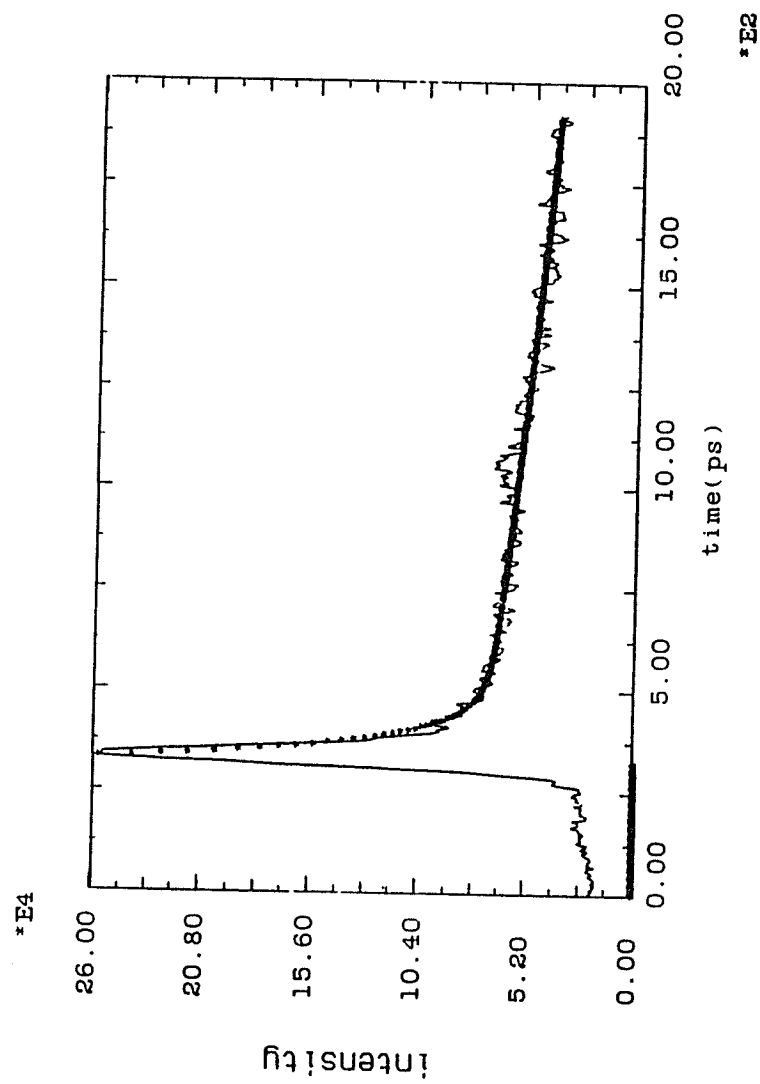


Figure 45 (b). Fluorescence lifetimes of NADH in water with $t_f=44\text{ps}$ and $t_s=2.6\text{ns}$

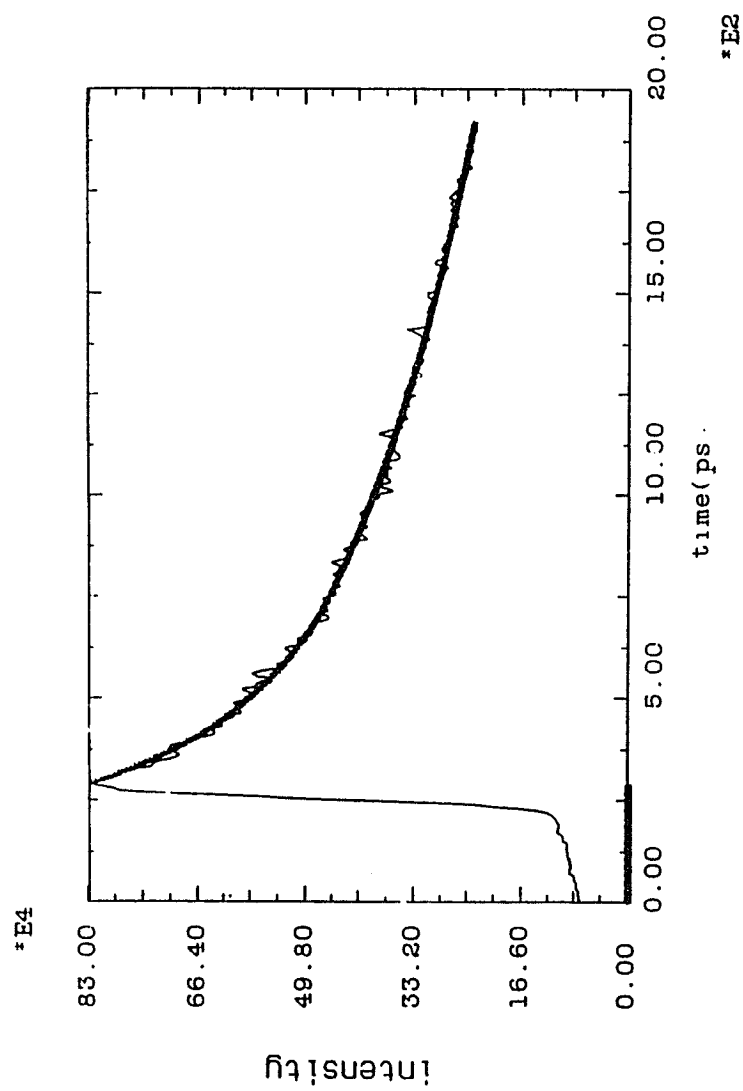


Figure 45(c). Fluorescence lifetimes of elastin in water with $t_f=169\text{ps}$ and $t_s=1.9\text{ns}$ and

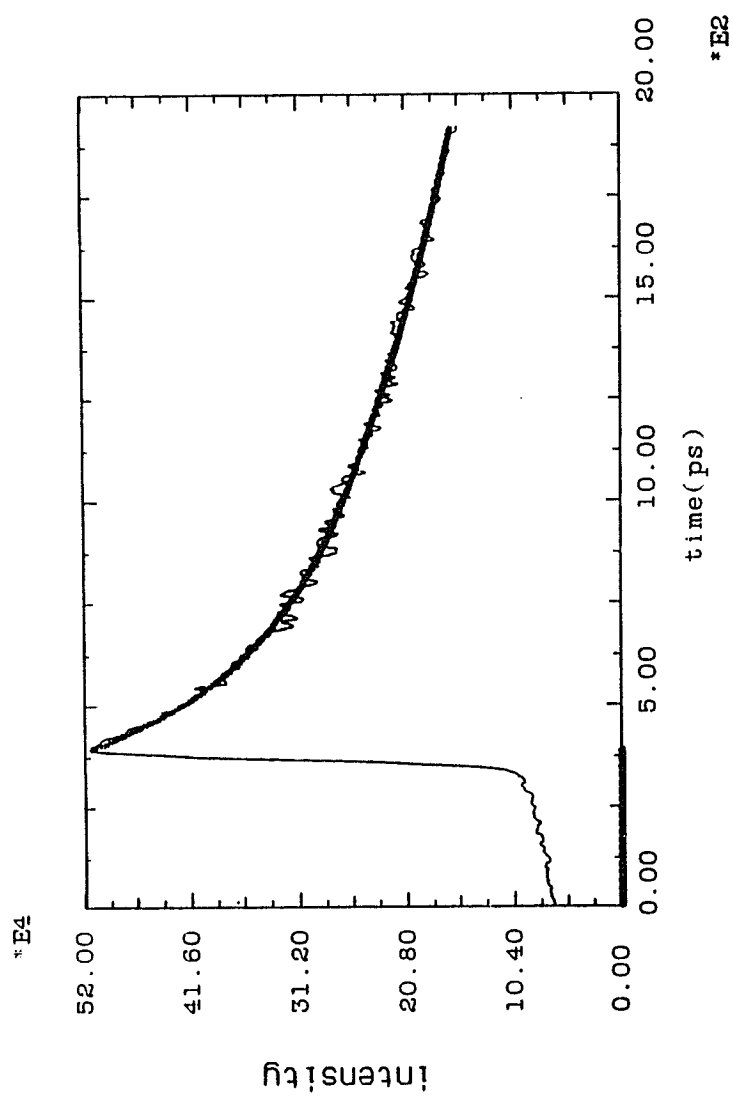


Figure 45 (d). Fluorescence lifetimes of collagen in water with $t_f=172$ ps and $t_s=1.9$ ns.

Hochstrasser and Negus [56] have shown that myoglobin with 265nm excitation emits fluorescence at 320nm. The emission is found to be dominated by tryptophan with double exponential decays. Thus protein fluorescence with double exponential decays at around 320nm appears to be from tryptophan. Tryptophan is an amino acid attached at different positions to form the protein. Trp-7 and trp-14 are examples of the different tryptophan residues present in myoglobin which decay with different fluorescence lifetimes. A combination of these tryptophan residues could give rise to a non-exponential decay profile.

Szabo and Rayner [54] have also studied tryptophan in solution and observed double exponential decays of 500ps and 3ns lifetimes. Non-exponential decays of tryptophan have been observed by Fleming and group [55], too.

Tryptophan is the most photochemically labile of the amino acids and plays an important role in photolysis of proteins and enzymes in the UV. It is well known that the fluorescence decay of tryptophan has great potential for use as a probe of the environments and motions of proteins. Thus it has been widely studied. The non-exponential decay of tryptophan is attributed to fluorescence from either tryptophyl residues in different environments or to one tryptophyl residue with multiple conformations of the molecule. Our observation of double exponential fluorescence decays in tissues in the 340nm band with a fast component in the subnanosecond range and a few nanosecond slow component suggests that the fluorescence could be from

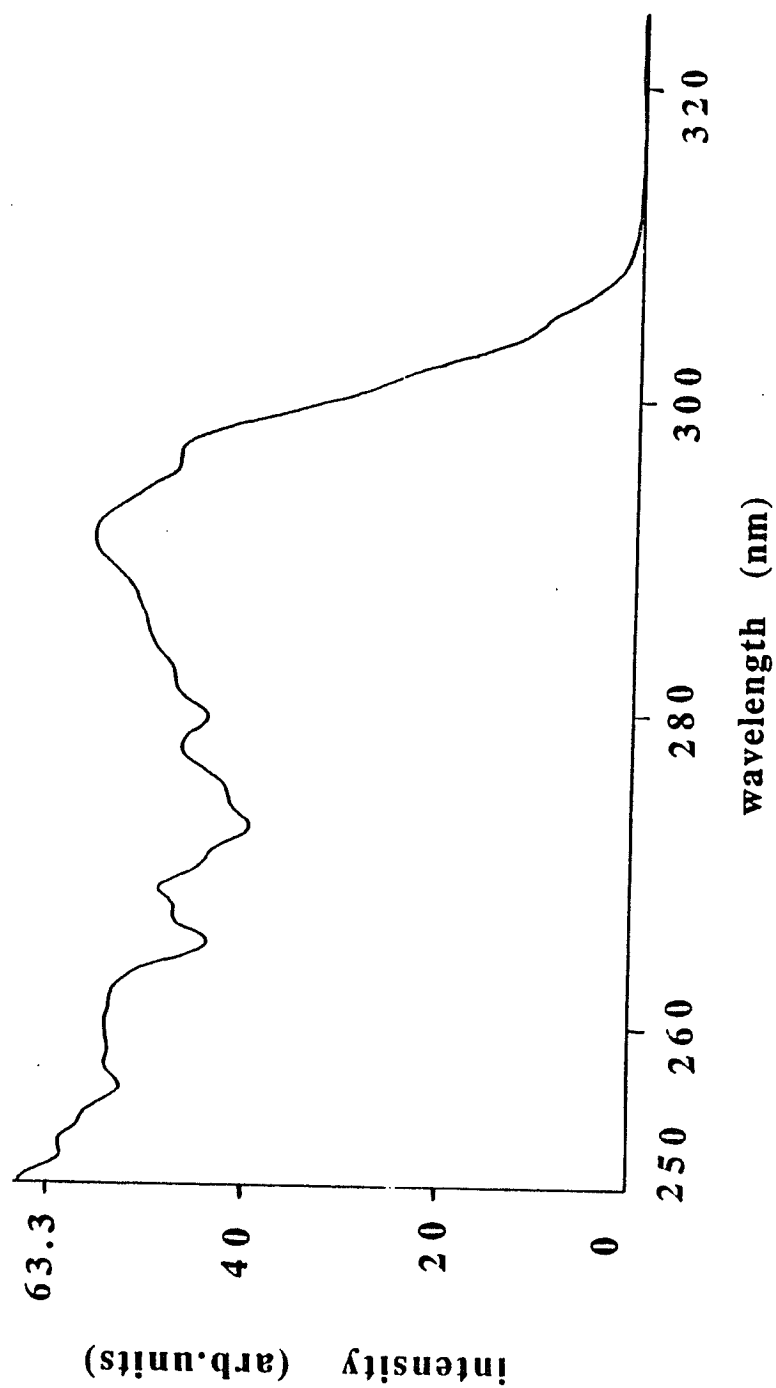


Figure 46(a). Excitation spectra with 340 nm emission of 10^{-4} M concentration of tryptophan with 2% filter in emission path and

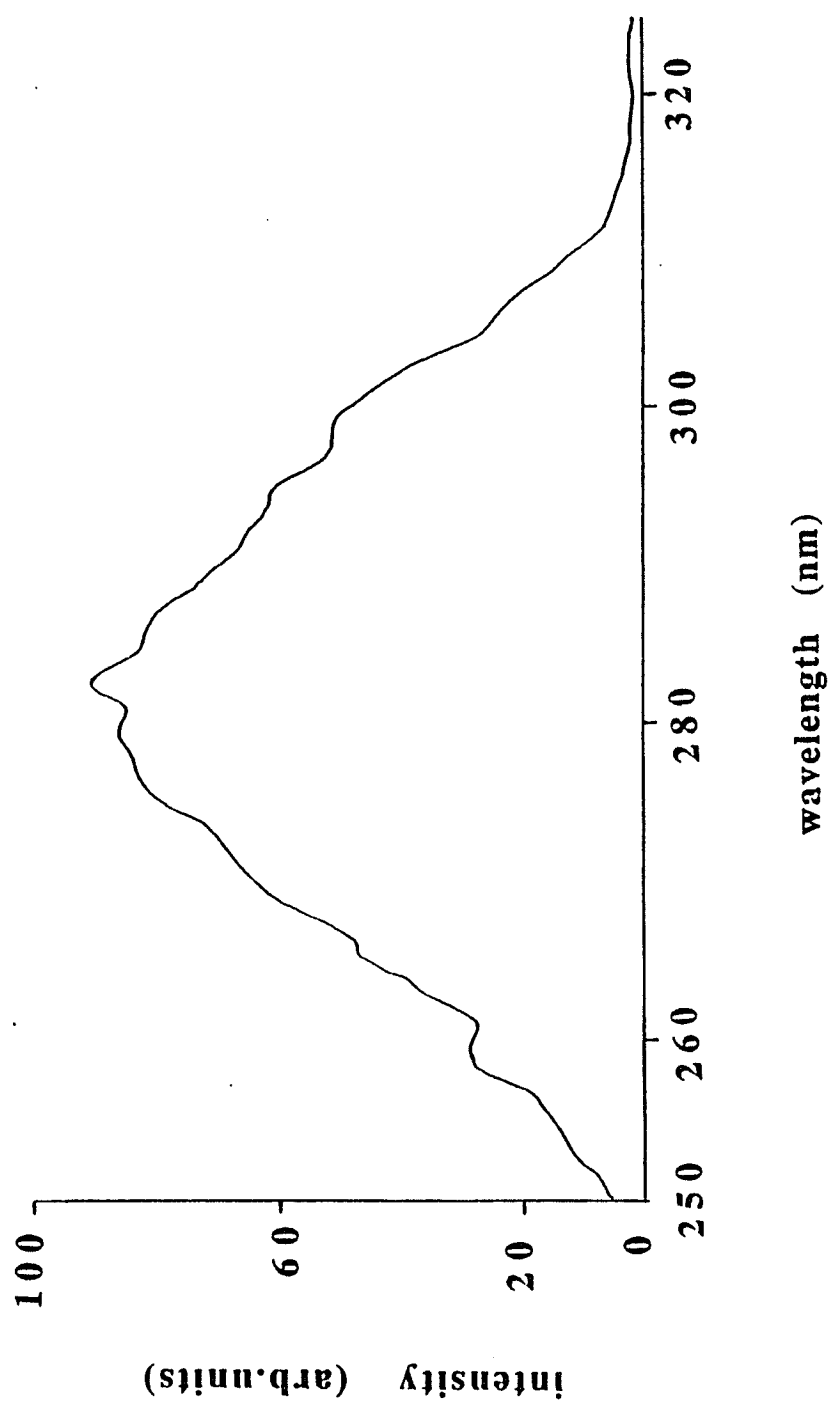


Figure 46(b). Excitation spectra with 340 nm emission of 10^{-4} M concentration of tyrosine with no filter in emission path.

excited indole rings in tryptophan residues. Due to the complex nature of the tissue, it is again difficult to specify the emitting chromophore definitely. However, from the available literature and current work on tryptophan fluorescence lifetimes, it can be partially confirmed that tryptophan is the emitting species in the 340nm band.

The double exponential fluorescence profile of tryptophan can be explained with different models. One of them (Szabo and Rayner [54]) invoked the rotamer model where the different conformers of the indole ring (The conformers are due to two or more different configurations of the alanyl chain in reference to the indole ring.) are assumed to interconvert very slowly or not at all during the excited state lifetime. If each conformer has a different lifetime, a given system will exhibit non-exponential decay.

The different steady state fluorescence of tryptophan has been assigned to different rotamers. This leads to change in the ratio of amplitudes of the fluorescence lifetimes as the emission wavelength is changed. This result, if observed in tissues, may confirm the tryptophan fluorescence from breast tissue with the various rotamers causing the differences between non-malignant and malignant tissues.

The fluorescence lifetimes of two benign tissues and one malignant tumor have been measured at emission wavelength bands $340 \pm 5\text{nm}$, $350 \pm 5\text{nm}$, $360 \pm 5\text{nm}$, $365 \pm 5\text{nm}$, $380 \pm 5\text{nm}$ and $390 \pm 5\text{nm}$. The temporal profiles of one benign samples is shown in Figures 47 (a), (b), (c), (d) and (e) in the above mentined wavelength bands, respectively. The fluorescence

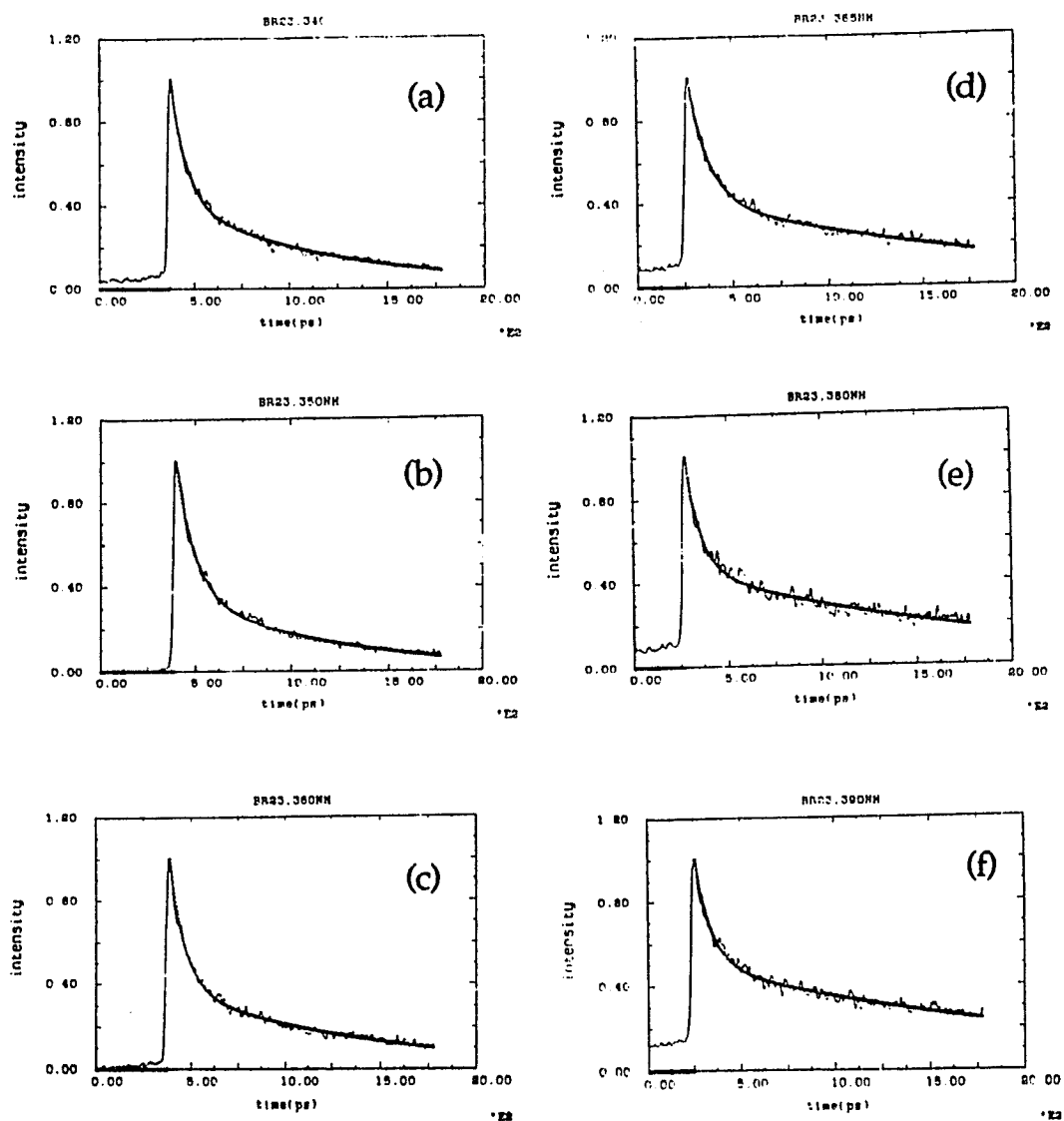


Figure 47. Emission dependence of fluorescence lifetimes and amplitudes of a benign tissue displayed in emission bands (a) 340 + 5 nm, (b) 350 + 5 nm, (c) 360 + 5 nm, (d) 365 + 5 nm, (e) 380 + 5 nm, (f) 390 + 5 nm.

Table 9: Emission wavelength dependence of fluorescence lifetimes and ratio of amplitudes of two benign tissues and one malignant tumor with 310nm excitation.

SAMPLE	tf(ps)	ts(ns)	af/as	emission wavelength (nm)
Benign tissue	84	0.9	1.4	340
	97	0.8	1.7	350
	77	1.0	1.5	360
	105	1.7	1.4	365
	78	1.6	1.1	380
	87	2.1	1.0	390
Benign tissue	56	0.9	1.3	340
	72	1.3	1.4	350
	77	1.7	0.9	360
	83	1.9	1.0	365
	68	1.9	0.8	380
	83	2.1	0.6	390
Malignant tissue	37	2.3	0.2	340
	38	2.4	0.4	350
	69	2.8	0.3	360
	6.8	2.1	0.3	365
	38	2.4	0.2	380
	24	2.2	0.2	390

lifetimes are seen to remain almost unchanged with emission wavelength. Table 9 lists the lifetimes and ratios which show no variation as emission wavelength is changed. This result implies that the fluorescence observed from human breast tissues most likely does not arise from rotamers of tryptophan.

Another alternative explanation may be the possibility of the short lifetime component arising from time-dependent diffusion-controlled intramolecular quenching mechanisms. Thus the decay will be governed by the following equation,

$$I(t) = I(0) \exp (-at-bt^{1/2})$$

Fitting the data with this equation gives values $a = 1/633.8\text{ps}^{-1}$ and $b = 3.99 \times 10^{-10}\text{ps}^{-1/2}$. Figure 48 displays the fluorescence temporal profile of a benign tissue with the above values of a and b . The least square fitting result shows that this model does not fit with experiment. As discussed earlier in Section II-Time-Rate Process, a non-radiative decay rate with $t^{1/2}$ dependence is not the correct form and further work with other forms of time-dependence is required.

Assuming the chromophore to be tryptophan, differences in the fluorescence lifetimes and the dominance of one decay amplitude to the other in benign and malignant tissues can be explained. Hochstrasser and Negus [56] have shown that different tryptophan residues in myoglobin exhibit different amplitudes and lifetimes. The ratio of amplitudes is seen to depend on the presence of one or more tryptophans at different positions of the

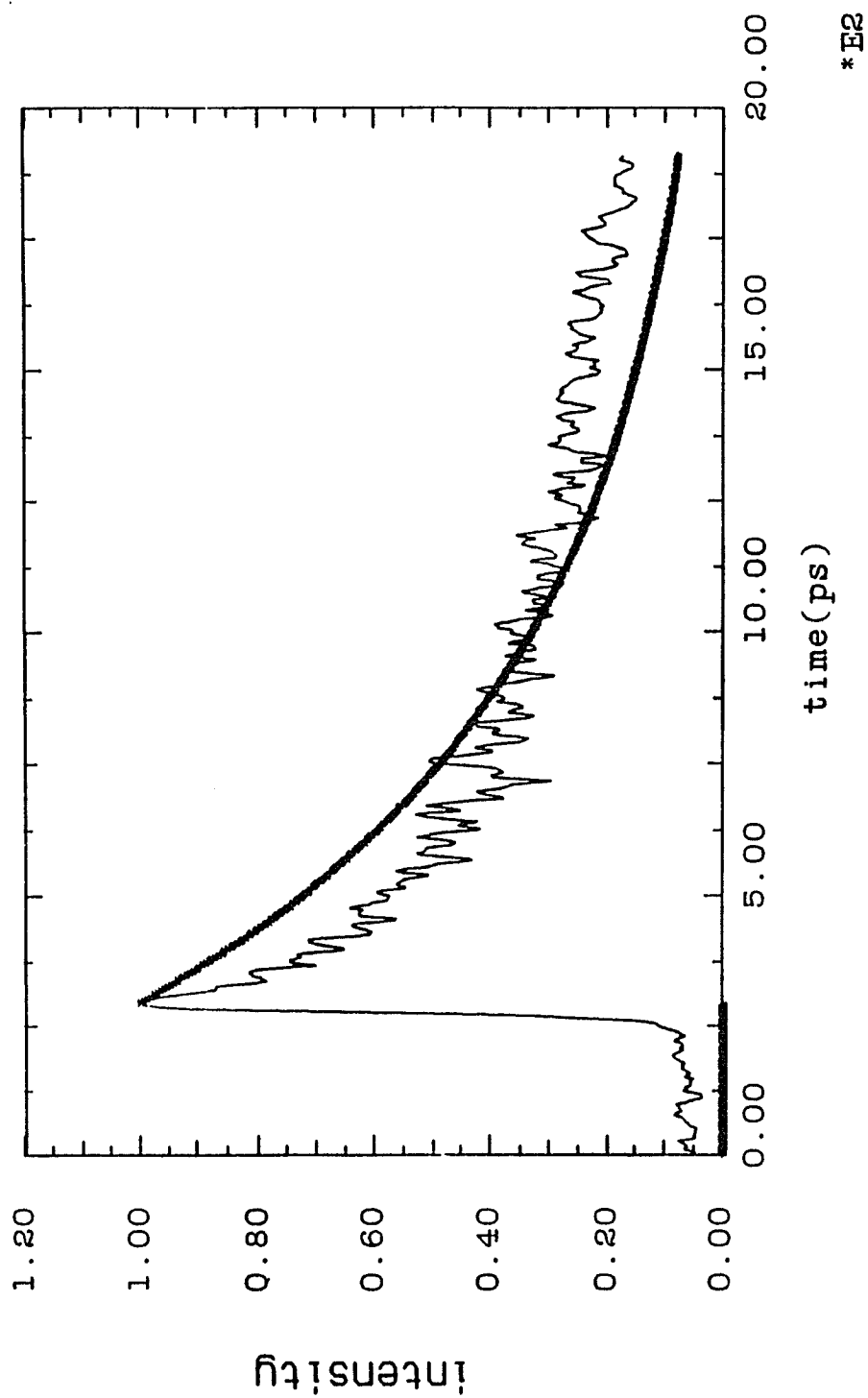


Figure 48. Time-resolved fluorescence profile of a benign breast tissue fitted to a time-rate equation with 310nm excitation; $a = 1/633.8\text{ps}^{-1}$ and $b=3.99\text{e-}10\text{ps}^{-1/2}$.

protein. One possibility could be the presence of two tryptophan residues in different proportions in cancerous and noncancerous tissues.

A second possibility could be due to the different micro-environments in benign and malignant tissues. Amplitudes and decay times of individual tryptophan conformers are strongly influenced by their micro-environments, such as different pH values and proximity of charged groups to the chromophores. Different pH values sometimes lead to charge transfers that give rise to enhanced intramolecular interactions. This results in larger non-radiative energy losses. Ratio of amplitudes is also known to change with pH of the tryptophan solution. Different pH may be one of the causes of the observed differences. The dominance of the fast amplitude in case of benign samples could be due to more non-radiative processes occurring in the tryptophan residues of benign tissues. The larger values of τ_s for cancerous tissues also suggests that more non-radiative processes occur in benign tissue environments. This result is in contradiction with our previous results. But in the previous case, a different set of molecules are excited and not tryptophan. In this case, tryptophan may be in a different environment from other molecules and give rise to opposite results.

The differences in the ratio of amplitudes between non-malignant and malignant tissues may be due to the intensity dependence of fluorescence. To test this, the tissue was excited with intensities of 0.1% and 0.01% of the original excitation intensity. No change in the profile was observed, as seen in Figure 49. This proves that the difference in the ratio of amplitudes is not

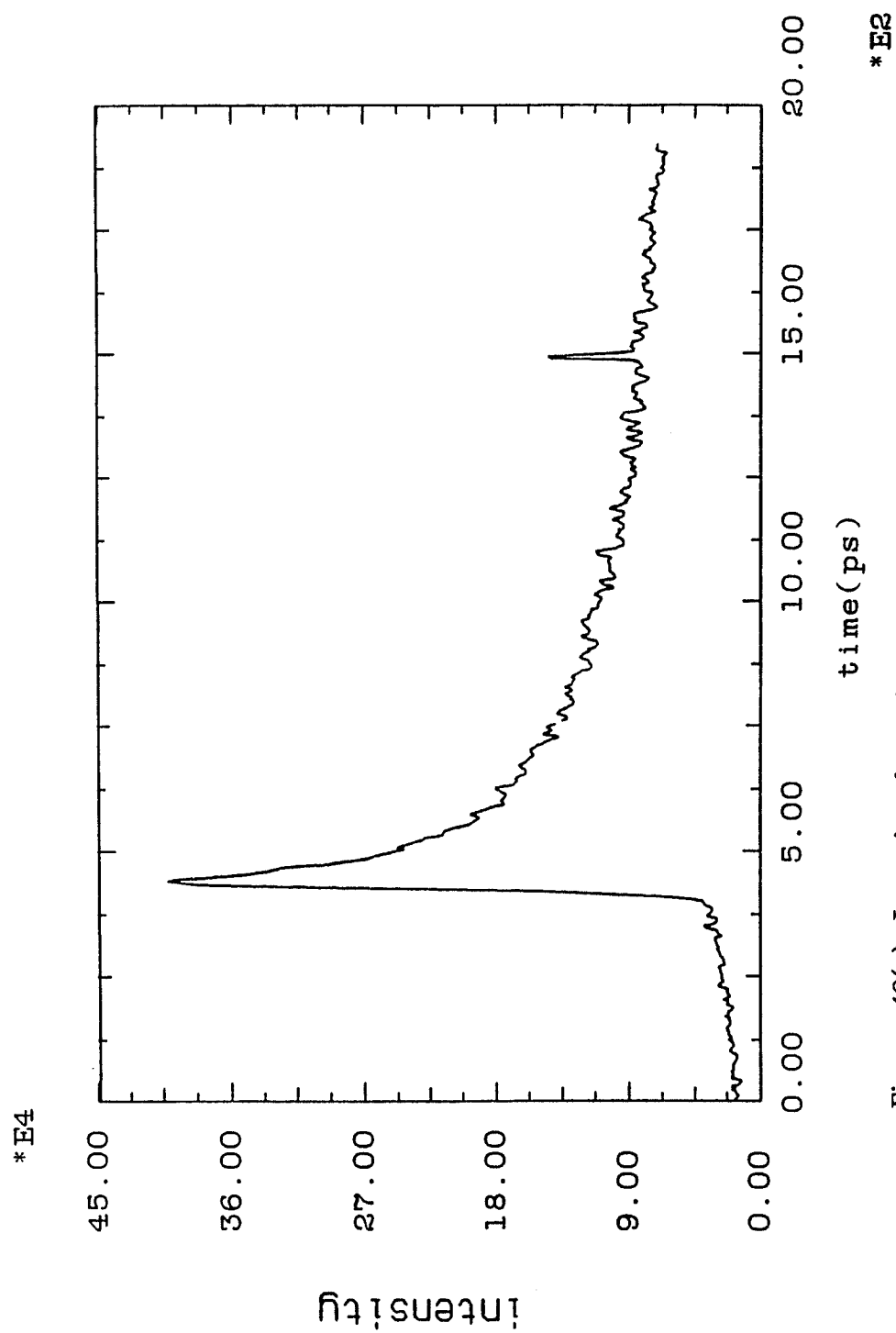


Figure 49(a). Intensity dependence of fluorescence temporal profile of a benign tissue with no filters,

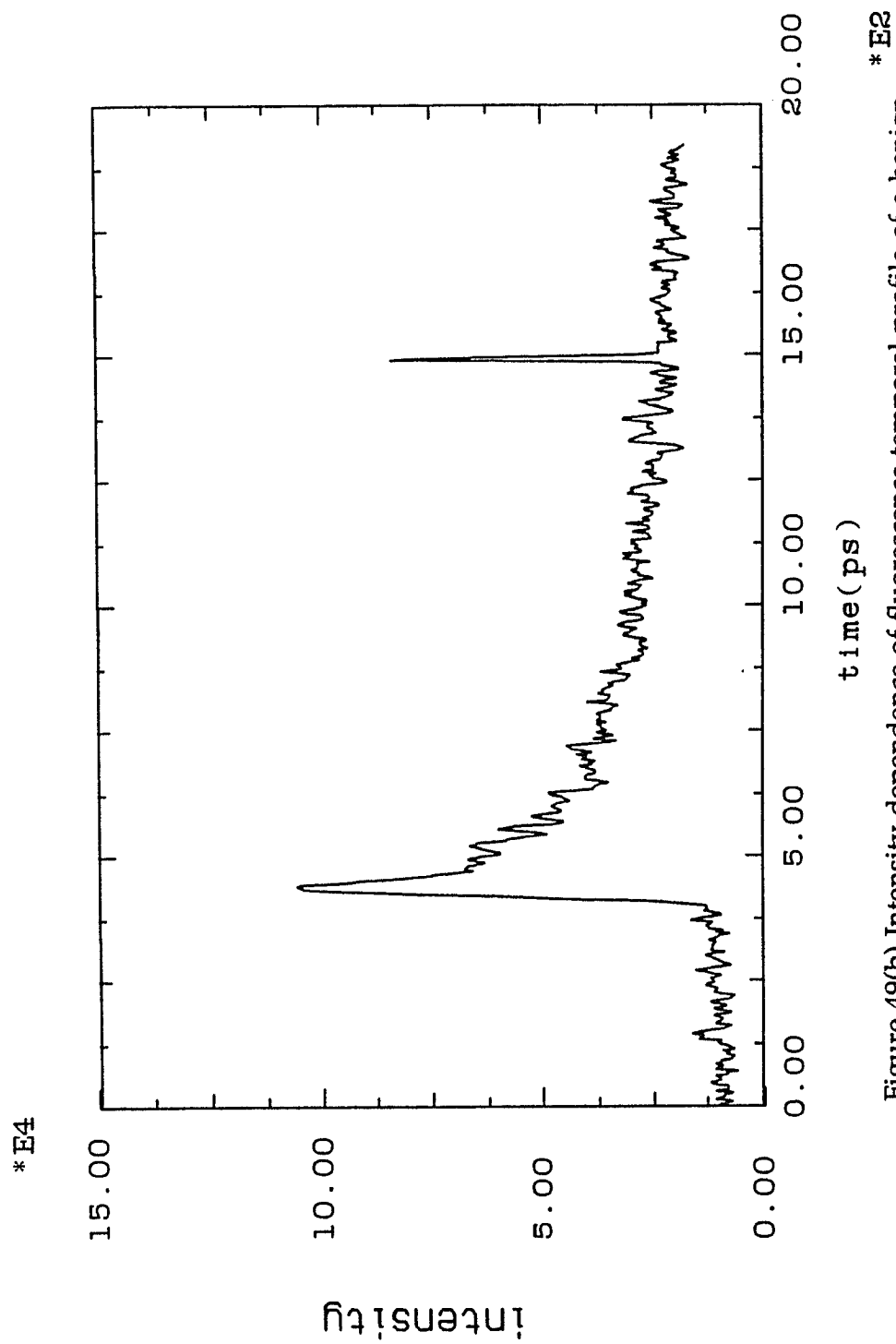


Figure 49(b) Intensity dependence of fluorescence temporal profile of a benign tissue with neutral density ND 25

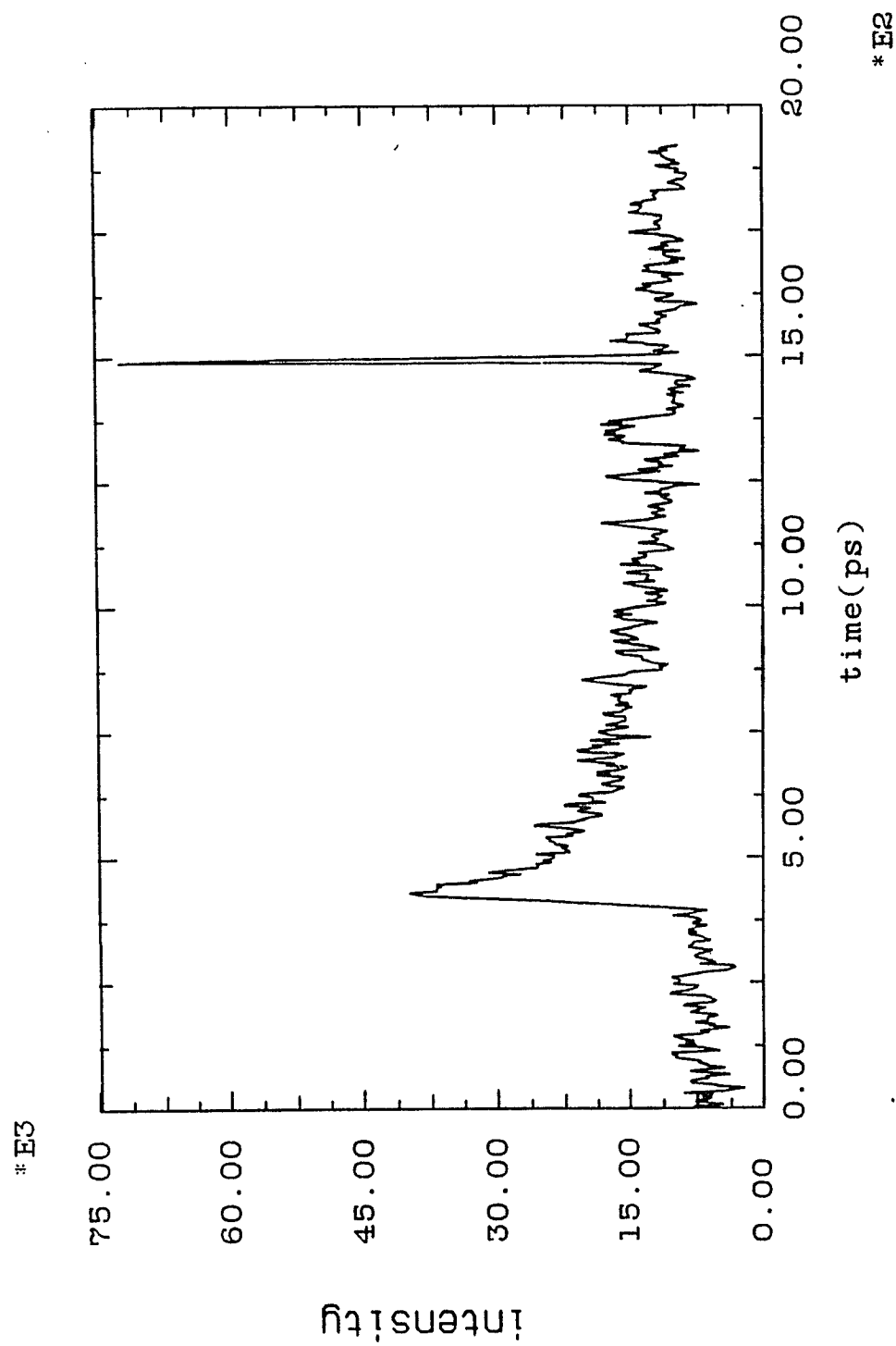


Figure 49(c) Intensity dependence of fluorescence temporal profile of a benign tissue with ND 13 in the excitation path.

due to intensity dependence.

The 440nm band has a spectral peak at around 460nm. It is known that NADH is a fluorescing coenzyme and has its peak at 460nm and an absorption band peaking at 350nm and weakly excited at 300nm. The weaker fluorescence of the 440nm band suggests that NADH could be the emitting nucleotide. The 440nm time-resolved profile is a double exponential which again could arise from two or more emitting species. The lifetimes of NADH with 310nm excitation are 44ps and 2.6ns (Table 10). Elastin and collagen may also contribute to the 440nm band. It could also be due to environmental conditions surrounding the NADH. NADH in bound and free states could show certain differences. This has not yet been tested and further research in the future may confirm the sources of the band emission. Fluorescence kinetics in the 440nm band vary in both amplitudes and lifetimes between the benign and malignant tissues. This shows that NADH, elastin and collagen are not the fluorophores responsible for any differences with this excitation wavelength. It should be noted, however, that with 351nm excitation NADH band showed significant differences between normal and malignant tissues but when this was extended to benign tissue no such differences were found. Since the 440nm band does not display any differences between non-malignant and malignant tissues, it appears that NADH is not the chromophore for diagnosis with 310nm excitation. From the lifetimes of the breast tissues (Table 8) and the fluorophores (Table 10) with 310nm excitation, it appears that tryptophan (148ps and 2.2ns) is responsible for the

Table 10: Fluorescence lifetimes and ratio of amplitudes of biomolecules with 310nm excitation.

SAMPLE	tf(ps)	ts(ns)	af/as
Tryptophan	148	2.2	0.5
NADH	44	2.6	0.7
Elastin	169	1.9	0.4
Collagen	172	1.9	0.4

fluorescence in the 340nm band (since it fluoresces around 320nm) with non-radiative processes shortening the fast component from 148ps to < 100ps. The slow component of tryptophan (2.2ns) is almost the same as that of the malignant tumors (2ns) and slower than that of non-malignant tumors (1-1.5ns). The 440nm band of the tissue fluorescence ($t_f < 100ps$ and $t_s = 1.5ns$ and $a_f/a_s = 0.5$), on the other hand appears to be from a combination of NADH ($t_f = 44ps$, $t_s = 2.6ns$ and $a_f/a_s = 0.7$), elastin (169ps and 1.9ns) and collagen (172ps and 1.9ns) may be contributing to the 440nm band but further research is necessary to accurately determine whether a combination of NADH, elastin and collagen contributes to the fluorescence.

It appears from this study that the two optically active chromophores with 310nm excitation are tryptophan and NADH, elastin and collagen. Much more work is necessary to confirm the environment they are in. The non-radiative decays may be a cause of the nonexponential forms observed, as before, with the other excitation wavelengths.

SECTION IV:

TIME-RESOLVED FLUORESCENCE DEPOLARIZATION WITH 310nm EXCITATION

It has earlier been mentioned that one of the physical processes which could affect the time decay of a biological system is depolarization effects. The internal molecular motions and energy transfer in tissues can be

determined by polarization measurements. Detecting fluorescence in the presence of a polarizer at an angle of 54.7 degrees from the parallel emission selects only the fluorescence decay with no orientational factor.

The total emission measured with no polarizer in front of the streak camera is

$$I_{\parallel} + I_{\perp}$$

According to Tao [57]

$$I_{\parallel} + I_{\perp} = ae^{-t/\tau_p} + be^{-(t/\tau_p + t/\tau_R)}$$

This shows that the rotational factor is a part of the emission observed.

The total fluorescence decay is

$$I(t) = I_{\parallel}(t) + 2I_{\perp}(t)$$

Observing the fluorescence with an analyzer set at some arbitrary angle α from the direction of parallel polarization,

$$I_{\alpha}(t) = \cos^2 \alpha I_{\parallel}(t) + \sin^2 \alpha I_{\perp}(t)$$

Thus, to obtain total fluorescence only, with no rotation, $\alpha = 54.7$ degrees.

Depolarization experiments were performed on the benign and

malignant samples at 310nm excitation and a double exponential curve observed as before by setting $\alpha = 54.7^\circ$. This result implies that the decay is not affected by any molecular rotation. But molecules present in biological tissues are in motion since they are not in a rigid medium. The question now arises as to how fast these reorientational motions are. Fluorescence depolarization is a method of determining the dynamics of molecular motion.

Dipoles preferentially selected for emission by polarized excitation light give partial polarization as some dipoles have two components. This absorption process is thus the first source of depolarization. The second source of depolarization is if the absorption and emission dipoles are not parallel. Fast motion causes the absorption dipole to change its direction before emission. The third depolarization factor is the Brownian rotation of the molecule. If the excited state lifetime is comparable to the time required for brownian motion to occur over an appreciable angle, significant depolarization occurs. This is the basic theory for using fluorescence depolarization to study the dynamics of the emitting fluorophores in tissues. The rotational freedom can be detected through a study of the time-resolved fluorescence and anisotropy. The time-resolved anisotropy is given by

$$r(t) = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$

An important parameter in the evaluation of time-resolved depolarization is the experimental value of the anisotropy at zero time, $r(0)$.

It is the intrinsic anisotropy of the chromophore and is a function of the relative orientations of the absorption and emission dipoles. For a chromophore with parallel absorption and emission dipoles, $r(0) = 0.4$. A value less than 0.4 indicates the existence of relaxation processes occurring at a time scale shorter than the resolution of the experiment [58]. This implies that the motion is very fast and so the absorption and emission dipoles may not be the same.

Rotational correlation times can be determined by fitting

$$r(t) = \sum r(0) e^{-t/\tau_r}$$

If $r(t)$ fits to a double exponential, the fast and slow components are usually interpreted as due to a very rapid internal motion and a slower motion of the molecule as a whole. The molecule motion could be restricted to a certain angle. This is particularly true in tissues where the environment is as viscous as water and so motion of molecules is hindered. Motion may also be hindered when molecules are in a bound state. Flavins and NADH are coenzymes bound to proteins in tissues. Tryptophan and tyrosine are amino acids which have bonds with at different sites. The motion of these molecules, hence may not be free. The rotational relaxation in these cases would be restricted to a certain angle depending on how tightly they are bound. $r(t)/r(0)$ gives the semi-angle of the cone within which the molecule rotates [59]. Measurements of parallel and perpendicular polarization fluorescence intensities have been performed using 310nm excitation. The figures 50 and

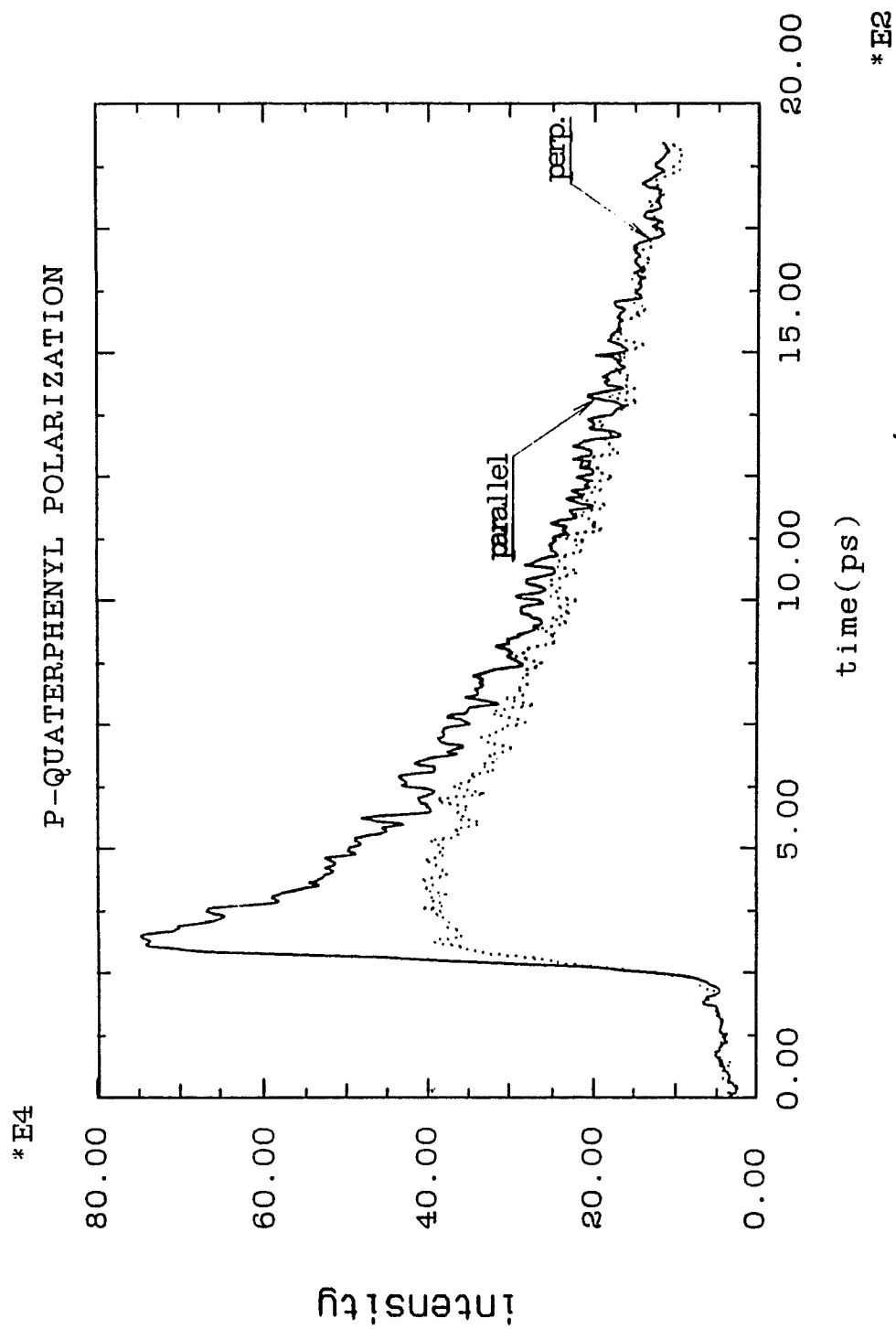


Figure 50. Time-resolved fluorescence profiles of 10^{-4} M concentration of p-Quaterphenyl with 310nm excitation and 340 band emission: (a) parallel polarization and (b) perpendicular polarization.

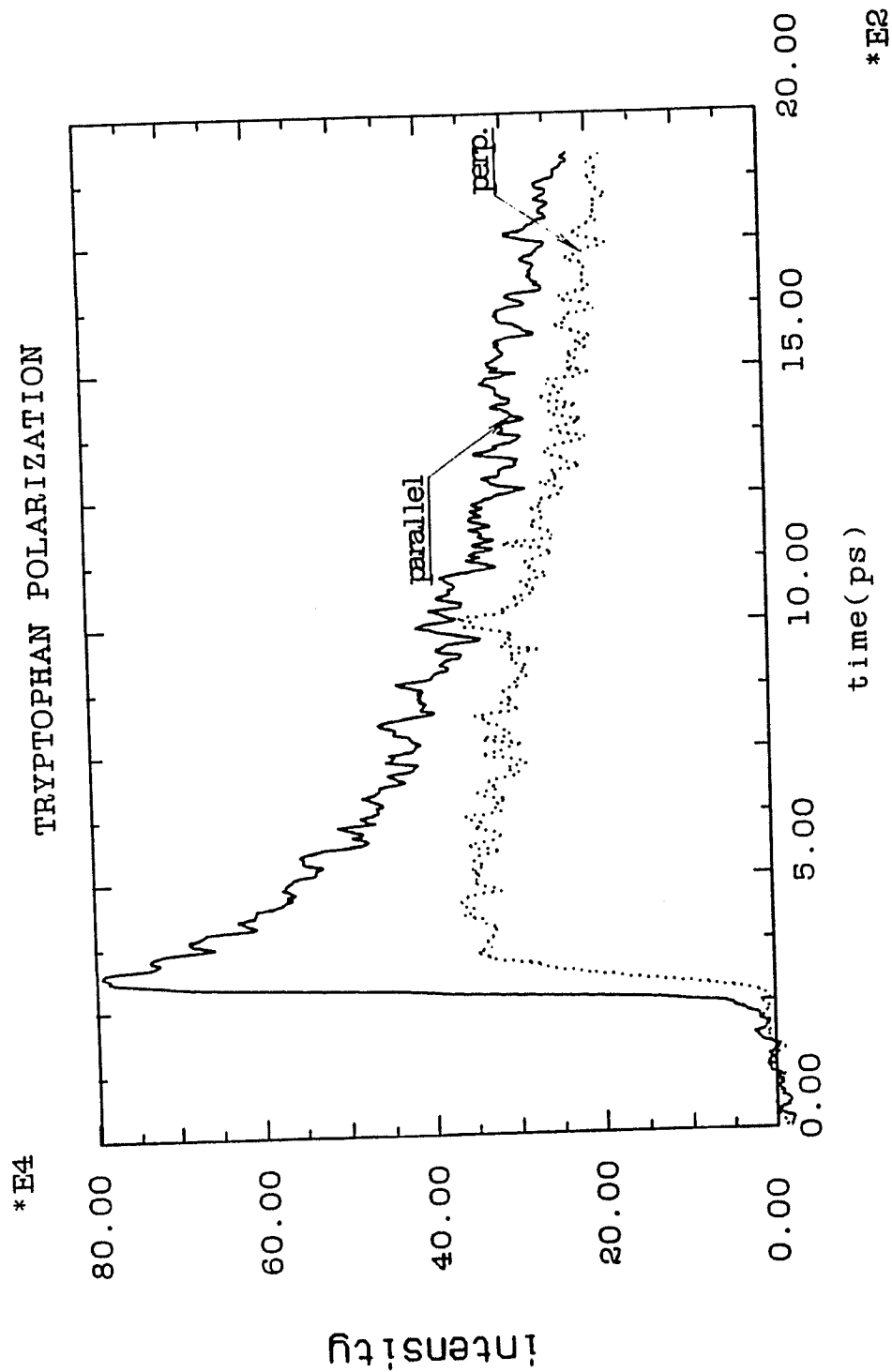


Figure 51. Time-resolved fluorescence profiles of 10^{-4} M concentration of L-tryptophan with 310nm excitation and 340 band emission: (a) parallel polarization and (b) perpendicular polarization.

51 show the parallel and perpendicular components of the time-resolved fluorescence of the p-Quaterphenyl dye and tryptophan. The parallel components in both cases show double exponential decay profiles while the perpendicular component shows a slower rise time with a single exponential decay. The intensities of the perpendicular components are about half the intensities of the parallel components. The zero time is chosen at the peaks of the temporal profiles. A value of $r(0) = 0.25$ was obtained for p-Quaterphenyl and 0.34 for tryptophan. Depolarization measurements of macromolecules have shown values between 0.4 and 0.2 [58]. These results confirm that our system is well set for the measurements from human tissues. It is known that rapid internal motions are responsible for deviations from $r(0) = 0.4$. The dye and tryptophan do show values lower than 0.4. Hence, this is attributed to fast motion of the molecules. In the case of benign tissue and malignant tissue, under the same conditions ($\lambda_{\text{exc}} = 310\text{nm}$ and $\lambda_{\text{em}} = 340\text{nm}$) it is seen that the fluorescence is completely and rapidly depolarized. Figures 52 and 53 display the parallel and perpendicular components of the time-resolved fluorescence of benign tissue and malignant tumor, respectively in the 340nm band. The parallel components in both cases are double exponential with the fast component of the malignant tumor being small. This is consistent with the results of time-resolved fluorescence with 310nm excitation and 340nm emission. Interestingly, the perpendicular components of both benign and malignant samples appear to be double exponentials with no change in shape. The rise times of the perpendicular components are fast

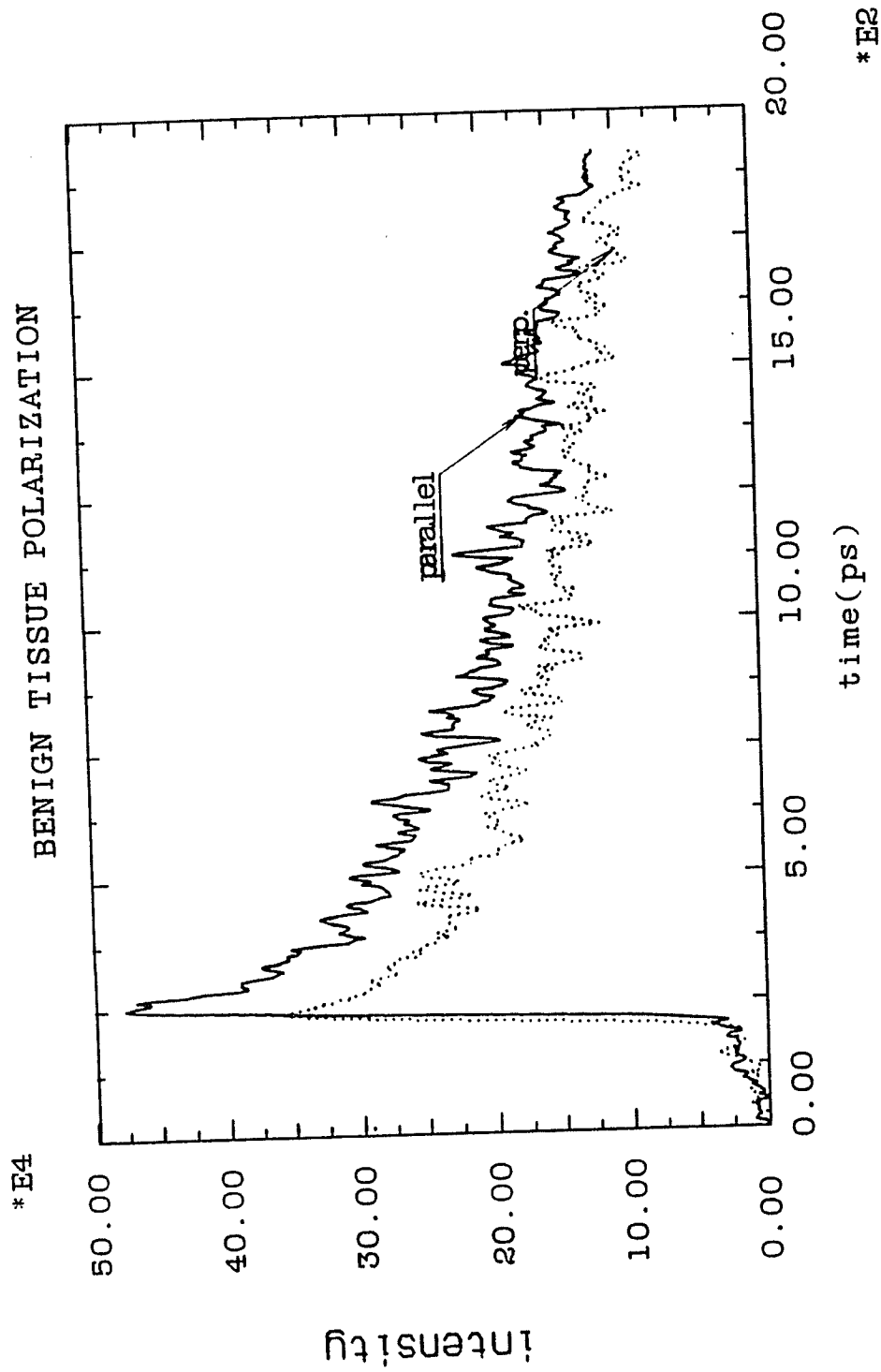


Figure 52. Time-resolved fluorescence profiles of a benign tissue with 310nm excitation and 340 band emission: (a) parallel polarization and (b) perpendicular polarization.

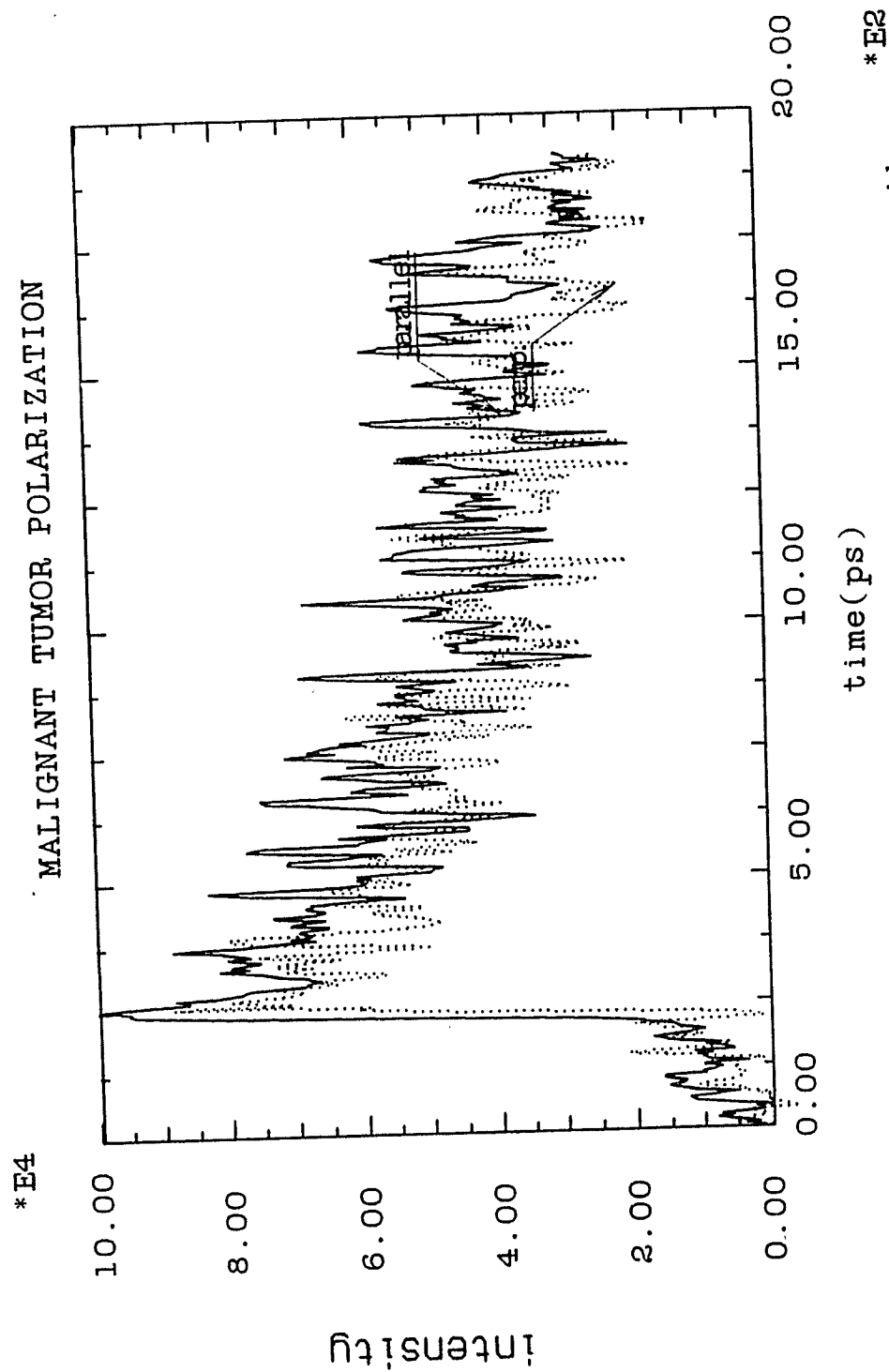


Figure 53. Time-resolved fluorescence profiles of a malignant tissue with 310nm excitation and 340 band emission: (a) parallel polarization and (b) perpendicular polarization.

and the same as that of the parallel components. The intensity of the perpendicular component of the benign tissue is lower than the intensity of the parallel component by less than a third. In the case of the malignant tissue, there is no difference in the intensities of the parallel and perpendicular components. This implies that the molecules undergo very rapid motional changes. Figure 54 (a), (b), (c) and (d) display the rotational anisotropy, $r(t)$ versus time of p-Quaterphenyl, tryptophan, benign tissue and malignant tissue, respectively. The double exponential forms of the dye and the fluorophore show slow decays and higher values of $r(t)$ as compared to those of the breast tissues. The orientational anisotropy cannot be measured with this time resolution of the detection system. The only conclusion that can be drawn here is that the molecular motions is less than 20ps. Further work with better time resolution is needed to probe into the exact speed of motion.

Summarizing, the fluorescence profiles using time-resolved techniques on breast tissue in the uv and visible ranges of excitation display a non-exponential profile in time. These profiles can be fitted to double exponentials and to time-dependent diffusion controlled phenomena and long-range energy transfer processes to obtain the fluorescence lifetimes and other non-radiative parameters. The experimental results do not fit the time-dependent rate process. The non-exponential forms have not been interpreted since it is not possible at this early stage to do so for such complex systems such as human tissues. It has been concluded that the non-exponential form does not

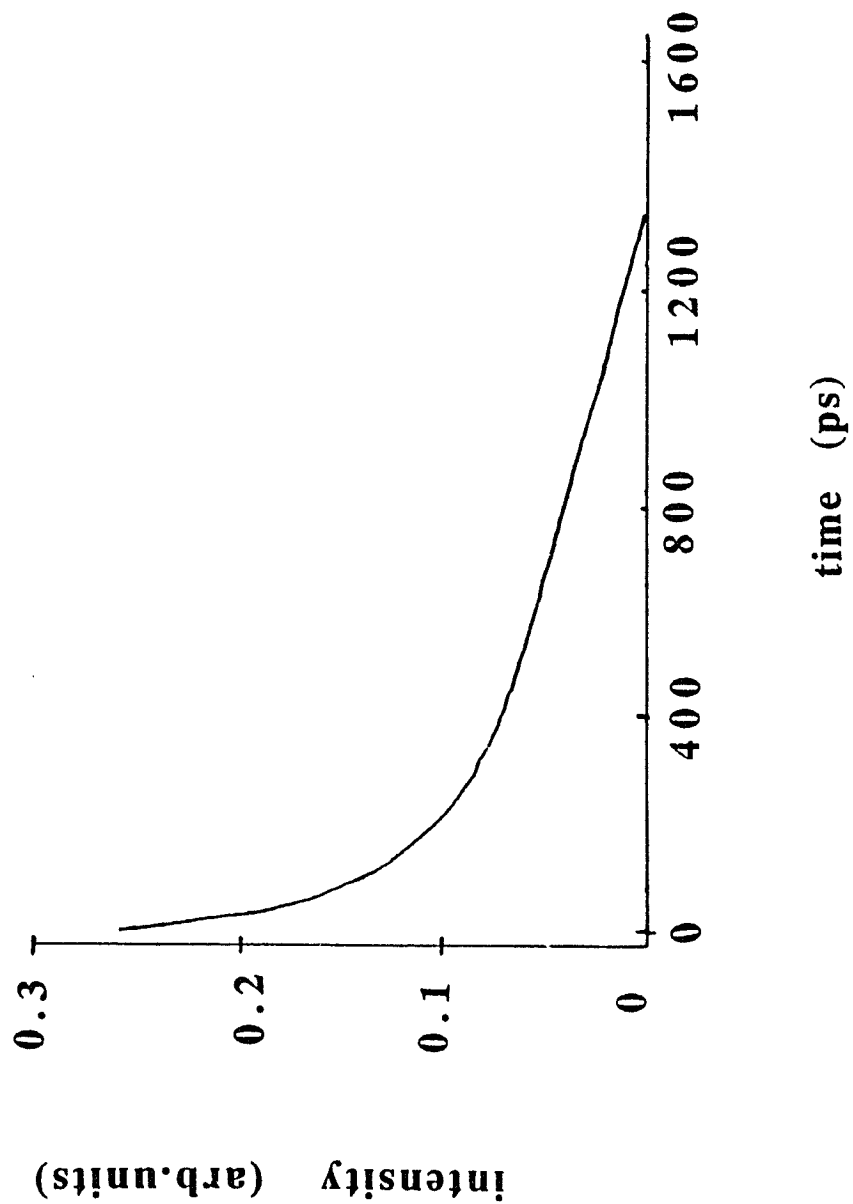


Figure 54(a). Fluorescence rotational anisotropy $r(t)$ versus time of p-Quaterphenyl with 310nm excitation and 340nm emission band.

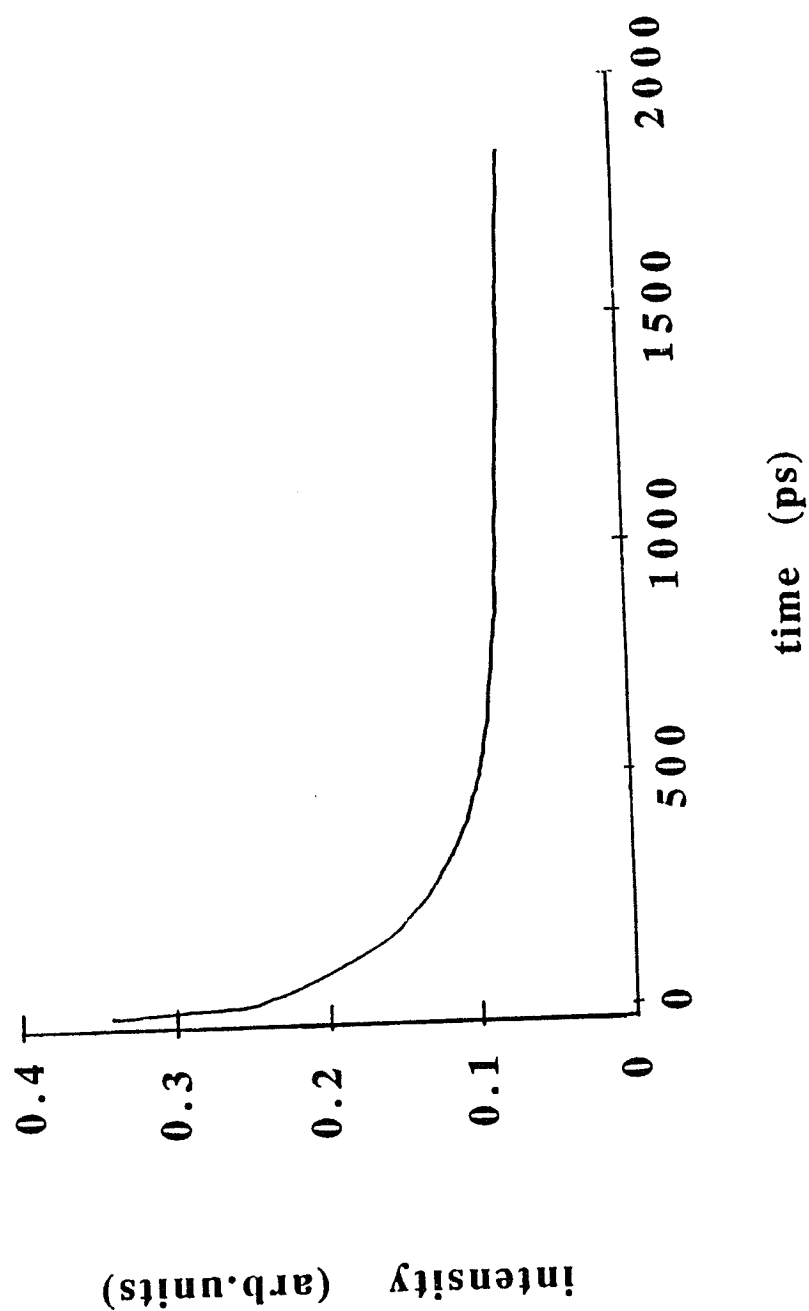


Figure 54(b). Fluorescence rotational anisotropy $r(t)$ versus time of tryptophan with 310nm excitation and 340nm emission band.

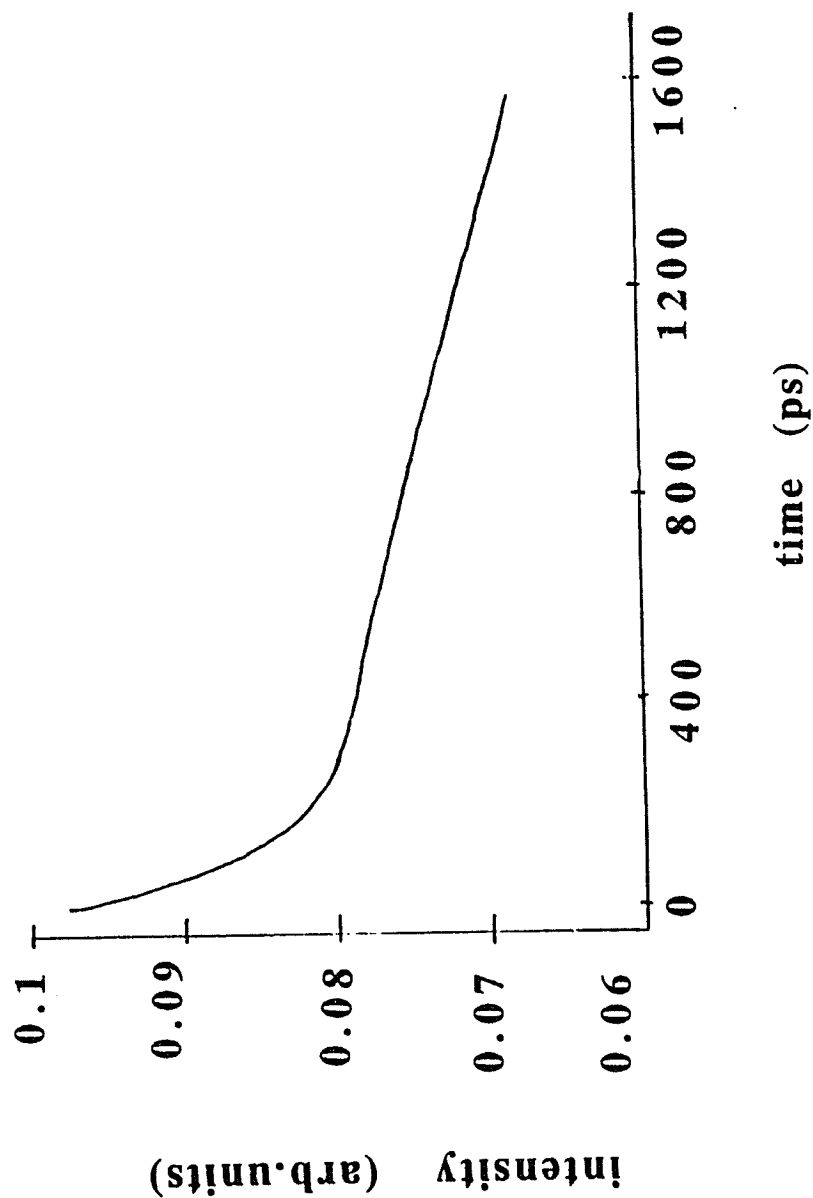


Figure 54 (c). Fluorescence rotational anisotropy $r(t)$ versus time of benign tissue with 310nm excitation and 340nm emission band.

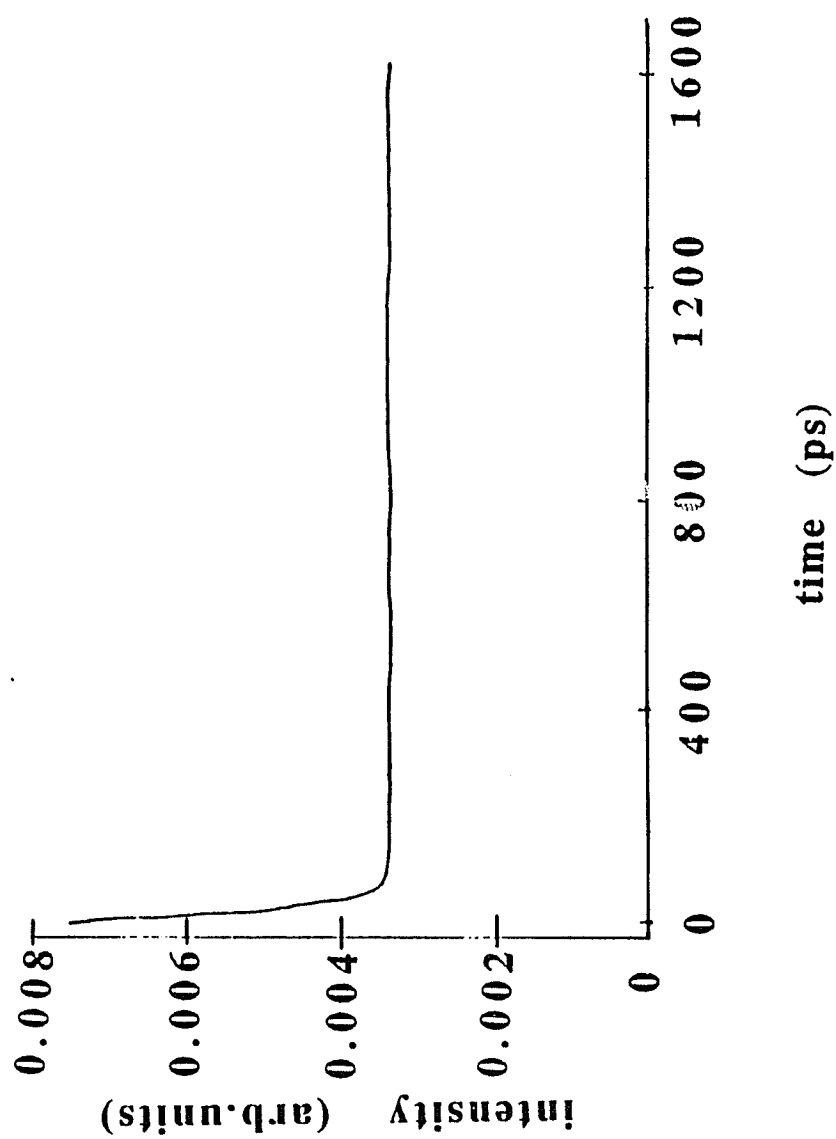


Figure 54 (d). Fluorescence rotational anisotropy $r(t)$ versus time of malignant tumor with 310nm excitation and 340nm emission band. Sharp spike may be due to noise

have any contribution from rotational motion of the molecules and that the molecular motions are much faster than the resolution of the detection system used. The chromophores that may be responsible for the observed signals, namely, NADH, flavins, elastin, collagen and tryptophan have been studied. From the lifetime measurements of the tissues and the fluorophores it has been suggested that NADH combined with elastin and collagen are responsible for the tissue fluorescence with 351nm excitation. With 310nm excitation, tryptophan is most likely the fluorophore responsible for the 340nm emission and NADH, that of the 440nm band. Elastin and collagen are also possible fluorophores contributing to the 440nm band. The differences obtained between benign and malignant samples have not yet been interpreted. It has been suggested that the differences most likely are due to more non-radiative processes occurring in the cancer environments with visible and near-uv excitation. UV excitation (310nm) is seen to be the key wavelength to observe differences in malignant and non-malignant human breast tissues in the time domain. Tryptophan is the molecule about which more non-radiative processes occur in the benign tissues than in cancer tissues. Much more work is necessary to draw any firm conclusions on the differences observed. A general understanding of the spectroscopic properties of normal and cancerous breast tissues has been obtained in this study.

CHAPTER 5: LOW TEMPERATURE EMISSION FROM TISSUES

When light is absorbed by an atom or a molecule, it is elevated to an excited state. The molecule can return to its ground state either by radiative or non-radiative transitions. The process of emission of light is known as luminescence. When the environmental conditions are such that the atom or molecule relaxes to the ground state almost immediately or after a sustained period of time one observes these phenomenon as fluorescence and phosphorescence, respectively.

Fluorescence is a transition from the first excited singlet state to the ground singlet state while phosphorescence is a transition from the triplet state to the singlet ground state. Since the $T_1 \rightarrow S_0$ transitions are forbidden, the lifetimes of the triplet states can be orders of magnitude longer than those of the singlet excited states. Typically, fluorescence lifetimes occur in the nanosecond range or less while phosphorescence can be as long as several seconds, particularly in rigid media. In fluid solutions, phosphorescence can range from tens of nanoseconds to hundreds of milliseconds, depending on experimental conditions. Prolongation of the luminescent state beyond the nanosecond range is greatly enhanced by cooling. It is known that non-radiative processes slow down at low temperatures and the fluorescence and phosphorescence are enhanced. The fluorescence and phosphorescence lifetimes are also increased at low temperatures. Aggregate formation is also enhanced at low temperature. Proteins were found to emit phosphorescence at liquid nitrogen temperatures [60]. Phosphorescence intensities of calcified

tissues and other biological materials have also been studied [61]. Studies have also been performed on carcinogens to determine if there existed a dependence between triplet state and carcinogenic activity [62]. No indication of a relationship had been found. It follows from these studies that the low temperature emission studies can provide information about the various physical processes occurring in malignant and non-malignant tissues from the changes in the luminescence decay lifetimes.

The fluorescence lifetime measurements at low temperature were performed with the third harmonic (351nm) 5ps pulse of an Nd-glass laser system which has been described in Chapter 2. The sample was placed in a glass dewar cooled by flowing nitrogen gas and temperature was monitored with a copper-constantan thermocouple. The fluorescence lifetimes were measured at room temperature (300 K) and at about 120 K. The lifetimes were also measured as a function of temperature. Measurements were taken at 5 temperature readings. The fluorescence spectra of the tissue was measured using a Perkin-Elmer spectrofluorometer before and after the low temperature measurements were performed.

The long lifetime > 100 nsec emission spectra were recorded using a Jarrel Ash spectrometer and SIT connected to a OMA II, described earlier in Chapter 2. The room temperature fluorescence was detected in the 404-555nm range. The phosphorescence spectra were recorded after the emission path was blocked for a fraction of a second at low temperature (120 K). The spectra were recorded in the 404-55nm range. Phosphorescence lifetimes were

obtained from photographs of temporal traces using a S-20 photomultiplier tube connected to a 7844 oscilloscope with appropriate filters to cut off the excitation pulse.

Results:

The results are divided into three parts: a) fluorescence lifetimes, b) phosphorescence spectra, c) phosphorescence lifetimes.

(a) Fluorescence Lifetimes:

Results:

Fluorescence profiles at room temperature consist of double exponential decays with 351nm excitation. Typical fluorescence profiles are shown in figure 55 (a) and (b) the room temperature fluorescence profiles of benign and malignant breast tissues respectively while figure 56 (a) and (b) display the corresponding profiles at low temperature (120 K). The fluorescence lifetimes and ratio of amplitudes of the benign sample are $t_1 = 294\text{ps}$, $t_2 = 3.3\text{ns}$, $a_1/a_2 = 0.9$ at room temperature (fig.55 (a)) and $t_1 = 733\text{ps}$, $t_2 = 3.5\text{ns}$, $a_1/a_2 = 0.5$ at low temperature (fig.56(a)). The fluorescence lifetimes and ratio of amplitudes of the malignant sample are $t_1 = 242\text{ps}$, $t_2 = 1.9\text{ns}$, $a_1/a_2 = 0.5$ at room temperature (fig.55 (b)) and $t_1 = 490\text{ps}$, $t_2 = 4.7\text{ns}$, $a_1/a_2 = 0.4$ at low temperature (fig.56(b)).

From Figure 56 one notices that the fluorescence profiles have a slower decay and the contribution of the fast component decreases at low

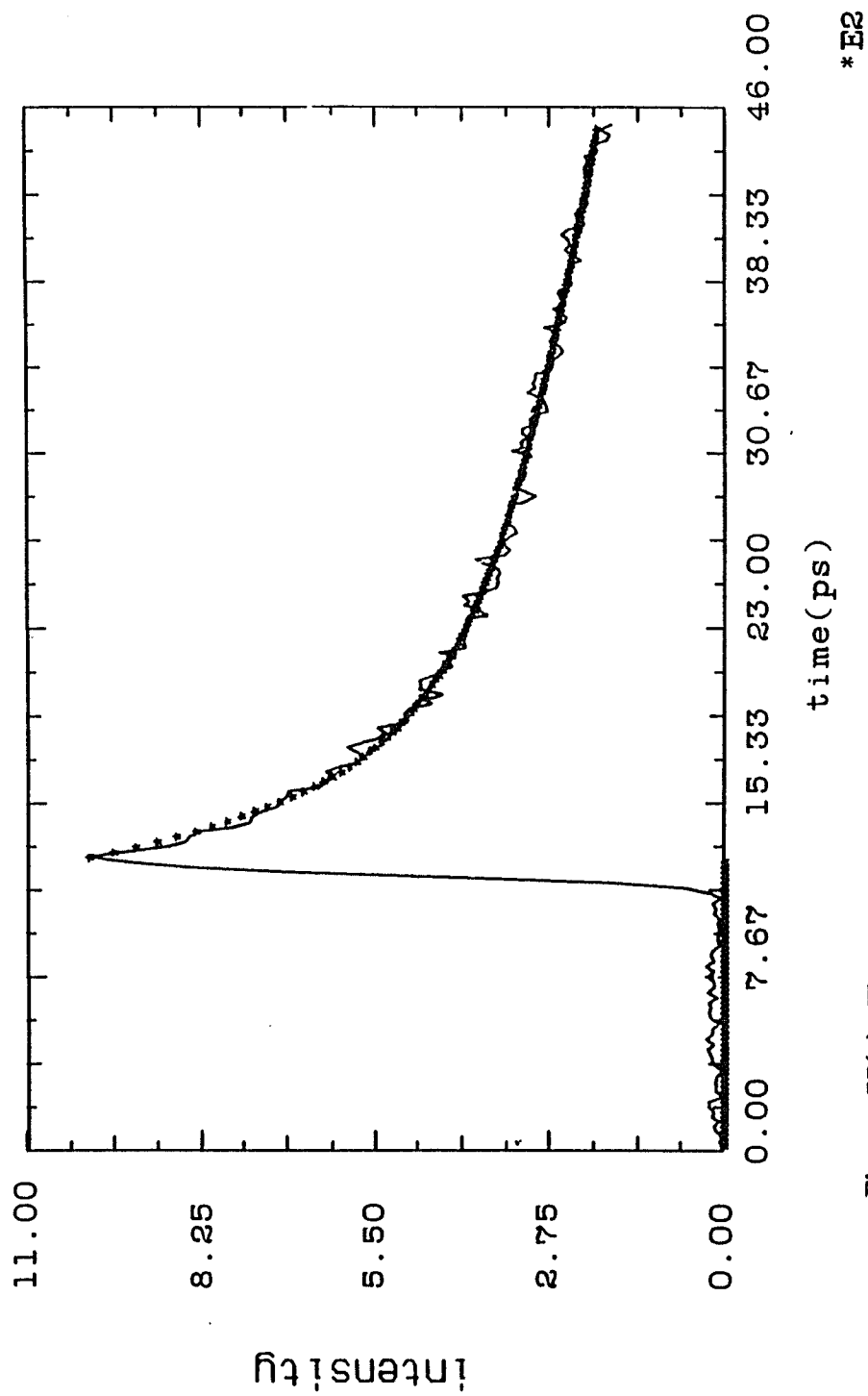


Figure 55(a). Fluorescence lifetime profiles at room temperature of benign breast tissue with $t_f = 294\text{ps}$, $t_s = 3.3\text{ns}$, $a_f/a_s = 0.9$

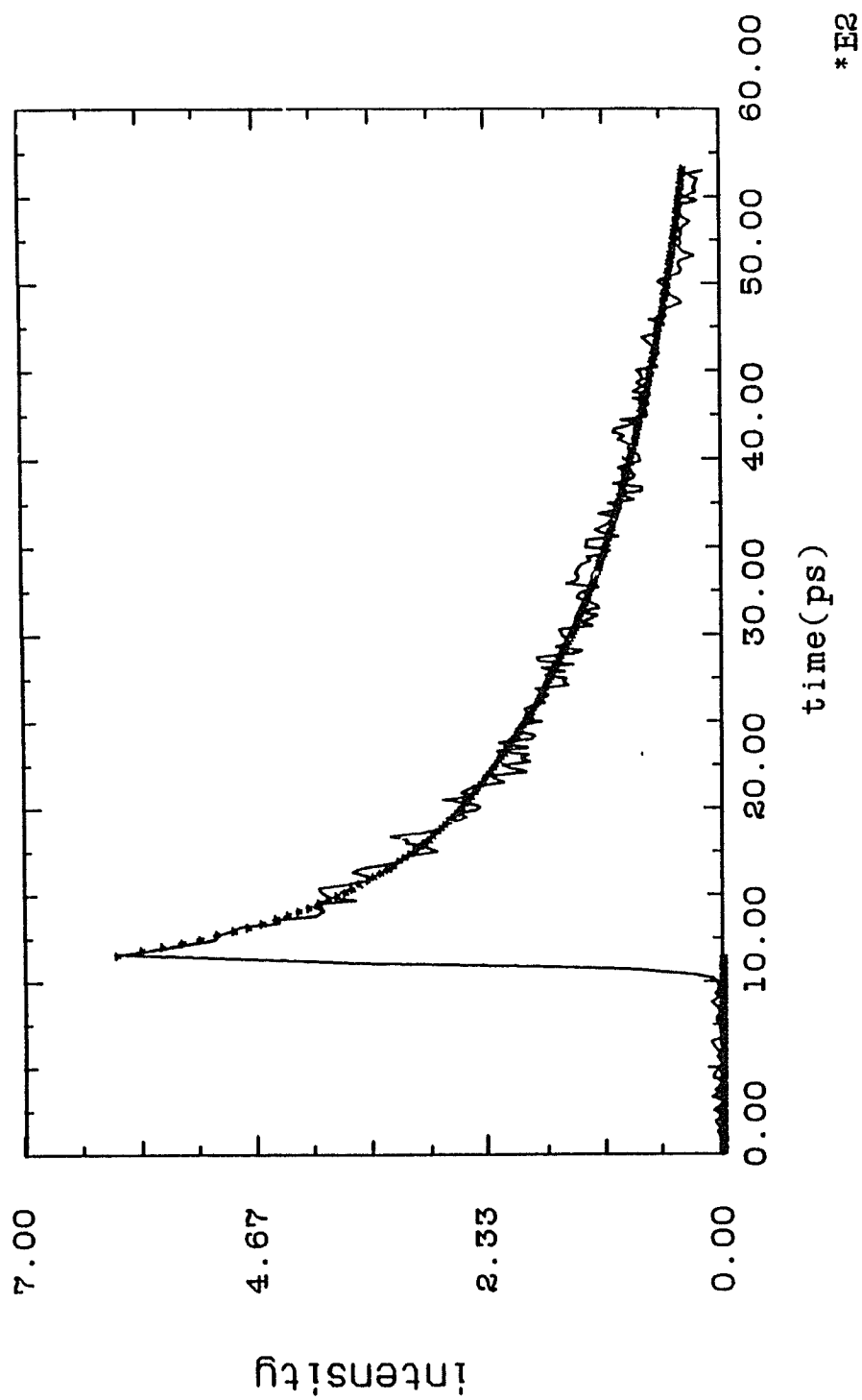


Figure 55 (b). Fluorescence lifetime profiles at room temperature of malignant breast tissue with $t_f = 242\text{ps}$, $t_s = 1.88\text{ns}$, $a_f/a_s = 0.5$.

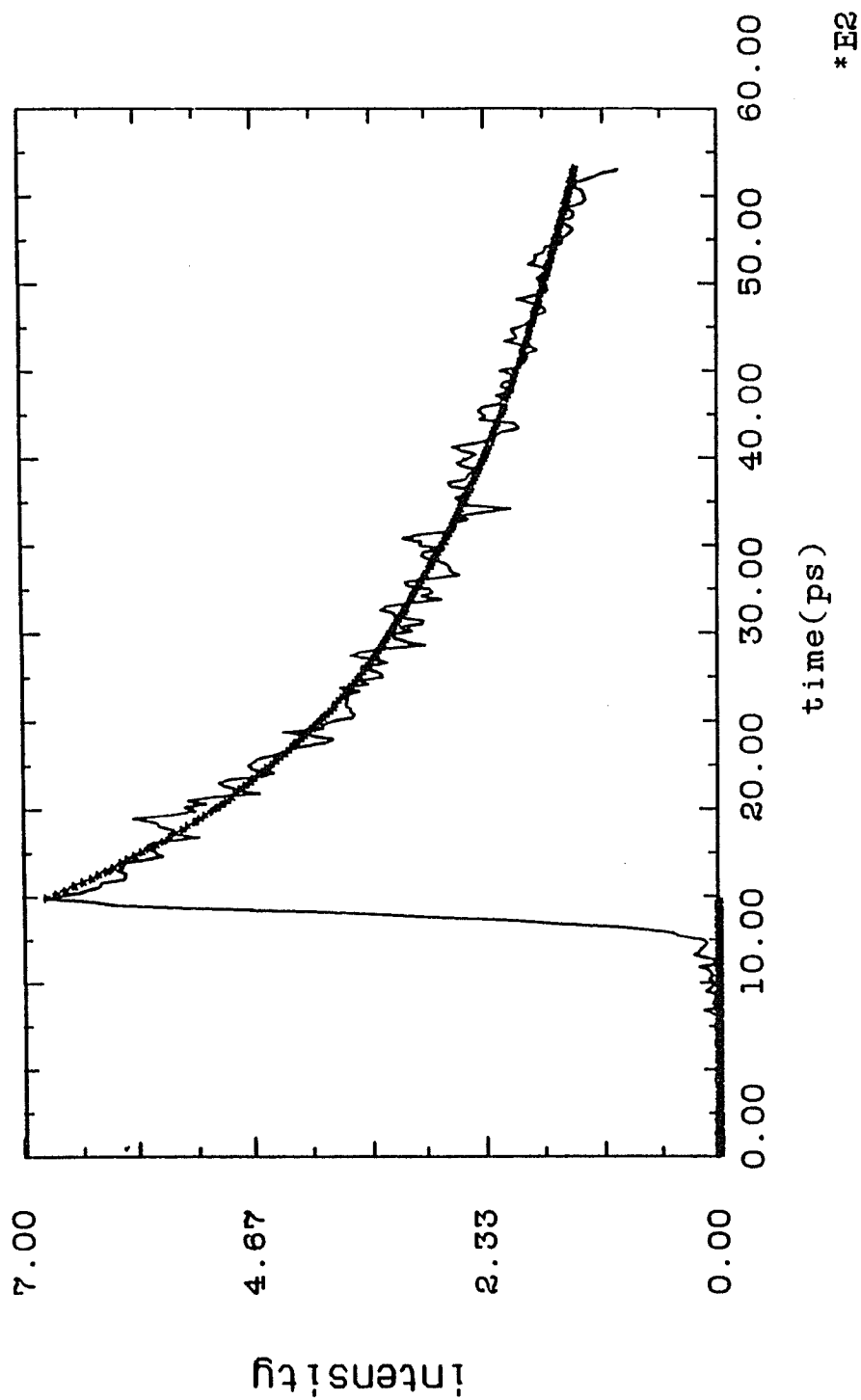


Figure 56 (a). Fluorescence lifetime profiles at low temperature (120°K) of benign breast tissue with $t_f = 733\text{ps}$, $t_s = 3.5\text{ns}$, $a_f/a_s = 0.47$.

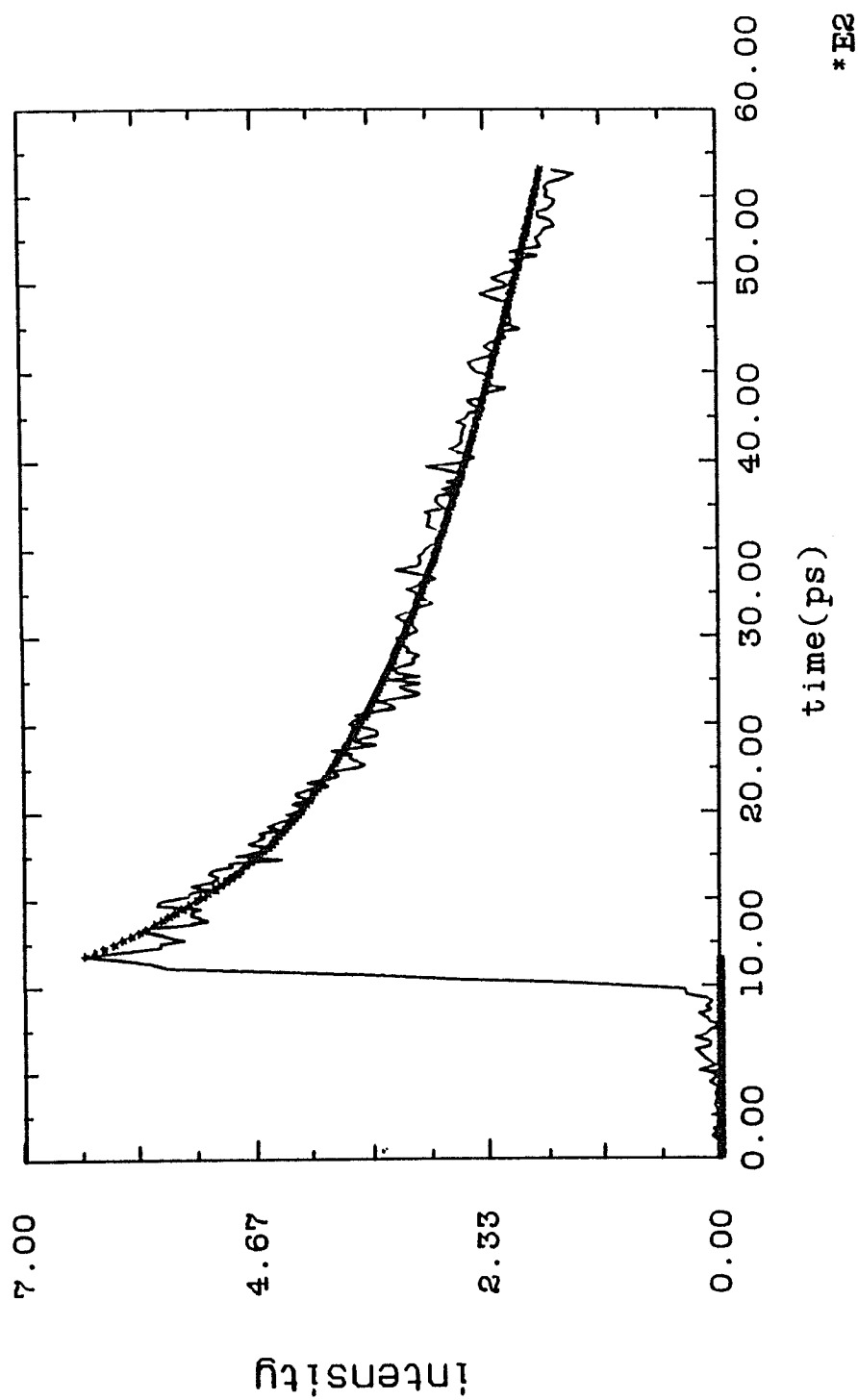


Figure 56 (b). Fluorescence lifetime profiles at low temperature (120 K) of malignant breast tissue with $t_f = 490\text{ps}$, $t_s = 4.7\text{ns}$, $a_f/a_s = 0.4$.

temperatures. The fluorescence intensity was more than two times stronger at low temperature as compared to that at room temperature. Fluorescence lifetimes were also measured at five temperature readings between room temperature and 120 K. Figure 57 (a) shows the change in the ratio of amplitudes of three samples while Figure 57 (b) shows the changes of the slow component of lifetimes. The ratio of amplitudes a_f/a_s showed a monotonic decrease till 150-200 K after which it remained constant. This was observed in two samples with ratio > 0.5 at room temperature. The third sample with ratio < 0.5 at room temperature did not show any decrease with temperature. The fluorescence lifetimes did not change at the different wavelength bands, 400nm, 450nm and 500nm at low temperature.

It is well known that non-radiative processes slow down at low temperatures. The fluorescence is seen to increase at low temperature and the ratio of the fast to slow component decreases for both benign and malignant tissues. Table 11 shows the change in fluorescence lifetimes and the ratio of fast to slow amplitudes. It is seen that the fast or the slow or both lifetimes appear to be slower than at room temperature. The ratio of amplitudes, a_s/a_f , shows a consistent decrease, implying a decrease in the contribution from the fast component, except in the case where ratio is already small at room temperature. These results indicate that non-radiative decay disappear or lessen at low temperatures. This proves conclusively that non-radiative processes do occur in tissues and give rise to the sharp component of fluorescence. A small but significant contribution from the fast component of

Table 11: Fluorescence lifetimes and amplitudes at room and low temperaure from benign and malignant breast tissues.

Tissue Type	tf(ps) [RT]	tf(ps) [LT]	ts(ns) [RT]	ts(ns) [LT]	af/as [RT]	af/as [LT]
Benign	294	733	3.3	3.5	0.9	0.5
Malignant	294	260	3.9	5	1.1	0.3
Benign	200	421	2.4	3.6	1.0	0.6
Benign	395	451	4.2	3.2	1.4	0.4
Benign	352	188	3.1	3.9	0.9	0.3
Malignant	242	490	1.9	4.7	0.5	0.4
Benign	252	168	4.0	3.9	0.5	0.4

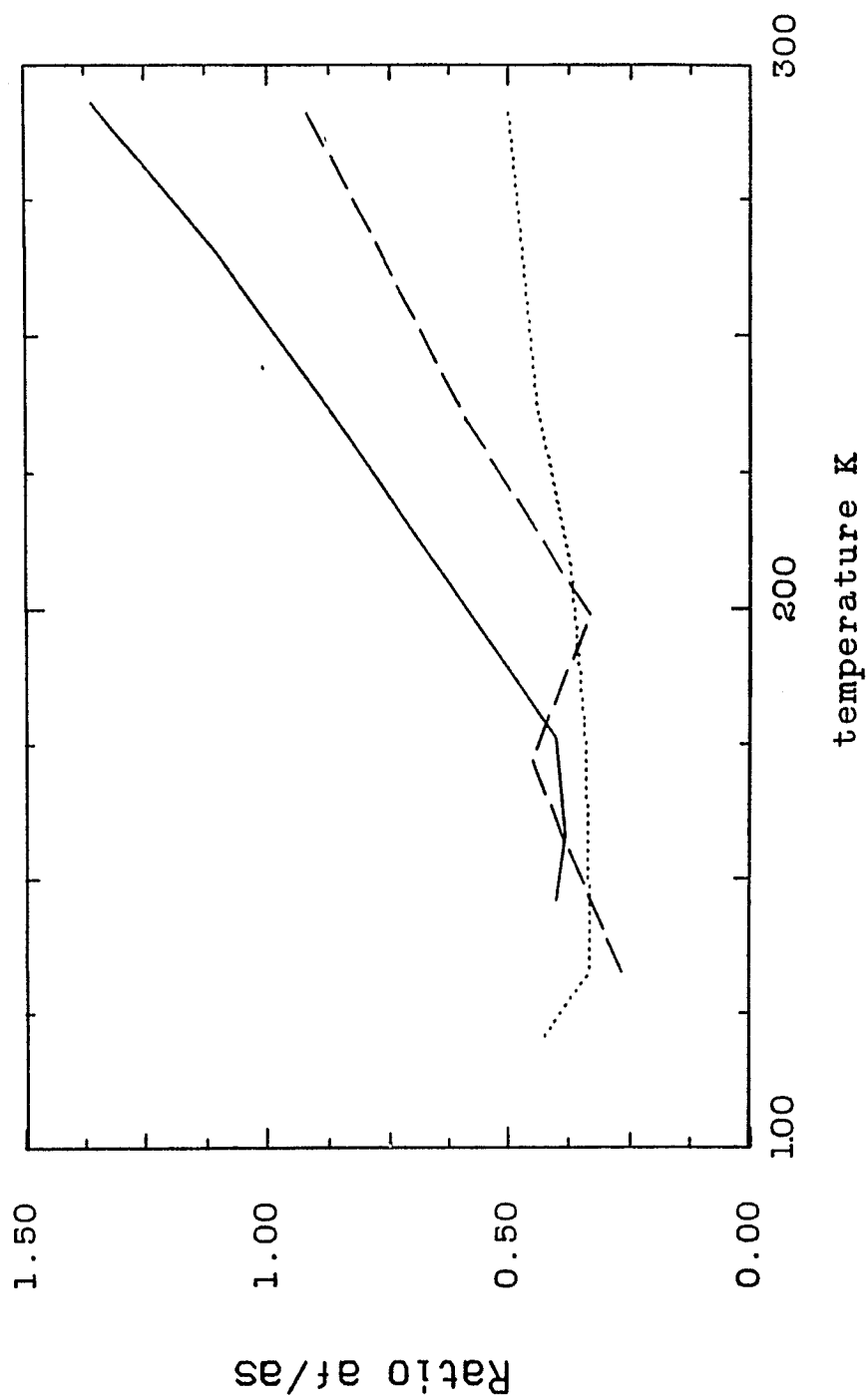


Figure 57 (a). Time-resolved fluorescence as a function of temperature ratio of amplitudes, af/as .

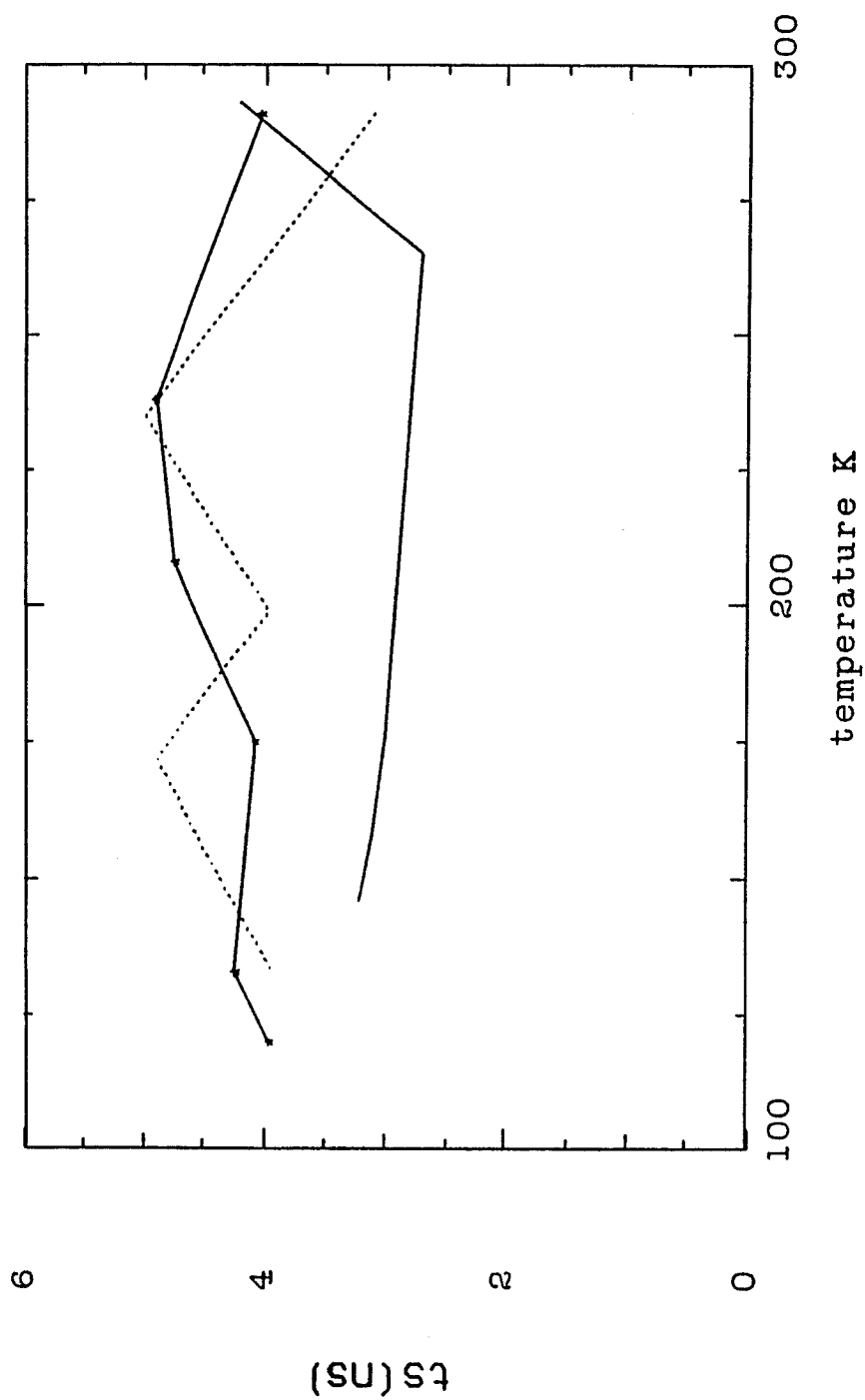


Figure 57 (b). Time-resolved fluorescence as a function of temperature ratio of slow component of lifetime, τ_s .

the fluorescence at low temperatures suggests that there could be more than one emitting species in the tissue environment.

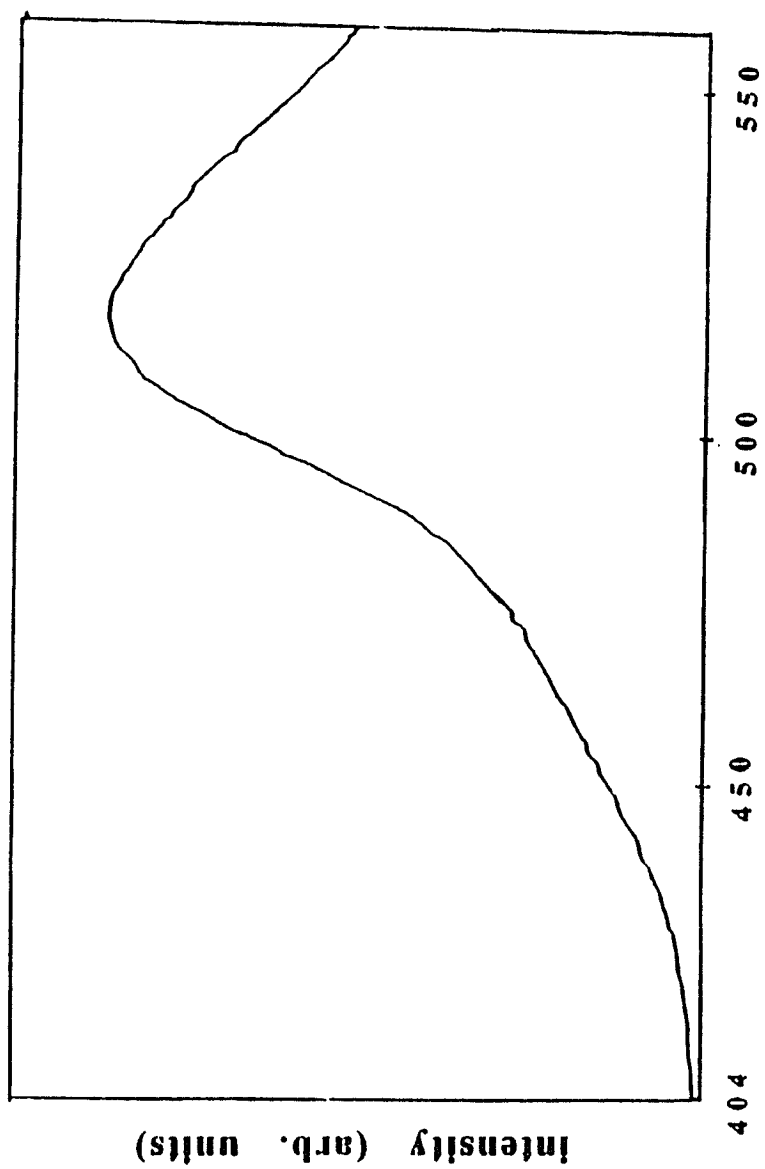
(b) Phosphorescence Spectra:

The low temperature measurements revealed emission with a very long decay (phosphorescence) from both benign and malignant breast tissues. At room temperature no signal could be observed when the emission path was blocked for a fraction of a second. At 120 K, the emission spectrum was detected.

Figures 58 (a) and (b) show the phosphorescence spectra from benign and malignant breast tissues, respectively in the 404-555nm region. Both benign and malignant tissues display phosphorescence peaks at 520nm, which is red-shifted from the fluorescence peak of 470nm of the benign tissue shown in Figure 58 (c).

(c) Phosphorescence Lifetimes:

Phosphorescence decay profiles from breast tissues were measured at 120 K and are displayed in Figure 59. The phosphorescence profiles from both benign and malignant tissues were fitted to double exponentials. The lifetimes were 28ms and 364ms for the benign tissues and 111ms and 617ms for the malignant tumor. For comparison with Figures 59 (a) and (b), the fluorescence lifetime at room temperature is shown in Figure 58 (c). The benign and malignant tissues do not show any differences in phosphorescence lifetimes.



wavelength (nm)

Figure 58 (a). Phosphorescence spectra in the 404nm to 555nm range of benign tissue with peak at 520nm

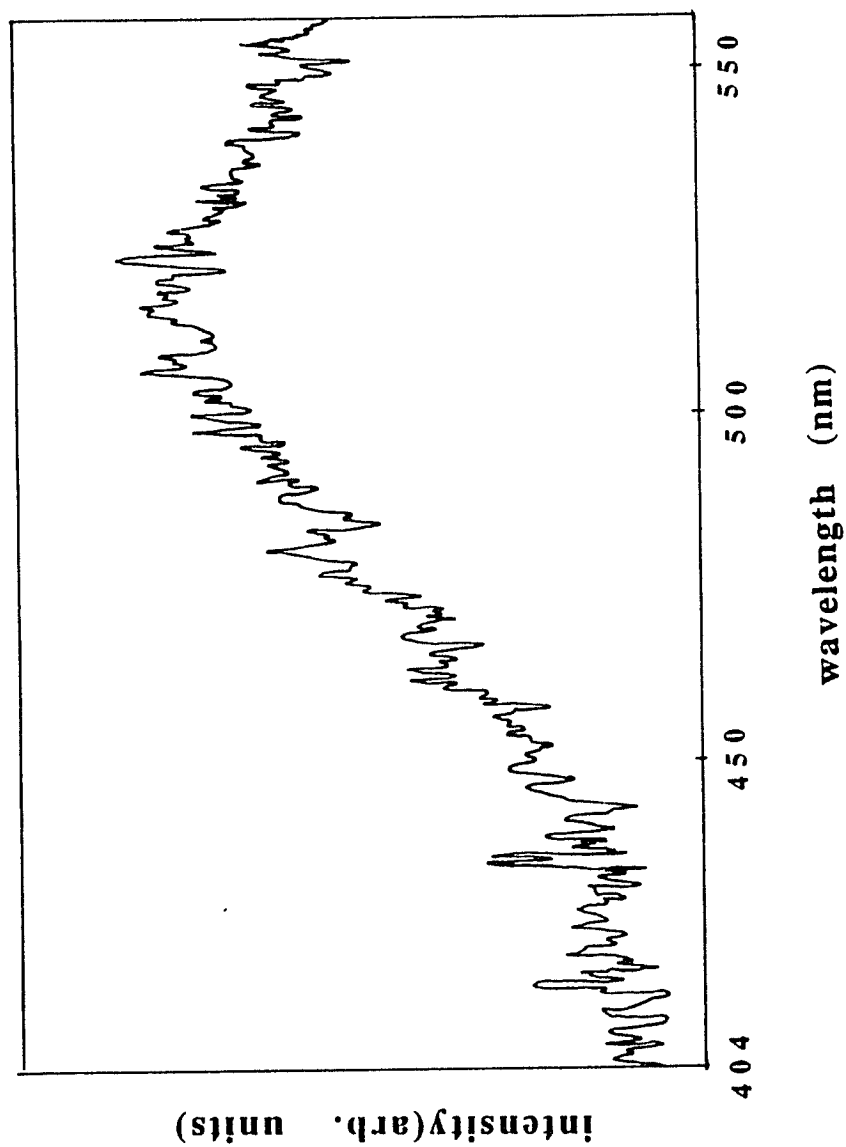


Figure 58 (b). Phosphorescence spectra in the 404nm to 555nm range of malignant breast tissue with peak 520nm

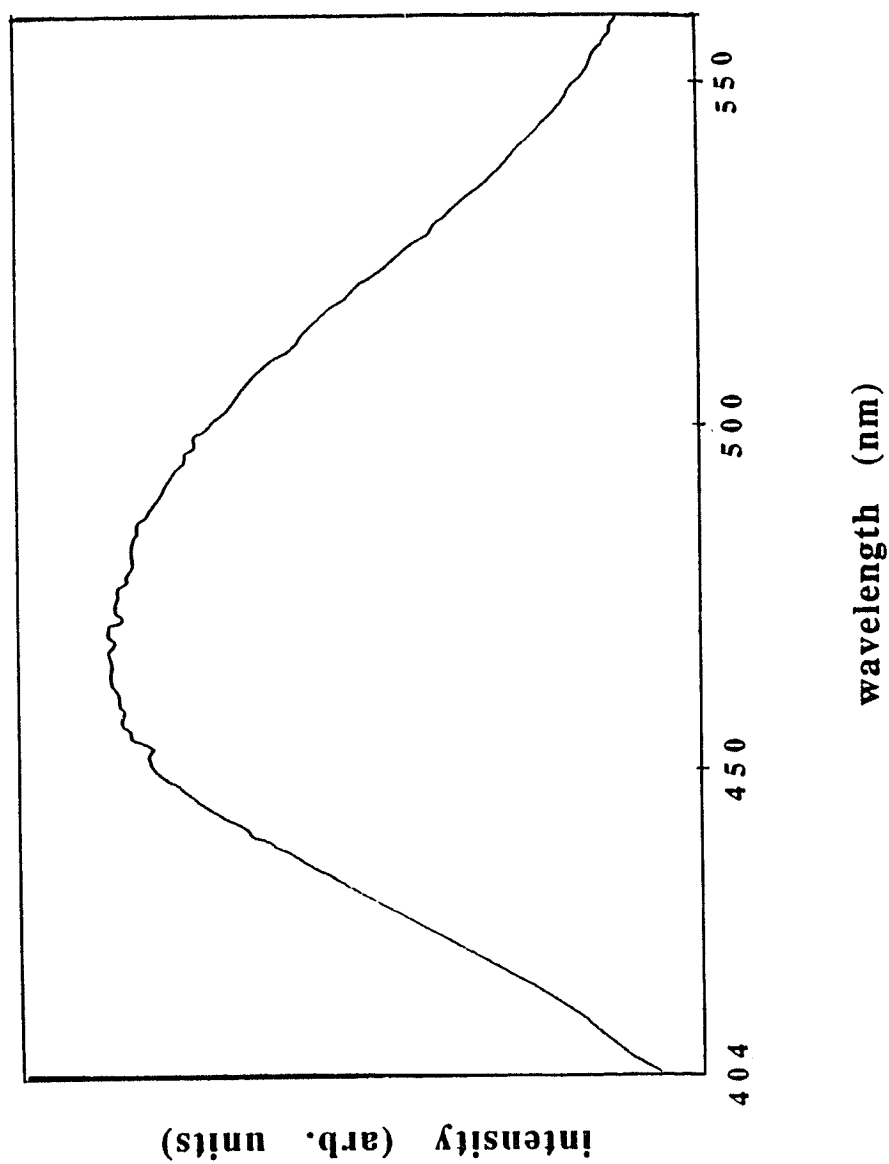


Figure 58 (c). Phosphorescence spectra in the 404nm to 555nm range of fluorescence spectra of benign tissue at room temperature with peak at 470nm.

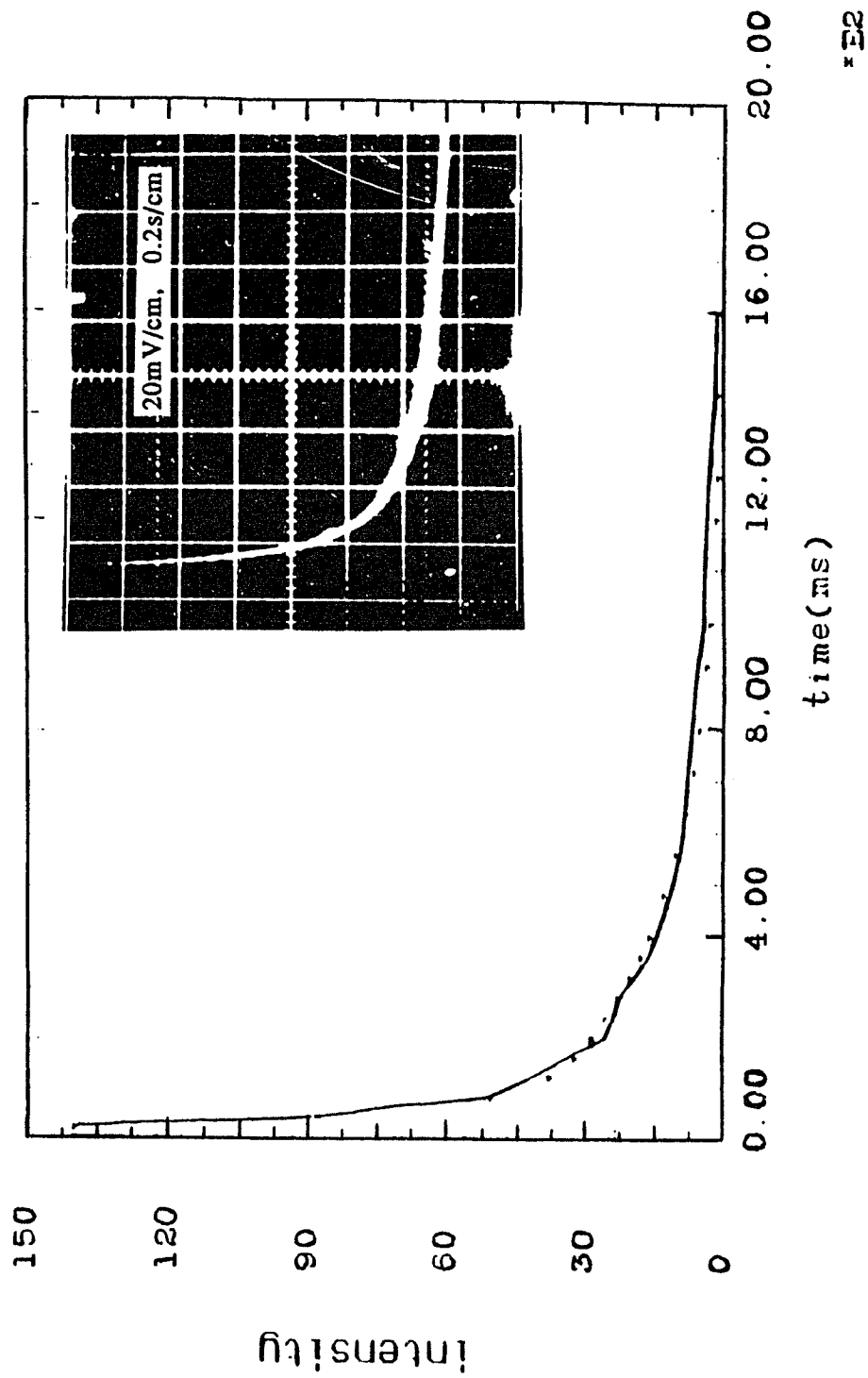


Figure 59 (a). Phosphorescence decay profiles of benign breast tissue with $t_f = 28\text{ms}$, $t_s = 364\text{ms}$, $a_f/a_s = 1.9$ (Inset are corresponding photograph).

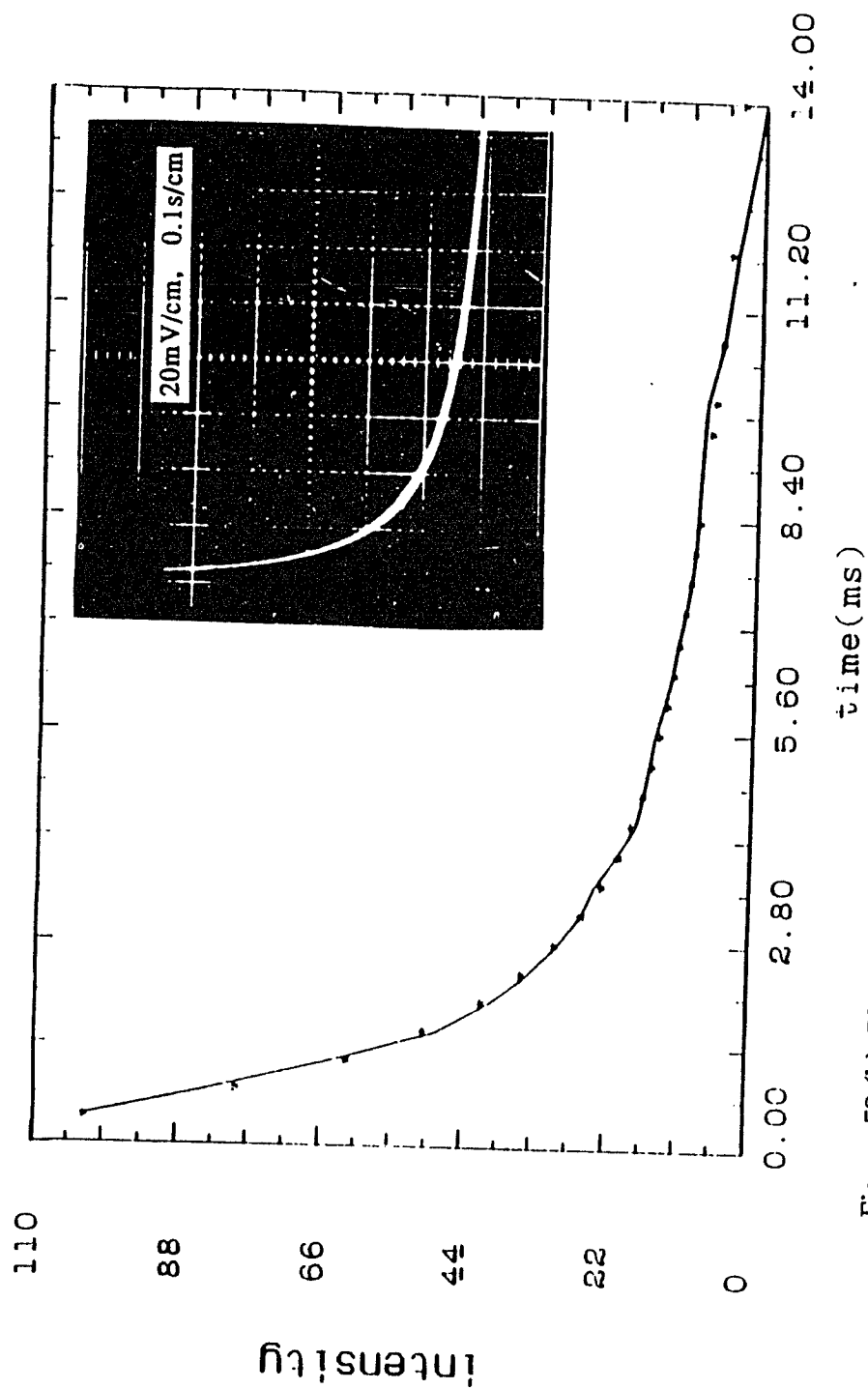


Figure 59 (b). Phosphorescence decay profiles of malignant breast tissue with $t_f = 110\text{ms}$, $t_s = 616\text{ms}$, $a_f/a_s = 2.1$ (Inset are corresponding photograph).

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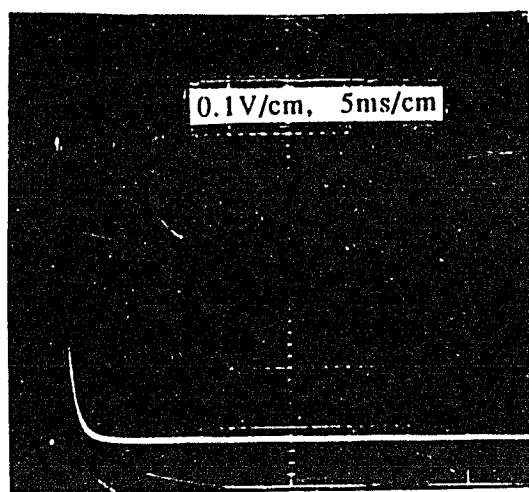


Figure 59 (c). Photograph of fluorescence lifetime at room temperature, in the same time scale, for comparison.

Table 12 lists the measured phosphorescence lifetimes and ratio of amplitudes of the three benign and three malignant breast tissues. The average phosphorescence fast and slow lifetimes of the three benign and three malignant samples measured are 72 ± 40 ms and 527 ± 181 ms for benign tissues 51 ± 52 ms and 360 ± 248 ms for malignant tissues. There appears to be a large variation in the lifetimes of the different samples with no significant difference between the benign and malignant tissues.

Discussion of Phosphorescence:

It is known that both fluorescence and phosphorescence are enhanced at low temperatures and that the non-radiative processes slow down. Phosphorescence is better studied at low temperature than at room temperature. The red shifted spectra with a few nanosecond delay and the long lifetimes (a few hundred milliseconds) suggest that phosphorescence does occur from human tissues. The appearance of phosphorescence from tissues at low temperatures suggests that intersystem crossing to a triplet state and the energy dissipates to the ground state non-radiatively occurs.

This work suggests that it may be possible to use the triplet manifold in native tissues to perform photodynamic therapy without dyes. The process of energy transfer from the higher excited triplet states of the tissue to the triplet ground state of the oxygen can be used to kill tumors. As with hematoporphyrin derivatives from where energy is transferred to the oxygen molecules, a similar process may occur in tissues. The oxygen molecules

Table 12: Phosphorescence lifetimes and ratios of amplitudes of benign and malignant breast tissues at low temperature with 351nm excitation.

SAMPLES	tf (ms)	ts (ms)	af/as
Benign	107	722	1.7
Benign	27	363	1.9
Benign	82	496	3.6
Malignant	32	344	2.8
Malignant	10	120	0.8
Malignant	110	616	2.2

present in the tissues can then be excited to the singlet state. Singlet oxygen is known to strongly react with the tissue through rapid oxidation and the tissue becomes necrotic.

Low temperature studies have shown that the non-exponential forms of fluorescence lifetimes are partially due to non-radiative decays, namely internal conversion and intersystem crossing, occurring in the molecular environment of normal and abnormal human breast tissues.

CHAPTER 6: TIME-RESOLVED FLUORESCENCE FROM ATHEROSCLEROTIC PLAQUE AND NORMAL AORTA

Atherosclerotic vascular disease has a high percentage of mortality like cancer. Hence diagnosis and cure of this disease is one of the important fields of research. Non-surgical techniques are being studied for safer methods of diagnosis and cure. Laser angioplasty, or the ablation of atherosclerotic plaque using laser energy has great potential for treatment of vascular disease. Although it is possible to ablate plaque using different laser sources, experimental studies show high rates of vessel perforation. This is due to the inability to limit ablative radiation precisely to the plaque. Laser-induced fluorescence spectroscopy has been shown to be capable of guiding selective plaque ablation during angioplasty.

Earlier work on normal coronary artery and white atherosclerotic plaque revealed a difference in the fluorescence spectra. These differences were used to guide selective ablation during in vitro laser angioplasty [63]. However, steady state fluorescence is not capable of accurately discriminating all subtypes of plaque, such as calcified plaque from normal aorta, which may limit its ability to accurately guide ablation. This drawback led to our study of the time-resolved fluorescence from normal aorta, fibrous and calcified atherosclerotic plaques. The temporal profiles of fibrous and calcified plaque being different from normal aorta would eliminate the diagnostic problem.

Tissue fluorophores can be excited by short pulses of laser light and

additional information may be obtained by this, as compared to steady state results. The purpose of this study is to investigate the potential of time-resolved fluorescence spectroscopy to identify different arterial tissue types, specifically the ability to discriminate fibrous plaque and calcified plaque from normal artery. The ability to time-resolve the fluorescence in the picosecond regime may provide further insight into the understanding and characterization of the nature of the emission and assist in identification of the different chromophores present, or obtain information of the physical processes occurring.

Time resolved fluorescence spectroscopy has been used to study the various dynamical processes in biological media. Tissues contain several fluorophores creating complex spectral emission profiles which cannot be easily identified to well-defined species, especially with steady state fluorescence alone. Time resolved fluorescence spectroscopy is thus another method which could help evaluate the heterogeneous nature of the emission from different types of aorta and its lesions.

Pathology

As described in Chapter 3, in the aorta, the intima or the inner layer consists typically of a smooth layer of endothelial cells situated on a delicate basement membrane. The middle layer is elastic in nature with elastin fibers separated by alternating layers of smooth muscle cells. The outer layer consists mainly of connective tissue with some elastic and nerve fibers. Figure

11 displays the cross sectional view of an artery with fibrous atherosclerotic plaque. The two kinds of atherosclerotic lesions studied here are fibrous and calcified plaque. These are lesions confined to the intima. The fibrous plaque consists of connective tissue including collagen, elastic fibers, proteoglycans and variable number of smooth muscle cells. The calcified plaque consists of deposits of calcium. Aorta is rich in elastin while coronary artery is more muscular.

Results

Picosecond time-resolved fluorescence from artery tissue [64] were measured using a streak camera with 10ps resolution upon excitation with a 5ps third harmonic at 351nm of a mode-locked Nd-glass laser. The energy of the pulse was about 20 J at the sample site. The sample size was about 5mm x 5mm x 1.5mm. The samples consisted of normal aorta, white atherosclerotic plaque and calcified atherosclerotic plaque.

The time-resolved fluorescence decay of normal aorta, white atherosclerotic plaque and calcified atherosclerotic plaque display double exponential profiles. Sixteen normal, seven fibrous plaque and six calcified plaque measurements were taken. Measurements were performed at two positions per sample in most of the samples. Bleaching effects were not investigated. The data were fitted to a double exponential,

$$I(t) = a_f \exp(-t/t_f) + a_s \exp(-t/t_s)$$

using the least square method of fitting.

The fluorescence spectra of the normal artery and the calcified plaque displayed a broad band peaked around 440nm when excited at 351nm. The bandwidth was 120nm. The fluorescence spectra of the white plaque displayed two bands, one peaked at 390nm and the other at 440nm (Figure 60). These spectral regions were chosen for measurements. The emission bands at $390 \pm 10\text{nm}$ (F1), $450 \pm 10\text{nm}$ (F2), $500 \pm 10\text{nm}$ (F3) and the entire bandwidth of 360nm to 650nm (F0) were time-resolved.

The typical curves of normal artery, white atherosclerotic plaque and calcified plaque are displayed in figures 61 (a), (b) and (c) in the F0 band. The results of each set were summarized and the data are displayed in Table 13.

The fast and slow components of each emission band were averaged for each type of sample in a set. The fast component of the white plaque is found to be shorter than that of the normal artery and calcified plaque in the both sets. The fast component for the fibrous plaque was different from the normal artery and calcified plaque with a 'p' value less than 0.03 for both cases. Using a threshold of 315ps for the fast component, it is possible to identify all fibrous plaque specimens and 12 out of the 16 normal artery or calcified plaque samples. The slow component on the other hand does not show any such distinction. The large standard deviation is caused by the variation in each group. The fast and slow components did not show consistency in numbers in each wavelength group as well as in the two sets. There were large variations in the fast component ranging from 80ps to 600ps in the two sets.

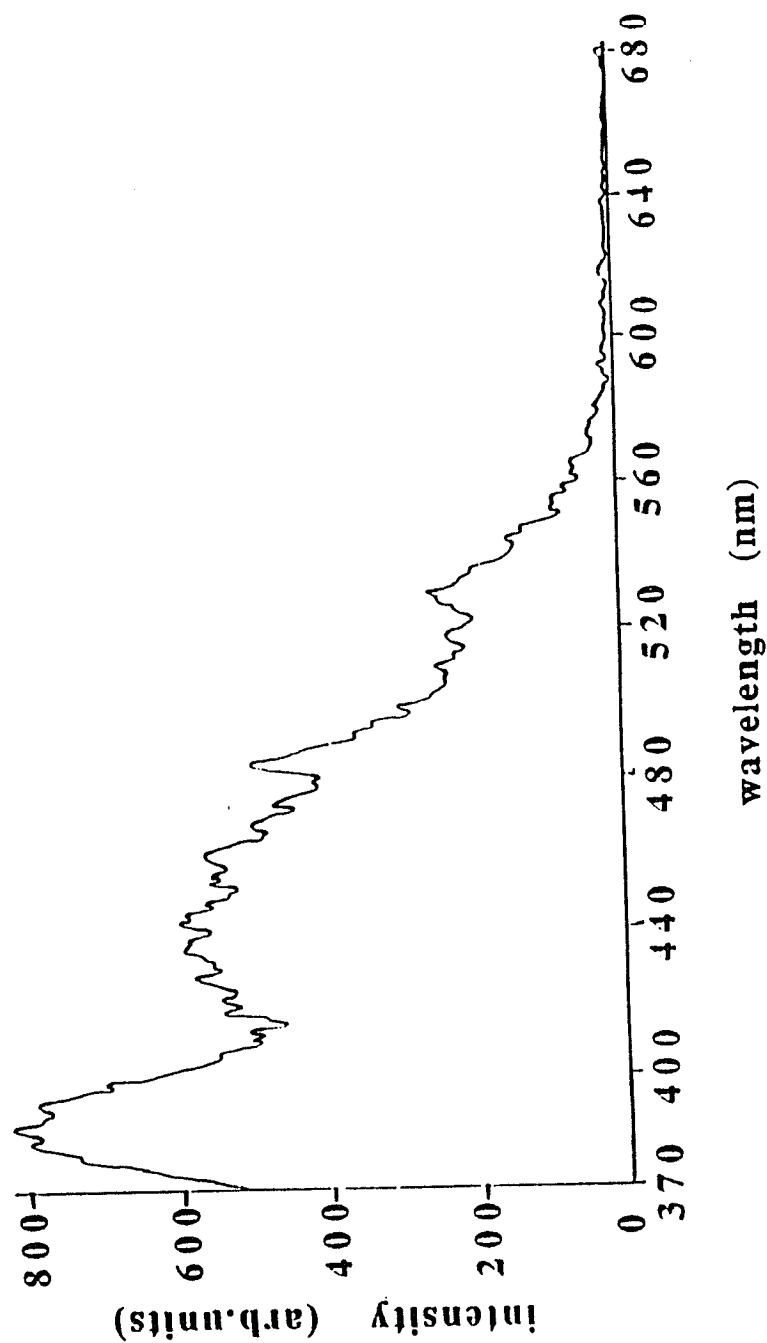


Figure 60. Typical fluorescence spectral profile of fibrous plaque with 351nm excitation.

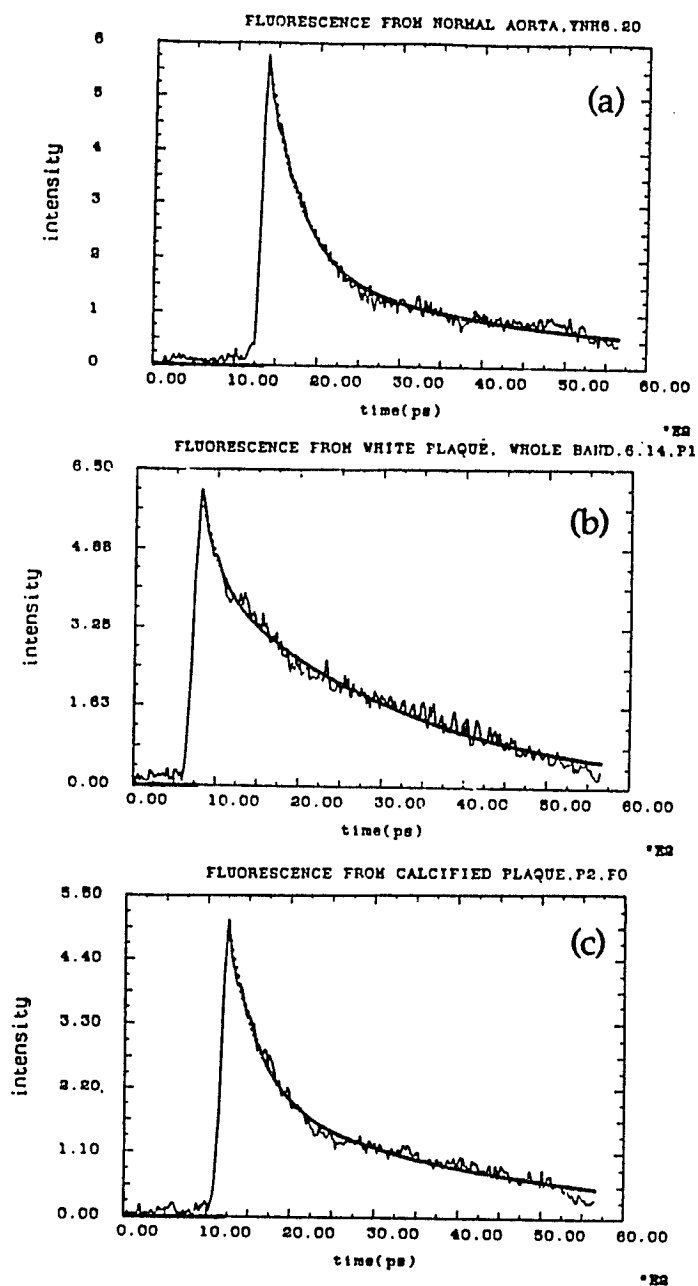


Figure 61. Kinetic fluorescence profiles in the F0 band excited by 351nm picosecond pulse, of (a) normal artery with $t_f = 404\text{ps}$, $t_s = 4\text{ns}$, $a_s/a_f = 0.43$, (b) fibrous atherosclerotic plaque with $t_f = 192\text{ps}$, $t_s = 2.3\text{ns}$, $a_s/a_f = 2.7$ and (c) calcified atherosclerotic plaque with $t_f = 404\text{ps}$, $t_s = 3.4\text{ns}$, $a_s/a_f = 0.63$.

Table 13: Fluorescence lifetimes and ratio of amplitudes of normal artery, fibrous atherosclerotic plaque and calcified atherosclerotic plaque in the emission bands 360nm to 650nm (F0), 390 $\pm$ 10nm (F1), 450 $\pm$ 10nm (F2) and 500 $\pm$ 10nm (F3) with 351nm excitation.

BAND	TISSUE	t_f (ps)	t_s (ns)	a_s/a_f	#of sites
F0	NORMAL	394 $\pm$ 143	2.8 $\pm$ 0.7	1.0 $\pm$ 0.6	16
	FIBROUS PLAQUE	231 $\pm$ 72	2.3 $\pm$ 0.4	1.98 $\pm$ 0.6	7
	CALCIFIED PLAQUE	416 $\pm$ 156	3.0 $\pm$ 0.7	1.1 $\pm$ 0.8	5
F1	NORMAL	205 $\pm$ 115	2.5 $\pm$ 0.5	1.4 $\pm$ 0.7	2
	FIBROUS PLAQUE	97.4	1.98	2.6	1
	CALCIFIED PLAQUE	314 $\pm$ 167	2.8 $\pm$ 0.6	1.06 $\pm$ 0.5	2
F2	NORMAL	327 $\pm$ 135	2.7 $\pm$ 0.6	0.9 $\pm$ 0.5	16
	FIBROUS PLAQUE	143 $\pm$ 47	2.3 $\pm$ 0.2	1.8 $\pm$ 0.4	7
	CALCIFIED PLAQUE	260 $\pm$ 146	2.4 $\pm$ 0.4	1.3 $\pm$ 0.6	6
F3	NORMAL	337 $\pm$ 168	2.9 $\pm$ 0.5	1.4 $\pm$ 0.7	7
	FIBROUS PLAQUE	154 $\pm$ 81	2.4 $\pm$ 0.1	2.2 $\pm$ 0.6	6
	CALCIFIED PLAQUE	305 $\pm$ 179	2.8 $\pm$ 0.7	1.3 $\pm$ 0.7	5

The ratio of amplitudes of slow to fast components show some difference, although not statistically significant. The results from the two sets indicate that the normal artery and calcified plaque have a ratio less than 1.5 while the white plaque has a ratio greater than 1.5.

Discussion:

The fluorescence lifetimes of normal artery, white atherosclerotic plaque and calcified atherosclerotic plaque display double exponential decays. The results obtained from several samples of normal artery, white plaque and calcified plaque indicate a difference in the fast component of lifetimes and a subtle difference in the ratio of amplitudes of slow to fast component.

The double exponential decay obtained could arise from two different molecules or due to environmental changes of one molecule type. The ratio of amplitudes could be an indication of the amount of the fluorophores present. Collagen and elastin are proteins known to be present in arterial tissue. It has been earlier noticed that the collagen content of white plaque is more than that of normal artery [65]. Collagen fluoresces at 390nm and elastin at 410nm. These could be contribute to the first band of the white plaque fluorescence spectra. The second band could be due to NADH (460nm). The fluorescence lifetimes of these substances have been measured to support these suggestions. The ratio of slow to fast components being larger in white plaque could imply the presence of two kinds of fluorophores in ratios that vary in the samples.

From the table of lifetimes of fluorophores (Table 7) and Figure 39 (h), it is seen that the presence of collagen increases the ratio, a_s/a_f . The fluorescence profile of NADH (10-4M) with saturated solutions of both collagen and elastin resembles that of the fibrous plaque profile. The temporal profile of NADH + elastin Fig.39 (f) resembles that of normal aorta and calcified plaque, though it is sharper. This may imply that there is some amount of collagen present but less than that seen in Figure 39 (h) (NADH +collagen+elastin). This result further supports the fact that the collagen content of fibrous plaque is more than that of normal aorta and calcified plaque.

The inconsistency in the lifetime numbers obtained for the lifetimes in the different sets could be due to several factors. The variation in numbers in the two sets could be due to variation of composition from sample to sample. Inconsistency in numbers were also obtained in different positions of each sample in a particular set. This could be due to bleaching effects when exciting the sample with the laser at the same spot several times. The variation in another position could be due to the variation of a certain fluorophore in different parts of the sample. Thus, it would be necessary to look for other excitation wavelengths.

Time-resolved fluorescence spectroscopy using 351nm excitation can differentiate fibrous plaque from normal artery, but cannot differentiate calcified plaque from normal artery. Since this agrees with the steady state fluorescence spectroscopy measurements, there appears to be no advantage

using time-resolved fluorescence at this excitation wavelength. In conclusion, it appears that excitation with 351nm is not the key wavelength for diagnostic purposes. This work gives us an idea of the heterogeneous nature of emission of the coronary artery. It indicates that there is probably a variation in the physical processes occurring and in the ratio of fluorophores in the white plaque as compared to the normal artery and calcified plaque. Further work with the right excitation wavelength is necessary for diagnostic and mapping purposes.

Time-Resolved Fluorescence Using 310nm:

Time-resolved fluorescence from atherosclerotic plaque and normal aorta using 310nm excitation were performed on 5 normal samples, 2 calcified plaque and 2 fibrous plaque samples. The number of sample sites measured in each case were at least two. Figure 62 (a),(b) and (c) display the fluorescence temporal profiles of normal aorta, fibrous plaque and calcified plaque, respectively. The fluorescence lifetimes displayed double exponential decays with no differences between the three types. Table 14 lists the lifetimes and amplitude ratios with this excitation. However, the ratio of amplitudes, a_s/a_f , could differentiate the calcified plaque from normal artery and fibrous plaque with a cut off ratio of 1. Calcified plaque consistently showed ratios less than 1 while normal aorta and fibrous plaque showed ratios greater than 1. This implies that a combination of 310nm and 351nm excitation may be able to distinguish normal aorta from the two types of plaque.

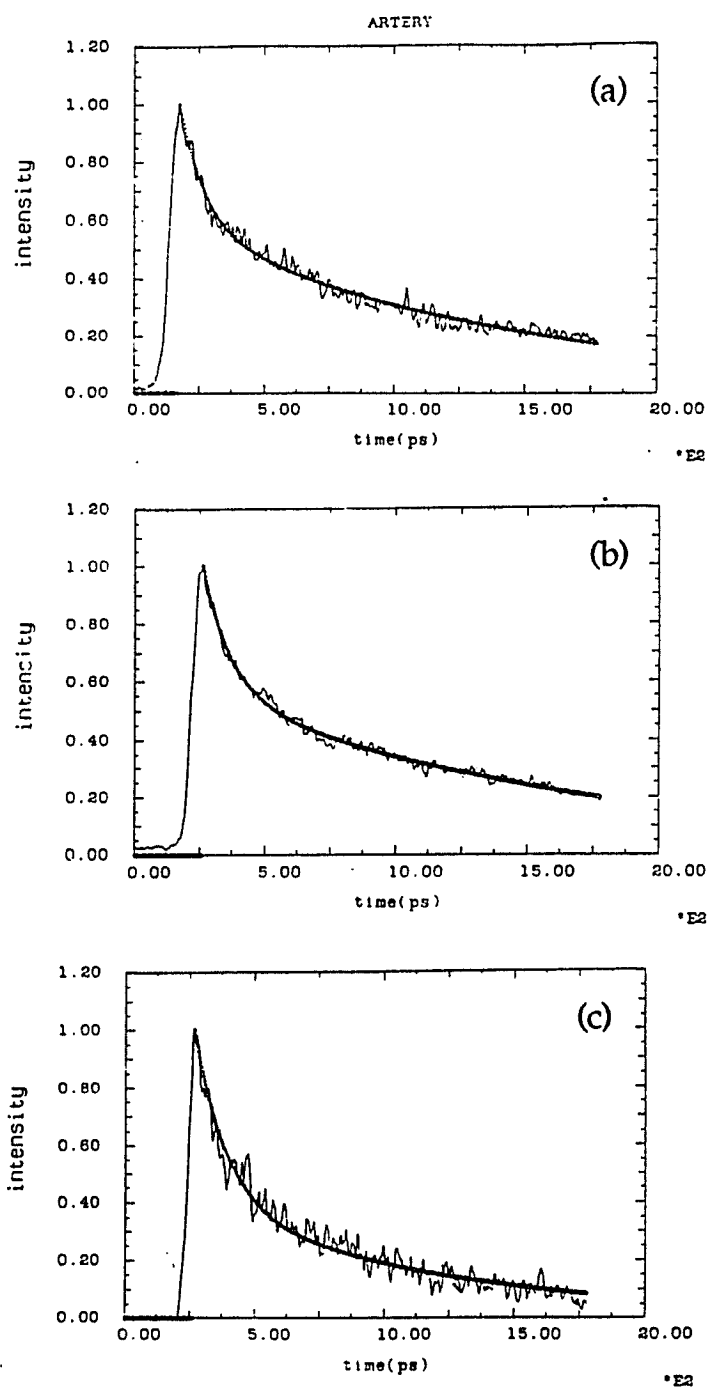


Figure 62. Kinetic fluorescence profiles in the $380 \pm 5\text{nm}$ band excited by 310nm picosecond pulse, of (a) normal artery with $t_f = 93\text{ps}$, $t_s = 2\text{ns}$, $a_s/a_f = 1.0$, (b) fibrous atherosclerotic plaque with $t_f = 102\text{ps}$, $t_s = 1.4\text{ns}$, $a_s/a_f = 1.4$ and (c) calcified atherosclerotic plaque with $t_f = 78\text{ps}$, $t_s = 728\text{ps}$, $a_s/a_f = 0.6$.

Table 14: Fluorescence lifetimes and amplitudes of normal aorta, fibrous and calcified atherosclerotic plaque with 310nm excitation and 380 + 10 nm emission.

# of samples	tf(ps)	ts(ns)	as/af
Normal	56	2.4	1.6
Normal	71	1.9	1.3
Normal	93	2.0	1.0
Normal	97	1.3	1.4
Normal	89	1.9	1.4
Fibrous Plaque	97	1.7	1.4
Fibrous Plaque	65	2.7	2.4
Calcified Plaque	100	1.7	0.7
Calcified Plaque	123	0.9	0.6
Calcified Plaque	101	1.1	0.8

To be able to use this technique as a diagnostic tool, better statistics should be obtained. But it appears that the combination of two excitation wavelengths is the key to solving the problem of atherosclerotic disease detection in human artery.

CHAPTER 7: DYNAMICS OF DOXYCYCLINE WITH TIME-RESOLVED FLUORESCENCE

Introduction

The bulk of this thesis deals with native fluorescence from biological materials. To complete the research, a minor part was devoted to fluorescence from external dyes. It has been established earlier [66] that certain dyes are retained longer by cancer cells than normal cells. Hematoporphyrin derivative (HPD) is a well-known photosensitizer which is being used in photodynamic therapy (PDT). Tetracyclines are another group of photosensitizers which show phototoxicity in cells. There has been evidence of generation of singlet oxygen due to tetracycline photosensitization [67]. For tetracycline to be used in PDT, the effects of laser pulse duration and peak irradiance on photochemical sensitization has been studied [68]. Studies on the photosensitized injury are usually performed with low intensity, CW irradiation which display independence of irradiance over several orders of magnitude. High powered pulsed lasers, on the other hand, display a dependence on irradiance. This is explained by sequential multiphoton absorption (or excited state absorption) which permits higher excited singlet or triplet states of the chromophore to be populated. Photochemical reactions from these higher states are quite different from those occurring from S_1 or T_1 states and in some cases have greater photobiological efficiency.

The purpose of this research is to obtain the intensity dependence of the fluorescence lifetimes and quantum yield of doxycycline, a specific

tetracycline, have been investigated to understand the dynamics of the process of sequential multiphoton absorption. A model has been developed to account for the observed phenomenon.

The fluorescence lifetimes and quantum yields were measured using a 351nm third harmonic 5ps pulse of a passively mode-locked Nd-glass laser. The fluorescence lifetimes were measured at $500 \pm 20\text{nm}$ using a 10ps streak camera combined with an SIT and a temporal analyzer and analyzed with a personal computer. The quantum yield measurements were performed using a 20ps third harmonic pulse of an actively mode-locked Nd-YAG laser at the repetition rate of 10Hz. The quantum yield was measured using a S-20 photomultiplier coupled to a boxcar integrator taking an average of 30 shots. This is described in Chapter 2, section 4. The doxycycline (DOTC) solution was prepared in buffered saline solution with a concentration of $2.2 \times 10^{-4}\text{ M}$. Figure 63 (a) and (b) shows the absorption and fluorescence spectra of doxycycline. The absorption of doxycycline is at 350nm while the emission peak is at 520nm.

Results

The fluorescence lifetimes of DOTC were measured with excitation intensities ranging from 10^7 W/cm^2 to 10^9 W/cm^2 . The fluorescence profile is a non-exponential and is fitted to a double exponential, assuming two emitting species and to a non-exponential with time-dependent non-radiative decay rates assuming quenching by another species.

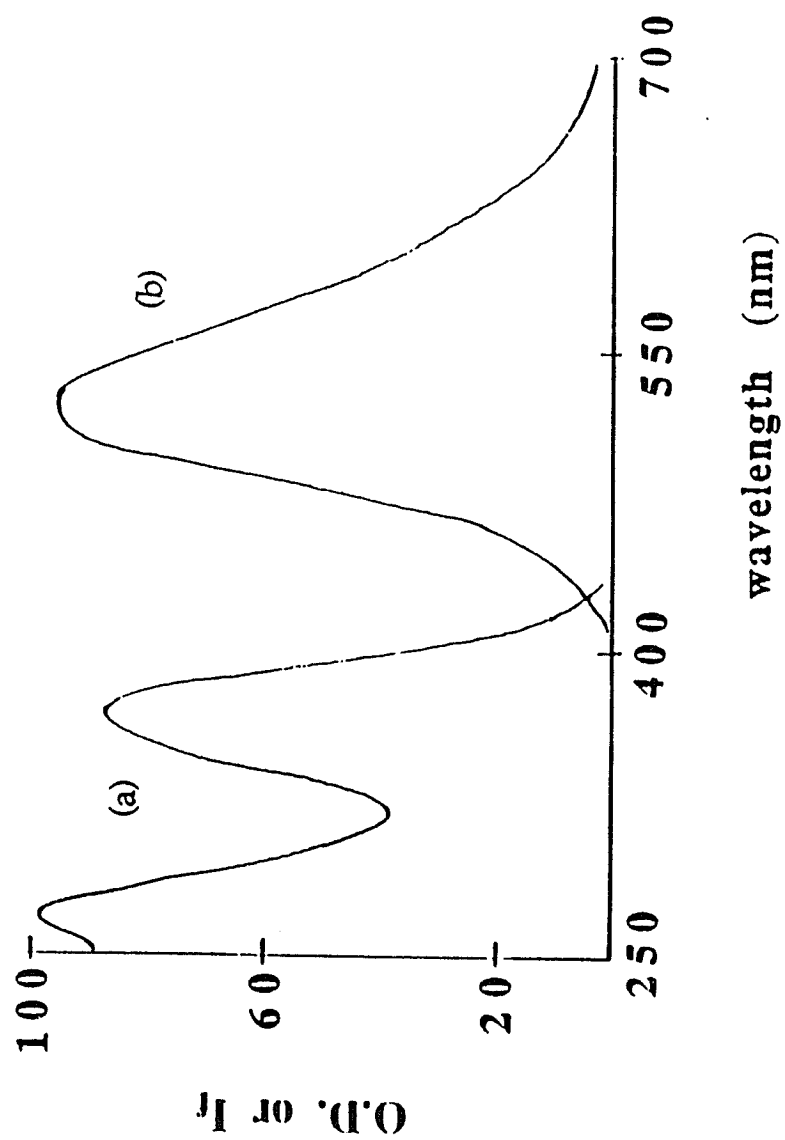


Figure 63. (a) Absorption and (b) emission spectra with 351nm excitation of DOTC.

Figure 64 shows a typical fluorescence profile in time at intensity 10^7 W/cm² fitted to a double exponential with $t_f = 20$ ps, $t_s = 239.5$ ps. No significant change was observed in the fluorescence temporal profiles over this intensity range. The non-exponential profiles were fitted to a double exponential using the least square method of fitting from a series of measurements. The average over the three intensities of the fast component was 21 ± 4 ps and of the slow components was 267 ± 28 ps.

The non-exponential decay can also be fitted to an equation with time-dependent decay rates:

$$I(t) = I_0 \exp [-(at + bt^{1/2})]$$

$$\text{with } a = 1/(270 \times 10^{-12})\text{ps}^{-1} \text{ and } b = 2.25 \times 10^{-3}\text{ps}^{-1/2}$$

as shown in Figure 65. This non-exponential form describes either diffusion quenching by another species or energy transfer to another species with fast Brownian rotational motion or energy transfer with diffusion.

Figure 66 (a) and (b) show the fluorescence profiles at room temperature and -120 C, respectively. The decay time changed at low temperature from 15ps and 350ps at $T = 28$ C to 43ps and 683ps at $T = -120$ C, respectively. The risetimes of the fluorescence at room temperature and -120 C are displayed in Figure 67 and is found to remain constant at about 30ps.

The quantum yield measurements over the excitation range of 10^{12} photons/cm² to 5×10^{15} photons/cm² is shown in Figure 69. A linear dependence on the incident intensity exists upto 10^{15} photons/cm² where it

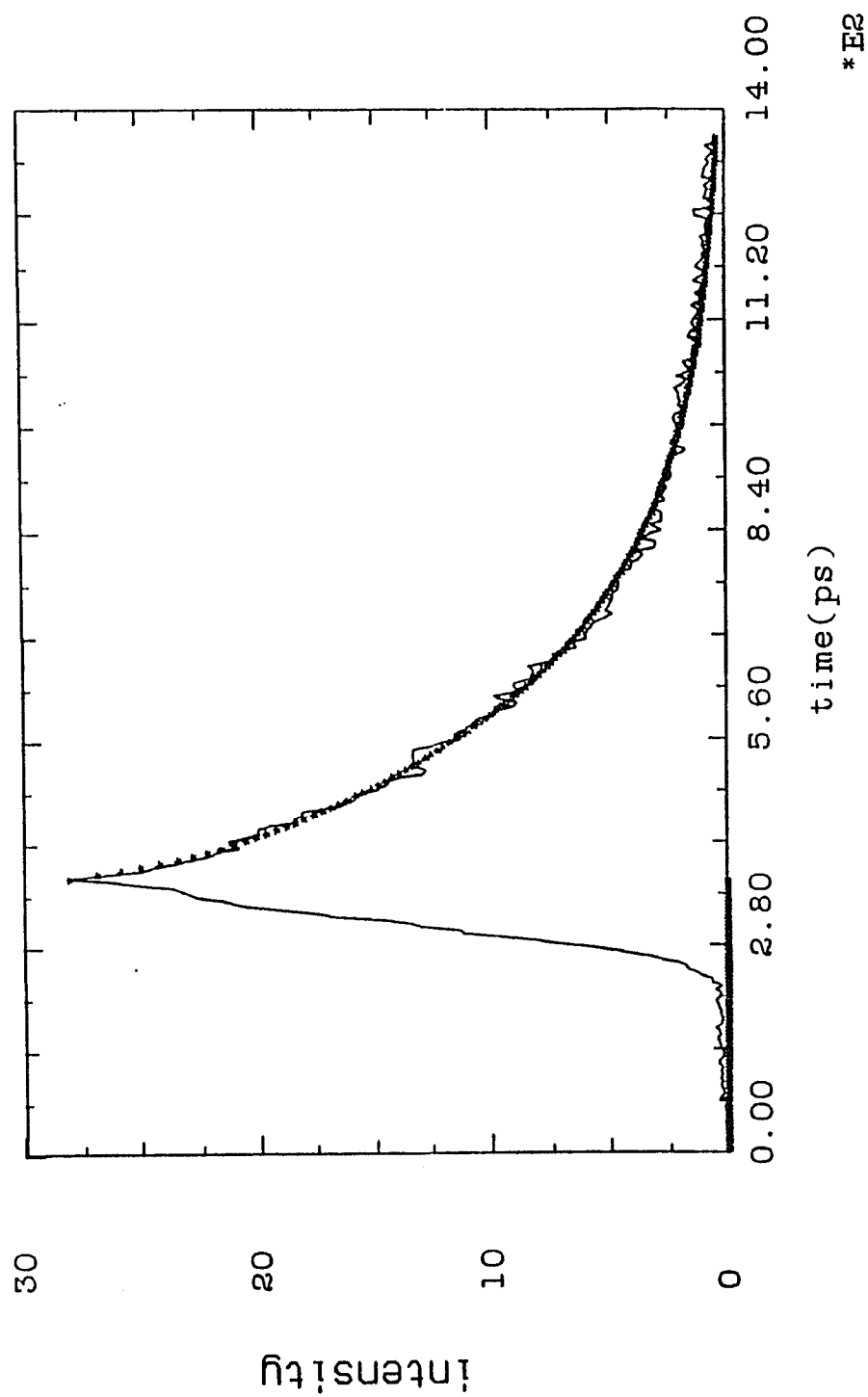


Figure 64. Typical fluorescence temporal profile of DOTC at intensity 10^7W/cm^2 , $t_f = 20 \text{ps}$ and $t_s = 234 \text{ps}$.

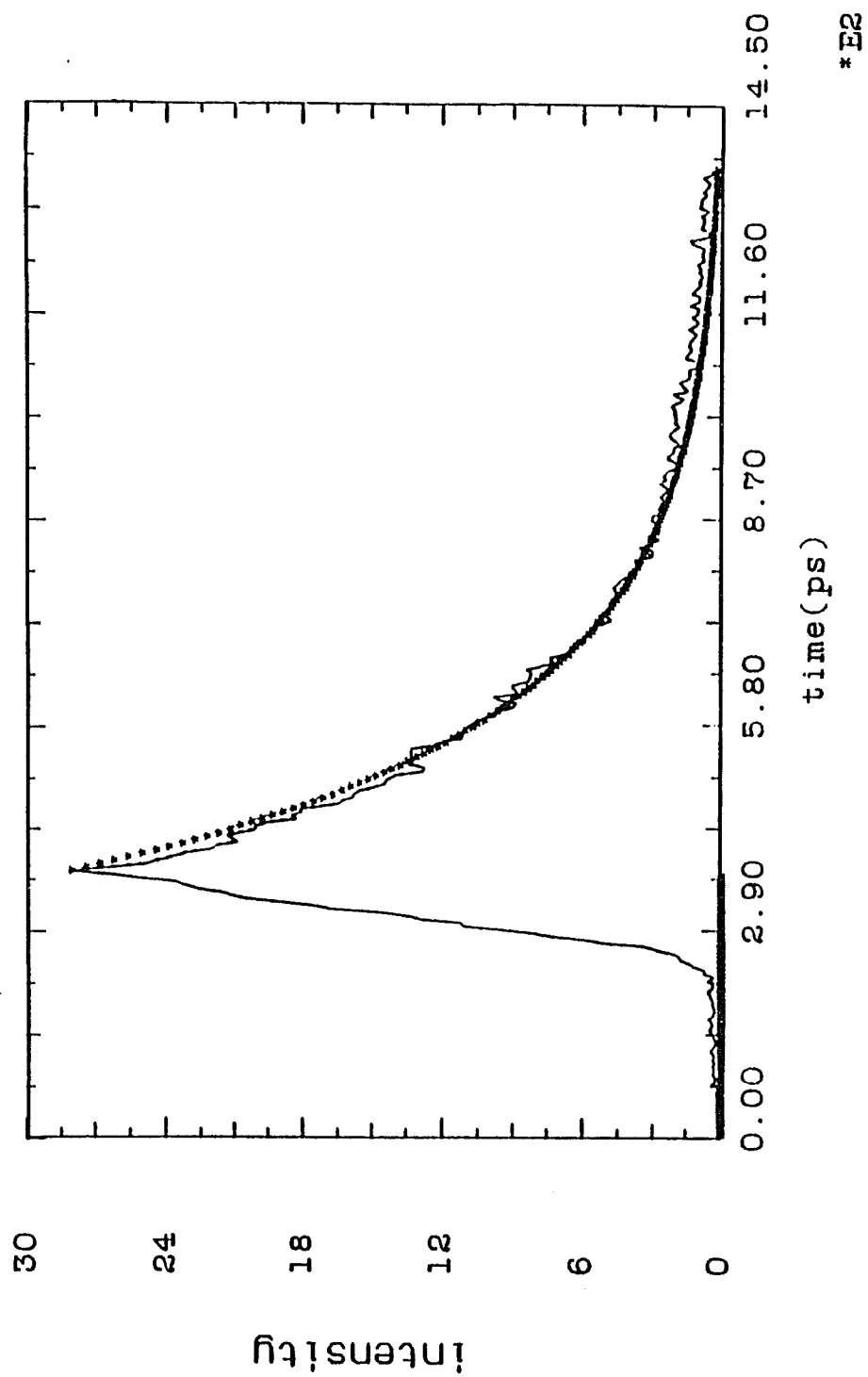


Figure 65. Fluorescence decay of DOTC fitted to a diffusion-controlled process with $a = 1/(280 \times 10^{-12})\text{ps}^{-1}$ and $b = 2.25 \times 10^{-3}\text{ps}^{-1/2}$.

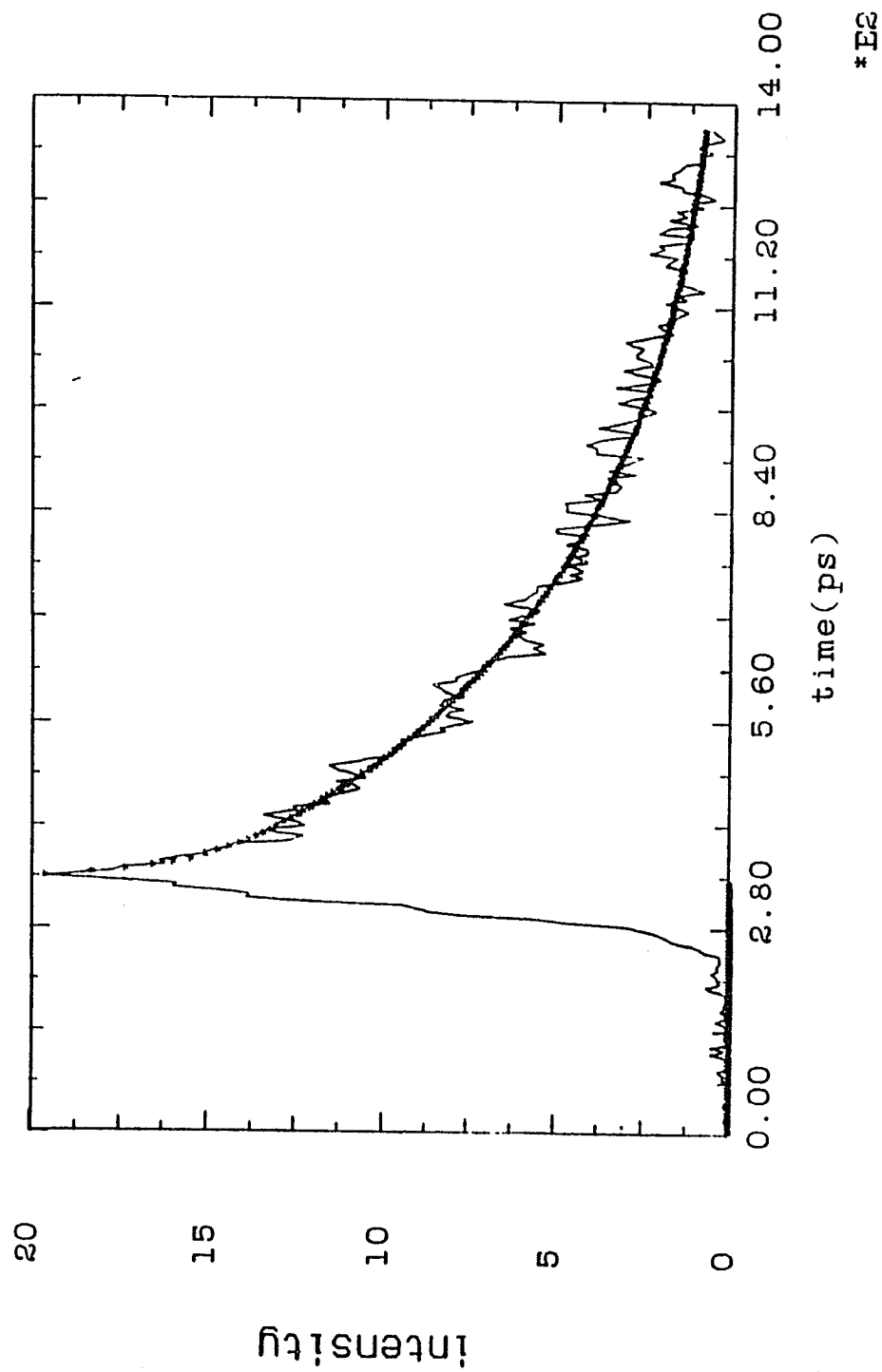


Figure 66 (a). Fluorescence decay profiles of doxycycline at room temperature, $t_f = 17\text{ps}$ and $t_s = 367\text{ps}$ and

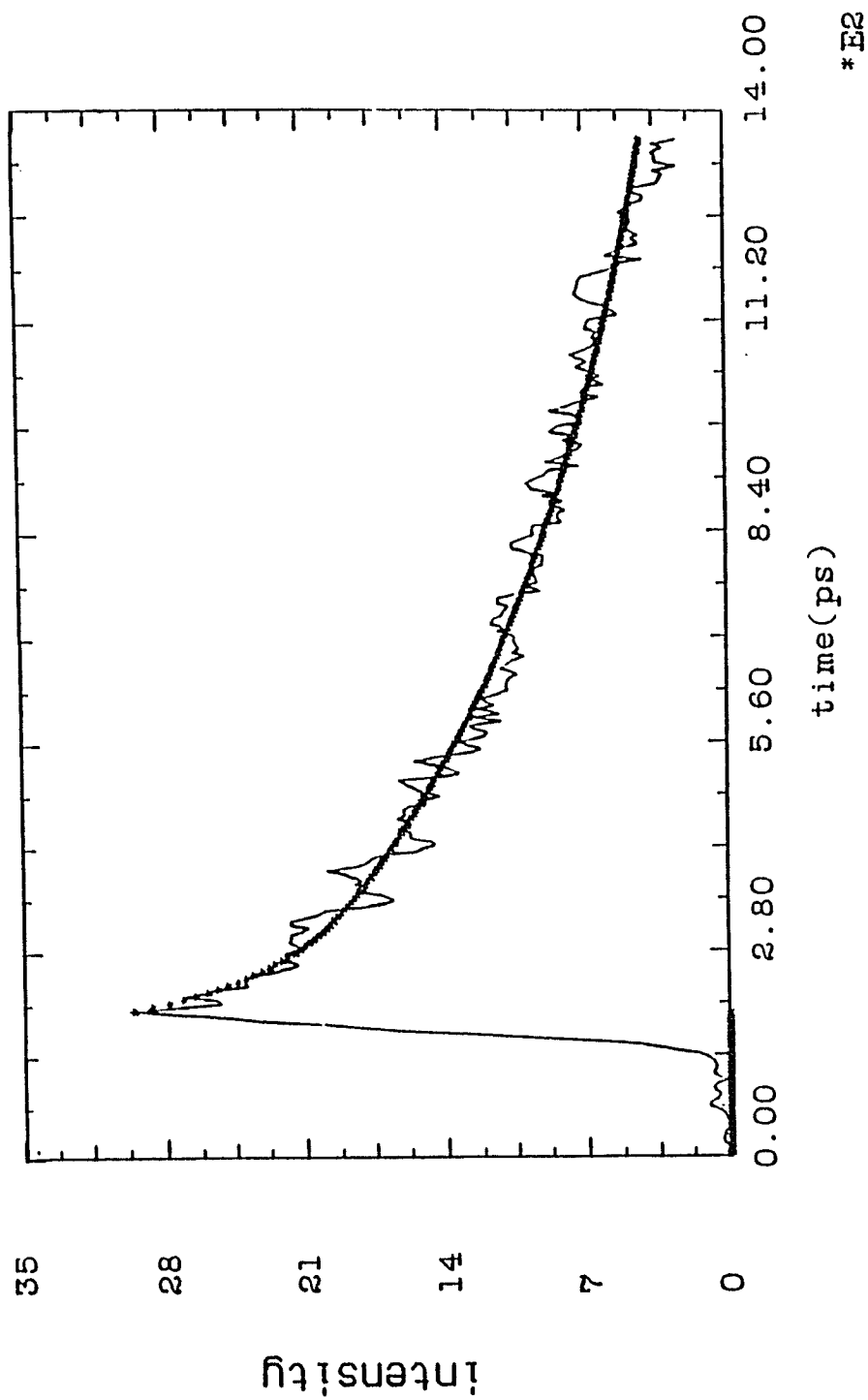


Figure 66 (b). Fluorescence decay profiles of doxycycline at-120 C, $t_f = 43$ ps and $t_s = 683$ ps.

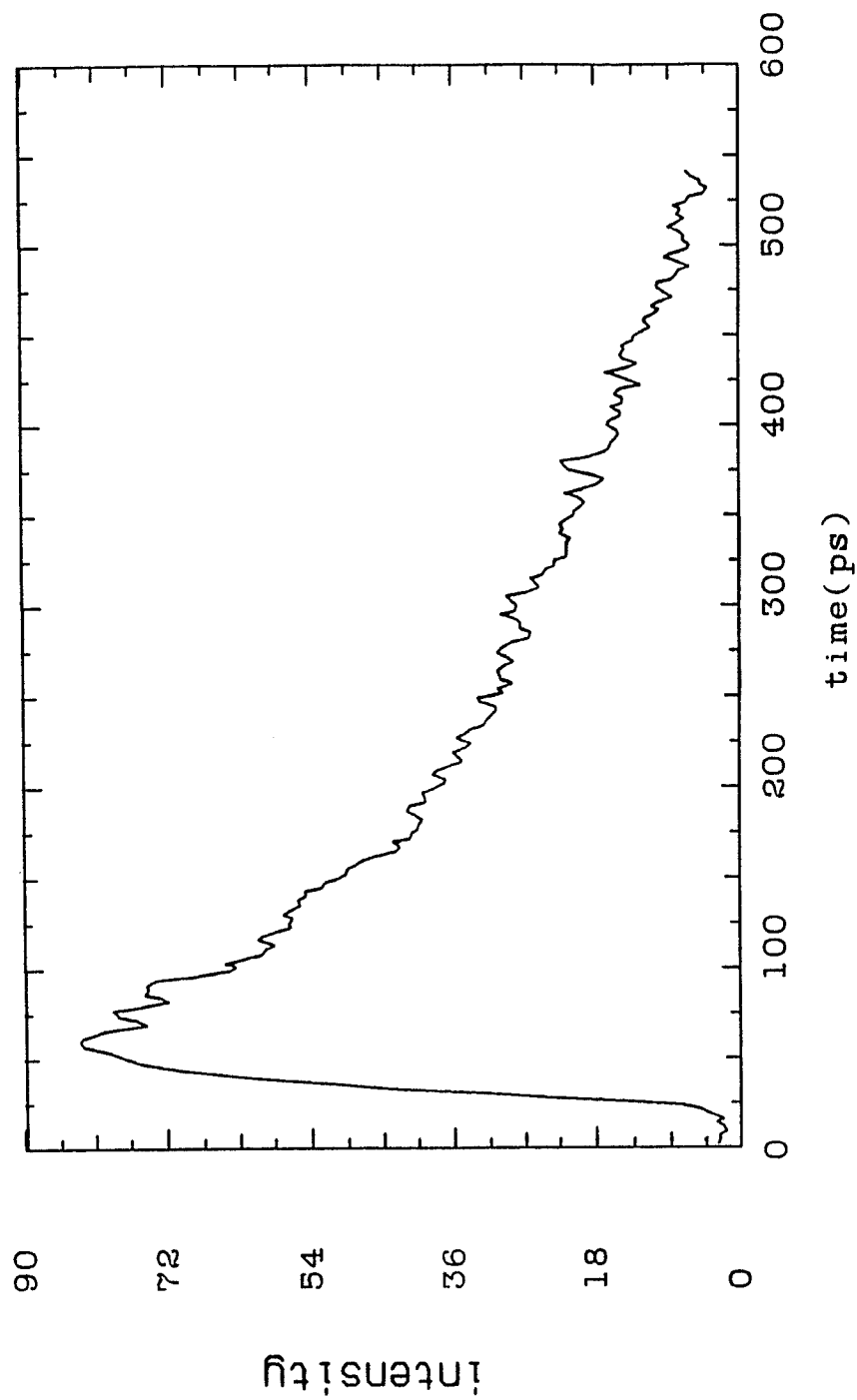


Figure 67 (a). Fluorescence decay profiles in the 225ps range to compare the rise times at room temperature; $t_{\text{rise}} \sim 30\text{ps}$.

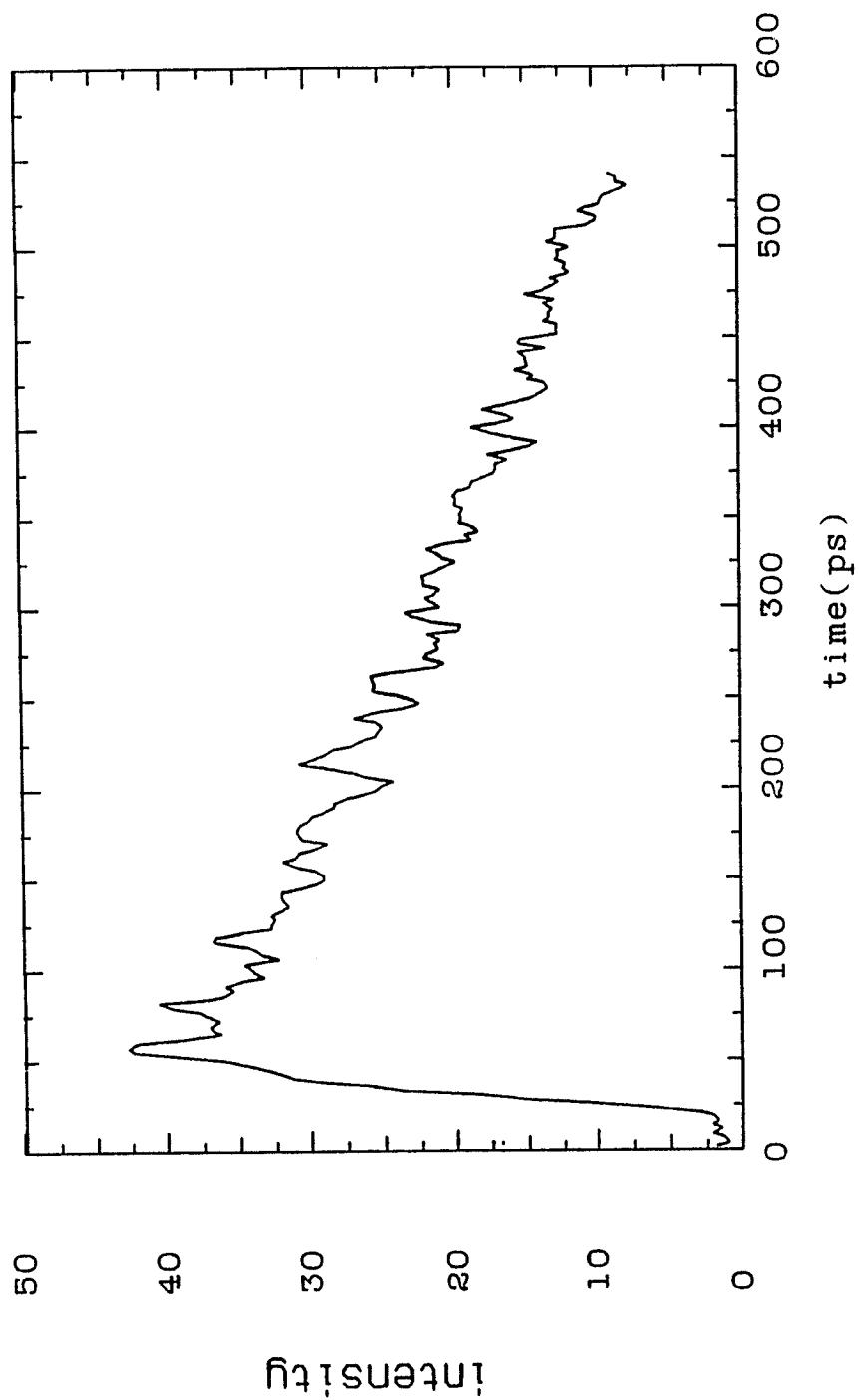


Figure 67 (b). Fluorescence decay profiles in the 225ps range to compare the rise times at low temperature (-120°C) ; $t_{\text{rise}} \sim 30\text{ps}$.

starts to decrease monotonically.

Two models are displayed in Figure 68 to describe the intensity dependence of the yield. A rate equation is used to describe the data involving absorption to the second excited state where (1) non-radiative relaxation occurs without feedback to the first excited state from the higher excited state and (2) non-radiative relaxation occurs as feedback to the first excited state.

It should be noted here that the time dependence of the non-radiative decay has been neglected for the purpose of calculation. A single exponential model is assigned for the overall profile.

Consider the first case where excited state absorption from S_1^* to S_2 with loss from S_2 . The rate equation to describe the depletion in the higher vibrational level of the first excited state (S_1^*). This is given by,

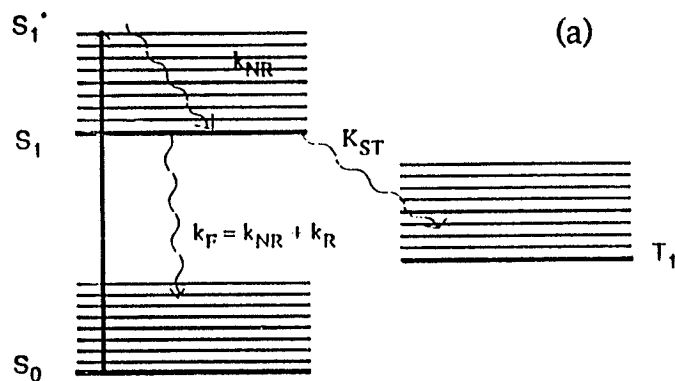
$$\frac{dS_1^*(t)}{dt} = \sigma_{01} I(t) S_0(t) - k_{NR} S_1^*(t) - \sigma_{12} I(t) S_1^*(t) \quad (1)$$

where non-radiative relaxation (k_{NR}) occurs from the state S_1^* to the lowest vibrational level of the first excited state (S_1) and absorption (σ_{12}) to the second excited state (S_2) from the state S_1^* .

The rate equation for the first excited state (S_1) is

$$\frac{dS_1(t)}{dt} = k_{NR} S_1^*(t) - \left(\frac{1}{\tau_F} + K_{ST} \right) S_1(t) \quad (2)$$

Energy level diagram of processes occurring at low intensities



Energy level diagram of processes occurring at high intensities

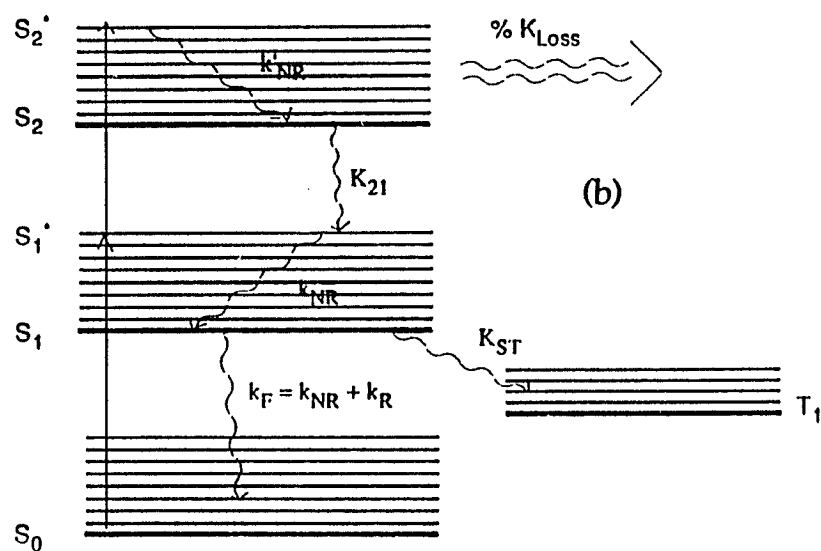


Figure 68. Energy level diagram of doxycycline with processes occurring at (a) low intensities and (b) high intensities.

where k_{NR} is the non-radiative decay rate from higher vibrational level, S_1^* , of the first excited state and $1/\tau_F$ is the radiative decay rate and K_{ST} is the intersystem crossing rate to the triplet state from S_1 .

For the second case where the excited state absorption from S_1^* and partial loss (K_{loss}) from S_2 and feed (K_{21}) to return to the state S_1 . The rate equations for the S_1^* and S_1 states are similar to equations (1) and (2) with the addition of the feedback term to S_1 from S_2 and are given by addition of $K_{21}S_2(t)$ to eqn. 2.

In this case, the rate equation for the second excited state includes absorption to the higher vibrational level and partial losses to the medium (K_{loss}) and to the first excited state (K_{21}):

$$\frac{dS_2(t)}{dt} = k'_{NR}S_2^*(t) - K_{21}S_2 - K_{loss}S_2 \quad (3)$$

Equations (1) and (2) have been solved for $S_1(t)$ [See Appendix II for expression of $S_1(t)$] to determine the quantum yield without feedback. Similarly, eqn.(1), eqn.(2) with feedback term and eqn.(3) have been solved for quantum yield with feedback. The quantum yield is given by,

$$QuantumYield = \frac{1}{I} \int S_1(t) dt \quad (4)$$

The expressions for quantum yields are given by,

Without feedback (Case 1),

$$Rel.Q.Y. = \frac{[\sigma_{01}m_0 \times (1 - \sigma_{12}I)]}{[\frac{1}{\tau_F} + K_{ST}]} \quad (5)$$

With feedback (Case 2),

$$Rel.Q.Y. = \frac{\sigma_{01}m_0 [1 - \sigma_{12}I \times (1 - \frac{K_{21}}{K_{21} + K_{loss}})]}{\frac{1}{\tau_F} + K_{ST}} \quad (6)$$

These expressions for the quantum yield obtained involve the first excited and the second excited absorption cross sections. Using the least square method to fit the data, the second excited state absorption cross section has been estimated to be $\sigma_{12} = 1.58 \times 10^{-16} \text{cm}^2$ without feedback and $2.6 \times 10^{-16} \text{cm}^2$ with 40% feedback to the first excited state. The fits are displayed in Figure 69 (a) and (b).

Discussion

The fluorescence lifetime profile of doxycycline is not dependent on excitation intensity within our experimental error. The linearity of the fluorescence quantum yield of DOTC upto 10^{15} photons/ cm^2 implies that the emission yield is proportional to the excitation intensity. However, the quantum yield decreases monotonically after an intensity of 10^{15} photons/ cm^2 . This decrease in quantum yield could be arise from a number of nonlinear processes such as ground state depletion (bleaching), exciton-exciton

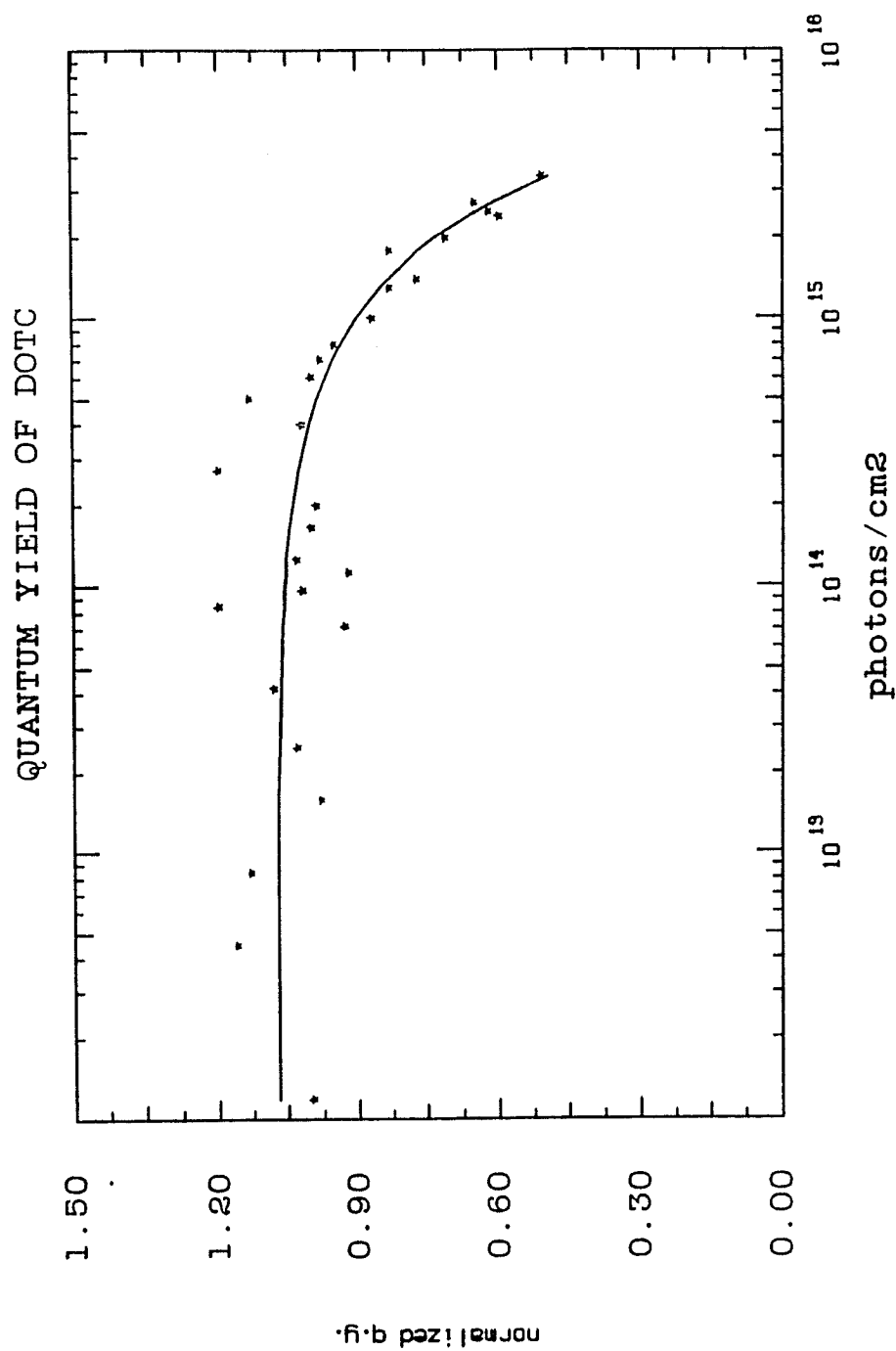


Figure 69 (a). The fluorescence relative quantum yield as a function of excitation intensity with no feedback

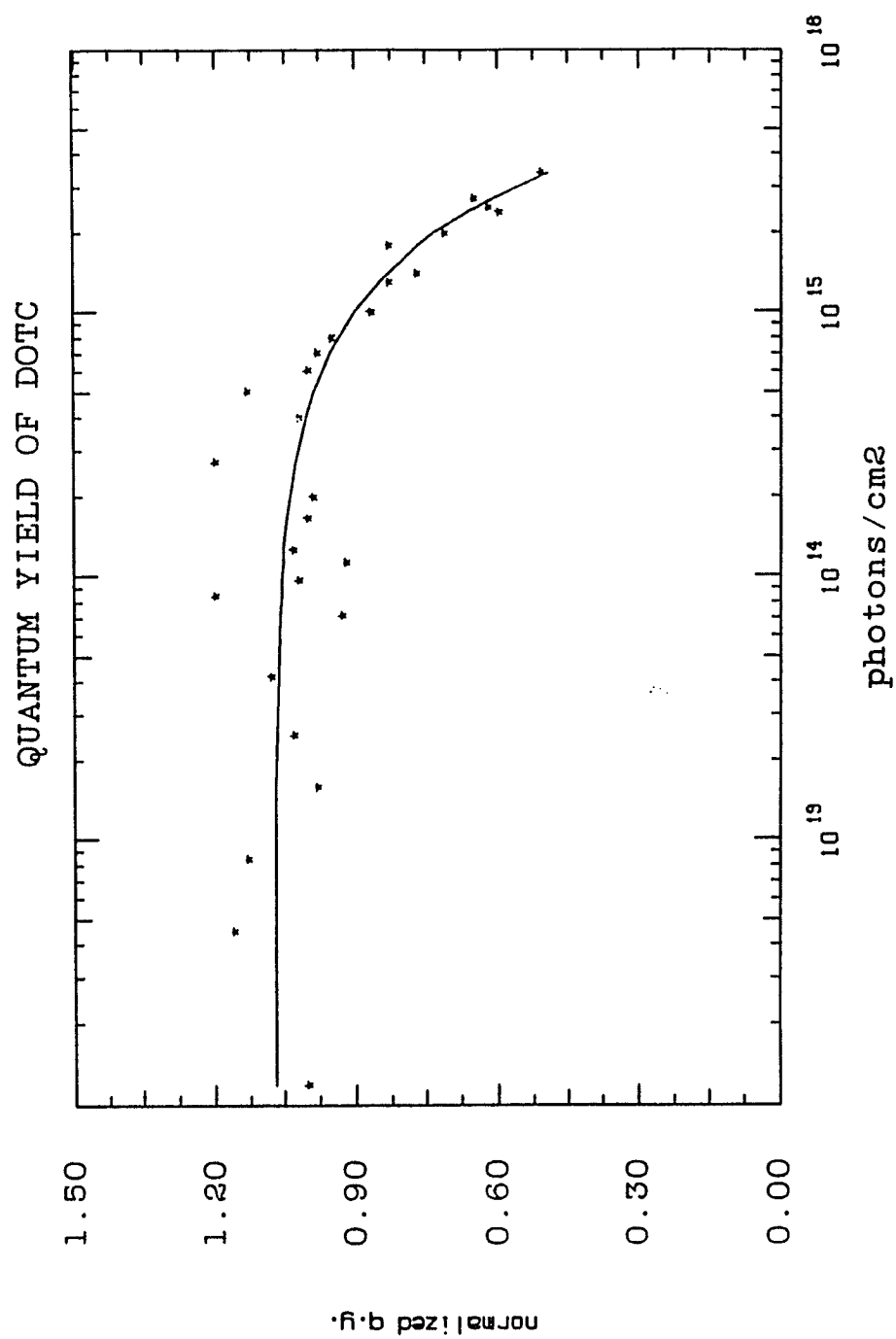


Figure 69 (b). The fluorescence relative quantum yield as a function of excitation intensity with 40% feedback.

annihilation, excited state absorption, photochemical bleaching. Ground state depletion is ruled out since transmission of light from DOTC does not decrease after the intensity at which quantum yield decreases. Since the concentration of DOTC is only 10^{-4}M , exciton-exciton annihilation is ruled out as this process requires the concentration greater than 10^{-3}M [4]. Photochemical bleaching would lead to change in the fluorescence spectra after photoexcitation with higher intensities, which did not occur here. Thus one points to excited state absorption to be the dynamical process at higher intensities.

The excited state absorption model fits (Figure 63 (a) and (b)) the experimental data with second excited state absorption cross section $1.58 \times 10^{-16}\text{cm}^2$ without feedback and $2.6 \times 10^{-16}\text{cm}^2$ with 40% feedback. Since the presence or the absence of feedback is not proven here, the values of absorption cross section for the second excited state have been presented with and without feedback. The size of the cross section is of the order of $2 \times 10^{-16}\text{cm}^2$.

Since the fluorescence lifetimes did not change appreciably over two orders of magnitude, this result supports the sequential excited state absorption theory. The non-exponential profile of the fluorescence lifetime of doxycycline implies activity of some non-radiative processes. The non-exponential fitting of data with a time dependent quenching rate describes the operation of either diffusional quenching by another species or energy transfer to another species with fast Brownian rotational motion of the dye or

energy transfer with diffusion. The transfer of energy to another species is suspected from the large shift of the fluorescence peak from the absorption peak. Proton transfer could be another form of energy transfer. It should be noticed that the fluorescence rise times do not change at liquid nitrogen temperatures. However, rise times should change at low temperatures if proton transfer occurs. To prove this, much lower temperature measurements should be attempted.

This study confirms the second excited state absorption in doxycycline at higher intensities and estimates the second excited state absorption cross section.

CHAPTER 8: CONCLUSION

In this thesis, luminescence spectroscopy is applied to human breast tissues and artery and to a photosensitizer. The fluorescence and phosphorescence properties of breast tissues have been studied using steady state and time-resolved methods. The intensity dependence of the fluorescence lifetimes and quantum yield of the photosensitizer, doxycycline, (tetracycline), was studied. These studies have shown that native fluorescence can be used to determine the optical properties of normal and abnormal tissues and determine the physical properties occurring.

The fluorescence spectra of breast tissue have been obtained at different excitation wavelengths, namely 488nm, 457.9nm and 514.5nm using an argon-ion laser and 527nm and 351nm of a Nd-glass laser. Depending on the excitation wavelength, different molecules are activated in the breast tissues, for example, flavins with 488nm excitation and NADH with 351nm excitation. Non-malignant and malignant breast tissues display differences in spectral shape. Normal breast tissues displayed spectral profiles with dips at 540nm and 580nm with visible excitation while cancer tissues displayed smooth profiles with no dips. The fluorescence spectra with 527nm and 351nm excitation also showed similar results, with dips in normal tissues at 480nm and 580nm, respectively. The marker here is found to be blood and the difference is interpreted as due to absorption of the fluorescence by hemoglobin in normal tissues. The fluorescence spectra from benign and

malignant tumors, however, did not show any differences. This led to the conclusion that visible excitation was not suitable for diagnostic purposes. But this study paved the way to pursuing this method further with the correct excitation wavelength for diagnostic purposes.

Time-resolved fluorescence of non-malignant and malignant tissues are a major part of this research with visible, near-UV and UV excitation. The temporal decays display non-exponential profiles. These non-exponential forms are interpreted as due to two or more fluorophores as well as due to certain non-radiative processes. The visible (527nm) and near-UV (351nm) excitation displayed differences in normal and cancer samples. The temporal profiles of the tissues have been fitted to double exponentials.

With 527nm excitation, normal and cancerous tissues displayed a difference in the fast component of the fluorescence lifetimes, the fast component of the cancer (100ps) being shorter than that of the normal tissue (200ps). The slow components of both types were around 1.5ns. The fluorescence from breast tissues with 527nm excitation may be from flavins. The fluorescence lifetimes of benign tissues and tumors did not show any differences in the fluorescence lifetimes as compared to malignant tumors. But, for diagnostic purposes, it is essential to distinguish benign tumors from malignant ones. This was a drawback with 527nm excitation.

With 351nm excitation, the fast components of the fluorescence lifetimes of benign tissues and tumors (>150ps) were found to be slower than that of malignant tumors (<150ps) only in the 450nm emission band. The slow

components in both cases were measured to be within 2ns to 3ns. The molecules most likely responsible for this fluorescence are NADH, elastin and collagen.

UV excitation at 310nm displayed significant differences between non-malignant and malignant tissues in the 340nm band. The slow components of the fluorescence lifetimes of benign tissues and tumors ($<1.6\text{ns}$) are found to be faster than those of malignant tumors ($>1.6\text{ns}$), in contrast with 527nm and 351nm excitation. The ratio of fast to slow amplitudes are also found to be larger (>0.5) for benign tissues and tumors as compared to that of malignant tumors (<0.5). The fast components in these cases varied from 20ps to 115ps. The molecule that may be responsible for the fluorescence in the 340nm band is tryptophan.

The fast component of malignant breast tumors have been observed to be shorter than that of the normal tissues, benign tissues and tumors with 527nm and 351nm excitation. This implies more non-radiative processes in the environment of the fluorophore in the malignant tumor. In the 310nm excitation, however, differences are observed in the slow component. The slow component of non-malignant tissues are found to be faster than that of the malignant tumors. This implies that the environment of another fluorophore (tryptophan) which is activated by 310nm excitation displays more non-radiative processes in the non-malignant samples.

The fluorescence spectra of benign and malignant tissues with 351nm excitation spans 370nm to 600nm. From the emission spectral peaks, it is

assumed that NADH (460nm), elastin (410nm) and collagen (390nm) are possible contributing fluorophores. The time-resolved fluorescence of the combination of these three molecules displays a profile similar to that of the benign tissue temporal profile with lifetimes of 292ps and 2.3ns. Selecting the possible fluorophores by comparing their spectral peaks with that of the breast tissue is the first step of determining the fluorophores responsible for the emission signal in tissues. To further ascertain the contribution of the fluorophores, time-resolved fluorescence profiles of the fluorophores are compared with that of the breast tissue. This method of determining the fluorophores responsible for the emission shows that with 351nm excitation, the most likely molecules are NADH, elastin and collagen combined. The malignant tumor lifetime is found to be similar to that of collagen or elastin. It is known that the collagen and elastin content of the breast tumors increase. Also, from the literature, studies performed in rat tumors show a decrease in the NADH concentration. Thus the ratio of NADH to collagen content or NADH to elastin content changes in tumors as compared to the normal breast tissue. More detailed work may confirm this view. The fluorescence profiles with 310nm excitation are measured in the 340nm and 440nm wavelength regions. This narrows down the possibility of fluorophores to tryptophan and NADH, elastin and collagen in the two wavelength regions. The fluorescence lifetimes of tryptophan and NADH with 310nm excitation are 148ps and 2.2ns and 44ps and 2.6ns, respectively. Average non-malignant tumor lifetimes are 73.6ps and 1.16ns and the average malignant tumor lifetimes are 67.5ps and

1.9ns in the 340nm band. The slow component of the tumor appears to be similar to that of tryptophan. However, the short component is faster than that of tryptophan. This may be due to the fact that the tryptophan is not free and is in the protein. The non-exponential decay profiles of the tissues with 351nm excitation are interpreted as partly due to more than one fluorophore, namely a combination of NADH, elastin and collagen and partly due to non-radiative processes. These are partially confirmed by time-resolved fluorescence studies of several biomolecules and low temperature luminescence studies. The 310nm excitation results in the 340nm and 440nm emission bands are interpreted as mainly due to tryptophan in the 340nm band and NADH, elastin and collagen in the 440nm band. The observed differences between non-malignant and malignant tissues could arise from environmental variations of tryptophan.

Low temperature luminescence studies have been performed with 351nm excitation. The fluorescence lifetimes slow down at almost liquid nitrogen temperatures. There occurs enhancement of fluorescence. Phosphorescence is observed for the first time in human breast tissues. Thus, low temperature studies also reveal the process of intersystem crossing to the triplet state of the fluorophore.

Time-resolved fluorescence depolarization studies have been performed with 310nm excitation. These studies show very fast motion of the molecules. The motion is found to be faster than the resolution of the detection system. The fluorescence detected at the 'magic angle' 54.7° also confirms that

orientational motion does not affect the fluorescence decay profile of the tissues. Scattering depolarizes the radiation quickly in the picosecond time scale.

Native fluorescence from tissues has been used to study cancer. This method has been extended to atherosclerotic disease. The steady state fluorescence studies, however, were unable to detect calcified plaque from normal artery with visible excitation. This led to probing the time domain for detection of various types of atherosclerosis. There did not appear to be any advantage of using time-resolved fluorescence over steady state methods with 351nm excitation. This work, however, gives an idea of the heterogeneous nature of emission of the aorta. It indicates that there is probably a variation in the physical processes occurring in fibrous plaque as compared to normal aorta and calcified plaque. The study with biomolecules confirms the presence of more collagen in fibrous plaque than in normal aorta and calcified plaque.

This study has been extended to the UV region with 310nm excitation. Interestingly enough, this wavelength appears to be the correct wavelength for the detection of calcified plaque. However, it does not differentiate fibrous plaque from normal artery. This work suggests that the different types of plaque can be distinguished by obtaining the key wavelength for a particular type and combining the results of all excitation and emission wavelengths.

A minor part of the thesis involves the understanding of the dynamics of the process of sequential multiphoton absorption in doxycycline which is a photosensitizer. The results obtained by studying the intensity dependence

of the fluorescence lifetimes and quantum yields of doxycycline supports the process of sequential excited state absorption theory as suggested by previous work. Non-exponential forms of the temporal profile are assumed to be either due to two emitting species or due to time-dependent non-radiative decay with quenching by another species. This is not confirmed or further pursued at this stage of the research. The absorption cross section for the second excited state has been estimated using a theoretical model.

There are certain limitations in using this technique for diagnostic purposes. Firstly, this thesis involves the use of native fluorescence *in vitro*. The presence of blood, body fluids and various other chemicals are features of the *in vivo* studies which could significantly change the fluorescence properties of tissues. The spectral and lifetime properties of breast tissues have to be studied *in vivo* before this method can be used as a diagnostic tool. The number of samples measured is also small for diagnostic purposes. One needs a clinical setting to evaluate this situation. However, the usefulness and advantages of native fluorescence in disease diagnosis and obtaining information on the dynamics of fluorophores is displayed in this thesis. With the key excitation and emission wavelengths or a combination of a few excitation wavelengths, the optical approach can be used as a diagnostic tool. Dynamical changes in the microscopic regime can be probed in further detail using this technique. This research is the first step in understanding optical properties of diseased tissue.

CHAPTER 9: FUTURE RESEARCH DIRECTION

This research on the native fluorescence of human breast tissues has displayed the optical properties of non-malignant and malignant tissues. However, interpretation of these results are not conclusive at this stage of the research. Further work along these lines is necessary to obtain more information and to substantiate the current interpretations. The following highlights some unanswered questions:

- (1) Is the complex spectral profile of normal tissue using visible excitation purely due to absorption by blood or are fluorophores other than flavins responsible for the secondary peaks ?
- (2) In what proportions are the various fluorophores present in non-malignant and malignant breast tissues ? Is there a variation of the proportions in non-malignant and malignant tissues ?
- (3) Is the non-exponential form of the fluorescence with 310nm excitation and 340nm emission due to different tryptophan residues or different environments of tryptophan ?
- (4) What is the time dependence of the non-radiative decay rate that is responsible for the non-exponential decays of tissues using 351nm and 310nm excitations ?
- (5) The molecular motion in tissues is found to be very fast; how fast is it ?
- (6) An important question is whether these results are consistent in *in vivo* studies.

The spectral difference between normal and cancer tissues with visible excitation have been observed to arise from absorption due to hemoglobin. The chemical analysis by removing blood does not eliminate the peaks completely. Thus, the peaks could partially arise from other fluorophores with emission peaks around 550nm and 600nm. The probable molecules that could be responsible for these peaks are melanin and porphyrins. This aspect of the research has not been pursued.

(a) A spectroscopic analysis of these fluorophores is necessary to confirm this.

(b) The fluorescence spectra from different tissue components, cells, individual cell components in different media may assist in determining the probable fluorophores.

The temporal profiles of the breast tissues are found to be non-exponential with visible, near-UV and UV excitations.

(a) More statistics with the 310nm excitation is required if this technique is to be used for diagnostic purposes.

(b) In this study, no differentiation has been made of the various stages of malignancy. A correlation of the various stages of cancer with their fluorescence properties can be used to detect the stage of malignancy. This is an important aspect that should be pursued for a complete study of the optical properties of cancer.

Certain fluorophores have been studied with time-resolved and steady state techniques. This work has suggested the contribution from a combination

of fluorophores to the tissue emission signal. However, no conclusive detection of the biomolecules and their contribution to the differences observed in tissue fluorescence has been made.

(a) A deeper analysis by breaking down the tissue into its constituents and studying them separately by optical means may lead to some conclusion.

(b) A biochemical study of the tissue components by breaking down the tissue will be able to determine the amounts of the various fluorophores present in normal and diseased tissue. Spectral and lifetime measurements of the emission of these molecules compared to the tissue emission will give a clearer picture of the fluorophores present in breast tissues. Combination of various molecules in varying proportions could be used to obtain the exact amounts of the fluorophores contributing to the tissue.

(c) Tissue simulation with various biomolecules in suitable environments with and without blood may be another approach of study.

The non-exponential form may be caused by non-radiative processes. This is confirmed by low temperature studies.

(a) Further detailed work by modelling with different time-dependent processes can be used to obtain a value for the non-radiative decay rate. This value may be different for non-malignant and malignant tissues and can be used as a diagnostic parameter.

(b) Low temperature luminescence studies should be extended to the UV regime since this excitation provides significant differences between non-malignant and malignant tissues.

The fluorophore responsible for the 340nm emission with 310nm excitation is tryptophan. There are several views which may be responsible for the double exponential profiles of the tissues and differences between non-malignant and malignant tissues, such as, the contribution of different tryptophan residues and conformations and the tryptophan environment. Further detailed work with tryptophan in different environments, different conformers of tryptophan and tryptophan residues have to be performed to be able to detect the cause of the observed differences.

The fluorescence depolarization studies on tissues with 310nm excitation have shown much faster motion of the molecules compared to the resolution of the detection system.

(a) This is another area that should be extended by studying the depolarization effects with shorter time resolution.

(b) Low temperature fluorescence depolarization studies may be used to study the motion of molecules since the motion will have slowed down at low temperatures.

Low temperature studies have revealed a long lifetime phosphorescence from human breast tissues. This study suggests a small difference in the phosphorescence lifetimes of benign and malignant tissues. This difference is very small and the number of samples studied is so few that this difference is not convincing. Many more samples have to be studied to detect a significant difference.

Time-resolved fluorescence from normal aorta and atherosclerotic

plaque with 351nm and 310nm excitation can be used to distinguish fibrous and calcified plaque from normal aorta. The 310nm excitation study should be further pursued to obtain information on the fluorophores and the physical processes occurring in diseased aorta as compared to normal aorta.

The intensity dependence of the fluorescence lifetimes and quantum yield of doxycycline has been investigated to understand the dynamics of the process of sequential multiphoton absorption. The second excited state absorption cross section has been estimated under two conditions, namely, with feedback and without feedback to the first excited state. It is not yet known which process occurs.

(a) This work has to be pursued to determine which process actually occurs and to confirm the value of the second excited state absorption cross section.

(b) Pump-probe absorption experiments should be performed to conclusively understand the dynamics of sequential multiphoton absorption in doxycycline.

This thesis has shown that spectroscopic methods can be used to determine physical properties and sources of diseased and non-diseased human tissues without the aid of external probes. Further detailed work along the same line may be able to produce a clearer picture of the dynamics of human tissues and their diseases and the contributing biomolecules. This work is the first step in that direction.

APPENDIX I

STEP BY STEP PROCEDURES TO USE THE PICOSECOND Nd-GLASS LASER SYSTEM:

The major experiments of this thesis have been performed using a picosecond mode-locked Nd- glass laser and a streak camera for detection. This section describes the step by step procedure of using the Nd-glass laser system.

1. Turn on the water cooler.
2. Turn on the pressure of nitrogen gas upto 100 lb (N_2 in Figure 1).
3. Switch on oscillator power supply (Figure 3).
4. Switch on pulse generator power (Figure 3).
5. Switch on amplifier power supplies .
6. Switch on triggering battery (D_2 in Figure 1).
7. Switch on both He-Ne lasers (H_1 and H_2 in Figure 1).
8. Turn on the pump of the mode-locking dye (DC_1 in Figure 1) for 3 minutes to circulate it (only on control).
9. Switch on streak camera, temporal analyzer, monitor and computer (Figure 4).
10. Raise the voltage of the spark gap to threshold ($\sim 16KV$); back off slightly from the threshold and fix it at that voltage (SG in Figure 1).

TO CHECK LASER SYSTEM

1. Place used polaroid photographic film on the path of the beam just outside the laser cavity for burn pattern (between WM_2 and P in Figure 1).
2. Charge laser and then fire when you hear the beeps.
3. The laser should burn the film at one spot if it works well.
4. Depending on the shape and depth of the burn, the laser functioning can be evaluated.
5. To obtain better laser output the 100% reflecting mirror should be changed slowly and the shape of the burn pattern on photographic film checked each time to obtain an almost circular shape.

TO CHECK PULSE SELECTION

1. Switch on the power supply for the photodiode D1 (see fig.1).
2. Connect signal cable from D1 to oscilloscope 519 (Make sure 125 Ω /50 Ω connector adapter is used).
3. Load film on camera attached to the oscilloscope.
4. Charge and then fire after opening the shutter.
5. Four pictures of the train with one missing pulse (the selected pulse) per train shows good selection (see Figure 5).
6. IF SELECTION IS BAD:
 - a) Change angle of mirror M3 such that the beam passes through the middle of the spark gap.
 - b) Check and change nitrogen pressure and oscillator voltage.
7. Photographs of pulse train with single pulse missing is shown in Figure 5.

IF OSCILLATOR DOES NOT CHARGE OR FIRE OR DUMP:

CAUTION: DUMP CAPACITOR OF ANY EXCESS CHARGE BEFORE LOOKING INSIDE POWER SUPPLY. SHUT OFF THE MAIN POWER SWITCH ON THE WALL AND ON THE UNITS.

1. If oscillator does not charge:
 - a) Check all connections inside the power supply.
 - b) Check board B (in Figure 3) in power supply (Charge it with spare).
2. If oscillator does not dump:
 - a) Check board B (in Figure 3).
 - b) Check transistor T (in Figure 3) on the inductor for dumping. Replace T and check again.
3. If oscillator does not fire:
 - a) Check all interlock connections.
 - b) Check board B (in Figure 3).
 - c) Check T (in Figure 3).
 - d) Check if the pulse generator is on external triggering or not.

IF LASER WORKS WELL AND PULSE SELECTION IS GOOD:

1. Go to Selection Mode in TA:
 - a) Change the persistence of integers to 10.
 - b) Select one window (or both windows as required)
2. Go to Calibration Mode in TA:
 - a) Press 3 and then 1 and wait for calibration to be over.
3. Go to Input Mode in TA.
4. Turn streak camera to Focus Mode.
5. Use appropriate CP gain and check alignment by observing He-Ne laser spot on the TV screen focussed in the middle of the window.
6. Use He-Ne laser (the one on the optics area-strong one) and let it overlap on the weak one on the sample. Then use it to align the scattering. Make sure to TURN OFF the strong beam before using the streak camera.
7. Check scattering on the monitor with slit of 30 mm-100 mm and obtain 3-7 channels.
8. Check to see if laser spot (530nm) falls at the center of the window with appropriate filters (0-54 and Nd. filters) in the luminescence path.
MAKE SURE THERE ARE ENOUGH Nd. FILTERS IN THE LUMINESCENCE PATH OR YOU WILL BURN THE STREAK CAMERA!
9. To obtain intensity and channel number of the laser spot disconnect monitor and trigger input cables from the streak camera and connect them together.
10. Next, press 1 in input mode and charge oscillator and fire with streak camera slit open. Then go to output mode and check shape and intensity after pressing 1. FWHM ~ 7 channels.

11. Place test sample at appropriate position and check fluorescence spot on monitor using appropriate filters (Ex-4-96,SP540 for 530nm excitation; 7-51,7-60 for 350nm excitation, Em-2(3-67) + Nd filters for 530nm exc. and 3-72 + Nd filters for 350nm exc.)
12. Change mode on streak camera to 'operate'.
13. Change the delay (appropriately chosen for a particular speed) in the delay box : see label in front of the streak camera panel.
14. Press 1 in input mode.
15. Charge , fire with streak camera slit open and see streak on the TV screen.
16. Go to output mode in TA.
17. Press 5 for gain selection and choose an appropriate gain and press 'enter'.
18. Reset and go back to output mode.
19. Then press 1 to check profile again.
20. Remain in output mode.
21. In the computer, go to QB directory by typing "cd qb".
22. In QB directory, type qb ta.bas ; this takes it to the program used to store data.
23. Run the program by pressing 'alt r' and then 'start'.
24. When program types ' Enter Data Filename (w/d) (a:jun011) ', turn knob to output mode and press 3 to store data in disk in drive A . Always use file name 'a:filename' to store data in floppy disk.
25. Before using floppy disk, format it using the command ' format a:/4 '.

TO MEASURE ENERGY:

1. Place detector of energy meter either at sample site or after the SHG or THG (depending on whether 527nm or 351nm is to be measured), after the cut-off filters.
2. Measure energy with appropriate scales (200 μ J) on the energy meter and sometimes with Nd filters when measuring 1054nm (since high energies would damage the meter).
3. Measure background for 527nm,351nm and 1054nm by altering nothing except turning down the spark gap voltage to zero.
4. Filters on beam path before detector :
 - a) For 527nm: 4-96, SP540 + Nd filters.
 - b) For 351nm: 7-51,7-60 + Nd filters.
 - c) For 1054nm: 7-99 + Nd filters.
5. Measure energy after cavity by placing a 4% reflecting mirror after the cavity such that the reflected beam falls into the detector.
6. To measure its background, place a card inside the cavity and in front of the spark gap to block the laser and measure energy.
7. Subtract background from all energy measurements to obtain true energies.

TYPICAL CHARACTERISTICS OF ENERGY OF LASER IN SINGLE SHOT

1. 1054nm - after SHG (in Figure 1):

a) Oscillator	100 μ J
b) Oscillator + amplifier2	1.5mJ
c) Oscillator + amplifier1 + amplifier2	3.0mJ

2. 1054nm - at sample site (S in Figure 1):

a) Oscillator	20 μ J
b) Oscillator + amplifier2	300 μ J
c) Oscillator + amplifier1 + amplifier2	1.0 m J

3. 527nm - after SHG:

a) Oscillator	1.3 μ J
b) Oscillator + amplifier2	90 μ J
c) Oscillator + amplifier1 + amplifier2	500 μ J

4. 527nm - at sample site:

a) Oscillator	15 μ J
b) Oscillator + amplifier2	40 μ J
c) Oscillator + amplifier1 + amplifier2	180 μ J

5. 351nm - at sample site:

a) Oscillator + amplifier1 + amplifier2	30 μ J
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To measure fluorescence kinetics, filters used in emission path after Ap4 in Fig. 1:

530nm excitation: 2 or 3 Corning 3-67 filters to cut off excitation pulse and relevant wide band (WB) or narrow band (NB) filters.

351nm excitation: (2) 0-51 filters to cut off excitation pulse and relevant WB or NB filters.

Notations for Fig. 1

	Lens
	Mirror (Cavity mirrors : dielectric)
	Wedged Mirror
	Aperture
	Amplifier ($A_1=1/2"$, $A_2=3/4"$) and laser
	Dye Cell (diameter= 5 cm)
	Cylindrical Lens
	Filters [$F_{527}=(2)3-67$; $F_{351}=(2)0-51$]
	Spark Gap
	Photodiode (D1 : Hadron 105C Photodiode, D2 : Hamamatsu C1083 Pin Diode)
	Cable

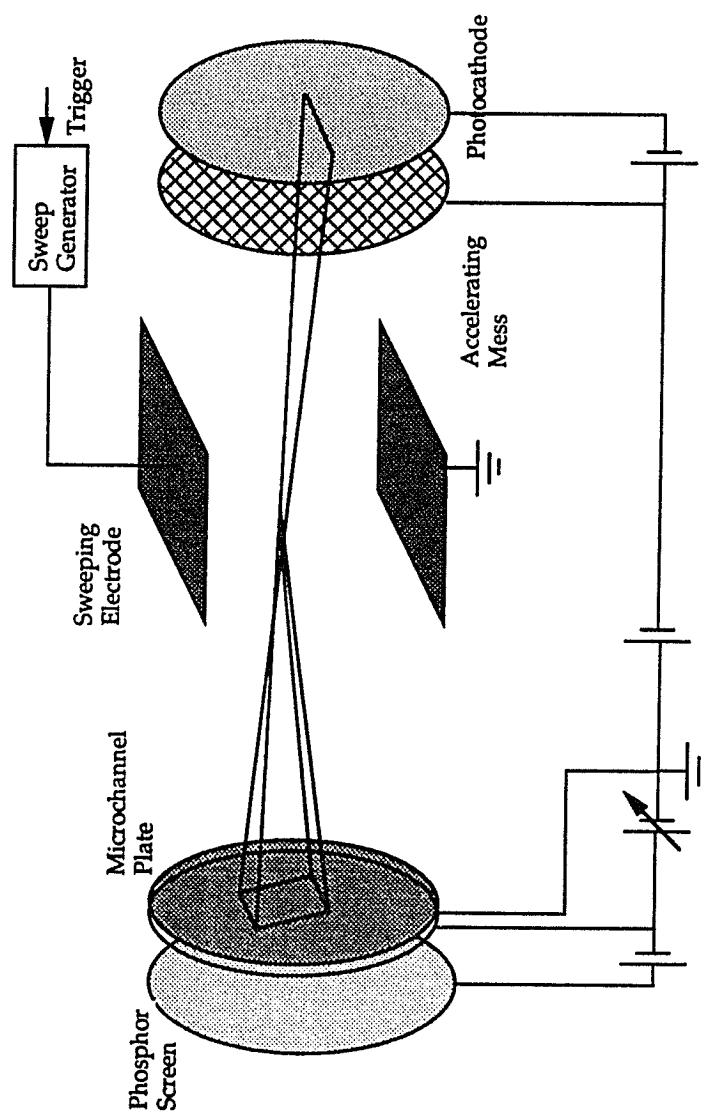


Figure 2. Schematic diagram of a streak camera

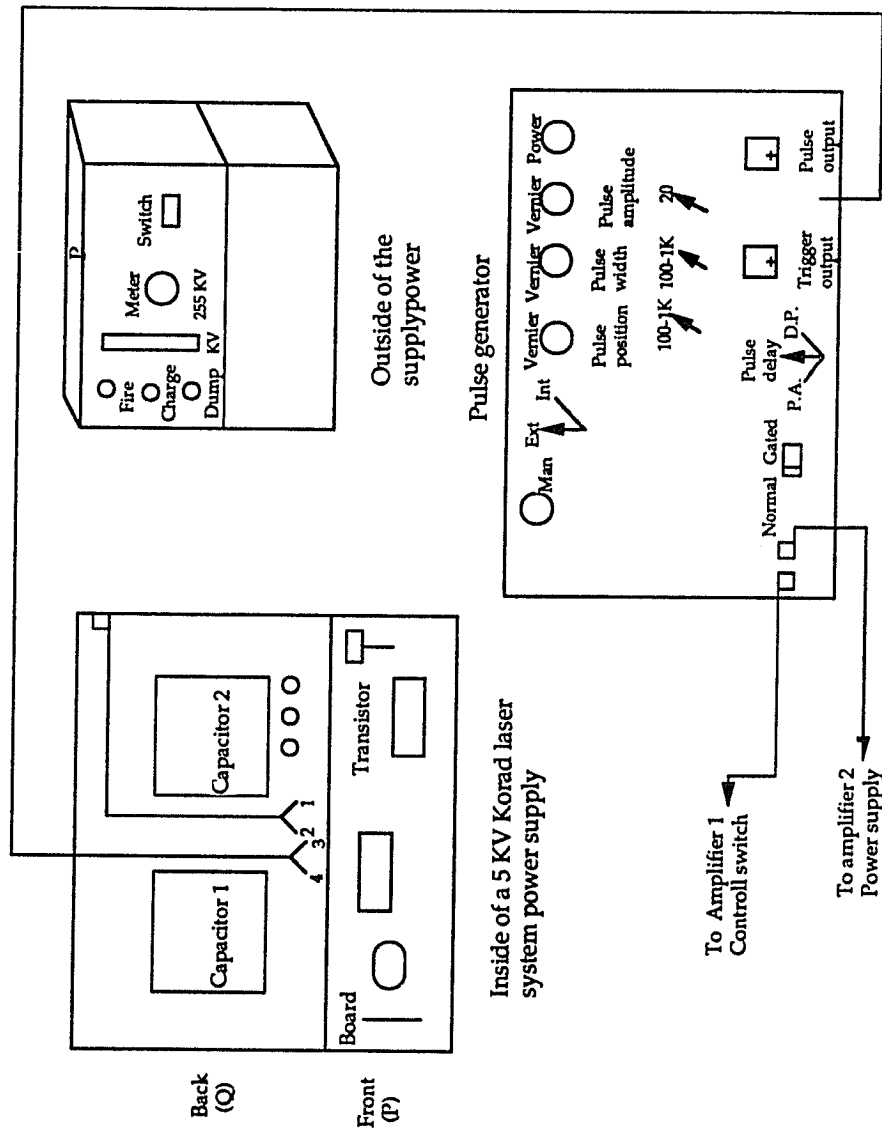


Figure 3. Schematic diagram of oscillator power supply and pulse generator

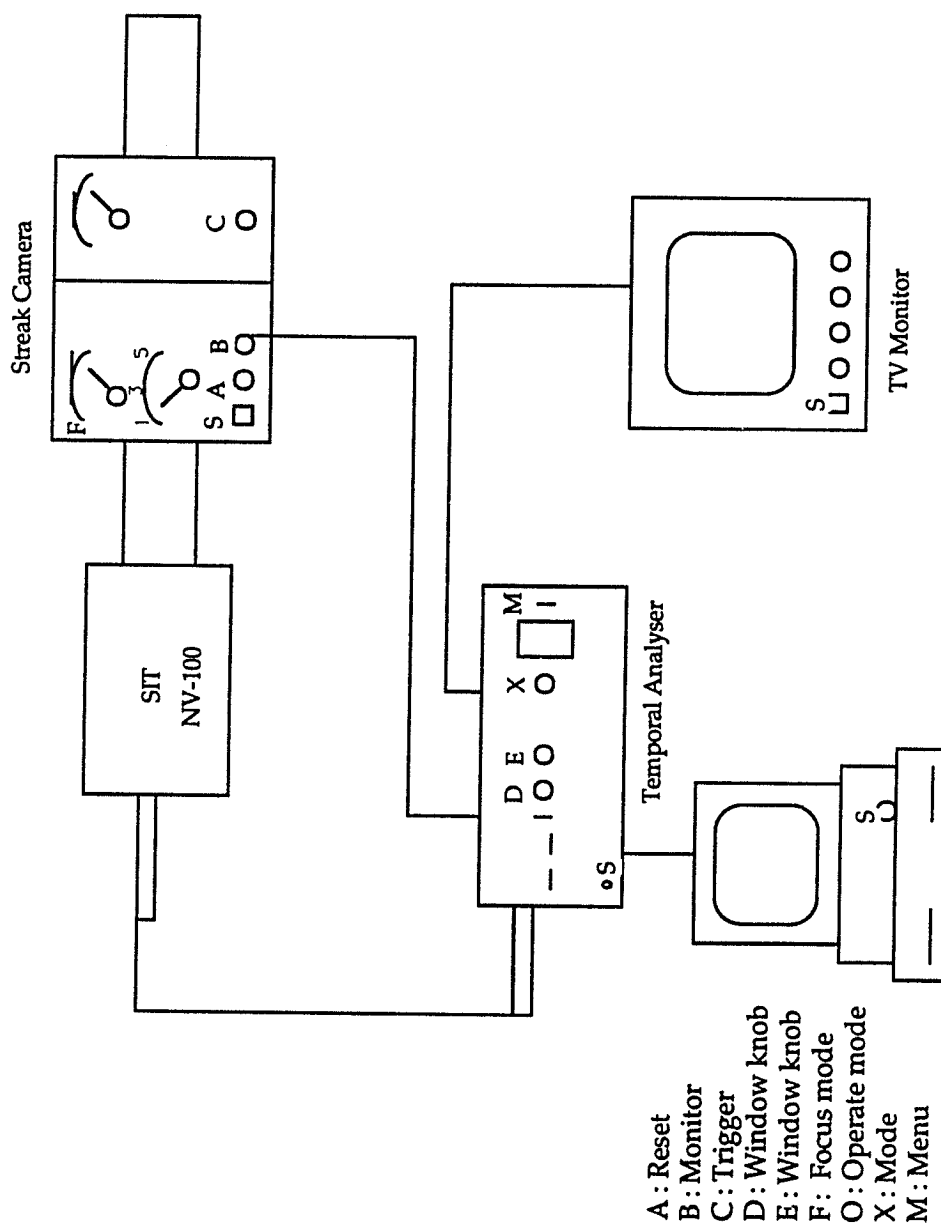


Figure 4. Nd-Glass laser detection system

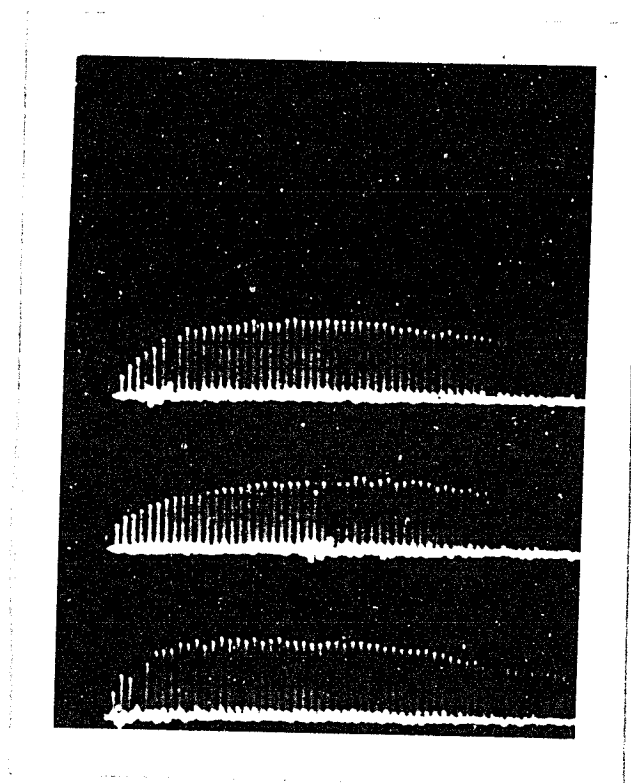


Figure 5: Pulse train with a single pulse selection

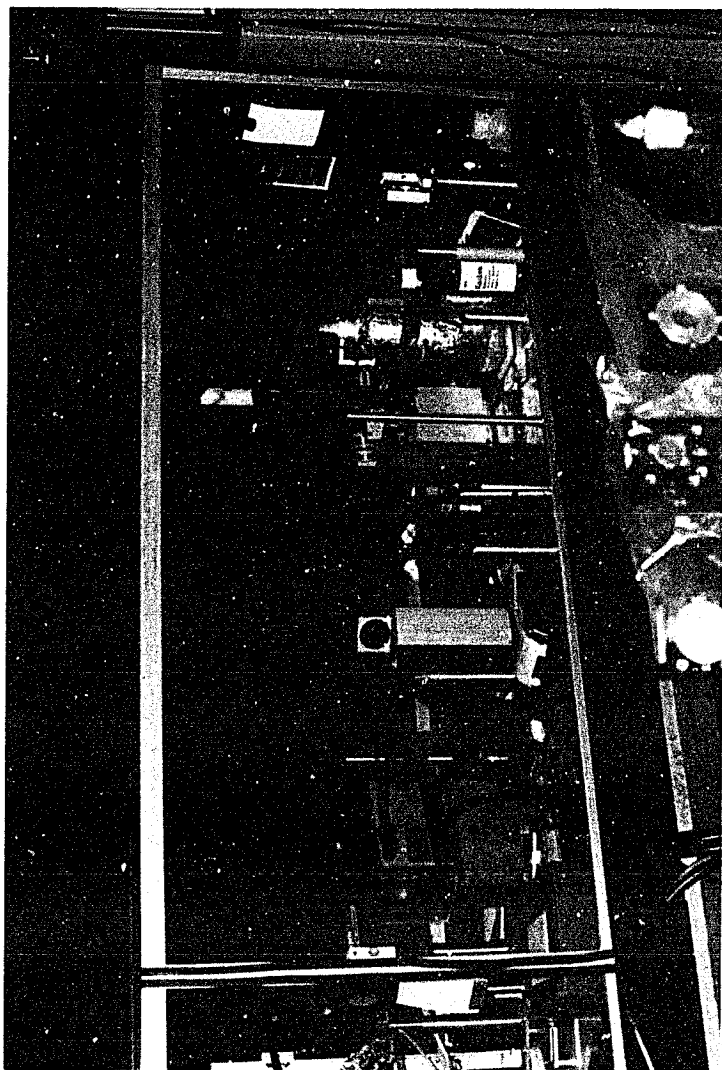


Figure 6: Photograph of Nd-glass laser oscillator system

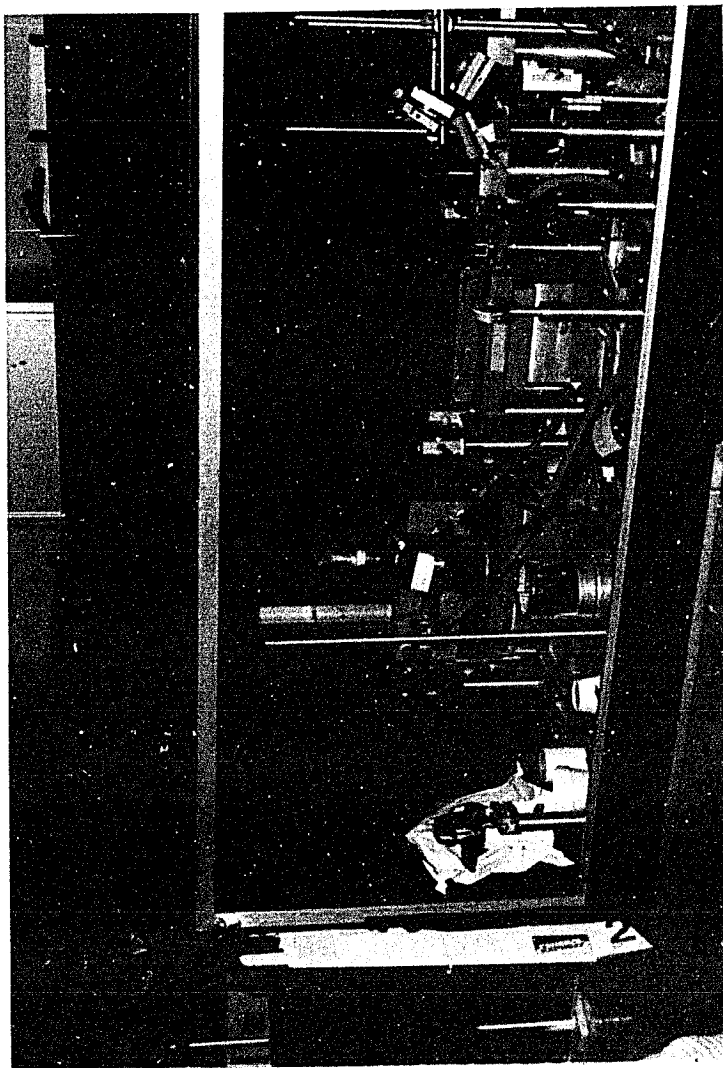


Figure 7: Photograph of amplifier system of Nd-glass laser

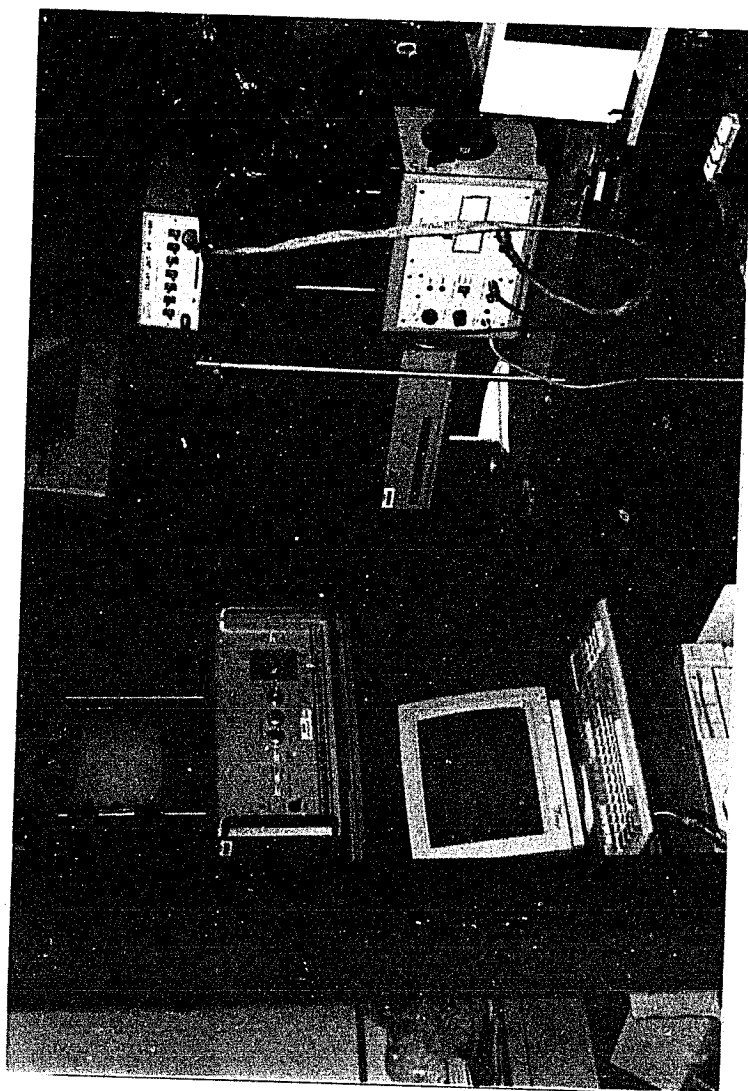


Figure 8: Photograph of detection system of Nd-glass laser

APPENDIX II

The solution for Equation (1) for $S_1(t)$ without feedback is:

$$S_1(t) = k_{NR} \sigma_{01} I m_0 (1 - \sigma_{12} I) \frac{e^{-k_{NR} t} - e^{-(\frac{1}{\tau_F} + K_{ST}) t}}{(\frac{1}{\tau_F} + K_{ST}) - k_{NR}} \quad (1)$$

The solution for Equation (2) for $S_1(t)$ with feedback is:

$$S_1(t) = k_{NR} \sigma_{01} I m_0 (1 - \sigma_{12} I) \frac{e^{-k_{NR} t} - e^{-(\frac{1}{\tau_F} + K_{ST}) t}}{(\frac{1}{\tau_F} + K_{ST}) - k_{NR}} + \frac{K_{21} k'_{NR} \sigma_{01} \sigma_{12} I^2 m_0}{K_{21} + K_{loss} - k'_{NR}} \left[\frac{e^{-k'_{NR} t} - e^{-(\frac{1}{\tau_F} + K_{ST}) t}}{(\frac{1}{\tau_F} + K_{ST}) - k'_{NR}} - \frac{e^{-(K_{21} + K_{loss}) t} - e^{-(\tau_F + K_{ST}) t}}{(\tau_F + K_{ST}) - K_{21} - K_{loss}} \right] \quad (2)$$

where k_{NR} is the non-radiative decay rate from S_1 to S_0 , k'_{NR} is the non-radiative decay rate from S_1^* (higher vibrational level of the first excited state) to S_1 , K_{loss} is the loss from S_2 , K_{21} is the feedback to S_1 from S_2 , τ_F is the fluorescence decay rate, K_{ST} is the intersystem decay rate and σ_{01} and σ_{12} are the absorption cross sections of the first and second excited states, respectively.

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